Processing of TRH Precursor Peptides in Rat Brain and Pituitary Is Zinc Dependent

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PEKARY, A. E., H. C. LUKASKI, I. MENA AND J. M. HERSHMAN. Processing of TRH precursor peptides in rat brain and pituitary is zinc dependent. PEPTIDES 12(5) 1025-1032, 1991.—The enzymes responsible for the posttranslational processing of precursor proteins to form alpha-amidated peptide hormones require the availability of several cofactors, including zinc, copper and ascorbate ions. Major changes in the availability of these cofactors, as well as the rate of hormone precursor conversion to active hormone, occur during neonatal development, aging and caloric restriction. The effects of 6 weeks of a zinc-deficient (ZD1) diet, pair feeding (PF) and partial zinc deficiency (ZD6) compared to a control diet on the enzymatic cleavage and processing of prepro-TRH to form TRH have been studied in the hypothalamus, brain, and pituitary of young adult male Sprague-Dawley rats. Reverse phase high pressure liquid chromatography (HPLC) revealed that TRH was the major TRH-IR component of the hypothalamus, brain and pituitary. The effect of zinc deficiency on the TRH-Gly-IR HPLC profile of rat brain was to reduce selectively the area of the peaks for TRH-Gly and other low molecular weight pre-pro-TRH peptide fragments with a C-terminal Gly compared to the corresponding TRH-Gly-IR peaks of the control group. We conclude that the processing of prepro-TRH to form TRH is zinc dependent via posttranslational processing enzymes such as carboxypeptidase H.

<table>
<thead>
<tr>
<th>TRH</th>
<th>TRH-Gly</th>
<th>Radioimmunoassay</th>
<th>Zinc deficiency</th>
<th>Brain</th>
<th>Hypothalamus</th>
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</table>

HYPOTHALAMIC and extrahypothalamic release and biosynthesis of TRH declines substantially during aging and nonthyroid illness and may be causally related to the profound decrease in circulating thyroid hormone levels and basal metabolism which accompany these conditions (12,30). The hypothalamic prepro-TRH mRNA (36) and prostatic TRH precursor levels (26), on the other hand, do not change significantly with aging, suggesting that posttranslational processing of TRH precursor peptides may be the step in TRH biosynthesis most vulnerable to the effects of aging. Prepro-TRH, which in the rat contains 5 repeated sequences of -Arg-Lys-Gln-His-Pro-Gly-(Lys/Arg)-Arg- (16), requires several enzymatic cleavage steps (20) to form TRH (pGlu-His-Pro-NH2): trypsin-like cleavage at the C-terminus of the paired basic residues, carboxypeptidase H removal of these C-terminal residues, alpha-amidation of the resulting C-terminal Gly and cyclization of the N-terminal Gin to form pyroglutamate.

The trypsin-like, lysine-arginine-cleaving enzyme is a Ca2+-dependent, zinc-sensitive, membrane-associated endopeptidase with specificity of cleaving on the carboxyl side of a pair of basic residues (33). Carboxypeptidase H is a zinc metalloenzyme which has been cloned and sequenced (7). The alpha-amidating enzyme, which has also been cloned and sequenced, requires copper and ascorbate for activity (20). Recent evidence suggests that the decline in TRH with aging may be attributable in part to a depletion of tissue copper (15), zinc (9,40) and ascorbic acid (24), essential cofactors of the TRH biosynthetic enzymes (7) which convert precursor peptides to the alpha-amidated and biologically active form (TRH, pGlu-His-Pro-NH2, for example). The membrane-associated endopeptidase, carboxypeptidase H and alpha-amidating reactions are general ones, responsible for the processing of most of the known peptide hormones. During the first postnatal week in rats, remarkable increases occur in the Cu2+, Zn2+ and ascorbic acid content of maternal milk, the efficiency of their uptake by the neonatal gut, the actual tissue levels of these cofactors and the rate of alpha-amidation of TRH-Gly (4, 6, 10, 14, 21, 36). Mild zinc deficiency has recently been identified as the major risk factor in the nutritional failure to thrive syndrome in human infants and toddlers (39).

We have measured in adult male rats the effects of zinc deficiency and pair feeding on: (a) the serum thyroid hormone levels, (b) the steady-state levels of TRH and TRH precursor peptides in extrahypothalamic brins, hypothalamus and pituitary and (c) the release of hypothalamic TRH during in vitro incubation. Our aim was to obtain a more complete understanding of the direct and indirect dependence of peptide hormone biosynthesis on this essential trace element.

METHOD

Animals

Forty weanling male Sprague-Dawley rats (Harlan-Sprague,
Madison, WI) weighing 40–50 g were housed individually in a controlled temperature environment (21–22°C) with a 12-h light-dark cycle. The animals were fed a semipurified diet adequate for all nutrients and containing approximately 26 ppm zinc and distilled-deionized water ad lib until their body weights reached 150 g. The rats, matched for weight, were divided into four groups of 10 animals per group.

**Experiment 1**

One group (control) was given ad lib a semipurified control diet containing 35.8 ppm zinc (see Fig. 2). Another group received a zinc-deficient (ZD1) diet ad lib which was similar to the control diet except that it contained less than 1 ppm zinc. The third group was pair fed (PF) the control diet in amounts equal to that consumed by the paired ZD1 rats. Another group was fed ad lib diet similar to the control diet but containing only 6 ppm zinc (ZD6). Body weights and food intake were determined daily.

Because zinc deficiency is known to cause a cycling threeday feeding pattern resulting in depressed food intake and reduction in body weight, it is common in nutritional studies to use a matched group of animals fed a diet adequate in zinc but in an amount equal to that consumed by the matched animal fed the zinc-deficient diet. This approach permits one to differentiate the effects of energy restriction from those of zinc deficiency. The pair-fed animal does not exhibit biological/biochemical signs of zinc deficiency.

The animals consumed these diets for 42 days, after which they were sedated with pentobarbital (50 mg/kg body weight). Blood was obtained by cardiac puncture and collected into heparinized plastic syringes.

**Experiment 2**

Experiment 2 was a repeat of Experiment 1 except that unanesthetized animals were decapitated and trunk blood was collected in unheparinized glass test tubes. This second experiment was carried out about one year after Experiment 1.

**Measurement of Tissue Zinc**

**Experiment 1.** Livers were obtained and wetashed using standard procedures (1). Liver zinc content was determined by atomic absorption spectrophotometry using a certified standard. Bovine liver standards (National Bureau of Standards, Washington, DC, Standard Reference Material 1577a) were analyzed for zinc concentration to evaluate our methodologic accuracy. The liver zinc content of the standard was 126 ± 1 µg/g as compared to the certified value of 130 ± 13 µg/g.

**Experiment 2.** Liver, femur, pancreas, one testis, and one epididymis (caput and caudate) were wetashed for zinc measurement as described above.

**Tissue Extraction and Chromatography**

Two methods for extracting TRH and TRH precursor peptides were compared in this study. A recently described reverse phase cartridge extraction procedure (22), which should provide good recoveries for all prepro-TRH fragments up to full-length prepro-TRH, was used in Experiment 1. A methanol extraction procedure, which the authors have used extensively for previous TRH and TRH-Gly studies (27, 28, 32), was used in Experiment 2 to determine whether large TRH-Gly-immunoreactive (IR) fragments can be recovered by this method.

**Experiment 1.** Tissues were weighed and boiled in 1.0 M acetic acid for 15 min, homogenized and centrifuged. The supematant for each tissue extract was passed through a Maxi Clear C18 reverse phase cartridge (900 mg size, Alltech Associates, Deerfield, IL) and eluted with 4 ml of 50% acetonitrile (Omnisolve grade, Mathew Coleman and Bell, Cincinnati, OH). The eluates were dried by lyophilization in a centrifugal evaporator (SpeedVac, Savant Instruments, Farmingdale, NY) connected to a high vacuum pump system (Virirs Freeze Mobile, Vitris Co., Gardiner, NY). The TRH-IR and TRH-Gly-IR were measured in the individual tissue extracts by RIA and the remaining extracts for each of the 10 tissues within each of the experimental treatment groups were pooled, dried, reconstituted in 1.0 ml of distilled water and particulates removed with a 25 mm diameter, 0.45 µm nylon 66 syringe filter (Alltech). The pooled tissue extracts were injected into a programmable HPLC system (Altex Scientific, Berkeley, CA) using a 4.6 x 25 cm Econosphere, 5 µm. C18 reverse phase column (Altex) previously equilibrated with 0.1% trifluoroacetic acid. At the time of injection, linear 0.5%/min gradient of acetonitrile at a flow rate of 1 ml/min was started. At 40 min, the gradient was increased to 2%/min. The 1 ml fractions collected were dried completely and reconstituted with distilled water just before measurement of TRH-IR and TRH-Gly-IR by the corresponding RIA.

**Experiment 2.** Tissues were weighed and boiled in 1.0 M acetic acid and homogenized as described above. Most tissues were then dried completely at 97°C with a fan blowing air into each test tube. This dried residue was homogenized with methanol, centrifuged and the supernatant dried completely at 60°C as above. One epididymis was taken for zinc measurement as described above. The remaining epididymal tissue was boiled, homogenized and divided into equal aliquots. One aliquot was extracted with a reverse phase cartridge (standard 300 mg Sep Pak C18, Waters Inc., Milford, MA) as described in Experiment 1. The remaining aliquot was extracted with methanol as described above.

**Radioimmunoassays**

The measurement of serum levels of thyrotropin (TSH) and concentrations of TRH-IR and TRH-Gly-IR in whole blood and tissue extracts has been described previously in detail (32). Thyroxine (T₄) and triiodothyronine (T₃) were measured with the IMX system (Abbott Laboratories, North Chicago, IL). Cross-reactivity curves for the TRH-Gly antiseraum used in the present study (898B4, 1:800 final dilution) are given in Fig. 1. This antibody has greatest affinity for peptides such as TRH-Gly (pGlu-His-Pro-Gly) or Arg-TRH-Gly (Arg-Gln-His-Pro-Gly) but <0.01% cross-reactivity for TRH (pGlu-His-Pro-NH₃) and related peptides. The within-assay coefficient of variation (CV) for the TRH-Gly RIA was 10.3% for 10,000 pg TRH/ml; the corresponding between-assay CV was 26.0%. The cross-reactivity of TRH-Gly and related peptides in the TRH RIA was <0.01% (Fig. 1). The within- and between-assay CV at 250 pg TRH/ml for the TRH RIA was 5.1% and 17.6%, respectively. All samples from a given experiment were measured together in the appropriate assays.

**In Vitro Incubation of Hypothalamic Fragments**

Hypothalamic fragments, bounded by the posterior margin of the optic chiasm and by the anterior margin of the mamillary bodies, and in a plane 1.5 mm lateral to the midline and 2 mm deep, were excised. Individual hypothalami were placed in 10 x 75 mm glass test tubes with 0.5 ml of Hank's balanced salt solution (Irvine Scientific, Santa Ana, CA) containing 50 µg/ml bac-
ZINC DEFICIENCY AND TRH BIOSYNTHESIS

Fig. 1. Cross-reactivity curves for rabbit antiserum to TRH (465B12) (left panel) and TRH-Gly (898B2) (right panel). Logit(B/Bo) = \ln[B/Bo/(1 - B/Bo)], where B/Bo is the ratio of the counts bound at a finite dose of unlabeled peptide divided by the counts bound at zero dose of unlabeled peptide. The units of the X axis refer to both the concentration of the assay standard and the concentration of the other related peptides.

icacin, 0.1% bovine serum albumin and 20 mM Hepes, pH 8.1, and equilibrated with 95% oxygen and 5% carbon dioxide. The test tubes were sealed with Parafilm, and after incubating 60 min at 37°C in a shaker bath, the medium was removed and replaced with 0.5 ml of O2-CO2 equilibrated Hank's medium containing 60 mM potassium chloride and 58 mM sodium chloride to maintain osmolality (31). After 30 min at 37°C, this medium was removed, the hypothalamic fragments were homogenized in methanol, centrifuged, the supernatant dried and the protein content of the pellet measured by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). The TRH-IR content of the media and hypothalamic extracts was measured by RIA.

Peptides

The following synthetic peptides were used to determine the specificities for the TRH and TRH-Gly RIA and their retention time on the reverse phase HPLC system used for characterizing the tissue extracts. TRH-Gly was synthesized by Bachem Fine Chemicals (Torrance, CA), Arg-TRH-Gly (Arg-Gln-His-Pro-Gly), Gln-His-Pro-Gly and Glu-His-Pro-Gly were synthesized by the UCLA Peptide Synthesis Laboratory (Los Angeles, CA). TRH-Gly-Lys, TRH-Gly-Lys-Arg, Lys-Arg-Gln-His-Pro-Gly and other TRH-related peptides were obtained from Peninsula Laboratories (Belmont, CA).

Statistical Methods

All results are reported as mean ± SD unless designated otherwise. Comparisons between the 4 treatment groups were carried out by one-way analysis of variance and the Scheffe contrast statistics using the Macintosh II computer (Apple Computer, Inc., Cupertino, CA) and the statistical program Statview 512+ (BrainPower, Inc., Calabasas, CA). To simplify the statistical presentation, only the significant (p<0.05) contrasts with the control group are presented in the tables of tissue hormone levels.

Zinc Content of Tissues

The effect of zinc deficiency (<1 ppm, ZD1), pair feeding (PF), partial zinc deficiency (6 ppm, ZD6) and zinc-adequate diet (20 ppm, control) on body weight and liver, femoral, pancreatic, testicular and epididymal zinc concentrations obtained in Experiment 2 are presented in Fig. 2. The body weights for the control and ZD6 groups did not differ significantly. The body weights for the ZD1 group were significantly less than the control, F(3,36) = 90, p<0.001, as were those for the PF group, F(3,36) = 4.5, p<0.01. The liver zinc concentrations for the ZD1 group were less than the control, F(3,36) = 6.6, p<0.005, and ZD6 values, F(3,36) = 11.7, p<0.001. Femoral zinc levels for the ZD1 and ZD6 groups were significantly less than the control group, F(3,36) = 71.3, p<0.001, and PF group, F(3,36) = 37.7, p<0.001, respectively. Epididymal zinc for the ZD1 group was less than the control values, F(3,36) = 37.2, p<0.001. Pancreatic zinc for the ZD1 and PF groups was less than the control group [F(3,36) = 5.8, p<0.005, and 7.55, p<0.001, respectively].

Serum Hormone Levels

The serum hormone levels for the 4 treatment groups in Experiment 2 are summarized in Fig. 3. The T4 levels in the ZD1, F(3,36) = 7.1, p<0.001, and PF groups, F(3,36) = 5.11, p<0.005, were all significantly decreased relative to the control group.

Tissue Content and Concentration of TRH-IR and TRH-Gly-IR

Hypothalamic TRH-IR content and concentration were not affected by zinc deficiency or pair feeding, while the TRH-Gly-IR content and concentration were both significantly reduced in the ZD1, F(3,36) = 13.5, p<0.001, and PF groups, F(3,36) = 9.86, p<0.001, compared to the control group (Table 1).

Brain weights were significantly reduced in the ZD1 group compared to the control group in Experiment 1, as seen in Table 2, F(3,36) = 6.9, p<0.01. Brain TRH-IR concentration, F(3,36) = 3.39, p<0.005, but not content was increased by zinc.
deficiency in Experiment 1 (Table 2) compared to controls. This effect may be secondary to the decrease in overall brain weight. The TRH-Gly-IR values in Experiment 2 were generally lower than the corresponding values in Experiment 1. This is attributable, in part, to the loss of high molecular weight TRH-Gly-IR peptides during the methanol extraction procedure (29).

Pituitary TRH-Gly-IR content declined significantly in the ZD1, F(3,36) = 2.86, p<0.05, and PF groups, F(3,36) = 3.63, p<0.025, compared to the control group in Experiment 1, without a significant change in the corresponding pituitary wet weight (results not shown). In Experiment 2, the pituitary weights for the ZD1 and PF groups were significantly decreased relative to the control group, as shown in Table 3, F(3,36) = 6.74, p<0.002.

HPLC Profile of TRH-IR and TRH-Gly-IR in Tissue Extracts

The TRH-IR of rat hypothalamus consists almost entirely of immunoreactivity which coelutes with synthetic TRH and is not affected by zinc deficiency (Fig. 4). The HPLC chromatograms of TRH-IR for pooled extracts of rat brain from the 4 treatment groups consist primarily of a single major peak with the same retention time as synthetic TRH, within experimental error (Fig. 5, upper panels). Other minor peaks are also evident. These TRH-IR profiles resemble those for the hypothalamus (Fig. 4). The corresponding profiles of brain TRH-Gly-IR (Fig. 5, lower panels) consist of a large number of peaks with the major peak eluting at the retention time corresponding to synthetic TRH-Gly. This peak is markedly diminished by zinc deficiency relative to the other 3 treatment groups, while the TRH-Gly-IR in the region of fractions 50–60 is relatively unaffected in comparison with the PF group (Fig. 5). These changes are consistent with the corresponding changes in TRH-Gly-IR content of rat brain in Experiment 1 (Table 2).

The pituitary profile of TRH-IR (Fig. 6) consists of a single peak with elution time corresponding to TRH, which was significantly decreased by zinc deficiency compared to the control group. The pituitary TRH-Gly-IR profile (Fig. 7) usually consisted of three major peaks. The peaks corresponding to TRH-Gly and Arg-TRH-Gly were significantly decreased by zinc deficiency compared to the PF group.

In Vitro Release of Hypothalamic TRH

No significant change in basal or K⁺-stimulated TRH release

| TABLE 1 |
|------------------|------------------|
| HYPOTHALAMIC WET WEIGHT AND CONTENT AND CONCENTRATION OF TRH-IR AND TRH-GLY-IR FROM EXPERIMENT 1 |
| Control | ZD1 | PF | ZD6 |
|------------------|------------------|
| Wet weight (mg) | 5.9 ± 3.1 | 6.0 ± 4.0 | 7.1 ± 4.0 | 8.6 ± 3.4 |
| TRH-IR (ng) | 5.8 ± 2.2 | 6.0 ± 1.2 | 5.1 ± 1.0 | 6.5 ± 0.8 |
| TRH-IR conc. (ng/g wet wt.) | 1169 ± 454 | 1526 ± 1034 | 921 ± 432 | 926 ± 505 |
| TRH-Gly-IR (ng) | 5.0 ± 1.7 | 1.2 ± 0.7* | 1.8 ± 0.9* | 3.9 ± 1.7 |
| TRH-Gly-IR conc. (ng/g wet wt.) | 1040 ± 532 | 377 ± 391* | 356 ± 366* | 527 ± 307 |
| TRH-IR/ TRH-Gly-IR | 1.2 ± 0.5 | 10.4 ± 15.7 | 3.6 ± 3.8 | 2.0 ± 0.9 |

*p<0.05 versus control group by one-way analysis of variance using Scheffe contrasts of the 4 groups.
from incubated hypothalamus was observed among the 4 treatment groups (Fig. 8). The rate of release following the addition of fresh medium containing 60 mM K⁺ was significantly increased (p<0.001) in comparison with the corresponding basal release rates by Student’s paired t-test.

**DISCUSSION**

The effect of zinc deficiency has been studied in young adult male Sprague-Dawley rats raised to sexual maturity on a zinc-adequate diet to avoid interference with developmental processes (26). Because tissue reserves of zinc are more difficult to deplete in adult animals, the treatment protocol extended over a 42-day period. Zinc deficiency, in addition to reducing body weight and inducing a hypogonadal state (23), also produces cold intolerance (19) attributable, in part, to a mild hypothyroidism, as seen in Fig. 3. A similar degree of suppression of serum TSH, T₄ and T₃ levels could be obtained by pair feeding to achieve the same mean body weight as for the ZD1 group (Fig. 3), in agreement with most previous reports on the thyroidal effects of caloric deprivation (5, 11, 34). These results suggest that the effects of zinc deficiency on thyroid hormone regulation in rats raised to sexual maturity on a zinc-adequate diet may be dominated by the concurrent hypocaloric state. The significant decrease in zinc levels of liver, femur, pancreas and epididymis of ZD1 animals suggests that zinc intake was inadequate to meet biologic needs.

An earlier 19-day study (23) using male 45 to 50 g weaning Sprague-Dawley rats reported that zinc deficiency reduces the hypothalamic concentration of TRH and serum T₄ and T₃ levels relative to an ad lib-fed control group. The serum T₃ values for the zinc-deficient group were also significantly lower than the corresponding values in a calorically restricted, weight-matched group which were, in turn, significantly below those for the ad lib controls. These results were consistent with a reduction of TRH biosynthesis and release by the hypothalamus and a possible inhibition of T₄ to T₃ conversion.

TRH biosynthesis in the hypothalamus is very efficient, resulting in a high concentration of TRH and levels of TRH precursor peptides which are too low for consistent chromatographic characterization with currently available antibodies (10,32). The absence of rate-limiting steps in TRH biosynthesis in this tissue tends to reduce its responsiveness to experimental manipulations.

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**TABLE 2**

EXTRAHYPOTHALAMIC BRAIN WET WEIGHTS AND CONTENT AND CONCENTRATION OF TRH-IR AND TRH GLY-IR

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ZD1</th>
<th>PF</th>
<th>ZD6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole brain (g)</td>
<td>0.88 ± 0.06</td>
<td>0.67 ± 0.19*</td>
<td>0.80 ± 0.048</td>
<td>0.86 ± 0.048</td>
</tr>
<tr>
<td>TRH-IR (ng)</td>
<td>1.7 ± 0.4</td>
<td>2.0 ± 0.5</td>
<td>1.9 ± 0.4</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>TRH-IR (ng/g wet wt.)</td>
<td>1.9 ± 0.5</td>
<td>3.4 ± 2.0*</td>
<td>2.3 ± 0.5</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>TRH-Gly-IR (ng)</td>
<td>51 ± 34</td>
<td>26 ± 20</td>
<td>90 ± 116</td>
<td>41 ± 28</td>
</tr>
<tr>
<td>TRH-Gly-IR (ng/g wet wt.)</td>
<td>58 ± 39</td>
<td>49 ± 57</td>
<td>114 ± 146</td>
<td>49 ± 34</td>
</tr>
<tr>
<td>TRH-IR/TRH-Gly-IR</td>
<td>0.07 ± 0.07</td>
<td>0.13 ± 0.09</td>
<td>0.06 ± 0.05</td>
<td>0.08 ± 0.12</td>
</tr>
</tbody>
</table>

**TABLE 3**

PITUITARY WET WEIGHTS AND CONTENT AND CONCENTRATION OF TRH-IR AND TRH GLY-IR (EXPERIMENT 2)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ZD1</th>
<th>PF</th>
<th>ZD6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet weight (ng)</td>
<td>8.8 ± 1.9</td>
<td>5.4 ± 2.0*</td>
<td>6.4 ± 0.9*</td>
<td>8.7 ± 1.8</td>
</tr>
<tr>
<td>TRH-IR (ng)</td>
<td>0.21 ± 0.18</td>
<td>0.10 ± 0.05</td>
<td>0.13 ± 0.03</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>(ng/g wet wt.)</td>
<td>25 ± 21</td>
<td>21 ± 10</td>
<td>21 ± 4</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>TRH-Gly-IR (ng)</td>
<td>1.3 ± 1.1</td>
<td>0.6 ± 0.3</td>
<td>1.1 ± 0.8</td>
<td>1.1 ± 1.4</td>
</tr>
<tr>
<td>(ng/g wet wt.)</td>
<td>154 ± 127</td>
<td>132 ± 75</td>
<td>173 ± 128</td>
<td>122 ± 125</td>
</tr>
<tr>
<td>TRH-IR/TRH-Gly-IR</td>
<td>0.3 ± 0.4</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0.5 ± 0.7</td>
</tr>
</tbody>
</table>

*p<0.05 versus the control group by one-way analysis of variance using the Scheffe contrast of the 4 groups.

**FIG. 4.** Effect of zinc deficiency and control diet on HPLC profile of TRH-IR for pooled rat hypothalamic extracts from Experiment 1.
For example, hypothalamic TRH concentration (Table 1 and Fig. 4) and basal and K⁺-stimulated release of TRH by in vitro incubated hypothalamic fragments from the ZD1, PF and ZD6 groups were not changed significantly relative to the control group (Fig. 8). These results contrast with the earlier report (23) that zinc deficiency suppresses hypothalamic TRH content and concentration, though experimental differences, noted above, may be relevant.

The extrahypothalamic brain and pituitary do have rate-limiting steps resulting in high concentrations of TRH precursor peptides. The combined use of high pressure liquid chromatography and TRH and TRH precursor RIA in these tissues can provide a readily quantitated overview of the steady-state distribution of peptide hormone precursors and products and the effect of hormonal status, cofactor availability and aging on peptide hormone biosynthesis (Figs. 4-7).

Hypothalamic TRH-Gly-IR content and concentration were significantly decreased in the ZD1 and PF groups compared to the control group (Table 1), suggesting that some interference with TRH precursor processing can occur without decreasing hypothalamic TRH content and basal and stimulated TRH release rates. These observations are consistent with a linkage of release rate of TRH with the intracellular TRH pool size (17).

The HPLC chromatographic profiles for extrahypothalamic brain TRH-IR, like those for hypothalamus, consist of a single major peak corresponding to TRH (Fig. 5, upper panels) which is not affected by zinc deficiency or caloric restriction, while the TRH-Gly-IR profile for brain is complex. The effect of zinc deficiency is to reduce the proportion of hydrophilic, low molecular weight TRH-Gly-IR peaks in the fraction 20 to 50 region (Fig. 5, upper left panel). This pattern is consistent with some partial inhibition of the processing of propro-TRH to form smaller peptides with C-terminal Gly residues which are cross-reactive in the TRH-Gly RIA (Fig. 1).

The HPLC profile of PF brain TRH-gly-IR (Fig. 5, lower left panel) reveals a significant increase in the area of peaks corresponding to low molecular weight TRH-Gly-IR peptides, such as Arg-TRH-Gly and Lys-Arg-TRH-Gly (peaks in the region of fractions 22 to 30), which are more immunoreactive than their C-terminally extended precursor forms (Fig. 1) which also elute in this same chromatographic region (32). The alpha-amidation of TRH-Gly is apparently rate limiting in brain tissue. Increased zinc uptake in the PF group (Fig. 2) should increase the activity of the zinc-dependent carboxypeptidase H enzyme which
cleeves the basic residues C-terminal to Gly, thereby increasing the total TRH-Gly-IR of the small TRH-Gly precursor peptides.

Pituitary content of TRH and low molecular weight TRH-Gly-IR peptides, including TRH-Gly, were decreased by zinc deficiency (Table 3 and Figs. 6 and 7). Eighty percent of pituitary TRH occurs in the posterior lobe and is of hypothalamic origin (28). This observation suggests that the pituitary is more susceptible to the effects of zinc deficiency than is the hypothalamus. Fasting has previously been reported to leave pituitary nuclear T₃ content and fractional occupancy of the T₃ nuclear receptor relatively unchanged relative to ad lib-fed controls (34). These effects of zinc deficiency and pair feeding on serum thyroid hormone levels do not, however, appear to be mediated by a reduction in hypothalamic TRH content and release rate, as noted above. Food deprivation in rats has been reported to increase the pituitary TSH response to IV TRH (5) and to reduce hypothalamic prepro-GH-releasing factor mRNA by 80%, while GHRH content and prepro-SRF mRNA and SRF levels were unchanged (2). Increased hypothalamic release rates for somatostatin and dopamine, which inhibit TSH secretion (13), remain as possible mediators of the calorice restriction-induced reduction in pituitary TSH release.

Conflicting results have appeared in the literature regarding the effect of zinc deficiency on T₃ to T₄ conversion in rat liver (8,25). Methodology problems resulting from the use of crude homogenates rather than microosomal preparations in some studies may be the cause of these reported discrepancies.

To summarize, serum T₃ levels decline significantly in young adult Sprague-Dawley rats fed a severely zinc-deficient diet or pair fed for 42 days compared to those fed a zinc-adequate diet ad lib. Total TRH-Gly immunoreactivity is significantly reduced in the hypothalamus and pituitary by zinc deficiency and the corresponding HPLC chromatographic profiles in brain and pituitary indicate this reduction occurs in the TRH-Gly-IR peaks corresponding to tetrapeptide TRH-Gly and other small peptide precursors to TRH-Gly. Pituitary TRH and TRH precursor peptide levels were decreased by zinc deficiency and pair feeding. The processing of TRH precursor peptides is not rate limited in the hypothalamus. Similarly, hypothalamic and extrahypothalamic brain TRH content and in vitro basal and K⁺-stimulated TRH release rates from hypothalamic fragments were not affected by zinc deficiency or pair feeding. The nearly identical suppressive effects of prolonged zinc deficiency and caloric restriction on serum TSH and thyroid hormone levels induced in sexually mature male rats appear to be secondary to the hypocaloric state of the pituitary and thyroid cells of these animals.

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