Bioassay of Avian Thyroid-stimulating Hormone

by

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Abstract

Thyroid-stimulating hormone (TSH) potency was measured as thyroxine-releasing activity in cultured quail thyroid glands, each of which was preincubated with shaking for 2 h in 1 ml of medium 199, pH 7.4, under a constant flow of 95% O₂ and 5% CO₂ at 37°C. The gland was then incubated for 3 h in 250 μl of the medium, which contained a reference or sample preparation. The thyroxine (T₄) concentration in the medium was determined by radioimmunoassay and used as an index of the TSH activity. In this bioassay, 7.8 μg of acetone-dried chicken pituitary gland (A1D) induced a significant increase in T₄ secretion. A linear log-dose-response relationship in a dose range between 7.8 and 500 μg was observed. The precision index of this assay was 0.19. Intra- and inter-assay coefficients of variation were 55% and 59%, respectively. Partially purified chicken pituitary glycoprotein showed higher TSH activity than A1D. Bovine TSH also showed TSH activity. Human chorionic gonadotropin showed slight activity. Chicken follicle-stimulating hormone and quail brain extracts both indicated negligible activity. Some pituitary extracts of Columba livia domestica, Melopsittacus undulatus, and Coturnix coturnix japonica showed the same TSH activity as that of chicken. This microbioassay system offers sufficient sensitivity and will be availavle for the specific measurement of avian TSH.

Introduction

For a comprehensive understanding of the regulation functions of vertebrates via adenohypophyseal hormones, a comparative knowledge of such functions is imperative even for lower vertebrates. One way of obtaining this information is to measure the plasma hormone concentrations of lower vertebrates. Also important is information about hormones and their receptors at the molecular level, and the fluctuations occurring in endogenous hormone levels.

Highly purified thyroid-stimulating hormone (TSH) from mammals has already been isolated and a radioimmunoassay method has also been developed. There are many reports on TSH purification from reptiles[9] and amphibians[10] and fish[11]. On the other hand, few reports have been published of studies on avian...
TSH\cite{12-13}, and although several bioassay procedures have been reported\cite{13-16}, they are not suitable for practical application in the purification of avian TSH. The author therefore devised a sensitive and specific homologous bioassay method for avian TSH, which is known to stimulate various types of activity in avian thyroid glands, and the TSH potency was measured as *in vitro* thyroxine (T4)-releasing activity.

**Materials and Methods**

**Thyroid glands**

Five-week-old male Japanese quails (*Coturnix coturnix japonica*) reared under long-day photoperiod conditions were used. The birds were sacrificed by decapitation and their thyroid glands were immediately dissected out. The capsules, comprising of connective tissue, were removed from the gland in cold physiological saline solution. Morphologically distorted thyroid glands were rejected, including those in which the left and right lobes differed in size or where one lobe was missing. Each lobe was cut into two equal parts under a stereoscopic microscope, so that it could be sufficiently submerged in incubation medium.

**Incubation**

Pairs of dissected lobes were incubated in medium 199 (Earl's balanced salt solution, Nissui), pH 7.4, under a constant flow of 95% O2 and 5% CO2 at 37°C in plastic tubes (tube size: 3.5 ml) with shaking. Incubated media were stored at -20°C until analysis.

**Hormones**

As an avian TSH preparation, A1D, an acetone-dried powder of chicken pituitary gland, and AGS112D, a fraction obtained by partially purifying A1D by ethanol precipitation\cite{16} and gel-filtration, were used. Bovine TSH (SIGMA), chicken follicle-stimulating hormone (AGCQSQ113445C)\cite{17} and human chorionic gonadotropin (HCG, Teikokuzouki) were also used.

**Radioimmunoassay**

For radioimmunoassay of T4, a commercially available T4 RIA Kit (Eiken) was employed, in which 125I-labelled T4 was used as a radioligand. Small modifications were made to the protocols recommended by the manufacturer. The T4 concentrations in the media were calculated by microcomputer using software developed by Wakabayashi\cite{18}.

**Statistical methods**

To test the significance of hormone levels, Mann-Whitney's U test or Student's t test was employed\cite{19}.

**Preincubation**

Single thyroid gland lobes, each comprising of two dissected pieces, were incubated in 1 ml of medium per tube, and changes in the levels of released T4 with time were measured by replacement of the medium at various intervals (0, 15, 30, 60, 120 and 180 min).

**Time course**

After the preincubation, medium in the tubes was replaced by 1 ml of medium containing 2.5 mg of A1D as the TSH preparation, and reincubation was done in
the new medium in order to measure any time-course changes in the release of T₄. As controls, thyroid glands were incubated in the same amount of new medium without TSH. Concentrations of T₄ in the medium were determined after 0, 0.5, 1, 2, 3, 6 and 9 h after reincubation.

**Tissue volume**

After a 2-h preincubation, 1/2, 1, 2 and 4 thyroid glands per tube were incubated for 6 h in 1 ml of medium containing 30 μg of A1D. A control group was prepared without TSH.

**Dose-response**

The relationship between the amount of added TSH and released T₄ was also studied. In a range from 1.95 μg to 2 mg, A1D was applied to the experimental group, while the control group was incubated in medium without TSH.

**Hormone specificity**

The possibility was evaluated of employing the whole system of activity, thus far attained from various incubation conditions, as a bioassay system for avian TSH. Using A1D as standard TSH, its slightly refined fraction (AGS112D), bovine TSH, chicken FSH, HCG, and brain extracts from quails were employed as additional components of the incubation medium.

**Results**

**Preincubation**

When endocrine organs are incubated, hormones are sometimes released by non-specific forms of stimulation such as excision or changes in temperature. Therefore, the amount of released T₄ without specific stimulation by TSH was first examined. The T₄ concentrations, showing great variety in their measured amounts, intensely increased immediately after incubation, and dropped rapidly thereafter, although a certain level of T₄ was still retained (Fig. 1). This led to the adoption of a precautionary measure, to avoid errors in measurement arising from T₄ secretion without hormonal stimulation, of a 2-h perincubation in 1 ml of media.

**Time course**

The time-course change in T₄ concentration following release by TSH stimulation was determined. Two hours after resumption of incubation, a statistically significant difference (t test, p=0.05) in the T₄ concentration was observed between the TSH-stimulated and non-stimulated groups. As the precision increased in accordance with the length of the incubation time, thyroid glands were incubated for 6 h (Fig. 2).

The above experiment was conducted in early spring in Tokyo, and the change in the amount of TSH in the incubation medium easily affected the amount of T₄ released, whereas the amount of T₄ in the control group, around the turn of June, was greatly increased, and the difference between the TSH-stimulated and non-stimulated groups became rather obscure when incubated for over 6 h (Fig. 3).

**Tissue volume**

When organs and tissues are incubated, the activity is sometimes affected by their volume. T₄ secretion, therefore, was measured using different volumes of thyroid gland. As expected, T₄ secretion increased in linear proportion to the in-
crease in the tissue volume. Comparison between the TSH-stimulated and non-stimulated groups revealed a difference in the increase in proportion to the increase in tissue volume (Fig. 4). It was concluded that four thyroid glands per tube was most appropriate for the present study. However, in order to economize on thyroid glands and samples, the actual experiment was conducted using one thyroid gland in 250 µl of medium per tube, thus retaining the appropriate ratio.

Dose-response

A statistically significant difference (t test, p=0.05) from the control group was observed in the TSH-stimulated group with more than 7.8 µg of A1D. A linear log-dose-response relationship was recognized in the dose range between 7.8 and 500 µg of A1D (Fig. 5). Further addition of TSH, more than 500 µg, sometimes resulted in a decrease in the amount of released T₄.

Hormone specificity

Higher activity was observed with AGS112D than with A1D; HCG showed activity when applied in a large quantity. Highly purified chicken FSH and brain
extracts from quails showed no TSH activity (Fig. 6). The same procedure was used for the pituitary extracts from other types of birds. The addition of pituitary extracts from *Columba livia domestica*, *Melopsittacus undulatus* and *Coturnix coturnix japonica* produced the same result as when A1D was used.

**Discussion**

In this study it was definitely shown that avian TSH can be measured as an index of *in vitro* T4 release from avian thyroid glands. Thyroid glands of 5-week-old quails were dissected and cultured. Radical release of T4 was observed without TSH stimulation, which subsided after 30 min of incubation. Therefore, all the thyroid glands employed in the present study were subjected to a 2-h preincubation, which was sufficient to abolish any influence of early release of T4.

It was confirmed that the amount of T4 thus released by TSH stimulation depended on the amount of thyroid gland in the culture. A large difference in the amount of released T4 between the TSH-stimulated and non-stimulated groups increased the precision of the assay, but for economy of TSH and the number of thyroid glands, the amount of culture medium per tube was limited to 250 µl per thyroid gland lobe.
The released T₄ concentrations in the media containing TSH preparations after 2-h incubation showed a statistically significant difference (t test, p = 0.05) from the control group. A seasonal change in the in vitro activity of avian thyroid glands was noted. The amount of T₄ released after 6 h of incubation in the control group increased in summer, so that any difference between the two groups was virtually eliminated. However, in any season of the year, a 3-h incubation produced a significant difference (U test, p = 0.01) between the two groups. Thus the measurement of TSH activity was shown to be practical.

The release of T₄ from avian thyroid glands has been specifically ascribed to TSH\textsuperscript{[20–21]}, which is uniformly observed not only in avian species but also in mammals\textsuperscript{[21]}. Higher T₄-releasing activity was observed in AGS112D than in A1D. This is ascribable to the increased potency of TSH following purification of A1D into AGS112D. This new method showed a sharp response not only to chicken TSH, but also to bovine TSH. It indicated almost no activity, or only a slight one, if any, to brain extract, FSH or HCG. As far as this study was able to show, pituitary extracts from other birds showed the same degree of TSH activity as those pro-
duced by chicken pituitary glands, the dose-response curve appearing to be almost parallel.

With this new method, the dose-response curve regressed to a linear line between 7.8 and 500 µg of AID. This method enables detection of TSH even in one hundredth of a chicken pituitary gland. Further addition of TSH, more than 500 µg, sometimes resulted in a decrease in the amount of released T4. The errors resulting in such cases due to inversion of the released amount might be avoided by applying a parallelization test, enabling the procedure to be interpreted as a bioassay method. In the present study, the same hormone concentration was determined with five repeat assays. The data thus obtained indicated that the average intra-assay coefficient of variation was 55%, inter-assay coefficient of variation 59%, and the index of precision 0.19. These figures are relatively higher than those expected for RIA and RRA, but they would be sufficient for a bioassay method used for determining physiological hormone activity. For the purpose of improving the measurement method, the experimental animals were changed from 5-week-old quails to newly hatched chickens, but no significant change was observed.

These facts indicate that this new microbioassay method, specific to avian TSH determination, offers an extremely high sensitivity and that it is further applicable to avian species in general. An extremely small sample could be determined if TSH activity were to be measured in a fraction obtained from chromatographic purification of TSH.

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