

# Evaluation of activation of G proteins in response to thyroid stimulating hormone in thyroid gland cells from euthyroid and hyperthyroid cats

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**Objective**—To evaluate alterations in ligand-stimulated activity of G proteins in thyroid gland cells of hyperthyroid cats.

**Sample Population**—Membranes of thyroid gland cells isolated from 5 hyperthyroid cats and 3 age-matched euthyroid (control) cats immediately after the cats were euthanatized.

**Procedures**—Isolated thyroid cell membranes were treated with thyroid-stimulating hormone (TSH), and activation of G protein was quantified by measurement of the binding of guanosine triphosphate  $\gamma$  labeled with sulfur 35 (GTP $\gamma^{35}$ S). The separate effects of G-protein inhibitory ( $G_i$ ) and G-protein stimulatory ( $G_s$ ) proteins were determined by the use of pertussis toxin and cholera toxin, respectively.

**Results**—Thyroid cell membranes from hyperthyroid cats had higher basal GTP $\gamma^{35}$ S binding than did thyroid cell membranes from euthyroid cats. Thyroid cell membranes from hyperthyroid and euthyroid cats had a concentration-dependent increase in TSH-stimulated GTP $\gamma^{35}$ S binding over the TSH range of 0 to 100 mU/mL, with maximal activity at 1 to 100 mU/mL for both. The percentage increase in GTP $\gamma^{35}$ S binding stimulated by TSH was similar in magnitude between the membranes from hyperthyroid and euthyroid cats. The TSH-stimulated activation of  $G_s$  and  $G_i$  was not different between euthyroid and hyperthyroid cats.

**Conclusions and Clinical Relevance**—Ligand-stimulated activation of G proteins was the same in thyroid cell membranes obtained from hyperthyroid and euthyroid cats. Therefore, alterations in inherent  $G_s$  or  $G_i$  activities did not appear to be part of the pathogenesis of hyperthyroidism in cats. (*Am J Vet Res* 2010;71:643–648)

Hyperthyroidism is the most common endocrinopathy in cats, and it affects a large percentage of middle-aged to older animals. Since its recognition as a clinical entity approximately 30 years ago, it has been diagnosed with increasing frequency; however, the cause of the disease remains unknown.<sup>1–4</sup> Studies<sup>5–8</sup> have indicated that the disease is at the level of the thyroid gland, with cells from hyperthyroid cats functioning autonomously to result in unregulated cell growth and hormone secretion. Although nutritional and environmental causes have been implicated in the etiology of the disease, no discrete factor has been identified that causes thyroid gland cells to become autofunctional.<sup>9–12</sup>

Synthesis and secretion of thyroid hormone is directly regulated by TSH that is released by the pituitary gland. The interaction of TSH with its receptor on the

## ABBREVIATIONS

CTX	Cholera toxin
$G_s$	G protein $\alpha$ subunit
GDP	Guanosine diphosphate
$G_i$	G-protein inhibitory
$G_s$	G-protein stimulatory
GTP	Guanosine triphosphate
PTX	Pertussis toxin
TSH	Thyroid-stimulating hormone

surface of thyroid cells results in activation of receptor-coupled heterotrimeric G proteins that control cAMP concentrations in the thyroid gland cells. Activation of this signal transduction system and subsequent elevation of intracellular cAMP concentrations result in growth and differentiation of thyroid gland cells as well as secretion of thyroid hormone.<sup>13–15</sup> Thus, abnormalities of any part of the receptor-G protein-cAMP signal transduction system could be postulated to result in the unregulated growth and hormone production seen in cats with hyperthyroidism.

The  $G_s$  and  $G_i$  families of G proteins control intracellular cAMP concentrations, with  $G_s$  proteins causing activation of adenylyl cyclase and elevation of cAMP concentrations and  $G_i$  proteins inhibiting adenylyl cy-

Received March 12, 2009.

Accepted June 8, 2009.

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clase and decreasing intracellular cAMP concentrations. Binding of TSH to its receptor causes a conformational change of the TSH receptor that results in replacement of GTP for GDP on the  $\alpha$  subunit of the coupled heterotrimeric G protein. This results in activation of the G protein such that  $\alpha$ -GTP separates from the  $\beta\gamma$  dimer. Both the  $\alpha$  subunit and  $\beta\gamma$  dimer interact with various effector molecules, including adenylyl cyclase, to cause cellular activation.<sup>16</sup> The  $G_\alpha$  has inherent GTPase activity and catalyzes the dephosphorylation of GTP, which results in reformation of the inactive GDP-bound  $\alpha\beta\gamma$  heterotrimer. By taking advantage of this activation-deactivation cycle, heterotrimeric G protein activity can be measured by use of a nonhydrolyzable analogue of GTP, GTP $\gamma$  labeled with sulfur 35 (<sup>35</sup>S).<sup>17</sup> This analogue binds to active  $G_\alpha$  and provides a stable radiolabel. The radioactivity then can be measured to quantify G protein activity.<sup>18</sup>

The TSH receptor is coupled to both  $G_s$  and  $G_i$  proteins.<sup>19</sup> Therefore, in thyroid gland cells, the relative expression or activation of  $G_s$  and  $G_i$  proteins in a cell ultimately determines the intracellular cAMP concentration that directly stimulates cell growth, proliferation, and hormone production.<sup>20</sup> For instance, if the ratio of  $G_s$  expression to  $G_i$  expression is higher for a disease state than for healthy tissue, the cAMP concentrations may be artificially increased. Specific bacterial toxins can affect the activities of  $G_s$  and  $G_i$  proteins through a process called ADP-ribosylation. These toxins catalyze the transfer of an ADP ribose group onto  $G_\alpha$  and cause permanent changes in G protein activity. Cholera toxin is specific for  $G_s$  proteins and results in constitutive activation of  $G_s$  protein. Similarly, PTX catalyzes a reaction specific to  $G_i$  proteins and results in an inactivation of  $G_i$  protein. These toxins are important for the pathogenesis of their particular diseases, and they can also be used experimentally to define the separate roles of  $G_s$  and  $G_i$  proteins in overall cellular activation of G proteins.<sup>21</sup>

Hyperthyroidism in cats is clinically and histopathologically similar to a hyperthyroid disease in humans (ie, toxic nodular goiter). Abnormalities of the G protein-cAMP signaling pathway have been implicated in the pathogenesis of toxic nodular goiter. Gain-of-function mutations of the  $\alpha$  subunit of  $G_s$  proteins and the TSH receptor that result in unregulated growth and function of the affected thyrocytes have been described.<sup>22-29</sup> Altered expression of the  $\alpha$  subunits of  $G_s$  and  $G_i$  proteins have also been detected in humans with toxic nodular goiter.<sup>30,31</sup> Similar mechanisms appear to be involved in the pathogenesis of hyperthyroidism in cats. Previous studies<sup>32,33</sup> conducted by our laboratory group have indicated a specific decrease of the expression of  $G_i$  protein (more specifically, the  $G_{12}$  subset) in membranes isolated from hyperthyroid cells. We postulate that a decrease in  $G_{12}$  expression leads to a relative increase of  $G_s$  expression and the unregulated hyperfunction of the thyroid cells seen in cats with hyperthyroidism.

Although the amount of expression appears to be affected, the activity of the TSH receptor-coupled G proteins in cats with hyperthyroidism has not been investigated. The study reported here was conducted to

examine activation of  $G_s$  and  $G_i$  proteins in response to TSH in thyroid cell membranes isolated from hyperthyroid cats to determine whether abnormal activation of G proteins is part of the molecular pathogenesis of hyperthyroidism in cats.

## Materials and Methods

**Sample population**—Thyroid gland tissue was surgically removed from 5 hyperthyroid cats (3 females and 2 males; 8 to 13 years old) and 3 age-matched euthyroid cats (2 males and 1 female; 8 to 14 years old) that were euthanized at our hospital because of traumatic injuries. Cats were euthanized by an IV injection of sodium pentobarbital. Thyroid glands were removed immediately after cats were euthanized.

Review of the medical record for each cat was performed by a veterinarian board-certified by the American College of Veterinary Internal Medicine (CRW), and a diagnosis of hyperthyroidism was determined on the basis of clinical signs and an increase in serum thyroxine concentration. Histologic examination of the thyroid gland tissue confirmed the diagnosis of hyperthyroidism. All hyperthyroid cats in this study had disease in both lobes of the thyroid gland. Euthyroid cats did not have clinical signs of hyperthyroidism, had serum thyroxine concentrations within the reference range, had thyroid gland tissue that was judged to be histologically normal, and did not have evidence of systemic disease, as determined on the basis of the medical history and results of physical examination and point-of-care biochemical analyses.

**Tissue preparation**—Thyroid glands were snap-frozen immediately after harvest in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use. Minced thyroid tissue (0.175 to 0.435 g) was placed in 2 mL of ice-cold buffer solution containing 50mM Tris, 1mM EDTA, and 250mM sucrose (pH, 7.4) and then was homogenized. Samples were centrifuged at  $2,500 \times g$  for 10 minutes at  $4^\circ\text{C}$ , and the resultant supernatant was collected and centrifuged at  $30,000 \times g$  for 30 minutes at  $4^\circ\text{C}$ . The supernatant from the second centrifugation was discarded, and the membrane-enriched pellet was resuspended in Tris-EDTA buffer, divided into aliquots, and stored at  $-80^\circ\text{C}$ . Protein concentration of the membranes was determined by use of a bicinchoninic acid protein assay,<sup>a</sup> with bovine serum albumin used as the standard.

**GTP $\gamma$ <sup>35</sup>S binding analysis**—The membrane-enriched pellets from the hyperthyroid and euthyroid cats were diluted in ice-cold buffer solution (50mM Tris-1mM EDTA [pH, 7.4]) to achieve equivalent amounts of protein in each sample to be assayed (25 to 35  $\mu\text{g}$ ). They were then added to a binding buffer solution containing 100mM Tris-2mM EDTA (pH, 7.4), 100mM  $\text{MgCl}_2$ , 100mM dithiothreitol, 500 $\mu\text{M}$  GDP, and 1nM GTP $\gamma$ <sup>35</sup>S.<sup>b</sup> To obtain the concentration-response curve, various concentrations of bovine TSH<sup>c</sup> (0 to 100 mU/mL) were added. These concentrations were selected on the basis of TSH concentrations used to stimulate cultured feline thyroid cells<sup>34</sup> and Wistar rat thyroid cells.<sup>35</sup> In some experiments, 50 $\mu\text{M}$  mastoparan,<sup>d</sup> a wasp venom peptide that nonspecifically activates all

classes of G proteins,<sup>36</sup> was added as a positive control group (data not shown). For each data point, a separate set of duplicate tubes containing a 1,000-fold excess of unlabeled GTP $\gamma$ S was included to quantitate non-specific GTP $\gamma$ S binding. The experimental samples were placed in a 30°C water bath for 15 minutes and then filtered through a vacuum-controlled 2- $\mu$ m filter.<sup>e</sup> Ice-cold buffer solution (50mM Tris–5mM MgCl<sub>2</sub> [pH, 7.5]) was used to stop the reaction. Filters were allowed to dry for 30 minutes and then placed into scintillation vials containing 1 mL of scintillation fluid.<sup>f</sup> Samples were analyzed for GTP $\gamma$ S binding by use of a scintillation machine.<sup>g</sup> Specific GTP $\gamma$ S binding was determined by subtracting the nonspecific binding obtained with the 1,000-fold excess of GTP $\gamma$ S from the total GTP $\gamma$ S binding. Triplicate determinations were made for each condition tested in each experiment. To examine the activation of G<sub>s</sub> and G<sub>i</sub> proteins, thyroid cell membranes were subjected to CTX- or PTX-catalyzed ADP-ribosylation.<sup>21</sup> Thyroid cell membranes were resuspended in ice-cold buffer solution (50mM Tris-HCl, 1mM EDTA, 20mM  $\beta$ -glycerophosphate,<sup>h</sup> 10  $\mu$ g of leupeptin<sup>h</sup>/mL, and 10  $\mu$ g of aprotinin<sup>h</sup>/mL [pH, 7.5]) to achieve a protein concentration of 100  $\mu$ g/mL. Activated toxin (CTX<sup>i</sup> or PTX<sup>i</sup>) and nicotinamide adenine dinucleotide<sup>h</sup> were added to the membranes to achieve a final concentration of 20  $\mu$ g/mL and 5.3 $\mu$ M for CTX and PTX, respectively. Membranes were incubated at 30°C for 60 minutes to allow the CTX- or PTX-catalyzed ADP-ribosylation to proceed. The treated membranes were then assayed for TSH-induced GTP $\gamma$ S binding as described previously. Untreated control samples were prepared identically, except that buffer solution was used in place of CTX or PTX in the reaction mixture.

**Statistical analysis**—Statistical analysis was performed by use of a commercial statistical program.<sup>k</sup> The dependent variable, GTP $\gamma$ S binding, was analyzed via a 2-way ANOVA by use of a general linear models procedure to assess effects of membrane type and concentration of TSH. The percentage change in GTP $\gamma$ S binding was also analyzed via a 2-way ANOVA by use of a general linear models procedure to assess effects of membrane type and treatment (TSH, CTX + TSH, or PTX + TSH). Differences between means were determined by use of the Duncan multiple range test. For all analyses, values of  $P < 0.05$  were considered significant.

## Results

Isolated thyroid cell membranes from euthyroid and hyperthyroid cats had a significant ( $P = 0.01$ ) quadratic relationship between GTP $\gamma$ S binding when stimulated by TSH at concentrations ranging from 0 to 100 mU/mL (Figure 1). At a TSH concentration of 0 mU/mL (unstimulated), membranes from hyperthyroid cats had significantly higher basal GTP $\gamma$ S binding than did membranes from euthyroid cats. Maximal response of 4.0 and 4.9 fmol of GTP $\gamma$ S binding/ $\mu$ g of protein was detected at a TSH concentration of 1 mU/mL for membranes from euthyroid and hyperthyroid cats, respectively. This response was significantly higher than the basal GTP $\gamma$ S binding for the unstimulated mem-

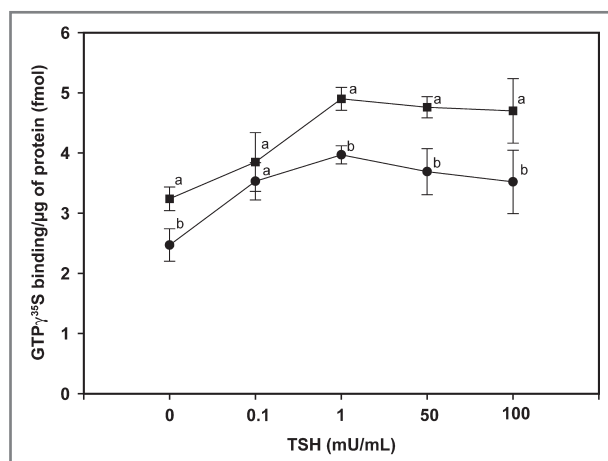


Figure 1—Concentration-response curve of binding of GTP $\gamma$  labeled with sulfur 35 (<sup>35</sup>S) to thyroid cell membranes obtained from 3 euthyroid (circles) and 5 hyperthyroid (squares) cats. Isolated thyroid cell membranes were incubated with various concentrations of TSH, and activation of G proteins was measured by quantification of GTP $\gamma$ S binding to the membranes. Values reported represent the mean  $\pm$  SD of 3 separate experiments. <sup>a,b</sup>Within a TSH concentration, means with different letters differ significantly ( $P < 0.05$ ).

Table 1—Effects of toxin treatment on TSH-stimulated binding of GTP $\gamma$  labeled with sulfur 35 (<sup>35</sup>S) to thyroid cell membranes obtained from 3 euthyroid and 5 hyperthyroid cats.

Variable	TSH	PTX + TSH	CTX + TSH	Membrane mean
Membrane				
Euthyroid cats	61 $\pm$ 6	34 $\pm$ 4	40 $\pm$ 2	45 $\pm$ 13
Hyperthyroid cats	54 $\pm$ 6	32 $\pm$ 3	36 $\pm$ 3	41 $\pm$ 11
Treatment mean	58 $\pm$ 6 <sup>a</sup>	33 $\pm$ 3 <sup>b</sup>	38 $\pm$ 3 <sup>b</sup>	—

Data reported represent the mean  $\pm$  SD percentage increase in GTP $\gamma$ S binding, as compared with basal (unstimulated) binding, for 3 separate experiments.  
 — Not applicable.  
<sup>a,b</sup>Means with different superscript letters differ significantly ( $P < 0.05$ ).

branes. Although basal and maximal GTP $\gamma$ S binding was higher in membranes from the hyperthyroid cats, the percentage increase of GTP $\gamma$ S binding did not differ significantly between membranes from euthyroid and hyperthyroid cats (Table 1). The GTP $\gamma$ S binding did not increase significantly as the concentration of TSH increased from 1 to 100 mU/mL.

In an effort to examine the separate effects of G<sub>i</sub> and G<sub>s</sub> proteins to the total GTP $\gamma$ S binding, membranes were incubated with PTX (an inactivator of all subgroups of G<sub>i</sub> proteins) or CTX (a specific activator of G<sub>s</sub> proteins). Thus, the separate contributions of G<sub>i</sub> and G<sub>s</sub> proteins could be quantified. Effects of toxin treatment on the percentage of TSH-stimulated GTP $\gamma$ S binding were determined (Table 1). The percentage of TSH-stimulated GTP $\gamma$ S binding was not significantly ( $P = 0.68$ ) different for the membrane type-by-toxin treatment interaction. However, there was a significant ( $P < 0.001$ ) effect of toxin treatment. In PTX-treated membranes, TSH-stimulated GTP $\gamma$ S binding was significantly inhibited, compared with binding in non-PTX-treated membranes, regardless of the membrane

type. In membranes incubated with CTX, there was also a significant decrease in TSH-stimulated GTP $\gamma$ <sup>35</sup>S binding, compared with binding in non-CTX-treated membranes. However, the inhibition of GTP $\gamma$ <sup>35</sup>S binding did not differ significantly between the PTX and CTX treatments. Inhibition of GTP $\gamma$ <sup>35</sup>S binding attributable to membrane type (euthyroid or hyperthyroid cats) did not differ significantly ( $P = 0.06$ ).

## Discussion

Analysis of results for the study reported here revealed that isolated feline thyroid cell membranes underwent measurable activation of G proteins when stimulated with their natural ligand, TSH. This was a concentration-dependent effect, which indicated a specific effect of the ligand-receptor interaction. Maximal ligand stimulation was seen at a TSH concentration of 1 mU/mL, with a plateau effect for concentrations up to 100 mU/mL. These data agree with those in another study<sup>34</sup> conducted by our laboratory group in which cultured feline thyroid cells were treated with TSH to induce the cellular responses of mitogenesis and hormone production. In the experiments in that study,<sup>34</sup> stimulation was detected at a TSH concentration of 1 mU/mL, although the maximal effect was detected at a TSH concentration of 50 mU/mL. It is not surprising that the maximal effect on GTP $\gamma$ <sup>35</sup>S binding was detected at a lower concentration in the present study because we used the activation of G proteins as the endpoint, rather than a total cellular response such as mitogenesis or hormone production. Additionally, the experiments in that previous study<sup>34</sup> were performed on cultured cells, compared with the experiments in the present study that were conducted by use of isolated membranes, which would be expected to respond to lower concentrations of ligand. Therefore, the ligand concentration that yielded optimal results in the study reported here appeared to be consistent with previous data. We believe that this is a physiologically appropriate system in which to quantify ligand-stimulated G protein responses in feline thyroid membranes.

Separate experiments were not conducted to examine TSH binding to the membranes from euthyroid or hyperthyroid cats. In another study,<sup>8</sup> researchers investigated mutations in the feline TSH receptor but were not able to identify any such mutations, and binding characteristics of cloned feline TSH receptors were identical to those of human TSH receptors. In addition, TSH specifically stimulates cultured thyrocytes from hyperthyroid cats and also stimulates a hormonal response in hyperthyroid cats.<sup>34,37</sup> Therefore, it is unlikely that differential binding of TSH to the receptors in membranes from euthyroid or hyperthyroid cats had a major role in the activation of G proteins that we measured.

Basal (ligand-unstimulated) GTP $\gamma$ <sup>35</sup>S binding was higher in membranes from hyperthyroid cats. This also is consistent with results of our previous study<sup>34</sup> in which we examined TSH-stimulation of cell activation in cultured feline thyroid cells. Analysis of those data revealed that thyroid cells from hyperthyroid cats had higher basal mitogenic and thyroid hormone-producing activity than did thyroid cells from clinically nor-

mal cats. We postulate that the cells from the hyperthyroid cats have a higher basal rate of G protein activity because of the inherent cellular changes that cause them to be autofunctional. In some cases of humans with toxic nodular goiter, gain-of-function mutations in G<sub>s $\alpha$</sub>  proteins have resulted in constitutively activated G<sub>s</sub> proteins.<sup>22,24,26</sup> Investigators have searched for these mutations in cats with hyperthyroidism, but such mutations have not been identified.<sup>8,38</sup> If such mutations do exist, they could cause an increase in basal activity, such as that detected in the present study; however, we postulate that it would be substantially higher than the activity that was observed. Also, we would not expect to detect ligand-stimulated increases in activation of G proteins because the mutated proteins are nonresponsive to typical receptor-ligand interactions.<sup>29</sup>

Interestingly, the TSH ligand was able to stimulate activity of G proteins in the membranes from the euthyroid and hyperthyroid cats to the same extent and at the same maximal TSH concentration. This indicates that the population of G proteins able to be stimulated by TSH is activated similarly between cells from euthyroid and hyperthyroid cats. These data further indicate a lack of mutations in the G<sub>s $\alpha$</sub>  subunit. Additionally, it can be inferred that there is typical receptor coupling of heterotrimeric G proteins in cells from hyperthyroid cats because the natural ligand, TSH, was used for activation in both systems.

In an attempt to examine the activation of G<sub>s</sub> and G<sub>i</sub> separately, thyroid membranes were incubated with CTX or PTX before TSH-stimulated activation of G proteins was measured. Both of these toxins irrevocably alter the G <sub>$\alpha$</sub>  function of a specific G protein class by catalyzing the transfer of an ADP-ribose group onto G <sub>$\alpha$</sub> . The ADP-ribosylation of G<sub>s $\alpha$</sub>  by CTX results in constitutive activation of G<sub>s $\alpha$</sub> . Treatment of membranes with PTX causes inactivation of G<sub>i $\alpha$</sub> . Therefore, in thyroid membranes incubated with CTX, G<sub>s</sub> proteins are not able to be stimulated with the TSH ligand. In thyroid membranes incubated with PTX, G<sub>i</sub> proteins are not able to be stimulated by TSH. Although there was a significant decrease in TSH-stimulated GTP $\gamma$ S binding in membranes treated with CTX and PTX, analysis of our data did not reveal a significant difference in activation of G proteins between thyroid membranes from euthyroid and hyperthyroid cats. This provides further evidence that G<sub>s</sub> and G<sub>i</sub> can be activated by the TSH ligand in thyroid cells from hyperthyroid cats.

Multiple environmental factors, including exposure to flame retardants,<sup>12</sup> canned foods,<sup>4,10,11</sup> and flavonoids,<sup>1</sup> have been implicated as a cause for hyperthyroidism in cats. The evidence for involvement of these factors in the disease is largely epidemiological<sup>4,10,11</sup>; however, all 3 studies implicated commercially available cat food as a risk factor for development of hyperthyroidism in cats. In another study,<sup>12</sup> investigators examined blood samples from cats with hyperthyroidism and from age-matched euthyroid (control) cats and found increased concentrations of some metabolites of flame retardants in some of the hyperthyroid cats. In a collaborative study<sup>12</sup> conducted by our laboratory group, we detected direct effects of a variety of flavonoid compounds on

the ability to stimulate cultured feline thyrocytes. The role these factors may have on the changes in G proteins seen in cells from hyperthyroid cats is not clear, but it remains a fertile area for future research.

Analysis of results from the study reported here indicated that TSH-stimulated activity of G proteins was the same in cells from euthyroid and hyperthyroid cats. This supports the hypothesis that it is a specific decrease in expression in G<sub>12</sub> and not a change in G protein activity that is part of the molecular pathogenesis of hyperthyroidism in cats. It is postulated that a decrease in expression of G<sub>i</sub> proteins causes cAMP to be increased in an unregulated manner. Reasons for this decrease in expression are unknown; however, the relationship with identified environmental factors implicated in causing hyperthyroidism needs to be investigated further. Also of interest is the effect of cAMP production in these cells. Future research efforts into this area need to be completed.

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- a. Micro BCA assay, Pierce Chemical Co, Rockford, Ill.
  - b. Perkin Elmer Radiochemicals, Boston, Mass.
  - c. Bovine thyrotropin, cell culture grade, Sigma-Aldrich, St Louis, Mo.
  - d. Synthetic peptide, EMD Chemicals, Gibbstown, NJ.
  - e. Millipore, Billerica, Mass.
  - f. Ecolite, MP Biomedicals, Irvine, Calif.
  - g. Beckman model LS6000, Coulter Inc, Fullerton, Calif.
  - h. Sigma-Aldrich, St Louis, Mo.
  - i. *Vibrio cholera*, EMD Chemicals, Gibbstown, NJ.
  - j. *Bordetella pertussis*, EMD Chemicals, Gibbstown, NJ.
  - k. SAS Stat, version 9.1, SAS Institute Inc, Cary, NC.
  - l. Ward CR, Rupp NC, Ralph AG. The flavonoid quercetin stimulates mitogenesis in feline hyperthyroid cells (abstr). *J Vet Intern Med* 2007;21:595.
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