for this vascular protection by estrogen since stilbestrol, which did not produce tubular regression in the doses used, also protected the testis against vascular injury caused by cadmium.

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Thyroidal Coupling of Tyrosine *in vitro*.* (30332)

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The mechanism by which tyrosines are coupled to form thyronines by living tissues is unknown. Coupling occurs spontaneously *in vitro* at alkaline pH(1) in the presence of oxygen, and it is possible that non-enzymatic coupling may occur *in vivo*. It is not known whether the spontaneous coupling that occurs at pH 7.0 is sufficient to account for thyronine synthesis in the intact thyroid gland. *In vivo*, synthesis of thyronines proceeds at a rapid rate, which may be faster than can be accounted for by spontaneous coupling.

The experiments described here were designed to investigate the ability of the thyroid gland to couple 2 molecules of tyrosine for formation of one molecule of thyronine. Attempts were made to demonstrate incorporation of tyrosine-UL-C^{14} into thyronine *in vitro* with calf and pig tissue slices and rabbit thyroid homogenates.

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Materials and methods. A. Preparation and incubation of tissue slices. (1) Fresh pig thyroids were obtained from a local slaughter house, chilled until processed and sliced on a Stadie-Riggs microtome. The slices were then weighed and placed immediately into 25 ml flasks containing 5.0 ml Krebs-Ringer phosphate buffer, pH 7.4. The flasks were incubated in a Dubnoff metabolic incubator at 38°C for 2 hours. The incubated material was then extracted exhaustively with n-butanol. The residue was digested with pancreatin, reextracted with n-butanol and chromatographed.

(2) Tissue slices from beef thyroid glands were prepared in the same way. In flask No. 1, 500 mg of thyroid tissue was incubated in 10 ml of Krebs-Ringer bicarbonate buffer. In flask No. 2, 50 μc (90 mc/mM) tyrosine-UL-C^{14} was added to 500 mg thyroid tissue and 10 ml Ringer bicarbonate buffer. The flasks were incubated in the Dubnoff incubator at 37°C for 3 hours. Additional buffer was then made up and pH adjusted to 9.0 with NaOH. To 12 ml of buffer 250 mg of pancreatin was added. Six ml of this mixture were added to each flask. Both were layered

with 3.0 ml of toluene. Hydrolysis was continued through 48 hours following which 5 butanol extractions were done.

A 20-tube counter current apparatus was used to prepare the radioactive butanol hydrolysates for chromatography. Preliminary counter current runs with non-radioactive standards were made with localization and identification by thin layer chromatography. Butanol (300 ml), water (280 ml) and NH_4OH (20 ml) were placed in a separatory funnel, shaken and allowed to stand overnight. Ten ml of the lower phase were added to each counter current tube followed by 10 ml of upper phase. Diiodotyrosine, monoiodotyrosine and tyrosine separated best in tubes 1 through 3, whereas thyronine was best separated in tube 4 and diiodothyronine and triiodothyronine in tubes 15 and 16. The radioactive butanol hydrolysate was then run through 19 transfers on the 20-tube counter current apparatus.

The material in tubes 4 through 8 was pooled as this was the distribution of thyronine on the run of non-radioactive standards. The pooled specimen was evaporated with air jets and chromatographed on paper with appropriate standards in a descending system of tertiary butanol (326 ml) and 2.0 N NH_4OH (74 ml) for 43 hours. The chromatographic strips were run through a Nuclear-Chicago strip scanner.

B. Preparation of homogenized rabbit thyroid glands. Fresh chilled rabbit thyroid glands were added to a solution containing 0.35 M sucrose, 0.004 M $MgCl_2$, 0.025 M Tris-HCl and 0.006 M 2-mercaptoethanol. The tissue was minced with scissors and the mixture poured into a prechilled Waring blendor. After blending for 30 seconds the mixture was transferred to a Potter-Elvehjem homogenizer for homogenization. The homogenate was then spun in a Spinco at 600 *g* for 20 minutes. The supernatant was removed, transferred to incubation flasks containing tyrosine-UL-C^{14} and incubated from zero to 60 minutes. Appropriate control flasks were incubated simultaneously. The reaction was stopped by addition of tricholoroacetic acid. After centrifugation the supernatant was removed and the remaining protein precipitates were prepared for counting by extraction with absolute ethanol and a 12:1 mixture of ether:chloroform. Formic acid was added to dissolve the protein and the digest was prepared for chromatography.

Results. Chromatography of hydrolysates of tissue slices from pig and beef thyroid failed to reveal the presence of thyronine. Using a descending system of tertiary butanol and 2.0 N NH_4OH a spot was detected in the same area as the thyronine standard when ninhydrin but not sulphanilic acid was used as a location reagent. However, further investigation using a butanol-dioxane-ammonia system failed to identify the substance as thyronine. Additional studies revealed the compound to be leucine, the most common amino acid in the thyroid gland. The possibility that thyronine might be destroyed by the experimental method was excluded by repeating the experiments with 200 μg of chromatographically pure thyronine added to the incubation flasks. Recovery was possible from both thyroid and liver tissue. These experiments were interpreted as indicating that thyronine, if present, in the tissue slices should not have been lost or broken down in the experimental procedures.

Because of the possibility that only minute amounts of coupled thyronines might be detected in the *in vitro* experiments, tissue slices of beef thyroid were incubated with 50 μc of tyrosine-UL-C^{14}. When the chromatographic strips for analysis were scanned for radioactivity, areas were detected but repeated elutions and chromatography with duplicate strips failed to identify one of them as thyronine. We concluded that by following the carbon skeleton there was evidence for formation of C^{14}-labeled compounds but no evidence for formation of C^{14}-labeled thyronine or thyronine analogs. Finally, thyroid homogenates were prepared and incubated with tyrosine-UL-C^{14} for 60 minutes. The trichloroacetic acid precipitable material contained radioactivity and was therefore digested with formic acid and chromatographed. No thyronine or thyronine analog was detected.

Discussion. The investigation of thyroid hormone synthesis dates back many years.

Harrington and Barger(2) suggested in 1927, that thyroxine was synthesized by the condensation of 2 molecules of diiodotyrosine.

The conversion of I^{131} iodide to thyroxine and diiodotyrosine by surviving slices of sheep, dog and rat thyroid glands was demonstrated by Morton and Chaikoff in 1943 (1). Harrington and Pitt Rivers(4) confirmed the non-enzymatic formation of thyroxine from diiodotyrosine in alkaline solution and showed an increased yield when oxidizing agents were added.

Taurog, Potter and Chaikoff(5) using homogenates and subcellular fractions of sheep thyroid then showed the conversion of iodide I^{131} to monoiodotyrosine I^{131} but not diiodotyrosine I^{131} or thyroxine I^{131}. The iodoamino acid was firmly bound to protein and not detectable until after hydrolysis with pancreatin.

Yip and Klebanoff(6,7) have incubated diiodotyrosine with myeloperoxidase in a cell-free suspension of beef thyroid in the presence of glucose and glucose oxidase. They were able to show the formation of a compound indistinguishable from thyroxine by paper chromatography, ultra-violet and infrared analyses and stimulation of tadpole metamorphosis. These studies supported the work of Igo and Mackler(8) who had previously reported thyroxine synthesis from diiodotyrosine by modified beef thyroid mitochondria in the presence of glucose and glucose oxidase. The mechanism of thyroxine synthesis by the enzyme myeloperoxidase or thyroid cell-free preparations has not as yet been demonstrated.

In our experiments, we were interested in starting with C^{14}-labeled tyrosine as a substrate hoping to demonstrate coupling to form the non-iodinated compound thyronine, in addition to the iodothyronines. Harrington and Pitt Rivers(4) had raised the possibility of this pathway of thyroxine synthesis but previous studies had failed to demonstrate this compound in non-enzymatic or enzymatic preparations. We were unable to identify thyronine in either tissue slices or homogenates incubated with tyrosine-UL-C^{14}. The systems used and the amount of radioactivity employed allow us to conclude that the order of magnitude of conversion, if it indeed did occur, was less than one part in a million.

In addition, no evidence was found of incorporation of tyrosine-UL-C^{14} into iodothyronines under the conditions of these experiments. This is in contrast to the established incorporation of I^{131} into iodothyronines in similar experiments(1). From this we conclude not only that thyronine is not an intermediate in the biosynthesis of thyroxine and triiodothyronine, but that there is, at present, no evidence that tyrosine itself supplies the carbon skeleton for the iodothyronine hormones. Maloof's data(9), showing recovery of only 200 counts per minute in thyroglobulin after intraperitoneal injection of 5 μc of tyrosine-UL-C^{14} is consistent with this interpretation, since tyrosine rapidly breaks down to precursors of other amino acids and carbohydrates, and since Maloof did not identify the counts he found in thyroglobulin as tyrosine or iodothyronine.

Summary. Attempts were made to demonstrate the presence of thyronine within thyroid tissue slices as well as its formation using as accepted precursor UL-C^{14}-tyrosine. Not only was thyronine not isolated but no evidence was found for the incorporation of tyrosine into any of the thyronine iodinated analogs.

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