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Binding Of Thyrotropin In Releasing Hormone By Thyrotropin-Secreting Cells.

Lynne Jones Eddy
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BINDING OF THYROTROPIN RELEASING HORMONE BY THYROTROPIN-SECRETING CELLS

by

LYNNE JONES EDDY

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Biophysics in The Graduate School of the University of Alabama in Birmingham

BIRMINGHAM, ALABAMA

1972

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INTRODUCTION

Recently, much research in endocrinology has been directed at elucidating the mechanism of action of hormones at the molecular level. The currently popular hypothesis holds that the two major processes in this action are 1) recognition of a specific cellular receptor by the hormone with interaction to form a hormone-receptor complex and 2) initiation by the hormone-receptor complex of metabolic processes culminating in the overt physiological response.

The receptor concept was devised by pharmacologists to explain drug action. In 1905 Langley (33) referred to a "receptive substance" in describing the effects of nicotine and curare in the fowl on abolishing nerve mediated, but not direct electrical, stimulation of muscle contraction. He stated that the drugs did not act directly on the contractile material but on the receptive substance, which received the drug stimulus and transferred it to the contractile material. He also demonstrated a difference in the action of high doses of nicotine on different types of striated muscle in the same animal in that nicotine stimulated the red muscle of the leg but did not stimulate the white muscle of the wing. He attributed this to differences in the receptive substance. In noting that adrenalin, an internal secretion, stimulated muscle

contraction by acting on a receptive substance, he proposed that other internal secretions such as thyroxine and secretin might also act on a receptive substance.

Endocrinologists and pharmacologists have further developed this concept and it now implies that a hormone, or a drug, interacts with a macromolecular species, usually assumed to be a protein, prior to producing a response. Since hormones are not thought to bind with their receptors covalently (27), weaker binding forces such as electrostatic, hydrogen, or disulfide bonds are probably involved. The interaction between a hormone and its specific receptor, through conformation and charge changes, produces an activated complex and constitutes the difference between specific and non-specific binding, the latter producing no response (2). This receptor may be located either within the cell or on its external membrane surface.

Initially, data obtained with thyrotropin releasing hormone (TRH) described only the overt physiological actions of this hormone on the anterior pituitary. Although this has been rapidly followed by studies of the subcellular processes under TRH influence, the first step, viz. the binding of TRH to a specific cellular receptor, has not been clearly defined. This dissertation will describe evidence available to hypothesize the location of the TRH receptor and the results of experiments designed to test this theory.

HISTORICAL REVIEW

The Discovery of Thyrotropin Releasing Hormone

Transplantation of the anterior pituitary away from its normal location is followed by loss of its structural and functional integrity (13). This observation led workers in the area to seek the factors controlling the anterior pituitary.

In 1935 Houssay et al (29) reported that sectioning of the pituitary stalk of toads resulted in necrotic areas in the anterior pituitary. They noted that these necrotic areas were vascularized by the hypothalamico-hypophyseal portal system and stated that the direction of blood flow in the portal system was from the hypothalamus to the anterior pituitary. Later, Green and Harris (19) directly observed this same direction of blood flow in living rats. Because of observations of the direction of blood flow in the portal system and the necessity of the portal system for anterior pituitary function, the hypothalamus was considered as a possible origin of the anterior pituitary control.

Harris and Jacobsohn (24) performed a series of experiments to localize this control. They noted that anterior pituitaries transplanted under the median eminence of hypophysectomized rats remained viable and were vascularized by the portal system.

Maintenance of normal weights of adrenals, gonads, and thyroids and the presence of normal estrus cycles, pregnancies, and lactation indicated that the anterior pituitary hormones were being adequately secreted. Anterior pituitaries transplanted under the temporal lobe or in the pituitary capsule but not in contact with the pituitary stalk also revascularized, although not by the hypothalamico-hypophyseal portal system, and did not maintain physiological integrity of the adrenals, gonads, or thyroids. Since the primary plexus of the portal system regenerated around the tissue transplanted under the median eminence, and the neural connection did not, it was hypothesized that the portal system transported a substance from the median eminence which maintained normal anterior pituitary function.

In 1962 Guillemin et al (22) definitely established for the first time the presence in an acid extract of sheep hypothalamic tissue of a thyrotropin (TSH) releasing factor which would release TSH in mice and rats.

Schally et al (47) suggested the term "releasing hormone" for the hypothalamic factors which regulated the specific anterior pituitary hormones. I have adopted this term and "TRH" will represent thyrotropin releasing hormone in this thesis.

Guillemin et al (20) reported a purification of TRH activity close to 60,000-fold for the active material obtained from sheep hypothalamic tissue; however, the recovery was very low, with only about 3 mg of material being obtained from 500,000 hypothalami.

Schally et al (48) recovered 7 mg from 200,000 porcine hypothalami. The minute amounts of TRH obtainable from hypothalamic tissue was a driving force behind the elucidation of TRH structure and possible large-scale synthesis of the hormone.

Burgus and Guillemin (11) and Schally et al (48) demonstrated that the amino acid residues of glutamic acid, histidine, and proline were present in equimolar amounts. Synthetic tripeptides of the residues in the six possible sequences, however, exhibited no biological activity (10, 47). Schally et al (48) showed there were no free carboxyl- or amino-terminal groups in native TRH.

Acetic anhydride treatment to protect the N-terminal amino acid of the tripeptides in varying sequences showed TRH activity only in the reaction products of Glu-His-Pro (10). Acetic anhydride treatment led to several derivatives, the major one being (pyro)Glu-His-Pro [(pyro)Glu = 2-pyrrolidone-5-carboxylic acid]. Other peptides such as immunoglobulins, fibrinopeptides, and gastrins have been shown to have (pyro)Glu as the N-terminus. The TRH activity of (pyro)Glu-His-Pro was very low, 10 units/mg, compared to ovine TRH, 60,000 units/mg; however, it was the first polypeptide of known structure to exhibit TRH activity.

Since it had been shown that TRH had no free C-terminus and it was known that vasopressin, oxytocin, and α-MSH have an amide on the C-terminus, Burgus et al (10) proceeded stepwise to form the amide derivative of (pyro)Glu-His-Pro. The carboxyl group was first methylated to form (pyro)Glu-His-Pro-OMe, which demonstrated TRH

activity of about half that of ovine TRH. Although both of these substances exhibited TRH activity, the low specific activity showed that neither was the structure of ovine TRH. The next step was ammonolysis of the methyl ester to form (pyro)Glu-His-Pro(NH_2). The biological activity of this amide derivative was nearly identical to that of ovine TRH.

(Pyro)Glu-His-Pro(NH_2) has since been shown to be the structure of porcine (4), rat (38), and probably bovine and human (7) TRH.

Biological Actions of TRH

Extracted ovine TRH caused an increased TSH release from the anterior pituitary, both in vivo (22) and in vitro (21), the latter indicating that TRH acted directly on the anterior pituitary.

The TSH synthesis response to TRH occurred much later than the release response. Sinha and Meites (49) demonstrated with rat anterior pituitaries incubated for 6 days that the TRH treated group contained 50 to 97% more TSH in the medium and explant than was present in the fresh pituitary. To prove that TSH synthesis is a direct response to TRH and not just secondary to TSH release as had been postulated (58), Wilber (56) incubated rat anterior pituitaries with TRH, either with added thyroxine (T_4) or with Ca^{++} omitted from the medium. With both of these treatments, which inhibited in vitro TSH release, TRH caused an increased

incorporation of radioactive precursors into intrapituitary TSH as compared to non TRH-treated controls.

Pretreatment of the intact animal with T_4 (6) or preincubation of the anterior pituitary with T_4 (21) inhibited the TSH release response to TRH, demonstrating a negative feedback of T_4 directly on the anterior pituitary. That this is not just a competition of T_4 for TRH binding sites was demonstrated by the lag period between T_4 administration and effect (6) and by failure of T_4 to displace TRH from its binding sites (31).

Cyclic AMP and Hormone Action

Many polypeptide hormone actions are now thought to be mediated through the adenyl cyclase system. Adenyl cyclase catalyzes the conversion of adenosine triphosphate (ATP) to adenosine 3',5'-monophosphate (cyclic AMP). Cyclic AMP, termed the "second messenger" by Sutherland and his associates (51), triggers a set of reactions which cause the overt actions attributed to the specific hormones. They proposed that the hormone-specific receptor on the plasma membrane becomes activated when complexed to a hormone and this activation then stimulates adenyl cyclase. The cell specificity to the hormone resides in the receptor, not the enzyme. Cyclic AMP is degraded by phosphodiesterase (PDE) to the inactive 5'-AMP. Since adenyl cyclase is a membrane-bound enzyme (14, 43), mimicking by cyclic AMP of a hormone's actions on a particular

tissue may be taken as presumptive evidence that the receptor for that hormone is on the plasma membrane.

Cyclic AMP has been implicated as the second messenger for TRH. Wilber et al (57) demonstrated that isolated rat anterior pituitaries would release TSH after N^6-2'-dibutyryl cyclic AMP (dibutyryl cyclic AMP), an analogue of cyclic AMP, was added to the medium and that theophylline, a PDE inhibitor, also stimulated TSH release. Labrie et al (32) and McKenzie et al (37) have also suggested that dibutyryl cyclic AMP stimulates TSH synthesis. Steiner et al (50) demonstrated that TRH caused a rapid increase in intracellular levels of cyclic AMP in isolated rat anterior pituitaries and that this increase was due to TRH stimulation of adenyl cyclase rather than to inhibition of PDE. On the basis of these observations, one may speculate that the receptor for TRH is located on the plasma membranes of TSH secreting cells.

Location of Hormone-binding Sites

Two methods have been used to localize the hormone receptors. One involves exposure of the tissue to radiolabeled hormone followed by determination of the subcellular species to which it specifically binds.

The other is use of substances which have no cellular effects per se but can inactivate the hormone even after it is bound to a specific receptor.

TSH has been shown to increase rapidly the intracellular cyclic AMP concentration in bovine thyroid slices (17). Exogenous dibutyryl cyclic AMP mimics TSH action in that it releases thyroid hormones in vivo (1). In order to demonstrate the presence of a TSH receptor on the plasma membrane of thyroid cells, Pastan et al (39) incubated canine thyroid slices with bovine TSH. The stimulatory effect of TSH on glucose-1-^{14}C conversion to $^{14}CO_2$ persisted even after the tissues had been washed free of unbound TSH, but was abolished by the addition of TSH antibodies or trypsin, which had no cellular effects themselves. They hypothesized that the specific TSH receptors must be located on the plasma membrane because the antibodies could not penetrate the cell membrane to inactivate TSH bound to an "internal" receptor and because trypsin did not enter the cell since it had no metabolic effects at the dose used.

Glucagon has been shown to stimulate adenyl cyclase in rat liver (42) and fat cells (3). This stimulation also appears to involve a membrane receptor since ^{125}I-glucagon was bound by plasma membranes of these responsive cells (46).

ACTH stimulates adenyl cyclase activity in adrenal cells and this activity has been localized to a particulate fraction (52). An extract of this particulate fraction, which reportedly contains all the adenyl cyclase activity of the tissue, has been shown to bind ^{125}I-ACTH (35).

In contrast to the above, at least some of the biological effects of insulin appear related to decreased adenyl cyclase activity. Ray et al (42) reported that insulin inhibited adenyl cyclase activity in rat liver membranes and Rodbell et al (45) have shown decreased intracellular levels of cyclic AMP in fat cells exposed to insulin. Since it had been shown that stimulation by bovine insulin of glucose-1-^{14}C incorporation into glycogen by rat diaphragms was abolished by exposure of the treated tissue to insulin antibody (39), it can be hypothesized that a plasma membrane receptor exists for insulin but that the "active complex" inhibits rather than stimulates adenyl cyclase. The former is supported by the observation that isolated rat liver plasma membranes specifically bind ^{125}I-insulin (28).

The mimicking of TRH action by dibutyryl cyclic AMP and the stimulation of adenyl cyclase by TRH suggest that the first step in the mechanism of action of TRH is binding to the plasma membrane of TSH secreting cells. To test this hypothesis, I have studied binding of radiolabeled TRH to thyrotropic cells in rat and bovine anterior pituitaries and in a mouse TSH-secreting tumor.

Study of the cellular location of the receptor can be done in two ways using radiolabeled hormone. One is separation of subcellular fractions which are then incubated with the hormone. The other is incubation of the intact cells and then separation of subcellular fractions. With both methods, it is necessary to separate bound from unbound hormone in the fractions. There are problems

involved with both methods. With the first method, one might see more non-specific, non-physiologic binding, such as binding to intracellular particles by a hormone which would normally not penetrate the plasma membrane. The problem with the latter method is that the fractionation procedures might physically disrupt the hormone-receptor complex. For preliminary experiments I thought the first method presented fewest difficulties; however, I was never able to show that the labeled TRH bound preferentially to any one subcellular fraction. The experiments reported in this dissertation involved incubation of the tissue with the labeled hormone and subsequent disruption of cells followed by fractionation by zonal centrifugation.

MATERIALS AND METHODS

Materials

Synthetic TRH (100 µl/ml) was supplied by Dr. Michael Anderson and ^{14}C-TRH, L-His^{14}C(U) (256.2 Ci/mole) by Dr. George Flouret, Abbott Laboratories. ^{3}H-TRH, L-(3,4-^{3}H)-Pro (40 Ci/mmole) was purchased from New England Nuclear. The labeled TRH was utilized in these experiments without addition of unlabeled TRH, unless otherwise specified. Sucrose, C.P., used in zonal centrifugation, was purchased from Pfanstiehl Laboratories. Sephadex G-50, medium beads, was purchased from Pharmacia. All other chemicals were reagent grade.

The TSH-secreting tumor, originally obtained from Dr. Jacob Furth, Columbia University, was grown as follows: LAF_1 mice (Jackson Laboratories) were maintained on a low iodine diet for 2 weeks prior to radiothyroidectomy with 150 µCi ^{131}I per animal, injected intraperitoneally. The animals were then given 0.05% 6-propylthiouracil (PTU) in the drinking water and two weeks later received subcutaneous tumor implants. Each donor tumor was examined histologically in order to verify viability. As the tumor transplants became palpable the PTU concentration was changed to 0.01%, and subsequently discontinued when the tumors reached diameters of 1 to 2 cm.

General Methods

Tissue incubations were for 30 min at 37 C in Krebs Ringer bicarbonate, pH 7.4, (KRB) (55), equilibrated with a 95% O_2 - 5% CO_2 mixture. Tissue homogenizations, zonal centrifugations, and gel filtrations were carried out at 0 - 5 C in a 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM $MgCl_2$.

Radioactivity was determined by dissolving 1 ml of the sample in 10 ml Aquasol® (New England Nuclear) and counting in a Nuclear Chicago Unilux liquid scintillation spectrometer. High concentrations of sucrose in the sample caused a two-phase system which was corrected by the addition of water in 0.2 ml increments until a one-phase system was established. Internal standards were used to correct for quenching.

Protein concentrations were determined using the Lowry method (36). Bovine serum albumin was used as the standard in all protein determinations.

Initial attempts to separate bound from unbound hormone utilized trichloroacetic acid (TCA) precipitation. TCA should precipitate only the large molecular weight protein but not free TRH. However, TCA could disrupt non-covalent TRH binding, so this method was discontinued in favor of a more gentle technique, gel filtration.

Gel filtration has been used to separate water-soluble substances on the basis of molecular size (41). This technique has been extended to the study of drug-protein binding for separation of

bound from unbound drugs (23). Sephadex G-50 excludes molecules larger than 10,000 MW, so TRH would be retained by the gel, while the protein would be excluded. Protein-bound TRH would be excluded together with the protein.

In these experiments, the Sephadex resin was equilibrated with the Tris buffer, poured into a 1 x 30 cm column, and allowed to settle. The 1 ml sample, either tissue homogenate or zonal fraction, was transferred to the top of the gel bed by means of a Pasteur pipette and fraction collection was started. As soon as the sample had entered the gel bed, 1 ml of the Tris was used to wash the sample from the sides of the column. As soon as this had entered the gel, elution with Tris buffer from an attached reservoir was started. Two ml fractions were collected using an LKB Ultrorac fraction collector. Protein appearance in the eluate was determined by measuring absorbance at 230 mμ using a Beckman DU spectrophotometer. Protein concentrations and radioactivity were determined as previously described.

Rat Anterior Pituitary

Male Sprague Dawley rats weighing 200 g were decapitated in such a way that entire pituitaries could be removed. The anterior pituitaries were dissected and placed in ice cold KRB. The pituitaries were weighed, quartered, and the equivalent of 2 glands incubated as described earlier in stoppered test tubes containing 1

ml KRB plus the radiolabeled TRH at concentrations of 25 pmoles ^{3}H-TRH/ml, 137 pmoles ^{14}C-TRH/ml, and 2700 pmoles ^{14}C- or ^{3}H-TRH/ml. Unlabeled TRH was used to lower the specific activity of ^{3}H-TRH at the highest concentration. After incubation the tubes were placed in ice water. The pituitary fragments of each tube were removed, blotted, and homogenized in 1 ml of the Tris buffer, using an all glass Potter-Elvehjem homogenizer. Following centrifugation at 500 x g for 8 min, the supernatant was filtered on a Sephadex G-50 column. After gel filtration 2 aliquots of the fraction with both radioactivity and protein were reincubated for 30 min at 37 C, one with a 2000-fold excess of unlabeled TRH. The incubation samples were then filtered on a Sephadex G-50 column to check stability of binding and displacement of the labeled hormone. Rat cerebral cortex pieces used as controls were treated identically to the anterior pituitaries.

The rat anterior pituitaries did not yield sufficient quantities of tissue for separation of subcellular fractions by zonal centrifugation. Therefore, the next series of experiments was done with bovine anterior pituitaries and a mouse TSH tumor, both of which were obtainable in more suitable quantities for zonal fractionation.

Bovine Anterior Pituitary and Mouse TSH Tumor

Bovine anterior pituitary, while suitable for zonal separation, has few thyrotropin-secreting cells. This tissue, for that reason,

was not appropriate for the more detailed studies. The TSH tumor, while not normal tissue in many respects, was better for detailed studies since the major anterior pituitary cell type was the thyrotroph. This tumor has been shown to be hormone dependent in vivo in that it regressed in the presence of thyroid hormones (16) and increased its TSH secretion after TRH administration (personal communication, Dr. J. M. Hershman).

Bovine anterior pituitaries removed from freshly decapitated calves, which had been pretreated with succinylcholine plus phenobarbital and subjected to experimental open heart surgery, were immediately chilled on ice. The dissected anterior pituitary either was minced with scissors or was sliced with a Stadie-Riggs tissue slicer yielding an estimated approximate thickness of 0.5 mm. Incubation was carried out as described earlier for rat pituitaries, but this time in 25 ml flasks, each containing 120 mg of tissue and 4 ml KRB plus 50 pmoles ^{3}H-TRH.

Mouse TSH tumors were sliced with a Stadie-Riggs tissue slicer, again about 0.5 mm, and 400 mg were incubated in each 25 ml Erlenmeyer flask containing 4 ml KRB plus 25 pmoles ^{3}H-TRH.

After incubation, the flasks were chilled in ice water and 500 mg of pooled bovine anterior pituitary or 1.5 g of pooled tumor slices were homogenized in a glass-Teflon Potter-Elvehjem homogenizer with 7 ml 8.55% sucrose. The homogenate was centrifuged at 500 x g for 8 min and the supernatant brought to a final volume of

20 ml with 8.55% sucrose. This was the starting sample for zonal centrifugation.

Zonal centrifugation (12) was carried out at 5 C using a B-XIV zonal rotor in a Beckman L2-65B preparative ultracentrifuge. The solutions were introduced into the rotor spinning at 3000 rpm. Solutions of sucrose in Tris buffer were introduced through the outside line into the rotor in the following order: 100 ml 9% (w/v); 100 ml 15%; 75 ml 25%; 75 ml 35%; 100 ml 43%; 100 ml 47%; 100 ml 50%; and sufficient 55% to fill the rotor. The tissue sample was then introduced through the line to the inside of the rotor. Sixty ml of 4.5% sucrose were introduced through the inside line to remove all sample from the lines and to move the sample away from the vanes. The rotor was accelerated to 30,000 rpm and run for 35 min. The rotor was then decelerated by the brake to 5000 rpm and allowed to coast to 3000 rpm. Unloading of the rotor was accomplished by pushing out the rotor contents through the inside line using a 56% sucrose solution in the outside line. The absorbance at 278 mμ was monitored during unloading of the rotor. Weight percent of sucrose was measured refractometrically on each 10 ml fraction and distribution of ^{3}H-TRH among the fractions was determined by counting 1 ml of each fraction in a liquid scintillation spectrometer. An aliquot of each fraction that contained both protein and radioactivity was then filtered on a Sephadex G-50 column to separate bound from unbound hormone.

In one tumor experiment after gel filtration of the 1.14 and 1.16 density fractions, the fractions containing both protein and ^{3}H-TRH were incubated at 37 C for 30 min both in the presence and absence of a 1000- to 2000-fold excess of unlabeled TRH. These samples were then filtered on a Sephadex G-50 column to check stability of binding and displacement of the labeled hormone. One ml of the bovine anterior pituitary sample was filtered on a Sephadex G-50 column to determine TRH binding in the 500 x g supernatant. Mouse liver slices used as controls were treated identically to the tumor slices.

Location of Plasma Membranes after Zonal Centrifugation

Plasma membranes generally sediment in a sucrose gradient between densities 1.14 and 1.18. This occurs with both equilibrium centrifugation and non-equilibrium techniques (54). Specific location of the plasma membrane fractions can be determined by analysis of the activity of 5'-nucleotidase (5'-AMPase), an enzyme specific to plasma membranes (15) or more specifically, by electron microscopy of the fractions of particular interest.

5'-AMPase determination (26) involved incubation of the tissue, or tissue fraction, with 5'-AMP. Specific activity of the enzyme was calculated from the amount of inorganic phosphate released (5) and protein in the incubation sample.

To prepare for electron microscopy, the zonal fractions were diluted two-fold with 0.2 M phosphate buffer, pH 7.4, and centrifuged at 20,000 x g to pellet the large molecular weight components. The pellets were washed several times with the buffer to remove all sucrose. The pellets were then fixed with 1% osmium in veronal acetate-HCl, pH 7.4, stained by uranyl acetate block staining, and embedded in Araldite® 502 resin. The sections were stained on the grid with Reynold's lead citrate and viewed with a Philips 200 electron microscope.

Equilibrium Filtration

Equilibrium filtration, similar in principle to equilibrium dialysis, has been used to study reversible protein binding by low molecular weight substances (30). The Sephadex G-50 column is equilibrated with the low molecular weight substance at a concentration that will produce less than maximal binding. The protein is then added to the column and eluted with the solution of the low molecular weight substance. Fractions are collected and the presence of protein and low molecular weight substance determined on each fraction. If there is binding a plot of the concentration of the low molecular weight substance vs. fraction number will show a peak above base line level when the protein is eluted. This peak is due to the low molecular weight substance which is bound to the protein. Since this bound substance is removed from solution, a trough in the elution profile will follow this peak.

For these experiments the Sephadex G-50 column was equilibrated with ^{3}H-TRH at concentrations of 1250, 95, and 3.8 femtomoles (10^{-15} moles) TRH/ml, respectively, in three separate experiments. Two rat anterior pituitaries were homogenized in 1 ml of the same concentration of ^{3}H-TRH, centrifuged at 500 x g, and the supernatant placed on top of the gel bed. The column was then eluted with the ^{3}H-TRH solution and 2 ml fractions were collected. Radioactivity in each fraction was determined and plotted vs. fraction number. Protein appearance in the eluate was determined by measuring absorbance at 230 mμ. Cerebral cortex pieces treated identically were used as controls.

RESULTS

Sephadex G-50 gel filtration adequately separated free TRH from the large molecular weight components. Bound TRH was eluted with the macromolecules, which although not chemically analyzed, contained protein. Protein concentrations and radioactivity were determined on each fraction. All the protein was eluted in 2 fractions, at 8 and 10 ml of effluent volume. The percent of ^{3}H-TRH eluted in each fraction was calculated. Bound TRH was expressed as femtomoles TRH bound per mg protein.

Rat Anterior Pituitary

Gel filtration of the rat anterior pituitary homogenate prepared from quartered pituitaries which had been incubated with radiolabeled TRH showed a small amount of the TRH bound to a macromolecule. The amount of TRH complexed to the macromolecule increased with increasing incubation concentrations of TRH (Table 1). The results at the highest concentration of TRH, 2700 pmoles/ml, showed that both the ^{14}C-TRH and the ^{3}H-TRH were bound to the same extent. Cerebral cortex pieces used as controls showed no detectable bound TRH.

Incubation of 1 ml aliquots of the gel filtration fractions which contained both radioactivity and protein followed by gel

TABLE 1

Incubation of rat anterior pituitaries with radiolabeled TRH. The equivalent of 2 pituitaries was incubated with the radiolabeled TRH. After incubation the tissue was homogenized, centrifuged, and the 500 x g supernatant was filtered on Sephadex G-50. Radioactivity and protein were determined on the hormone-bound fraction and the results expressed as fmoles bound per mg protein.

incubation conc. of TRH (pmoles/ml)	no. experiments	radiolabel	fmoles TRH bound per mg protein ($\bar{x} \pm$ S.D.)
25	4	^{3}H	15 ± 1.5
137	2	^{14}C	292 ± 13.5
2700	4	^{3}H	814 ± 134
2700	1	^{14}C	1190

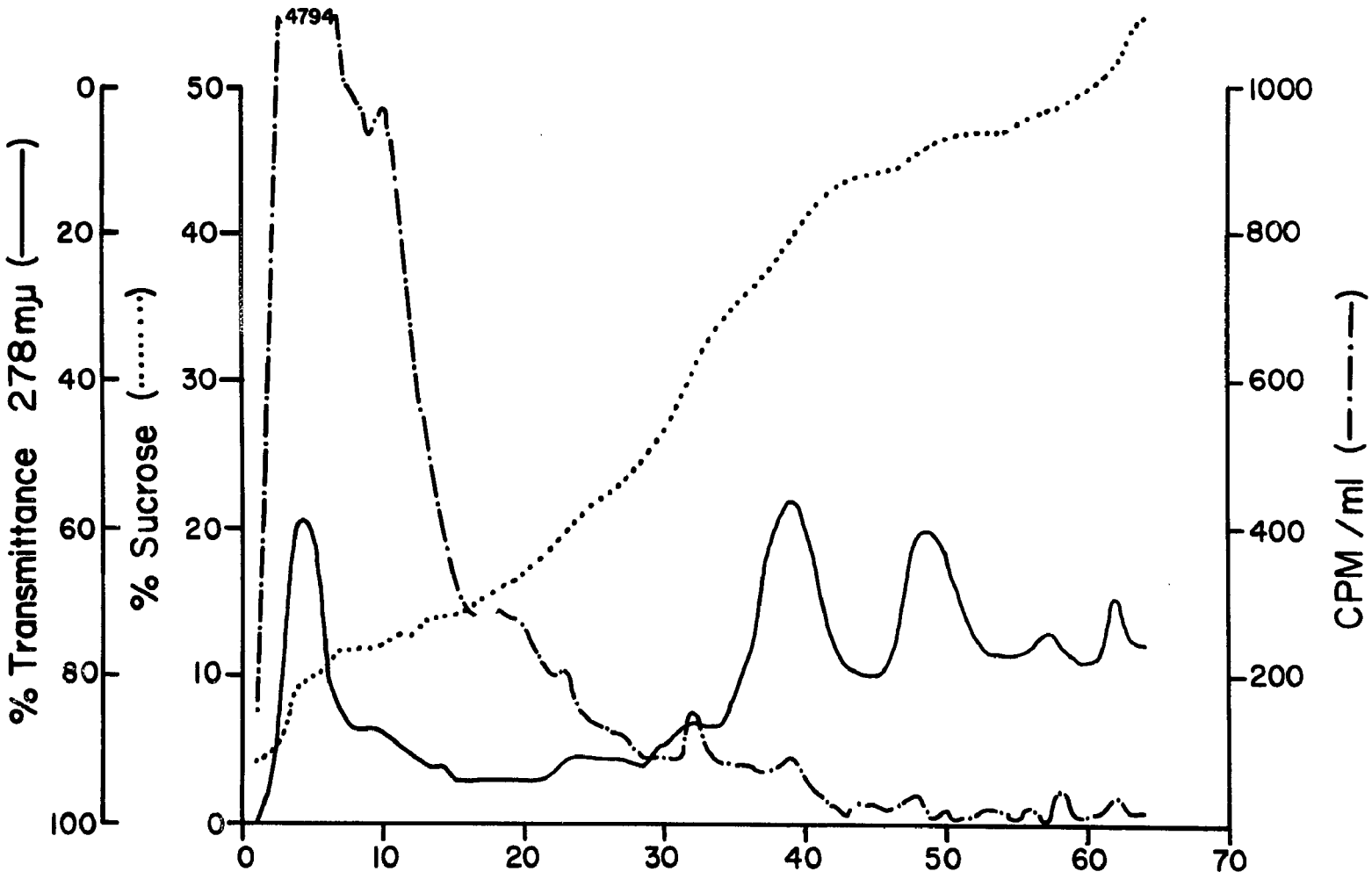
filtration of the incubation samples resulted in a 40 to 60 percent displacement of labeled TRH. This demonstrated that either some of the binding was labile to this treatment or the binding was non-specific. Incubation of the second 1 ml aliquot with a 2000-fold excess of unlabeled TRH demonstrated displacement of an additional 20 to 30 percent of the labeled TRH. These experiments indicated that, of the TRH which was stable to reincubation and refiltration, 50 percent was displaceable by this concentration of unlabeled TRH.

Bovine Anterior Pituitary

Gel filtration of the 500 x g supernatant of bovine anterior pituitaries incubated with ^{3}H-TRH demonstrated about 3% of the TRH complexed.

To identify the subcellular location of the complexing macromolecule, the 500 x g supernatant was subjected to zonal centrifugation. After zonal centrifugation most of the TRH remained with a protein peak at the lighter end of the gradient, 9% sucrose (density 1.03), but some migrated with two protein peaks, at 33% sucrose (density 1.14) and 37% sucrose (density 1.16). A representative experiment is shown in Figure 1. The TRH at density 1.03 was demonstrated by gel filtration not to be bound, while the hormone at densities 1.14 and 1.16 was shown to be bound to a macromolecule. The fractions contained plasma membranes, identified by the presence of 5'-AMPase activity. Both TRH binding and

Figure 1. Zonal fractionation of bovine anterior pituitary slice homogenate after incubation with ^{3}H-TRH. Relative amount of protein was expressed as percent transmittance (——) and recorded as the rotor was unloaded. Percent sucrose (···) and cpm/ml (— — —) were measured on each 10 ml fraction.


4794
% Transmittance 278mμ (——)
% Sucrose (·····)
CPM / ml (—·—·—)
0
20
40
60
80
100
50
40
30
20
10
0
1000
800
600
400
200
0
10
20
30
40
50
60
70

5'-AMPase activity were higher at density 1.14 than at 1.16 (Table 2).

The purification by zonal centrifugation of the TRH-macromolecule and the 5'-AMPase activity was the same. At density 1.14 there was a five-fold increase over the 500 x g supernatant in both TRH binding and the specific activity of the enzyme. At density 1.16 there was a doubling of both enzyme activity and binding response over the 500 x g supernatant.

Mouse TSH Tumor

After zonal centrifugation of the tumor homogenate most of the TRH remained with the protein peak at the lighter end of the gradient, sucrose density 1.03. However, small quantities of TRH migrated with two protein peaks at densities 1.14 and 1.16. In the representative experiment shown in Figure 2, these three peaks contained approximately 57% (density 1.03), 14% (density 1.14), and 11% (density 1.16) of the total radioactivity. Gel filtration of the density 1.03 fraction showed separation of labeled TRH and protein (Figure 3), indicating that the TRH was not bound.

However, gel filtration of the density 1.14 fraction showed that 67% of the ^{3}H-TRH came off the column with the protein (Figure 4). One ml of the gel filtration fraction which contained both ^{3}H-TRH and protein was incubated without added unlabeled TRH. Gel filtration of this incubation sample demonstrated that 78% of the TRH

TABLE 2

^{3}H-TRH binding and 5'-AMPase activity in bovine anterior pituitary glands. The pituitary slices were incubated with ^{3}H-TRH, homogenized, and centrifuged at 500 x g. The supernatant was subjected to zonal centrifugation and TRH binding and enzyme activity were measured on the 1.14 and 1.16 sucrose density fractions. TRH binding and enzyme activity were also measured on the 500 x g supernatant.

	TRH binding fmoles/mg protein	No. observations	5'-AMPase μmoles/P_i/mg protein/hr	No. observations
500 x g supernatant	7.7±1.6*	6	3.07±0.12*	3
1.14 density fraction	44.5	1	16	1
1.16 density fraction	11.9	1	6	1

*Mean ± S.D.

Figure 2. Zonal fractionation of mouse TSH-secreting tumor slice homogenate after incubation with ^{3}H-TRH. Percent transmittance (——) was recorded as the rotor was unloaded. Percent sucrose (···) and cpm/ml (— — —) were measured on each 10 ml fraction.

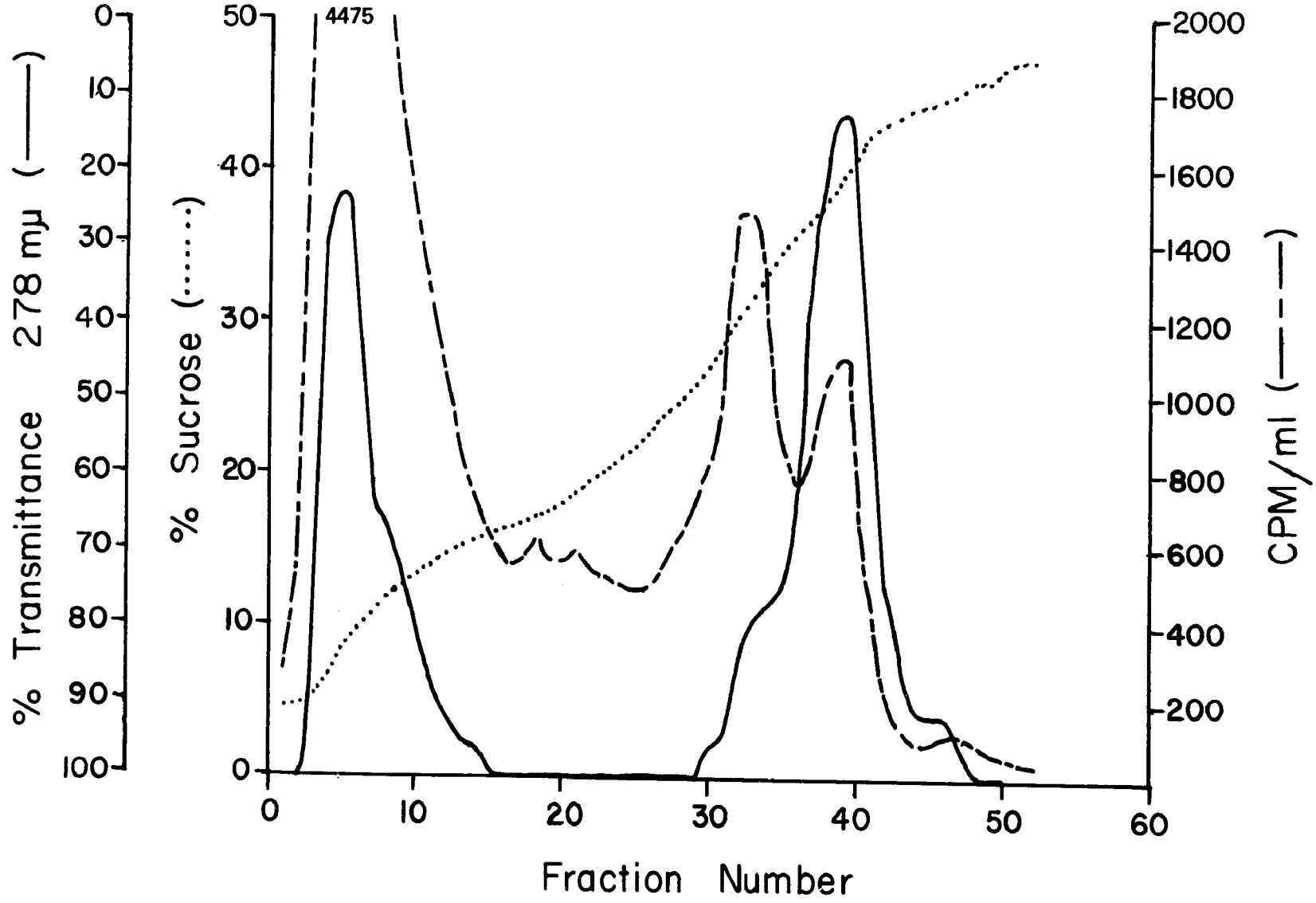

% Transmittance 278 mμ (——)
% Sucrose (·····)
CPM/ml (— — —)
4475
Fraction Number
0
10
20
30
40
50
60
70
80
90
100
200
400
600
800
1000
1200
1400
1600
1800
2000

Figure 3. Gel filtration of sucrose density 1.03 fraction from zonal fractionation of TSH tumor slice homogenate. Protein appearance in the eluate, reported as percent transmittance (——), was measured at 230 mμ on each 2 ml fraction. ^{3}H-TRH, expressed as percent of total ^{3}H-TRH eluted (— — —), was determined by counting 1 ml of each fraction in a liquid scintillation spectrometer.

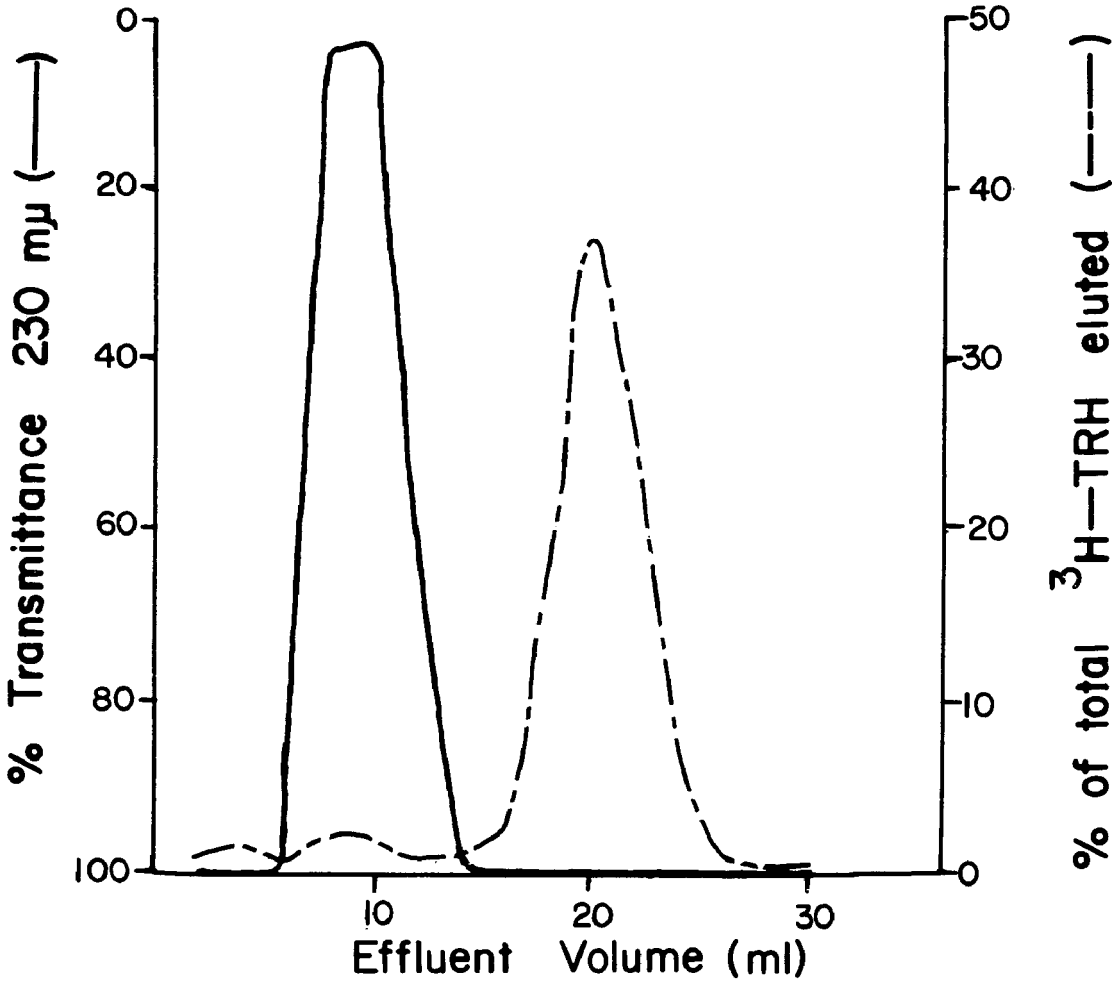
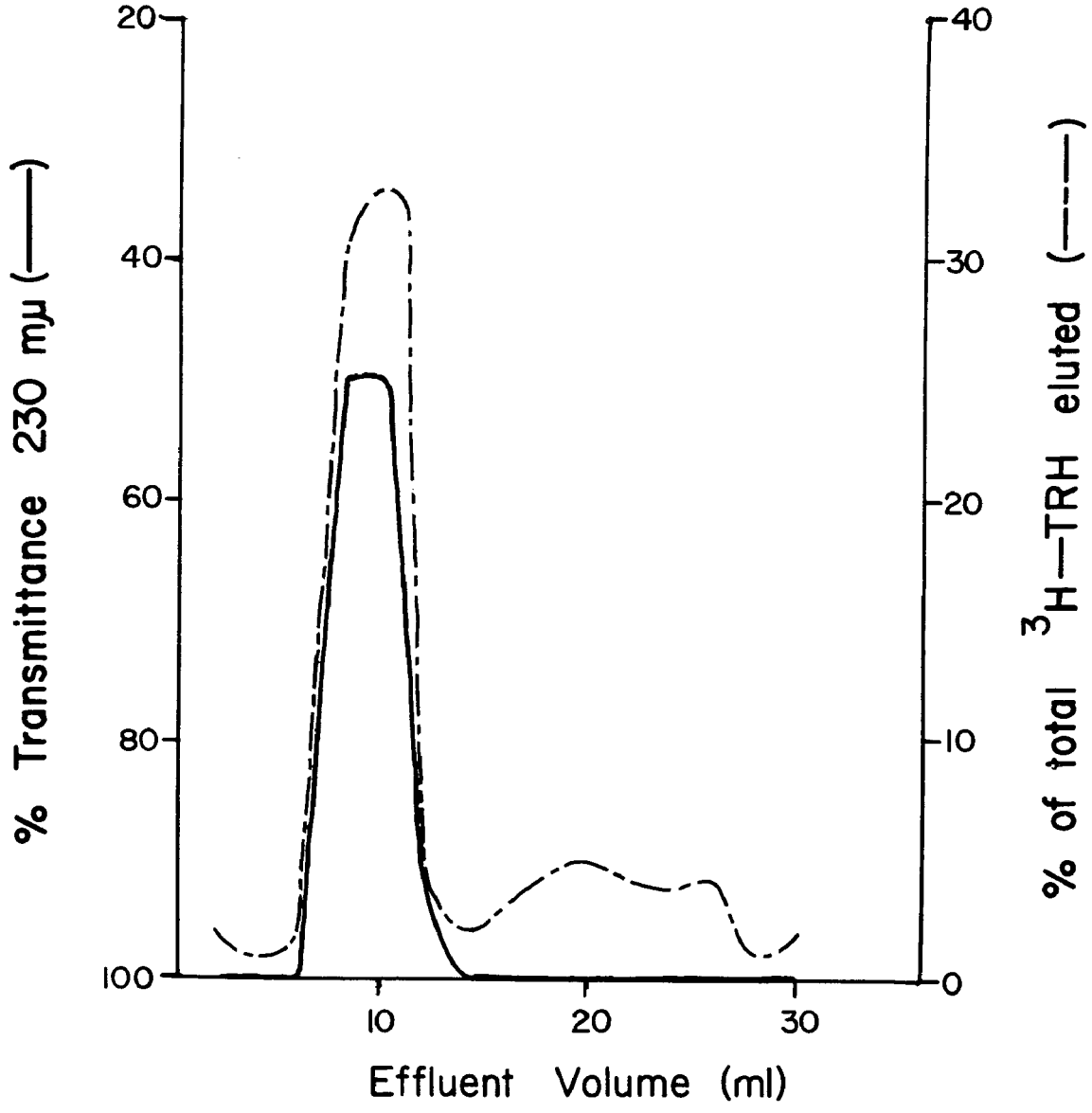

0
20
40
60
80
100
% Transmittance 230 mμ (——)
50
40
30
20
10
0
% of total ^{3}H—TRH eluted (– – –)
10
20
30
Effluent Volume (ml)

Figure 4. Gel filtration of sucrose density 1.14 fraction from zonal fractionation of TSH tumor slice homogenate. Percent transmittance (——) was measured at 230 mμ on each 2 ml fraction. ^{3}H-TRH, expressed as percent of total ^{3}H-TRH eluted (— — —), was determined by counting 1 ml of each fraction in a liquid scintillation spectrometer.


20
40
60
80
100
40
30
20
10
0
10
20
30
% Transmittance 230 mμ (——)
% of total ^{3}H—TRH eluted (– – –)
Effluent Volume (ml)

eluted remained bound (Figure 5). A second ml of the gel filtration fraction was incubated with a 2000-fold excess of unlabeled TRH and after gel filtration only 26% of that which was originally in the 1.14 fraction remained bound (Figure 6). This demonstrated that of the TRH that remained bound after reincubation and refiltration, 67% ($\frac{78\% - 26\%}{78\%}$) was displaceable by a 2000-fold excess of unlabeled TRH.

Gel filtration of the density 1.16 fraction indicated that 51% of the labeled TRH was eluted with the protein (Figure 7); however, after incubation of the gel filtration fraction containing both ^{3}H-TRH and protein without added unlabeled TRH followed by gel filtration of the incubation sample only 32% of the ^{3}H-TRH remained bound (Figure 8). When the gel filtration fraction was incubated with a 1200-fold excess of unlabeled TRH, 87% of the original ^{3}H-TRH was apparently displaced (Figure 9). Thus, of the TRH which remained bound after refiltration, 60% ($\frac{32\% - 13\%}{32\%}$) was displaceable by a 1200-fold excess of unlabeled TRH.

Although there was considerable variability in the amount of TRH bound per mg protein among the experiments, there was always greater binding to the macromolecule at density 1.14 than at density 1.16 (Table 3).

Attempts to measure 5'-AMPase activity in the mouse TSH tumor homogenate or zonal fractions were totally unsuccessful. Because of this failure of biochemical identification, electron microscopy was used to further delineate the subcellular species responsible for

Figure 5. Gel filtration of the bound fraction of density 1.14 (Figure 4) after incubation without added unlabeled TRH. Percent transmittance (——) at 230 mμ was measured on each 2 ml fraction. ^{3}H-TRH, expressed as percent of total ^{3}H-TRH eluted (— — —), was determined by counting 1 ml of each fraction in a liquid scintillation spectrometer.

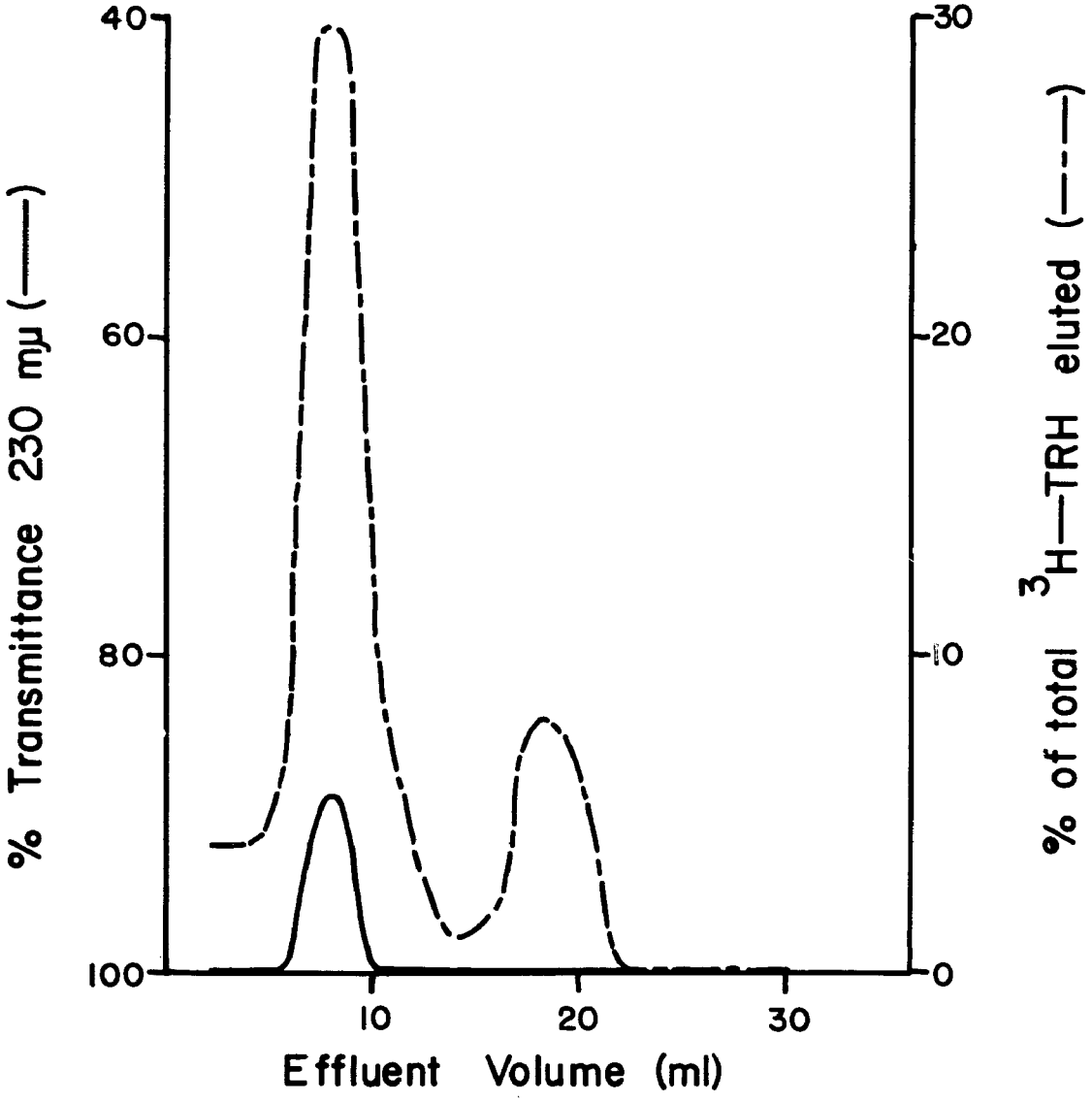

40
60
80
100
30
20
10
0
10
20
30
% Transmittance 230 mμ (——)
% of total ^{3}H—TRH eluted (- - -)
Effluent Volume (ml)

Figure 6. Gel filtration of the bound fraction of density 1.14 (Figure 4) after incubation with added 2000-fold excess of unlabeled TRH. Percent transmittance (——) was measured at 230 mμ. ^{3}H-TRH, expressed as percent of total ^{3}H-TRH eluted (— — —), was determined by counting 1 ml of each fraction in a liquid scintillation spectrometer.

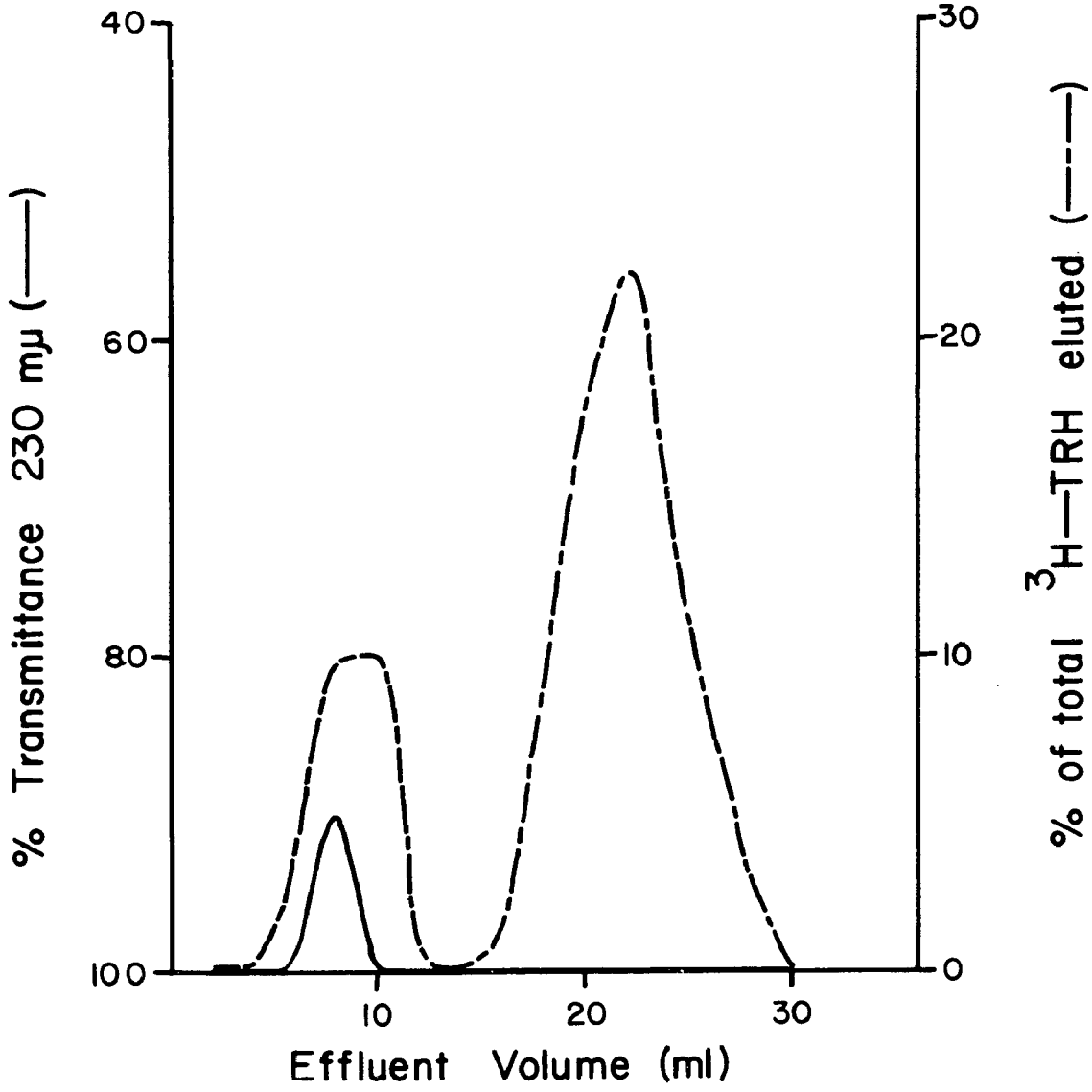

40
60
80
100
30
20
10
0
10
20
30
Effluent Volume (ml)
% Transmittance 230 mμ (——)
% of total ^{3}H—TRH eluted (– – –)

Figure 7. Gel filtration of sucrose density 1.16 fraction from zonal fractionation of TSH tumor slice homogenate. Percent transmittance (——) was measured at 230 mμ on each 2 ml fraction. ^{3}H-TRH, expressed as percent of total ^{3}H-TRH eluted (— – —), was determined by counting 1 ml of each fraction in a liquid scintillation spectrometer.

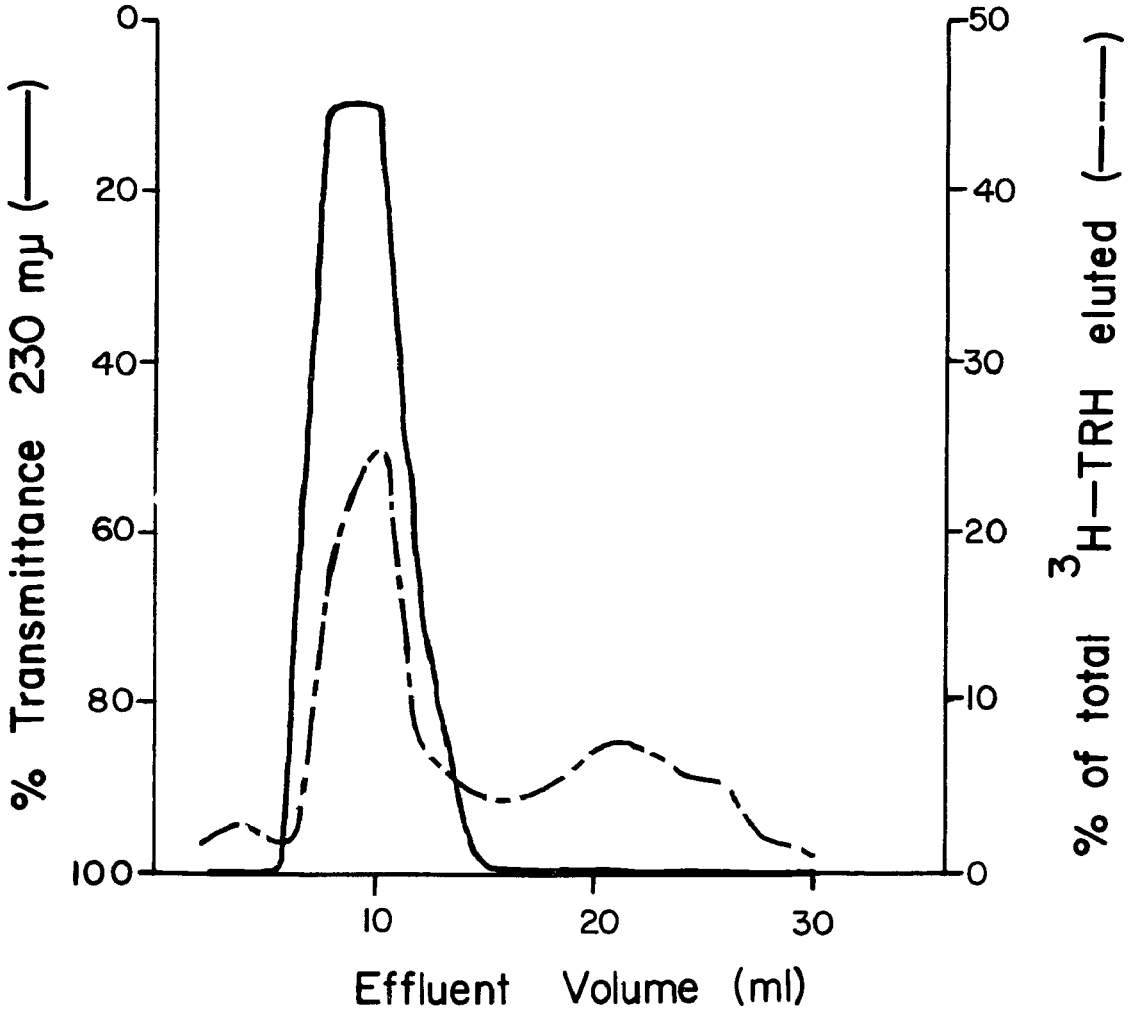

0
20
40
60
80
100
% Transmittance 230 mμ (——)
50
40
30
20
10
0
% of total ^{3}H—TRH eluted (– – –)
10
20
30
Effluent Volume (ml)

Figure 8. Gel filtration of the bound fraction of density 1.16 (Figure 7) after incubation without added unlabeled TRH. Percent transmittance (——) at 230 mμ was measured on each 2 ml fraction. ^{3}H-TRH, expressed as percent of total ^{3}H-TRH eluted (— – —), was determined by counting 1 ml of each fraction in a liquid scintillation spectrometer.

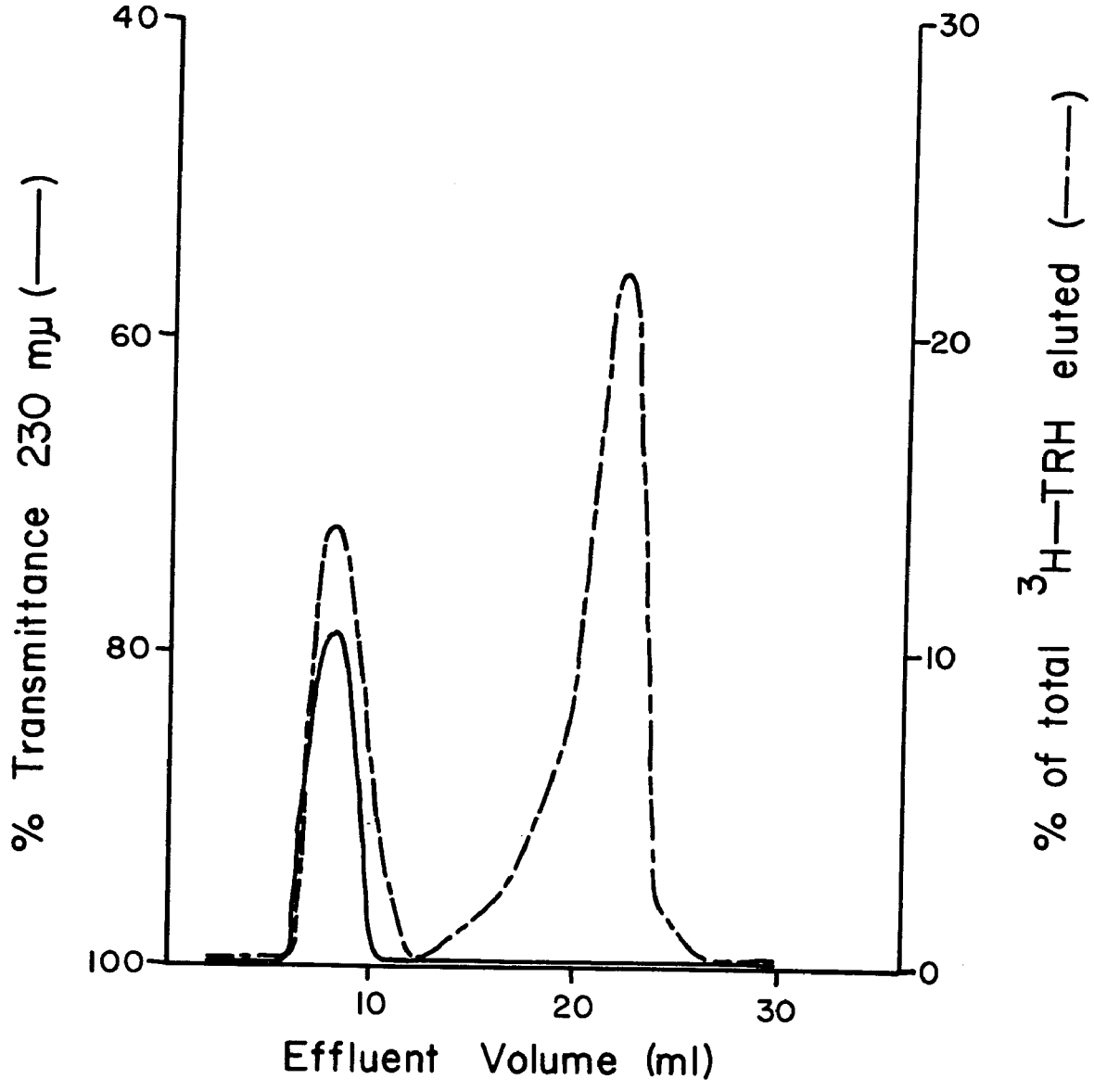

40
60
80
100
% Transmittance 230 mμ (——)
30
20
10
0
% of total ^{3}H—TRH eluted (- - -)
10
20
30
Effluent Volume (ml)

Figure 9. Gel filtration of the bound fraction of density 1.16 (Figure 7) after incubation with added 1200-fold excess of unlabeled TRH. Percent transmittance (——) was measured at 230 mμ. ^{3}H-TRH, expressed as percent of total ^{3}H-TRH eluted (— – —), was determined by counting 1 ml of each fraction in a liquid scintillation spectrometer.

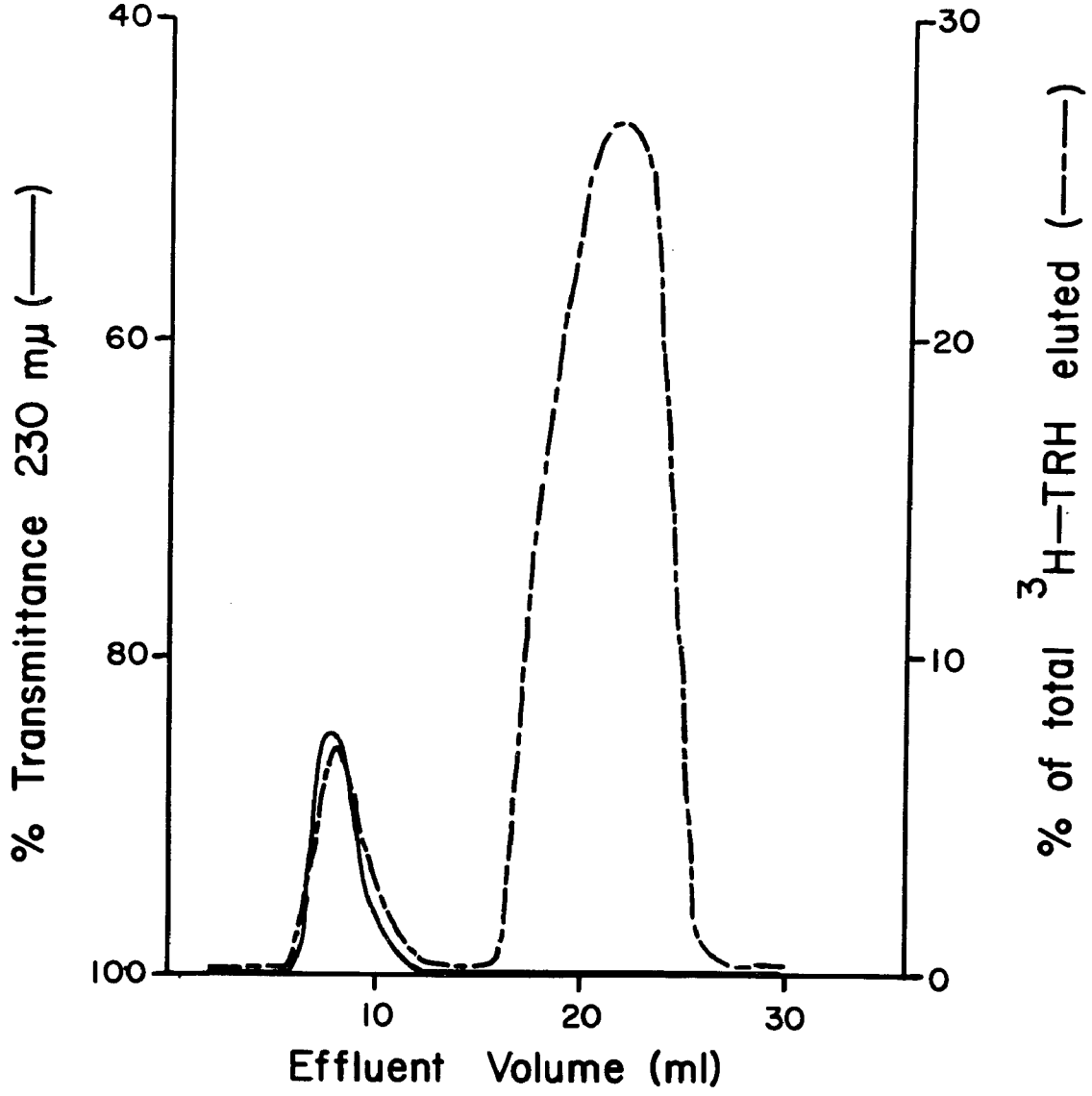

40
60
80
100
30
20
10
0
10
20
30
% Transmittance 230 mμ (——)
% of total ^{3}H—TRH eluted (– – –)
Effluent Volume (ml)

TABLE 3

^{3}H-TRH binding to plasma membranes of TSH tumor. TSH tumor slices were incubated with ^{3}H-TRH, homogenized and centrifuged at 500 x g. The supernatant was subjected to zonal centrifugation. The results are of individual fractionations and show the difference in specific binding between the membrane fractions at sucrose densities 1.14 and 1.16.

experiment number	fmoles TRH/mg protein	
	1.14 sucrose density	1.16 sucrose density
1	100	60
2	280	100
3	450	58
4	280	130
Mean	278	87

TRH binding. Electron microscopy of the 20,000 x g pellet of the density 1.14 fraction revealed mostly membranous vesicles, probably derived from plasma membranes (Figure 10). This fraction showed only a few mitochondria and no rough endoplasmic reticulum. The 20,000 x g pellet from density 1.16 showed mostly the membranous vesicles with some mitochondria, but no rough endoplasmic reticulum (Figure 11). The identity of the granular structures seen in both sections was not positively known, but they appeared to be either TSH granules or viruses (Figures 10 and 11, arrows). They measured about 80 mμ in diameter and were surrounded by a smooth membrane.

To further elucidate the nature of these granular structures, tumor tissue was stained with periodic acid Schiff (PAS) and with peroxidase labeled TSH-antibody. Both treatments very positively stained the tissue, proving that the tumor contained large amounts of TSH. Electron microscopy of the tumor, not pretreated with TRH, showed these granules scattered throughout the cytoplasm with only a few at the plasma membrane (Figure 12). The tumor membrane fractions (Figures 10 and 11) were fractionated from tumor slices which had been pretreated with TRH. Many of these granules seem to be attached to the membranes. It is tempting to hypothesize from these electron micrographs that under the influence of TRH these granules migrate to the plasma membrane and release their content of TSH.

Mouse liver slices were treated identically to the tumor slices and after zonal centrifugation all of the ^{3}H-TRH remained at

Figure 10. Electron micrograph of 20,000 x g pellet from sucrose density 1.14 fraction of TSH tumor; osmium fixation, uranyl acetate and lead citrate stain (42,500 X). Line bar in lower left hand corner represents 200 mμ. Arrow points to granular structure referred to in text.

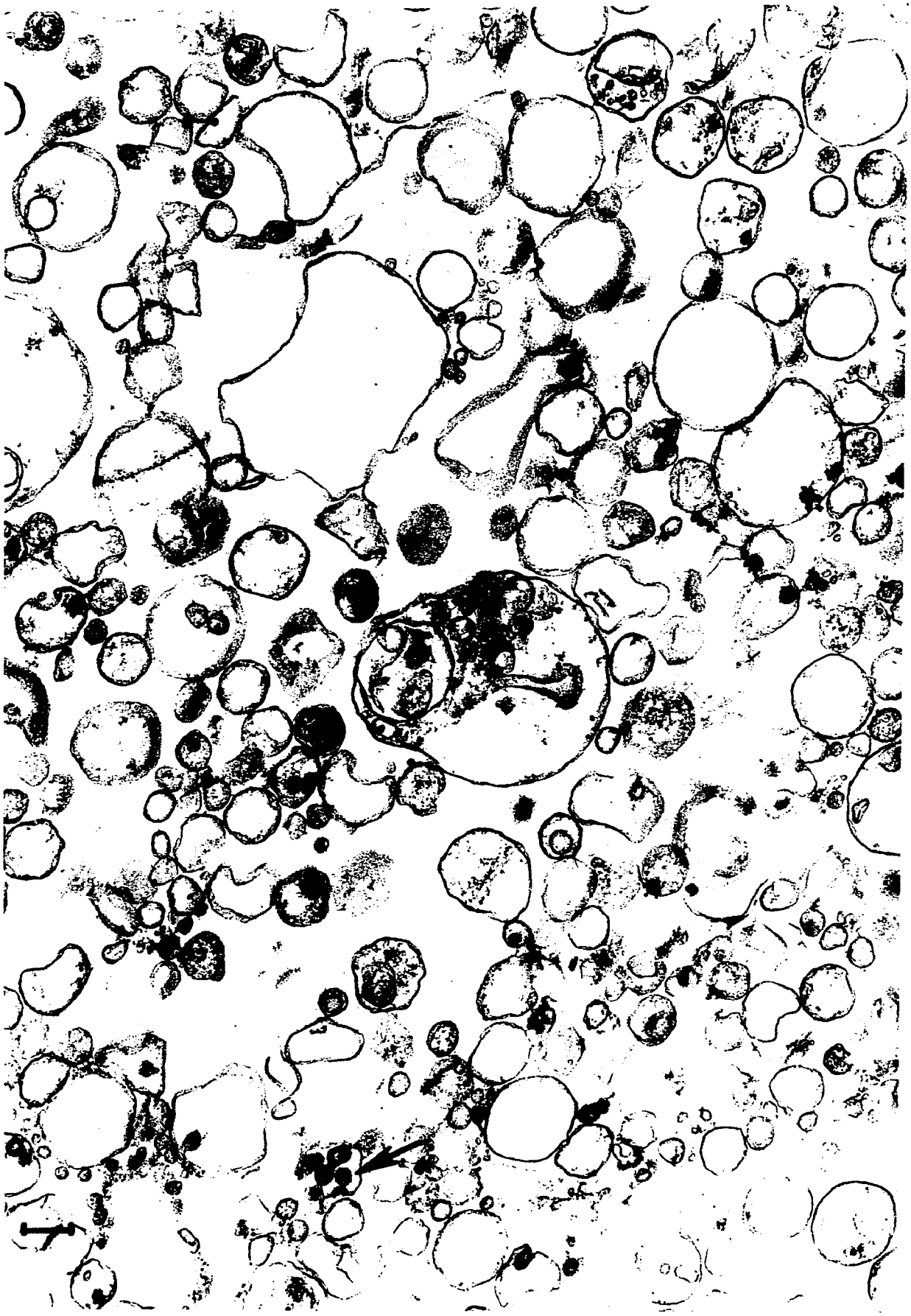


Figure 11. Electron micrograph of 20,000 x g pellet from sucrose density 1.16 fraction of TSH tumor; osmium fixation, uranyl acetate and lead citrate stain (42,500 X). Line bar in lower left hand corner represents 200 mμ. Arrow points to granular structure referred to in text.

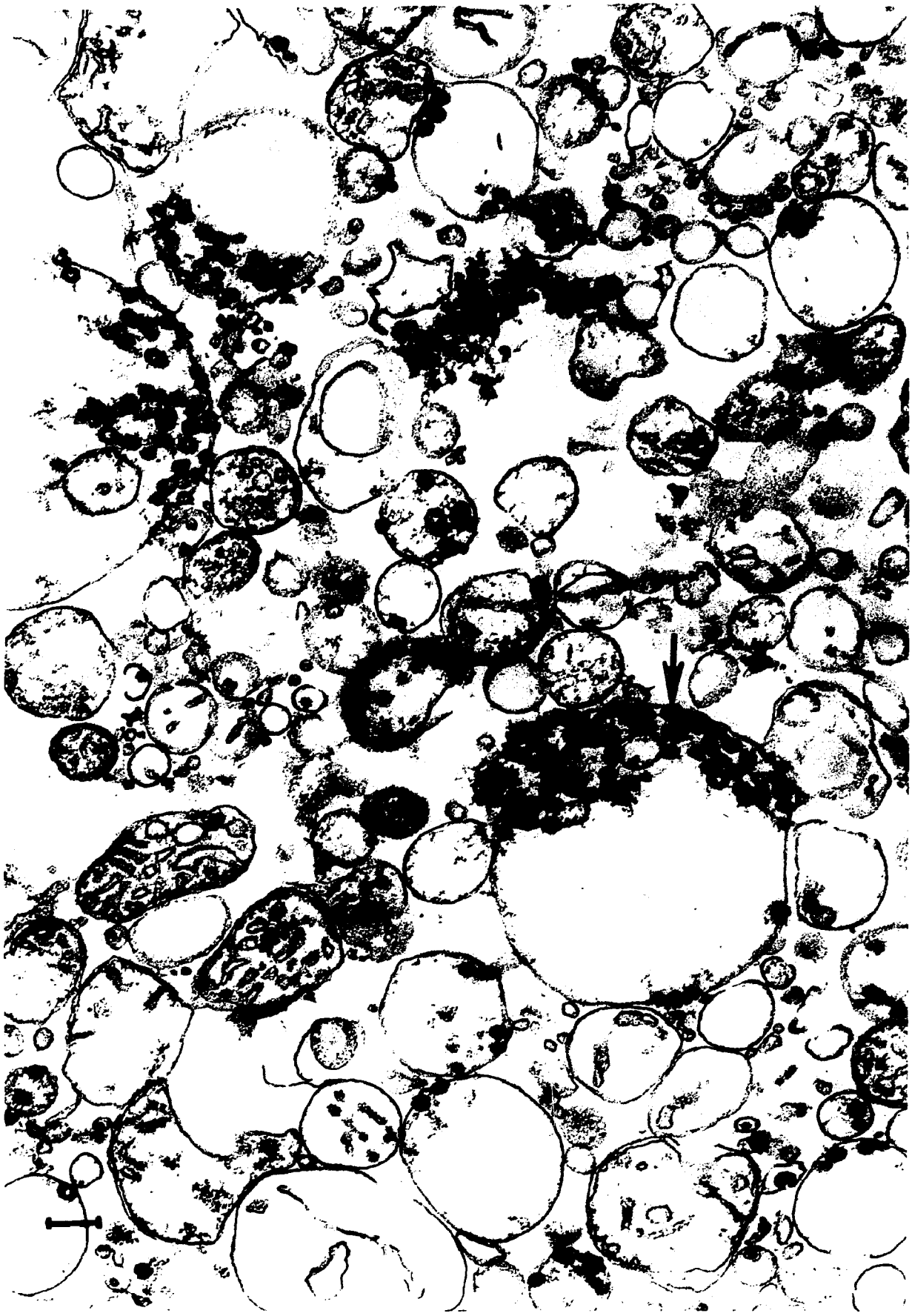
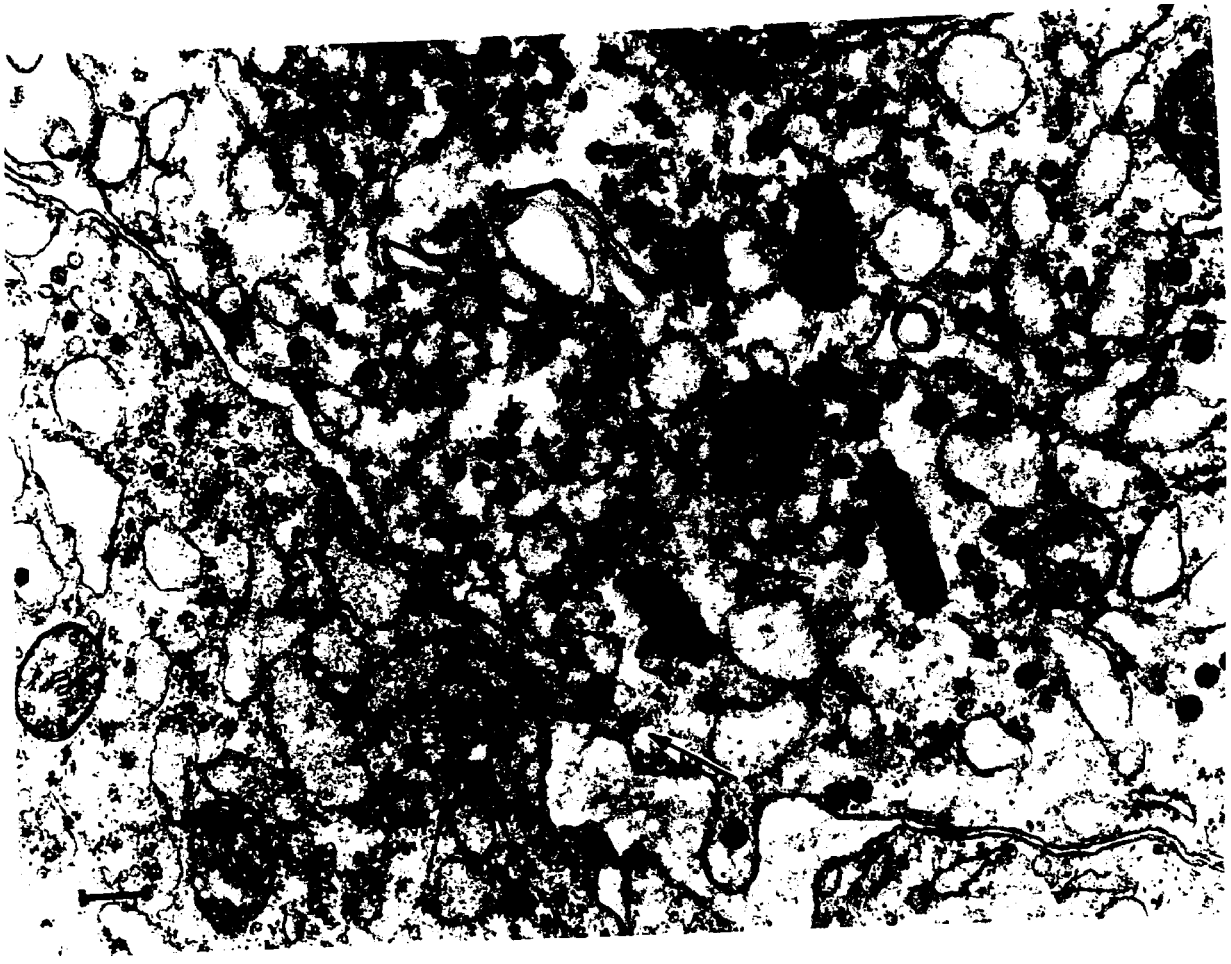


Figure 12. Electron micrograph of mouse TSH tumor; osmium fixation, uranyl acetate and lead citrate stain (32,000 X). Line bar in lower left hand corner represents 300 mμ. Arrow points to granular structure referred to in text.



the lighter end of the gradient, density 1.03 (Figure 13). There was no migration of TRH with any particulate fraction. Gel filtration of the density 1.03 fraction showed negligible binding of TRH to protein (Figure 14).

Equilibrium Filtration

Using three different concentration of ^{3}H-TRH I was never able to show positive results with this method. It is possible the lack of prior incubation and/or the low temperature (4 C) could have inhibited the TRH binding.

Figure 13. Zonal fractionation of mouse liver slice homogenate after incubation with ^{3}H-TRH. Percent transmittance (——) was recorded as the rotor was unloaded. Percent sucrose (•••) and cpm/ml (— — —) were measured on each 10 ml fraction.

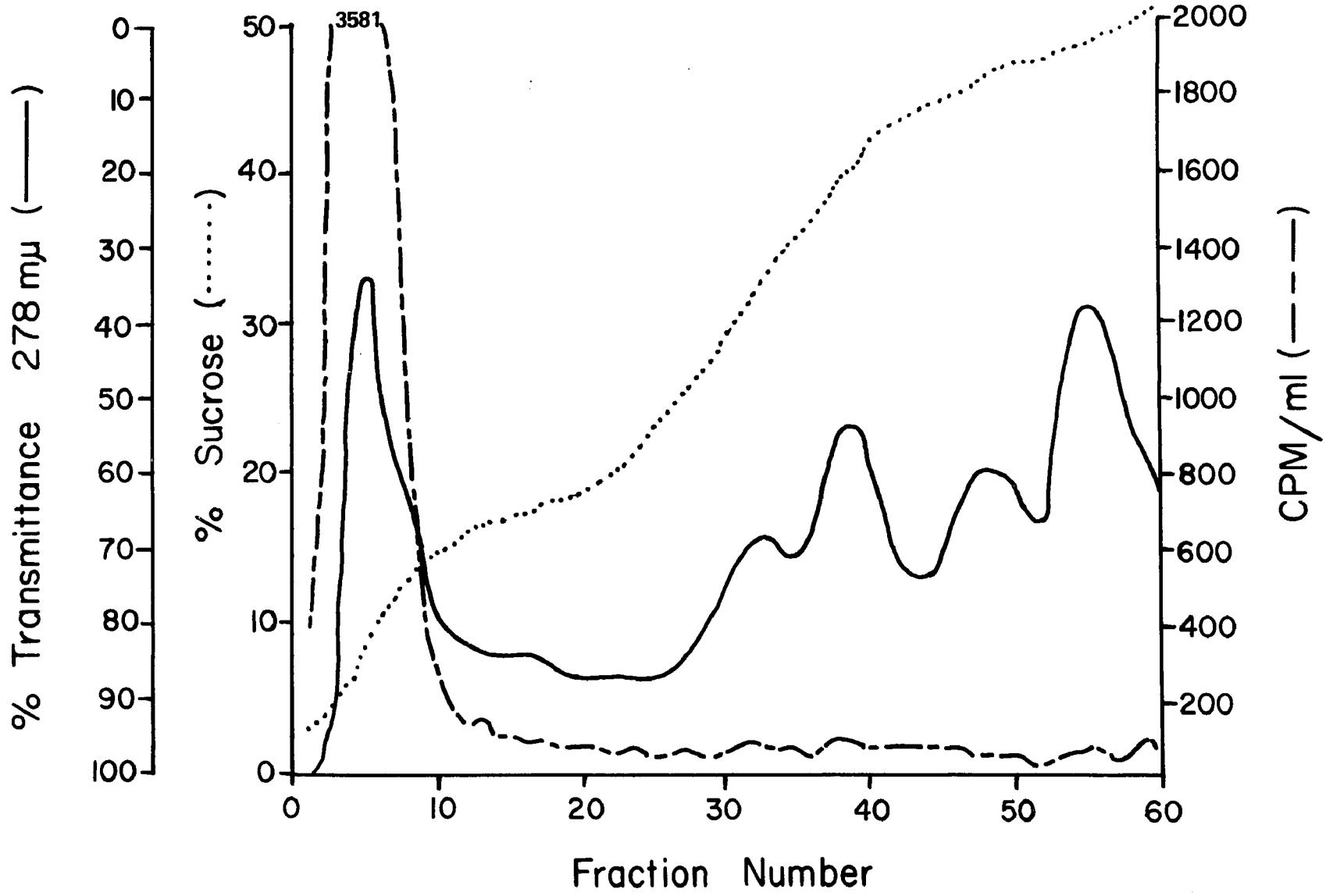
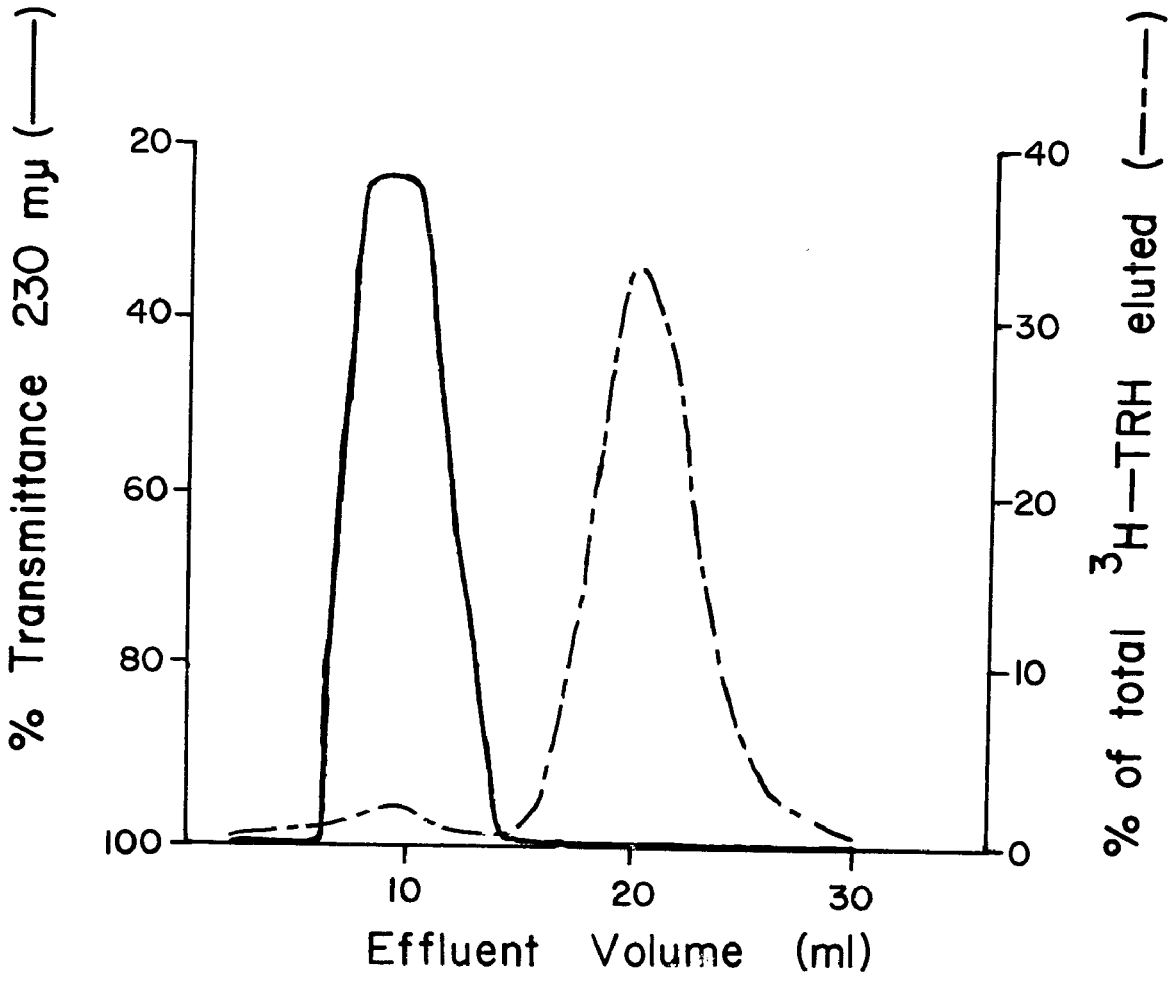

% Transmittance 278 mμ (——)
0
10
20
30
40
50
60
70
80
90
100
% Sucrose (·····)
50
40
30
20
10
0
3581
CPM/ml (— — —)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
10
20
30
40
50
60
Fraction Number

Figure 14. Gel filtration of sucrose density 1.03 fraction from zonal fractionation of liver slice homogenate. Percent transmittance (——) was measured at 230 mμ on each 2 ml fraction. ^{3}H-TRH, expressed as percent of total ^{3}H-TRH eluted (— — —), was determined by counting 1 ml of each fraction in a liquid scintillation spectrometer.


% Transmittance 230 mμ (——)
% of total ^{3}H—TRH eluted (– – –)
20
40
60
80
100
40
30
20
10
0
10
20
30
Effluent Volume (ml)

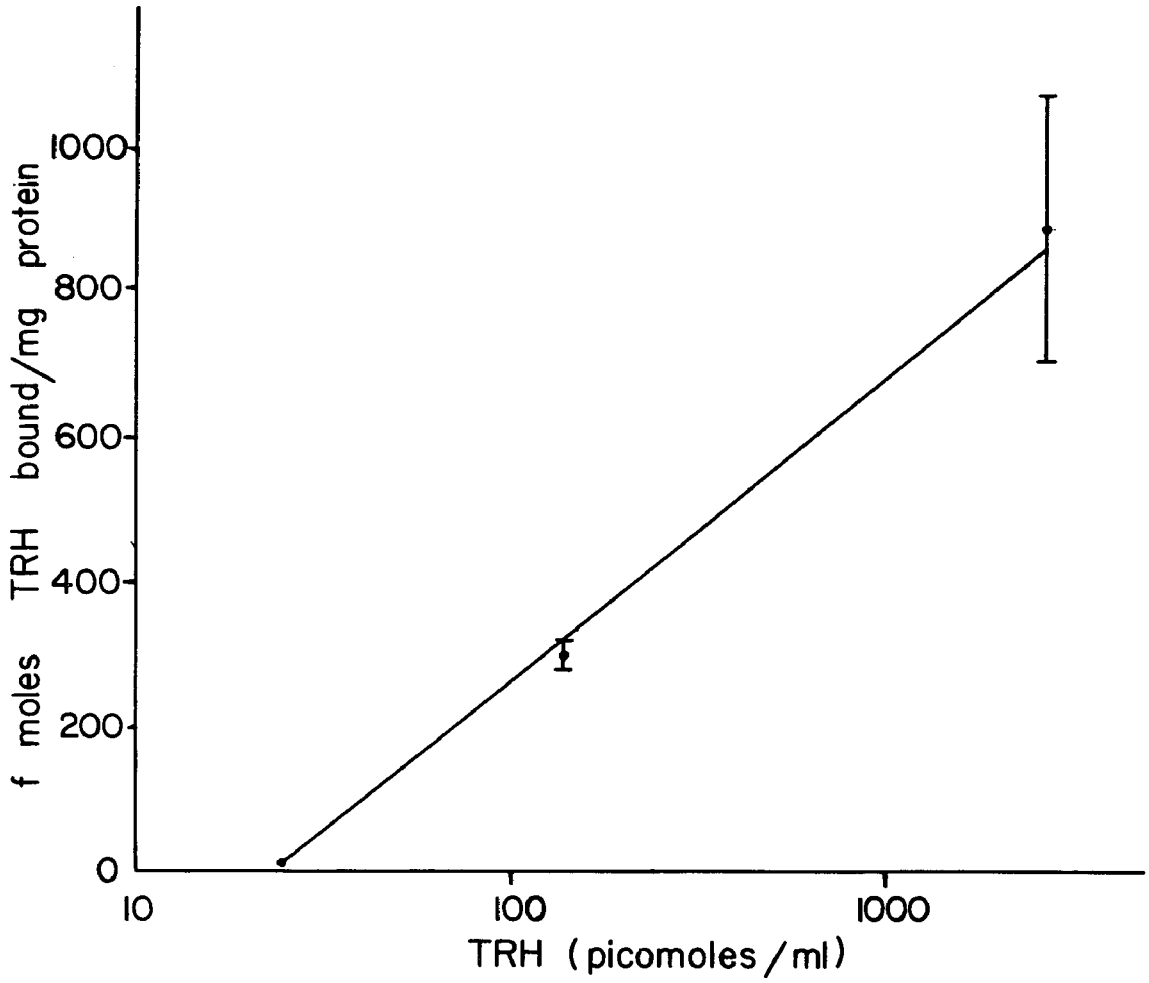
DISCUSSION AND CONCLUSIONS

TSH release from the anterior pituitary has been demonstrated to be linearly related to the log of the TRH dose both in vivo (22) and in vitro (21). The experiments reported here with rat anterior pituitaries showed that the binding of TRH might also be linearly related to the log of the TRH concentration (Figure 15); however, these results are equivocal since only three concentrations were used.

Comparison of the TRH binding to bovine and tumor plasma membrane fractions showed greater binding to the tumor plasma membranes, even though the incubation concentration was lower. This difference between the two tissues could be expected due to the lower percentage of thyrotrophs in the anterior pituitary tissue and supports the concept of specificity in binding of the hormone.

Labrie et al (31) and Grant et al (18) recently reported that plasma membranes of TRH responsive cells bind TRH. Labrie et al studied the binding of ^{3}H-TRH to plasma membranes isolated from bovine anterior pituitaries. This laboratory reported (40), in agreement with my results, greater specific binding to the material sedimenting at the interface between sucrose densities 1.14 and 1.16 than at the interface between 1.16 and 1.18. Additionally, they found that the subcellular material between density 1.16 and 1.18

Figure 15. Effect of increasing the concentration of radiolabeled TRH on the binding of the hormone to incubated quartered rat anterior pituitaries. The data are from Table 1 and the points show Mean $\pm$ S.D. At 2700 pmoles/ml both the ^{14}C- and the ^{3}H-TRH results were combined.


f moles TRH bound/mg protein
1000
800
600
400
200
0
10
100
1000
TRH (picomoles/ml)

was contaminated by particles other than plasma membranes. There was also greater fluoride-sensitive adenyl cyclase activity in the 1.14 to 1.16 fraction than in the 1.16 to 1.18 fraction. Grant et al studied TRH binding to plasma membranes isolated from TSH-secreting tumors and pituitary cells in tissue culture and noted greater binding of ^{3}H-TRH to the tumor plasma membranes compared to the pituitary cells.

The results reported in this dissertation showed that TRH binding to plasma membranes of thyrotropic cells is a physiological phenomenon in that TRH was bound to plasma membranes of intact cells in a way which survived cell disruption and zonal centrifugation. In these studies TRH has been demonstrated to bind to plasma membranes of cells of thyrotropin secreting tissues but not to those of non-TSH secreting cells from liver and cerebral cortex tissue.

My lack of success with incubation of isolated subcellular fractions with labeled TRH is unexplainable in light of the recently published reports by Labrie et al (31) and Grant et al (18). It could have been due to the low concentration of subcellular components in the incubation samples or to the low specific activity of the ^{14}C-TRH used in these experiments.

In order to state that a hormone's actions are mediated by cyclic AMP, Robison, Butcher, and Sutherland (44) have defined the following criteria which should be satisfied.

1. The hormone should stimulate adenyl cyclase in the particular tissue while hormones not active on this particular tissue should not.

2. The hormone should increase intracellular cyclic AMP levels and this increase should precede the physiological response.

3. Exogenous cyclic AMP, or a derivative, should mimic the physiological effect of the hormone.

4. The hormone should be potentiated by phosphodiesterase inhibitors.

Since adenyl cyclase is a plasma membrane enzyme, it might be fitting to add:

5. The hormone should be capable of binding specifically to the plasma membranes of responsive cells.

The first three of the above have been demonstrated for TRH. Steiner et al (50) showed stimulation of adenyl cyclase by TRH. TRH rapidly increased intracellular cyclic AMP levels (50). Dibutyryl cyclic AMP stimulated TSH release (57) and, possibly, synthesis (32, 37), in TRH responsive cells. The potentiation of TRH by a PDE inhibitor has only been implied. Theophylline increased TSH release by itself (57); however, the effects of the combination of TRH and theophylline have not been reported.

These experiments demonstrate the binding of TRH to TSH secreting cells and lend support to the sequence of events of TRH action being initial binding of TRH to the plasma membrane receptor, following which adenyl cyclase is stimulated. This stimulation increases the intracellular levels of cyclic AMP which directly control the physiological actions attributed to TRH, namely, release and synthesis of TSH.

The characterization of the receptor and enzyme macromolecules for TRH has not been accomplished; however, the two subunits have been functionally separated in other cells. Lefkowitz et al (34) demonstrated that in the absence of Ca^{++}, ACTH binding to adrenal tumor plasma membranes remained intact but ACTH stimulation of adenyl cyclase was inhibited. Since fluoride stimulation of adenyl cyclase was not inhibited in the absence of Ca^{++}, they suggested a requirement for Ca^{++} for ACTH stimulation of adenyl cyclase. Tomasi et al (53) showed a difference in thermal lability of the receptor and catalytic subunits of the glucagon responsive adenyl cyclase system in liver plasma membranes. After boiling for 1 min the adenyl cyclase activity was destroyed, but the glucagon binding was not. Boiling for 5 min destroyed the binding capability of the membrane. They also used Sephadex G-200 gel filtration of liver membranes, pretreated with sonication and in the presence of deoxycholate, to separate epinephrine receptors from epinephrine-sensitive adenyl cyclase. Application of some of these methods to thyrotropin cell plasma membranes might yield more information about the TRH sensitive receptor and catalytic subunits.

The types of binding forces between TRH and its receptor are not known; however, displacement of labeled TRH by unlabeled TRH indicates that the binding is not covalent. No experiments were designed to determine structural requirements for TRH activity, but from the literature possible conclusions can be drawn.

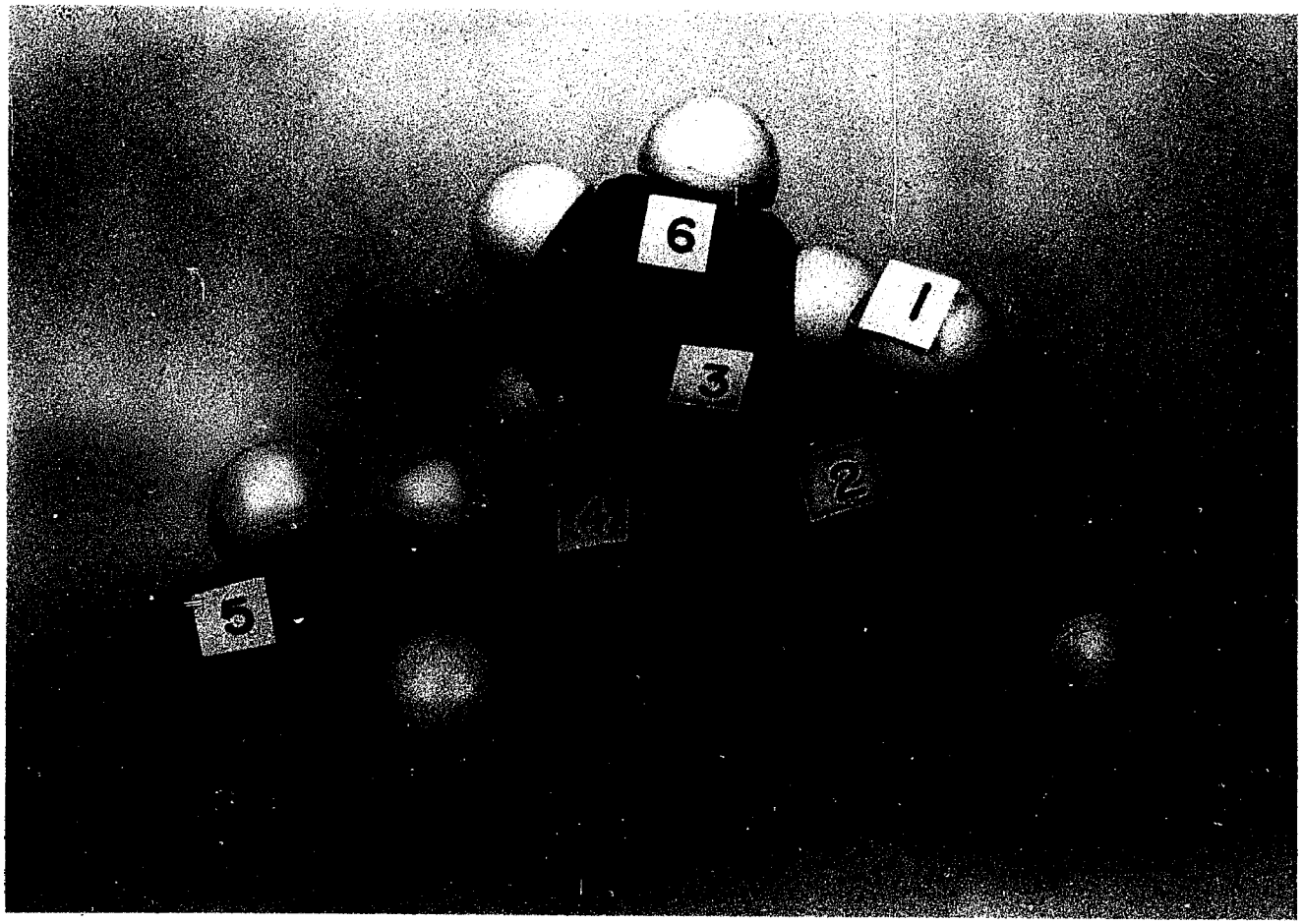
Experiments with TRH analogues have demonstrated some of the specific structural requirements for TRH activity. Bowers et al (7) reported that substitution of proline amide with alanine amide, valine amide, leucine amide, or proline methyl amide decreased the activity of the substance relative to TRH. Phenylalanine amide or glycine amide substituted analogues showed no TRH activity. Modifications of the carboxyl end of the peptide which decreased activity indicate that not only is the amide group involved in binding to the receptor but also there is a spatial requirement for this amide group.

Benzyl derivatives of the imidazole ring of histidine of the above analogues showed no activity, except the N^{im}-benzyl-TRH, which was slightly active. Inactivation of TRH by the Pauly reagent (47) (diazotized sulfanilic acid which couples to histidine in the TRH tripeptide) suggests that an unsubstituted histidine is necessary for TRH action.

Burgus et al (10) have reported that although Glu-His-Pro was not active, (pyro)Glu-His-Pro was slightly active. This same laboratory has also reported that another product of acetic anhydride treatment of Glu-His-Pro, N-acetyl-Glu-His-Pro, is not active (9). Both these reports support the theory that the cyclization of the glutamate is required for biological activity.

A stereo model construction of TRH is shown in Figure 16. The presence of several carbonyl and amino groups on one surface of the molecule allows speculation that this is the active site of the

Figure 16. Stereo model of TRH. The (pyro)glutamate is on the left; the imidazole ring of histidine, shown in the protonated state, is perpendicular to the molecule; the proline amide is on the right. Carbon, black; hydrogen, white; oxygen, red; nitrogen, blue. The numbers are explained in the text.


6
1
3
2
4
5

molecule. This model has five sites for hydrogen bonding (numbers refer to the model in Figure 16): 1 and 2, the amide N and the carbonyl C in proline amide, respectively; 3, the carbonyl C in histidine; 4 and 5, the two carbonyl C's in (pyro)glutamate. At physiological pH the imidazole ring of histidine (6 on model) would have a net positive charge, allowing for electrostatic binding to the receptor. This model is in accord with the published experimental results, but there is insufficient evidence to state that it is a correct representation of the conformation of TRH.

SUMMARY

TRH has been demonstrated to bind to plasma membranes of intact thyrotropin secreting cells. This binding is specific for the plasma membrane fraction in that TRH does not bind to any other major subcellular fraction. Since TRH actions have previously been shown to be mediated through the plasma membrane adenyl cyclase, it is quite possible that the TRH receptor on the membrane is in close association to the enzyme. The binding of TRH to its receptor, in accord with the theory proposed by Hechter and Halkerson (25), should then stimulate adenyl cyclase. The stimulation of adenyl cyclase causes an increased level of intracellular cyclic AMP which then initiates the intracellular reactions leading to the overt physiological response of the thyrotropin secreting cell.

TRH binds specifically to thyrotropin secreting tissue and not to such non-TSH secreting tissues as cerebral cortex and liver, demonstrating that the specificity of binding is for plasma membranes of thyrotropin secreting cells.

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