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The Thyroid Hormone Nuclear Receptor: Interactions with Histones and Photoaffinity Labelling

by

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DISSERTATION

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This dissertation is dedicated to my husband,

Ken,

in appreciation of his never-ending support and understanding.

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ABSTRACT

The thyroid hormone nuclear receptor is a chromatin-localized DNA-binding protein which is considered to mediate the effects of thyroid hormones on gene expression. To elucidate the receptor's role in hormone action, it is important to understand the structure and interaction of the receptor with other nuclear constituents. The current studies investigate the interaction of the receptor with histones and the receptor's structure using photoaffinity labelling techniques.

Recently it was proposed that the receptor consists of two subunits: a core-subunit which binds thyroxine (T_4) with greater affinity than triiodothyronine (T_3); and a regulatory factor, possibly a histone or histone-like protein, which confers to the core-subunit the ability to bind T_3 with greater affinity than T_4, thereby creating the holo-receptor. I have investigated the validity of the "Core-Holo" receptor model and studied the interactions of histones with the receptor. I have determined that the "Core-Holo" receptor model is incorrect and that there is no regulatory factor that determines the relative affinity of the thyroid hormone nuclear receptor for T_3 and T_4. However, histones were found to be necessary in some circumstances for obtaining measurable T_3 binding activity by the receptor and for stabilizing the affinity-purified receptor. While these features were shared by other proteins tested, the potential of histones to concentrate the receptor in the nucleus must be further investigated.

Photoaffinity-labelling studies of rat liver receptor preparations with ^{125}I-T_3 resulted in the specific labelling of a 46,000 MW protein. Several lines of evidence suggest that this protein is the

thyroid hormone receptor. First, on the basis of inhibition of photolabelling studies, the 46,000 MW protein has a 10-fold higher affinity for T_3 than T_4, which is consistent with the characteristics of the thyroid hormone receptor and correlates with the greater biological potency of T_3 as compared to T_4. Secondly, the increased intensity of the 46,000 MW protein observed when affinity-purified receptor preparations were photoaffinity-labelled correlates with the increased specific activity of the thyroid hormone receptor in these preparations. Thirdly, the 46,000 MW protein is not specifically photolabelled in rat serum, indicating that it is not a serum thyroid hormone binding protein or another serum contaminant. Additionally, there appeared to be lower levels of specifically photolabelled bands within the molecular weight range of 47,000 to 60,000. Although the identity of these proteins is not known, they may represent modified forms of the 46,000 MW protein or other gene products. Alternatively, the 46,000 MW protein may represent a proteolytic cleavage product of the higher molecular weight form.

Additional evidence supporting my claim that the 46,000 MW protein may be the thyroid hormone receptor comes from the photoaffinity labelling studies performed with GH_3 and HTC cells. A protein of similar molecular weight (48,000) was photoaffinity-labelled in GH_3 cells, which have been demonstrated to be responsive to thyroid hormones, but not in HTC cells, which have not been observed to be responsive to these hormones.

John D. Baxter
Chairman

INTRODUCTION

Thyroid hormones are important for the normal growth and differentiation of many tissues. They exert profound effects on the development of the central nervous system and the skeletal system as well as the cardiac, renal and respiratory systems. Thyroid hormones regulate numerous metabolic processes, including tissue oxygen consumption, calorigenesis, mineral balance, and the metabolism of carbohydrates, lipids, and proteins. They are therefore important in the maintenance of metabolic homeostasis (for review, see references 1 and 2).

A number of hypotheses regarding the molecular basis for thyroid hormone action have been investigated. One site of thyroid hormone action may be the plasma membrane, where thyroid hormones have been demonstrated to affect the uptake of carbohydrates and amino acid transport (3-5). Pliam and Goldfine (6) have identified high-affinity, low-capacity binding sites on the plasma membrane which may serve some receptor function or may be involved in mediating the transport of T_3 into the cell, as suggested by Cheng *et al.* (7). Thus, the exact role of the plasma membrane T_3-binding site and the actions of T_3 at the cell surface need to be defined. Another possible site of thyroid hormone action may be the mitochondrion, since thyroid hormones exert a striking effect on oxygen consumption (8). Lo and Edelman (9) have suggested that the effect of thyroid hormones on calorigenesis may result from increased levels of Na^+/K^+ ATPase molecules. However, other studies claimed that high levels of Na^+/K^+ ATPase cannot totally account for the thyroid hormone thermogenic response and that increases in flavin co-enzyme-requiring

enzymes may also be responsible for increased oxygen consumption (10). This increase in enzyme levels suggests that thyroid hormones influence calorigenesis by acting at the nuclear level. Sterling has proposed that thyroid hormones act directly on the mitochondrion through thyroid hormone binding sites on the inner mitochondrial membrane (11-13). While Sterling and collaborators have demonstrated displaceable T_3 binding to a Triton X-100 extract of a mitochondrial membrane preparation after both *in vitro* (11) and *in vivo* administration of T_3 (14), neither Oppenheimer *et al.* (15) nor Grief and Sloane (16) were able to demonstrate saturability of mitochondrial T_3 binding. Thus, the physiological significance of the mitochondrial binding sites and a direct effect of thyroid hormones on the mitochondrion have yet to be defined.

A large body of evidence suggests that thyroid hormones can regulate the expression of specific genes at the nuclear level, and correlative evidence suggests that these effects may be mediated by nuclear receptors. While nuclear receptors for thyroid hormones have been identified and are known to be intrinsic, non-histone, chromosomal proteins, the mechanisms by which these receptors mediate thyroid hormone action have yet to be elucidated. In an effort to broaden our understanding of the molecular steps involved in thyroid hormone action, I have studied the interaction of the nuclear receptor with histone proteins and have further characterized the receptor by photoaffinity labelling techniques.

A. Thyroid Hormone Effects on Gene Expression

Tata and Widnell (17) first examined the effects of thyroid hormone administration on RNA synthesis in rat liver by measuring the activity

of DNA-dependent RNA polymerase in isolated nuclei. They found that, following the administration of T_3 to hypothyroid rats, RNA polymerase I activity increased within 10 h while RNA polymerase II activity was elevated by 24 h. Similarly, Variengo et al. (18) demonstrated that the increase in nuclear RNA polymerase I activity always preceded the rise of RNA polymerase II activity. In contrast, Jothy et al. (19) observed an early, albeit transient, elevation of polymerase II activity between 40 and 80 min after thyroid hormone treatment.

Tata and Widnell (17) also studied the effects of thyroid hormones on nuclear functions by measuring the rate of precursor (^{14}C-orotic acid) incorporation into RNA. The rate of precursor incorporation was 30-40% greater than that of control within 3 - 4 h after T_3 injection into hypothyroid rats and reached 200% of control values by 16 h after hormone administration. In a more recent study, Dillmann et al. (20) found that T_3 administration to hypothyroid rats increased the rate of formation of both poly(A)-containing nuclear RNA and polysomal mRNA by 60% over control levels at the earliest time point measured (6 h). Twenty-four hours after T_3 administration, the rate of RNA formation had reached the levels found in euthyroid rats. Thus, these studies clearly established that thyroid hormones influence RNA polymerase activity and the rate of RNA formation.

To study the mechanism(s) by which thyroid hormones regulate gene expression, it is important to have a biological endpoint with which to assess its action. A number of gene products whose production is influenced by thyroid hormones have been identified: 1) α_{2u}-globulin, a protein found in the urine of adult male rats (21-23) as well as in

the submaxillary gland (24); 2) malic enzyme and α-glycerophosphate dehydrogenase, enzymes expressed in liver (25-28); 3) growth hormone (29-32); 4) Na^+/K^+ ATPase (9); and 5) β-adrenergic receptors in cardiac tissue (33). Since the majority of work in our laboratory has involved thyroid hormone regulation of the growth hormone gene, I will concentrate the rest of this discussion on its regulation.

Thyroid hormones are known to influence the production and secretion of growth hormone (GH) in the rat adenohypophysis *in vivo* (34,35). With the establishment by Yasumura *et al.* (36) of rat pituitary tumor cells in culture, the effects of thyroid hormones on GH synthesis could be studied *in vitro*. Using a subline of these cells, GH_1, Samuels and Shapiro (37) showed that the addition of thyroid hormones to medium containing serum from a thyroidectomized calf increased growth hormone production 4- to 6-fold over untreated cells. In these studies thyroid hormones appeared to affect the production of GH specifically, as the incorporation of radioactive amino acids into total proteins remained unaltered in the presence of T_3 while incorporation into GH was elevated. Similar findings have subsequently been confirmed by a number of investigators (31,32,38,39).

Using cell-free translation systems, Martial *et al.* (32) were the first to demonstrate that the T_3-mediated increase in GH synthesis was due to increased levels of GH mRNA. Using cDNA-RNA hybridization assays, these investigators subsequently confirmed that thyroid hormones influence GH mRNA levels (40). A cDNA probe prepared from partially purified GH mRNA hybridized more quickly to RNA extracted from T_3-treated cells than to RNA from untreated cells, suggesting

that thyroid hormones increase the copy number of GH mRNA molecules. Since a cDNA probe to unfractionated poly(A)$^+$ RNA showed similar kinetics when hybridized to total cytoplasmic RNA from hormone-treated or control cells, it was concluded that thyroid hormone does not increase the levels of all RNA species but exerts a specific effect on GH mRNA. Using a cloned GH cDNA probe, Wegnez et al. (41) also demonstrated that thyroid hormones given to GH_3D_6 cells increased the number of GH mRNA molecules/cell 9-fold over control values. In these studies, the increase in GH mRNA levels could be ascribed to an effect of thyroid hormones either on mRNA synthesis or on stabilization of GH mRNA, or both.

To determine whether thyroid hormones increase GH mRNA synthesis or stabilize existing GH mRNA, Spindler et al. (42) performed "nuclear run-off" experiments to quantify the number of RNA polymerase molecules engaged in transcription. In these experiments, nuclei were isolated from T_3-treated and untreated cells (GC), and those RNA polymerase molecules that had initiated transcription in vivo were allowed to continue RNA synthesis in the presence of radiolabelled ribonucleoside triphosphates. RNA was extracted and subsequently hybridized to a cloned GH cDNA probe to quantify the levels of GH mRNA. The results indicated that, by 4 h, T_3 increased the transcriptional activity of the GH gene 20-fold over control levels. This increase in transcriptional activity did not fully account for the increase in GH mRNA molecules; thus, thyroid hormones may have some effect on stabilizing GH mRNAs as well. Evans et al. (43) likewise reported an increase in GH gene transcription after T_3 exposure to GC cells.

Additional studies have shown that thyroid hormones exert pleiotropic effects on pituitary tumor cells and rat liver. Ivarie et al. (44) analyzed ^{35}S-methionine pulse-labelled proteins from T_3-treated and control GH_3D_6 cells by two dimensional gel electrophoresis and demonstrated that thyroid hormones modulated the synthesis of 15 proteins. The synthesis of 10 proteins was induced and that of 5 was repressed. Thus, approximately 1% of greater than 1000 gene products which were detected by two-dimensional gels were sensitive to T_3. Seelig et al. (45) extracted poly(A)$^+$ RNA from T_3-treated and control rat liver and subsequently translated the RNA in a reticulocyte/lysate translation system in the presence of ^{35}S-methionine. Analysis of these products on two-dimensional gels demonstrated that T_3 stimulated the RNA activity of 11 genes and inhibited the activity of 7 genes. In this study, thyroid hormones influenced 8% of the 231 translation products detected. In a separate study, 8 of the 18 mRNA sequences responsive to thyroid hormone were also shown to be increased by the administration of growth hormone (46). Thus, in the studies of Seelig et al. (45) some of the effects of thyroid hormones were due to its influence on increasing growth hormone production, which subsequently increased specific gene sequences. Thus, thyroid hormones appear to have multiple effects on pituitary tumor cells and rat liver.

B. Nuclear Receptors for Thyroid Hormones

1. Detection of Nuclear Receptors

The first evidence for the probable existence of specific nuclear receptors for thyroid hormones was provided by Oppenheimer et al.

(15). In their studies, rat liver and kidney cells were fractionated after in vivo injection of the animals with a small amount of ^{125}I-T_3 and increasing quantities of non-radioactive T_3. They found that the proportion of ^{125}I-T_3 bound to the nuclear fraction decreased with increasing unlabelled T_3 concentrations, suggesting specific binding. Such specific T_3 binding activity was not detected in the mitochondrial, microsomal or cytosolic fractions. In an earlier study, Schadlow et al. (47) found evidence for specific T_3 binding in the rat pituitary, but not in rat liver, brain or kidney. These investigators did not subfractionate the rat tissues and therefore may have failed to detect specific T_3 binding in liver, brain and kidney due to a large amount of non-receptor binding. The specific nuclear binding sites for T_3 were localized to chromatin, while non-specific binding within the nucleus was demonstrated by Surks et al. (48) to be present on the outer nuclear membrane. The receptor's interaction with chromatin will be discussed below.

Samuels and Tsai (49) subsequently demonstrated the existence of specific T_3 and T_4 binding sites in the nuclei of GH_1 cells. These binding sites are probably similar to those of rat liver, as they exhibit similar equilibrium dissociation constants for T_3 binding (1.65×10^{-10}M and 2.1×10^{-10}M for pituitary cells and rat liver, respectively) (50). Other laboratories have since confirmed the presence of putative nuclear receptors for thyroid hormones (51-54).

Based on the following evidence, these nuclear binding sites for thyroid hormones are considered to represent receptors that are

involved in mediating some of the actions of thyroid hormones:

i. The sites bind thyroid hormones with an apparent dissociation constant similar to physiologically active concentrations of these hormones.

ii. The relative binding affinities of a number of thyroid hormone analogs parallel their biological potencies.

iii. Binding sites are found in thyroid hormone-responsive tissues and cells.

iv. Hormonal occupancy of these sites is correlated with a biological response.

v. The localization of these sites in the nucleus is consistent with the findings that thyroid hormones regulate gene expression.

Since these data are correlative and therefore do not constitute proof that the binding sites identified are actual thyroid hormone receptors, it is worthwhile to examine these correlations in greater detail.

2. Binding of Various Thyroid Hormone Analogs by the Nuclear Receptor

The relative binding affinities of a number of thyroid hormone analogs for nuclear binding sites have been shown to parallel their biological potency. The nuclear binding of T_3 and T_4 has been studied in a number of conditions: in the intact rat (55); in rat pituitary tumor cells in culture (49); and in isolated nuclei and solubilized nuclear extracts from various tissues (56-64). In all instances, the affinity of T_3 for nuclear binding sites was 10-fold

greater than that of T_4. This higher affinity of T_3 correlates with its greater physiologic potency and underscores the possible physiological relevance of the nuclear receptors. In vivo binding studies indicated that analogs such as isopropyl T_2, tetrac, triac and reverse T_3 also have relative binding activities that correlate with their biological potency when their metabolism, fractional removal rates and distribution are taken into account (55). In an in vitro study, the relative affinities of 35 thyroid hormone analogs for nuclear binding sites were also found to be in excellent agreement with their reported thyromimetic potencies (55).

3. Tissue Distribution of Receptors

Specific nuclear binding sites for thyroid hormones have been found in thyroid hormone-responsive tissues such as the pituitary, liver, kidney, lung, heart, cerebral cortex, and thyrotropic cells (65-68). They are found only in low concentration in the spleen and testis, which are not known to be responsive to thyroid hormones (65). In the case of cell culture systems, both the GH clonal cell lines and H35 cells, developed from Reuber hepatoma H35, contain thyroid hormone receptors and are responsive to thyroid hormones (44,69). In contrast, cells (HTC) developed from a Morris hepatoma (7288C) do not contain significant levels of intranuclear T_3 binding sites and are not known to be responsive to T_3 (69).

4. Relationship of Hormonal Occupancy and Biological Response

The occupancy of nuclear receptors by thyroid hormones has been correlated with biological responses in a number of studies. Oppenheimer et al. (70) examined the relationship between the occupany of

rat liver nuclear sites with T_3 and the induction of the hepatic enzymes, α-glycerophosphate dehydrogenase (α-GPD) and malic dehydrogenase. When receptor sites were more than 95% saturated, the rate of enzyme accumulation was found to be maximal and, as fewer sites were desaturated due to decreasing plasma T_3 levels, the accumulation of new enzyme ceased. When the relationship between fractional nuclear occupancy and biological response was examined, the induction of α-GPD and malic dehyrogenase was not linearly related to nuclear occupancy (71). At 100% occupancy, the rate of hepatic enzyme induction was 10- to 20-fold greater than the rate of induction at euthyroid levels of occupancy (47% saturation). Therefore, with increasing receptor occupancy there was an amplification of enzyme synthesis by the liver. The mechanism underlying the apparent amplification of enzyme induction in the liver is unknown. In contrast, the induction of GH production in the rat pituitary was linearly related to receptor occupancy, when 30-70% of the sites were occupied (72). Samuels et al. (73) also found that T_3 occupancy of nuclear sites in GH_1 cells was linearly related to the rate of GH synthesis. From these results it appears that there may be differences in the way thyroid hormone receptors mediate hormonal action in the liver compared with the pituitary. Nonetheless, the data are consistent with an involvement of these receptors in both tissues in the actions of thyroid hormone.

5. Physical Characteristics of the Solubilized Nuclear Receptor

As mentioned previously, the thyroid hormone nuclear receptor has been localized to chromatin. For further characterization, the receptor can be solubilized from chromatin by salt extraction of nuclei.

Surks et al. (48) demonstrated that up to 70% of the nuclear receptor could be extracted from rat liver nuclei with 0.4 M KCl, and this has since become a standard receptor solubilization procedure used in several laboratories (74,75). Latham et al. (63) used 0.2 M $(NH_4)_2SO_4$, commonly used to extract RNA polymerase from chromatin, to solubilize receptors from rat liver nuclei. Salt extraction of rat liver and GH_1 cell nuclei yields a receptor species that sediments in sucrose density gradients at 3.5S and 3.8S, respectively (63,76).

Gel exclusion chromatography of the salt-extracted receptor from rat liver nuclei on Sephadex G-100 indicated that the receptor has a molecular weight of between 50,000 - 70,000 (49). Based on this finding and that of equilibrium density gradient centrifugation, the receptor's Stokes' radius has been calculated to be 35 Å and the frictional ratio (f/f_o) to be 1.4, indicating that the receptor is slightly asymmetric (63). Characterization of the GH_1 cell nuclear receptor demonstrated that it has a molecular weight of 54,000, a Stokes' radius of 33 Å, and a frictional ratio of 1.212 (76,77). Nuclear extracts from rat liver have been affinity labelled with N-bromo-acetyl-^{125}I-T_3 or ^{125}I-T_4 and subsequently analyzed on SDS-polyacrylamide gels (78). Although control experiments were lacking (which precludes the conclusion that the radioactive protein band observed in these studies is the thyroid hormone receptor), the studies indicated that the major binding protein has a molecular weight of 56,000. Radiation inactivation studies performed on rat liver chromatin preparations demonstrated that the receptor has a molecular weight of 59,000 (79).

Treatment of the receptor with proteolytic enzymes destroys its thyroid hormone binding activity, whereas nuclease treatment is without effect, confirming its protein nature (48,74). The receptor is retained by a column of DEAE-Sephadex, an anionic exchanger, and not by the cation exchanger BioRex 70, suggesting that it is an acidic protein (62,64). Additionally, the receptor was shown to be less sensitive to digestion by trypsin than chymotrypsin. Finally, the isoelectric point of the receptor, as reported by Apriletti et al. (80), was found to be 5.8. Therefore, the receptor appears to be an acidic chromatin protein with an apparent molecular weight between 50,000 and 70,000 and a slightly asymmetric shape.

6. Purification of the Nuclear Thyroid Hormone Receptor

Investigators have attempted to purify the rat liver nuclear receptor; to achieve homogeneity, a 25,000- to 50,000-fold purification is needed . Silva et al. (62) developed a purification procedure that included, as a first step, the dialysis of nuclear extracts to precipitate non-essential proteins. This was followed by chromatography of the dialyzed extract on a column of DEAE-Sephadex. Using this procedure, they achieved a 60- to 125-fold purification of the rat liver receptor. Utilizing successive chromatographic steps on Sephadex G-100, DEAE-Sephadex and DNA-Sepharose, Torresani and Anselmet (81) obtained rat liver receptor preparations which were purified 280-fold. Additionally, Nikodem et al. (78) have achieved a 100-fold purification using high-pressure liquid chromatography.

To date, the most highly purified receptor preparations have been obtained by Apriletti et al. (64) using affinity chromatography. The

affinity support matrix used by these investigators was prepared by coupling T_3 through its amino group to the terminal primary amino groups of diaminohexane-Sepharose using the deactivated ester of glutaric acid (64,82). The purification scheme initially involves the chromatography of nuclear extracts on a column of Sephadex G-100, which results in an included receptor fraction that has been purified up to 6-fold. Chromatography of the partially purified receptor on the affinity matrix first and followed by separation on a column of DEAE-Sephadex results in a final purification of approximately 500-fold. Although the best purification has been obtained with this procedure, a further 50- to 100-fold purification is required to achieve homogeneity.

7. The Receptor is an Intrinsic Nuclear Protein

The thyroid hormone receptor appears to be an intrinsic nuclear protein based on the following evidence. Binding proteins with characteristics similar to those of the nuclear receptor have not been detected in the cytoplasm of rat liver or GH cells (15,49,53,54). Cytosolic thyroid hormone binding sites have been found, but these proteins have affinities for the hormones that do not correlate with their biological potencies. However, the possibility that a small number of thyroid hormone binding proteins similar to the nuclear receptor are present in the cytoplasm cannot be excluded as they may have escaped detection due to a high background of binding contributed by low-affinity, high-capacity sites.

The thyroid hormone nuclear receptor is associated with the nucleus in the presence or absence of the hormone. As an example, the amount

of nuclear T_3 binding present after *in vivo* administration of radioactive T_3 is similar to the amount measured when isolated nuclei or nuclear extracts not previously exposed to T_3 are incubated with radioactive T_3 in *in vitro* binding assays (52,83). Furthermore, euthyroid and hypothyroid rats, which exhibit differing serum concentrations of thyroid hormones, have equivalent concentrations of hepatic thyroid hormone nuclear receptors when assayed *in vitro* (52). These results suggest that hormone-mediated translocation of the thyroid hormone receptor from cytoplasm to nucleus does not occur and that the nuclear receptor is an intrinsic chromosomal protein.

8. Interactions of the Receptor with Chromatin and DNA

The distribution of thyroid hormone receptors within chromatin has been studied with a number of methods. Charles *et al.* (83) used formaldehyde, which fixes histones and about 10% of non-histone chromosomal proteins to DNA (84), to fix receptors to chromatin. When HeLa cell chromatin containing ^{125}I-T_3-receptor complexes was treated with formaldehyde, radioactivity was fixed to DNA; the investigators suggested that the radioactivity represented ^{125}I-T_3-receptor complexes. Additionally, the radioactivity was observed to co-migrate on sucrose density gradients with slowly sedimenting chromatin, which was characterized as containing a low ratio of protein to DNA and most of the endogenous RNA polymerase activity found in the nucleus (83). These results suggested that the T_3-receptor was closely associated with DNA and appeared to be distributed non-randomly within chromatin.

A chromatin fractionation procedure established by Gottesfeld *et al.* (85,86) has been used to determine if thyroid hormone receptors

reside in transcriptionally active or inactive chromatin. Incubation of chromatin with DNase II preferentially digests chromatin enriched in actively transcribed gene sequences. Using this method, Levy and Baxter (87) found only small differences in the distribution of thyroid hormone receptors between active and inactive chromatin fractions from GC cells. In contrast, Samuels et al. (88) demonstrated an eight-fold enrichment of thyroid hormone receptors in the active chromatin fraction of GH_1 cells. In a more recent study, Levy-Wilson (89) used micrococcal nuclease to form transcriptionally active (nuclease-sensitive) and inactive (nuclease-resistant) fractions from GH_3 cells. Hybridization of the digested chromatin to a rat GH cDNA probe demonstrated that the transcriptionally active region was enriched for GH sequences and for thyroid hormone receptors.

Nucleases have also been employed to characterize the organization of the receptor itself. Analysis of micrococcal nuclease digestion products by sucrose density gradient sedimentation indicated that the rat liver nuclear receptor was excised as an approximately 6.0S form (90-92). Similar experiments performed with GH_1 cell nuclei produced an abundant 6.5S species and a less abundant 12.5S species (76). The 6.5S receptor form is converted to a 4.9S form upon further digestion with DNase I and to a 3.8S form with salt treatment. The data accumulated to date suggest that the 6.5S species represents the 3.8S receptor in association with linker DNA and/or other chromatin components, while the 12.5S form represents the receptor in association with a mononucleosome having intact linker DNA attached. Based on kinetic studies, Samuels et al. (1) suggested that the 6.5S and 12.5S forms

come from different chromatin domains. He noted that the 12.5S species was formed rapidly, attained a maximal level by 10 min of digestion, and remained at this level for the duration of the experiment. In contrast, formation of the 6.5S form occurs less rapidly and the amount of the 6.5S form increases with time in parallel with the excision of mononucleosomes from chromatin. Samuels et al. (1) suggested that the 12.5S receptor form is derived from a chromatin domain that is exceedingly sensitive to micrococcal nuclease digestion. The investigators did not entertain the possibility that the 6.5S species is formed from the 12.5S species.

The 6.5S form has been characterized in greater detail. The sedimentation profile of the 6.5S form with increasing salt concentrations from 10 mM to 200 mM KCl exhibits a stepwise decrease in the sedimentation coefficient from 6.5S at 10 mM KCl to 5.3S at 100 mM KCl and finally to 3.9S at 200 mM KCl (77). This stepwise reduction in sedimentation coefficient indicates that the 3.9S form is associated with at least two other components that are progressively and sequentially disaggregated by high-salt conditions. One of these components appears to be a DNA fragment because complete digestion of the 6.5S form with DNase I produces the 3.8S form. Furthermore, Perlman et al. (77) observed that the 6.5S species, when sedimented through a D_2O-containing sucrose gradient, has a density value that falls between that of protein and DNA. A comparison of the density value for the 6.5S receptor with the density values of protein and DNA suggests that the 6.5S species contains 15% DNA and 85% protein. The molecular weights of the 6.5 and 3.8S forms were calculated using their respective

sedimentation coefficients, Stokes' radii (calculated from their elution characteristics on Sepharose CL-6B), and estimated partial specific volumes. The molecular weights are 149,000 for the 6.5S form and 54,000 for the 3.8S receptor form. Thus, the 6.5S species, which contains 15% DNA and 85% protein, is calculated to have a DNA component of 22,000 MW (equivalent to 36 base pairs) and a protein component of 127,000 MW. Perlman et al. (77) proposed that the 6.5S form consists of a 54,000 MW receptor in dimeric form alone or in association with other unique protein components bound to 35-40 base pairs of DNA. The nature of these proteins has not yet been determined.

MacLeod and Baxter (93,94) demonstrated that partially purified receptor preparations bind to DNA in cell-free assays. The solubilized receptor was bound equally well by native or denatured DNA and by DNA from a variety of eukaryotic species as well as from prokaryotes. These studies suggested that the receptor itself possesses a DNA binding site. However, the possibility cannot be excluded that the receptor does not have a DNA binding site and that it interacts with a DNA binding protein present in the nuclear extracts, causing the receptor to bind to DNA. Recently, Dr. Apriletti of our laboratory has demonstrated that the affinity-purified receptor binds to regions of the GH gene.

9. Modulation of Nuclear Receptor Levels

Samuels et al. (73,76,88,95,96) have reported that thyroid hormone administration to GH_1 cells alters the concentration of nuclear receptors in a dose- and time-dependent manner. Incubation of cells with high doses of T_3 for 24 h decreased receptor numbers by as much

as 60%, while growth hormone synthesis, measured by immunoprecipitation of ^{14}C-leucine-labelled proteins, was found to be at its highest level. Thus, after being in the presence of T_3 for 24 h, receptor levels were at their lowest and growth hormone synthesis was maximal, suggesting to Samuels and collaborators that the thyroid hormone receptor may be acting as a repressor (73). In this way, thyroid hormone action would be secondary to hormone-mediated receptor depletion and be a result of de-repression of a specific nuclear response; however, there is no evidence to support this notion. As a matter of fact, Samuels et al. (88) subsequently reported that GH_1 cells grown under high-cell-density conditions had low receptor levels as well as diminished levels of growth hormone synthesis. These data suggest that the induction of growth hormone synthesis by T_3 was proportional to the number of T_3-receptor complexes and are not consistent with the repressor model. Clearly, the mechanism(s) by which the nuclear receptor mediates thyroid hormone effects on gene expression is not understood.

Based on an investigation of the half-life of thyroid hormone nuclear receptors, Samuels et al. suggested that receptors from GH_1 cells actually constitute two functional classes, a depletable and non-depletable fraction (96). Alternatively, it appears that the results could be due to the simple establishment of a new steady state. Both high concentrations of T_3 and high-cell-density growing conditions reduced the depletable receptor fraction (88,96). Further evidence for the existence of functionally different classes of thyroid

hormone nuclear receptors comes from studies which probed the effect of butyrate on these receptors.

Butyrate, when given to GH_1 or GH_3 cells, decreased the concentration of nuclear thyroid hormone receptors to 20% of control levels (76,97). Samuels et al. (76) suggested that this reduction was due to a lowered affinity of chromatin for the receptor due to an increase in the extent of histone acetylation caused by butyrate. While Samuels et al. (76) did not report an effect of butyrate on total protein synthesis in GH_1 cells, Eberhardt et al. (97) noted that longer-term butyrate treatment of GH_3 cells decreased overall protein synthesis. Two-dimensional gel electrophoretic analysis indicated that a number of specific proteins were induced or repressed. Therefore, Eberhardt and investigators (97) suggested that butyrate may have a direct effect on repressing receptor synthesis in the GH_3 cells. Eberhardt et al. (97) noted that, even though receptor levels were decreased by butyrate treatment, T_3 given to butyrate-treated GH_3 cells or T_3 given simultaneously with butyrate to GH_3 cells resulted in equivalent rates of GH synthesis as compared with administration of T_3 alone. This led them to propose that a subclass of receptors, those remaining after butyrate treatment, was responsible for inducing GH synthesis. In order to determine whether the receptors remaining after T_3-induced receptor depletion and/or butyrate-induced receptor reduction mediate the action of thyroid hormone on GH gene expression, the instantaneous rate of GH mRNA should be measured and correlated with receptor levels. In preliminary experiments conducted by Dr. Nancy Lan (University of California, San Francisco; personal communication),

the levels of pre-GH mRNA paralleled the level of T_3-receptor complexes (i.e., when receptor number was reduced due to the presence of high concentrations of T_3, the amount of pre-GH mRNA was also reduced). These preliminary findings suggest that the depletable receptor fraction represents the physiologically active receptors.

10. Evidence for a Receptor Subunit with a Different Hormone-Binding Specificity

In general, nuclear extracts prepared from either rat liver or GH_1 cells contain equivalent concentrations of T_3 and T_4 binding sites as determined by Scatchard analysis (49,63). Additionally, ^{125}I-T_3 binding by nuclear extracts is inhibited by unlabelled T_4, and ^{125}I-T_4 binding is completely inhibited by both unlabelled T_3 and T_4, with T_3 being the more avid competitor (49,58,63,65,75). Thus, in nuclear extracts, both T_3 and T_4 appear to be bound by the same protein(s) with a higher affinity for T_3 than T_4. However, Latham et al. (63) observed that, following chromatography of rat liver nuclear extracts on QAE-Sephadex, a component not detected in the original extract was present which bound T_4 more avidly than T_3. In view of the evidence supporting the concept that T_3 and T_4 are bound by the same protein, this result suggested that a property of the receptor important for T_3 binding activity was lost during purification.

In order to study this effect, Eberhardt et al.(98,99) performed a number of experimental manipulations of nuclear extracts, and their influences on T_3 and T_4 binding were examined. When nuclear extracts were heated for various times at 50°C, there was a marked

loss of T_3 binding activity but essentially no loss of T_4 binding activity. Scatchard analysis of heat-treated extracts indicated that the number of high-affinity T_4 binding sites and their affinity for T_4 were unaltered compared to control experiments; however, the concentration of high-affinity T_3 binding sites was reduced by 90%. Competition studies indicated that the ability of T_4 to inhibit ^{125}I-T_4 binding in heated extracts was similar to control values, but the ability of T_3 to inhibit ^{125}I-T_4 binding had been reduced 1000-fold. When extracts were acidified to pH 6.0, essentially similar results were observed: at pH 7.6, the number of T_3 and T_4 binding sites was equivalent; at pH 6.0, the concentration of high-affinity T_4 binding sites exceeded that for T_3. Results from competition studies demonstrated that T_4 inhibited ^{125}I-T_4 binding at pH 6.0 and at pH 7.6 to the same extent, while the ability of T_3 to inhibit ^{125}I-T_4 binding at pH 6.0 compared to pH 7.6 was greatly reduced. Thus, both heating and acidification appear to affect the receptor's ability to bind T_3 with high affinity but not its ability to bind T_4 with high affinity.

It was considered unlikely that heating and acidification led to a loss of a binding species which preferentially bound T_3, but not T_4, because the original T_3 binding species also bound T_4. Furthermore, the number of residual T_4 binding sites and their affinity for T_4 after these manipulation were the same as that of the T_3/T_4 binding species in the original extract. It was also considered unlikely that heating and acidification destroyed the receptor and simultaneously generated a new T_4 binding protein.

sedimentation coefficients, Stokes' radii (calculated from their elution characteristics on Sepharose CL-6B), and estimated partial specific volumes. The molecular weights are 149,000 for the 6.5S form and 54,000 for the 3.8S receptor form. Thus, the 6.5S species, which contains 15% DNA and 85% protein, is calculated to have a DNA component of 22,000 MW (equivalent to 36 base pairs) and a protein component of 127,000 MW. Perlman et al. (77) proposed that the 6.5S form consists of a 54,000 MW receptor in dimeric form alone or in association with other unique protein components bound to 35-40 base pairs of DNA. The nature of these proteins has not yet been determined.

MacLeod and Baxter (93,94) demonstrated that partially purified receptor preparations bind to DNA in cell-free assays. The solubilized receptor was bound equally well by native or denatured DNA and by DNA from a variety of eukaryotic species as well as from prokaryotes. These studies suggested that the receptor itself possesses a DNA binding site. However, the possibility cannot be excluded that the receptor does not have a DNA binding site and that it interacts with a DNA binding protein present in the nuclear extracts, causing the receptor to bind to DNA. Recently, Dr. Apriletti of our laboratory has demonstrated that the affinity-purified receptor binds to regions of the GH gene.

9. Modulation of Nuclear Receptor Levels

Samuels et al. (73,76,88,95,96) have reported that thyroid hormone administration to GH_1 cells alters the concentration of nuclear receptors in a dose- and time-dependent manner. Incubation of cells with high doses of T_3 for 24 h decreased receptor numbers by as much

as 60%, while growth hormone synthesis, measured by immunoprecipitation of ^{14}C-leucine-labelled proteins, was found to be at its highest level. Thus, after being in the presence of T_3 for 24 h, receptor levels were at their lowest and growth hormone synthesis was maximal, suggesting to Samuels and collaborators that the thyroid hormone receptor may be acting as a repressor (73). In this way, thyroid hormone action would be secondary to hormone-mediated receptor depletion and be a result of de-repression of a specific nuclear response; however, there is no evidence to support this notion. As a matter of fact, Samuels et al. (88) subsequently reported that GH_1 cells grown under high-cell-density conditions had low receptor levels as well as diminished levels of growth hormone synthesis. These data suggest that the induction of growth hormone synthesis by T_3 was proportional to the number of T_3-receptor complexes and are not consistent with the repressor model. Clearly, the mechanism(s) by which the nuclear receptor mediates thyroid hormone effects on gene expression is not understood.

Based on an investigation of the half-life of thyroid hormone nuclear receptors, Samuels et al. suggested that receptors from GH_1 cells actually constitute two functional classes, a depletable and non-depletable fraction (96). Alternatively, it appears that the results could be due to the simple establishment of a new steady state. Both high concentrations of T_3 and high-cell-density growing conditions reduced the depletable receptor fraction (88,96). Further evidence for the existence of functionally different classes of thyroid

hormone nuclear receptors comes from studies which probed the effect of butyrate on these receptors.

Butyrate, when given to GH_1 or GH_3 cells, decreased the concentration of nuclear thyroid hormone receptors to 20% of control levels (76,97). Samuels et al. (76) suggested that this reduction was due to a lowered affinity of chromatin for the receptor due to an increase in the extent of histone acetylation caused by butyrate. While Samuels et al. (76) did not report an effect of butyrate on total protein synthesis in GH_1 cells, Eberhardt et al. (97) noted that longer-term butyrate treatment of GH_3 cells decreased overall protein synthesis. Two-dimensional gel electrophoretic analysis indicated that a number of specific proteins were induced or repressed. Therefore, Eberhardt and investigators (97) suggested that butyrate may have a direct effect on repressing receptor synthesis in the GH_3 cells. Eberhardt et al. (97) noted that, even though receptor levels were decreased by butyrate treatment, T_3 given to butyrate-treated GH_3 cells or T_3 given simultaneously with butyrate to GH_3 cells resulted in equivalent rates of GH synthesis as compared with administration of T_3 alone. This led them to propose that a subclass of receptors, those remaining after butyrate treatment, was responsible for inducing GH synthesis. In order to determine whether the receptors remaining after T_3-induced receptor depletion and/or butyrate-induced receptor reduction mediate the action of thyroid hormone on GH gene expression, the instantaneous rate of GH mRNA should be measured and correlated with receptor levels. In preliminary experiments conducted by Dr. Nancy Lan (University of California, San Francisco; personal communication),

the levels of pre-GH mRNA paralleled the level of T_3-receptor complexes (i.e., when receptor number was reduced due to the presence of high concentrations of T_3, the amount of pre-GH mRNA was also reduced). These preliminary findings suggest that the depletable receptor fraction represents the physiologically active receptors.

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This would require that the formation of a T_4 binding protein be quantitatively and qualitatively coupled to the loss of T_3 binding activity. Studies of the inhibition of T_4 binding by iodoacetamide indicated that the binding protein(s) exhibited identical inhibition characteristics before and after heat treatment, suggesting that a new T_4 binding protein was not generated. Thus, the results are most consistent with the possibility that the receptor was being altered in such a way that its high-affinity T_3, but not T_4, binding was affected.

Further studies demonstrated that dilution of partially purified receptor preparations (nuclear extracts purified by chromatography of Sephadex G-100), also resulted in a loss of T_3, but not T_4, binding activity (99). When a comparison was made between the concentration of hormone binding sites and the protein concentration of the receptor preparation, it was observed that the concentration of T_4 binding sites was linearly related to protein concentration. However, the number of high-affinity T_3 binding sites varied non-linearly at low protein concentrations and approached linearity at higher protein concentrations. Thus, the authors suggested that dilution as well as heating and acidification promoted the dissociation from the receptor of some factor important for the maintenance of high-affinity T_3 binding.

Conditions were sought which would reconstitute high-affinity T_3 binding from a receptor fraction which had lost this activity. The addition of heated nuclear extracts to diluted partially purified receptor preparations increased the concentration of T_3-binding

sites to a level that equalled, but did not exceed, the concentration of T_4 binding sites. No significant influence on the number of T_4 binding sites was observed. Treatment of the heated nuclear extracts with either DNase or RNase did not change its ability to stimulate T_3 binding activity. Likewise, the addition of DNA or RNA to diluted receptor preparations did not stimulate binding. However, treatment with trypsin obliterated this stimulatory ability, suggesting that proteins were required for reconstitution. The nature of the protein-protein interaction was studied by testing the ability of ovalbumin, poly(L)-lysine, cytochrome C or lysozyme to reconstitute T_3 binding. None of these polypeptides or proteins stimulated T_3 binding activity. However, the addition of a protein preparation enriched for core histones (H2A, H2B, H3 and H4) did increase the concentration of T_3 binding sites to a level equaling but not exceeding the concentration of T_4 binding sites (99). The addition of histones did not affect the affinity of these sites for T_3, nor did they significantly change the concentration of T_4 binding sites or their affinity for T_4. Therefore, it appeared that the chromatin factor(s) responsible for conferring high-affinity T_3 binding activity on the receptor was a histone or histone-like protein. These results led to the proposal that the receptor (holo-receptor) consisted of at least two subunits, a core-receptor and regulatory factor. The core receptor was found to bind T_4 more avidly than T_3. Upon association with the regulatory factor, a histone or a histone-like species, the core-receptor was converted to the holo-receptor, which binds thyroid hormones with affinities that parallel their thyromimetic potencies. The holo-receptor was therefore considered to be the physiologically important receptor.

C. Rationale for the Thesis Project

1. Analysis of the Interaction of Histones with the Intranuclear Thyroid Hormone Receptor

The "Core-Holo" receptor model provides a possible explanation for the lack of observable receptor translocation from cytoplasm to nucleus. The model suggests that the mature nuclear receptor is synthesized in the cytoplasm as the core-receptor, which has different binding characteristics from the holo-receptor. This may explain why a binding protein with the characteristics of the holo-receptor has not been identified in the cytoplasm. Additionally an investigation of the model may identify the protein components that compose the regulatory factor. Furthermore, an understanding of the receptor's interaction with the chromosomal protein and in turn the interaction of that complex with DNA may help elucidate the mechanism resulting in thyroid hormone effects on gene expression. Therefore, it was my intention: 1) to test the validity of this model; 2) if correct, to characterize the regulatory factor; and 3) to study the interaction of the core-receptor with this factor and the interaction of the complex with the genome.

2. Photoaffinity Labelling of the Thyroid Hormone Nuclear Receptor

Photoaffinity labelling is a new approach which is being used to study hormone-receptor interactions. The hormone (ligand), which is derivatized with a photoreactive group, is incubated with a preparation of the corresponding receptor and subsequently irradiated.

Irradiation leads to the formation of covalent bonds between the photosensitive group of the hormone, which in the case of peptide hormones is either a diazo- or azido-function, and reactive chemical groups of the receptor or other nearby protein components (100-102).

This technique has been used to investigate the molecular weight and subunit structure of the receptors for corticotropin (ACTH) (103), epidermal growth factor (104), insulin (105), parathyroid hormone (PTH) (106), melanocyte-stimulating hormone (107), cholecystokinin (108), angiotensin II (109), β-adrenergic agonists (110), glucagon (111), ecdysterone (112), glucocorticoids (113) and progesterone (114, 115). Recently, investigators have begun studying the functional consequences of the covalent attachment of hormones to target tissues. Ramachandran et al. (116) showed that photolysis of (2-nitro-5-azido-phenyl-sulphenyl)-Trp^9-ACTH led to the persistent activation of corticosterone production. Likewise, the photo-induced covalent attachment of (2-nitro-5-azido-phenyl-sulphenyl)-bovine PTH to the PTH receptor on canine renal plasma membranes resulted in the activation of adenylate cyclase (106). An irreversible discharge of secretory proteins from guinea pig pancreatic acini was obtained when the cholecystokinin receptor was covalently modified with a photoreactive analog of cholecystokinin octapeptide (108). Additionally, the ability to photoaffinity-label the insulin receptor has enabled investigators to demonstrate that the radioactive insulin localized to the plasma membrane of adipocytes disappeared upon heating, suggesting that the receptors were internalized (117-119).

Photoaffinity labelling of thyroid hormone binding proteins has been demonstrated by Somack et al. (120), using the photoaffinity

analog N-(ethyl-2-diazo-malonyl)thyroxine (EDM-T_4). These investigators demonstrated that irradiation of ^{125}I-EDM-T_4 in the presence of thyroxine-binding prealbumin (TBPA) at 254 nm resulted in covalent linkage of ^{125}I-EDM-T_4 to TBPA. This photolabelling was totally dependent on light and was demonstrated to be specific for high-affinity hormone binding sites since labelling was inhibited when thyroid hormone analogs such as T_3, T_4 and tetraiodothyroacetic acid were present during photolysis. The mechanism for photolabelling was assumed to involve photo-induced conversion of the diazo group of EDM-T_4 to a reactive carbene with subsequent insertion into C-H bonds. However, after destruction of the diazo group by pre-irradiation, ^{125}I-EDM-T_4 was found to label TBPA after further irradiation; this carbene-independent photo-attachment was also specific for a high-affinity hormone binding site. It was proposed that the mechanism for the carbene-independent process may involve attachment via radical formation following photo-induced loss of thyronine ring iodine. This hypothesis was supported by the recent demonstration by van der Walt and Cahnman (121) that irradiation of thyroxine at 330 nm leads to progressive deiodination, with the initial iodine being removed from the phenolic ring. On the basis that T_4 itself may be photo-reactive, Somack et al. (120) irradiated TBPA in the presence of ^{125}I-T_4 at 254 nm and obtained specific photo-induced labelling of TBPA. As a result of the demonstration that ^{125}I-T_4 could serve as a photo-reactive probe for a thyroid hormone binding protein, I intended to determine: 1) whether ^{125}I-T_3 could serve as a photo-reactive probe for the thyroid hormone nuclear receptor; 2) the molecular weight of the receptor; and 3) the receptor's isoelectric point.

II. MATERIALS AND METHODS

A. Histone Protein Isolation and Purification

1. Materials and Buffers: Buffer A: 0.25 M sucrose, 10 mM Tris•HCl (pH 7.0), 50 mM sodium bisulfite, 0.24 mM spermine, 2 mM $MgCl_2$, 1 mM PMSF; Buffer B: 20 mM Tricine•HCl (pH 7.6), 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% Triton X-100; Buffer C: 0.14 M NaCl, 50 mM sodium bisulfite (pH 4.0), 1 mM PMSF; Buffer D: 50 mM sodium bisulfite, 1 mM PMSF. Fresh bovine thymus glands were obtained from McDermott Meat Co., Berkeley, California.

2. Isolation of Histones from Bovine Thymus: Histones were isolated according to the procedure of R. D. Cole, Berkeley, CA. (personal communication). 1200 gm frozen tissue was pulverized and washed with 1 L of Buffer A (warmed to 37°C). The following steps were all carried out at 4°C. The buffer was decanted and the tissue was added to 1.5 L of fresh cold Buffer A. This mixture was homogenized for 15 sec at each of the four speeds of a Waring Blender and then twice for 2 min using a Polytron (Brinkman Instruments). The homogenate was then diluted to a total volume of 3.0 L, and it was strained successively through 1, 2, 4, 6 and finally 8 layers of cheesecloth. The homogenate was centrifuged for 10 min at 700 x g. The supernatant was discarded and the resulting nuclear pellet was weighed and suspended in a volume of Buffer D equal to the pellet's weight (i.e., 1 ml buffer/g tissue). To this nuclear suspension was added 20% TCA with stirring until a final concentration of 5% was reached. The nuclei were extracted for 15 min and the suspension was then centrifuged for 10 min at 8000 x g. Both the supernatant and

pellet were saved. The pellet was further extracted by stirring with 200 ml 5% TCA for 15 min. Following centrifugation, this supernatant was combined with the previously prepared supernatant and filtered through a coarse scintered glass funnel, without suction. This filtrate, which contained histone H1, was dialyzed extensively versus water, lyophilized, and stored at -20°C. The pellet was extracted with 200 ml of 0.25 M HCl for 15 min and then centrifuged for 10 min at 8000 x g. The supernatant was saved and the pellet was re-extracted with 0.25 M HCl. Both supernatants were combined and filtered through a coarse scintered glass funnel prior to dialysis and subsequent lyophilization. This supernatant fraction was enriched in the core histones (H2A, H2B, H3 and H4) and is referred to in the following text as the core histone extract.

3. Separation of Histones on Bio-Gel P-60: A combination of the reported methods of Bohm et al. (122), Westhuyzen et al. (123), and von Holt and Brandt (124) was followed with some minor modifications. Approximately 60-85 mg of lyophilized core histone extract was dissolved in 4.0-4.5 ml of a freshly prepared 8 M urea-1% β-mercaptoethanol solution and stored overnight at 4°C. The sample was applied to a column of Bio-Gel P-60 (2.5 x 160 cm), which had been equilibrated with 0.2 N HCl-0.02% NaN_3 (pH 1.7). The column was eluted with HCl-NaN_3 buffer at room temperature. The pressure head of the buffer reservoir was maintained at 40 cm and the column's flow rate was adjusted to 30 ml/hr. Column fractions were monitored for histones by their absorbance at 230 nm. Chromatography resulted in the separation of the crude histone extract into four fractions.

These four fractions contained H1, H2A-H3, H2B and H4, respectively, and were pooled, dialyzed against water and subsequently lyophilized.

4. Purification of H2A and H3 by Sephadex G-100 Chromatography: H2A was separated from H3 by chromatography on Sephadex G-100, as described by Bohm et al. (122), Westhuyzen et al. (123), and von Holt and Brandt (124) with the following modifications. Approximately, 20-60 mg of lyophilized H2A-H3 from Bio-Gel P-60 chromatographic fractions was dissolved in 2-4 ml of 0.05 M sodium acetate-0.05 M sodium bisulfite (pH 5.4) and stored at 4°C overnight. The sample was applied to a column of Sephadex G-100 (2.5 x 160 cm) and eluted with sodium acetate-sodium bisulfite at room temperature. The pressure head of the buffer reservoir was maintained at 40 cm and the column's flow rate was adjusted to 50 ml/hr. The H2A fraction, which eluted after the H3 fraction, was effectively separated from H3. Both fractions were dialyzed against water before lyophilization.

5. Preparation of UV-Transparent Guanidine: 1 kg of guanidinium carbonate (Gu_2CO_3, MCB, practical grade) was dissolved in 2 L of distilled water with stirring for 30 min at room temperature and then placed in a 37°C bath for 2-4 hr. The suspension was filtered through two layers of filter paper to remove undissolved particulate matter and the filtrate was stirred overnight with 110 gm of activated charcoal. The charcoal suspension was filtered through 4 layers of filter paper on top of a 1-inch layer of moist diatomaceous earth. To the clear filtrate was added 6 L of chilled absolute ethanol to precipitate Gu_2CO_3. The precipitation process was carried out for 2 days at 4°C. The precipitated Gu_2CO_3 was collected by filtration on a Buchner funnnel and washed with 2 L of cold absolute ethanol

and air-dried overnight. The dried precipitate was mixed with a small quantity of distilled water and concentrated HCl was added until the pH of the solution reached 1.0. The solution was left overnight to allow complete evolution of CO_2. The pH of the solution was adjusted to 6.8 with concentrated NaOH and the final volume was measured. Solid $Na_2HPO_4 \cdot H_2O$ and Na_2HPO_4 were added to a final concentration of 0.05 M each (total phosphate concentration, 0.1 M) and the pH was adjusted to 6.8. The concentration of guanidine•HCl was measured by refractive index, using standard solutions of guanidine•HCl for comparison. Guanidine•HCl in 0.1 M $NaPO_4$ buffer will be referred to as GuCl-PO_4.

6. Purification of H2A and H3 by Bio-Rex 70 Chromatography: The procedure of Spring and Cole (125) for the fractionation of total histones was used to separate H2A from H3, with the following modifications. Approximately 7 mg of H2A/H3, a fraction resulting from purification of total core histones on Bio-Gel P60, was dissolved in 1 ml 8% GuCl-PO_4-1 mM PMSF and applied to a column of Bio-Rex 70 (1.6 x 60 cm) equilibrated to 8% GuCl-PO_4. The column was washed with 100 ml 8% GuCl-PO_4 and the proteins were eluted first with 450 ml of a linear 8-15% GuCl-PO_4 gradient, followed by two separate washes of 200 ml of 15% GuCl-PO_4 and then 120 ml 40% GuCl-PO_4 at approximately 20 ml/hr. H2A eluted from the column at approximately 9.5% GuCl, while H3 eluted at the transition between the 15% and 40% GuCl-PO_4 washes. These two fractions were dialyzed against water and lyophilized.

7. Purification of H2B by Bio-Rex 70 Chromatography: The procedure of Spring and Cole (125), with the following modifications, was

used to purify H2B (Bio-Gel Fraction III) further . Approximately 7 mg of BioGel Fraction III (H2B) was dissolved in 1.5 ml 7% GuCl-PO_4 and applied to a column of Bio-Rex 70 (1.6 x 60 cm) equilibrated with 7% GuCl-PO_4. The column was washed with 100 ml 7% GuCl-PO_4 and protein eluted with a 320-ml linear 8-13% GuCl-PO_4 gradient at a flow rate of 20 ml/hr. H2B which eluted at approximately 10.5% GuCl was dialyzed against water and lyophilized.

B. Purification of Thyroid Hormone Nuclear Receptor

1. Buffers: Solution A: 0.34 M sucrose, 2 mM $MgCl_2$, 0.24 mM spermine; Solution B: 2.1 M sucrose, 6.5 mM $MgCl_2$, 0.1 mM spermine; Buffer C: 20 mM Tricine•HCl (pH 7.6), 2 mM $CaCl_2$, 1 mM $MgCl_2$ containing 5% Triton X-100; Buffer D: 20 mM Tris•HCl (pH 8.0), 0.25 M sucrose, 1 mM EDTA, 0.1 mM dithiothreitol (DTT), 15% glycerol; Buffer E: 30 mM Tris•HCl (pH 8.0), 1 mM EDTA, 30% glycerol; Buffer G: 50 mM sodium phosphate (pH 7.6), 0.2 M ammonium sulfate, 1.0 mM EDTA, 0.2 mM DTT, 5% glycerol; Buffer H: Buffer G containing 30% glycerol.

2. Isolation of Rat Liver Nuclei: Frozen rat livers obtained from PEL-FREEZE (Rogers, Arkansas) were pulverized in a liquid nitrogen-cooled Waring blender in two 500-gm batches and subsequently thawed in 2 L Solution A (warmed to 37°C). Once thawed, the livers were maintained on ice and all procedures were carried out at 4°C. 1 kg of rat livers was washed three times with 667 ml cold Solution A. 3 L of Solution B was added to the drained livers and the mixture was homogenized for 1.5 min at full speed, allowed to cool for 1 min, and then homogenized for 1 min with a Polytron (Brinkman Instruments).

The homogenate was filtered through a laminate of 8 layers of cheesecloth and 1 layer of Miracloth (Calbiochem). Nuclei were isolated from the filtrate by continuous flow (7-8 L/hr) in a TZ-28 rotor (DuPont/Sorvall) at 18,000 rpm. The rotor initially contained 500 ml water underlayered with 800 ml 2.1 M sucrose, which was re-oriented by slow rotor acceleration before starting homogenate flow. When all the homogenate had been processed through the rotor, the residual homogenate was displaced with 0.53 M sucrose. Nuclei were washed by resuspending in Buffer C, which effectively removed the outer nuclear membrane, and centrifuging for 10 min at 2000 x g in a GSA rotor (Dupont/Sorvall). The nuclear pellet was resuspended in Buffer D to a volume of approximately 25 ml and stored in liquid nitrogen.

3. Preparation of Nuclear Extract: Nuclei from 1 kg of rat liver were thawed and resuspended to a volume of 120 ml with Buffer D. DTT was added to a final concentration of 10 mM. The suspension was divided in half and to each half was added 3 ml 4 M $(NH_4)_2SO_4$. Nuclear suspensions were sonicated twice for 15 sec, separated by 1 min of ice cooling, using 85 watts average power (Model 185; Heat Systems Ultransonics, Inc.; Plainview, N.J.). The sonicated mix was centrifuged for 3 hr at 55,000 x g. The supernatant, referred to as Fraction II (FII), was directly applied to a column of Sephadex G-100.

4. Partial Purification of Fraction II (FII) by Sephadex G-100 Chromatography: Approximately 100 ml of FII was chromatographed on a column of Sephadex G-100 (5 x 90 cm) (at $4^{\circ}C$), which had been equilibrated with Buffer G-0.2 mM DTT. The column was eluted with Buffer G at a flow rate of approximately 100 ml/hr. Generally, 0.100 ml from

each column fraction was analyzed for receptor content by standard binding assays, as described (Materials and Methods B-8). Chromatography on Sephadex G-100 resolved the nuclear extract into two fractions of T_3 binding activity, Peak I and Peak II. Peak I (which eluted in the void volume) contained 90% of the protein and between 30% and 45% of T_3 binding activity, while the included fraction, Peak II, contained the majority of T_3 binding. Peak II was pooled and fresh DTT was added to a final concentration of 0.2 mM DTT and stored in liquid nitrogen.

5. Affinity Chromatography of Partially Purified Receptor Preparations: T_3 affinity resins were prepared by Dr. James Apriletti of our laboratory. Partially purified receptor preparations were applied to the T_3 affinity matrix at room temperature. Generally, 10 to 20 ml of receptor preparation containing 4000 fmol T_3 binding sites was applied per ml bed volume at a flow rate of approximately 3 to 7 ml/ml bed volume/hr. The column was washed with three different buffers at a flow rate of approximately 10 ml/ml bed volume/hr as follows: 1) 2 to 5 column volumes of Buffer G and 50 µg/ml core histone extract; 2) 5 to 10 column volumes of Buffer H containing 0.3 M ammonium sulfate and 50 µg/ml histones; 3) 2 to 5 column volumes of Buffer H containing 0.1 M ammonium sulfate and 50 µg/ml core histones. The column was eluted with 6 to 9 column volumes of Buffer H containing 0.1 M ammonium sulfate, 50 µg/ml core histones and 10^{-7} M T_3 at a flow rate of approximately 2.5ml/ml bed volume/hr. Fractions (5.5 ml) were collected, 0.5 ml was removed for binding assays, and the remainder was frozen in liquid nitrogen.

6. Removal of Free T3 from the Affinity-Purified Receptor: Before binding assays could be performed on column fractions from affinity gel chromatography, free T_3 had to be removed. Free T_3 was removed by passing 0.4 ml of the column eluates over a column of Sephadex G-25 (1.8 ml bed volume) prepared in Pasteur pipettes and equilibrated at room temperature with Buffer G. The column was washed with 0.4 ml Buffer G and the protein was collected in 0.8 ml Buffer G. Specific ^{125}I-T_3 binding activity was measured by the standard binding assay (Materials and Methods).

7. DEAE-Sephadex Chromatography of Affinity-Purified Receptor: To remove histones from affinity-purified receptor preparations, chromatography on DEAE-Sephadex was performed according to the method of Apriletti et al. (64). As a first step, the salt concentration of affinity-purified receptor preparations was lowered by filtration over Sephadex G-25 assay columns (Materials and Methods) equilibrated with Buffer E. The Sephadex G-25 column eluate was applied to a small column of DEAE-Sephadex A-25 (0.5 to 1.0 ml bed volume) equilibrated with Buffer E. The column was washed with several column volumes of Buffer E and the receptor was eluted with either a linear gradient from 0 to 0.4 M NaCl in Buffer E or stepwise with 0.2 M NaCl in Buffer E, at a flow rate equal to gravity.

8. Binding Assay: Total hormone binding was measured in incubation mixtures containing receptor, ^{125}I-T_3 (high specific activity, 500 to 1200 mCi/mg; or carrier-free, 2400 to 3400 mCi/mg) or ^{125}I-T_4 (high specific activity, 600 to 1500 mCi/mg, New England Nuclear) and Buffer G in a total volume of 0.5 ml. Incubations were

generally performed for 4 hr at room temperature or until equilibrium was reached (unpublished observation). The samples were then chilled and 0.4 ml portions were filtered over Sephadex G-25 assay columns (Materials and Methods, B-6) at 4°C. The columns were washed with 0.4 ml of Buffer G, and the excluded peak containing bound ^{125}I-T_3 or ^{125}I-T_4 was eluted with 0.8 ml of Buffer G. Non-specific binding was measured by adding a 1000-fold excess (or as otherwise stated) of non-radioactive T_3 or T_4 to parallel incubation mixtures. Specific binding was calculated by subtracting the amount of non-specifically bound hormone from the total hormone bound . When hormone binding was measured in affinity-purified preparations, incubations were performed at 25°C to allow for exchange of bound T_3 with ^{125}I-T_3.

9. Stimulation Assay (Effect of Histones on Hormone Binding): Stimulation assays were performed by incubating histones, receptor preparations, ^{125}I-T_3 or ^{125}I-T_4 in the presence or absence of unlabelled hormone brought to a final volume of 0.5 ml. The ionic strength of the buffer was adjusted to be equivalent to 0.15 M NaCl with a combination of Buffer G (Section B-1) and 0.28 M Na(PO_4). Assays were incubated at 22°C for 4 hr and subsequently chilled on ice for 10 min. Aliquots (0.4 ml) were filtered over Sephadex G-25 assay columns according to the method outlined above.

C. Cell Culture

1. Maintenance of GH_3W5 and Cultured Hepatoma Cells (HTC): GH_3W5 cells were obtained from the American Type Culture Collection, Rockville, Maryland, and HTC cells were obtained from the Cell Culture

Facility, University of California, San Francisco. Both cell lines were maintained as monolayer cultures in Dulbecco's Modified Eagle's Medium containing 4.5 g glucose in 500 ml (DME-21), 10% calf serum (Rockland Inc.; Gilbertsville, Pennsylvania) 100 μg/ml streptomycin sulfate, 60 μg/ml of penicillin (100 U/ml) and 2 mM glutamine. This nutrient medium will be referred to as complete medium. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed every 3 days.

2. Buffers for the Preparation of Receptor-Containing Nuclear Extracts From Cells: Buffer I: 0.25 M sucrose, 20 mM Tris•HCl (pH 7.85), 1.1 mM $MgCl_2$,; Buffer J: Buffer I containing 0.5% Triton X-100; Buffer K: 0.4 M KCl, 20 mM Tris•HCl (pH 7.8), 1 mM EDTA, 5 mM DTT; PBS: 1 mM $CaCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2 \cdot 6H_2O$, 137 mM NaCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$ (pH 7.4); PBS-CMF: PBS without $CaCl_2$ and $MgCl_2$.

3. Preparation of GH_3W5 and HTC Cell Nuclear Extracts for Photoaffinity Labelling: Prior to the preparation of nuclear extracts from GH_3 or HTC cells, their complete medium was replaced with medium without fetal calf serum for 3 hr to facilitate the dissociation of T_3 contained in fetal calf serum from T_3 receptors. At the end of this 3-hr period, the cells were placed on ice and their medium was aspirated. All subsequent procedures were carried out at 4°C. Cells were washed three times with PBS-CMF and lysed by incubation with Buffer J. The resultant cellular suspension was centrifuged for 10 min at 800 x g to obtain nuclear pellets. Nuclei were further washed once with Buffer J and once with Buffer I; this procedure produced purified intact nuclei devoid of their outer nuclear

membrane. Nuclei were extracted by incubation with Buffer K for 30 min. Generally, nuclei isolated from an 80% confluent T-150 flask were incubated with 4.5-6.0 ml Buffer K. The nuclear suspension was subsequently centrifuged in an Eppendorf microcentrifuge (Brinkman Instruments) for 10 min to separate the nuclear extracts from chromatin. Nuclear extracts were stored in liquid nitrogen.

4. Partial Purification of GH_3W5 and HTC Cell Nuclear Extracts: Partial purification of GH_3 and HTC cell nuclear extracts was obtained by chromatography on Sephadex G-100 as described in Materials and Methods, B-4, with the following changes. 5 ml of nuclear extract, adjusted to 10 mM DTT, was applied to a column of Sephadex G-100 (1.5 x 90 cm) equilibrated with Buffer G-0.2 mM DTT and subsequently eluted with Buffer G-0.2 mM DTT. Column fractions were analyzed for T_3 binding activity as described in Materials and Methods. A similar elution profile was obtained for GH_3 cell nuclear extracts as for rat liver nuclear extracts. Column fractions comprising PII were pooled and stored in liquid nitrogen.

Samples of HTC cell nuclear extracts were chromatographed on Sephadex G-100 by a procedure similar to that followed for GH_3 cell nuclear extracts. T_3 binding assays were not performed on the column fractions because Ivarie et al. (69) have shown that HTC cells lack thyroid hormone receptors. Thus, the same column fractions that constitute GH_3 cell PII were pooled to form HTC cell "PII." These samples were stored in liquid nitrogen.

D. Photoaffinity Labelling

1. Photoaffinity Labelling of Soluble Extracts: 180-230 µg of a partially purified receptor preparation (PII) was incubated with

^{125}I-T_3 (carrier-free, 2400 to 3400 mCi/mg; New England Nuclear) with or without excess non-radioactive T_3 or T_4 in a total volume of 2.3 ml for 3.5 hr at room temperature. The samples were chilled and 0.4 ml of the incubation mixture was filtered over a Sephadex G-25 assay column to determine the amount of ^{125}I-T_3 bound. The remainder of the sample was irradiated with either 254 nm or 300 nm light by using an RPR 2537 Å or RPR 3000 Å lamp, respectively. For irradiation at 254 nm, the samples were placed in quartz cuvettes and irradiated in a Rayonet mini-photochemical reactor for 100 sec at a distance of 2.5 cm from the light source. At the longer wavelength of 300 nm, samples were irradiated in borosilicate glass tubes for three 20-min periods, separated by 5-min cooling periods on ice. Irradiation at both wavelengths of light was performed at room temperature. Samples were then dialyzed against water and lyophilized prior to preparation for gel electrophoresis. Borosilicate glass tubes, which absorbed a minimal amount of light below 295 nm (allowing light above 295 nm to pass through), were kindly provided by Dr. Martin Shetlar.

2. Photoaffinity Labelling of Cell Monolayers: GH_3W5 and HTC cells grown in 10-cm tissue culture dishes were "deinduced" by maintenance in "hypo" medium, which is complete medium supplemented with 10% calf serum from a thyroidectomized calf (Rockland Farms, Inc.) for 16 hr. This incubation period facilitates the dissociation of T_3 from T_3 receptors. After the deinduction period, the "hypo" medium was removed. Cells were washed once with PBS and subsequently incubated with ^{125}I-T_3 with or without excess non-radioactive T_3 or T_4 in DME-H21-glutamine-penicillin-streptomycin for approximately

2.5 hr at 37°C. Following the hormone-binding period, cells were placed on a bed of ice. The hormone-containing medium was removed and the cells were washed twice with PBS; additionally, 5 - 10 ml PBS was left on the cells to protect them from drying. They were irradiated for 60 min using a Fotodyne light box (Model 3-3000, Fotodyne, Inc. New Berlin, Wisconsin) equipped with 3 lamps that emit light of predominantly 300 nm, placed approximately 10 cm from the cells. To prevent light below 295 nm from reaching the cells, pyrex watch glasses, which absorb light below 295 nm and only minimally absorb light above 295 nm, were placed over the cells. Cells were harvested with the aid of a rubber policeman and pelleted by centrifugation for 10 min at 800 x g. Two washes with PBS followed. Nuclei were subsequently isolated by incubating cells with Buffer I for 10 min and then subjecting them to two rounds of snap freezing in a mixture of dry ice and ethanol followed by thawing. The resultant suspension was centrifuged for 10 min at 800 x g to separate nuclei from other cellular components. The resultant nuclear pellet was incubated with Buffer J for 10 min followed by centrifugation at 800 x g for 10 min to remove outer nuclear membranes. Isolated nuclei were washed twice with Buffer I. Nuclear extracts were prepared by a procedure similar to that outlined in Materials and Methods. Protein concentrations were determined according to the methods provided by BioRad (Oakland, California), which are based on the method of Bradford (126). Equal amounts of protein, from different experimental conditions, were dialized and lyophilized in preparation for analysis by gel electrophoresis.

E. Gel Electorphoresis

1. Solutions for Acid-Urea-Triton X-100 (AUT) Gels (127): Acrylamide stock: 60% acrylamide, 0.4% bis-acrylamide, N,N,N',N',-tetramethyl-ethylene-diamine (TEMED)/acetic acid: 4% TEMED, 43.2% acetic acid (T/AA); 10% Triton X-100; 10% ammonium persulfate (APS); AUT Sample Buffer: 6 M urea, 0.90 M acetic acid, 5% β-mercapto-ethanol; Running Buffer: 0.9 M acetic acid; Pre-running Buffer: 1 volume glycerol, 3 volumes 0.9 M acetic acid; Dyes: Basic Fuschia, 0.2%; α-benzo-azo-napthylamine, 0.2%; Gel Stain: 50% trichloro-acetic acid, 80 mM EDTA, 0.1 % Coomasie Blue (R).

2. Preparation of Acid-Urea-Triton X-100 (AUT) Gels: Discontinuous gels consisting of a 12% acrylamide running gel and an 8.4% acrylamide stacking gel were prepared for the analysis of histone proteins. For one running gel, 8.16 g of ultrapure urea (Schwartz-Mann) was dissolved in a solution of 4.54 ml water, 3.4 ml acrylamide stock, and 2.13 ml T/AA. This solution was filtered in a Millipore filtration unit, degassed for approximately 2 min, and 0.63 ml 10% Triton X-100 and 0.10 ml 10% APS were added. The gel (12.5 x 14.5 cm) was poured immediately and overlaid with water. Once polymerized, the water was removed from the gel's surface and the stacking gel prepared. The stacking gel solution was prepared by dissolving 2.72 g urea in a mixture of 1.8 ml water, 0.79 ml acrylamide stock, 0.71 ml T/AA. The solution was degassed, 0.21 ml 10% Triton X-100 and 0.030 ml 10% APS were added, and then the stacking gel was poured. A lucite comb was used to create the sample lanes. When polymerization was complete,

the gel was secured in a standard vertical gel electrophoresis apparatus; running buffer was added to the lower buffer reservoir, and prerunning buffer to the upper reservoir. The cathode was secured to the bottom chamber and the anode to the upper chamber. The gels were generally pre-electrophoresed at 150 V until constant current was reached (approximately 10 hr). The upper and lower buffer chambers were emptied, as were the sample lanes. Lyophilized samples were dissolved in AUT Sample Buffer, to which had been added fresh β-mercaptoethanol to 5%. The samples and two dyes, α-benzo-azo-napthylamine and Basic Fuschia, were applied to separate lanes; α-benzo-azo-napthylamine co-migrates with histone H4 and Basic Fuschia runs at the ion front. Both upper and lower chambers were refilled with running buffer, and electrophoresis was carried out at 110 V for 10-11 hr or until the Basic Fuschia dye had just electrophoresed out of the gel. Gels were stained in Gel Stain for 20 min and destained in 7% acetic acid overnight. Gells were dried onto Whatmann 3MM paper using a gel dryer (Schadel, Inc.).

3. Solutions for Sodium-Dodecyl-Sulfate Gels: Acrylamide Stock: 30% acrylamide, 0.8% bis-acrylamide; 4X Lower Gel Buffer (LGB): 1.5 M Tris•HCl (pH 8.8), 0.4% SDS; 4X Upper Gel Buffer (UGB): 0.5 M Tris•HCl (pH 6.8), 0.4% SDS, 69% glycerol, 10% ammonium persulfate (APS), TEMED; SDS Sample Buffer: 0.188 M Tris•HCl (pH 6.8), 10% glycerol, 5% B-mercaptoethanol, 2.3% SDS; 1X LGB: 0.375 M Tris•HCl (pH 8.8), 0.1% SDS, 0.03% APS, 0.01% TEMED; 1X UGB: 0.125 M Tris•HCl (pH 6.8), 0.1% SDS, 0.03% APS, 0.01% TEMED; Running Buffer: 0.025M Tris (pH 8.3), 0.192 M glycine, 0.1% SDS, Bromophenol Blue, 0.2%.

4. Preparation of Sodium-Dodecyl Sulfate Slab Gels (128): A discontinuous SDS gel system consisting of a lower running gel (12 x 14.5 cm) and an upper stacking gel (2 x 14.5 cm) was used. Histone proteins were analyzed on 15% running gels, while all other proteins were analyzed on 10% lower gels. A 10% running gel was cast with the following solution: 8.34 ml water, 6.67 ml acrylamide stock, 5 ml 4X LGB, 66 µl 10% APS, and 10 µl TEMED. The solution was degassed for 2 min prior to the addition of TEMED. After casting, the gel was overlaid with 0.1% SDS and allowed to polymerize. Directly after the gel had polymerized, the 0.1% SDS solution was replaced with approximately 5 ml of 1X LGB. Before casting the upper stacking gel, the 1X LGB was removed and the top of the running gel was rinsed twice with approximately 5 ml of 1X UGB. The upper gel solution consisted of: 2.65 ml water, 0.75 ml acrylamide stock, 1.25 ml 4X UGB, 0.35 ml 69% glycerol, 15 µl 10% APS, and 5 µl TEMED. Again the solution was degassed before the addition of TEMED. The gel was poured and a Teflon comb was inserted to form sample application lanes. Samples were dissolved in 20 - 30 µl SDS Sample Buffer and boiled for 2 min. Once samples had been applied, the upper and lower buffer reservoirs of the gel electrophoresis apparatus were filled with Running Buffer, the cathode was connected to the upper reservoir and the anode to the lower one. Electrophoresis was conducted at 17.5 mamps/gel until the Bromophenol Blue dye, which had been added to the upper reservoir, had reached the bottom of the gel. Gels were stained in Gel Stain, destained overnight in 7% acetic acid, and then dried onto Whatmann 3MM paper. Autoradiograms were prepared by exposing

Kodak X-OMAT AR-5 film to these dried gels. Intensifying screens (Dupont Lightning Plus) were used to enhance the image, and exposure was performed at -70°C.

RESULTS

A. Analysis of the Interaction of Histones with the Intranuclear Thyroid Hormone Receptor

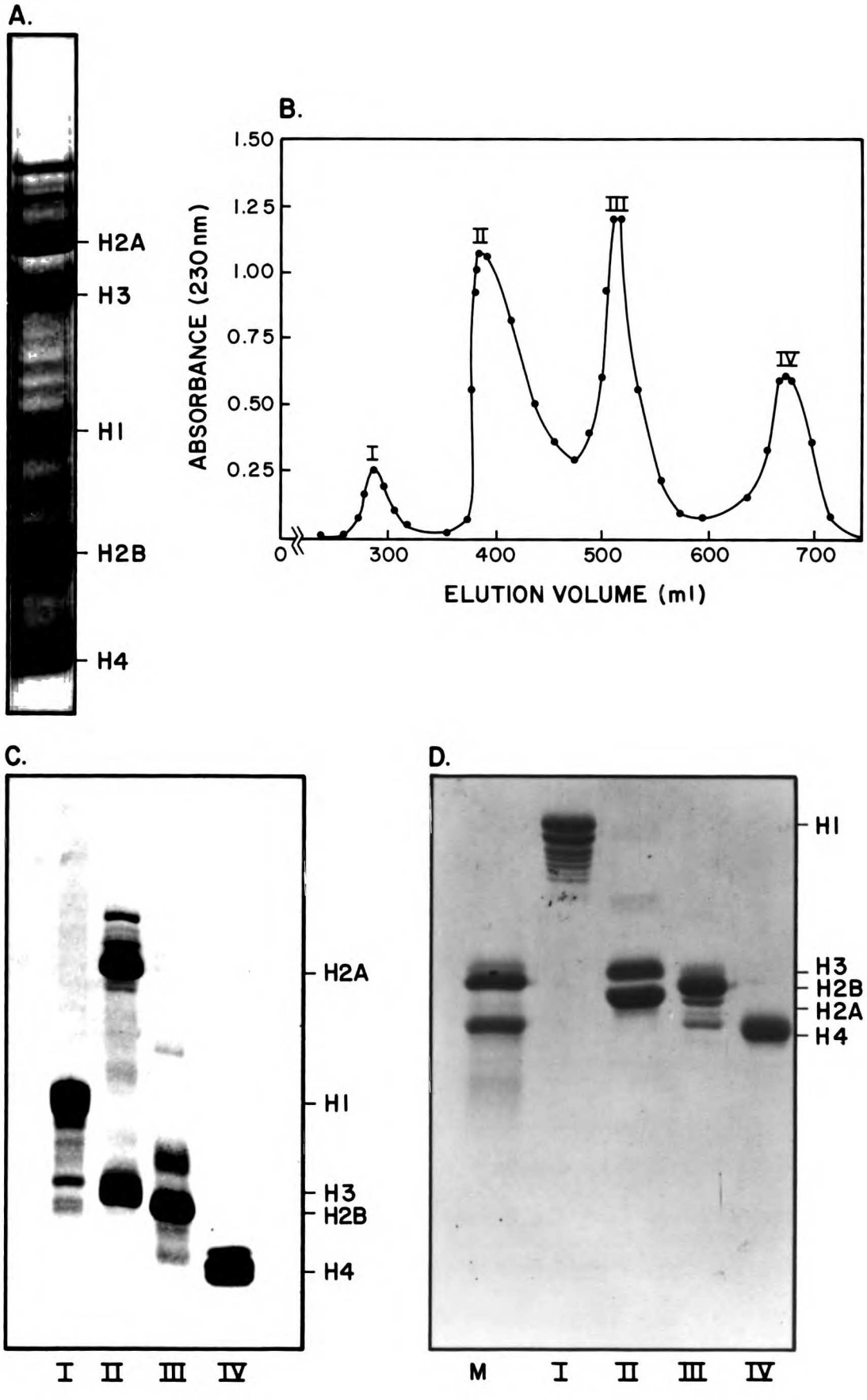
In the studies performed by Eberhardt et al. (98,99), which led to the "Core-Holo" receptor hypothesis, a protein fraction enriched for core histones (H2A, H2B, H3 and H4) was shown to be the most effective in restoring T_3 binding activity to the thyroid hormone receptor. An attempt was made to define further the nature of the regulatory factor(s) responsibe for this effect by subfractionating core histone extracts in order to obtain a homogenous protein subfraction that retained stimulatory activity. Utilizing differential solvent extraction procedures, these investigators obtained three fractions: 1) H4 and H2A; 2) H3 and H2A; and 3) H2B and H2A. Preliminary results indicated that none of these fractions possessed stimulatory activity; however, when the fractions were recombined and chromatographed on Sephadex G-200, the fractions corresponding to a size of 60,000 MW had 90% of the stimulatory activity of the original core histones. Fractions enriched for H1 had less stimulatory activity than the core histone fractions. Although these studies suggested that some complex of the mixture of core histones was responsible for stimulating T_3 binding activity, the identity of the regulatory factor had not been determined. Consequently, I prepared purified fractions of each core histone species to reexamine this point in more detail.

1. Purification of Histones

Since the solvent extraction procedures used to isolate individual histones did not yield pure fractions, chromatographic

techniques involving gel exclusion and ion exchange chromatography were used to subfractionate the crude core histone extract (122-125). At each step of the purification procedure, fractions were tested for their stimulatory activity. The initial purification step involved chromatography of core histone extracts on a column of Bio-Gel P60. As shown by the chromatographic profile in Fig. 1B, this procedure separated the proteins into four fractions. Acid-urea-Triton X-100 (AUT) gel electrophoresis of these fractions (Fig. 1C) indicated that Fraction I contained H1 proteins, Fraction II contained a mixture of H2A and H3, Fraction III contained H2B, and Fraction IV contained relatively pure H4. The AUT gel electrophoretic pattern of starting material (Fig. 1A) compared with that of the column fractions (Fig. 1C) demonstrates that adequate separation was obtained. AUT gel electrophoresis of histone fractions separates proteins according to their charge and hydrophobicity (Fig. 1C). Thus, variants that differ from each other by amino acid substitutions and modified histones that are phosphorylated or acetylated can be separated (129). However, such minor species are not separated by SDS gel electrophoresis (compare Fig. 1C and 1D). For example, evidence for the presence of modified forms of H4 was found in the data shown in Fig. 1C, lane IV, but not in Fig. 1D, lane IV. Additionally, the protein bands that migrate more slowly than the major H2A band (Fig. 1C, lane II) were identified as H2A variants by comparison with reported findings (130). The H2B-containing fraction shows evidence of minor contamination (Fig. 1C and 1D, lane III). These results pointed out the necessity for further purification of Bio-Gel Fractions II (H2A and H3) and III (H2B).

Figure 1: Chromatographic separation of core histones on Bio-Gel P60 and electrophoretic analysis of Bio-Gel P60 column fractions. A. Crude core histones were prepared by HCl extraction of steer thymus tissue as described in Materials and Methods. An aliquot of crude core histones was analyzed by acid-urea-triton X-100 gel electrophoresis (Materials and Methods). B. 60 mg of crude core histones in 4.0 ml of 8 M urea - 1% β-mercaptoethanol was applied to a column of Bio-Gel P60 (2.5 x 160 cm). Proteins were eluted with 0.2 N HCl-0.02% NaN_3 (pH 1.7) at a flow rate of 30 ml/hr. 5-ml fractions were collected and analyzed for their absorbance at 230 nm. Protein peaks I, II, III and IV were pooled, dialyzed against water, and subsequently lyophilized. C. Equivalent amounts of protein from Bio-Gel P60 Fractions I, II, III and IV were analyzed by acid-urea-Triton X-100 gel electrophoresis. Lanes: I-IV, Fractions I-IV, respectively, from B. D. 5 μg of Bio-Gel P60 Fractions I, II, III and IV were analyzed by SDS gel electrophoresis (Materials and Methods). Lanes: M, H2B and H4 (Boehringer-Mannheim); I-IV, Fractions I-IV, respectively, from B.


A.
H2A
H3
H1
H2B
H4
B.
ABSORBANCE (230 nm)
1.50
1.25
1.00
0.75
0.50
0.25
0
300
400
500
600
700
ELUTION VOLUME (ml)
I
II
III
IV
C.
H2A
H1
H3
H2B
H4
I II III IV
D.
H1
H3
H2B
H2A
H4
M I II III IV

The Bio-Gel Fraction II was further purified on a column of Bio-Rex 70 (Fig. 2). This step produced a fraction of pure H2B as assessed by AUT electrophoretic criteria (Fig.2, Inset).

Two different procedures were used to separate H2A from the H3 contained in Bio-Gel Fraction II. Chromatography of this fraction was performed on a column of either Sephadex G-100 or Bio-Rex 70. The elution profile after Sephadex G-100 chromatography exhibited two major protein peaks (Fig. 3A), and AUT electrophoresis (Fig. 3A, Inset) demonstrates that an H3-enriched fraction elutes ahead of H2A. The H3-enriched fraction, Fraction I (Sephadex G-100), always contained contaminating H2A, while Fraction II (Sephadex G-100) contained H2A and H2A variants (Fig. 3A, Inset). Chromatography of Bio-Gel Fraction II on a column of Bio-Rex 70 resulted in a more complex elution profile. AUT gel electrophoresis (Fig. 3B, Inset) showed that the H2A fraction (Fraction I, Bio-Rex) was free of all contaminating proteins, including H2A variants, while a second fraction (Fraction II, Bio-Rex) was enriched for H3 but still contained H2A (Fig. 3B, Inset). The nature of the minor peaks eluting before Peak I (Fig. 3B) was not examined further. The difference in the migration pattern of H3 between these two gels (compare Fig. 3A and 3B, Insets) was due to the presence of fresh β-mercaptoethanol in Bio-Rex Fraction II (131).

2. Stimulatory Activity of Various Histone Fractions

The ability of the various histones to increase T_3 binding activity when added to diluted partially purified receptor preparations (PII receptor, containing diminished high-affinity T_3 binding activity) was determined. Figure 4 demonstrates that H2A (Fig. 4A,

Figure 2: Purification of H2B (Bio-Gel P60 Fraction III) by chromatography on Bio-Rex 70. 7 mg of Bio-Gel P60 Fraction III, enriched for H2B, was applied to a column of BioRex 70 (1.6 x 60 cm) equilibrated to 7 % $GuCl \cdot PO_4$ (0.1 M). The proteins were eluted with 100 ml 7% $GuCl \cdot PO_4$ and a 300-ml linear gradient of 8 to 13% $GuCl \cdot PO_4$. 5-ml fractions were collected and analyzed for histone protein content by measuring their absorbance at 230 nm. Peak protein fractions were pooled, dialyzed against water and then lyophilized. (Inset): Equivalent amounts of protein were analyzed by acid-urea-Triton X-100 gel electrophoresis. Lanes: A, Bio-Gel P60 Fraction III; B, Bio-Rex 70-purified H2B.

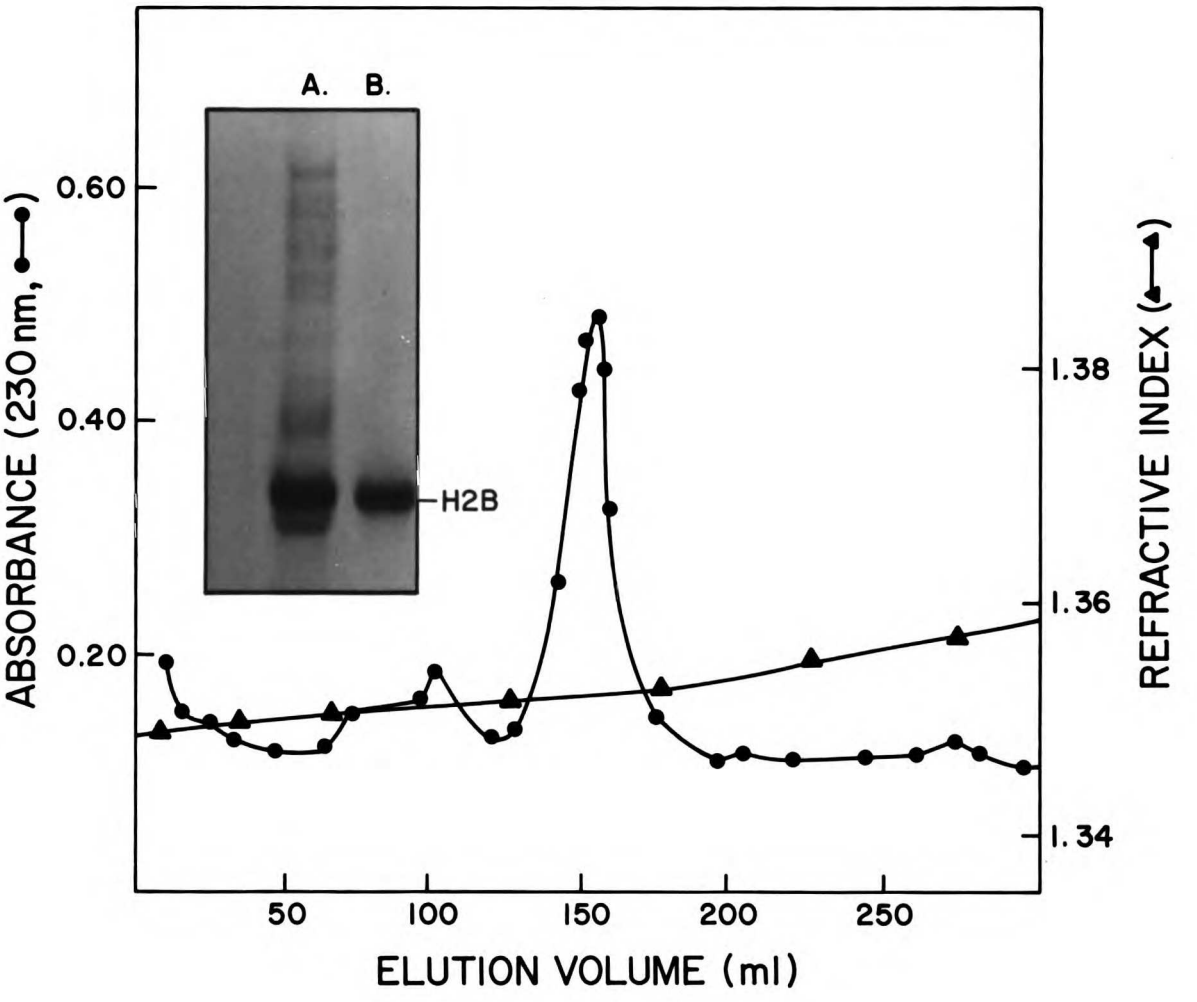
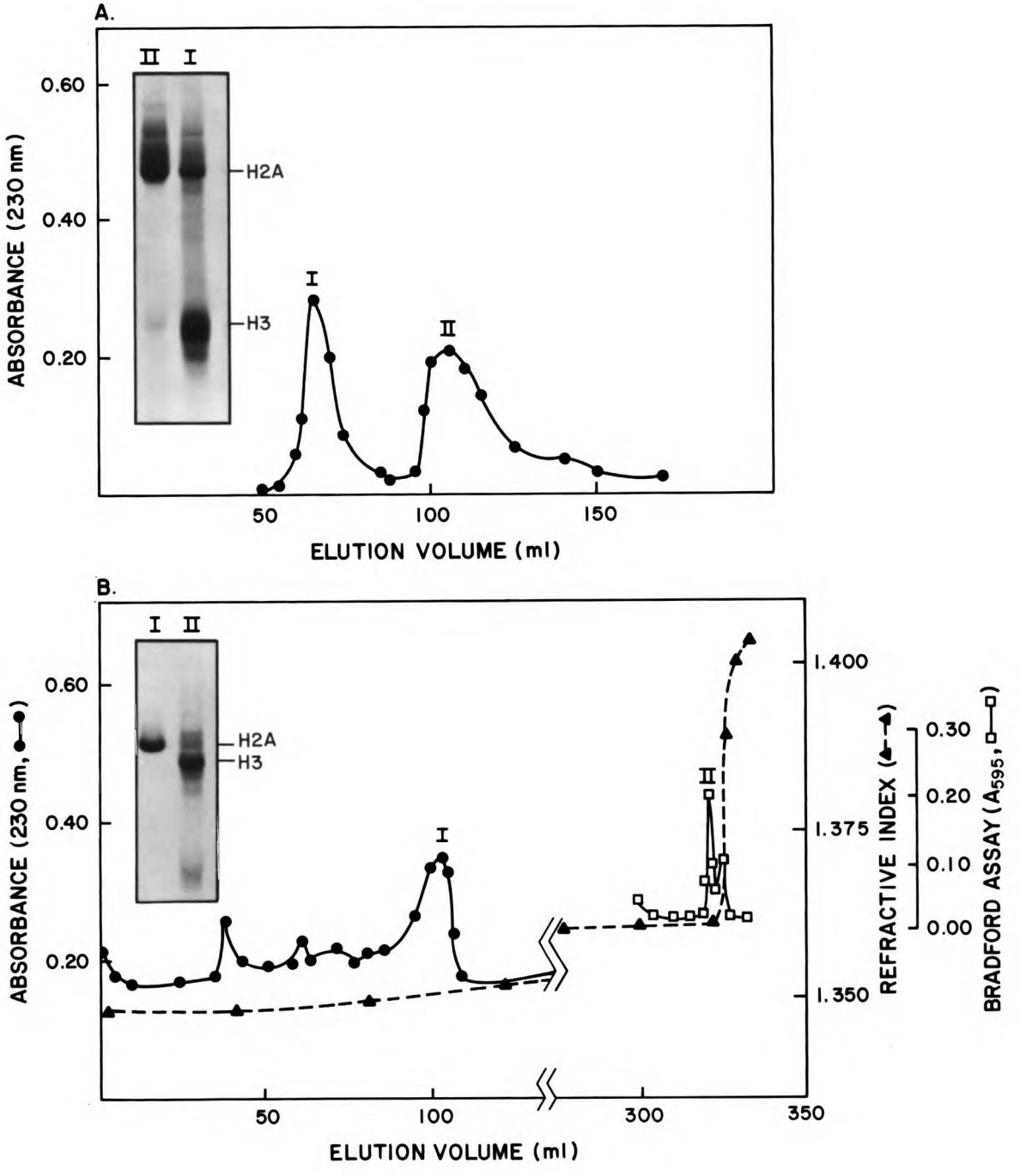

A.
B.
H2B
0.60
0.40
0.20
ABSORBANCE (230 nm, ●—●)
1.38
1.36
1.34
REFRACTIVE INDEX (▲—▲)
50
100
150
200
250
ELUTION VOLUME (ml)

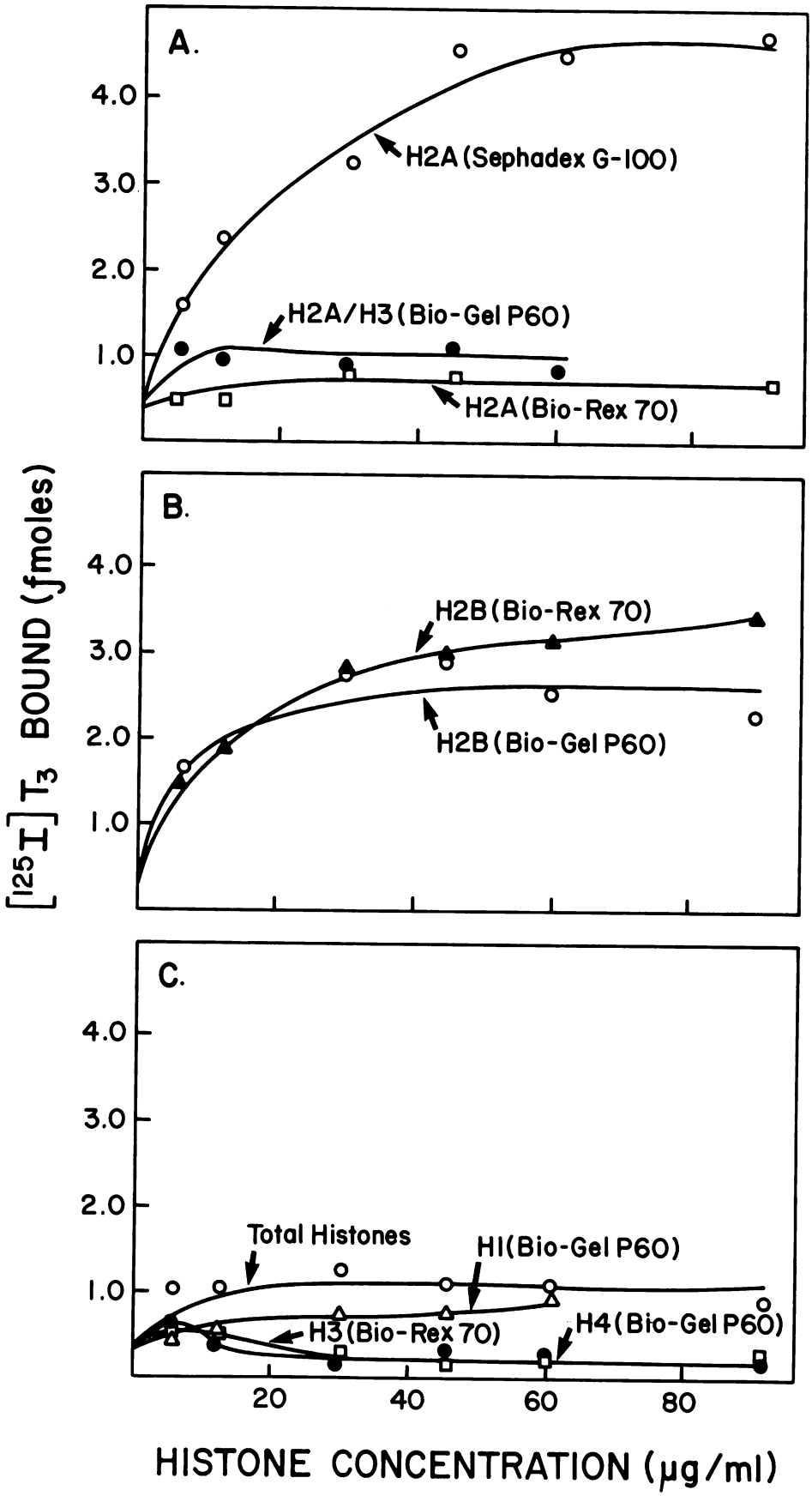
Figure 3: Chromatographic separation of H2A/H3 (Bio-Gel Fraction II) on Sephadex G-100 (A) and Bio-Rex 70 (B). A. 20 mg of Bio-Gel Fraction II was applied to a column of Sephadex G-100 (2.5 x 160 cm). The proteins were eluted with 0.05 M sodium acetate-0.05 M sodium bisulfite (pH 5.4) at a flow rate of 50 ml/hr. 35-ml column fractions were collected and their absorbance at 230 nm was measured. The two protein peaks were pooled, dialyzed against water and lyophilized. Inset: Equivalent amounts of protein from Sephadex G-100 Fraction I and Fraction II were applied to an acid-urea-Triton X-100 gel. AUT gel electrophoresis was performed as described in Materials and Methods. Lanes: I, Sephadex G-100, Fraction I; II, Sephadex G-100, Fraction II. B. Chromatography of 7 mg H2A/H3 (Bio-Gel Fraction II) was conducted on a column of Bio-Rex 70 (1.6 x 60 cm). The column washed with 100 ml 8% $GuCl \cdot PO_4$ (0.1 M) and the proteins were eluted with 450 ml 8% $GuCl \cdot PO_4$, followed with a 450-ml linear gradient of 8 to 15% $GuCl \cdot PO_4$, 200 ml 15% $GuCl \cdot PO_4$, and finally 120 ml 40% $GuCl \cdot PO_4$. Column fractions of 3.5 ml were collected and their absorbance at 230 nm was measured. However, when the percentage of GuCl in the column buffer increased to 15%, the buffer's absorbance at 230 nm interfered with the detection of proteins. Therefore, the presence of protein was also monitored by performing a Bradford Protein Assay as supplied by Bio-Rad Laboratories (Oakland, CA). Inset: The two protein peaks were analyzed by acid-urea-Triton X-100 gel electrophoresis. Lanes: I, Bio-Rex, Fraction I; II, Bio-Rex, Fraction II.


A.
II I
H2A
H3
I
II
0.60
0.40
0.20
ABSORBANCE (230 nm)
50
100
150
ELUTION VOLUME (ml)
B.
I II
H2A
H3
I
II
0.60
0.40
0.20
ABSORBANCE (230 nm, ●—●)
1.400
1.375
1.350
REFRACTIVE INDEX (▲—▲)
0.30
0.20
0.10
0.00
BRADFORD ASSAY (A595, □—□)
50
100
300
350
ELUTION VOLUME (ml)

Sephadex G-100) and H2B (Fig. 4B) have greater stimulatory activity than H1, H3, H4 and total histones (Fig. 4c). Of interest was the finding that H2A (Sephadex G-100) which contained H2A variants had a greater effect on increasing T_3 binding activity than H2A (Bio-Rex 70) which lacked the variants (Fig. 4A). This suggests that the stimulatory activity of H2A-containing preparations may reside with the H2A variants. The possibility that a minor component (not evident on the gel) may actually possess the stimulatory activity cannot be excluded. Thus, until the stimulatory activity of isolated H2A variants is measured and compared with that of purified H2A, the significance of this finding is not clear. Further purification of H2B on a column of Bio-Rex 70 did not significantly alter its ability to stimulate T_3 binding (Fig. 4B). In addition, commercially available H2B (Boehringer-Mannheim) was just as effective as these two H2B preparations in stimulating T_3 binding activity (data not shown).

Several observations suggested that the original total histone extract possessed inhibitory factors. First, the total histone extract (Fig. 4C, total histones) was not as effective in stimulating receptor T_3 binding activity as H2A (Fig. 4A, Sephadex G-100) and H2B fractions (Fig. 4B). Furthermore, H2A in combination with H3 (Bio-Gel P60) had much less stimulatory activity than H2A without H3 (Sephadex G-100) (Fig. 4A), suggesting that the inhibitory activity migrates with H3. Indeed, H3 added to PII receptor preparations appeared to reduce T_3 binding activity, as did the addition of H4 (Fig. 4C). In preliminary experiments, the addition of H4 to incubations of H2B with PII receptor preparations led to less stimulation of T_3 binding activity than obtained with H2B alone.

Figure 4: The effects of various histone subfractions on the binding of ^{125}I-T_3 by partially purified thyroid hormone nuclear receptor. Receptor preparations (PII, Sephadex G-100 chromatography) were incubated with 1 nM ^{125}I-T_3 with or without a 100-fold excess of unlabelled T_3 in the presence of increasing concentrations of histones for 4 h at 22°C in a total volume of 0.5 ml. Samples were chilled and 0.4 ml aliquots were analyzed for the amount of ^{125}I-T_3 bound by filtration over Sephadex G-25 assay columns by the standard binding assay (Materials and Methods). The amount of ^{125}I-T_3 specifically bound was determined by subtracting nonspecific binding (^{125}I-T_3 bound in the presence of unlabelled T_3) from the total amount of ^{125}I-T_3 bound. Panels A, B and C display the effects of (i) various H2A-contain-ing fractions, (ii) H2B-containing fractions, and (iii) total histones, H1, H3, H4, respectively, on ^{125}I-T_3 binding by PII receptor preparations.


A.
4.0
3.0
2.0
1.0
H2A (Sephadex G-100)
H2A/H3 (Bio-Gel P60)
H2A (Bio-Rex 70)
B.
4.0
3.0
2.0
1.0
H2B (Bio-Rex 70)
H2B (Bio-Gel P60)
[125I] T3 BOUND (fmoles)
C.
4.0
3.0
2.0
1.0
Total Histones
HI (Bio-Gel P60)
H3 (Bio-Rex 70)
H4 (Bio-Gel P60)
20
40
60
80
HISTONE CONCENTRATION (µg/ml)

3. Stimulatory Effects of Histones on T_3 and T_4 Binding

To examine whether H2A and H2B were possible candidates for the regulatory factor postulated to exist by the "Core-Holo" receptor model, their effects on both T_3 and T_4 binding had to be determined. However, a reevaluation of the effects of crude core histone extracts on T_3 and T_4 binding by PII and affinity-purified receptor preparations resulted in data that were contradictory to the model. Table I shows that the addition of core histone extracts produced an approximate 3-fold stimulation of both T_3 and T_4 binding activity of the PII receptor preparation. In addition, the T_3 and T_4 binding activities of the affinity-purified receptor were stimulated approximately 11- and 6-fold, respectively. Scatchard analysis of ^{125}I-T_3 and ^{125}I-T_4 binding by PII receptor preparations demonstrated that the stimulation was due to an increase in the concentration of both T_3 and T_4 binding sites with no significant change in their dissociation constants (K_ds) (Fig. 5). This finding is in contrast to the previously reported results where only the T_3 and not the T_4 binding activity of PII receptor preparations was stimulated by the addition of core histones (99). Thus, the data were not consistent with the previously reported data used to generate the "Core-Holo" receptor model, and therefore the nature of the histone effect on hormone binding by the thyroid hormone receptor originally reported needed further evaluation.

4. Nature of Histone Effect on T_3 Binding by Receptors

The protocol for the stimulation assays used in these studies, which was similar to that used by Eberhardt et al. (99), required the

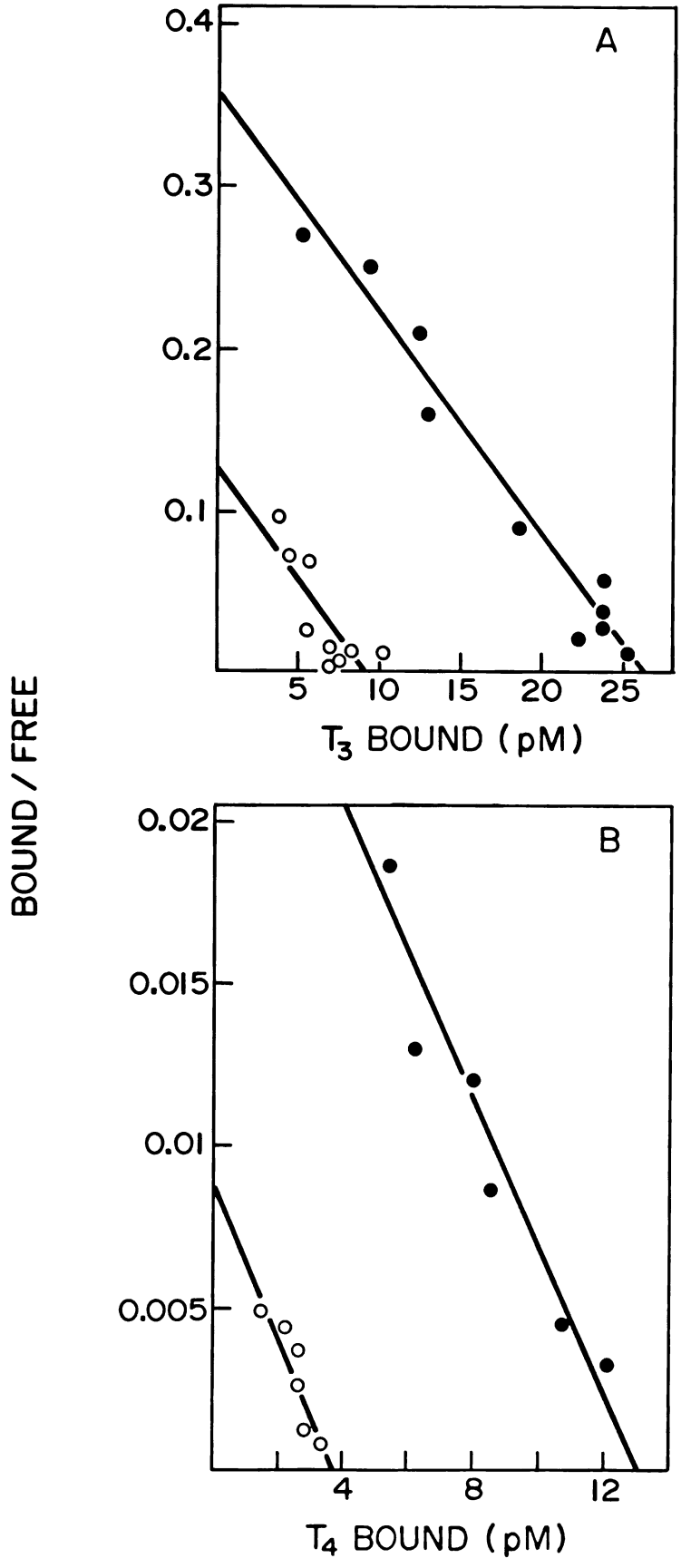
TABLE I

Stimulation of ^{125}I-T_3 and ^{125}I-T_4 Binding by Core Histones

Receptor Preparation	Hormone	Histone Addition	Fmol Bound (± S.D.)	Fold-Stimulation By Histones
Sephadex G-100-Purified				
	T_3	-	0.40 ± 0.06	
	T_3	+	1.17 ± 0.28	2.9
	T_4	-	0.09 ± 0.02	
	T_4	+	0.28 ± 0.01	3.0
Affinity-Purified				
	T_3	-	0.19 ± 0.03	
	T_3	+	2.10 ± 0.18	11
	T_4	-	0.12 ± 0.02	
	T_4	+	0.73 ± 0.05	6

Sephadex G-100-purified receptor preparations (3µg protein) were incubated with 1 nM ^{125}I-T_3 or 0.8 nM ^{125}I-T_4 ± 1 µM unlabelled T_3 or T_4 in the absence or presence of 22 µg of histones for 2 h at 25°C before filtering over Sephadex G-25. Affinity-purified receptor preparations (25 ng protein) were incubated with 0.5 nM ^{125}I-T_3 or ^{125}I-T_4 ± 1 µM unlabelled T_3 or T_4 in the absence or presence of 12.5 µg core histones in a total volume of 0.5 ml. After incubation for 1 h at 25°C and 16 h at 4°C, samples were filtered over Sephadex G-25. In this representative experiment, all assays were performed in triplicate. Specifically bound hormone was calculated by subtracting nonspecific binding (the amount bound in the presence of unlabelled hormone) from total hormone bound.

Figure 5: Scatchard analysis of the binding of ^{125}I-T_3 (A) and ^{125}I-T_4 (B) by Sephadex G-100-purified receptor preparations in the presence (●) or absence (o) of core histones. Receptor was assayed by the standard binding assay. The concentrations of ^{125}I-T_3 and ^{125}I-T_4 varied from 40 to 2,000 and 300 to 6,500 pM, respectively. Nonspecific binding was determined in parallel assays containing a 1000-fold excess of unlabelled T_3 or T_4. The assay incubations (0.5 ml total volume) were performed for 4 h at 22°C and contained 3 µg of protein from the Sephadex G-100-included fraction and 25 µg of core histones, when added. The apparent K_ds and maximum binding capacities were calculated by linear regression analysis. For T_3 binding, the maximum binding capacity was 9.1 pM without histones and 26.2 pM with histones, while the K_ds were 70.6 pM and 72.3 pM, respectively. For T_4, the maximum binding capacity was 3.7 pM without histones and 13.0 pM with histones, and the K_ds were 417 pM and 463 pM, respectively. Similar results were obtained in three other independent experiments (data not shown).


A
0.4
0.3
0.2
0.1
5
10
15
20
25
T3 BOUND (pM)
B
0.02
0.015
0.01
0.005
4
8
12
T4 BOUND (pM)
BOUND / FREE

addition of histones at the start of the assay. It had been assumed that histones had to be present for the entire length of the incubation assay to exert their stimulatory effect. This assumption was investigated in order to understand the nature of the histone effect. Histones were added at the start of the incubation with receptor and $^{125}I\text{-}T_3$, 1 min before filtration over Sephadex G-25, or directly to the Sephadex G-25 assay column. As shown in Table II (Experiment I), the same level of stimulation of $^{125}I\text{-}T_3$ binding activity was obtained irrespective of the time of histone addition. Moreover, when histone-pretreated columns were extensively washed with buffer (prior to the application of sample) to reduce the concentration of histones present in the Sephadex matrix to negligible levels, stimulation of hormone binding was still observed (Table II, Experiment 2). Stimulation also occurred when an excess of T_3 was added prior to the gel filtration step, indicating that the histones do not act by influencing the ability of the receptor to bind hormone while both are present on the assay column. The results suggest that histones may be acting by preventing receptor binding to or degradation by the Sephadex G-25 assay columns. This notion is supported further by the data of Table III. In this experiment, diluted receptor preparations were filtered through Sephadex G-25 columns that were either untreated or pretreated with core histones. The column eluates were then assayed for T_3 binding. As shown, passage of the receptor through untreated Sephadex resulted in a loss of approximately 75% of the control T_3 binding activity, and pretreatment of the column with histones prevented this loss. Thus, the histones appear to prevent loss of receptors during their filtration over Sephadex G-25 columns.

TABLE II

Effect of Histones on Receptor Binding Assay

Treatment	Relative T_3 Binding (± S.D.)
Experiment 1	
No histones (control)	1.0 ± 0.1
Histone addition at start of reaction	4.1 ± 0.9
Histone addition 1 min before filtration over assay column	4.8 ± 0.3
Histone addition directly to the assay column	4.9 ± 0.7
Experiment 2 (excess unlabelled T_3 added to receptor prior to filtration)	
Addition of histones directly to the assay column:	
with no wash before sample application	8.0 ± 0.4
followed by a 2.5-ml wash with Buffer G	7.5 ± 0.6
followed by a 10-ml wash with Buffer G	7.5 ± 0.3

In Experiment 1, Sephadex G-100-purified receptor preparations (PII) (1.5 µg protein) were incubated with 1 nM ^{125}I-T_3 ± 1 µM unlabelled T_3 in a final total volume of 0.5 ml for 4 h at room temperature; 15 µg of crude core histones in 50 µl were added at various times to the reaction mixtures or in 400 µl to the assay column. The amount of ^{125}I-T_3 bound was determined by Sephadex G-25 assay. The control value of 0.63 ± 0.06 fmol ^{125}I-T_3 specifically bound was assigned the value 1.

In Experiment 2 (performed separately), Sephadex G-100-purified receptor preparations (3.1 µg protein) were incubated with 1 nM ^{125}I-T_3 ± 1 µM unlabelled T_3 in 25 µl Buffer G for 4 h at room temperature and

then placed on ice. The reactions were then diluted to 500 µl with Buffer G containing 1 µM unlabelled T_3. To determine the bound ^{125}I-T_3, 0.4-ml aliquots were filtered over Sephadex G-25 assay columns, which had been pretreated with 20 µg of crude core histones in 400 µl of Buffer G, and washed with buffer as indicated above. The control value was 0.65 $\pm$ 0.04 fmol ^{125}I-T_3 bound. All assays were performed in triplicate, and the average value is indicated.

TABLE III

Prevention of Receptor Loss by Pretreatment of Sephadex G-25 with Core Histones

Conditions	% Control (± S.D.)
1. Control	100 ± 9
2. Filtration over untreated Sephadex G-25	24 ± 7
3. Filtration over histone-treated Sephadex G-25	88 ± 8

Sephadex G-100-purified receptor preparations (PII) (2 - 4 µg protein/ 0.4 ml) were filtered over Sephadex G-25 assay columns (conditions 2 and 3). The excluded 0.8-ml "eluate" was collected as in the standard T_3-binding assay. The assay column in condition 3 had been pretreated with 0.4 ml of a 50-µg/ml core histone extract in Buffer G followed by a 2-ml wash with Buffer G. The assay column in condition 2 was washed with 2.4 ml Buffer G. To control for an effect by simple dilution of the receptor due to chromatography, the receptor preparation in condition 1 was diluted two-fold with Buffer G. Then, 0.4-ml aliquots of the column eluates or the receptor preparation (condition 1) were assayed for specific T_3 binding as described in Materials and Methods (B-8). The amount of bound hormone was determined by filtering samples over histone-treated assay columns. All assays were performed in triplicate and the specific binding by the control (0.85 fmol) was assigned to be 100%.

5. Stimulation of Receptor Hormone Binding Activity by Various Proteins

Whether this stimulatory effect of histones was a specific characteristic of histone proteins or a feature of randomly chosen proteins was investigated by pretreating Sephadex G-25 assay columns with a number of proteins prior to filtration of the receptor. As shown in Table IV, pretreatment of Sephadex G-25 columns with the core histones (H2A, H2B, H3 and H4) increased $^{125}I\text{-}T_3$ binding activity approximately 4.8- and 5.9-fold over control values. Of the other proteins tested, only catalase and BSA increased T_3 binding to the same level obtained with pretreatment by histones. These results suggest that the ability of histones to increase T_3 binding activity was not a characteristic of all the proteins tested and, therefore, suggest that this stimulatory activity may be a specific feature of histone proteins.

6. Stabilization of Affinity-Purified Receptors

While the presence of histones was not required during incubation of PII receptor preparations with $^{125}I\text{-}T_3$, the presence of core histones was required during incubations of affinity-purified receptor with $^{125}I\text{-}T_3$ to enable detection of T_3 binding activity, as shown in Fig. 6. Core histones (H2A, H2B, H3 and H4) were added to receptor preparations at various times during a 4-hr pre-incubation period at 25°C, and then $^{125}I\text{-}T_3$ or $^{125}I\text{-}T_4$ and histones were added to all samples. The standard binding assay was performed. Figure 6 (time 0) shows that the affinity-purified receptor in the absence of histones during the pre-incubation period had approximately

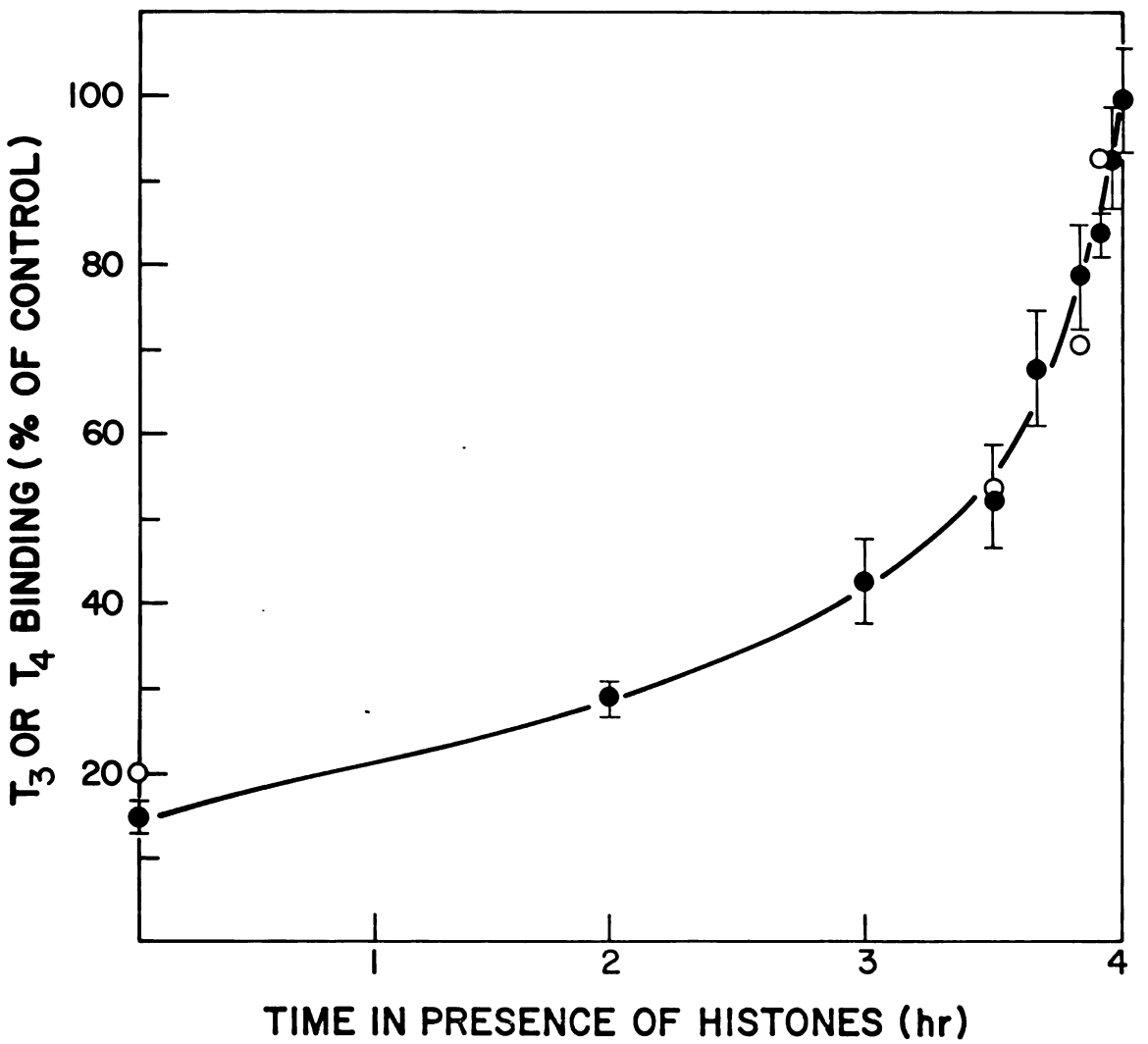
TABLE IV

Effect of Pretreatment of Sephadex G-25 with Various Proteins on T_3 Binding

Protein	Concentration (μg/ml)	Moles Applied (x 10^{-9})	Fold-Stimulation
H1	60	1.12	3.2 ± 0.5
H2A	60	1.71	5.9 ± 2.1
H2B	60	1.74	5.8 ± 2.0
H3	60	1.57	5.3 ± 1.5
H4	60	2.12	5.9 ± 1.8
Urease	200	0.17	3.5 ± 0.4
Catalase	200	0.32	5.4 ± 1.7
Gamma-globulin	200	0.52	3.2 ± 0.2
Bovine Serum Albumin	200	1.18	4.8 ± 1.6
Ovalbumin	200	1.90	2.0 ± 0.7
Soybean trypsin inhibitor	200	3.48	3.4 ± 0.4
Chymotrypsin	200	3.70	1.3 ± 0.1
Cytochrome C	200	5.97	1.7 ± 0.2
Ribonuclease	200	6.35	1.5 ± 0.3
Protamine	200	8-16	1.5 ± 0.2
Insulin	200	13.30	2.4 ± 1.2

Sephadex G-100-purified receptor was incubated with 1 nM ^{125}I-T_3 in the presence or absence of 1 μM unlabelled T_3 for 4 h at 25°C. Before filtration of this incubation mixture over Sephadex G-25 assay columns, the Sephadex G-25 matrix was pretreated by adding 400 μl of 200-μg/ml solutions of the indicated proteins or polypeptides (except in the case of the histones, where 400 μl of 60-μg/ml solutions were added). The fold-stimulation was calculated as the specifically bound T_3 eluted from the pretreated columns divided by that eluted from non-treated columns. The values are an average of two to five separate experiments, with the assays in each experiment done in triplicate. Shown are means ± S.D. The number of moles applied was calculated based on the molecular weight of the individual proteins.

Figure 6: Effect of core histones on T_3 (●) and T_4 (o) binding by affinity-purified receptors. Histones were removed from affinity chromatography-purified receptors by chromatography on DEAE-Sephadex, as described in Materials and Methods. The receptor-containing DEAE-Sephadex eluate (3.2 ml) was diluted to 32 ml with Buffer G at $4^{o}C$. Samples (0.35 ml) of the diluted receptor were pre-incubated for 4 h at $25^{o}C$ with 25 µg of core histones in 50 µl buffer added at the start of incubation (control) or at various times during the incubation. The control sample is represented on the graph as the 4-h time point. After pre-incubation, ^{125}I-T_3 or ^{125}I-T_4 and histones were added; incubation was continued at $4^{o}C$ for 14 h before filtering on Sephadex G-25 assay columns. All assays were in triplicate, with nonspecific binding subtracted. The binding in the control samples (histones present for 4 h) was 6.75 fmol for T_3 and 1.25 fmol for T_4.


100
80
60
40
20
T_3 OR T_4 BINDING (% OF CONTROL)
1
2
3
4
TIME IN PRESENCE OF HISTONES (hr)

15% of the T_3 and T_4 binding activity observed when histones were present for the entire period; thus, 85% of the hormone binding activity was lost in 4 hr. The data further indicate that relatively short incubation periods in the absence of histones led to a dramatic loss of T_3 and T_4 binding activity; at 3.25 hr (Fig. 6), which reflects the presence of histones for 45 min, 50% of the binding activity was lost . Thus, the affinity-purified receptor appeared to be unstable in the absence of histones. A similar loss in binding activity was observed when the affinity-purified receptor was pre-incubated in the absence of histones at 4°C. However, in the presence of histones, the receptor was relatively stable at either 4 or 25°C. These results suggest that the addition of core histones was necessary to stabilize hormone binding activity of the affinity-purified receptor.

Other proteins were tested for their ability to stabilize the affinity-purified receptor. As shown in Table V, core histones, at 10 μg/ml (7.4×10^{-7}M), increased the amount of ^{125}I-T_3 bound 59-fold over control values; at 50 μg/ml (1.8×10^{-6}M), a maximum stimulatory effect approximately 78-fold had been reached. Higher concentrations of ovalbumin, soybean trypsin inhibitor, H1, lysozyme, and insulin (200 μg/ml {0.5 to 1.4 $\times 10^{-5}$M}) were required to increase T_3 binding to a similar level (43.9- to 63-fold). Thus, the data suggest that the different proteins vary in their receptor-stabilizing activities and that the core histones are among the most active, if not the most active, in this respect. However, more extensive dose-response studies, especially with lower concentrations of

TABLE V

Stabilization of the ^{125}I-T_3 Binding Activity of Affinity-Purified Receptor by Various Proteins

Protein	Concentration (μg/ml)	Molarity ($\times 10^{-5}$)	T_3 Bound (fmol) (± S.D.)
Control			1.0 ± 0.3
Core histones	10	0.074	59.0 ± 3.8
	25	0.18	78.0 ± 2.9
	50	0.37	79.0 ± 6.9
Urease	200	0.04	49.1 ± 4.0
Ovalbumin	200	0.48	53.6 ± 1.5
Soybean trypsin inhibitor	200	0.87	55.5 ± 4.4
H1	200	0.93	51.1 ±10
Lysozyme	200	1.44	43.9 ± 8.0
Cytochrome C	200	1.49	17.4 ± 2.6
Insulin	25	0.42	46.6 ± 2.3
	50	0.83	63.2 ± 1.2
	100	1.66	70.0 ±10

Histones were removed from affinity-purified receptors by chromatography on DEAE-Sephadex as described in Materials and Methods (B-7). The receptor was incubated for 4 h at 25°C in a total volume of 100 μl with various proteins at the indicated concentrations. After 4 h, 350 μl of Buffer G containing 64 μg/ml core histones and 0.5 nM ^{125}I-T_3 was added to each 100-μl incubation, and the samples were incubated at 4°C for 16 h before performing the standard T_3-binding assay using columns pretreated with 25 μg of core histones. Binding by T_3 to the

proteins alone was subtracted. This representative experiment was performed in triplicate. Shown are means $\pm$ S.D. The molarity of the individual proteins present during the 4-h incubation at 25^{o}C was calculated based on their molecular weight.

some of the other proteins such as ovalbumin, need to be performed to assess more fully the specificity of these actions.

7. Effect of Heat on Receptor Binding of T_3 and T_4

The discrepancy between the results of the histone stimulation studies presented here and those of Eberhardt et al. (99) led to a reevaluation of their heating experiments (98). Figure 7A shows the effect of heat treatment at 50°C on thyroid hormone binding activity in a crude nuclear extract (FII) prepared by the method outlined in Materials and Methods, referred to in this discussion as Procedure I. In agreement with the results of Eberhardt et al. (98), this heating led to a rapid reduction in T_3 binding activity, while T_4 binding remained relatively constant (Fig. 7A). Scatchard analysis of T_3 and T_4 binding by heated nuclear extracts (FII) showed that the remaining T_3 binding sites (22% of control) had the same apparent equilibrium dissociation constant as controls (Fig. 8A). The remaining T_4 binding sites were predominantly low-affinity, high-capacity sites (Fig. 8B), which is in contrast to the report of Eberhardt et al. (98). In their studies, the T_4 binding sites remaining in heat-treated FII receptor preparations were similar to the original T_4 binding sites. When the effect of heat on more purified receptor preparations (PII receptor) was examined, a pattern different from that shown in Fig. 7A was observed (as shown in Fig. 7B); there was a rapid reduction of both T_3 and T_4 binding activities.

The effects of a 50°C heat treatment on the hormone binding activities of crude nuclear extracts prepared by a different procedure (Procedure II) (in which nuclei were sonicated for three 15-sec periods,

Figure 7: Effect of heating on binding of ^{125}I-T_3 (solid symbols) and ^{125}I-T_4 (open symbols) by thyroid hormone receptors. Samples were incubated at 50°C and aliquots were removed at the specified times, chilled on ice, and centrifuged at 12,000 x g for 10 min at 4°C to remove precipitated protein. Binding assays were performed on the supernatants using 1 nM ^{125}I-T_3 or ^{125}I-T_4 $\pm$ 1 μM unlabelled hormone. The amount bound in the heated sample is plotted as the percentage of the amount bound in the unheated control. The 100% values for ^{125}I-T_3 and ^{125}I-T_4, respectively, were: A, 20.5 and 5.1 fmol; B, 4.3 and 0.88 fmol; C, 29.0 and 10.4 fmol; D, 0.65 and 1.1 fmol. All assays were performed in triplicate, and the standard deviation is indicated by the error bars. A: Crude nuclear extract, prepared by Procedure I (sonication for two 15-sec periods). B: The included peak from Sephadex G-100 chromatography of extract prepared by Procedure I. C: Crude nuclear extract prepared by Procedure II (sonication for three 15-sec periods). D: The excluded peak from Sephadex G-100 chromatography of nuclear extract prepared by Procedure II.

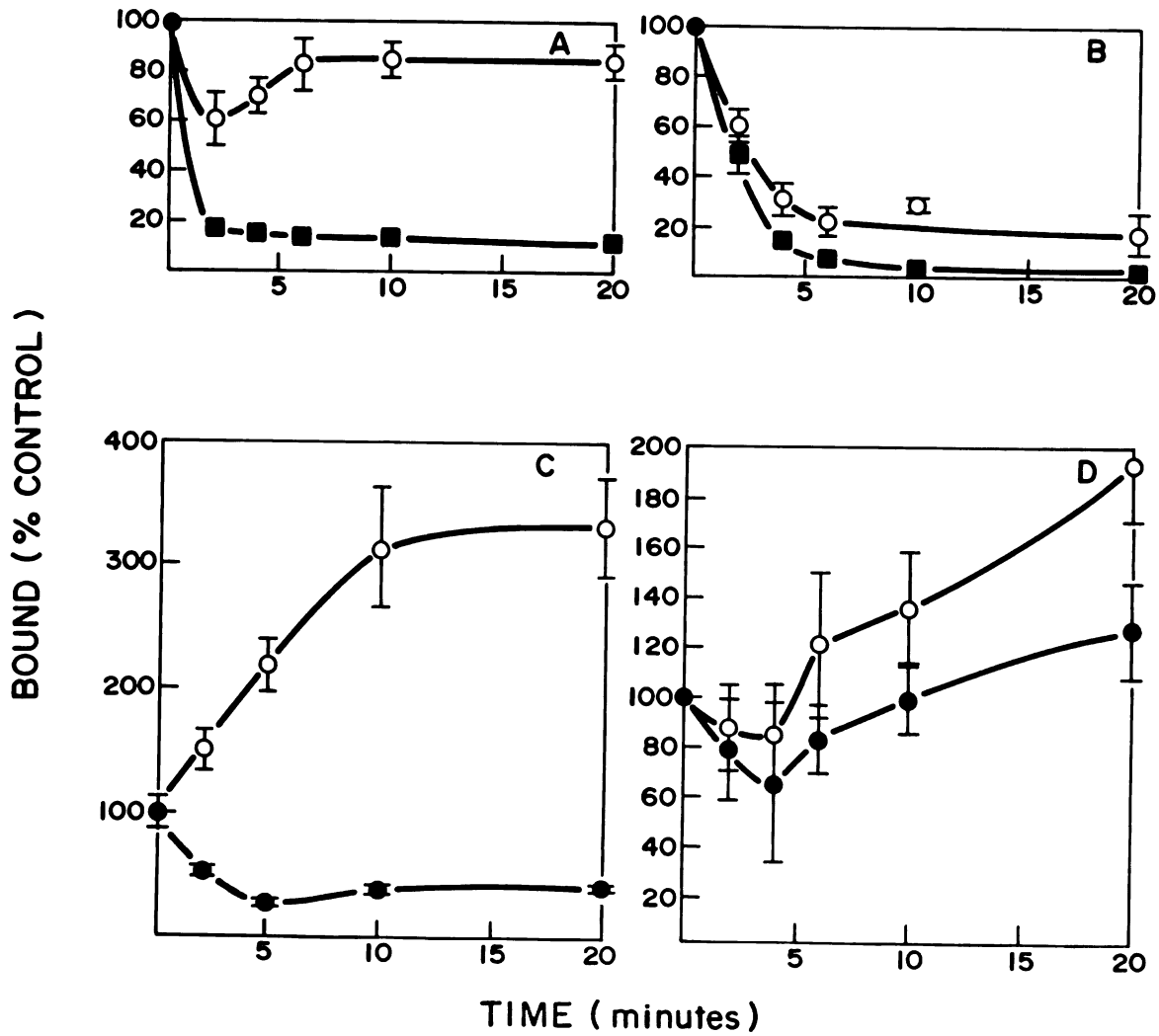
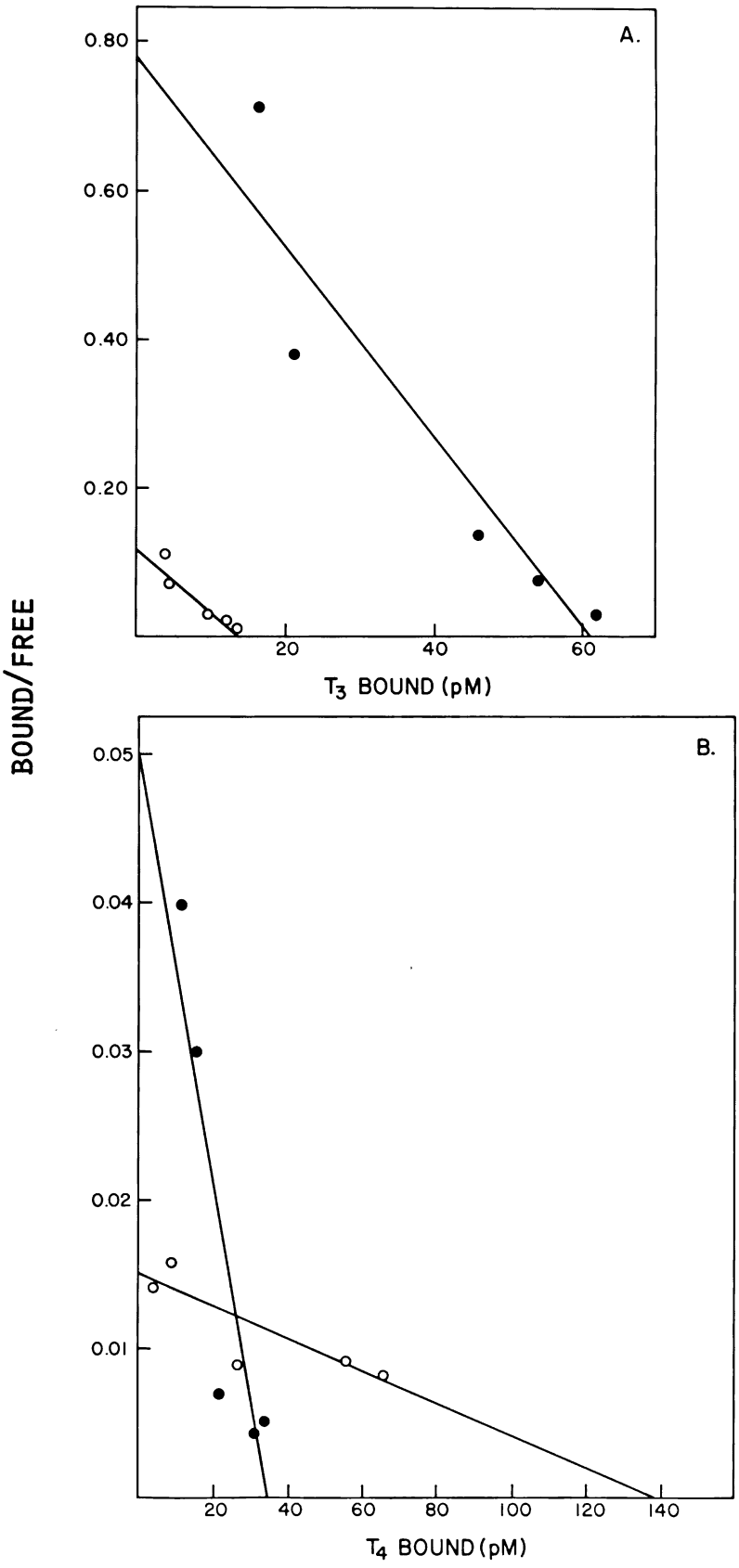

A
B
C
D
BOUND (% CONTROL)
TIME (minutes)

Figure 8: Scatchard analysis of ^{125}I-T_3 (A) and ^{125}I-T_4 (B) binding to crude nuclear extracts before (●) and after (o) heating for 10 min at 50°C. Nuclear extracts were prepared by Procedure I (sonication for two 15-sec periods). Heated samples were treated as described in the Legend to Fig. 7. Binding assays were conducted with concentrations of ^{125}I-T_3 and ^{125}I-T_4, which varied from 40 to 2000 and 40 to 1000 pM, respectively. All assays were performed in duplicate, and non-specific binding (in the presence of 1 μM unlabelled hormone) was subtracted from total hormone bound. The K_ds for ^{125}I-T_3 and ^{125}I-T_4 binding by the control extracts were 0.077 and 0.687 nM, respectively; for the heated extracts, they were 0.111 and 9.40 nM, respectively.

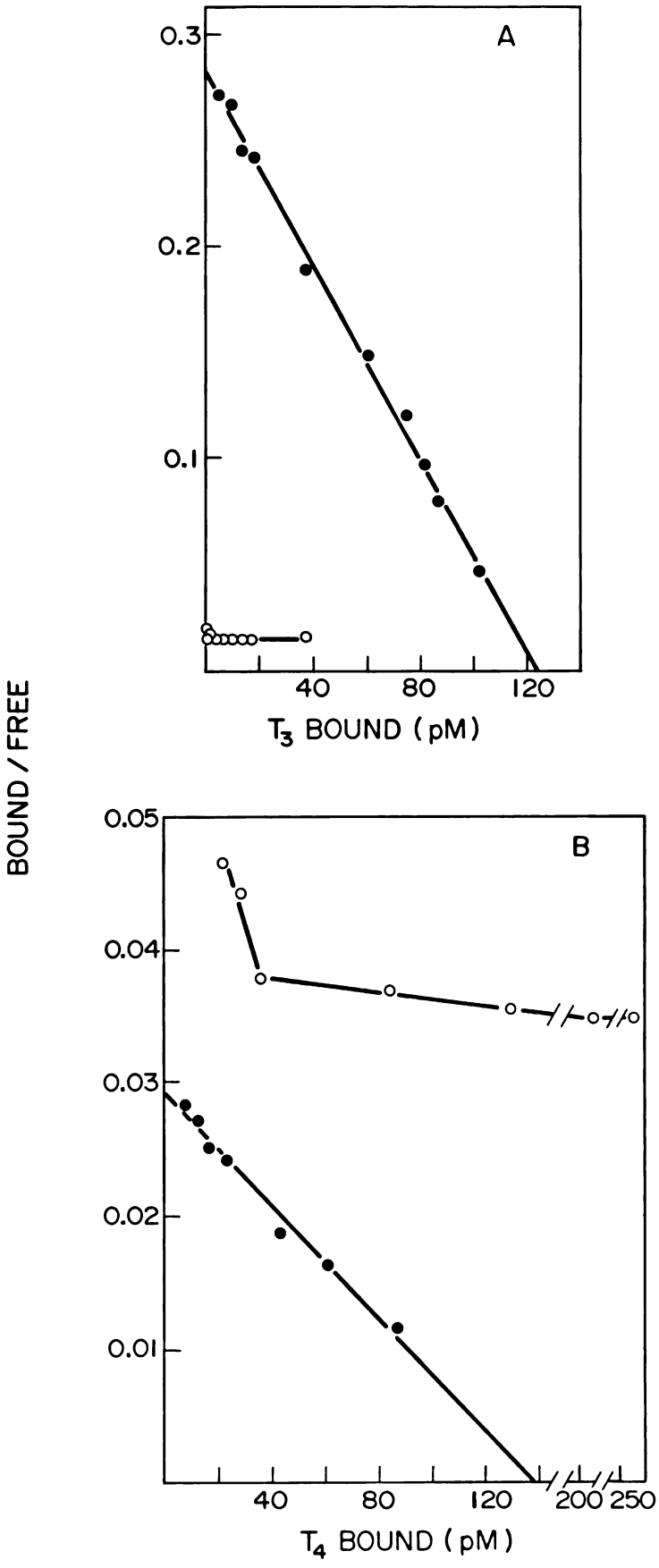

A.
0.80
0.60
0.40
0.20
20
40
60
T3 BOUND (pM)
BOUND/FREE
B.
0.05
0.04
0.03
0.02
0.01
20
40
60
80
100
120
140
T4 BOUND (pM)

as opposed to two periods) were somewhat different from those observed on extracts prepared by Procedure I. With the crude extract (Fig. 7C), whereas there was a decrease in T_3 binding, heat treatment resulted in a three-fold increase in T_4-binding activity. Thus, it appears that there is a simultaneous generation of new T_4 binding sites in association with a loss of T_3 binding sites. Scatchard analysis of T_3 and T_4 binding by the crude nuclear extracts prepared by Procedure II showed that heat treatment decreased the concentration of high-affinity binding sites for both T_3 and T_4 (Fig. 9, A and B). The majority of the T_3 and T_4 binding sites remaining after heat treatment were low-affinity, high-capacity sites (Fig. 9, A and B).

The nuclear extract prepared by Procedure II was also chromatographed on Sephadex G-100, and the effect of heat on binding activity in excluded (PI) and included (PII) fractions was examined. Heat treatment of the PI fraction, which contained approximately 95% of the protein with little hormone binding activity, resulted in an increase of both T_3 and T_4 binding (Fig. 7D). As mentioned above, heating of the PII receptor preparation led to a decrease in both binding activities similar to the effect observed for PII receptor preparations prepared by Procedure I (Fig. 7B). Thus, the heating appears to stimulate binding activity in fractions that do not contain the original binding activity.

In summary, these results indicate that high-affinity T_3 and T_4 binding sites are inactivated during heat treatment and that, simultaneously, low-affinity T_4 binding sites are generated or exposed. These conclusions are in disagreement with those of

Figure 9: Scatchard analysis of ^{125}I-T_3 (A) and ^{125}I-T_4 (B) binding to crude nuclear extracts before (●) and after (o) heating for 10 min at 50°C. Nuclear extracts were prepared by Procedure II (sonication for three 15-sec periods). Heated samples were treated as described in the legend to Fig. 7. Binding assays were conducted with concentrations of ^{125}I-T_3 and ^{125}I-T_4, which varied from 20 to 2,000 and 300 to 9,500 pM, respectively. All assays were performed in duplicate, and nonspecific binding (1000-fold excess of unlabelled hormone) was subtracted from total hormone bound. The K_ds for ^{125}I-T_3 and ^{125}I-T_4 binding by the control extracts were 0.438 and 4.75 nM, respectively.


A
0.3
0.2
0.1
40
80
120
T3 BOUND (pM)
BOUND / FREE
B
0.05
0.04
0.03
0.02
0.01
40
80
120
200
250
T4 BOUND (pM)

Eberhardt et al. (98,99), who reported that heat treatment of FII receptor preparations decreased the concentration of high-affinity T_3 sites while not affecting the number of T_4 sites or their affinity for T_4. Thus, an extension of the experiments performed by Eberhardt et al. has produced results that are not consistent with the "Core-Holo" receptor model.

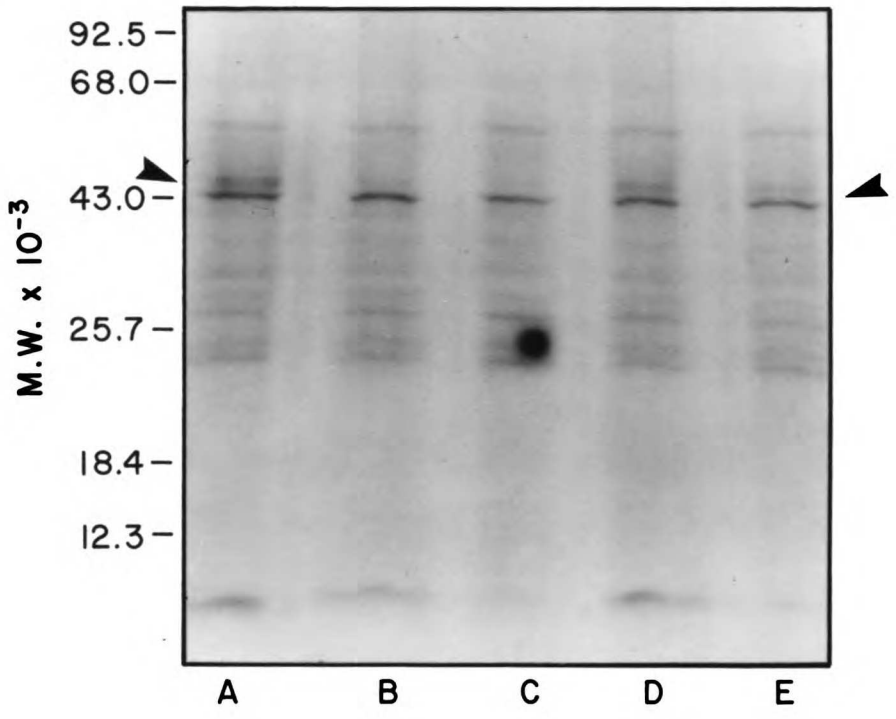
B. Photoaffinity Labelling

1. Photoaffinity Labelling of the Rat Liver Thyroid Hormone Nuclear Receptor

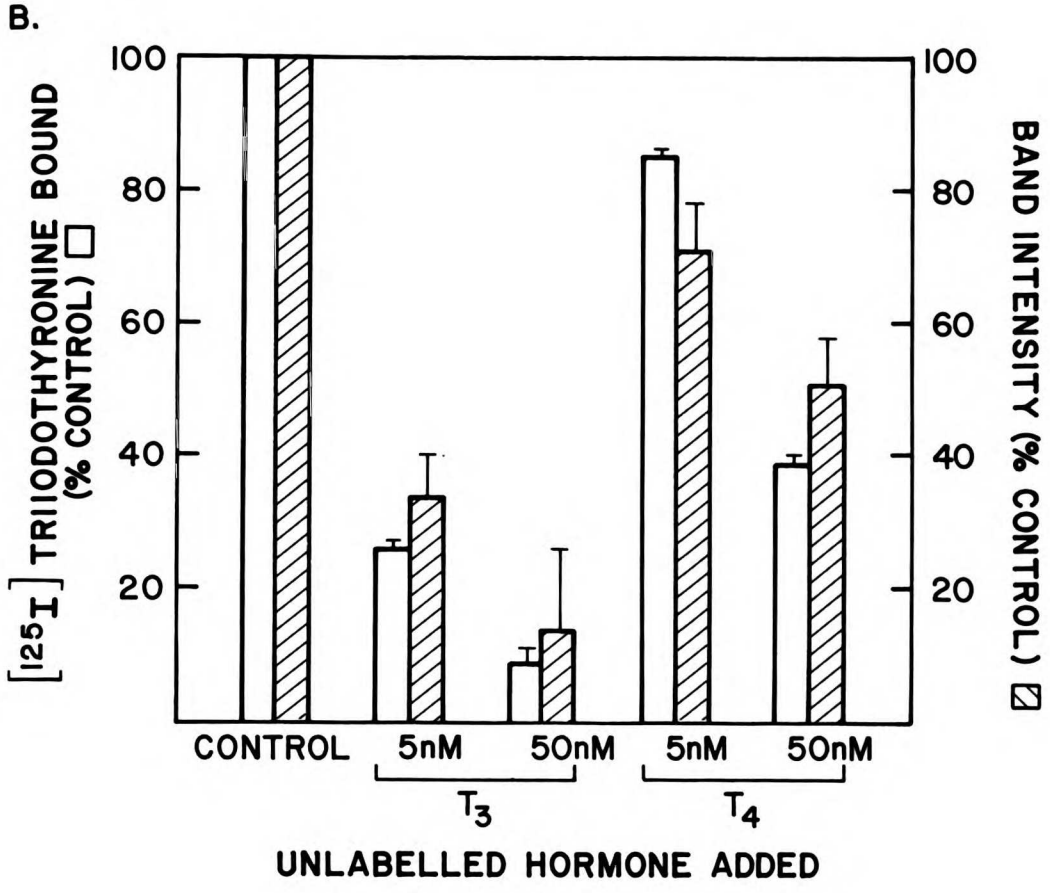
Direct evidence that ^{125}I-T_3 can serve as a photoreactive probe was obtained by irradiating 1 nM ^{125}I-T_3 in the presence of partially purified thyroid hormone nuclear receptor preparations followed by SDS polyacrylamide gel electrophoresis and autoradiography. These initial experiments were conducted according to the method of Somack et al. (120), which involved irradiating samples for 100 sec with 254 nm light. As shown in Fig. 10A (lane A), a number of protein bands were covalently labelled and, in experiments not shown here, the photolabelling was found to be light-dependent. That this extensive labelling was due to nonspecific binding of T_3 to numerous proteins was supported by the finding that the addition of 5 or 50 nM unlabelled T_3 (Fig. 10A, lane B and C, respectively) or T_4 (Fig. 10A, lane D and E, respectively) did not inhibit the photolabelling of most of these with ^{125}I-T_3. However, there was a band corresponding to a molecular weight of 46,000 whose labelling was inhibited by the addition of excess unlabelled T_3 and T_4 (Fig. 10A, arrow). This inhibition of the photolabelling process indicated that the 46,000 MW protein

Figure 10: Irradiation of partially purified rat liver thyroid hormone nuclear receptor in the presence of ^{125}I-T_3 with or without unlabelled T_3 or T_4. Partially purified receptor preparations (PII, Materials and Methods) were incubated with 1 nM ^{125}I-T_3 in the presence or absence of unlabelled T_3 or T_4 for 4 h at 22°C. Samples were chilled and an aliquot was analyzed, by standard procedures, for the amount of ^{125}I-T_3 reversibly bound (Materials and Methods). The remaining receptor preparations were irradiated with 254 nm light for three 20-min periods, separated by 5 min of cooling on ice. A. Autoradiogram of SDS polyacrylamide gel analysis of irradiated receptor samples. Irradiated samples were dialyzed against water, lyophilized and analyzed by SDS gel electrophoresis as described in Materials and Methods. Lanes: A, ^{125}I-T_3; B, + 5 nM T_3; C, + 50 nM T_3; D, + 5 nM T_4; E, + 50 nM T_4. Approximately 60 fmol of receptor was applied to each lane. B. The effect of unlabelled T_3 and T_4 addition on reversible ^{125}I-T_3 binding to the partially purified receptor and on the photolabelling of the 46,000-dalton protein. The amount of ^{125}I-T_3 reversibly bound by the receptor as analyzed by Sephadex G-25 assay columns is depicted by the open bars. The density of the band (on the autoradiogram in Fig. 10A) corresponding to a molecular weight of 46,000 daltons was determined by scanning with a densitometer and is depicted by the hatched bars.

A.


92.5
68.0
43.0
25.7
18.4
12.3
M.W. x 10^{-3}
A
B
C
D
E

B.


[^{125}I] TRIIODOTHYRONINE BOUND (% CONTROL) □
BAND INTENSITY (% CONTROL) ▨
100
80
60
40
20
CONTROL
5nM
50nM
5nM
50nM
T_3
T_4
UNLABELLED HORMONE ADDED

has a higher affinity for the added unlabelled ligands than other proteins in the preparation. Furthermore, the results in Fig. 10A demonstrate that the addition of as little as 5 nM unlabelled T_3 greatly diminished the intensity of the 46,000 MW band (lane B), whereas 50 nM unlabelled T_4 was required to achieve comparable inhibition of the ^{125}I-T_3-dependent labelling (lane E). Thus, the 46,000 MW protein band has approximately a 10-fold higher affinity for T_3 than T_4, a property similar to that of the thyroid hormone nuclear receptor.

The intensity of the 46,000 MW bands obtained with the addition of various concentrations of unlabelled T_3 or T_4 was evaluated by densitometry. These results, expressed as a percentage of the control (which did not contain unlabelled T_3 or T_4), were compared with the amounts of ^{125}I-T_3 bound to the thyroid hormone receptor measured in reversible binding assays (Fig. 10B). As indicated, the addition of 5 and 50 nM unlabelled T_3 reduced photolabelling of the 46,000 MW band to 34 and 14%, respectively, while these concentrations of T_3 decreased the reversible binding of ^{125}I-T_3 to the receptor to 25 and 10%, respectively, of the control. Addition of 5 and 50 nM T_4 reduced photolabelling of the 46,000 MW band to 71 and 51%, respectively, while reversible binding was reduced to 91 and 44%, respectively, of the control. This analysis further supports the notion that the photolabelled 46,000 MW protein has similar binding affinities for T_3 and T_4 as does the thyroid hormone nuclear receptor.

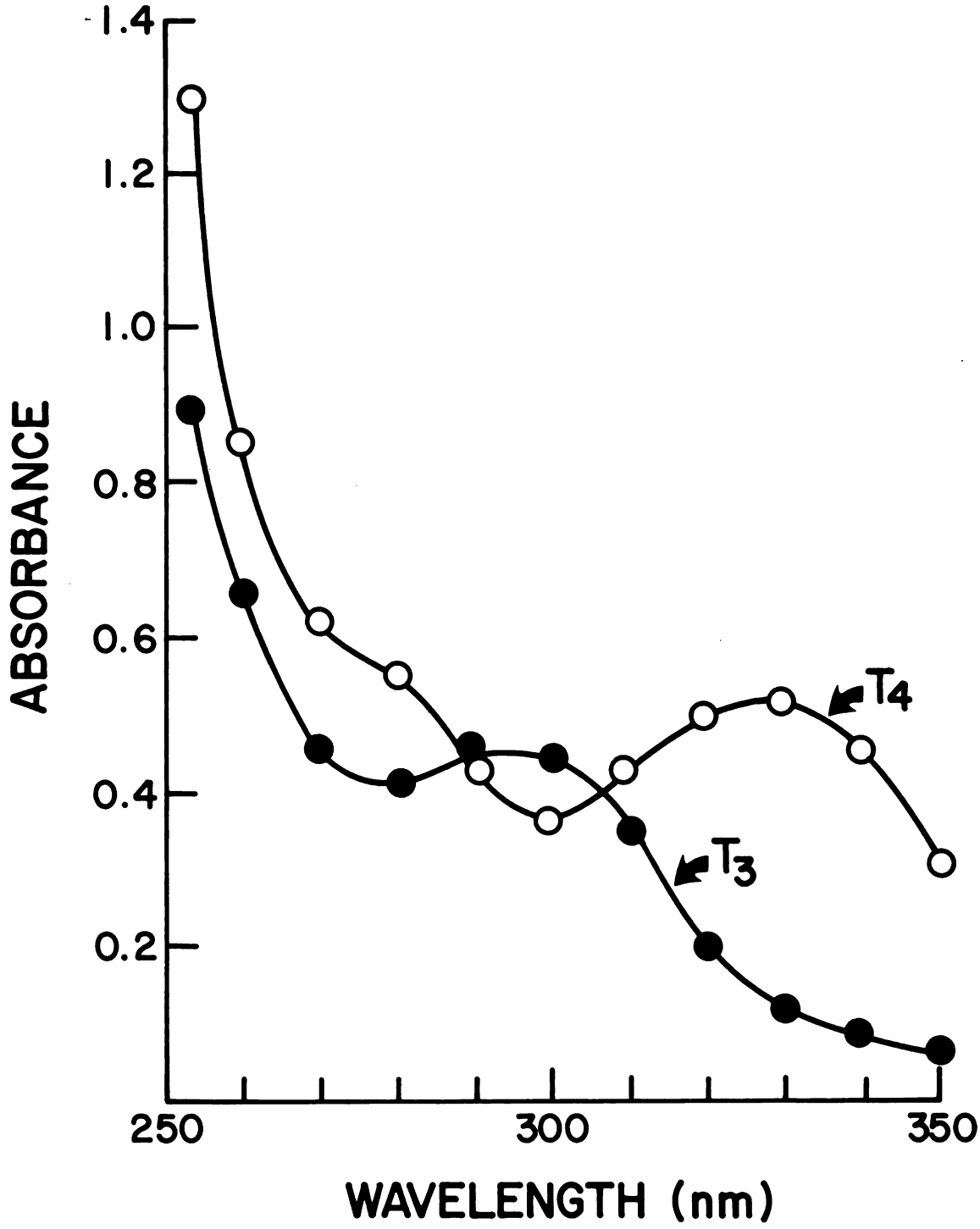
The incorporation of radioactive label into the 46,000 MW protein was found to be very low, approximately 0.01% of the ^{125}I-T_3 added. The low incorporation of label may have been due to two factors.

First, light of 254 nm is very damaging to iodothyronines (132), suggesting that the effective concentrations of T_3 may have been reduced during the photolabelling process. Secondly, receptor-containing preparations absorbed 254 nm light (and only minimally absorbed 280 - 310 nm), which may have reduced the intensity of light available to photoactivate T_3. It was therefore considered more beneficial to use light of another wavelength.

To determine the wavelength of light that should be used, an absorption spectrum of T_3 was determined. Figure 11 demonstrates that 1 nM T_3 (in Buffer G) absorbs light most effectively in two ranges: 1) from 250 to 270 nm; and 2) from 280 to 310 nm. For the reasons given above, light in the range of 280 to 310 nm was chosen for subsequent photolabelling experiments. Therefore, an RPR-3000 Å lamp (Southern New England Ultraviolet Company; Hamden, CT), which emits light with a peak emission at 254 nm and also at 300 nm was used; 254 nm light was effectively blocked out by borosilicate glass tubes provided by Dr. Martin Shetlar, University of California, San Francisco.

The ability of ^{125}I-T_3 to serve as a photoreactive probe using 300 nm light was investigated. Because Somack *et al.* (120) suggested that photolabelling of TBPA with ^{125}I-T_4 may have occurred through the attachment of thyronine or iodide radicals formed upon photo-induced deiodination, it was necessary to determine whether irradiation of ^{125}I-T_3 with 300 nm light would induce deiodination. The extent of deiodination was monitored by filtering irradiated ^{125}I-T_3 over Sephadex G-25 assay columns to which ^{125}I-T_3 adsorbs, while free

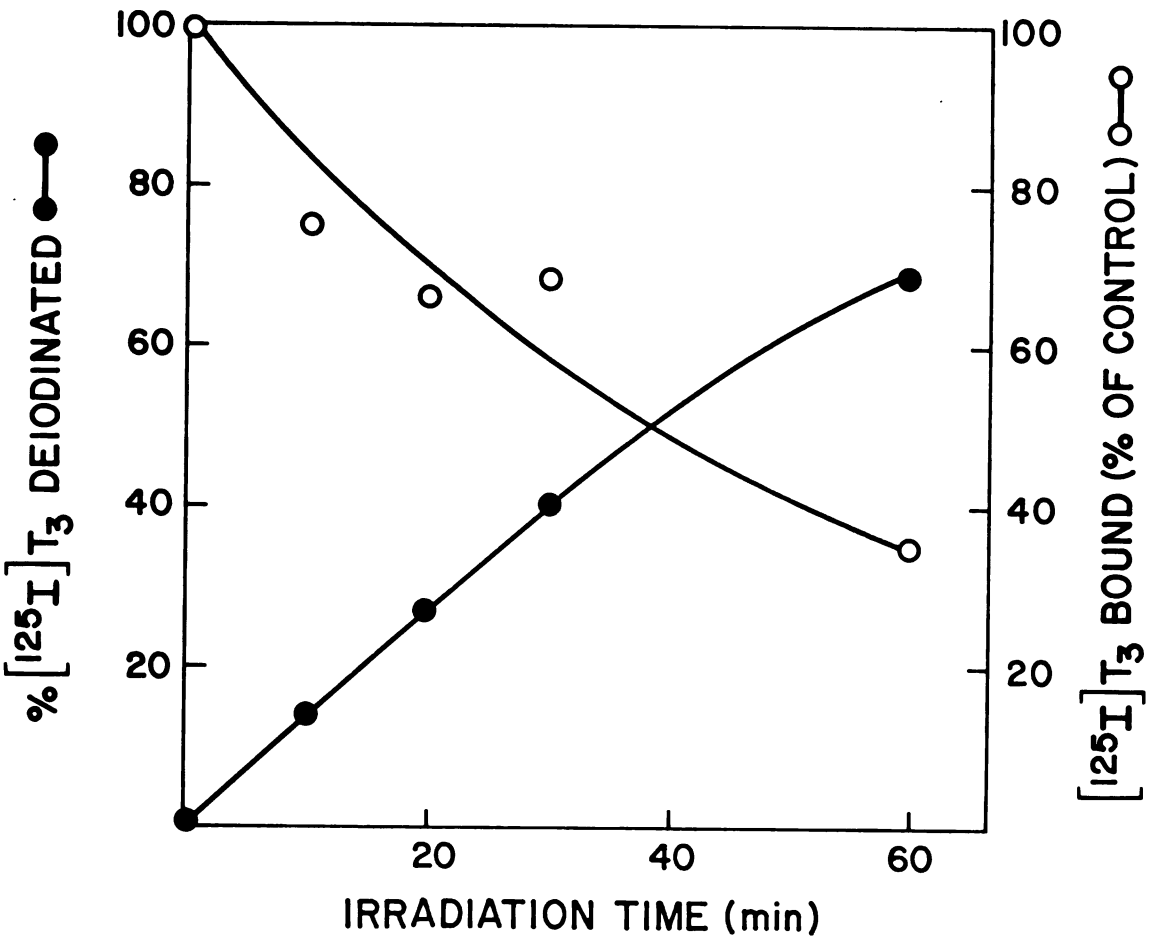
Figure 11: Absorption spectra of triiodothyronine and thyroxine. The absorbance of light, with a wavelength from 250 to 350 nm, by T_3 and T_4 (1 nM in Buffer G) was measured in relation to air. The amount of light absorbed by 1 nM T_3 or T_4 in Buffer G (Section II, B) was measured in a Gilford spectrophotometer (Gilford Instruments, Oberlin, Ohio). The measurements were determined at room temperature.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE
T4
T3
250
300
350
WAVELENGTH (nm)

iodide elutes in the included fraction. The amount of radioactive iodide which eluted from the Sephadex columns after different periods of irradiation was compared to the amount of ^{125}I-T_3 present in non-irradiated samples to obtain a percentage of ^{125}I-T_3 that was deiodinated. As shown in Fig. 12, irradiation of ^{125}I-T_3 with 300 nm light led to increasing levels of deiodination; after 60 min of irradiation, approximately 70% of ^{125}I-T_3 had been deiodinated. Furthermore, irradiated ^{125}I-T_3 preparations lost their ability to bind to the thyroid hormone receptor in proportion to the loss of the iodide groups (Fig. 12); ^{125}I-T_3 preparations that had been irradiated for 60 min were able to bind the receptors with only 40% the ability of a similar concentration of non-irradiated ^{125}I-T_3. Therefore, as shown by these two procedures, ^{125}I-T_3 was deiodinated with 300 nm light and could possibly serve as a photoreactive probe. Thus, subsequent photoaffinity labelling experiments were conducted with 300 nm light.

Figure 13 demonstrates that, when receptor preparations in the presence of ^{125}I-T_3 were irradiated with 300 nm light, many proteins were labelled. Similar to the results obtained after irradiation with 254 nm light (Fig. 10), many of these protein bands were nonspecifically labelled since the addition of 50 nM unlabelled T_3 did not block their photolabelling (Fig. 13). However, a protein band at 46,000 MW (Fig. 13, arrow) was specifically labelled, because the addition of 50 nM T_3 blocked its photolabelling. The incorporation of radioactive label was greater when irradiation of receptor-^{125}I-T_3 incubation mixtures was conducted for 60 min as opposed to 20 min

Figure 12: Time course of light-dependent deiodination of ^{125}I-T_3 and subsequent binding of irradiated ^{125}I-T_3 to receptor preparations. A solution of 1 nM ^{125}I-T_3 in Buffer G was irradiated with 300 nm light. At various times, 0.40-ml aliquots were removed to determine the amount of deiodination incurred and to attain a measure of the irradiated T_3's ability to bind to the receptor. The columns were washed with a total of 2.4 ml, the first 0.80 ml was discarded and the next 1.6 ml was saved and counted. The radioactivity present in the 1.6-ml fractions represented free ^{125}I. (Intact ^{125}I-T_3 was still attached to the Sephadex G-25 columns.) The amount of free ^{125}I (●--●) is expressed as a percentage of the amount of ^{125}I-T_3 present in 0.4 ml of 1 nM ^{125}I-T_3 prior to irradiation. Additionally, 0.4 ml aliquots of ^{125}I-T_3 irradiated for various times were added to test tubes with partially purified receptor (PII) in the presence or absence of unlabelled T_3 (100-fold excess). Incubation was conducted for 4 h at 22°C. The amount of specifically bound ^{125}I-T_3 was determined by filtration on Sephadex G-25 assay columns by the standard procedure. The amount of irradiated ^{125}I-T_3 bound was compared with the control, the amount of non-irradiated ^{125}I-T_3 bound (o--o).


100
80
60
40
20
% [125I] T3 DEIODINATED
[125I] T3 BOUND (% OF CONTROL)
20
40
60
IRRADIATION TIME (min)

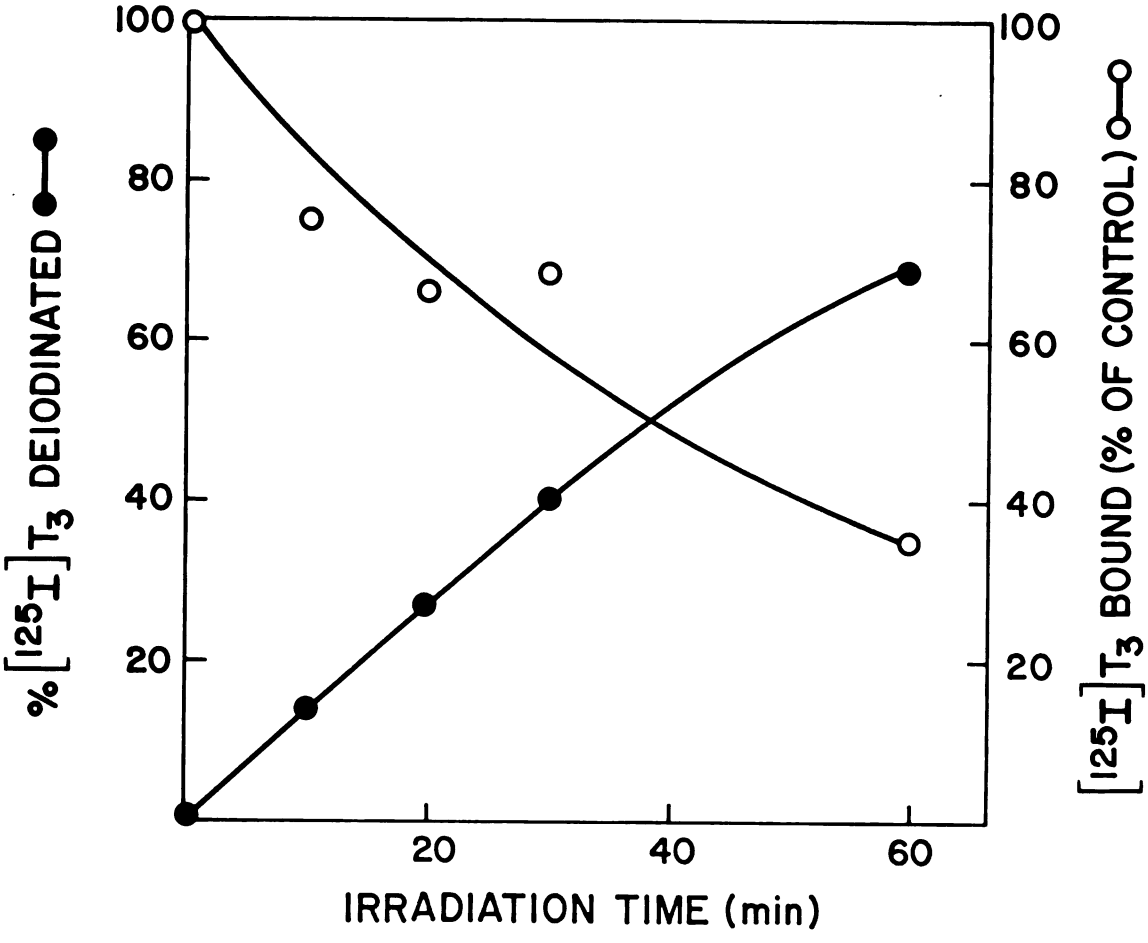
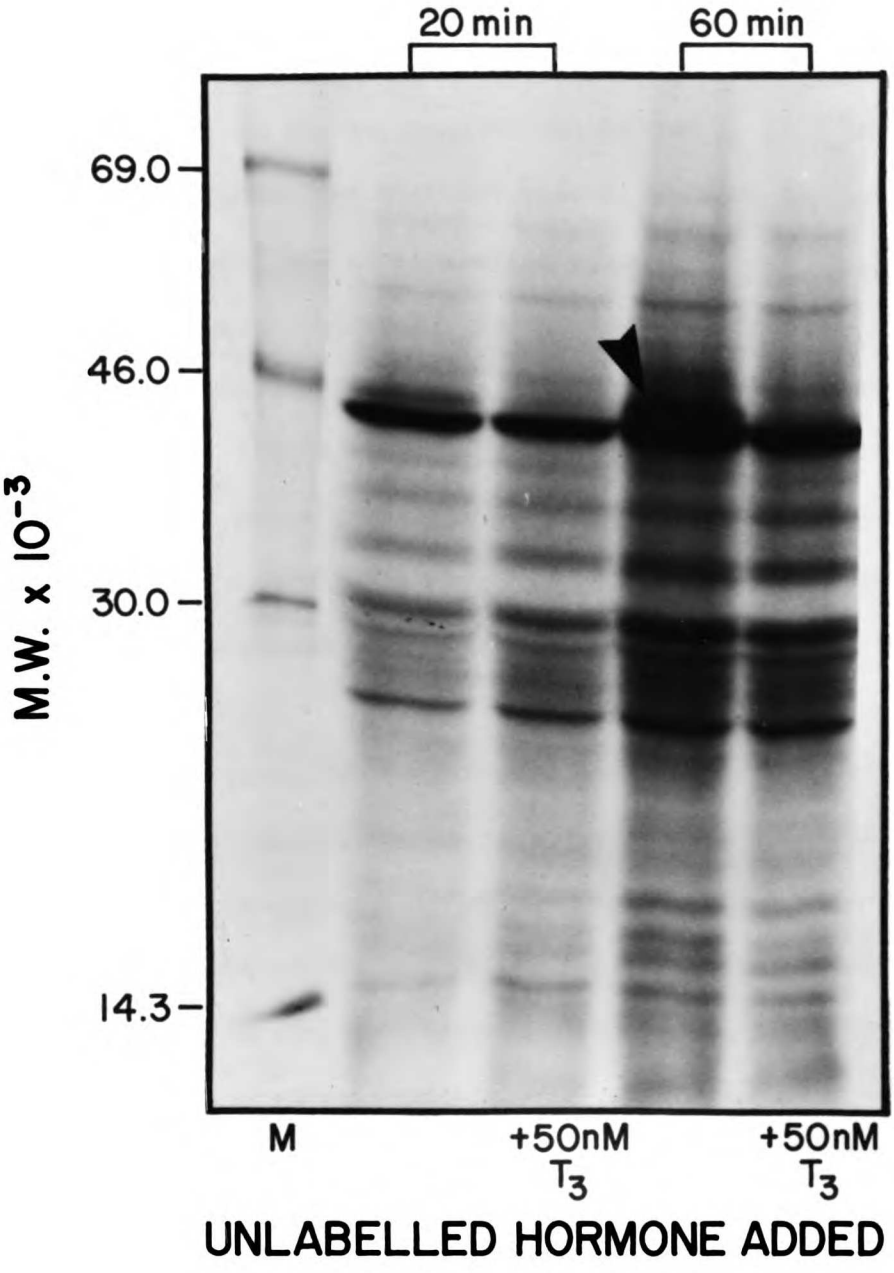

100
80
60
40
20
% [125I] T3 DEIODINATED
[125I] T3 BOUND (% OF CONTROL)
20
40
60
IRRADIATION TIME (min)

Figure 13: Kinetic analysis of the photolabelling of partially purified rat liver thyroid hormone nuclear receptor with ^{125}I-T_3. PII receptor preparations were incubated with 1 nM ^{125}I-T_3 in the presence or absence of 50 nM unlabelled T_3 for 3.5 h at room temperature. Samples were chilled and a 0.4-ml aliquot was removed to determine the amount of ^{125}I-T_3 bound by the standard binding assay. The remaining samples were irradiated with 300 nm light for either one 20-min period or three 20-min periods, separated by 5 min of cooling on ice. Reaction products were analyzed on SDS polyacrylamide gel electrophoresis and an autoradiogram of the SDS gel is presented. Approximately 300 fmol of total binding activity were present in the control samples. Lanes: M, ^{14}C-methylated protein molecular weight standards (Amersham/Searle Corp.).


20 min
60 min
69.0
46.0
30.0
14.3
M.W. x 10^{-3}
M
+50nM T_3
+50nM T_3
UNLABELLED HORMONE ADDED

and also greater than when photolabelling experiments were performed with 254 nm light (Fig. 10). The efficiency of photolabelling (at 60 min) was approximately 0.1%.

Competition analysis, as previously described in this section, was performed using 300 nm light. Rat liver receptor preparations incubated with ^{125}I-T_3 in the presence of unlabelled T_3 or T_4 were irradiated for 60 min. The autoradiographic pattern in Fig. 14 demonstrates that the addition of unlabelled hormone diminished the labelling intensity of a protein band (arrow) which migrated at 46,000 MW. Similar to the photoaffinity labelling experiments performed with 254 nm light (Fig. 10), a 10-fold higher concentration of unlabelled T_4 was required to block photolabelling to an equivalent extent as with 5 nM unlabelled T_3. The higher affinity of the 46,000 MW protein for T_3 than T_4 again suggests that this protein is the thyroid hormone receptor.

Photoaffinity labelling experiments were also conducted with the affinity-purified receptor, which is approximately 250-fold more purified than the PII receptor preparations that were used in the previous studies. It was reasoned that, if the specifically photolabelled 46,000 MW protein is the thyroid hormone receptor, then irradiation of affinity-purified receptor preparation in the presence of ^{125}I-T_3 should result in a predominantly labelled protein of 46,000 MW. Figure 15 indicates that a 46,000 MW protein was specifically labelled; its labelling was blocked by the addition of unlabelled T_3 (Fig. 15, lane B, arrow). Furthermore, the results indicated an enrichment in the photolabelling of the 46,000 MW protein in relation to other

Figure 14: Photoaffinity labelling of partially purified thyroid hormone rat liver nuclear receptor with ^{125}I-T_3. PII receptor preparations were incubated with 1 nM ^{125}I-T_3 in the presence or absence of unlabelled T_3 or T_4 for 4 h at room temperature. Aliquots (0.4 ml) were removed for a determination of ^{125}I-T_3 bound by the standard binding assay. Samples were irradiated with 300 nm light for three 20-min periods, separated by 5 min of cooling on ice. They were subsequently analyzed by SDS gel electrophoresis and an autoradiogram of the SDS gel is presented. Approximately 320 fmol of receptor binding activity was applied to the control lane. Lanes: M, ^{14}C-methylated protein molecular weight standard.

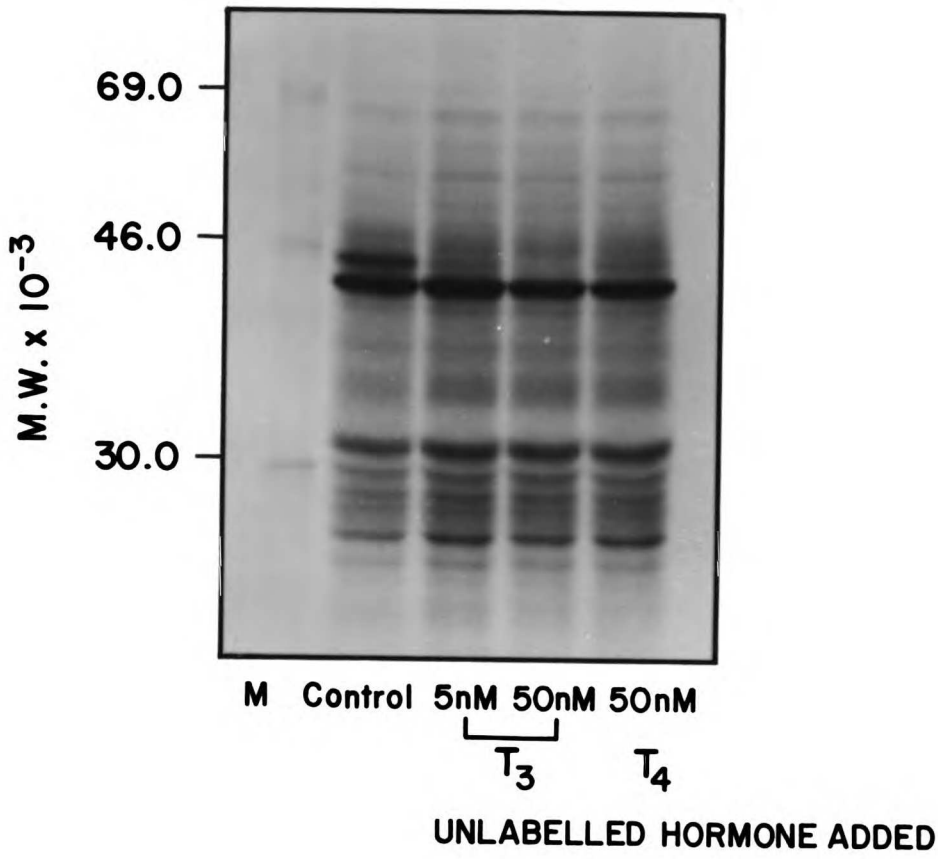
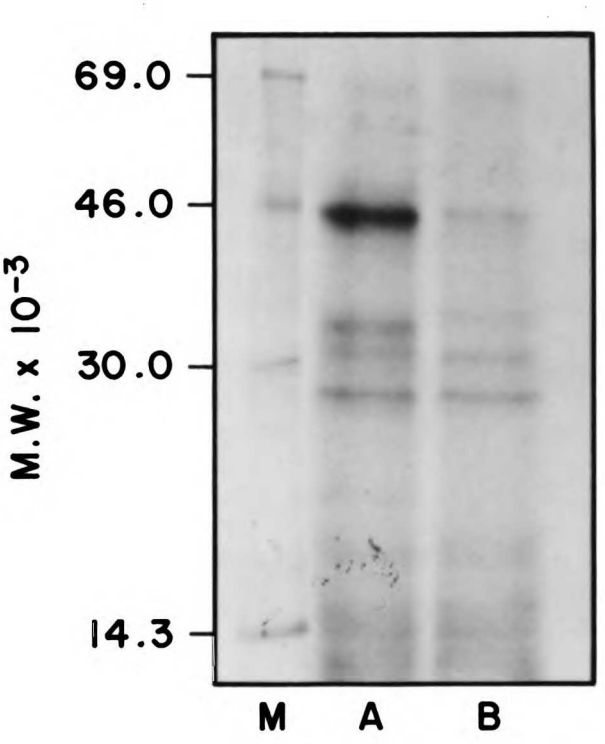

69.0
46.0
30.0
M.W. x 10^{-3}
M
Control
5nM
50nM
50nM
T_3
T_4
UNLABELLED HORMONE ADDED

Figure 15: Photoaffinity labelling of the affinity-purified receptor with ^{125}I-T_3. Free T_3 was removed from affinity-purified receptor by filtration over Sephadex G-25 assay columns as described in Materials and Methods. Column eluates containing the affinity-purified receptor were incubated with 1 nM ^{125}I-T_3 with or without unlabelled T_3 (50-fold excess) for 4 h at room temperature. The amount of ^{125}I-T_3 bound to the receptor preparation was determined for a 0.4-ml aliquot by the standard binding assay. Samples were irradiated with 300 nm light for three 20-min periods, separated by 5 min of cooling on ice and subsequently analyzed by SDS gel electrophoresis. An autoradiogram of the SDS gel is presented. Approximately 550 fmol of receptor binding activity was applied to lane A. Lanes: M, ^{14}C-methylated protein molecular weight standards; A, control; B, + 50 nM T_3.

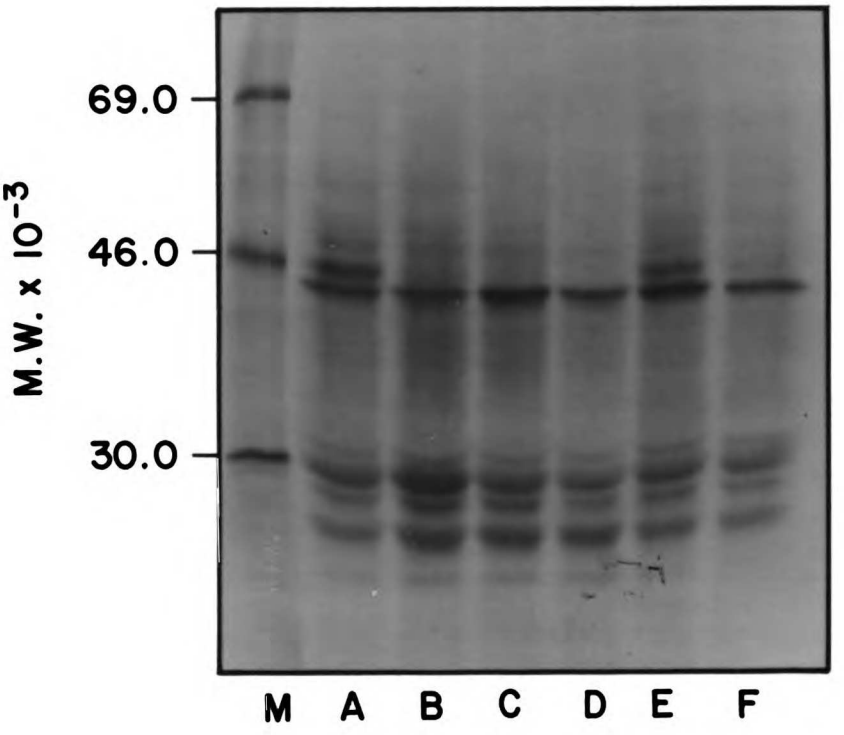

69.0
46.0
30.0
14.3
M.W. x 10⁻³
M
A
B

labelled bands in this receptor preparation, which was enriched for thyroid hormone nuclear receptors. A protein band that migrates at a molecular weight of approximately 33,000 MW also appeared to be specifically photolabelled; the identity of this protein band is not known.

In a corollary experiment, PII receptor preparations were incubated with T_3-affinity matrices under conditions that would adsorb virtually all of the thyroid hormone nuclear receptor. The supernatant was separated from the affinity matrix, incubated with 1 nM ^{125}I-T_3 in the presence or absence of unlabelled T_3, and subsequently irradiated with 300 nm light. As shown in Fig. 16 (lanes C and D), treatment of the PII receptor preparation with the T_3-affinity matrix prevented labelling of the 46,000 MW protein, presumably due to adsorbtion of the 46,000 MW protein to the affinity matrix. This result further supports the hypothesis that the 46,000 MW protein is the thyroid hormone receptor. Incubation of PII receptor preparations with Sepharose 6B did not prevent labelling of the 46,000 MW band (Fig. 16, lanes E and F).

As shown by the data in Fig. 15, photolabelling of the affinity-purified receptor resulted in minimal non-specific labelling. This reduced background labelling should greatly facilitate further characterization of the receptor. However, these experiments were not routinely performed with the affinity-purified receptor because of the relative unavailability of sufficient amounts of this preparation. Therefore, experimental conditions were sought that would decrease the level of non-specific labelling obtained with PII receptor preparations.

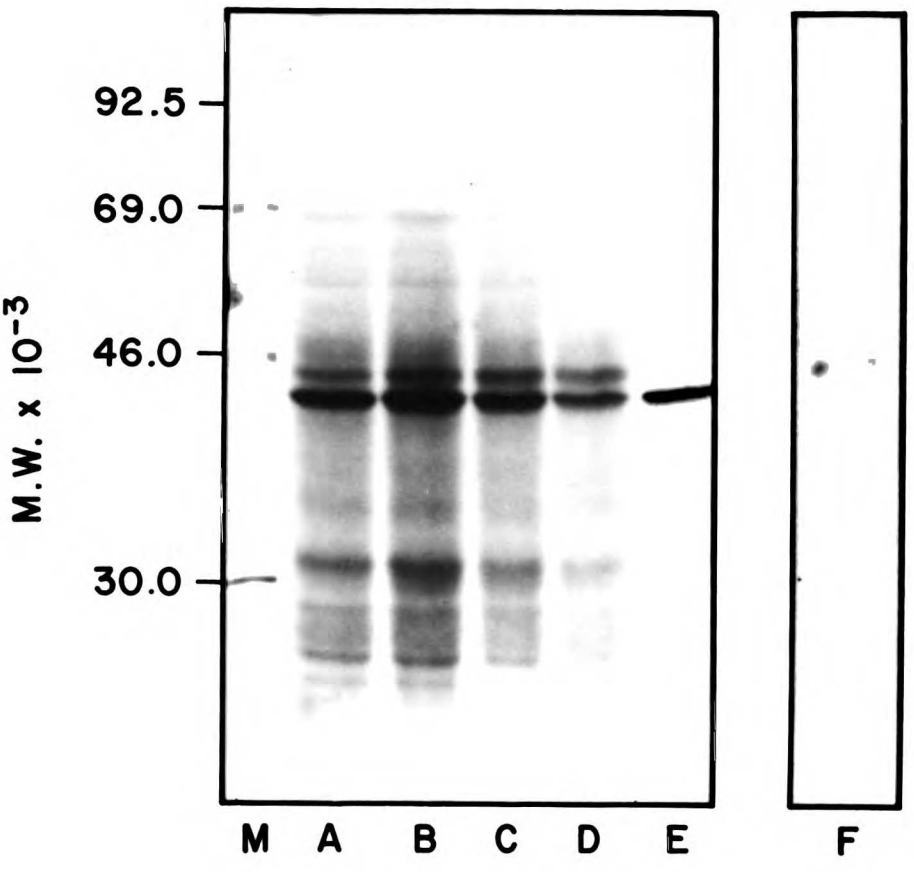
Figure 16: Photoaffinity labelling of partially purified receptor treated with T_3-affinity matrix. PII receptor preparations were incubated with either T_3-affinity matrix or Sepharose 6B at a ratio of 4000 fmol T_3 binding sites/ ml bed volume for 2 h at room temperature with gentle shaking. Incubation mixtures were transferred to Quik-Sep columns (Isolab) and the eluates were collected by gravity. Treated and control receptor preparations were incubated with 1 nM ^{125}I-T_3 in the presence or absence of 50nM unlabelled T_3 for 4h at room temperature. Samples were irradiated with 300nm light for three 20-min periods separated by cooling on ice for 5 min. Irradiated samples were analyzed by SDS gel elctrophoresis and the autoradiogram is represented. Lanes: M, ^{14}C-methylated protein molecular weight standards; A, control; B, + 50nM T_3; C, PII treated with T_3-affinity matrix control; D, + 50nM T_3; E, PII treated with Sepharose 6B control; F, + 50nM T_3.


69.0
46.0
30.0
M.W. x 10^{-3}
M
A
B
C
D
E
F

In an effort to reduce background labelling, irradiation of ^{125}I-T_3 pre-bound to PII receptor preparations was performed. ^{125}I-T_3 was incubated with PII receptor preparations for 4 hr and then filtered over Sephadex G-25 assay columns pretreated with histones to remove free ^{125}I-T_3 (Materials and Methods). The resulting 0.8 ml column eluate, which contained ^{125}I-T_3-receptor complexes, was irradiated with 300 nm light, and an autoradiogram of the reaction products is shown in Fig. 17 (lane F). Under these conditions, there was the predominant labelling of a protein with a molecular weight of 46,000 and a drastically reduced background of photolabelling. However, this protocol also reduced the efficiency of photolabelling. For example, the intensity of the 46,000 MW band in Lane F, which represents 335 fmol of binding activity that had been filtered over Sephadex prior to irradiation, is considerably less than the intensity of the 46,000 MW band in Lane A, which reflects 220 fmol of unfiltered receptor. The reason for this difference in photolabelling efficiency is not clear. It was considered a possibility that the ^{125}I-T_3-receptor complexes were not stable during the 60-min irradiation period; therefore, in a subsequent experiment the irradiation period was reduced from 60 to 20 min. Irradiation of ^{125}I-T_3-receptor complexes for 20 min did not enhance the intensity of the 46,000 MW band (data not shown). This finding suggests that a large excess of ^{125}I-T_3 is necessary to obtain efficient photolabelling. These results necessitated finding another method that would reduce non-specific labelling.

The irradiation of rat liver receptor preparations (PII) in the presence of 0.25 to 1.0 nM ^{125}I-T_3 was performed to determine if

Figure 17: Photoaffinity labelling of partially purified rat liver thyroid hormone nuclear receptor preparations with various concentrations of ^{125}I-T_3 (A-E) and photolabelling of ^{125}I-T_3-receptor complexes (F). Lanes A - E: Autoradiogram of an SDS-gel of PII receptor irradiated in the presence of varying concentrations of ^{125}I-T_3. The irradiation procedure was similar to that outlined in the legend to Fig. 14. A, 1.0 nM ^{125}I-T_3; B, 0.75 nM ^{125}I-T_3; C, 0.50 nM ^{125}I-T_3; D,. 0.25 nM ^{125}I-T_3; E, 0.25 nM ^{125}I-T_3 + 50 nM T_3. Approximately 220 fmol of T_3 binding activity was applied to lane A. Lane F: Autoradiogram of an SDS gel of photoaffinity labelled ^{125}I-T_3-receptor complexes prepared by described below. PII receptor was incubated with 1 nM ^{125}I-T_3 for 4 h at room temperature, after which the entire sample was filtered over Sephadex G-25 assay columns according to the standard binding assay. All assay columns were pretreated with histones. The 0.8-ml column eluates (Materials and Methods) were combined and an aliquot was removed for a determination of bound T_3. The remaining sample was then irradiated for 60 min at 4°C. Reaction products were analyzed on SDS gel electrophoresis, and the autoradiogram of the SDS gel is presented. Approximately 335 fmol of T_3 binding activity was applied to this lane.


92.5
69.0
46.0
30.0
M.W. x 10⁻³
M
A
B
C
D
E
F

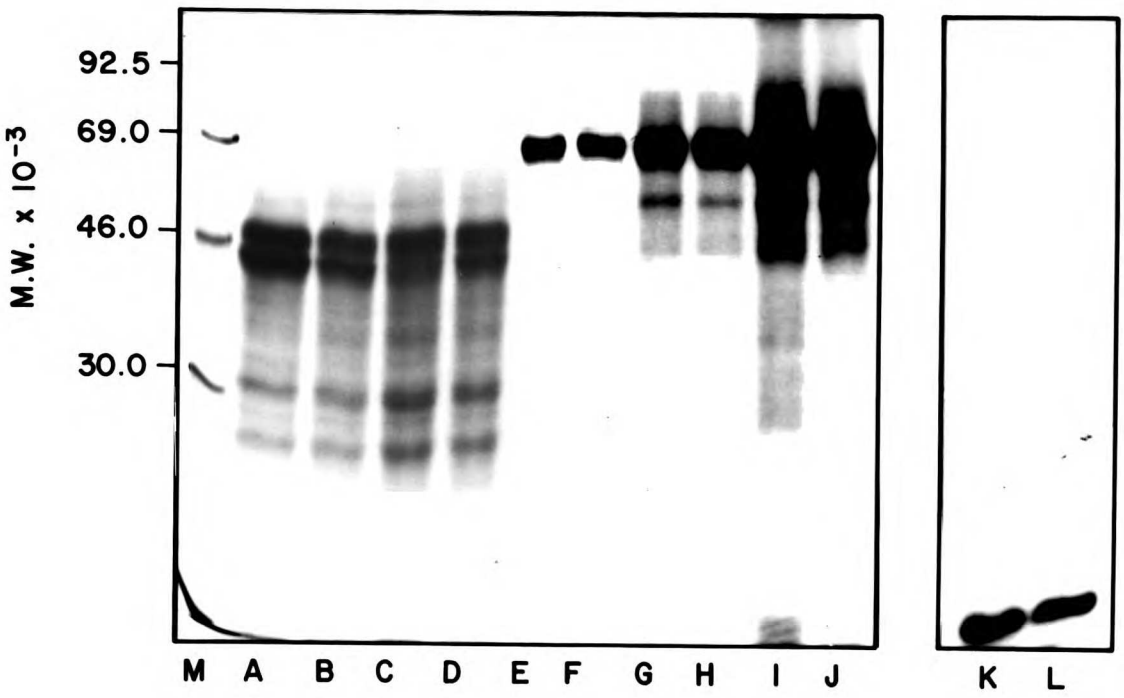
using lower concentrations of ^{125}I-T_3 would decrease non-specific photolabelling. Indeed, the photolabelling of PII receptor preparations in the presence of 0.25 nM ^{125}I-T_3 resulted in less non-specific photolabelling than when 1 nM ^{125}I-T_3 was present (Fig. 17, compare lanes D and A). Thus, both procedures (removal of free ^{125}I-T_3 from ^{125}I-T_3-receptor complexes prior to irradiation and irradiation of the receptor in the presence of reduced concentrations of ^{125}I-T_3) produced less non-specific labelling, but an intensely labelled 46,000 MW band was only observed when the receptor was irradiated in the presence of free ^{125}I-T_3.

In many of the autoradiograms, there was evidence of less intensely photolabelled proteins whose labelling appeared to be inhibited upon the addition of excess unlabelled T_3 or T_4. The proteins migrated at a molecular weight between 47,000 and 50,000 (Figs. 10 and 13-16).

2. Photoaffinity Labelling of Rat Serum Proteins

In order to determine whether the 46,000 MW band specifically labelled in rat liver nuclear extracts might be a serum contaminant, photoaffinity labelling of rat serum proteins in the presence of ^{125}I-T_3 was performed. Rat serum proteins were irradiated in the presence of ^{125}I-T_3 with or without unlabelled T_3, and the reaction products were analyzed by SDS gel electrophoresis. The results presented in Fig. 18, lanes E-J, indicate that protein bands (including that which migrated at 46,000 MW) were not specifically photolabelled (Fig. 18, lanes I and J). In experiments not shown here, the irradiation of concentrations of rat serum higher than that

Figure 18: Photoaffinity labelling of partially purified rat liver thyroid hormone nuclear receptor (Lanes A - D), rat serum (Lanes G - J) and human thyroxine binding prealbumin (TBPA) (Lanes K and L) with ^{125}I-T_3. Samples were incubated with 1 nM ^{125}I-T_3 in the presence or absence of unlabelled T_3 or T_4 for 4 h at room temperature. Aliquots (0.4 ml) were removed for a determination of ^{125}I-T_3 bound by standard procedures. Samples were irradiated with 300 nm light for three 20-min periods, separated by 5 min of cooling on ice. Irradiated samples were analyzed by SDS gel electrophoresis and the autoradiogram is presented. Relative amounts of proteins used (PII receptor:rat serum:TBPA) were 1:0.02-0.09:38,000. Lanes: M, ^{14}C-methylated protein molecular weight markers; A, receptor in the presence of 1 nm ^{125}I-T_3; B, + 5 nM T_3; C, + 50 nM T_3; D, + 50 nM T_4; E, serum (relative concentration, 0.02) in the presence of 1 nM ^{124}I-T_3; F, + 50 nM T_3; G, serum (relative concentration, 0.04) in the presence of ^{125}I-T_3; H, + 50 nM T_3; I, serum (relative concentration, 0.09) in the presence of 1 nM ^{125}I-T_3; J, + 50 nM T_3; K, TBPA in the presence of 1 nM ^{125}I-T_3; L, same as K + 50 nM T_3.


M.W. x 10⁻³
92.5
69.0
46.0
30.0
M A B C D E F G H I J
K L

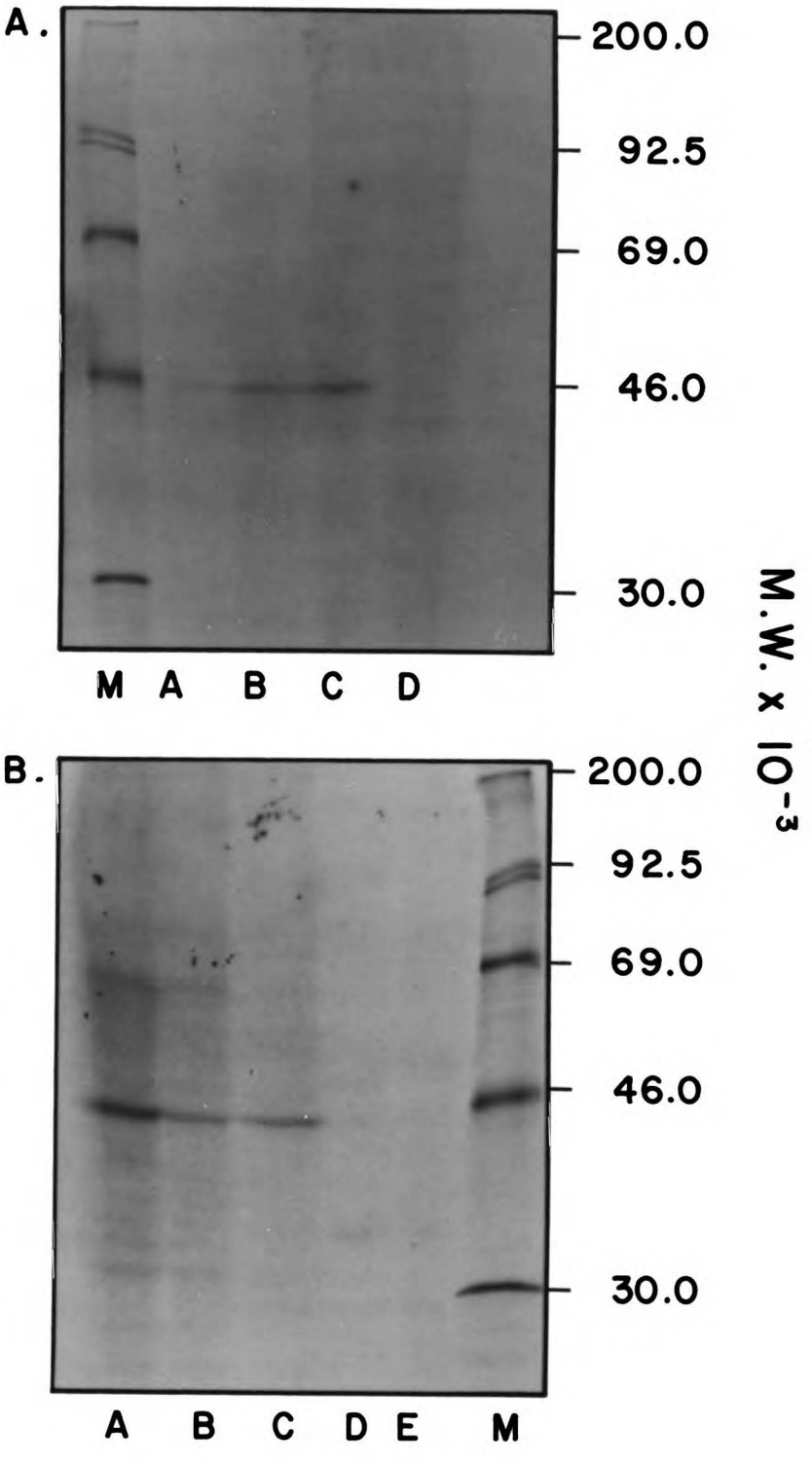
used in Fig. 18 (lanes I and J) did not yield a specifically photolabelled 46,000 MW protein band. Irradiation of purified TBPA in the presence of ^{125}I-T_3 resulted in its photolabelling (Fig. 18, lanes K and L). The addition of 50 nM unlabelled T_3 (Fig. 18, lane L) did not block photolabelling (50 nM T_3 was not expected to block photolabelling of TBPA, since the affinity of TBPA for T_3 is very low, 1 x $10^{-7}M^{-1}$ {133}). Specific photolabelling of a 46,000 MW protein was observed in rat liver receptor preparations (compare lanes A, B, C and D, Fig. 18). This indicates that the 46,000 MW protein is not a serum contaminant and supports the claim that it is the thyroid hormone nuclear receptor.

3. Photoaffinity Labelling of GH_3W5 and HTC Cells

It was of interest to determine whether the 46,000 MW protein photolabelled in rat liver nuclear extracts, and presumed to be the receptor, would also be photolabelled in GH_3W5 cells, a cell line responsive to thyroid hormones and demonstrated to contain approximately 20,000 thyroid hormone receptors per cell (69). Likewise, photoaffinity labelling experiments were conducted with HTC cells, which have not been demonstrated to be responsive to thyroid hormones and contain about 200 binding sites per cell (69).

A kinetic analysis of the photoaffinity-labelling of intact GH_3 cells with ^{125}I-T_3 was performed as described in Materials and Methods. Figure 19A demonstrates that, after a 10-min irradiation period (lane C), a 46,000 MW protein was covalently labelled. The addition of 1 μM unlabelled T_3 blocked its photolabelling (Fig. 19A, lane D), indicative of a specific photolabelling event.

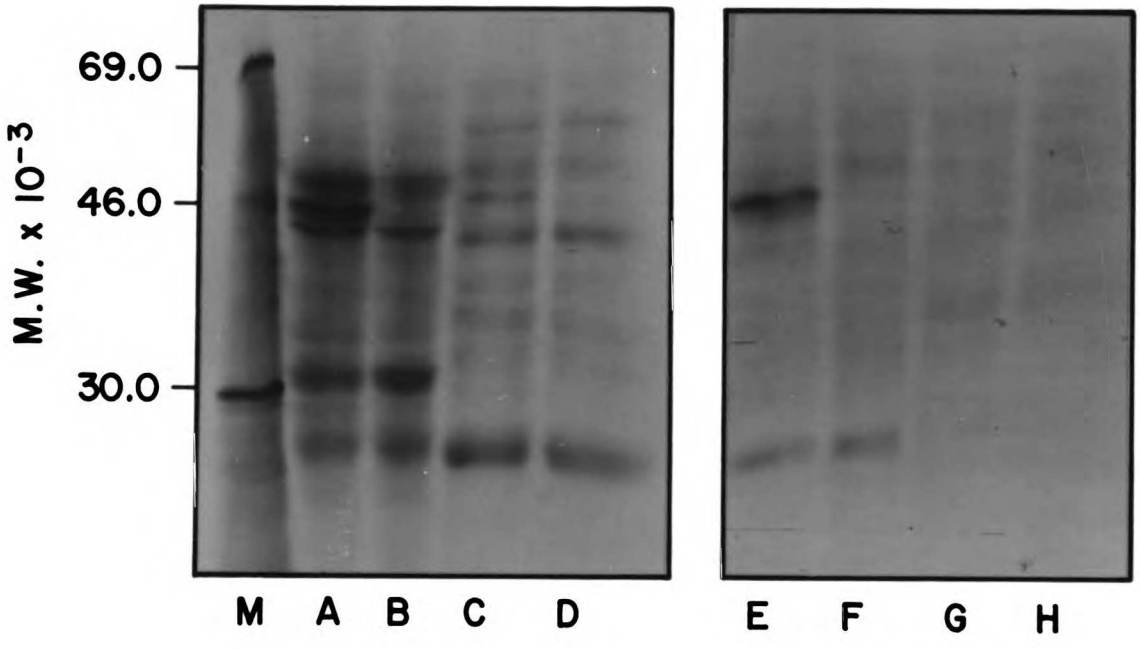
Figure 19: Photoaffinity labelling of intact GH_3 and HTC cells with ^{125}I-T_3. Cells, GH_3 and HTC, were incubated with 1 nM ^{125}I-T_3 with or without unlabelled T_3 or T_4 for 2.5 h at 37°C. They were then kept cold by placement on a bed of ice and washed with PBS. 10 ml of PBS was left to cover the cells to prevent drying and a Pyrex glass watch glass was placed over the cells to prevent penetration of light with a wavelength below 295 nm. The intact cells were irradiated with 300 nm light for various times, after which nuclear extracts were prepared according to the methods described in Materials and Methods. Nuclear extracts of equal protein concentrations were dialyzed against water and subsequently lyophilized. Samples were analyzed by SDS gel electrophoresis and autoradiograms are presented. Panel A: GH_3 cells which had been incubated with 1 nM ^{125}I-T_3 in the presence or absence of unlabelled T_3 were irradiated for various times. Lanes: M, ^{14}C-methylated protein molecular weight standards; A, 2 min irradiation; B, 5 min irradiation; C, 10 min irradiation; D, 10 min irradiation + 1 μM T_3. Panel B. GH_3 and HTC cells which had been incubated with 1 nM ^{125}I-T_3 with or without unlabelled T_3 or T_4 were irradiated for 10 min. Lanes: A, control GH_3 cells; B, + 50 nM T_3; C, + 50 nM T_4; D, control HTC cells; E, + 50 nM T_3; M, ^{14}C-methylated protein molecular weight standards.


A.
200.0
92.5
69.0
46.0
30.0
M A B C D
B.
200.0
92.5
69.0
46.0
30.0
A B C D E M
M.W. x 10-3

Results obtained from irradiating both GH_3 and HTC cells in the presence of ^{125}I-T_3 are presented in Fig. 19B. A 46,000 MW protein was photolabelled in GH_3 cells (Fig. 19B, lanes A-C) but not in HTC cells (Fig. 19B, lanes D and E), which suggests that the 46,000 MW protein is the receptor. The addition of 50 nM unlabelled T_3 or T_4 to GH_3 cells (Fig. 19B, lanes B and C, respectively) blocked photolabelling, albeit poorly. Again, there is evidence for specifically photolabelled bands which migrated at molecular weights between 68,000 and 47,000. To obtain a definitive answer (on the basis of competition analysis) as to whether the photolabelled 46,000 MW protein of GH_3 cells is the receptor, it was necessary to prepare partially purified receptor from GH_3 and HTC cells.

Irradiation of partially purified nuclear extracts from GH_3 cells in the presence of ^{125}I-T_3 with or without unlabelled T_3 produced a specifically photolabelled band of 48,000 MW (Fig. 20, compare lanes C with D and E with F). Also shown in this figure is the specifically photolabelled 46,000 MW protein from rat liver receptor preparations (compare lane A with B). Of interest was the finding that the specifically labelled protein band in GH_3 cell nuclear extracts has a higher molecular weight than the specifically labelled protein from rat liver receptor preparations. This may point to an inherent difference in the structure of these receptors or may be due to partial proteolysis of the rat liver receptor preparations. The autoradiogram of HTC cell nuclear extracts irradiated in the presence of ^{125}I-T_3 (Fig. 20, lanes G and H) demonstrates that specific thyroid hormone binding proteins do not exist in the nuclei

Figure 20: Photoaffinity labelling of partially purified receptor from rat liver, GH_3 and HTC cells with ^{125}I-T_3. Nuclear extracts prepared from rat liver, and GH_3 and HTC cells were individually chromatographed on Sephadex G-100 according to the procedures described in Materials and Methods. Peak II fractions were incubated with 1 nM ^{125}I-T_3 with or without 50 nM unlabelled T_3 for 4 h at room temperature. Aliquots (0.4 ml) were removed for a determination of ^{125}I-T_3 bound by standard procedures. Samples were irradiated with 300 nm light for three 20-min periods, separated by 5 min of cooling on ice. Irradiated samples were analyzed on SDS gel electrophoresis and the autoradiograms from two separate experiments are presented. Lanes: M, ^{14}C-methylated protein molecular weight markers; A, rat liver PII receptor; B, rat liver PII receptor + 50 nM T_3; C and E, GH_3 cell PII; D and F, GH_3 cell PII + 50 nM T_3; G, HTC cell PII; H, HTC cell PII + 50 nM T_3.


69.0
46.0
30.0
M.W. x 10⁻³
M
A
B
C
D
E
F
G
H

of these cells or exist at a level too low for detection by this method. The finding that a 48,000 MW protein is specifically photolabelled in nuclear extracts from GH_3 cells and not from HTC cells and that it is similar in size to the protein specifically labelled in rat liver nuclear extracts suggests that this 46,000 MW protein photolabelled in rat liver receptor preparations may be the thyroid hormone receptor.

DISCUSSION

It is widely accepted that thyroid hormones exert a profound effect on gene expression, and it is considered that these hormonal actions are mediated by a nuclear receptor. Although receptors appear to bind to DNA, there was a previous suggestion that they bind to histones. In order to understand more about the nature of the interaction of receptors with nuclear constituents and receptor structure, I have in the current studies investigated the interaction of the receptor with histones and have photoaffinity-labelled the receptor.

A. Analysis of the Interaction of the Thyroid Hormone Nuclear Receptor with Histones

The first series of experiments was focused on the effect of histones on the hormone binding and other properties of the nuclear thyroid hormone receptor. The initial experiments were designed to extend the "Core-Holo" receptor model previously proposed by Eberhardt *et al.* (98,99), who suggested that the receptor consisted of a core subunit whose T_3 binding activity was stimulated by a regulatory factor. The regulatory factor was postulated to be a histone or histone-like protein(s).

Eberhardt *et al.* (98,99) demonstrated that the dilution of PII receptor preparations reduced high-affinity T_3 binding activity, which could be restored upon the addition of core histones; T_4 binding activity was not reduced by dilution nor was it influenced by the addition of core histones. However, in the present studies, dilution of PII receptor preparations decreased both high-affinity

T_3 and T_4 binding and the addition of core histones increased both binding activities (Table I)--a finding dissimilar to those of Eberhardt et al. The addition of histones also increased both T_3 and T_4 binding activities of the affinity-purified receptor (Table I).

A re-investigation of the effect of heat treatment on nuclear extracts also presented findings contradictory to those of Eberhardt et al. (98). In their studies, heat treatment of crude nuclear extracts was found to decrease the number of high-affinity T_3 binding sites without altering the binding characteristics of the T_4 sites. In the present studies, heat treatment decreased both high-affinity T_3 and T_4 sites (Fig. 8, A and B). Thus, these contradictory findings do not support the hypothesis proposed by Eberhardt et al. (98,99) that dilution or heat treatment of receptor preparations promoted the dissociation from the receptor of a "regulatory factor" important for maintaining high-affinity T_3 binding. Instead, there was evidence from the heat treatment experiments to suggest that the thyroid hormone receptor was lost as a result of heat treatment and that new binding sites were generated or exposed. As an example, the T_4 sites remaining after heat treatment of crude nuclear extracts (Fig. 7A) were different from the original sites; they had a lower affinity for T_4 (Fig. 8B). Additionally, heat treatment of crude nuclear extracts prepared by Procedure II (Fig. 8C and 9B) clearly demonstrated that low-affinity T_4 sites were generated or exposed. Furthermore, low-affinity T_3 binding sites were generated as well upon heating (Fig. 9A).

Why these present results are different from those of Eberhardt et al. is not clear. Perhaps the reason is that in their studies the level of non-specific T_4 binding was higher and the affinity of the binding site for T_4 was lower than the values presented here. Thus, it is possible that, in their studies, dilution could have reduced both high-affinity T_3 and T_4 sites, but that this was not detected because of the presence of high levels of non-specific T_4 binding. This high level of non-specific binding would probably have also masked any stimulation of T_4 binding activity by histones. Likewise, if in their studies the T_4 binding site had a relatively low affinity for T_4 prior to heating, then the generation of low-affinity sites after heating could have gone unnoticed. That this may have been the case is supported by the finding that the K_d values for T_4 binding were 6.3 and 9.1 nM, respectively, before and after heating in the studies of Eberhardt et al. (98). The values presented in the current study were 0.69 and 9.4 nM, respectively. Thus, it is possible that the receptor preparations used by Eberhardt et al. (98,99) may have been less pure than those currently used and therefore led to their results.

For whatever reason, the results presented here are different from those presented by Eberhardt et al. (98,99). The present results did not support the "Core-Holo" receptor model and, thus, the model was rejected as being incorrect. However, the results did demonstrate that histones and several other proteins increased the receptor's ability to bind hormone. Therefore, the nature of the stimulatory effect observed with histones was investigated.

The results indicated that the ability of histones to increase T_3 and T_4 binding by the receptor could be obtained when histones were applied directly to Sephadex G-25 columns (Table II). This result suggested that the observed stimulatory effect occurred during filtration of receptor-^{125}I-T_3 incubation mixtures over Sephadex G-25. Confirmation of this concept came from the finding that receptors could be recovered in much higher yield from eluates of columns preincubated with histones than from untreated columns (Table III). Therefore, histones appeared to prevent adsorption or degradation of receptors during filtration of receptor preparations over Sephadex G25 assay columns. Based on data to date, it is not possible to differentiate between these two possibilities. However, several reports indicated that Sephadex has adsorption characteristics. In low-salt conditions, the presence of terminal carboxylic groups on the dextran chains of Sephadex enabled it to act as a weak cation exchanger (134). In high-salt conditions, tightly cross-linked resins such as Sephadex G-10, -15, and -25 were found to adsorb aromatic proteins through interactions with ether linkages present in the carbohydrate chains of Sephadex (135). Thus, it is possible that, when receptor preparations in Buffer G (which has an ionic strength equivalent to 0.70 M NaCl) are filtered through Sephadex G-25, the receptor is adsorbed to the resin through hydrophobic interactions. The receptor has been observed to adhere to many surfaces, such as glass, Sepharose, Sephacryl, and cellulose (J. Apriletti, personal communication). Therefore, it is possible that the receptor interacts with Sephadex as well.

Based on the finding that Sephadex can adsorb proteins, it is possible that histones may increase hormone-binding activity by preventing the adsorption of receptors to Sephadex. How this is accomplished is not clear. The primary amino acid sequence of histone proteins indicates the presence of a relatively high concentration of hydrophobic amino acids (136), which could facilitate an interaction with Sephadex in high-salt conditions. However, in high salt, histones snap into extremely tight complexes (137), which may preclude an interaction of histones with Sephadex. Thus, the interaction of histones with Sephadex in the salt conditions used is not known.

In an attempt to determine the specificity of the stimulatory effect observed with histones, I tested the ability of other proteins to increase T_3 binding activity and discovered that, with the exception of BSA and catalase (Table IV), most of the proteins tested did not stimulate T_3 binding to the same extent as did histones. Whether these findings argue for or against a specific interaction of histones with the thyroid hormone nuclear receptor is not clear, but it should be kept in mind that the nucleus where the receptor resides has a high concentration of histone proteins. Thus, it is conceivable that within the nucleus the receptor does interact specifically with histones and that, in <u>in vitro</u> conditions, the receptor may and does interact with other proteins such as BSA and catalase. How histones, BSA and catalase stimulate T_3 binding by the receptor is not known. These proteins have different characteristics: histones are low-molecular-weight basic proteins (approximately 13,000 MW); BSA is a 68,000 MW acidic protein; and catalase is an acidic protein of 250,000 MW.

Therefore, it is not possible to determine the mechanism used by these proteins on the basis of their overall charge.

In the course of these studies, it was found that the partially purified receptor was stable in the absence of histones at either 25^{o} or 4^{o} C. However, the affinity-purified receptor rapidly lost its ability to bind T_3 in the absence of histones, while in their presence the receptor's binding activity was stable (Fig. 6). Whether this stabilization effect observed with histones was a property shared by all proteins was investigated by comparing the stabilizing ability of histones with that of randomly chosen proteins. The results indicated that the proteins tested (Table V) could stabilize the receptor; however, in the majority of the cases a higher concentration of these proteins as compared with histones was necessary to obtain the same stabilizing effect. Thus, histones are more efficient in stabilizing the receptor. The mechanism by which histones and other proteins stabilize the receptor is not known. It is possible that the addition of histones and some other proteins prevented proteolysis of the receptor by acting as a "sink" for proteases. While the existence of proteases in the receptor preparations has not been demonstrated, and the addition of the protease inhibitors, PMSF and soybean trypsin inhibitor, did not change the stabilization of the receptor, other protease inhibitors have not been tested. Conversely, it is conceivable that, as a result of an interaction of the receptor with histones, auto-proteolysis or random denaturation of the receptor is prevented. In order to further characterize the receptor and to understand its

role in mediating thyroid hormone action on gene activity, the stability of the receptor must be enhanced.

Another investigation into the interaction of the receptor with histone proteins was conducted by Dr. J. Apriletti of our laboratory. In preliminary experiments, he demonstrated that partially purified receptor preparations bind to histone-Sepharose matrices but not to ovalbumin- or insulin-Sepharose. This suggested that there was a unique interaction between histones and the receptor. Supporting this notion was the finding that core histone extracts could elute the receptor from the histone-Sepharose matrix. However, a solution of 0.5 M NaCl could also elute the receptor from histone-Sepharose and, in fact, similar concentrations of salt could elute the receptor from either histone-Sepharose or DEAE-Sephadex. Thus, the interaction between histones and the receptor was ionic in nature.

Experiments performed at the outset of this study presented evidence which was considered indicative of an interaction between two of the core histones and the thyroid hormone receptor. It was observed that H2A and H2B consistently displayed the greatest ability to stimulate T_3 binding by the receptor (Fig. 4) and this was considered to have important physiologic implications in light of recently published information regarding the structure of nucleosomes. Nucleosomes were known to consist of DNA wound about a histone core (138); however, the spatial arrangement of histones in the nucleosome core had not been identified until the report of Klug et al. (139). They presented a model which proposed that, "The central role of the $(H3)_2(H4)_2$

tetramer in a nucleosome structure...is to organize the DNA into the central turn of a primary particle.' H2A and H2B could then add to this intermediate as two heterodimers, stabilizing it by binding firmly the two extra half-turns of DNA." Their study suggested that two heterodimers of (H2A-H2B) are located on the periphery of the central $(H3)_2(H4)_2$ tetramer, thereby making H2A-H2B more accessible for interactions with other chromosomal proteins. It was therefore considered possible that the thyroid hormone receptor could interact with H2A and H2B in vivo.

It should be pointed out that the greater stimulatory activity obtained with H2A and H2B was only observed when the histones were added to the incubation mixture of receptors and T_3 prior to filtration over Sephadex G-25 columns (Fig. 4). When the different core histones were applied directly to the assay column, they had equivalent stimulatory activities (Table IV). These different findings may have been due to the use of different salt conditions in these two assays. Stimulation assays were performed at an ionic strength equivalent to 0.15 M NaCl, while the filtration assays were performed with a buffer whose ionic strength was equivalent to 0.7 M NaCl. Thus, at an ionic strength equivalent to physiologic conditions there appeared to be a specific interaction between the thyroid hormone receptor and H2A and H2B.

In summary, the study has demonstrated that there is an interaction of the thyroid hormone nuclear receptor with histones. Evidence has been presented which indicates that histones are important for: 1) obtaining measurable T_3 binding activity by the thyroid hormone

nuclear receptor; and 2) stabilizing the affinity-purified receptor. Furthermore, the results demonstrate that interactions of the receptor with H2A and H2B occur under physiologic conditions, which suggests that they may also occur *in vivo*. These interactions were shown to be ionic in nature. The results do not support the "Core-Holo" receptor model or the notion that histones affect the binding specificity of the receptor.

This interaction of the nuclear receptor with histones is not unique to the thyroid hormone receptor; the estrogen receptor has also been found to interact with histones. Puca *et al.* (140) suggested that histones were the acceptor proteins of chromatin for these receptors and that an interaction between the histones and the estrogen receptor led to an alteration of gene expression. In a more recent study, Kalos *et al.* (141) demonstrated that the estrogen receptor preferentially interacted with H2A- and H2B-Sepharose as compared to H3- and H4-Sepharose matrices. Thus, in light of these findings with the estrogen receptor, it is important to determine whether the thyroid hormone receptor interacts more strongly with H2A- and H2B-Sepharose compared to H3- or H4-Sepharose. Furthermore, it would be interesting to compare the interaction of the receptor with H2A-Sepharose with H2A variants linked to Sepharose. These results may provide further information regarding the physiologic importance of the interaction of the thyroid hormone nuclear receptor with histone proteins.

B. Photoaffinity Labelling of the Thyroid Hormone Nuclear Receptor

Recently, photoaffinity labelling techniques have been used to study low-abundance proteins such as hormone receptors. Therefore, it

was of interest to use the technique to characterize the nuclear thyroid hormone receptor further. The studies were undertaken to determine the size of the fundamental hormone binding unit of the receptor and its isoelectric point. It was anticipated that this photolabelling technique would produce a covalently modified form to be used as a marker in purification schemes using denaturing conditions.

In this study, I have demonstrated that photolysis of rat liver receptor preparations in the presence of ^{125}I-T_3 resulted in the specific labelling of a 46,000 MW protein, which, on the basis of several lines of evidence, is considered to be the thyroid hormone nuclear receptor. The addition of unlabelled T_3 or T_4 to reaction mixtures diminished the photo-induced covalent attachment of ^{125}I-T_3 to this 46,000 MW protein (Figs. 10 and 14). It was demonstrated to have a 10-fold higher affinity for T_3 than T_4, which is consistent with the characteristics of the thyroid hormone receptor and correlates with the greater biological potency of T_3 as compared with T_4 (Figs. 10 and 14). Furthermore, Figure 10 demonstrates that the addition of unlabelled hormone decreased ^{125}I-T_3 reversibly bound to the receptor to the same extent that it inhibited the photolabelling of the 46,000 MW protein. When affinity-purified receptor preparations, in which the thyroid hormone nuclear receptor is purified 500-fold, were photolysed, the 46,000 MW protein was predominantly labelled. Thus, the increased labelling of the 46,000 MW band observed when affinity-purified receptor preparations were photolysed was consistent with the increased specific acitivity of the receptor preparation. That the 46,000 MW protein was not specifically labelled in rat

serum (Fig. 18) indicated that this protein is not a serum-binding protein for thyroid hormones or any other serum contaminant, and thus indirectly supports the evidence that it is the nuclear receptor. Additionally, a protein with a similar molecular weight to that of the specifically labelled protein of rat liver receptor preparations was specifically photolabelled in GH_3 cells, which are responsive to thyroid hormone and have 20,000 receptors/cell nucleus (69). This protein was not photolabelled in HTC cells, a cell line which has not been found to be responsive to thyroid hormones (69). The molecular weight of the specifically photolabelled band from GH_3 cells is 48,000, while that observed from rat liver is 46,000. This difference may be due to proteolysis having occurred in the rat liver preparations, although this has not been proven. Thus, the data suggest that the 46,000 MW protein specifically labelled in rat liver receptor preparations may be the thyroid hormone nuclear receptor.

It appears that the nuclear receptor is a single polypeptide chain; however, the possibility that the receptor is composed of subunits (i.e., hormone binding or non-hormone binding) cannot be excluded. Analysis of photolysed reaction products was performed under denaturing electrophoretic conditions; thus, it is possible that an unlabelled subunit may have been dissociated under these conditions and have gone undetected. Therefore, to determine the exact molecular weight of the receptor and possible subunit structure using the photoaffinity labelling technique, the reaction products needed to be analyzed in a non-denaturing gel electrophoresis system. The molecular weight obtained for the putative receptor in these studies is similar

to that estimated by Latham et al. (50,500 MW {63}), who used non-denaturing gel filtration methods, which suggests that the receptor may not consist of subunits. However, their studies were performed with high-salt buffers, which could have dissociated any possible subunits. Thus, the native structure of the receptor still needs to be determined.

In an earlier study I had reported that the pI of the receptor was approximately 6.2 (142); in the current study this result could not be reproduced. A specifically labelled spot was not observed on autoradiography of two-dimensional gels, regardless of whether isoelectric-focusing or non-equilibrium-pH-gradient gels were used as the first dimension. It appears therefore that the photolabelled receptor did not enter into the first dimension. Similar results were observed in studies concerning the characterization of the photoaffinity-labelled β-adrenergic receptor; the photolabelled receptor migrated into an SDS gel but not into an isoelectric focusing gel (T. Lavin, personal communication).

The mechanisms by which ^{125}I-T_3 labelled the thyroid hormone receptor are not well understood. It is known that aryl halides, especially bromo-aryl and iodo-aryl groups, are extremely photoreactive (143). Somack et al. (120) reported that photolysis of T_4 resulted in the loss of either inner or outer ring iodides, forming iodothyronine and iodide radicals. This dehalogenation process was shown by van der Walt and Cahnmann (121) to occur preferentially at the outer phenolic ring and then at the inner non-phenolic ring of T_4 and T_3, although not exclusively in that order. Additionally, Cysyk and

Prusoff (144) have reported that ultraviolet irradiation of 5-iodo-2'-deoxyuridine resulted in the formation of free deoxyuridine and iodide radicals and, when 5-iodo-2'-deoxyuridine was photolysed in the presence of thymidine kinase, the deoxyuridine (but not the iodide) radical covalently attached to the enzyme. If the thyroid hormone receptor were labelled by an iodothyronine radical in accordance with the mechanism proposed by Cysyk and Prusoff (144), deiodination of the ^{125}I-T_3 would have had to occur at the inner non-phenolic ring, because deiodination of ^{125}I-T_3 at the outer ring would have removed the radioactive iodide and not resulted in the labelling of the receptor. We cannot exclude the possibility that the radioactive iodide radical alone may be covalently labelling the receptor.

While these studies were in progress, Pascual et al. (145) reported that, with the use of N-2-diazo-3,3,3-trifluoropropionyl-L-^{125}I-T_3, two proteins in nuclear extract of GH_1 cells with molecular weights of 47,000 and 57,000 were specifically photolabelled. Initially, these investigators reported that both bands were observed only when intact cells were irradiated with 254 nm light and not with 300 nm light; using 300 nm light only the 47,000 MW protein was photolabelled. Pascual et al. (145) therefore suggested that the 57,000 MW form represented the 47,000 MW protein crosslinked to a protein of 10,000 MW; crosslinking could have been caused by 254 nm light acting as a zero-length crosslinker. In recent studies, as a result of having increased the coupling efficiency of the photolabelling event, both the 47,000 and 57,000 MW bands were photolabelled, regardless of the wavelength (H. Samuels, personal communication). Thus, the 57,000 MW

protein is most likely not the 47,000 MW protein crosslinked to a 10,000 MW protein. An investigation into the relationship of the 57,000 MW with the 47,000 MW protein indicated that the latter was not a proteolytic cleavage product of the 57,000 MW protein (145). Peptide mapping of the 57,000 and 47,000 MW labelled proteins suggested that these two receptor forms were similar. Using S. aureus V8 protease, similar peptide maps of the 57,000 and 47,000 MW proteins were obtained; i.e., there was a 24,000 MW intermediate peptide product that was further converted to a 12,000 MW peptide upon complete protease digestion (145). Only the radioactive fragments were monitored, leaving open the possibility that unlabelled peptides of the 57,000 and 47,000 MW proteins may be very dissimilar. (The difference in the molecular weight of these two receptor forms predicts that their peptide maps will most certainly be different.) Based on these findings, the 57,000 MW protein may be a postsynthetically modified form of the 47,000 MW protein or may be independent of it. (There is evidence for receptor modification, such as glycosylation of the insulin receptor {146}.)

In the studies presented here, a 57,000 MW labelled protein was not observed when either rat liver or GH_3 cell receptor preparations were photo-affinity labelled. This may have been due to the fact that the coupling efficiency obtained in these studies was less than that obtained in the studies by Samuels' group (personal communication). However, in the present studies there was evidence for faintly labelled bands in the molecular weight range of 47,000 to 50,000 (Figs. 10, 13-16, and 20) or 47,000 to 68,000 (Fig. 19B), whose

labelling intensity diminished with the addition of excess unlabelled T_3. The identity of these higher molecular weight species is not known. They may represent modifications of the 46,000 MW protein, such as glycosylated forms; the 46,000 MW protein may be a proteolytic cleavage product of these other forms; or the two protein forms may be separate gene products. The identity of these higher molecular weight proteins and their relationship to the 46,000 MW receptor need to be determined.

Additionally, while these studies were in progress, van der Walt et al. (147) demonstrated that photolysis of crude rat liver nuclear extracts in the presence of ^{125}I-T_3 led to the photo-induced labelling of 45,000 and 12,000 MW proteins. They did not investigate whether this photolabelling event was specific in nature by using excess unlabelled T_3 to inhibit labelling. Furthermore, in my experience the photolabelling of crude nuclear extracts (as opposed to partially purified receptor preparations {PII}) with ^{125}I-T_3 always led to non-specific photolabelling of many proteins and, thus, specific labelling of individual proteins was never demonstrated. I therefore wonder how these investigators were able to obtain predominantly labelled 45,000 and 12,000 MW bands. Additionally, because of the lack of supporting evidence, I disagree with their claim that they have demonstrated photolabelling of the rat liver thyroid hormone nuclear receptor.

In summary, this study has demonstrated that photolysis of receptor preparations from rat liver or GH_3 cells in the presence of ^{125}I-T_3 results in the predominant and specific labelling of a 46,000 and

48,000 MW protein, respectively. Many lines of evidence suggest that these two proteins may be the thyroid hormone nuclear receptor. However, whether there are other forms of the receptor or whether the receptor consists of subunits remains to be determined.

It was initially anticipated that photolabelling receptor preparations would result in the specific labelling of the receptor with enough intensity that its isolation from polyacrylamide gels would yield a radioactive receptor probe. This receptor probe was then to be used in the further purification of the receptor using denaturing conditions. The photolabelling procedure and gel electrophoresis systems as presented here, if unmodified, cannot be used to prepare a radioactive receptor probe from partially purified receptor preparations. The coupling efficiency of the photolabelling event is extremely low (0.1%) and needs to be increased in order to be able to monitor within a reasonable time period the radioactivity covalently attached to the receptor in a gel slice using a gamma counter. Secondly, the 46,000 MW band cannot be isolated from a gel from other separate labelled proteins because of the high level of non-specific background labelling. However, radioactive receptor probes can be prepared from affinity-purified receptor preparations. Furthermore, because the receptor can be localized to a certain position on an SDS-gel, it can be extracted from the gel, and when enough material is obtained it can be analyzed for its amino acid sequence. Knowledge of the receptor's amino acid sequence could lead to the determination of its nucleotide sequence. With sequence information, DNA primers can be synthesized and ultimately these primers can be used to clone the thyroid hormone nuclear receptor.

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