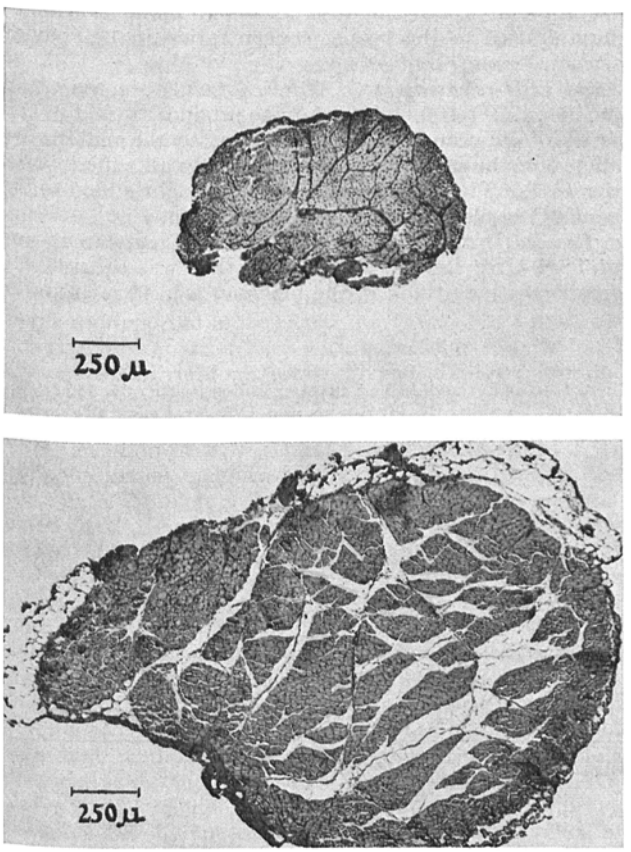
Discussion. Wasting of the MLA after castration can be prevented by testosterone[3] and a series of papers have shown that androgens, in addition to their responsibility for development and maintenance of the male accessory sex organs, stimulate an increase in body weight and growth[4]. Indeed the increase in weight of the MLA of castrated male animals after testosterone has been suggested and widely used as a biological indicator of the myotropic activity of androgens[5]. However, the use of the MLA as a test for anabolic activity in castrated male animals has been criticized[6] and the lack of a standard method which would allow determination or comparison of the relations of myotropic and androgenic effects of anabolic steroids, has made therapeutical applications difficult[7]. The persistence of the MLA in female rats has the character of a qualitative finding and may help to find new methods of evaluation of androgenic and myotrophic actions of anabolic steroids.



Cross section of the largest part of MLA in a female rat 1 and 2 months after birth. During this time testosterone propionate 1 mg was injected s.c. twice weekly.

Zusammenfassung. Testosteronapplikation bei neugeborenen weiblichen Ratten (1 mg Testosteron propionat zweimal wöchentlich s.c.) führt zur Erhaltung des M. levator ani, der normalerweise sich bei beiden Geschlechtern gleichartig entwickelt, bei fortlaufender Reduktion vom 18. Tag der Embryonalentwicklung an. Die Möglichkeit der Erhaltung des Muskels als Test für die Wirkung anaboler Steroide wird diskutiert.

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Presence of Calcium Ions as a Requisite for the in vitro Stimulation of TSH-Release by Hypothalamic TRF

We have recently undertaken a series of experiments to explore the mechanisms of action of the hypothalamic factor TRF (TSH-releasing factor) when it stimulates acutely the secretion of adenohypophysial TSH (thyroid-stimulating hormone). Thus, we have already observed that neither cycloheximide nor actinomycin-D prevent the in vitro release of TSH induced by TRF, in conditions in which we could demonstrate the efficacy of the antibiotics to inhibit protein and RNA synthesis[1,2]. In vivo we have shown[3] that TRF injected i.v. stimulates release of TSH in an extremely rapid manner, evidence for increasing plasma TSH concentration being observed less than 120 sec following injection of the hypothalamic material. These results have led us to several working hypotheses considering a possible effect of TRF on the cellular membrane potential in its stimulating release of TSH. One of the corollaries of these hypotheses would be that alteration of the K^+, Ca^{++} and Mg^{++} concentrations of the incubation milieu should modify the activity (release of TSH) of the mediator (TRF).

The results presented here will show that indeed the effect of TRF in stimulating the release of TSH when added to pituitary tissues incubated in vitro[1,4] can be completely prevented by prior incubation of the pituitary in Ca^{++}-free medium. Experiments in which Ca^{++} is re-added to the medium show further that the responsiveness to TRF is restored when Ca^{++} is re-introduced in the incubation fluid.

Materials and methods. (1) *Incubation of the pituitary glands.* The anterior pituitary gland of rats (males, body

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weight 100–110 g) is rapidly removed after decapitation and split at the isthmus in 2 identical halves. Each half goes to 1 of 2 Pyrex 20 ml beakers containing 2.0 ml of incubation fluid equilibrated with 95% O_2–5% CO_2; 3 pituitaries are similarly prepared. Thus, 2 populations of pituitary halves are obtained; they are identical in origin and practically of identical weight, as confirmed at the end of each incubation. We have shown earlier[4,5] that this design is the only one which so far has been demonstrated to allow a valid comparison between control and experimental tissues regarding secretion of pituitary hormones in the short-term incubation system. The incubations are run in a Dubnoff metabolic shaker at 100 strokes/min, 37°5C, in a water-saturated atmosphere of 95% O_2–5% CO_2. The duration of the various stages of incubation varied within the series of experiments and will be indicated for each experiment. When collected for TSH assay at the different times selected for each experiment, the incubation fluids were stored in polypropylene tubes at – 20 °C.

(2) *Incubation fluids.* The basic 'control' incubation fluid is the solution known as Krebs-Ringer bicarbonate glucose, described in UMBREIT et al.[6]. When the molarity of one of the ions was varied from this control medium, isotonicity with the control medium was maintained by altering the solution's molarity of NaCl.

(3) *Bioassay for TSH and statistical calculations.* The method used was that of MCKENZIE as practiced in this laboratory[7]; calculations for statistical analysis as in [7], all fluids being assayed for TSH concentration in complete 4-point assays. For the TSH assays all incubation fluids were diluted to the proper concentration in 1% bovine serum albumin.

(4) *TSH-releasing factor, TRF.* A single preparation of purified TRF (100 U/mg) of ovine origin was used in this series of experiments. It corresponds to the material of stage 3 of the sequence described in [8]. In all experiments, the dose of TRF used was 2.0 µg dry weight added to the incubation system in 0.1 ml 0.9% NaCl.

Results. In this series of experiments, the minimal dose of TSH USP Reference Standard that was consistently significantly detected in the bioassay was 40 microunits,

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Time in min	Treatments N	T	TSH released T/N	TSH released, mU/incubation N	T
Protocol No. 8009					
0	KR	KR			
10	KR	O Ca^{++}	1.09 (0.73–1.71)	0.96	0.80
30	KR	O Ca^{++}			
50	KR + TRF	O Ca^{++} + TRF	0.52 (0.27–0.83)	2.10	0.83
70 end					
Protocol No. 8017					
0	KR	O Ca^{++}			
10	KR	O Ca^{++}			
30	KR	O Ca^{++}			
50	KR	O Ca^{++}			
70	KR + TRF	O Ca^{++} + TRF	0.36 (0.17–0.59)	1.96	0.60
90 end					
Protocol No. 8037					
0	KR	KR			
15	KR	O Ca^{++}	3		
35	KR	O Ca^{++}			
75	KR + TRF	O Ca^{++} + TRF	0.45 (0.24–0.63)	3.12	1.2
95	KR	KR			
125	KR	KR + TRF		< 0.6	2.20
					2.0 (1.36–4.27)[a]
145 end					
Protocol No. 8040					
0	KR	O Ca^{++}	with changes every 10 min between 0 and 60 min		
60	KR + TRF	O Ca^{++} + TRF	0.55 (0.28–0.88)	1.4	< 0.6
80	KR	KR			
100		KR + TRF		–	1.90
		–			2.55 (1.75–5.1)[a]
120 end					

N, T, normal control, treated experimental pituitary, respectively. KR, regular Krebs-Ringer bicarbonate glucose medium. O Ca^{++}, incubation medium free of Ca^{++}. Treatments, indicates the various additions or fluids added at the time indicated. TSH released, corresponds to the amount of TSH measured in the incubation fluids *at the end* of the incubation period starting at the time shown in the same horizontal row. [a], the ratios reported here with their true confidence limits represent the ratios of the amount of TSH released by the same pituitaries in response to TRF in presence of Ca^{++} to that observed in Ca^{++}-free medium.

with a linear response up to 0.2 milliunits. In the experiments reported here, various dilutions of the experimental fluids were used depending on the amounts of expected TSH released in the fluids, this having been appreciated usually in preliminary experiments. As shown in the Table, the results are expressed as ratios of the amounts of TSH released by the experimental pituitaries (T, treatment) over that released by the control pituitaries (N, normal). These ratios are obtained by complete 4-point assays between T and N fluids; thus true confidence limits can be calculated for these ratios. As an indication of the amounts of TSH involved in these experiments, *approximate* values (milliunits of TSH) released during the incubation are also reported from calculations in 3-point assays.

Duration of incubations and times of addition of treatments are reported in details in the Table. The 4 multiple experiments reported here with somewhat different designs gave confirmatory results. From the first experiment (protocol No. 8009) it can be seen that incubation in Ca^{++}-free medium did not modify, per se, secretion of TSH; addition of TRF stimulated acute release of TSH from the tissues incubated in Krebs-Ringer, whereas the effect was completely prevented in the pituitaries incubated in Ca^{++}-free medium. The results obtained from the second experiment (protocol No. 8017) with a similar design, were identical. In the third and fourth experiments (protocol No. 8037 and No. 8040), with closely related designs, the response to TRF usually observed was obtained in the control group, whereas it was again greatly diminished in absence of Ca^{++}. The pituitaries were then re-incubated in regular Krebs-Ringer; when TRF was added to the tissue previously incubated in Ca^{++}-free medium, TSH-release was stimulated, thus demonstrating that the prior altered response to TRF was not due to irreversible alterations to the pituitary tissue caused by the prolonged incubation in absence of Ca^{++}.

Discussion. It would appear from the results presented here that Ca^{++} plays an important and necessary role in the intimate mechanisms whereby hypothalamic TRF stimulates acutely the release of TSH. Similar results in the past have been interpreted as involving Ca^{++} in the 'stimulus-secretion coupling'[9,10] for electrical stimulation, high K environment, locally elevated concentrations of acetylcholine and the secretory activity of the tissues to which these various electrical or chemical informations were applied[9-11]. The results reported here are compatible with the concept that the same hypothesis may apply to the adenohypophysial tissue and a specific mediator of its secretion, one of the hypothalamic releasing factors. A possible role of Ca^{++} as implicated in the secretion of the adenohypophysis had been anticipated some years ago by DOUGLAS and POISNER[12,13].

Résumé. L'incubation in vitro de fragments d'hypophyse (rat) dans le liquide de Krebs-Ringer sans calcium (Ca^{++}) inhibe l'action du facteur hypothalamique TRF (TSH-releasing factor) qui normalement stimule la sécrétion de l'hormone thyréotrope (TSH). La ré-introduction de l'ion Ca^{++} (2.54 m*M*) rétablit la réponse (sécrétion de TSH) au facteur TRF.

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Potassium-Induced Stimulation of Thyrotropin Release in vitro. Requirement for Presence of Calcium and Inhibition by Thyroxine

We have shown in a previous note that calcium ions (Ca^{++}) are necessary for the hypothalamic hormone TRF (thyrotropin-releasing factor) to stimulate in vitro the secretion of thyrotropin (TSH)[1]. With availability of the above results and in keeping with the hypothesis presented in [1] that led us to investigate the possible requirement for Ca^{++} on the action of TRF, we decided to study the effects of elevating potassium (K^+) content in the fluid of the in vitro pituitary incubation, on TSH secretion. The results presented below will demonstrate that K^+ can stimulate the in vitro secretion of TSH, that this effect, like that of TRF, requires the presence of Ca^{++} and also that it can be inhibited by pre-incubation with thyroxine.

Materials and methods. The methods for incubation of the pituitaries, for the bioassays of TSH and their statistical analysis, for the characterization of the TRF used here, have all been described in detail in a previous note[1]. The solutions of L-thyroxine Na (Calbiochem. Los Angeles, California) used here were prepared freshly in alkalinized 0.9% NaCl for each experiment, at a concentration of 27.0 μg/ml. At the end of the incubation period, fluids were diluted with the proper medium so that the same concentrations of electrolytes were achieved in the fluids from controls and treated tissues to be injected in the animals used in the TSH assay.

Results. (1) *Effects of high K^+ on TSH-release.* Several experiments were conducted with various designs as reported in the Table. All studies show that when the K^+ molarity of the incubation fluid is raised to 25 meq/l or above (protocol Nos. 7973, 7990a, 7998, 8029, 8002a), release of TSH takes place.

(2) *Effects of high K^+ on TSH-release in absence of Ca^{++}.* The release of TSH induced by elevated K^+ (25 meq/l) is completely abolished when Ca^{++} is omitted from the incubation fluid (protocol Nos. 8017, 8026). The response

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