Translation of thyroglobulin 33S messenger RNA as a means of determining thyroglobulin quaternary structure*

(*Xenopus* oocyte/tryptic peptides/19S thyroglobulin)

G. VASSART[†], S. REFETTOFF[‡], H. BROCCAS[§], C. DINSART, AND J. E. DUMONT

Institut de Recherche Interdisciplinaire, School of Medicine, Free University of Brussels, 2 Rue Evers, B-1000 Brussels, and Biology Department, Euratom, Belgium

Communicated by J. Brachet, June 10, 1975

ABSTRACT Thyroglobulin is a 19S protein of approximately 660,000 daltons and unknown quaternary structure. We have previously shown that a 33S mRNA purified from mammalian thyroids promoted synthesis in the *Xenopus* oocyte of a peptide immunologically related to thyroglobulin. Chemical identity to the native protein is now presented by means of a tryptic peptide analysis. Moreover, the 33S mRNA is shown to contain all the information required for the synthesis of a complete 19S thyroglobulin molecule. Gel filtration in Sepharose under denaturing conditions indicates that the reduced polypeptide encoded by the 33S mRNA is larger than 210,000 daltons. A model of a dimeric thyroglobulin with about 300,000 dalton subunits is presented.

Thyroglobulin is a huge multimeric glycoprotein of approximately 600,000 daltons and a sedimentation coefficient of 19 S (1). It is the major product of protein synthesis by the thyroid gland (2), which after iodination and coupling of a fraction of its tyrosyl residues, serves as storage for inactive thyroid hormones. A very specific structure of the molecule is required to allow efficient coupling of iodotyrosines into the hormonal iodothyronine moieties. Indeed, when compared with other iodoproteins, iodinated thyroglobulin yields higher amounts of thyroid hormones (3, 4).

In spite of numerous studies during the last 10 years, the quaternary structure of thyroglobulin has eluded its investigators. Molecular weights ranging from 25,000 to 200,000 have been obtained for thyroglobulin protomers (5-9). Recently, strong arguments have been proposed in favor of a structural heterogeneity of purified thyroglobulin preparations (9), suggesting that specific or nonspecific cleavages of the molecule occur either *in vivo* or during the purification procedure. This would explain the difficulties encountered in work dealing with the quaternary structure of thyroglobulin, and suggests that the solution to the problem might not be derived by standard analytical techniques.

Our initial aim was the isolation and characterization of RNA preparations possessing thyroglobulin messenger activity. Thus, a 33S mRNA had been well characterized (10-12) while the possible existence of smaller mRNAs had been proposed (10, 13, 14). In this work, we present evidence that the 33S thyroglobulin mRNA promotes the synthesis of a complete 19S thyroglobulin. Estimation of the molecular weight of the encoded peptide strongly suggests that, contrary to all previous guesses, thyroglobulin is a dimer of equal-sized about 330,000 dalton subunits.

* Publication no. BIO 1197 of the Biology Department, Euratom.

[†] Fonds National de la Recherche Scientifique (Belgium).

[‡] On sabbatical leave from the Pritsker School of Medicine, Chicago.

[§] Laboratory of Organic Chemistry, Faculty of Sciences, Free University of Brussels.

MATERIAL AND METHODS

mRNA preparations

Poly(A)-rich RNA was purified from a beef thyroid fraction enriched in membrane-bound polysomes (10, 11). The total RNA extracted with phenol-chloroform (15) from the cellular material sedimenting between 800 and 27,000 g was ethanol precipitated and subjected to chromatography through poly(U)-Sepharose. Poly(A)-rich RNA eluted from the column at 50° was thus obtained, and a 33S fraction was purified by centrifugation at 2° through a 5-30% linear sucrose gradients in 10 mM Tris-HCl, pH 7.5; NaCl, 10 mM; EDTA 1 mM (60,000 rpm, 150 min, Beckman rotor SW65Ti).

Handling of the oocytes

Xenopus laevis oocytes were injected with the 33S RNA or buffer according to the procedure of Gurdon *et al.* (16). Usually 30 oocytes were incubated for 16 hr in Barth's medium (17) containing 1 mCi/ml of [³H]leucine (Amersham, 5 Ci/mmol). Following homogenization in phosphate-buffered saline (NaCl, 0.15 M; sodium phosphate buffer, 0.02 M, pH 7.5) an oocyte supernatant was prepared by centrifugation for 30 min at 40,000 rpm (Beckman rotor SW56Ti) and stored in small aliquots at -20°.

Characterization of the products synthesized by the oocytes

Oocyte supernatant was subjected to centrifugation at 20° through 5-20% linear sucrose gradients prepared in phosphate-buffered saline (60,000 rpm, 150 min, Beckman rotor SW65Ti). Fractions were collected and were either precipitated with 5% trichloroacetic acid for 15 min at 80° in the presence of 50 µg of bovine serum albumin, or assayed for the presence of labeled material precipitable by specific anti-thyroglobulin serum using the sucrose cushion method (18).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoreses were performed as previously described (11).

Noniodinated [¹⁴C]Leucine-Labeled Thyroglobulin was prepared by incubating 10 g of beef thyroid slices for 6 hr in 100 ml Krebs-Ringer bicarbonate buffer containing 2 mM mercaptoimidazole and 50 µCi of [¹⁴C]leucine (Amersham, 300 mCi/mmol of CFB.67). Thyroglobulin was purified by (NH₄)₂SO₄ precipitation between 37% and 42% saturation (20).

Tryptic Peptides were prepared and analyzed essentially as described by Hayashi *et al.* (19) (see legend to Fig. 4).

Gel Filtration under Denaturing Conditions. Samples were prepared by reduction for 3 hr in 6 M guanidine-HCl, 0.3 M Tris-HCl, pH 8.6, 0.1 M 2-mercaptoethanol followed by carboxymethylation with iodoacetamide (20% molar ex-

FIG. 1. (A) Sedimentation in sucrose of poly(A)-rich RNA from beef thyroids. The absorbance at 260 nm was continuously monitored and fractions were collected (33). Ribosomal RNA was centrifuged simultaneously in a separate bucket (arrows). (B) Resedimentation in sucrose of the 33S RNA peak (broken lines in A). Broken lines in B indicate material used for the injection of *Xenopus* oocytes.

cess) for 15 min. They were then applied onto a 0.9×85 cm Sepharose 2B (Pharmacia) column equilibrated with 6 M guanidine-HCl, pH 5, and gel filtered (25). The column was calibrated with radioiodinated markers of known molecular weight (myosin, phosphorylase B, bovine serum albumin, human thyroxine-binding globulin, and ovalbumin). Radioiodination with a single atom of iodine per molecule of protein was performed by a modification of the method of Hunter and Greenwood (35). Incorporation of iodine was shown not to alter the sedimentation (myosin) or biologic properties (thyroxine-binding globulin) of the proteins.

RESULTS

Purification of 33S mRNA

Fig. 1A shows a typical sedimentation pattern of poly(A)-rich RNA prepared from a beef thyroid homogenate. The pattern was qualitatively highly reproducible (10–12). RNA in the 33S peak was harvested by ethanol precipitation, heated for 1 min to 80° in a low ionic strength buffer (10 mM Tris-HCl, pH 7.5; NaCl, 10 mM; EDTA, 1 mM) followed by quick chilling. This procedure should reveal hidden nicks in the RNA molecules (21) and dissociate possible mRNA polymers (22). Following such treatment or after exposure to 90% formamide (not shown), the RNA still sedimented as a sharp 33S peak (Fig. 1B).

Characterization of the product of 33S mRNA translation by the oocytes

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. The purified 33S mRNA collected by ethanol precipitation (broken lines, Fig. 1B) was injected into *Xenopus* oocytes. Fig. 2 shows the migration pattern in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the immunoprecipitated material from mRNA-injected oocytes after

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the peptide encoded by the 33S mRNA. Oocytes were injected with 33S mRNA (fractions 16–21, Fig. 1B). The oocyte supernatant was immunoprecipitated with antithyroglobulin serum. The resulting immunoprecipitate was dissociated by boiling for 2 min in 0.3 M Tris, 0.1 M dithiothreitol, 1% sodium dodecyl sulfate, alkylated with iodoacetamide, and subjected to electrophoresis in 4% sodium dodecyl sulfate-polyacrylamide gel (28). Gels were cut in 2 mm sections (Gilson gel fractionator) and radioactivity of the slices was measured (11). Unlabeled beef thyroglobulin (20 μ g) was similarly treated but in the absence of dithiothreitol. It was run on a separate gel colored with Coomassie blue (top of the figure).

full reduction and alkylation. A single peak of radioactivity associated with high-molecular-weight material and migrating slightly slower than the 12S unlabeled thyroglobulin marker was evidenced. The marker thyroglobulin had been alkylated but had not been reduced, since reduction is known to yield a pattern showing at least 10 bands (9, 23, 24). The fact that the material in the radioactive peak on Fig. 2 is made of monomeric polypeptide chains is supported by the observation that three different methods (8, 25, 26) of reduction and alkylation yielded identical results.

Sucrose Density Gradient Centrifugations. Extracts from buffer- and 33S mRNA-injected oocytes were layered onto 5–20% linear sucrose density gradients (in PBS) and centrifuged for 150 min at 20° . This temperature was chosen because of the known tendency of noniodinated 19S thyroglobulin to dissociate in the cold into two 12S subunits

FIG. 3. Sedimentation in sucrose of the native protein encoded by the 33S mRNA. Extracts were from 33S-mRNA-injected (●) or buffer-injected (○) oocytes. Fractions were either trichloroacetic acid precipitated (A), or immunoprecipitated (B). Radioactivity was counted in a toluene base scintillation mixture (4 g of Omni-fluor per liter of toluene).

FIG. 4. Tryptic peptide analysis of the protein encoded by the 33S mRNA. (A) Specific immunoprecipitates prepared from 33S-mRNA-injected oocyte extracts and containing [^3H]leucine-labeled material were mixed with purified [^{14}C]leucine-labeled noniodinated beef thyroglobulin (Tg). Following reduction in the presence of 8 M urea and alkylation, the mixture was digested with crystalline trypsin in NH_4HCO_3 , pH 8.1, for 2 hr. After lyophilization, it was dissolved in the elution solution and applied to a 0.7×95 cm SP Sephadex C-25 column. The peptides were eluted by means of a discontinuous pyridine-acetate gradient (19). Two milliliter fractions collected from the column were counted simultaneously for ^{14}C and ^3H radioactivity. Correction for the contribution of ^{14}C in the ^3H channel and for quenching by pyridine were made using the internal standard method. (B) [^3H]Leucine-labeled total proteins from buffer-injected oocytes were mixed with [^{14}C]leucine-labeled thyroglobulin synthesized by thyroid slices, and the mixture was subjected to the procedure described in (A).

(27). As shown on Fig. 3B, immunoprecipitations performed with antithyroglobulin serum on each fraction collected from the bottom of such gradients revealed the presence of a

19S peak only in samples from 33S-mRNA-injected oocytes. A variable amount of 12S material was also present. The distribution of the acid-precipitable radioactivity in similar

gradients also revealed a small 19S peak. The great majority of the oocyte endogenous proteins migrated in the 4–5S region (Fig. 3A). Similar results were obtained with two different mRNA preparations, tested on oocytes from two different animals.

Tryptic Peptide Analysis. Since the identification of the protein encoded by the 33S mRNA rested only on its immunological (Figs. 2 and 3B) and sedimentation properties (Fig. 3A), it was of interest to demonstrate its chemical identity with thyroglobulin from beef thyroid. Accordingly, noniodinated [^{14}C]leucine-labeled beef thyroglobulin and the [^3H]leucine-labeled material present in specific immunoprecipitates prepared from 33S-mRNA-injected oocytes were mixed and, after full reduction and carboxymethylation, were codigested with trypsin (19). Analysis of the resulting peptides, developed on sulfopropyl (SP) Sephadex C-25, showed complete qualitative correspondence of the 23 observed peaks (Fig. 4A). The stability of the ratio between ^{14}C and ^3H radioactivity calculated for each peak was used as a measure of quantitative correspondence. Except for four peaks, labeled in Fig. 4A as number 1, 2, 4, and 5, it was shown that this ratio varied by less than 15%. In a control experiment (Fig. 4B) is shown the complete lack of correspondence between the chromatographic patterns of tryptic peptides from [^3H]leucine-labeled oocyte endogenous proteins and [^{14}C]leucine-labeled beef thyroglobulin. As in the preceding experiment, the original materials were codigested and cochromatographed.

Gel Filtration of the Peptide under Denaturing Conditions. Estimation of the size of thyroglobulin peptides by their migration during sodium dodecyl sulfate–polyacrylamide gel electrophoresis has failed, since the observed molecular weight has been shown to be a function of the acrylamide concentration of the gel (9, 12). A method using the retardation coefficient (28) has given an apparent molecular weight of 185,000 for the peptide encoded by the 33S mRNA (11). The discrepancy between this figure and the fact that the peptide migrated slightly slower than 12S thyroglobulin (molecular weight 330,000) (Fig. 2) prompted us to approach the problem in a different way. The labeled thyroglobulin peptide from immunoprecipitates was fully reduced in 6 M guanidine-HCl, pH 8.6, and alkylated, and its size was estimated by gel filtration in Sepharose 2B equilibrated with 6 M guanidine-HCl, pH 5 (25, 29). All iodine-labeled markers used for the calibration of the column were similarly treated and analyzed by multiple passages through the same column. Fig. 5A reveals that the elution volume of the peptide is significantly smaller than that of ^{131}I -labeled myosin applied simultaneously to the column. Estimation of the molecular weight of the peptide ranges from 270,000 to 340,000 (Fig. 5B).

DISCUSSION

The application of conventional analytical methods in the study of thyroglobulin structure has been frustrating because of disagreements between results obtained by different laboratories (1, 5–9). As an alternative approach, efforts were devoted to the isolation of thyroglobulin mRNA(s) and to the characterization of its (their) translation product(s). However, in the absence of reliable information on the molecular weight of thyroglobulin protomers, the identification of the translation product(s) was uncertain and rested mainly upon immunological techniques.

Earlier work from this laboratory suggested the presence of two RNA fractions with thyroglobulin messenger activity. A 16S fraction and a well-defined 33S poly(A)-rich RNA

FIG. 5. Gel filtration of the reduced and alkylated peptide encoded by the 33S mRNA in 6 M guanidine-HCl. (A) Specific immunoprecipitates prepared from 33S-mRNA-injected oocyte extracts were dissociated, reduced, and alkylated. The samples were then mixed with radioiodinated ^{131}I myosin and ^{125}I albumin which had been similarly treated. Before layering on the column, sucrose (100 mg/ml) and Blue Dextran (6%) were added. The 1 ml fractions collected from the column were first counted for ^{131}I and ^{125}I radioactivity in a double channel γ -spectrometer. They were then mixed with a liquid scintillation cocktail (Omnifluor, 4 g; Triton X-100, 30%; toluene 70%) and ^3H radioactivity was measured. Corrections were made for the contribution of ^{131}I activity in the ^{125}I channel during the γ -scintillation counting, and for the contribution and decay of ^{131}I and ^{125}I during the liquid scintillation counting. The peak at fraction 52 represents free iodine resulting from partial deiodination of the markers. The second small peak of ^3H radioactivity of molecular weight slightly smaller than albumin eluted on some runs as a trail and most likely represents degradation products of the peptide. Alternative explanations for its identity are given in the text. (B) Estimation of the molecular weight of the peptide. V_e = elution volume of the proteins; V_0 = elution volume of the Blue Dextran. The flow of the column remained constant at 1.5 ml/hr for 40 days. The same column was used for eight separate analyses. The thyroglobulin peptide was passed three times through the column (broken lines), along with various iodine labeled markers. BSA, bovine serum albumin; TBG, thyroxine-binding globulin; Tg, thyroglobulin.

peak (12). While we were able to demonstrate that, when injected into *Xenopus* oocytes, the 33S mRNA coded for a heavy peptide immunologically related to thyroglobulin (11, 12), De Nayer *et al.* (30) obtained the synthesis of an 87,000 dalton peptide in *Xenopus* oocytes injected with total thyroid polysomal RNA. Di Lauro *et al.* (14), using an acellular protein synthesizing system, found only the synthesis of 15,000 dalton immunoreactive peptides. The mRNA preparations in these last studies were, however, poorly characterized, so that translation of fragmented mRNAs could well account for the results. The existence of very large thyroid polysomes (31, 32) synthesizing preferentially thyroglobulin (33, 34) supported the existence of heavy thyroglobulin subunits.

The first conclusion of the present work is that all information required for the synthesis of a complete 19S thyroglobulin molecule is present in the 33S mRNA. Indeed, a small but reproducible amount of 19S radioactive material is

demonstrated in extracts from oocytes injected with 33S mRNA (Fig. 3A). That this material is related immunologically to thyroglobulin is evidenced by the results reported in Fig. 3B. Our inability to obtain 19S material in previous studies was due to the use of Triton X-100 in the homogenization medium of the oocytes (12).

Results from the tryptic peptide analysis (Fig. 4) allow the chemical identification of the peptide encoded by the 33S mRNA. It is evident that each of the 23 peaks separated chromatographically must contain many different tryptic peptides. Indeed, an estimate would indicate a maximum of 300 tryptic peptides containing leucine in a 300,000 dalton polypeptide with the composition of thyroglobulin. Still, the close qualitative and quantitative agreement between the patterns obtained from the beef thyroglobulin and the peptide synthesized by the 33S-mRNA-injected oocytes strongly supports chemical identity. The slight quantitative discrepancies could be explained by differences in glycosylation of the proteins, since it is not known if oocytes do perform that particular posttranslational modification. Other possible explanations are: (1) contaminants in the immunoprecipitate; (2) synthesis by the oocyte of a thyroglobulin precursor with additional amino acids at the N-terminus; and (3) nonuniformly labeled thyroglobulin fragments in the bovine thyroid gland slices.

The following observations indicate that the 19S thyroglobulin synthesized in the oocyte must be a polymer composed of $2n$ equal sized subunits: (1) 19S thyroglobulin can be dissociated into two half-sized 12S subunits (1); (2) after reduction and alkylation of the peptide a single peak is observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2); and (3) when centrifuged under non-denaturing conditions, the oocyte-translated product presents both 12S and 19S thyroglobulin species (Fig. 3). These results are at variance with those we have previously reported (10) as well as with recent results from De Nayer *et al.* (13). We would suggest that translation of fragments of 33S mRNA could be responsible for this discrepancy in the case of De Nayer's experiments (13), while, in our case (10), we would incriminate the specificity of the antithyroglobulin serum previously used. Indeed, with antisera against ultracentrifugally pure thyroglobulin, only 33S mRNA is found to promote the synthesis of immunoreactive thyroglobulin.

The majority of the published models for thyroglobulin structure agree with the $2n$ equal-sized subunits model. Estimated values for n have ranged from 2 (1) to 20 (14). Due to its abnormal behavior during sodium dodecyl sulfate polyacrylamide gel electrophoresis, the molecular weight of the peptide encoded by the 33S mRNA is difficult to estimate. The observation that it migrates more slowly than 12S (330,000 daltons) thyroglobulin (Fig. 2) suggests that our previous estimation of 185,000 daltons (11) is probably far too small. Indeed, the results of gel filtration in 6 M guanidine-HCl after full reduction and alkylation of the peptide indicates that it is larger than the 210,000 dalton heavy chain of myosin. The only model of thyroglobulin quaternary structure which would be compatible with our results is thus the simplest one, where n equals 1. Interestingly, a peptide of about 300,000 daltons has recently been characterized in thyroglobulin from normal guinea pigs as well as from guinea pigs treated with antithyroid drugs (36). Thus, contrary to all previous suggestions, it appears that 19S thyroglobulin is a dimer composed of equal-sized 300,000 dalton polypeptides. Further studies will show if the two protomers have or have not the same primary structures.

We would like to thank Drs. Haeberli, Edelhoch, and Salvatore for having communicated their results prior to publication; Dr. Tel-

lerman for gift of myosin; Dr. Van de Werve for a gift of phosphorylase B; and Mrs. Ch. Borrey and Mrs. D. Legrand for preparing the manuscript. This work was realized under Contract of the Ministère de la Politique Scientifique within the framework of the Association Euratom-University of Brussels-University of Pisa. S.R. was supported in part by U.S. Public Health Service Grant AM-15,070.

1. Edelhoch, H. (1965) *Rec. Prog. Horm. Res.* **21**, 1-31.
2. Seed, R. W. & Goldberg, I. H. (1963) *Proc. Nat. Acad. Sci. USA* **50**, 275-281.
3. Taurig, A. (1970) *Rec. Prog. Horm. Res.* **26**, 189-247.
4. Rolland, M., Montfort, M. F. & Lissitzky, S. (1973) *Biochim. Biophys. Acta* **303**, 338-347.
5. Charlwood, P. A., Pitt-Rivers, R. & Schwarz, H. L. (1970) *Biochem. J.* **116**, 769-772.
6. Pierce, J. G., Rawitch, A. R., Brown, D. M. & Stanley, P. G. (1965) *Biochim. Biophys. Acta* **111**, 247-256.
7. Vecchio, G., Carlomagno, M. S. & Consiglio, E. (1971) *J. Biol. Chem.* **246**, 6676-6682.
8. Lissitzky, S., Rolland, M., Reynaud, J., Savary, J. & Lasry, S. (1968) *Eur. J. Biochem.* **4**, 464-471.
9. Spiro, M. J. (1973) *J. Biol. Chem.* **248**, 4446-4460.
10. Vassart, G., Brocas, H., Nokin, P. & Dumont, J. E. (1973) *Biochim. Biophys. Acta* **324**, 575-580.
11. Vassart, G., Lecocq, R. E., Brocas, H. & Dumont, J. E. (1974) *Biochim. Biophys. Acta* **353**, 261-265.
12. Vassart, G., Brocas, H., Lecocq, R. E. & Dumont, J. E. (1975) *Eur. J. Biochem.* **55**, 15-22.
13. De Nayer, Ph. (Sept. 1974) *Proc. Americ. Thyroid Assoc.*, p. T4.
14. Di Lauro, R., Metafora, S., Consiglio, E., Gentile, G. & Varone, S. (1975) *J. Biol. Chem.* **250**, 3267-3272.
15. Perry, R. P., La Torre, J. & Greenberg, J. R. (1972) *Biochim. Biophys. Acta* **262**, 220-226.
16. Gurdon, J. B., Lane, C. D., Woodland, H. R. & Marbaix, G. (1971) *Nature* **233**, 177-182.
17. Barth, L. G. & Barth, L. J. (1959) *J. Embryol. Exp. Morphol.* **7**, 210-222.
18. Rhoads, R. E., McKnight, G. S. & Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 2031-2039.
19. Hayashi, T., Iwai, K. & Ui, N. (1971) *Biochim. Biophys. Acta* **251**, 208-216.
20. Robbins, J. & Rall, J. E. (1960) *Physiol. Rev.* **40**, 415-429.
21. Ricard, B. & Salser, W. (1974) *Nature* **248**, 314-317.
22. Haines, M. E., Carey, N. H. & Palmiter, R. D. (1974) *Eur. J. Biochem.* **43**, 549-560.
23. Rolland, M. & Lissitzky, S. (1972) *Biochim. Biophys. Acta* **278**, 316-336.
24. Bilstad, J. M., Edelhoch, H., Lippoldt, R., Rall, J. E. & Salvatore, G. (1972) *Arch. Biochem. Biophys.* **151**, 341-350.
25. Fish, W. W., Mann, K. G. & Tanford, C. (1969) *J. Biol. Chem.* **244**, 4989-4994.
26. Stevens, R. H. & Williamson, A. R. (1972) *Nature* **239**, 143-146.
27. Schneider, A. B. & Edelhoch, H. (1970) *J. Biol. Chem.* **245**, 885-893.
28. Banker, G. A. & Cotman, C. N. (1972) *J. Biol. Chem.* **247**, 5856-5861.
29. Tanford, C. (1968) *Adv. Protein Chem.* **23**, 121.
30. De Nayer, Ph., Durieux, B., Nyssen-Gautier, F. & de Visscher, M. (1974) *Life Sci.* **14**, 625-630.
31. Keyhani, F., Claude, A., Lecocq, R. E. & Dumont, J. E. (1971) *J. Microsc. (Paris)* **10**, 269-282.
32. De Nayer, Ph. & Labaw, L. W. (1971) *Endocrinology* **88**, 783-786.
33. Vassart, G. & Dumont, J. E. (1973) *Eur. J. Biochem.* **32**, 322-330.
34. De Nayer, Ph., Labrique, M. & de Visscher, M. (1973) *Biochim. Biophys. Acta* **294**, 309-321.
35. Refetoff, S., Faug, V. S. & Marshall, J. S. (1975) *J. Clin. Invest.* **56**, 177-187.
36. Haeberli, A., Salvatore, G., Edelhoch, H. & Rall, J. E. *J. Biol. Chem.*, in press.