tive of primary myocardial damage. These changes, may in part, be due to the accumulation of ganglioside in the heart. The abnormal but characteristic ganglioside pattern in heart muscle may be duplicated in striated muscle. If this is proven to be the case, then biopsied striated muscle can be used instead of brain biopsy for biochemical identification of certain ganglioside disorders.

*Summary.* The finding of ganglioside in heart is further evidence of the widespread distribution of these lipids. The increase of $GM_2$ ($G_5$) ganglioside in the heart of two patients with $GM_2$ gangliosidosis suggests that the metabolic defect is not limited to the nervous system.

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## Stimulation of Thyrotropin (TSH) Secretion by TSH-Releasing Factor (TRF) in Organ Cultures of Anterior Pituitary* (33566)

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Guillemin *et al.* (1) reported purification of a thyrotropin releasing factor from ovine hypothalami. Schally *et al.* (2, 3) isolated porcine TRF by procedures including gel filtration on Sephadex, phenol extraction, carboxymethylcellulose chromatography, countercurrent distribution, free-flow electrophoresis, and partition chromatography. These porcine preparations stimulated the release of TSH in codeine–thyroxine treated mice at a dose of 1 ng and depleted pituitary TSH in the same animals at a dose of 4 ng (2–5). These preparations also raised plasma TSH levels in rats (6) and stimulated TSH release *in vitro* (7).

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McKenzie (8) recently demonstrated that crude hypothalamic extracts caused increased incorporation of radioactive amino acids into TSH *in vitro*. We utilized our porcine TRF preparation, which was considered essentially homogeneous (2, 3), to undertake studies on the question of whether or not TRF can cause the stimulation of synthesis as well as release of TSH.

*Materials and Methods.* Tissue cultures of male rat anterior pituitaries were carried out by the method of Nicoll and Meites (9) with slight modifications.

Adult male rats of the Sprague-Dawley strain (Cheek-Jones Co., Houston, Texas) were used as donors for cultured tissue. The animals were decapitated and each posterior pituitary was removed and discarded. The anterior pituitary was removed and cut into

TABLE I. Effects of TRF on TSH Contents of Media and Tissues.

| Expt. no. | No. of pit. halves | Dose of TRF (pg) | TSH released, in terms of standard[a] Mean (mU) | 95% Fiducial limits | Pituitary TSH[a] Mean (mU) | 95% Fiducial limits | Total TSH (mU) in pit. and medium |
|---|---|---|---|---|---|---|---|
| 1 | 4 | — | 144 | 94–270 | 188 | 133–261 | 332 |
| | 4 | 40 | 224 | 168–298[b] | 326 | 188–573[b] | 550 |
| 2 | 3 | — | 40 | 33– 48 | 128 | 65–252 | 168 |
| | 3 | 40 | 120 | 103–165 | 222 | 122–404[b] | 242 |
| 3 | 4 | — | 28 | 23– 33 | 43 | 32– 59 | 71 |
| | 4 | 40 | 91 | 76–103 | 110 | 85–144 | 201 |

[a] Assay in terms of the USP reference standard.

[b] Although the 95% fiducial limits for TSH contents overlapped in three groups when they were calculated against the TSH standard, a direct comparison of the TSH levels, using the levels in the controls as 1.00, showed that the potencies of the experimental group were significantly above the control in each case.

six to eight explants, of approximately equal size, with a scalpel.

The cultures were performed in 3.5 × 1 cm sterile disposable plastic petri dishes (Falcon Plastics, Inc.) each containing 3 ml of Trowell's T8 medium (Microbiological Associates, Bethesda, Maryland), used in Expt. 1, or medium 199 (Difco, Detroit, Michigan), used in Expts. 2 and 3. In each dish, explants from 3 or 4 pituitary halves from different rats were placed upon a strip of washed lens paper which was supported on a stainless steel mesh platform. An atmosphere of 95% $O_2$–50% $CO_2$ and a temperature of 36° were maintained. After a 5- (Expt. 2) or 6-day preculture period, the medium was replaced with fresh medium and the culture was continued for another 24–27 hr.

Each addition of TRF was 40 pg (dry wt.) of AVS 11-48 no. 6-9 in 50 μl of isotonic saline. Thyroxine was added as 3 μg dissolved in 2 μl of 0.1 *M* NaOH to make a final concentration of 1 μg/ml. Puromycin dihydrochloride (Sigma) was neutralized with NaOH and added, dissolved in culture medium to make a concentration of 0.0002 *M*. Puromycin, thyroxine, and TRF were added in that order at 1-hr intervals.

Experiments were terminated 24 hr after the addition of TRF and media and tissues were frozen until assay. In the first two experiments reported, TRF was added to control media after termination of culture but before assay. This "compensation" procedure was designed to cancel out any possible direct effect of TRF in the TSH assay (7). The viability of certain of the tissue samples used in this study was confirmed by histological examination at the end of the experiment.

The TSH was assayed by the method of McKenzie (10) using the USP thyrotropin reference standard and 5 assay mice/point. When preparations were assayed at two dose levels, mean potency and 95% confidence limits were calculated by factorial analyses (11). Some results were read off dose–response curves and presented as mean mU ± SE; levels of significance between groups were then determined using the Student's *t* test.

*Results.* Data presented in Table I show that three or four control pituitary halves released 144 (94–270), 40 (33–48), or 28 (23–33) mU of TSH during the last day of culture. These values are approximately 40% of the average release per day during the first 5 or 6 days. Control tissues at the end of the experiments still contained appreciable amounts of TSH.

The stimulatory effect of TRF is also shown in Table I. Opposite halves from the same sets of 3 or 4 rat pituitaries provided matched tissue samples for each experiment. Addition of 40 pg of TRF consistently re-

TABLE II. Effects of Thyroxine and Puromycin on TSH Secretion.

| No. of pit. halves | Addition | TSH contents (mU of TSH ± SE) Medium | Tissue |
|---|---|---|---|
| Expt. 2 | | | |
| 3 | — | 42 ± 3 | 166 ± 41 |
| 3 | Thyroxine (1 μg/ml) | 16 ± 4[a] | 165 ± 19 |
| 3 | Thyroxine + TRF (40 pg) | 15 ± 2[a] | 236 ± 20[b] |
| 3 | Puromycin (0.0002 *M*) | 24 ± 2[a] | 151 ± 35 |
| 3 | Puromycin + TRF | 30 ± 3[a] | 88 ± 18 |
| 3 | Puromycin + Thyroxine + TRF | 14 ± 2[a] | 135 ± 13 |
| Expt. 3 | | | |
| 4 | — | 42 ± 5 | 43 ± 3 |
| 4 | Thyroxine | 16 ± 1[a] | 62 ± 6 |
| 4 | Thyroxine + TRF | 20 ± 3[a] | 108 ± 26[b] |
| 4 | Puromycin | 25 ± 3[a] | 58 ± 22 |
| 4 | Puromycin + TRF | 15 ± 1[a] | 56 ± 6 |

[a] Medium samples significantly lower in TSH activity than controls ($p < .025$).

[b] Tissue samples significantly greater than the controls and also to tissues subjected to thyroxine alone, when probabilities from the two sets of data are combined ($p < .01$).

sulted in increased TSH contents in both medium and tissue samples 24 hr later. The data are presented in terms of the USP reference standard with 95% confidence limits. The mean total increases in medium and tissue TSH activity produced by 40 pg of TRF were 218, 174, and 130 mU, or around 4 mU/pg of TRF.

Some effects of thyroxine and puromycin on TSH secretion are shown in Table II. Prior addition of thyroxine (1 μg/ml) resulted in a reduced TSH release and prevented any significant effect of TRF on TSH release. However, increased TSH contents were observed in tissues exposed to both TRF and thyroxine. No significant effect of TRF was observed when it was added after puromycin, under the conditions employed here.

*Discussion.* During the last day of a 6- or 7-day period, control tissues released 72 (47–135), 27 (22–32), and 14 (11–17) mU of TSH/two pituitary halves in three respective experiments. These figures are surprisingly high in view of the results of Bakke and Lawrence (12) who estimated the normal secretion rate in rats as 16.8 mU/day.

The total increases in medium and tissue TSH activity were around 4 mU of TSH/pg of TRF added. If we assume that pure TSH has an activity of 60 units/mg (13), then this increase is 0.7 μg of TSH/pg of TRF, or a "multiplier effect" of about 70,000. Since the molecular weight of TRF is approximately 2000, or one tenth that of TSH, these results may be interpreted as indicating that around 7000 molecules of TSH are produced for each molecule of TRF, under these experimental conditions.

The "multiplier effect" observed in this work is further circumstantial evidence for the hormonal nature of this substance (3). Furthermore, we feel that this probably reflects processes in the intact animal.

The work reported here is consistent with preliminary data from this laboratory (3) indicating that increased incorporation of labeled amino acids into the pituitary occurs within 4 hr after the addition of TRF. Since essentially pure TRF was used in this and in previous (2–7) work, which showed stimulation of release of TSH, these results suggest that one substance is responsible for both release and synthesis of TSH. These results also support the conclusions of Sinha and Meites (14) and of McKenzie (8) that the hypothalamus stimulates the production of TSH. However, unequivocal differentiation between emptying of the pituitary cells as a stimulus for synthesis and a direct effect of TRF on synthesis is not yet possible.

Previous reports by Guillemin *et al.* (15), Sinha and Meites (16), Bowers *et al.* (17), Schally and Redding (7), Vale *et al.* (18), Wilber and Utiger (19) and others were confirmed when thyroxine (1 μg/ml) inhibited TSH release and blocked the stimulatory effect of 40 pg of TRF on release in 2 experiments. However, an unanticipated effect was observed in 2 experiments when TRF seemed to increase TSH activity in the tissue in the presence of thyroxine. A dissociation between effects on synthesis and on release is suggested by these results.

In the work presented here, puromycin added 1 hr before thyroxine, which in turn was added 1 hr before TRF, depressed secretion of TSH and blocked the action of TRF. This is in contrast to previous work by Bowers *et al.* (20) which indicated that puromycin, an inhibitor of protein synthesis, blocks the inhibitory effects of thyroxine without preventing the response to TRF using systems *in vivo* and acutely *in vitro*.

*Summary.* Effects of pure TRF on male rat pituitary tissue were studied using standard organ culture techniques and chemically defined media. Addition of 40 pg of TRF caused increased TSH activity in both media and tissues, in comparison with controls. Under the conditions used, TRF seemed to stimulate the production of about 4 mU of TSH activity/pg of TRF used. This suggests that TRF can induce both the release and synthesis of TSH.

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