ences in oxidation–reduction potentials or steric restrictions.

References

Averill, B. A., Herskovitz, T., Holm, R. H., and Ibers, J. A. (1973), *J. Am. Chem. Soc. 95,* 3523.

Bartsch, R. G. (1963), in Bacterial Photosynthesis, San Pietro, A., Gest, H., and Vernon, L. P., Ed., Yellow Springs, Ohio, Antioch Press, p 315.

Bartsch, R. G., Kakuno, T., Horio, T., and Kamen, M. D. (1971), *J. Biol. Chem. 246,* 4489.

Carter, C. W., Jr., Freer, S. T., Yuong, Ng. H., Alden, R. A., and Kraut, J. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 381.

Carter, C. W., Jr., Kraut, J., Freer, S. T., and Alden, R. A. (1974b), *J. Biol. Chem. 249,* 6339.

Carter, C. W., Jr., Kraut, J., Freer, S. T., Alden, R. A., Sieker, L. C., Adman, E., and Jensen, L. H. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3526.

Carter, C. W., Jr., Kraut, J., Freer, S. T., Yuong, Ng. H., Alden, R. A., and Bartsch, R. G. (1974a), *J. Biol. Chem. 249,* 4212.

Castro, C. E. (1975), in The Porphyrins, Dolphin, D., Ed. (in press).

DeKlerk, H., and Kamen, M. D. (1966), *Biochim. Biophys. Acta 112,* 175.

Dus, K., Tedro, S., and Bartsch, R. G. (1973), *J. Biol. Chem. 248,* 7318.

Dutton, D. L., and Leigh, J. S. (1973), *Biochim. Biophys. Acta 314,* 178.

Evans, M. C. W., Lord, A. V., and Reeves, S. G. (1974), *Biochem. J. 138,* 177.

Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, New York, N.Y., Wiley.

Herskovitz, T., Averill, B. A., Holm, R. H., Ibers, J. A., Phillips, W. D., and Weiher, J. F. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2437.

Hori, K. (1961), *J. Biochem. 50,* 481.

Jacks, C. A., Bennett, L. E., Raymond, W. N., and Lovenberg, W. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1118.

Kennel, S. J., Bartsch, R. G., and Kamen, M. D. (1972), *Biophys. J.* 12, 882.

Lambeth, D. O., and Palmer, G. (1973), *J. Biol. Chem. 248,* 6095.

Meyer, T. E. (1970), Ph.D. Thesis, University of California at San Diego.

Miller, W. G., and Cusanovich, M. A. (1975), *Biophys. Struct. Mech. 1,* 97.

O'Reilly, J. E. (1973), *Biochim. Biophys. Acta 292,* 509.

Velick, S. F., and Strittmatter, G. (1956), *J. Biol. Chem. 221,* 265.

Wood, F. E., and Cusanovich, M. A. (1975), *Bioinorg. Chem. 4,* 337.

Phenylalanyl-tRNA Synthetases of Rat Liver: Differential Effects of Thyroid Hormone†

Jennifer B. Nielsen and Audrey E. V. Haschemeyer*

ABSTRACT: Thyroxine and analogues inhibit rat liver aminoacyl-tRNA synthetase activity for phenylalanine and tyrosine. A high yield purification of the major cytoplasmic form of phenylalanyl-tRNA synthetase (C_1) and its characterization is reported. Polyribosome-bound and other sedimentable forms are found to be indistinguishable from soluble enzyme by immunoprecipitation. Mitochondrial phenylalanyl-tRNA synthetase (M) and cytoplasmic activity (C_2) resistant to anti-C_1 antibody have been partially purified and characterized. Tissue levels of the three forms are estimated at 22, 1.8, and 4.1 units/g of liver for C_1, C_2, and M, respectively [1 unit = 1 nmol of Phe-tRNA/min, 30°C]. Charging capability toward rat liver and yeast tRNA, kinetic parameters, and physical properties are compared. Only enzyme C_1 is hormone inhibited [$K_I = 4 \times 10^{-6}$ M for triiodothyronine]. The data indicate that C_2 and M are not structurally related to C_1; C_2 may represent an independent cytoplasmic pool of M. Implications of C_1 inhibition in relation to effects on liver protein synthesis are discussed.

The thyroid hormones thyroxine and 3,3′,5-triiodothyronine are recognized to play a significant role in protein metabolism, although the tissue-specific and dose-dependent interplay of anabolic and catabolic effects have obscured the primary mechanism of action of the hormone. Recently, by use of rapid kinetic methods, it has been possible to show a correlation between thyroid hormone level and the rate of polypeptide chain assembly and release during protein synthesis in rat liver in vivo (Mathews et al., 1973). A 40% depression in elongation rate was found in surgically thyroidectomized animals compared to euthyroid controls; rates were restored to normal by triiodothyronine injections. A small elevation in synthetic rate occurred in normal animals given similar hormone injections. Data for other systems, however, indicate opposite effects in more acute hyperthyroidism (Kivirikko et al., 1967; Nadkarni and Samuel, 1973; Griffin and Miller, 1973), suggesting the possibility of multiple effects on the protein synthetic pathway particu-

† From the Department of Biological Sciences, Hunter College of the City University of New York, New York, New York 10021. *Received August 7, 1975.* This research has been supported by Grant HD 04670 from the U.S. Public Health Service. A fellowship from the Helen Hay Whitney Foundation (to A.E.V.H.) in the early stages of this work is gratefully acknowledged.

larly at pharmacologic doses of the hormone. In conjunction with the in vivo studies cited above, we have undertaken examination of the direct effects of thyroxine and its analogues on a number of protein synthetic enzymes in vitro.

The aminoacyl-tRNA synthetases are of particular interest because of their central role in the translation of genetic information from nucleotide sequences to amino acid residues and the fact that thyroxine itself is an amino acid. Considerable effort has been made to identify regulatory roles for these enzymes or their cognate tRNAs (Neidhardt, 1966; Peterson, 1967), and many reports of possible multiple enzymes for activation of a particular amino acid have appeared. These studies, however, have been limited to systems at low levels of purification. More recent reports indicate that the production of chromatographically distinct forms of these enzymes may result from proteolytic activity or other preparation artifacts (Rouget and Chapeville, 1971; Rymo et al., 1972). Thus, with the exception of the distinct category of mitochondrial aminoacyl-tRNA synthetases (Barnett et al., 1967; Buck and Nass, 1968), the presence of multiple forms of these enzymes capable of regulatory responses to hormones or other factors has not been convincingly demonstrated. More recently, the possibility has been raised that sedimentable complexes of aminoacyl-tRNA synthetases (Bandyopadhyay and Deutscher, 1971; Roberts and Coleman, 1972; Irvin and Hardesty, 1972; Vennegoor and Bloemendal, 1972) may reveal enzyme species different from soluble forms.

To examine the question of possible regulatory mechanisms for protein synthesis controlled by the aminoacyl-tRNA synthetases in relation to thyroxine, we first surveyed the effects of low levels of the hormone in vitro on tRNA charging activity of partially purified enzyme preparations from liver. Preliminary results suggested the existence of multiple forms of phenylalanyl-tRNA synthetase (Haschemeyer, 1968), the enzyme found to be most susceptible to inhibition by low levels of triiodothyronine or thyroxine. Subsequent work has been focussed on high-yield purification and characterization of these enzymes (Nielsen and Haschemeyer, 1973) and examination of the differential action of thyroid hormone in vitro.

Materials and Methods

Animals. Male Sprague-Dawley rats (Charles River Laboratories), 150–200 g, were used for preparation of liver aminoacyl-tRNA synthetases as pH 5 enzyme; male Long-Evans rats, 200–250 g, bred from stock at Hunter College, were used for isolation and characterization of purified phenylalanyl-tRNA synthetases.

Materials. L-[G-^{3}H]Phenylalanine, L-[3,5-^{3}H]tyrosine, L-[4,5-^{3}H(N)]leucine, L-[^{14}C(U)]arginine, L-[^{14}C(U)]serine, L-[^{14}C(U)]proline, and L-[^{14}C(U)]amino acid mixture were obtained from New England Nuclear Corp. The tritiated amino acids were adjusted to specific activity 1.00 Ci/mmol with unlabeled L-amino acids (Calbiochem). L-Thyroxine (3,3′,5,5′-tetraiodo-L-thyronine), 3,3′,5-triiodo-L-thyronine, 3,5-diiodo-DL-thyronine, DL-thyronine, and 3,5-diiodo-L-tyrosine were obtained from Sigma and Nutritional Biochemicals. No differences among the various preparations used were noted. Stock solutions of these compounds were prepared in 1 m*M* KOH and stored at 4°C for not more than 2 weeks.

Total rat liver tRNA was prepared according to Petrissant et al. (1971). After deacylation in 1.8 *M* Tris (pH 8.0) at 37°C for 1 hr, the preparations were dialyzed exhaustively against distilled water and lyophilized. Phenylalanine acceptance was about 60 pmol/A_{260} unit (absorbance measured in 1 m*M* $MgCl_2$), or about 3% of total tRNA on a molar basis. Similar acceptance levels were found for tRNA obtained from pH 5 enzyme preparations (Holley et al., 1961) fractionated on DEAE-cellulose (Barnett, 1965) and eluted with 0.6 *M* NaCl. Mitochondrial tRNA was prepared as indicated for total tRNA, starting with purified mitochondria (O'Brien and Kalf, 1967). Yield was about 1 mg/100 g of liver, and phenylalanine acceptance was about 12 pmol/A_{260}. Yeast tRNA (General Biochemicals) with phenylalanine acceptance of 4 pmol/A_{260} was treated with partially purified liver tRNA nucleotidyl transferase (CCA pyrophosphorylase) according to Deutscher (1970) to obtain a product with acceptance activity of 40 pmol/A_{260}. Purified yeast tRNA^{Phe} (Mannheim-Boehringer) has an acceptance level of 1300 pmol/A_{260}.

Assay of Phe-tRNA Synthetase Activity. For enzyme purification and in all studies of purified fractions, enzyme activity was assayed by the formation of [^{3}H]Phe-tRNA in a reaction mixture containing 100 m*M* Tris (pH 8.0), 10–40 m*M* $MgCl_2$, 0.1 m*M* ethylenediaminetetraacetate (EDTA), 8 m*M* adenosine 5′-triphosphate, 1 m*M* 2-mercaptoethanol, 10 m*M* KCl, 10 μM [^{3}H]phenylalanine (1 Ci/mmol), 2 mg/ml of total tRNA (or 0.05 mg/ml of tRNA^{Phe}), and 0.002–0.015 enzyme unit (1 unit = 1 nmol of Phe-tRNA/min) in a final volume of 120 μl. Incubation was carried out at 30°C for 2 min. All enzyme preparations showed linear kinetics for at least 5 min. Duplicate aliquots of 50 μl were analyzed for [^{3}H]Phe-tRNA on Whatman 3MM filter paper discs (Bollum, 1959; Barnett and Jacobson, 1964) in a Packard scintillation spectrophotometer in Omnifluor (New England Nuclear) toluene solution (efficiency of ^{3}H on discs = 22%). Apparent Michaelis constants for phenylalanine, ATP, and tRNA were determined at 10 m*M* Mg^{2+}. K_M's for rat tRNA^{Phe} (total and mitochondrial) and yeast tRNA^{Phe} were obtained with unfractionated tRNA; tRNA^{Phe} concentration was determined from maximal charging levels. K_M for yeast tRNA^{Phe} was obtained with the Mannheim-Boehringer product; in these experiments filters were pretreated with 0.2 mg/ml of uncharged tRNA to provide carrier for quantitative precipitation of low levels of charged tRNA^{Phe}. K_M values were obtained from Lineweaver-Burk plots using at least five different substrate concentrations. Inhibitor when present was added to the assay tube prior to enzyme addition at zero time.

Assays for the degradation of aminoacyl-tRNA were carried out in 100 m*M* Tris (pH 8.0), 0.1 m*M* EDTA, 1 m*M* 2-mercaptoethanol, 0.2–40 m*M* $MgCl_2$, 0.02–0.15 enzyme unit/ml, and 250 pmol/ml (400 cpm/pmol) of [^{3}H]Phe-tRNA (rat liver total tRNA or mitochondrial tRNA). The fully charged tRNA was prepared with excess pH 5 enzyme and isolated by phenol extraction, alcohol precipitation, and Sephadex G-25 filtration in 0.01 *M* potassium acetate buffer (pH 5.7). Ribonuclease was assayed according to Gagnon and de Lamirande (1973); addition of liver ribonuclease inhibitor (Lalonde and de Lamirande, 1974) inhibited mitochondrial ribonuclease and [^{3}H]Phe-tRNA degradative activity by 70%.

pH 5 Enzyme. Liver aminoacyl-tRNA synthetases precipitated at pH 4.9 according to Holley et al. (1961) were dissolved in 0.01 *M* Tris (pH 7.8) containing 1 m*M* $MgCl_2$ and 3.5 m*M* 2-mercaptoethanol, dialyzed for 2 hr against the same buffer, and frozen in small aliquots in liquid nitro-

gen. Activity for the formation of aminoacyl-tRNA was assayed in a reaction mixture containing 0.03 mg/ml of enzyme protein, 1–10 A_{260} units/ml of rat liver tRNA, 5×10^{-5} *M* of each amino acid at 10 mCi/mmol, and other ingredients of the standard assay system above. Reaction volume was 0.50 ml and 100-μl aliquots were taken at 5-min intervals during incubation at 25° and analyzed on filter paper discs as above. Pyrophosphate exchange was assayed at the same enzyme level using the system described by Calendar and Berg (1966).

Purification of Liver Cytoplasmic Phe-tRNA Synthetase C_1. Overnight-starved rats were stunned and decapitated; the livers were rapidly removed and homogenized in cold medium containing 0.25 *M* sucrose, 10 m*M* Tris (pH 7.4), 10 m*M* $MgCl_2$, 25 m*M* KCl, and 2 m*M* 2-mercaptoethanol, using a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 15000*g* for 10 min to sediment nuclei, debris, and mitochondria, the pellets were washed once with $\frac{1}{5}$ the original volume of medium and recentrifuged, and the two supernatants were combined to constitute the post-mitochondrial supernatant (PMS).[1] Assays of cytochrome oxidase (Margoliash and Walasek, 1967), glutamic dehydrogenase (Beaufay et al., 1959), and mitochondrial ribonuclease (Gagnon and de Lamirande, 1973) in the PMS and crude homogenate indicated that contamination of the PMS by mitochondrial enzymes did not exceed 8% of total mitochondrial activity.

Post-mitochondrial supernatant preparations were treated with 0.5% Triton X-100 (Sigma) to free membrane-bound enzyme and 2 mg/ml of protamine sulfate (Schwarz/Mann) to remove tRNA and ribosomes. After the mixture was stirred 10 min at 0°C, the precipitate was removed by centrifugation and the supernatant was applied to a column of Sephadex G-25 (Pharmacia) equilibrated with 0.01 *M* potassium phosphate (pH 7.5), 2 m*M* 2-mercaptoethanol, and 20% glycerol. The material eluting in the void volume was loaded on phosphocellulose P11 (Whatman) at a level of 70 mg of protein/ml settled bed in the same medium. After thorough washing of the column with the starting buffer, elution was carried out with a linear gradient of 0–0.5 *M* KCl in the same buffer. Active fractions at about 0.35 *M* KCl were pooled and loaded directly (final concentration 0.4 mg of protein/ml) onto a column of hydroxylapatite prepared according to Miyazawa and Thomas (1965) and equilibrated with 0.01 *M* potassium phosphate (pH 6.8) containing 0.001 *M* 2-mercaptoethanol and 20% glycerol. The final load was 5 mg of protein/ml settled bed. Elution was carried out with a linear gradient of 0.01–0.5 *M* potassium phosphate (pH 6.8). The major peak of activity subsequently labeled C_1 was recovered at about 0.4 *M* potassium phosphate. Active fractions of this peak were pooled, concentrated to about 2 mg/ml using an Amicon ultrafiltration cell with a PM30 membrane, and stored in small aliquots in liquid nitrogen in 0.01 *M* potassium phosphate (pH 6.8), 20% glycerol, and 0.001 *M* 2-mercaptoethanol. Activity was stable for up to 6 months. The concentrated enzyme was finally chromatographed on a column (1.8 × 50 cm) of Sepharose 4B (Pharmacia) equilibrated with 0.25 *M* KCl, 0.05 *M* Tris (pH 7.5), 20% glycerol, and 0.001 *M* 2-mercaptoethanol.

A similar purification procedure, omitting the Triton X-100 step, was used with liver post-ribosomal supernatant, prepared by centrifugation of the PMS at 100 000*g* for 90 min. All steps in the enzyme purification were carried out at 4°C except where otherwise indicated. Protein assays were made by the biuret or Lowry methods and by absorbance measurements. Interference in the Lowry technique by mercaptoethanol was eliminated by preincubation of the protein solution with H_2O_2, as described by Geiger and Bessman (1972).

Antibody Preparation. Antibody to the purified enzyme C_1 was prepared after removal of salts, glycerol, and mercaptoethanol from the enzyme by passage through Sephadex G-25 equilibrated with 0.05 *M* potassium phosphate (pH 7.5). 1.0 ml of enzyme solution at 0.5 mg/ml was mixed with an equal volume of methylated serum albumin (Mandell and Hershey, 1960) at 0.5 mg/ml, homogenized with 1 ml of Freund's Complete Adjuvant (Calbiochem), and injected subcutaneously, intramuscularly, and into the footpads of a young white female New Zealand rabbit. After weekly injections for 4 weeks, anti-C_1 activity was found in serum collected from marginal ear veins. The titer was maintained by fortnightly injections; blood was collected 10 days after each injection. The serum was treated with an equal volume of 1% glycerol, 0.15 *M* NaCl, and then with saturated ammonium sulfate solution to 33% saturation. After 10 min at 0°C precipitated γ-globulins were washed with 50% saturated ammonium sulfate, dissolved in 1% glycerol–0.15 *M* NaCl, dialyzed overnight at 4° against the same medium, and stored in small aliquots at −70°.

Total inhibition of enzyme C_1 activity by anti-C_1 γ-globulin was achieved by incubation for 20 min at 0°C at a concentration ratio of 50 μg of immunoglobulin protein/μg of enzyme protein, prior to carrying out the standard assay for Phe-tRNA formation. Preparations of γ-globulin from control rabbits (untreated with enzyme C_1) had no effect on the assay system. Attempts to prepare antibody to the mitochondrial enzyme M were unsuccessful.

Cytoplasmic Phenylalanyl-tRNA Synthetase C_2. Post-mitochondrial supernatant containing 0.5% Triton X-100, prepared as described above, was incubated for 20 min at 0°C with anti-C_1 γ-globulin at a concentration ratio of 20 mg of supernatant protein/mg of γ-globulin protein. After centrifugation at 15 000*g* for 10 min to remove the immunoprecipitate, the resultant supernatant was treated with protamine sulfate and chromatographed on Sephadex G-25, phosphocellulose, and hydroxylapatite, as described above for enzyme C_1. Preparations of C_2 were also obtained without prior immunoabsorption of C_1 by isolation of the small but distinct peak of activity eluting at about 0.2 *M* KCl from phosphocellulose P11 (Figure 1). Subsequent chromatography of this fraction on hydroxylapatite (Figure 2) showed little C_1 contamination. Enzyme C_2 was highly stable during storage in liquid nitrogen in the final buffer containing 0.2 *M* potassium phosphate (pH 6.8), 20% glycerol, and 1 m*M* 2-mercaptoethanol.

Mitochondrial Phenylalanyl-tRNA Synthetase M. Mitochondria were purified from the 15 000*g* pellet of the liver homogenate according to the method of O'Brien and Kalf (1967). Resuspension and centrifugation were repeated five times to eliminate supernatant enzymes and microsomal contamination. Final yield was 3 mg of mitochondrial protein/g of liver (wet weight), representing about 7% of total liver mitochondrial protein (Dinziani and Viti, 1955). Bacterial contamination, as tested on nutrient plates, was less than 100 viable cells per mg of protein.

The purified mitochondrial pellet was suspended in 10

[1] Abbreviations used are: PMS, post-mitochondrial supernatant; PRS, post-ribosomal supernatant.

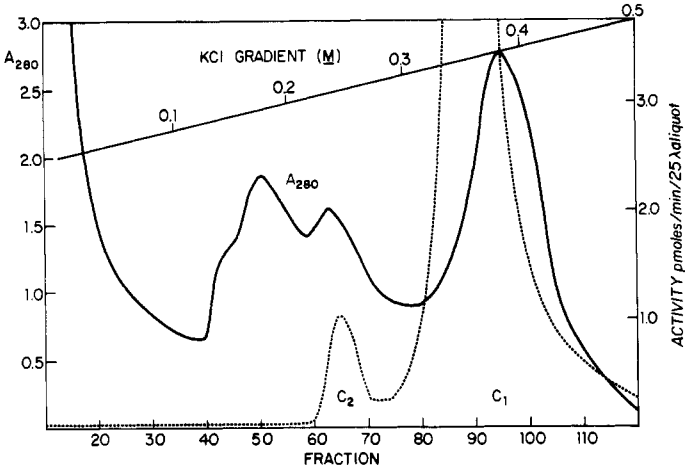

FIGURE 1: Phosphocellulose chromatography of post-mitochondrial supernatant treated with Triton X-100 and protamine sulfate. Activity (- - -) of the two components designated C_1 and C_2; A_{280} (—). 17.5 g of protein was applied to a 250-ml bed volume column (3 × 40 cm) of Whatman P-11.

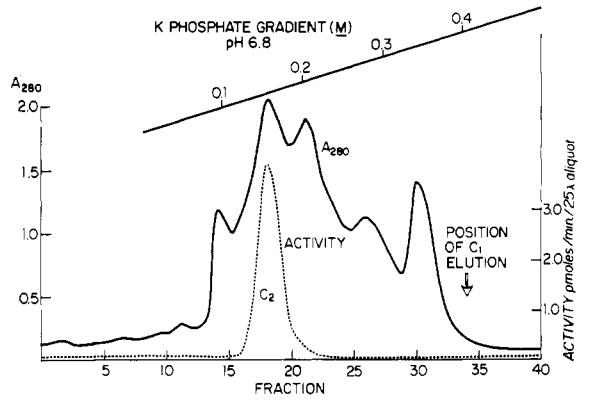

FIGURE 2: Hydroxylapatite chromatography of enzyme C_2 (fractions 61–70 of Figure 1) on a 1 × 13 cm column (10-ml settled bed) prewashed with 0.01 *M* potassium phosphate (pH 6.8) containing 20% glycerol and 1 m*M* 2-mercaptoethanol, and developed with a potassium phosphate gradient with the same additions.

volumes of 0.01 *M* potassium phosphate (pH 6.8) containing 20% glycerol, 1 m*M* 2-mercaptoethanol, and 0.5% Triton X-100, and homogenized with five strokes in a tight-fitting glass Tenbroeck homogenizer. The combined supernatants obtained after centrifugation at 15 000*g* for 10 min and reextraction of the pellet with 5 volumes of the same buffer were chromatographed on hydroxylapatite as described for enzyme C_1, and assayed for Phe-tRNA formation and ribonuclease. The synthetase activity eluted at about 0.2 *M* potassium phosphate whereas ribonuclease eluted at 0.3 *M*. Fractions free of nuclease activity were pooled and stored in liquid nitrogen. About 30% activity loss was associated with freezing and thawing, but no further loss occurred with storage.

Liver Polyribosomes. Liver homogenates were prepared as described for enzyme purification except that the freshly prepared medium contained 2 mg/ml of total yeast RNA (PL Biochemicals) as excess substrate for endogenous ribonuclease. Purified polyribosomes were prepared from 0.5% deoxycholate treated post-nuclear supernatant (Haschemeyer and Gross, 1967) by centrifugation through 2 *M* sucrose at 340 000*g* for 4 hr. Recovery of liver RNA in the polyribosome pellet was 40%.

Results

Effect of Thyroxine on Aminoacylation of tRNA with pH 5 Enzyme. As shown in Table I, thyroxine and other iodothyronines have their greatest effect on the activating reactions of the amino acids most closely related to them structurally. The largest inhibition was that of triiodothyronine on the charging of phenylalanine; thyroxine and diiodothyronine were almost as effective while thyronine, diiodotyrosine, and tyrosine were without effect. The tyrosine activation reaction was also strongly inhibited, 40 and 90% at triiodothyronine concentrations of 1×10^{-5} and 2×10^{-4} *M*, respectively.

In the standard assay for Phe-tRNA formation the inhibition by iodinated thyronines was independent of tRNA concentration over a tenfold range. Comparable levels of inhibition were observed in the assay for Phe-AMP-enzyme complex formation by pyrophosphate exchange. Thus, inhibition occurs at the first step of the activation and transfer sequence; assay at the stage of Phe-tRNA formation, however, was more convenient and reproducible. Further study required isolation of the affected enzyme at a much higher level of purification.

Table I: Effect of Thyroxine and Related Compounds at 5×10^{-5} *M* on the Kinetics of Formation of Various Aminoacyl-tRNAs by Rat Liver pH 5 Enzyme.[a]

Amino Acid	Compound Tested	% Inhibition
Phenylalanine	L-Thyroxine	50 ± 10
	L-Triiodothyronine	70 ± 10
	DL-Diiodothyronine	55 ± 10
	DL-Thyronine	0
	L-Diiodotyrosine	0
	L-Tyrosine	0
Tyrosine	L-Triiodothyronine	40 ± 5
Leucine	L-Triiodothyronine	15 ± 5
Arginine, proline, serine	L-Triiodothyronine	5
15 amino acid mixture (Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, Val)	L-Triiodothyronine	15 ± 5

[a] Assay conditions for pH 5 enzyme are given in Materials and Methods.

Major Cytoplasmic Enzyme (C_1). Initial activity of the major phenylalanyl-tRNA synthetase activity in rat liver post-mitochondrial supernatant (PMS) averaged 22 units/g of liver; yield at 800-fold purification was 36% (Table II). Purification from the post-ribosomal supernatant (PRS) gave a similar result although final yield was reduced to 22% due to loss in the 100 000*g* centrifugation. The enzyme showed comparable charging activity toward unfractionated rat liver or yeast tRNA, provided the latter had been pretreated with tRNA nucleotidyl transferase for restoration of terminal nucleotides. When the nucleotidyl transferase step was omitted, yeast tRNA acceptor activity disappeared as the enzyme was purified. The final product was unstable to assay at temperatures greater than 30°C or times beyond 5 min. Increasing Mg^{2+} concentration above that of the standard assay system (10 m*M*) also caused a decline in activity.

C_1 shows chromatographic properties similar to those of an enzyme isolated from liver PRS by another group (Lanks et al., 1971; Tscherne et al., 1973a,b); however, activity and recovery are about 4000 times greater. Sedimentation coefficients estimated from sucrose density gradients

Table II: Purification of Major Cytoplasmic Rat Liver Phenylalanyl-tRNA Synthetase (C_1) from Post-Mitochondrial Supernatant.

Fraction	Protein Recovery (mg/g of liver)	Specific Activity vs. yeast tRNA (units[b]/mg)	Specific Activity vs. Rat tRNA (units[b]/mg)	Yield[a] (%)	-Fold Purification
Supernatant	102	0.25	0.21	100	1
After Sephadex G-25	55	0.37	0.38	98	1.8
Phosphocellulose P11	3.8	3.1	3.4	61	16
Hydroxylapatite	0.11	65	97	51	450
Sepharose 4B	0.05	109	164	36	790

[a] Set at 100% for post-mitochondrial supernatant vs. rat tRNA. [b] One unit of synthetase activity is that amount which produces 1 nmol of Phe-tRNA per min in the standard assay system, 10 m*M* Mg^{2+}, 30°C.

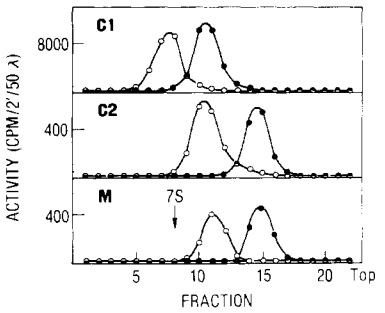

FIGURE 3: Sucrose density gradient sedimentation of cytoplasmic and mitochondrial rat liver phenylalanyl-tRNA synthetases in the absence (●) or presence (○) of added rat liver tRNA (1 A_{260} unit/sample). 0.1 ml of enzyme solution freed of glycerol by Sephadex G-25 chromatography was layered on a 5.0-ml 5–20% sucrose gradient containing 0.01 *M* potassium phosphate buffer (pH 6.8) and 2 m*M* 2-mercaptoethanol. Sedimentation was carried out in a Spinco SW 50.1 rotor at 48000 rpm for 13 hr. Human γ-globulin was run as a marker; calculations of $s_{20,w}$ relative to a marker (7.0) were made according to McEwen (1967).

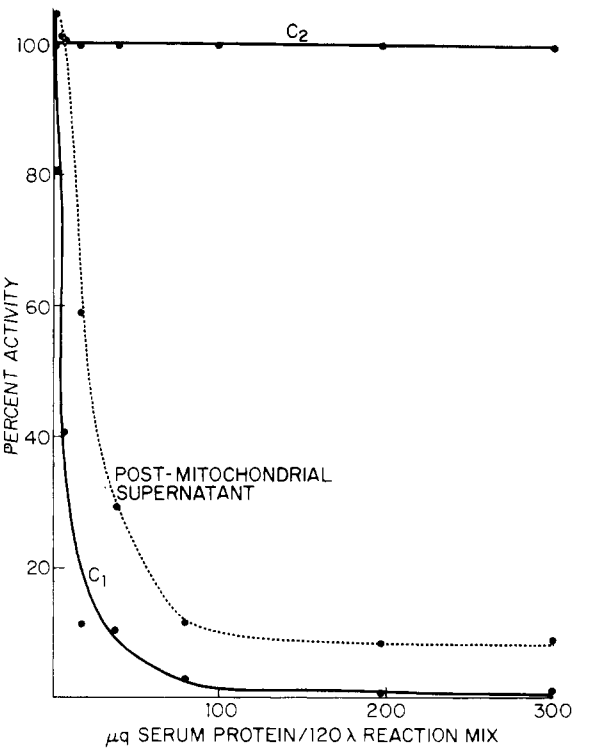

FIGURE 4: Inhibition of phenylalanine-tRNA synthetase activity by anti-C_1 antibody. The enzyme was preincubated with antibody for 20 min at 0°C in the presence of all assay ingredients except tRNA. Reaction was started by the addition of tRNA and warming to 30°C. Post-mitochondrial supernatant was inhibited 90%; purified C_1 (hydroxylapatite product) was inhibited 99%; purified C_2 (hydroxylapatite product) was unaffected.

(McEwen, 1967) were 5.5 S for enzyme alone and 7.2 S in the presence of tRNA (Figure 3), compared with values of 7.7 S and 11.6 S for the activity studied by Lanks et al. (1971). The latter may represent dimers of C_1 and the C_1–tRNA complex. Molecular weight ratios for the two sets of data are 1.65 and 2.05, based on spheres of equal hydration (Haschemeyer and Haschemeyer, 1973). [Deviation from spherical shape would account for some of the difference between these ratios and the theoretical value of 2.0 for a monomer–dimer system.] Antibody prepared to enzyme C_1 (anti-C_1 γ-globulin) produced total inhibition of Phe-tRNA formation by purified enzyme obtained from PMS or PRS (Figure 4) and of polyribosome-bound activity (0.5 unit/mg of RNA), as shown in Figure 5.

Inhibition by triiodothyronine of the activity of purified C_1 in the standard assay system with rat total tRNA was noncompetitive with respect to phenylalanine (Figure 6) and independent of tRNA concentration, as observed for pH 5 enzyme. Inhibitor constants for 3,3′,5-L-triiodothyronine and 3,5-DL-diiodothyronine are presented in Table III. These values are comparable to the inhibitory concentrations of thyroid hormones found for a variety of other enzymes not involved in protein synthesis and are well above physiological tissue concentrations (Wolff and Wolff, 1964). No inhibition of C_1 was found with 3,5-L-diiodotyrosine or DL-thyronine up to 2×10^{-4} *M*.

The levels of thyroid hormone required for 50% inhibition of activity were similar at all stages of purification of C_1. Inhibition was reversible, however, with bovine serum albumin added either before or after treatment of the enzyme with triiodothyronine. Inhibition by 4×10^{-6} *M* triiodothyronine (50% in the absence of bovine serum albumin) was reduced to 20% by the addition of bovine serum albumin at 0.2 mg/ml and to 10% at 2 mg/ml of bovine serum albumin, compared to T_3-free controls. Controls were unaffected by bovine serum albumin up to 1 mg/ml. Inhibition was reversible, moreover, upon removal of the inhibitor, eliminating the possibility of specific and irreversible iodination of residues in the active site. C_1 was preincubated under standard assay conditions with 1×10^{-5} *M* triiodothyronine and chromatographed on Sephadex G-25 to eliminate the hormone. The specific activity of enzyme eluted at the void volume was identical with that of a control preincubated without triiodothyronine. Both were inhibited to the same extent by 4×10^{-6} *M* triiodothyronine as the enzyme not subjected to preincubation. Some experiments suggested a possible protective effect of T_3 on the enzyme, particularly at low concentrations. Inclusion of T_3 throughout preparation, however, produced no more than a 10% greater recovery of C_1.

Residual Cytoplasmic Activity (C_2). Phosphocellulose chromatography of post-mitochondrial supernatant treated with anti-C_1 antibody revealed a small peak of phenylalanyl-tRNA synthetase activity eluting at about 0.24 *M* KCl. This activity (C_2) was also discernible in untreated post-mitochondrial supernatant (Figure 1). It separated from C_1

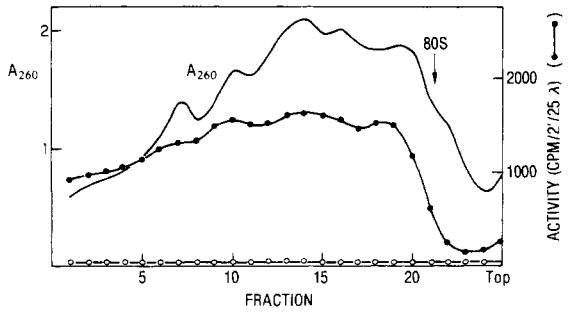

FIGURE 5: Sucrose density gradient analysis of rat liver polyribosomes, associated phenylalanyl-tRNA synthetase activity (●), and activity after treatment with anti-C_1 antibody (○). Polyribosomes prepared as described in Materials and Methods were gently resuspended by use of a loose-fitting Dounce homogenizer in 20 m*M* Tris (pH 7.4), 10 m*M* KCl, 5 m*M* $MgCl_2$, and 2 m*M* mercaptoethanol; 0.1 ml was layered on a 15–30% sucrose gradient in the same medium and centrifuged 20 min at 50 000 rpm in a Spinco SW 65 rotor at 4°C. Ribosome monomers were run separately as a marker.

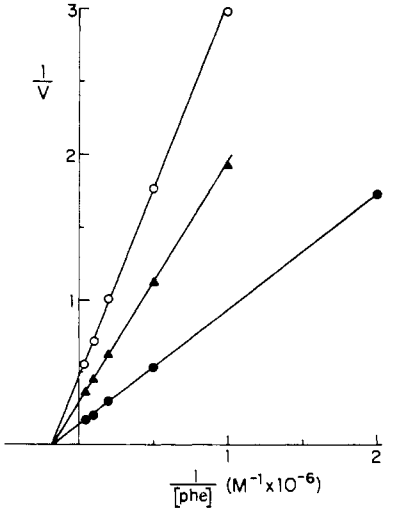

FIGURE 6: Double reciprocal plot of phenylalanyl-tRNA synthetase activity of purified enzyme C_1 and the inhibition by 3,3′,5-triiodo-L-thyronine. Reaction rate (*V*) is given in units of picomoles of [^{3}H]Phe-tRNA formed per minute. (●) Standard assay system, no additions; (▲) 5×10^{-6} *M* triiodothyronine; (○) 1.1×10^{-5} *M* triiodothyronine.

on hydroxylapatite (Figure 2), but because of its elution with the bulk of the protein only a twofold purification was achieved (Table IV) at this step. Sedimentation coefficients of C_2 in the absence and presence of tRNA were 3.5 S and 5.6 S, respectively (Figure 3). Purified C_2 was unaffected by anti-C_1 γ-globulin (Figure 4).

Approximately 30% of C_2 in anti-C_1 treated PMS sedimented at 100 000*g*, 90 min, but was recoverable from the pellet with Triton X-100. No C_2 sedimented from 0.5% deoxycholate-treated PMS. In contrast, about 40 and 20% of C_1 sedimented from untreated and deoxycholate-treated PMS, respectively. No activity attributable to C_2 was found associated with polyribosomes (Figure 5).

The possibility that C_2 was derived from C_1 as an artifact of preparation appears unlikely. Rechromatography of purified C_1 on phosphocellulose yielded no activity in the C_2 region. Purified C_1 treated with post-mitochondrial supernatant (as a possible source of proteolytic activity) showed no C_2 activity above that associated with the PMS alone. In contrast, multiple forms of C_1 were found to be generated by ammonium sulfate fractionation, a methodology which was discontinued. These forms were separable on Sepharose 4B, but were antigenically indistinguishable.

Table III: Characteristics of Rat Liver Phenylalanyl-tRNA Synthetases.

	C_1	C_2	M
Total tissue activity assayed vs. rat tRNA (units/g of liver)	22 ± 1.5 (10)	1.8 ± 0.1 (4)	4.1 ± 0.2 (3)
Specific activity of purified enzyme (units/mg of protein)	164	0.36	0.39
K_M(app), phenylalanine	6 μ*M*	10 μ*M*	8 μ*M*
K_M(app), ATP	0.2 m*M*	0.3 m*M*	0.2 m*M*
K_M(app), rat $tRNA^{Phe}$	1×10^{-7} *M*	2.4×10^{-7} *M*	6.3×10^{-7} *M*
K_M(app), yeast $tRNA^{Phe}$ (purified)	7×10^{-8} *M*	1.3×10^{-7} *M*	2.6×10^{-7} *M*
K_M(app), rat mitochondrial $tRNA^{Phe}$	7×10^{-8} *M*	7×10^{-8} *M*	6×10^{-8} *M*
Inhibition by anti-C_1 γ-globulin	99%	0	0
Inhibition by iodinated thyronines	+	–	–
K_I (triiodothyronine)	4×10^{-6} *M*		
K_I (diiodothyronine)	1×10^{-5} *M*		

[a] Conditions were standard except for variation of concentration of the component whose K_M (app) was being determined.

Table IV: Purification of Minor Cytoplasmic Rat Liver Phenylalanyl-tRNA Synthetase (C_2) from Post-Mitochondrial Supernatant Treated with Anti-C_1 γ-Globulin.

Fraction	Protein Recovery (mg/g of liver)	Specific Activity vs. Rat tRNA (units[b]/mg)	Yield[a] (%)	-Fold Purification
Anti-C1 supernatant	110	0.016	8	1
Sephadex G-25	70	0.022	7	1.4
Phosphocellulose P11	7.7	0.14	5	9
Hydroxylapatite	1.8	0.36	3	23

[a] Based on total phenylalanyl-tRNA synthetase activity of post-mitochondrial supernatant. [b] 1 unit = 1 nmol/min of Phe-tRNA in standard assay system, 10 m*M* Mg^{2+}.

Table III summarizes the properties of C_2 compared with C_1. Total tissue activity for these enzymes is based on levels in the post-mitochondrial supernatant (crude homogenates showed only 85% of these levels, presumably because of interfering substances). Substrate affinities, as estimated by K_M's, were generally less for C_2 than for C_1, but this may be due to the lower level of purification. Higher K_M values for $tRNA^{Phe}$ in unfractionated rat liver and yeast tRNA compared to purified yeast $tRNA^{Phe}$ may reflect competition by other tRNAs. Maximal charging levels of total rat liver tRNA indicated that either enzyme C_1 or C_2 alone was capable of charging all tRNA charged by the unfractionated post-mitochondrial supernatant.

Mitochondrial Enzyme. To test for a possible mitochon-

rial origin of the residual cytoplasmic activity, partial purification of mitochondrial phenylalanyl-tRNA synthetase (M) was carried out. Tissue concentration, based on final specific activity (Table IV) and mitochondrial protein content, is estimated at 4.1 units/g of liver. The presence of ribonuclease in the mitochondrial homogenate produces a high Mg^{2+} requirement (>40 m*M*), previously ascribed to the synthetase activity (Lietman, 1968). Upon addition of rat liver ribonuclease inhibitor, however, or after fractionation on hydroxylapatite, Mg^{2+} dependency for formation of Phe-tRNA is like that for the cytoplasmic enzymes. Affinity for mitochondrial tRNA was similar to that of the cytoplasmic enzymes, but affinity for cytoplasmic tRNA was reduced. No inhibition by anti-C_1 γ-globulin or by iodinated thyronines was observed. Sedimentation behavior (Figure 3) was similar to that of C_2.

Discussion

A number of studies have suggested that thyroid hormones may have inhibitory effects on protein synthesis. Reduced levels of incorporation of radioactive amino acids into collagen (Kivirikko et al., 1967) and plasma proteins (Nadkarni and Samuel, 1973) are found in experimental hyperthyroidism in rat; thyroxine addition to perfused normal rat liver reduced albumin synthesis (Griffin and Miller, 1973). These findings contrast with the stimulation of polypeptide chain elongation rate by thyroid hormone in liver protein synthesis in vivo (Mathews et al., 1973) and the correlation of plasma protein synthesis in perfused liver with donor thyroid status (Griffin and Miller, 1973). The present results raise the possibility that under circumstances of hormonal excess, the inhibition of aminoacyl-tRNA formation for phenylalanine, and possibly also tyrosine, may cause precursor availability to become rate-limiting in protein synthesis, thus overriding stimulatory effects.

In the case of phenylalanine activation, the inhibitory action of the hormone is directed only against the major cytoplasmic enzyme (C_1). Inhibition is reversible, noncompetitive with respect to both phenylalanine and tRNA, and requires both the thyronine moiety and the presence of iodine on the tyrosine portion of the molecule. Triiodothyronine, which was the most effective inhibitor, is considered by some investigators to be more important than thyroxine for hormonal function (Sterling, 1970); its effectiveness in vitro, however, may be due to greater solubility. 3,5-Diiodothyronine, which also inhibits C_1, is formed in liver homogenates by deiodination of triiodothyronine (Plaskett, 1961), and has been suggested as a possible intermediate in the action of thyroid hormone (Barker, 1964). Related compounds (e.g., diiodothyroacetic acid) are hormonally active in certain systems (Kaltenbach, 1970). Similarity in structure to the amino acid substrate of the inhibited enzymes (Table I) suggests a binding site at the amino acid binding site. However, in the case of the phenylalanine enzymes inhibition occurred with only one of the enzymes studied here, and was noncompetitive.

The phenylalanyl-tRNA synthetase of liver mitochondria, freed from mitochondrial ribonuclease, differs markedly in chromatographic and physical properties from the major cytoplasmic enzyme C_1. It is not inhibited by thyroxine or its analogues up to concentrations of 2×10^{-4} *M* nor by anti-C_1 antibody. The results indicate that C_1 and M are not closely related structurally. An absence of immunologic cross-reaction between cytoplasmic and mitochondrial enzymes has also been shown with the leucyl-tRNA synthetases of *Tetrahymena* (Chiu and Suyama, 1973).

The origin of the cytoplasmic activity C_2, whose properties closely resemble the mitochondrial enzyme, is of interest. Assays for other mitochondrial enzymes in the postmitochondrial supernatant showed levels amounting to only 7–8% of that associated with mitochondria. In contrast, C_2 in the PMS amounts to 30% of total M + C_2 activity. This may reflect extraordinary leakage of this enzyme. Alternatively, C_2 may represent an independent cytoplasmic pool of M, presumably a product of synthesis by the cytoplasmic protein synthetic system. C_2 has full charging activity toward liver $tRNA^{Phe}$, and therefore should itself be able to function in cytoplasmic protein synthesis. The fact that it is not polyribosome-bound does not necessarily limit its role, since most aminoacyl-tRNA synthetases do not show this property (Moline et al., 1974). The high tissue concentration of enzyme C_1 and its proximity to the protein synthetic apparatus, however, suggest that C_1 is likely to dominate liver cytoplasmic Phe-tRNA production. Another possibility is that C_2 may be associated with a minor cell type, e.g., the Kupfer cells which account for 10% of liver volume.

The present findings suggest a possible correlation between depression of protein synthetic activity under strongly hyperthyroid conditions and the inhibition of aminoacyl-tRNA formation. Studies in vivo in this laboratory indicate a 70% reduction in [^{3}H]phenylalanine incorporation into liver protein and a prolongation of polypeptide chain assembly time in rats given a high dose (400 μg/100 g body weight) of triiodothyronine for a 2-hr period compared to animals receiving saline or 40 μg of hormone (100 g body weight). Kinetic analysis of this system by the method of Mathews et al. (1973) and examination of possible qualitative effects will be reported subsequently.

Acknowledgments

We thank Dr. Allyn Rule of Mt. Sinai School of Medicine for advice on immunological procedures. The encouragement of Dr. Jerome Gross is gratefully acknowledged.

References

Bandyopadhyay, A. K., and Deutscher, M. P. (1971), *J. Mol. Biol. 60,* 113.

Barker, S. B. (1964), in The Thyroid Gland, Pitt-Rivers, R., and Trotter, W. R., Ed., Washington, D.C., Butterworths, p 199.

Barnett, W. E. (1965), *Proc. Natl. Acad. Sci. U.S.A. 53,* 1462.

Barnett, W. E., Brown, D. H., and Epler, J. L. (1967), *Proc. Natl. Acad. Sci. U.S.A. 57,* 1775.

Barnett, W. E., and Jacobson, K. B. (1964), *Proc. Natl. Acad. Sci. U.S.A. 51,* 642.

Beaufay, H., Bendall, D. S., Baudhuin, P., and de Duve, C. (1959), *Biochem. J. 73,* 623.

Bollum, F. J. (1959), *J. Biol. Chem. 234,* 2733.

Buck, C. A., and Nass, M. M. K. (1968), *Proc. Natl. Acad. Sci. U.S.A. 60,* 1045.

Calendar, R., and Berg, P. (1966) *Proced. Nucleic Acid Res. 1,* 384.

Chiu, A. O. S., and Suyama, Y. (1973), *Biochim. Biophys. Acta 299,* 557.

Deutscher, M. P. (1970), *J. Biol. Chem. 245,* 4225.

Dinziani, M. U., and Viti, I. (1955), *Biochem. J. 59,* 141.

Gagnon, C., and de Lamirande, G. (1973), *Biochem. Biophys. Res. Commun. 51,* 580.

Geiger, P. J., and Bessman, S. P. (1972), *Anal. Biochem.*

49, 467.
Griffin, E. E., and Miller, L. L. (1973), *J. Biol. Chem. 248,* 4716.
Haschemeyer, A. E. V. (1968), *Biol. Bull. 135,* 406–407.
Haschemeyer, A. E. V., and Gross, J. (1967), *Biochim. Biophys. Acta 145,* 76.
Haschemeyer, R. H., and Haschemeyer, A. E. V. (1973), Proteins: A Guide to Study by Physical and Chemical Methods, New York, N.Y., Wiley-Interscience, p 139.
Holley, R. W., Brunngraber, E. F., Saad, F., and Williams, H. H. (1961), *J. Biol. Chem. 236,* 197.
Irvin, J. D., and Hardesty, B. (1972), *Biochemistry 11,* 1915.
Kaltenbach, J. C. (1970), *J. Exp. Zool. 174,* 55.
Kivirikko, K. I., Laitinen, O., Aer, J., and Halme, J. (1967), *Endocrinology 80,* 1051.
Lalonde, G., and de Lamirande, G. (1974), *Biochim. Biophys. Acta 353,* 323.
Lanks, K. W., Sciscenti, J., Weinstein, I. B., and Cantor, C. R. (1971), *J. Biol. Chem. 246,* 3494.
Lietman, P. S. (1968), *J. Biol. Chem. 243,* 2837.
Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1,* 66.
Margoliash, E., and Walasek, O. F. (1967), *Methods Enzymol. 10,* 339.
Mathews, R. W., Oronsky, A., and Haschemeyer, A. E. V. (1973), *J. Biol. Chem. 248,* 1329.
McEwen, C. R. (1967), *Anal. Biochem. 20,* 114.
Miyazawa, Y., and Thomas, C. A. (1965), *J. Mol. Biol. 11,* 223–237.
Moline, G., Hampel, A., and Enger, M. D. (1974), *Biochem. J. 143,* 191.
Nadkarni, G. B., and Samuel, A. M. (1973), *Biochem. Med. 7,* 226.
Neidhardt, F. C. (1966), *Bacteriol. Rev. 30,* 701.
Nielsen, J. B., and Haschemeyer, A. E. V. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 459.
O'Brien, T. W., and Kalf, G. F. (1967), *J. Biol. Chem. 242,* 2172.
Peterson, P. J. (1967), *Biol. Rev. 42,* 552.
Petrissant, G., Boisnard, M., and Puissant, C. (1971), *Biochimie 53,* 1105.
Plaskett, L. G. (1961), *Biochem. J. 78,* 652.
Roberts, K., and Coleman, W. H. (1972), *Biochem. Biophys. Res. Commun. 46,* 206.
Rouget, P., and Chapeville, F. (1971), *Eur. J. Biochem. 23,* 452–459.
Rymo, L., Lindvik, L., and Lagerkvist, U. (1972), *J. Biol. Chem. 247,* 3888.
Sterling, K. (1970), *Recent Prog. Horm. Res. 26,* 249.
Tscherne, J. S., Lanks, K. W., Salim, P. D., Grunberger, D., Cantor, C. R., and Weinstein, I. B. (1973a), *J. Biol. Chem. 248,* 4052.
Tscherne, J. S., Weinstein, I. B., Lanks, K. W., Gersten, N. B., and Cantor, C. R. (1973b), *Biochemistry 12,* 3859.
Vennegoor, C., and Bloemendal, H. (1972), *Eur. J. Biochem. 26,* 462.
Wolff, E. C., and Wolff, J. (1964), in The Thyroid Gland, Pitt-Rivers, R., and Trotter, W. R., Ed., Washington, D.C., Butterworths, p 237.