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Mechanism of Action of TSH Studied by Organ Culture of Fetal Rat Thyroid Gland.* (31654)

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Using the organ culture technique, we reported recently(1) that addition of TSH§ to the culture medium greatly improved ^{131}I utilization by embryonic thyroid explants obtained from 21-day rat fetuses. Total ^{131}I content as well as incorporation of the isotope into protein-bound iodotyrosines and thyroxine were higher in the presence of TSH; the most striking response observed being the increase in incorporation of ^{131}I into thyroxine. We reported earlier(2) a similar effect of TSH on organ culture of thyroid glands removed from younger fetuses.

It was of interest to determine, under similar conditions, whether TSH stimulation of thyroid hormone production is dependent upon synthesis of proteins. It is shown here that addition of TSH to the medium failed to influence the ^{14}C incorporation of [^{14}C] leucine into protein and that puromycin, at a concentration that inhibited protein formation, failed to abolish the TSH-induced stimulation of ^{131}I incorporation into protein-bound iodotyrosines and thyroxine.

In order to explore the mechanism of action of TSH, we have made use of Tapazole‖ which blocks the protein binding of iodine without affecting the ability of thyroid gland to concentrate iodide(3,4) and of sodium perchlorate which is thought to interfere with the active transport of iodide without affecting its organification(4,5).

Materials. Penicillin G and puromycin were purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio. L-leucine, uniformly labeled with ^{14}C, was obtained from the New England Nuclear Corp., Boston, Mass. Amorphous insulin, 20 units/mg and practically devoid of glucagon (less than 0.0003%) was kindly provided by Otto K. Behrens of Lilly Laboratories, Indianapolis, Ind. The culture medium used in this study was medium TC-199(6), obtained from Hyland Laboratories, Los Angeles, Calif., to which penicillin G was added to provide a concentration of 50 units/ml. The basal culture medium was enriched also by insulin since our previous findings indicated that addition of insulin to the organ culture medium greatly improved the ^{131}I utilization by embryonic thyroid tissue(1,7,8) and also enhanced the incorporation of ^{14}C-labeled L-amino acids into protein(9). Amorphous insulin was dissolved in a minimum volume of 0.003 N HCl. A stock solution of this hormone made to contain 1 mg of insulin per ml of medium was sterilized by passing through a Millipore® filter with an average porosity of 0.45 μ. An amount of the filtrate was added to the medium to yield a concentration of 5 μg of hormone per ml. TSH (Thytropar, obtained from Armour and Co., Kankakee, Ill.) was dissolved in a measured volume of the medium (5 units/ml) and the solution thus obtained was sterilized by passage through the Millipore filter. 2An aliquot of the filtrate was then added to the culture

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§ Abbreviations: TSH for thyrotropic hormone; MIT for monoiodotyrosine; DIT for diiodotyrosine; T_4 for thyroxine.

‖ 1-Methyl-2-mercaptoimidazole.

medium to provide a concentration of 0.05 unit of hormone per ml.

Culture procedures. Thyroid glands were excised under sterile conditions from 21-day fetuses from Long-Evans rats, and immediately thereafter were placed in the culture media. They were cultured by the method of Chen(10) as modified by Elias and Rivera (11). Details of the culture have been described(1,2). Each culture dish contained 1 ml of medium on which a piece of dacron polyester organdy was floated to support 2 separate thyroid lobes (one gland).

Two media were used: the basal medium (199 + penicillin + insulin) and the medium to which TSH had been added (199 + penicillin + insulin + TSH).

The culture dishes were kept in an humidified incubator maintained at 37°. A mixture of 95% O_2 and 5% CO_2 that had been bubbled through distilled water was passed continuously into the culture chamber, the pH of the medium remained at 7.4-7.6. The medium was removed at the end of the second day and replaced with fresh medium with or without TSH and the culture was left in the incubator for another 24 hours. Exactly 6 hours before terminating the experiments, 10 μc of carrier-free ^{131}I or 0.5 μc of L-[U-^{14}C]leucine were added to each culture dish. Puromycin (0.2 mM/ml), when used, was added 15 minutes before the radioactive compounds. Sodium perchlorate (2 mM/ml), when used, was added 2 hours before addition of ^{131}I. In the experiments where iodide concentrating abilities of the explants were studied, 25 μl of a freshly prepared Tapazole solution (2 mM/ml) were added to each culture dish 2 hours before terminating the culture; 30 minutes thereafter 10 μc of ^{131}I were added.

Analytical procedures. At the end of incubation, the explants from each dish were removed, washed once with fresh medium and transferred to a centrifuge tube containing about 1 ml of 10% trichloroacetic acid. One ml of an aqueous solution containing 2 mg of bovine serum albumin was added as carrier, and the tissue was homogenized. The homogenate was centrifuged and the protein precipitate was prepared for ^{14}C counting as described by Singh *et al*(12).

For ^{131}I measurements, upon termination of the culturing the explants were removed from the dish, washed once with fresh medium, and immediately homogenized with 300 μl of a solution (pH 8) consisting of 0.15 M NaCl, 0.05 M Tris,¶ 0.014 M $CaCl_2$ and 0.002 M Tapazole. Total ^{131}I present in the thyroid explants was determined by counting a 20 μl aliquot of this homogenate in a well-type scintillation counter. To determine the ^{131}I-labeled compounds in unhydrolyzed tissue, 100 μl of each homogenate were placed on Whatman No. 3MM filter paper, and chromatographic analysis was carried out in collidine : 3NNH_4OH (100 : 37) mixture. Chromatography was begun immediately after delivery of the homogenate onto the paper to minimize destruction of iodoamino acids (13). The rest of the homogenate was then digested for 8 hours with Pronase-p** (5 mg/ml of homogenate) at 37° under toluene(14). One hundred μl of each digest were also chromatographed immediately after delivery onto filter paper in the collidine : 3 N NH_4OH mixture.

Radioautograms were prepared from the dried paper chromatograms with Kodak Blue Brand X-ray film. For quantitative estimation of ^{131}I in the various components, sections of the chromatograms corresponding to the bands observed on the radioautograms were cut out, folded, and placed in vials for counting in the well-type scintillation counter.

Results. Six hours after addition of 10^7 counts/min of ^{131}I to the medium 92 $\times$ 10^3 counts/min were recovered in the 2 thyroid lobes that had been cultured in the basal medium (Table I). Chromatographic analysis of the unhydrolyzed homogenate showed that protein-bound ^{131}I, 72 $\times$ 10^3 counts/min, constituted about 78% of the recovered ^{131}I. The protein-bound iodoamino acids formed by the explants in 6 hours were MIT, DIT and T_4; the ratio [^{131}I]MIT to [^{131}I]DIT averaged 1.4. As a result of the presence of in-

¶ Tris (hydroxymethyl) aminomethane.

** Pronase-p *(Streptomyces griseus* protease) 45 proteolytic units/mg was obtained from California Corp. for Biochemical Research, Los Angeles.

TABLE I. Effect of TSH and Puromycin on ^{131}I Uptake and Metabolism of Fetal Rat Thyroid Glands in Organ Culture for 3 Days. Six hr before the end of the culture, 10 μc (10^7 counts/min) of carrier-free ^{131}I were added to medium. Each value is expressed as counts/min $\times$ 10^{-3} recovered in one whole thyroid gland (2 lobes) and is the mean $\pm$ S.E. of the results of 6 separate experiments.

	Culture in basal medium		Culture in basal medium cum TSH	
^{131}I measurement	No addition	100 μg puromycin added	No addition	100 μg puromycin added
Total uptake	92.2 $\pm$ 7.57	93.3 $\pm$ 8.40	282.6 $\pm$ 20.35	279.3 $\pm$ 15.90
Protein-bound	72.2 $\pm$ 6.65	71.3 $\pm$ 6.24	248.3 $\pm$ 21.27	237.3 $\pm$ 13.45
MIT	43.0 $\pm$ 3.80	44.9 $\pm$ 3.70	126.9 $\pm$ 8.78	123.5 $\pm$ 8.29
DIT	29.7 $\pm$ 3.69	28.4 $\pm$ 3.37	103.6 $\pm$ 10.56	102.7 $\pm$ 7.15
T_4	1.4 $\pm$ .19	1.4 $\pm$ .16	10.8 $\pm$ 1.44	9.4 $\pm$ .69
I^-	15.5 $\pm$ 2.20	15.9 $\pm$ 2.04	27.6 $\pm$ 2.20	31.2 $\pm$ 2.22

sulin in the basal medium [^{131}I]T_4 was detectable in substantial amounts in the chromatograms of hydrolyzed homogenates (1,7, 8). When TSH was added to the medium, there was a considerable increase in ^{131}I recoveries in thyroid explants as well as a considerable increase in the incorporation of ^{131}I into protein. Table I shows that ^{131}I recovered in 6 hours in one thyroid gland (2 lobes) cultured in the medium to which TSH had been added was 282 $\times$ 10^3 counts/min, about 300% of the control values. Chromatographic analysis of the unhydrolyzed homogenate revealed that 248 $\times$ 10^3 counts/min, about 88% of the total ^{131}I was protein-bound. Chromatographic analysis of the hydrolyzed homogenate showed that the amounts of labeled MIT, DIT and T_4 were considerably greater in the experiments carried out with the media to which TSH had been added. The value for the ratio [^{131}I]MIT to [^{131}I]DIT was 1.2.

The effect of TSH on incorporation of ^{14}C of ^{14}C-labeled leucine into protein was negligible. Table II shows that the ^{14}C of [^{14}C] leucine added to the basal medium was readily incorporated into protein. The 6-hour incorporation of leucine carbon into thyroidal protein was about the same in the basal medium and in the TSH-containing medium.

Table II shows also that puromycin blocked almost completely the 6-hour incorporation of leucine carbon into protein by the glands cultured for 3 days in the basal medium. The inhibition is the same when the media used contained TSH. However, under similar conditions, this antibiotic did not affect the 6-hour ^{131}I uptake and its organification by the explants cultured in the basal medium (Table I)—nor did the antibiotic abolish the TSH-induced increase in the incorporation of ^{131}I into iodotyrosines and thyroxine.

Pretreatment of the explants with sodium perchlorate lowered the recovery of total ^{131}I in the glands cultured in the basal medium. The 6-hour ^{131}I uptake per one embryonic thyroid gland was 0.12% *versus* 0.92% in the basal medium without perchlorate. Of the 11.9 $\times$ 10^3 counts/min recovered in the thyroid explants (2 lobes) 9.1 $\times$ 10^3 counts/min (about 76.5%) were protein-bound (Table III). Chromatographic analysis of the hydrolyzed homogenate showed that MIT, DIT and T_4 were synthesized. The iodoamino acids formed were mainly MIT and DIT, and the ratio [^{131}I]MIT to [^{131}I]DIT was about 3.6 (*vs* 1.4 in the basal medium without perchlorate). Our results show that treatment with perchlorate 2 hours before addition of ^{131}I did affect the ability of the explants to concentrate iodide with little effect on total PB^{131}I; however, the disposition of the ^{131}I within the lobes is altered.

When embryonic thyroid glands were

TABLE II. Effect of TSH and Puromycin on Incorporation of ^{14}C of L-[U-^{14}C] Leucine into Protein by Fetal Rat Thyroid Glands. Six hr before termination of the culture the labeled leucine was added to each dish (0.5 μc/mc). Each value is the mean $\pm$ S.E. of the results of 5 experiments.

Addition to medium	^{14}C recovered in protein of one whole gland (counts/min)
None	3050 $\pm$ 87
Puromycin (.2 mM/ml)	39 $\pm$ 5
TSH (.05 unit/ml)	3060 $\pm$ 240
Puromycin + TSH	36 $\pm$ 4

TABLE III. Effect of TSH on ^{131}I Uptake and Metabolism of Perchlorate-Pretreated Fetal Rat Thyroid Glands. Thyroid glands from 2 fetuses (2 culture dishes) were used for each chromatographic analysis. Each value is expressed as counts/min × 10^{-3} recovered in one whole gland (2 lobes) and is the mean ± S.E. of the results of 5 separate experiments.

^{131}I measurement	Culture in basal medium	Culture in basal medium cum TSH
Total uptake	11.9 ± .59	17.1 ± 1.15
Protein-bound	9.1 ± .24	13.5 ± .94
MIT	7.3 ± .27	10.7 ± .87
DIT	2.0 ± .14	2.8 ± .15
T_4	.2 ± .01	.3 ± .02
I^-	1.9 ± .18	2.7 ± .21

cultured for 3 days in the TSH-containing medium and were pretreated with perchlorate 2 hours before addition of ^{131}I, the ^{131}I uptake of the explants was slightly greater than in those cultured without TSH (Table III). Of the 17 × 10^3 counts/min recovered in a whole thyroid gland, 13.5 × 10^3 counts/min were protein-bound (79.1%). The ratio [^{131}I] MIT to [^{131}I]DIT was about 3.8. In the TSH-containing medium, perchlorate also lowered the recovery of total ^{131}I in the glands and altered the distribution of ^{131}I in the enzymatically hydrolyzed tissue. In the presence of perchlorate, the important effect of TSH on ^{131}I metabolism of embryonic thyroid gland explanted into organ culture is not seen; indeed, in the presence or absence of TSH in the culture media, the ratio [^{131}I] MIT to [^{131}I]DIT was approximately the same as was the amount of T_4 synthesized by the explants.

The effect of TSH on ^{131}I recoveries in the Tapazole-pretreated fetal thyroid explants is shown in Table IV. In order to block effectively the organification of iodide, Tapazole was added 30 minutes before the addition of ^{131}I(8). Protein-bound ^{131}I was not detected on the chromatograms. Apparently TSH affected the [^{131}I]iodide recoveries in the Tapazole-treated glands. The ^{131}I contents of the explants cultured in the TSH-containing medium were always higher than in those cultured in the basal medium. However, the differences were statistically significant only at 10, 20 and 40 minutes after addition of ^{131}I. Thus it seems reasonable to admit that TSH has a small influence on the ability of the explants to concentrate iodide.

Discussion. The findings reported here extend our previous observations on the influence of TSH on iodine metabolism of embryonic thyroid glands transplanted into organ culture(1,2). The 6-h ^{131}I uptake as well as the incorporation of the isotope into protein-bound iodotyrosines and thyroxine were higher in the presence of TSH; the most striking response observed being the incorporation of ^{131}I into thyroxine which increased more than 7-fold in the explants cultured for 3 days in the medium containing TSH. Under similar conditions the results of the present study show that TSH failed to influence the 6-h ^{14}C incorporation of [^{14}C]leucine into protein. It has been shown that slices of guinea pig thyroid glands readily incorporate the ^{14}C of a variety of uniformly ^{14}C-labeled amino acids and that this process is under control of TSH(15). It has been demonstrated also that TSH added *in vitro* stimulated the incorporation of [^{14}C]leucine into protein by isolated bovine thyroid cells (16). However, using sheep thyroid slices, Raghupathy *et al*(17) showed that TSH added *in vitro* had no effect on incorporation of [^{14}C]leucine into protein. Our results on organ culture of embryonic thyroid glands support these latter observations: it is shown here that fetal rat thyroid explants readily incorporated the ^{14}C of [^{14}C]leucine into protein and that this process is not under the influence of TSH.

We have already shown(9) that puromycin, a potent inhibitor of protein synthesis(18,19), appreciably inhibited the 24-hour incorporation of leucine carbon into protein by fetal thyroid gland cultured for 2 days. In the

TABLE IV. Effect of TSH on ^{131}I Recoveries in Tapazole-Pretreated Fetal Rat Thyroid Glands. Each value is expressed as counts/min × 10^{-3} per one whole thyroid gland (2 lobes) and is the mean ± S.E. of the results of 5 separate experiments.

Min after addition of ^{131}I	Culture in basal medium	Culture in basal medium cum TSH
5	8.3 ± .64	11.1 ± 1.37
10	9.6 ± .90	16.4 ± 2.66
20	14.1 ± 1.61	21.9 ± 2.22
40	16.9 ± 1.40	24.5 ± 1.44
60	17.9 ± 1.91	22.2 ± 1.57
90	18.6 ± 3.52	24.2 ± 2.73

present study, we found that puromycin blocked almost completely the 6-hour incorporation of leucine carbon into protein by fetal glands cultured for 3 days in the presence and absence of TSH. Since puromycin did not have any influence on the incorporation of ^{131}I into protein-bound iodoamino acids, iodination of tyrosine must have taken place within the matrix of preformed thyroglobulin molecules. Several workers have arrived at a similar conclusion from experiments with intact rats(20), slices prepared from thyroid gland of various animals(21-24), isolated cells obtained from bovine thyroid glands(25) and subcellular fractions obtained from pig and sheep thyroid glands(26,27).

Since the TSH-induced increase in the incorporation of ^{131}I into iodotyrosines and thyroxine was observed even when *de novo* formation of protein was blocked by puromycin, it seems therefore that TSH stimulation of thyroid hormone synthesis is not dependent on increased synthesis of protein. In bovine thyroid isolated cells, Tong(25) found that puromycin reduced slightly the ^{131}I incorporation into iodoamino acids and that the addition of TSH increased [^{131}I] amino acid formation above non-puromycin-blocked control levels. In addition, the observations of Taurog and Thio(28) on intact rabbits suggest that TSH-induced thyroxine release from the thyroid gland does not depend on prior stimulation of protein synthesis.

Perchlorate is know to interfere with active transport of iodide and to have little effect on the organification of iodide(4,5). Indeed, when the fetal glands were cultured for 3 days in the basal medium the addition of perchlorate, 2 hours before that of ^{131}I, lowered considerably the ^{131}I uptake but did not change significantly the PB^{131}I in the explants. However, in our hands, the distribution of iodotyrosines and thyroxine was strongly affected by the pretreatment with perchlorate. When the explants were cultured in the TSH-containing medium, pretreatment with perchlorate provoked approximately the same effect. Since we found that perchlorate disturbs strongly the internal distribution of iodoamino acids within the glands cultured in the basal medium as well as in TSH-containing medium, these experiments with perchlorate do not allow us to shed any light on the mechanism of action of TSH.

TSH had a small influence on the recoveries of ^{131}I in the glands that were treated with Tapazole before addition of the isotope to the medium. Since Tapazole blocks the organification of iodide without affecting its accumulation in the gland(3,4), TSH apparently has an influence on the ability of the explants to concentrate iodide.

It seems reasonable to admit that the effect of TSH on organ culture of embryonic thyroid glands is not dependent only on the stimulation of iodide concentrating ability of the explants. There is no doubt that TSH also increases significantly the incorporation of ^{131}I into iodoamino acids and especially into thyroxine.

Summary. Thyroid glands excised from fetuses of 21-day pregnant rats were cultured for 3 days in the absence and presence of TSH. The 6-hour ^{131}I uptake as well as the incorporation of the isotope into protein-bound iodotyrosines and thyroxine were higher in the presence of TSH. Under similar conditions TSH failed to influence the 6-hour ^{14}C incorporation of [^{14}C]leucine into protein. Puromycin blocked almost completely the 6-hour incorporation of leucine carbon into protein by the fetal glands cultured in the presence and absence of TSH, but did not abolish the TSH-induced increase in the incorporation of ^{131}I into protein-bound iodotyrosines and thyroxine. It seems therefore that TSH stimulation is not dependent on increased synthesis of protein. Treatment with sodium perchlorate affected the ability of the explants to concentrate iodide with little effect on total PB^{131}I; however, the disposition of ^{131}I within the lobes is strongly altered. In the presence or absence of TSH, pretreatment with perchlorate provoked approximately the same effect. Although TSH had an influence on the ^{131}I recoveries in the glands treated with Tapazole, it is suggested that the effect of TSH on organ culture of embryonic thyroid glands is not dependent only on the stimulation of iodide concentrating ability of the explants.

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Reaction of Infectious RNA with Mammalian Cells.* I. Attachment to Cells. (31655)

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In the course of studies to explain the marked discrepancy between the degree of infectivity in mammalian cells of whole polio virus and the infectious RNA derived from it, we noted(1) that infectious RNA apparently enters the host cell in at least two steps, 1) "attachment" which is independent of duration of exposure to monolayers, temperature, osmolarity and pH within the limits tested, and 2) "uptake" which is markedly influenced by these factors. Weil(2) using polyoma viral DNA and Borriss and Koch(3) using polio viral RNA, have reported similar findings. Experiments designed to study "attachment" of free polio virus RNA to mammalian cells in monolayers are described here.

The data to be reported suggest that the comparatively low titers of infectious RNA cannot be explained by inefficiency of attachment to cells. An average of 75% of the infectious units exposed to cell monolayers is removed by them in one minute. Previous investigators(4,5,6) who have reported poor attachment to cells used isotopically labelled cellular and viral RNA rather than infectivity as their quantitative tool.

Materials and methods. RNA—cell system.

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