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STUDIES ON THE BIOACTIVITY OF RADIOLABELED, HIGHLY-PURIFIED BOVINE THYROTROPIN

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SUMMARY: Bovine thyrotropin radiolabeled stoichiometrically with chloramine T was subjected to high pressure liquid chromatography on a Waters' Protein I-125 column. More than 80% of the radioactivity eluted after the $Na^{125}I$ ("salt") peak. In contrast, thyrotropin bioactivity eluted before the salt peak. Radiolabeled thyrotropin affinity-purified with thyroid plasma membranes eluted after the salt peak. Discordance between the thyrotropin bioactivity and radioactivity elution profiles was confirmed by labeling thyrotropin with ^{125}I by lactoperoxidase and then measuring both bioactivity and radioactivity in each chromatographic fraction. These data suggest that the bioactivity in radiolabeled thyrotropin may not be inherent in the radiolabeled molecules.

INTRODUCTION

Studies on the binding of radiolabeled thyrotropin (TSH)** to its receptors in thyroid tissue have been fraught with difficulty (reviewed in references 1 and 2). For example, in many studies the affinity of the ligand for its binding sites has been lower than expected. TSH-binding to thyroid tissue is greatly reduced at physiological salt concentrations, maximal at an unphysiological pH, and there are increasing reports of reversible and hormone-specific binding of TSH to a variety of non-thyroid tissues (3-10). Recent technical advances in thyroid membrane preparation hold promise for resolving these issues (11). In terms of the radioactive TSH ligand used in studies on binding to thyroid tissue, little loss of bioactivity has been observed following radioiodination by different procedures (1,12,13). On this basis, such studies have been conducted on the premise that radiolabeled TSH is largely bioactive.

Because of the recent availability of a simple, highly sensitive bioassay for TSH using cultured thyroid cells (14) and of new high pressure liquid

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** Abbreviations: TSH: thyrotropin; bTSH: bovine TSH; HPLC: high pressure liquid chromatography; BSA: bovine serum albumin.

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chromatography (HPLC) columns capable of separating large protein molecules (15), we studied further the nature of the radiolabeled TSH ligand. The results indicate that although radiolabeled TSH retains bioactivity, these two functions elute in separate peaks on HPLC.

MATERIALS AND METHODS

High Pressure Liquid Chromatography: The mobile phase used was 140mM NaCl, 10mM sodium phosphate, with or without 0.1% BSA, pH 7.4, at a flow rate of 1.0 ml/minute. BSA in the mobile phase was used initially in some experiments in an attempt to minimize adsorption of tracer quantities of [^{125}I]TSH or of TSH bioactivity to the column. However in later experiments this was not found to be necessary. Fractions of 15 and of 30 seconds duration were collected for the measurement of radioactivity and bioactivity respectively. Optical density was monitored at 280nm.

Bioassay: TSH was bioassayed using cultured human thyroid cells as previously described for dog cells (14). The entire HPLC fraction was diluted 1:1 with L-15 medium containing 40mM Hepes, 1.0 mM 3-isobutyl-1-methylxanthine, to a final volume of 1 ml. The mixtures were then added to 35mm diameter culture dishes containing approximately 10^5 human thyroid cells. Recovery of TSH bioactivity from the HPLC column (using bovine TSH standards) was calculated as the sum of bioactivity in each fraction (30 second) as a percentage of the injected dose.

Iodination Procedures: Stoichiometric chloramine T iodination of highly purified bTSH was performed as previously described (12). Iodination with stable Na^{127}I was stopped after 10 minutes. Affinity purification of [^{125}I] TSH was by the method of Smith and Hall (16) except that 2M NaCl was used. Lactoperoxidase iodination with ^{125}I was by the procedure of Thorell and Johansson (17), as modified by Pekonen and Weintraub (4).

Materials: A Waters Associates (Milford, Mass.) Protein I-125 Column was used with a Model 6000A Solvent Delivery System, U6K Injector, and a Series 440 Absorbance Detector. Highly purified bTSH was kindly supplied by Dr. J.G. Pierce. Partially purified bTSH (Thytropar) was purchased from Armour Pharmaceutical Co. (Chicago, Ill.). Lactoperoxidase (61 U/mg), bovine serum albumin (BSA) and protein standards were purchased from Sigma Chemical Company (St. Louis, Mo.). Carrier free Na^{125}I (17 Ci/mg), Na^{131}I (25 Ci/mg) were purchased from New England Nuclear Co. (Boston, Mass.).

RESULTS

HPLC of a tracer amount of highly-purified bovine TSH radiolabeled with ^{125}I by the stoichiometric chloramine T method (12) showed that about 80% of the radioactivity was retarded on the column beyond the Na^{125}I ("salt") peak (Figure 1). The column therefore was not functioning only as a molecular sieve, but also had some affinity for the radiolabeled TSH. Injection of unlabeled bTSH revealed a single TSH bioactivity peak between the void volume of the column and the salt peak. The position of this peak was identical for both highly-purified bTSH (Pierce) and partially purified bTSH (Thytropar). Based on more than 20

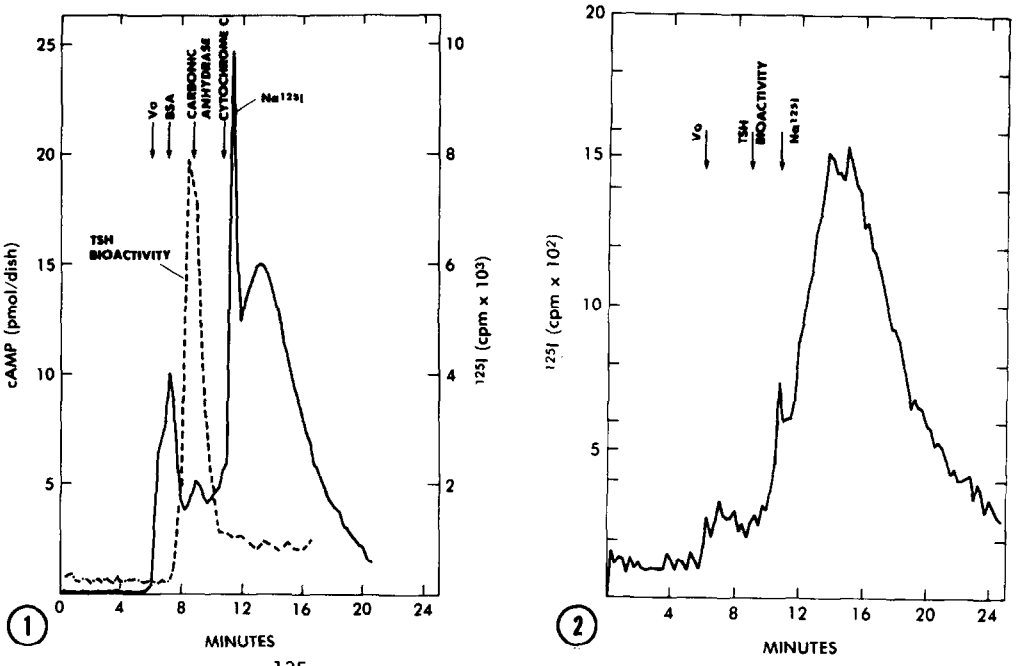

Figure 1: HPLC of [^{125}I]TSH. Highly-purified bovine TSH (Pierce) was iodinated by the stoichiometric chloramine T method (11). After preparative Sephadex G-25 and G-100 gel filtration, a 25 µl aliquot of the peak fraction of the latter was injected on a calibrated Waters Protein I-125 column. The mobile phase was phosphate-buffered saline, 0.1% bovine serum albumin, pH 7.4, and the flow-rate was 1.0 ml. per minute. Fractions of 0.25 minute were collected. In a separate application on the same day, 8µg of highly-purified bTSH was injected and 0.5 minute fractions were collected and assayed for bioactivity, as described in Materials and Methods. Subsequent reinjection of the radiolabeled TSH produced the same profile as prior to the injection of bTSH for measurement of bioactivity. The arrows in this and subsequent Figures indicate the elution peaks of separately injected marker proteins (mobile phase without bovine serum albumin), as measured by absorption at 280 nm. BSA-bovine serum albumin.

Figure 2: HPLC profile of thyroid membrane affinity-purified [^{125}I]bTSH. Highly-purified bTSH was iodinated stoichiometrically with chloramine T, followed by Sephadex G-25 gel filtration. The radiolabeled bTSH was affinity-purified as described in Materials and Methods. After preparative Sephadex G-100 gel filtration, a 25 µl aliquot of radiolabeled material was subjected to HPLC and 0.25 minute fractions were collected and measured for radioactivity. The arrows indicate the void volume, positions of elution of unlabeled TSH bioactivity and Na^{125}I as determined before and after the injection of [^{125}I]TSH.

injections a typical recovery of TSH bioactivity from the column was 80%. Less than 5% of the [^{125}I]TSH eluted in the fractions corresponding to those with TSH bioactivity. Elution profiles of TSH bioactivity and radioactivity were no different when these substances were injected separately or together.

Radiolabeled bTSH was affinity-purified using human thyroid plasma membranes. HPLC of this material revealed it to elute almost entirely after the salt peak, not corresponding to the elution zone of TSH bioactivity (Figure 2).

The [^{125}I]TSH eluting in the "post-salt" region rebound extremely well to human thyroid plasma membranes, with binding characteristics (affinity, inhibition by NaCl) identical to those previously reported (10) (data not shown).

Because of the separate elution positions of tracer-TSH (in which bioactivity cannot be determined) and bioactivity in un-iodinated TSH (Figure 1), it was important to determine the elution position of bioactivity in an iodinated TSH preparation. That is, it was possible that the iodination procedure may have shifted the elution position of TSH bioactivity to after the "salt peak", corresponding to that of the ^{125}I-tracer TSH. To enable detection of TSH bioactivity following iodination, 10 µg highly-purified bTSH was iodinated by the stoichiometric chloramine T method (12) using stable ^{127}I and then subjected to HPLC. TSH bioactivity eluted in the same region as shown in Figure 1 (data not shown). Recovery of TSH bioactivity from the column was approximately 80% of the bioactivity present prior to iodination.

Highly-purified bTSH was iodinated with ^{125}I using lactoperoxidase (17). Half of the reaction mixture was subjected to HPLC without prior gel filtration and bioactivity and radioactivity were then measured in the same individual fractions (Figure 3A). Approximately 70% of the original TSH bioactivity was recovered. However less than 5% of the radioactivity eluted in the fractions containing bioactivity. Unreacted Na^{125}I was 60% of the injected dose. The remaining 40% eluted in a very broad, low smear, plateauing well beyond the Na^{125}I peak before declining. This change in column performance is discussed below.

For comparison, the other half of the same iodination reaction mixture was applied to a Sephadex G-25 column (Figure 3B). The elution profile confirmed a 40% incorporation of ^{125}I into TSH in the void volume. Figure 3C depicts the radioiodine profile on Sephadex G-100 of the material eluting in the void volume on Sephadex G-25 (Figure 3B). The peak fraction, utilized by most laboratories as the radiolabeled TSH ligand for binding studies, was subjected to HPLC. Only about 5% of the injected radioactivity emerged prior to the Na^{125}I peak in the position of elution of bioactive TSH, (Figure 3D).

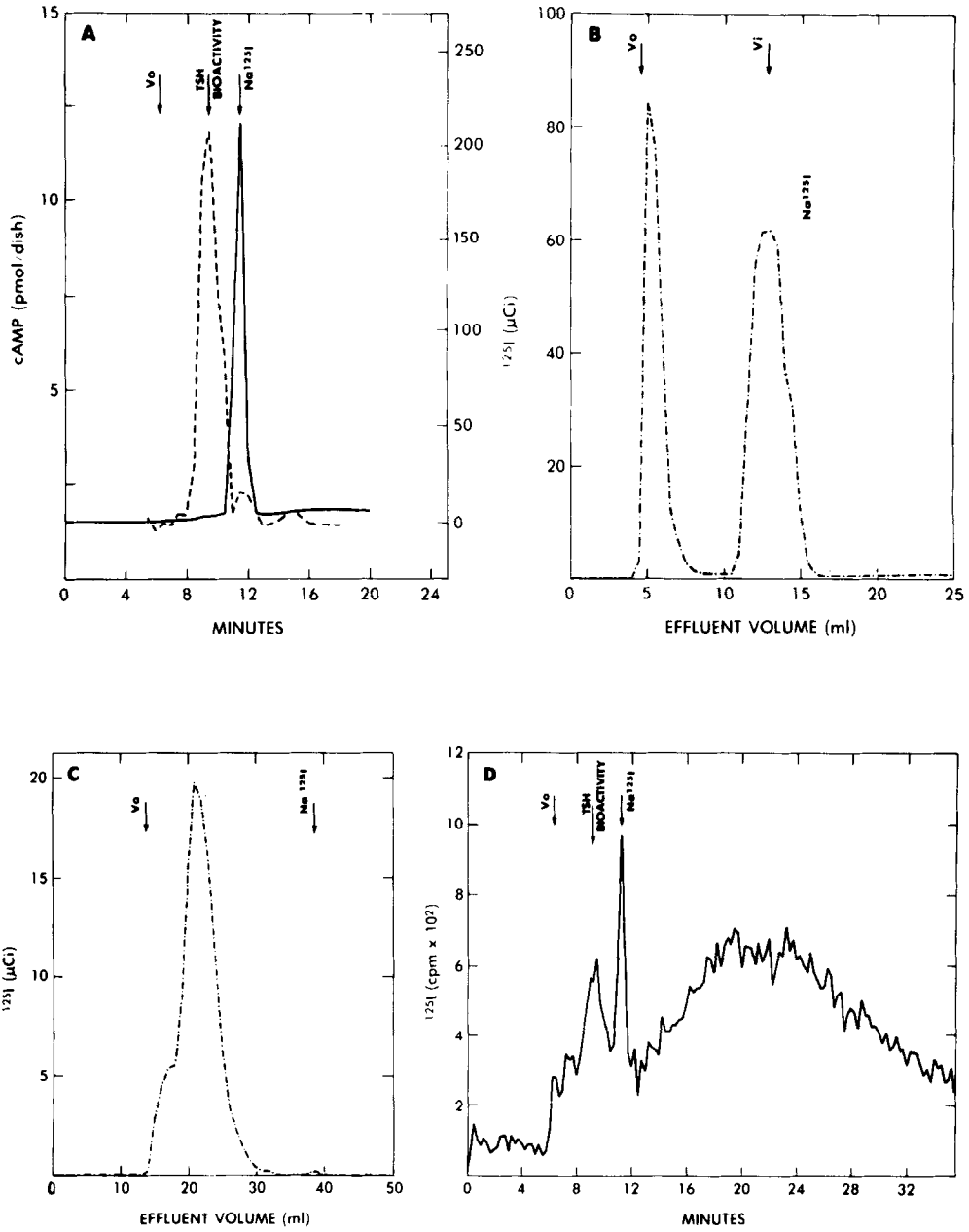

Figure 3A Bioactivity of [^{125}I]bTSH on HPLC. Eight μg of highly-purified bTSH (Pierce) was labeled with ^{125}I with lactoperoxidase. Half of this material was applied directly to the Protein I-125 column without preparative gel filtration. The mobile phase was phosphate buffered saline, 0.1% bovine serum albumin, pH 7.4. 60% of the injected radioactivity eluted in the Na ^{125}I peak (12 mins). The remaining 40% eluted in a broad, low peak after the Na ^{125}I. After determination of radioactivity, the same fractions (0.5 minute) were also assayed for TSH bioactivity as described in Materials and Methods. Na ^{131}I was used to label the ligand in the cyclic AMP radioimmunoassay. TSH bioactivity (70% recovered) eluted together with less than 5% of the radioactivity.

The preceding experiments were conducted using the same column over a 6 month period. During this time, and as can be seen in the preceding Figures, there was a progressive broadening and flattening of the profile of the [^{125}I] labeled TSH after the Na^{125}I peak. Similar data were obtained with a second column. Further studies with a third and fourth Waters' Protein I-125 column differed in that bioactivity was retarded to a greater extent than was the radioactivity. However, consistent with the first 2 columns there was clearly nonidentity between the TSH bioactivity and radioactivity peaks.

DISCUSSION

Retention of biological activity in TSH iodinated by different methods is between 57% and 100% (1,12,13). In the most extensive study, iodination of TSH by the lactoperoxidase method yielded an average bioactivity recovery of 76% (range 59%-100%; n=6) (13). Consistent with these data, in different iodinations we observed bioactivity recovery of 70%-90%. In all previous studies, bioactivity has been determined in the entire, unfractionated iodinated-TSH preparation. To our knowledge, however, radiolabeled TSH has not previously been subjected to chromatography, and the profiles of bioactivity and radioactivity then determined simultaneously in each individual fraction. Goldfine et al subjected radiolabeled TSH to Sephadex G-25 gel filtration, but examined bioactivity in the pooled fractions from the entire void volume peak (12). Kotani et al observed concordance of the elution profiles on Sephadex G-100 gel filtration of bioactivity in unlabeled TSH, and radioactivity in a separately iodinated TSH preparation (1).

3B: The half of the iodination reaction mixture not subjected to HPLC was applied to Sephadex G-25 (11) in order to separate radiolabeled TSH from free Na^{125}I. Fractions (1 ml) in the void volume peak were pooled and applied to a Sephadex G-100 column (11).

3C: Sephadex G-100 gel filtration (11) of the [^{125}I]TSH eluting in the void volume on Sephadex G-25 (Figure 3B). Fractions (1.0 ml) were measured for radioactivity.

3D: HPLC of [^{125}I]TSH in the Sephadex G-100 (Figure 3C) peak fraction (number 22). A 25 µl aliquot was applied to the Protein I-125 column. Each fraction was 0.25 minutes. The elution position of TSH bioactivity in a preceding and subsequent injection on this column is indicated by an arrow.

The present study demonstrates that when the iodinated TSH preparation is subjected to HPLC, bioactive TSH and radioactive TSH do not elute coincidently. This separation of TSH bioactivity and radioactivity with the Waters' I-125 column was fortuitous since the column is marketed as having the function of a molecular sieve. Clearly, however, radiolabeled and biologically active TSH had different affinities for the solid phase of the column which consists of silica to which is bonded hydrophilic diol compounds.

The reason for the separate elutions of TSH bioactivity and radioactivity is not known but could be explained by either of two alternatives. First, the introduction of iodine, or the oxidative effect of the process, may alter the TSH molecule so that its affinity for the column is changed. In the typical experiment shown, 70% of the (pre-iodination) bioactivity was recovered in fractions containing only 5% of the radioactivity. No bioactivity was observed in the major iodinated-TSH peak. This would have been detected had it been present because microgram quantities of TSH were injected, entire HPLC fractions were assayed, and the sensitivity of the assay is under 1 ng/ml TSH (14).

A second possible explanation for the different elutions of TSH bioactivity and radioactivity is that only a small proportion of the TSH molecules in the TSH preparation are biologically active, even before iodination. Further studies are necessary to resolve this issue. It is noteworthy, however, that highly-purified TSH is polymorphic on polyacrylamide gel electrophoresis (18-20). Isoelectric focusing of rat (21), human (22), bovine (23) and whale (24) TSH also reveals multiple iso-hormones. Another factor to be considered is that immediately following TSH purification, TSH bioactivity is high, and then falls rapidly to a relatively stable level (18,25). The reason for this initial decline in TSH bioactivity is unknown.

It must be emphasized that the present data do not invalidate the physiological significance of previous data on TSH binding since biological activity is not a prerequisite for a ligand, as is well-established with catecholamine analogues. However in the case of TSH, it now seems necessary to examine further the nature and purity of the different TSH isohormones.

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