of hypothalamic PIF, and presumably thereby depress pituitary prolactin secretion.

Addendum. In agreement with the data presented here, we (Clemens, J. A., Meites, J., *The Physiologist,* 1967, v10, 144) found that implantation of prolactin into the *median eminence* of intact and castrate female rats reduced significantly pituitary weight and pituitary prolactin concentration, and increased hypothalamic PIF content. Profound involution of the mammary glands was also observed.

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In vitro Studies with Thyrotropin Releasing Factor.* (32436)

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The presence of a thyrotropic hormone (TSH)-releasing factor (TRF) in hypothalamic extracts of several species has now been well established(1-7). The isolation in our laboratory of what we think is essentially pure thyrotropin releasing factor(8) provided material for various *in vivo* and *in vitro* investigations. *In vivo* studies have shown that 1 nanog of porcine TRF will stimulate the release of TSH in codeine-thyroxine treated mice and that a dose of 4 nanog will deplete pituitary TSH content in the same animals (8-11). TRF has no effect in hypophysectomized mice(10). The present report describes *in vitro* studies carried out with porcine TRF and the effect of thyroxine, triiodothyronine, Actinomycin D as well as α- and β-melanocyte-stimulating hormone (MSH) on TSH release *in vitro* from isolated rat pituitaries in response to TRF. Preliminary reports of some of this work were briefly presented at recent meetings(12,13).

Materials and methods. Highly purified preparations of TRF were obtained from lyophilized defatted pig hypothalami by extraction with 2 N acetic acid, followed by heating and lyophilization, reextraction with glacial acetic acid and gel filtration on Sephadex(8). Further purification was effected by phenol extraction, chromatography on carboxymethylcellulose (CMC), countercurrent distribution (CCD) and free flow electrophoresis (FFE). The final purification step consisted of partition chromatography on Sephadex(8) and the TRF thus obtained was homogeneous in 6 thin-layer chromatography and electrophoresis systems(8,11). The doses of TRF are given in terms of dry weight.

Actinomycin D (Act D, Merck and Co.) was administered to rats in doses of 200 μg/100 g body weight, 3 hours before removal of pituitaries or added to preincubation and incubation media at a concentration of 1-10 μg/ml. The sodium salts of triiodothyronine (T_3) and thyroxine (T_4) were dissolved in 0.1 N sodium hydroxide, diluted with saline and added to preincubation and incubation media

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at a concentration of 1 μg/ml. Pure porcine α- and β-MSH prepared as described previously(14) were also added to preincubation and incubation media at a concentration of 13 μg/ml.

Pituitary incubation and detection of TRF activity *in vitro* was carried out by the method of Saffran and Schally(15) with minor modifications(3). Male rats of Sprague-Dawley strain, 200 g body weight, served as pituitary donors in all experiments. After removal each pituitary was cut in half and transferred to a 15 ml beaker containing 1.5 ml Krebs-Ringer bicarbonate medium with 200 mg% glucose. One or two pituitary halves were placed in each of 2 beakers, one serving as a control and the other with an equal number of pituitary halves from the same animals, as experimental. After a preincubation period of 30-60 minutes, the preincubation media were discarded and replaced with fresh medium(16). Depending on experimental requirements, various test materials, but not TRF, were added at this stage and a second preincubation period of 30-60 minutes followed. The medium was again discarded, fresh medium, TRF and other materials as required were added and the pituitaries were incubated for 1 hour. After incubation of the pituitary halves, and before the TSH assay, the same materials which had previously been added to "experimental pituitaries" were added to "control pituitaries". This "compensation procedure" cancelled out any possible direct effect of TRF and other materials in the TSH assay(3).

The TSH released by the pituitaries was assayed by the (3 point) McKenzie assay(17) and degree of stimulation was assessed by comparing the amount of TSH released (recorded as mean CPM) by the stimulated and unstimulated pituitaries(3). Two to 4 doses of USP reference standard were included in each assay and there were 5-6 animals in each group. Statistical analyses were carried out as described previously (3,10) and the probabilities were calculated on the basis of Wilcoxon's rank sum test. In some cases the differences were further analyzed by the more exacting Student's t test.

Results. Previous results showed that porcine TRF purified by Sephadex gel filtration was active *in vitro* at doses of 100 μg(3). After repurification by phenol extraction and CMC chromatography the material showed TRF activity at levels of 0.1-1 μg(11). Subsequent concentration by CCD and FFE produced a material which caused a significant increase in TSH release *in vitro* at doses of

TABLE I. Effect of TRF Purified by FFE on Release of TSH from Rat Pituitaries *in vitro*.

Dose TRF added to experimental nanog	TSH assay, change in blood ^{131}I (cpm) at 2 hr ± S.E. Control	Exp	Mean Δ cpm	p
.1	35 ± 5	93 ± 12	49	.01
.1	27 ± 9	67 ± 9		.01
.3	30 ± 9	78 ± 18	48	.01
1	17 ±10	95 ± 16	91	.01
1	27 ± 4	131 ± 8		.01
3	−8 ± 6	111 ± 19	139	.01
3	58 ±19	218 ± 51		.01

TABLE II. Effect of 10 Picrograms of Porcine TRF on Release of TSH from Rat Pituitaries *in vitro*.

Dose TRF added to experimental nanog	TSH assay, change in blood ^{131}I (cpm) at 2 hr ± S.E. Control	Exp	Mean Δ cpm	p
.01	201 ± 31	335 ± 37	134	.01
.01	−8 ± 10	84 ± 22	92	.01
.01	−3 ± 12	47 ± 11	44	.05
.01	1 ± 16	31 ± 11	30	.05

TABLE III. Effect of Porcine TRF on Release of TSH from Rat Pituitaries *in vitro*.

Dose TRF added to experimental nanog	TSH assay, change in blood ^{131}I (cpm) at 2 hr ± S.E. Control	Exp	Mean Δ cpm	p
.03	−18 ± 13	70 ± 9	77	.01
.03	− 5 ± 9	61 ± 9		.01
.09	98 ± 28	178 ± 17	82	.01
.09	−23 ± 21	62 ± 19		.05
.27	130 ± 15	456 ± 98	340	.01
.27	142 ± 15	457 ± 116		.01
.27	−11 ± 8	369 ± 124		.01
.54	65 ± 14	741 ± 163	653	.01
.54	47 ± 32	727 ± 154		.01
.54	4 ± 34	653 ± 117		.01
.54	60 ± 25	668 ± 117		.01
1.08	20 ± 20	1390 ± 198	1174	.01
1.08	−43 ± 21	944 ± 239		.01

TABLE IV. Lack of Effect of Actinomycin D on Stimulation of TSH Release *in vitro* After TRF.

Treatment	Dose TRF added to experimental nanog	TSH assay, change in blood ^{131}I (cpm) at 2 hr ± S.E. Control	Experimental	Mean Δ cpm	p
None	5	124 ± 46	702 ± 69	578	.01
Act D *in vivo**	5	29 ± 10	314 ± 70	244	.01
" " *	5	41 ± 22	388 ± 90	347	.01
Act D *in vivo,** *in vitro*†	5	82 ± 26	537 ± 158	455	.01
Act D *in vitro*†	5	−24 ± 22	416 ± 101	440	.01
" " †	5	− 5 ± 8	295 ± 84	300	.01
None	.5	65 ± 14	741 ± 163	676	.01
"	.5	47 ± 32	727 ± 154	680	.01
Act D *in vitro*‡	.5	862 ±206	1566 ± 150	704	.05
" " ‡	.5	54 ± 10	812 ± 157	758	.01

* 500 μg Act D/animal/3 hr. † 1 μg Act D/ml medium. ‡ 10 μg Act D/ml medium.

TABLE V. Inhibition by Triiodothyronine (T_3) and by Thyroxine (T_4) of the *in vitro* Stimulation of TSH Release Induced by TRF.

Additions	Dose TRF added to experimental nanog	TSH assay, change in blood ^{131}I (cpm) at 2 hr ± S.E. Control	Experimental	Mean Δ cpm	p
None	.5	100 ± 55	818 ± 92	718	.01
"	.5	60 ± 25	668 ± 117	608	.01
1 μg T_3	.5	144 ± 27	184 ± 54	41	NS
"	.5	162 ± 23	155 ± 38	−7	NS
None	.5	−43 ± 21	944 ± 239	987	.01
"	.5	4 ± 34	653 ± 93	649	.01
1 μg T_4	.5	72 ± 36	84 ± 117	12	NS
"	.5	−31 ± 28	8 ± 30	−39	NS

0.1 nanog (Table I). This table also shows that when larger amounts of TRF were added more TSH was released.

After the final purification step by partition chromatography, TRF elicited a small but statistically significant stimulation of TSH release *in vitro* at doses of 0.01 nanog in 4 different experiments. The results of these experiments are shown in Table II. Table III shows that the amount of TSH released into the medium was related to the doses of added TRF, and that by increasing the doses of TRF greater stimulation of TSH release was effected. The dose response curve to TRF appeared to be sigmoidal. Doses of 0.03-0.09 nanog seemed to lie on the lower portion of the curve. TSH release appeared to be related to log-dose of TRF over the range of 0.27-1.08 nanog. This middle portion of the curve is approximately linear and a further increase in doses of TRF does not result in greater responses.

Table IV shows that pretreatment of pituitary donor animals with Act D *in vivo* or addition of Act D to incubation media in doses of 1-10 μg/ml or the combined *in vivo* and *in vitro* treatments do not block the release of TSH which follows the addition of TRF.

The results shown in Table V indicate that addition of 1 μg T_3 or T_4/ml to preincubation and incubation media completely blocked the stimulation of release of TSH regularly seen after 0.5 nanog TRF. Since Act D has been shown not to interfere with the stimulation of TSH release *in vitro* (Table IV) and *in vivo* (18) but inhibits the effect of T_4(19), it was of interest to determine what would happen if these two substances were added together. Table 6 shows that the inhibition by T_3 or T_4

TABLE VI. Reversal by Actinomycin D of the T_3 or T_4 Inhibition of the Stimulatory Effect of TRF on TSH Release *in vitro*.

Additions	Dose TRF added to experimental nanog	TSH assay, change in blood ^{131}I (cpm) at 2 hr ± S.E. Control	Experimental	Mean Δ cpm	p
1 μg T_4	.5	−31 ± 28	8 ± 30	39	NS
"	.5	72 ± 36	84 ± 117	12	NS
1 μg T_4 + 1 μg Act D	.5	−25 ± 30	649 ± 106	674	.01
Idem	.5	29 ± 22	695 ± 120	665	.01
"	.5	−8 ± 12	495 ± 141	503	.01
1 μg T_3	.5	106 ± 51	166 ± 36	60	NS
"	.5	92 ± 17	118 ± 24	26	NS
1 μg T_3 + 1 μg Act D	.5	30 ± 13	805 ± 34	775	.01
Idem	.5	18 ± 20	1380 ± 199	1362	.01
"	.5	108 ± 16	941 ± 83	833	.01

of the stimulation of TSH release after 0.5 nanog TRF is reversed by preincubation of the pituitaries with 1 μg/ml Act D.

α- and β-MSH have been shown to stimulate thyroid activity in mice, rabbits and guinea pigs(20-22). It was considered to be of interest to determine whether α- and β-MSH could also reverse the inhibitory effect of T_4 on the release of TSH *in vitro* induced by TRF. Table VII shows that α- or β-MSH did not stimulate the release of TSH *in vitro*. Addition of T_4 as in experiments shown in Table V inhibited the response to TRF and the presence of α- and β-MSH in the medium did not reverse this inhibition.

Discussion. The results reported here indicate in an unequivocal fashion that TRF acts directly on the adenohypophysial tissue. Amounts as small as 10 picog TRF can significantly stimulate the release of TSH. By increasing the doses of TRF, greater stimulation of TSH release is obtained. Simple calculations show that if we assume the potency of pure thyrotropin to be 60 U/mg (23), then TRF induced the stimulation of 200-2000 times its own weight of thyrotropin. Such a significant "multiplier effect" would strongly support the concept of hormonal action of TRF.

Purified growth hormone-releasing factor (GRF), luteinizing hormone releasing factor (LRF) and follicle-stimulating hormone-releasing factor do not stimulate TSH release *in vivo* or *in vitro*(5,11). Schreiber was the first to use the *in vitro* system of Saffran and Schally(15) for detection of stimulation of TSH release *in vitro*(24). However, his criteria for TSH release were very indirect and based on activation of hypophysial phosphatases. Solomon and McKenzie(16) and Sinha and Meites(25) used the pituitary incubation technique(15) for measuring TRF activity of crude rat hypothalamic extracts and Guillemin *et al*(26) and our laboratory

TABLE VII. Effect of α- and β-MSH and T_4 on the Release of TSH *in vitro* After TRF.

Additions	Dose TRF added to experimental nanog	TSH assay, change in blood ^{131}I (cpm) at 2 hr ± S.E. Control	Experimental	Mean Δ cpm	p
α-MSH	—	352 ± 136	293 ± 49	−59	NS
β-MSH	—	669 ± 267	581 ± 198	−88	NS
T_4	.5	48 ± 73	95 ± 64	47	NS
None	.5	133 ± 73	585 ± 34	452	.01
α-MSH + T_4	.5	371 ± 150	299 ± 124	−72	NS
" "	.5	611 ± 143	632 ± 108	21	NS
β-MSH + T_4	.5	791 ± 215	784 ± 104	−7	NS
" "	.5	311 ± 41	244 ± 35	−67	NS

(27) utilized it for partially purified TRF preparations from various species. However, the TRF preparations used in studies reported here are 10,000-100,000 times more active than those reported previously(26,27). The effect of TRF on pituitary TSH release can be contrasted with the action of α- and β-MSH, which stimulate the thyroid (20-22), presumably by a direct action, since they are active in hypophysectomized animals(20) and have no effect on release of TSH from rat pituitaries *in vitro*(26,27). Cehovic(22) suggested that α- and β-MSH control at the level of pituitary tissue the entry of the thyroid hormones, an effect which would regulate the secretion of TSH. Our results do not conclusively disprove this theory, but the lack of suppression by α- and β-MSH of the inhibitory effects of T_4 on the stimulation of TSH release after TRF, combined with an effect of MSH in hypophysectomised animals(20) strongly suggests that MSH acts directly on the thyroid gland.

It has been previously reported that addition of T_4 to incubation media blocks the stimulation of TSH release *in vitro* after the addition of crude rat hypothalamic extracts(25) or of purified ovine TRF preparations(26). Others have reported that T_4 blocks the *in vivo* responses to TRF(5-7,11, 18). This is in agreement with the reports on the inhibitory action of T_4 on TSH release *in vivo* reported by various investigators(5-7,11,25,28). The inhibitory effect of T_4 *in vivo* is dose dependent since the stimulation of release of TSH can be reinitiated by increasing the doses of TRF(11,18,29).

The inability of Act D to block the release of TSH *in vitro* (this paper) and *in vivo*(18) seems to suggest that in spite of supression of DNA dependent formation of messenger RNA there is still enough RNA left to permit protein synthesis, or that the stimulation of release of TSH does not require *de novo* synthesis of TSH protein and that TRF stimulates the release and not the synthesis of TSH.

Tata(19) and others found that Act D blocks the biological effects of T_3 and T_4 *in vivo*. It was interesting to find, in agreement with *in vivo* data(18), that Act D prevents the thyroxine inhibition of the TRF induced stimulation of TSH release *in vitro*. The mechanism of this action is not clear. Although a direct interaction of Act D and thyroid hormones cannot be excluded(30) it is more likely that T_3 and T_4 induce in the pituitary the formation of substances, possibly protein or nucleic acid, which inhibit the action of TRF on the release of TSH and that Act D suppresses the formation of these substances (18).

Summary. Porcine TRF stimulates the release of TSH from rat anterior pituitaries *in vitro* at doses as small as 0.01 nanog. By increasing the doses of TRF, greater amounts of TSH are released into the incubation media. The pituitary response to TRF is inhibited by small amounts of T_3 and T_4. Act D does not abolish the response to TRF, indicating that *de novo* synthesis of TSH is not required for TRF to exert its effect. α- and β-MSH do not stimulate the release of TSH *in vitro* and did not reverse the inhibitory effect of T_4 on TSH release *in vitro*. Preincubation with Act D reverses the inhibition of TSH release induced by T_3 and T_4. This may indicate that Act D interferes with the formation of inhibitory substances, induced by T_3 and T_4, which suppress the release of TSH after TRF.

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