Metabolism of Thyroxine in Larva of Rice Moth (*Corcyra cephalonica* St.) (25315)

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Our earlier investigations(1,2) have clearly established that the insect, *Corcyra cephalonica* St., responds to administration of thyroxine in a manner strikingly similar to that observed in mammals. This similarity could be extended to alterations in the catalase and amylase levels as well as to existence of a direct thyroxine-Vit. B_{12} antagonism(3). The greater effectiveness of triiodothyronine in the insect as well as in mammals raises the question whether thyroxine might be deiodinated to triiodothyronine before exerting its action. *In vitro* conversion of thyroxine to triiodothyronine occurs in mammalian tissues(4,5,6). The results of our study of *in vitro* and *in vivo* metabolism of thyroxine in *Corcyra* are here presented. Excretory metabolism of thyroxine in *Corcyra* studied by chromatographic methods, showed iodide to be the major metabolite. *In vitro* experiments with homogenates of *Corcyra* larvae established that deiodination of thyroxine was not a normal feature of insect metabolism, but occurred only in larvae reared on thyroxine-supplemented diet.

Materials and methods. Chromatographically pure radioactive l-thyroxine (labeled in the 3′ and 5′ positions) was obtained from Abbott Labs, Chicago, Ill., and had a specific activity of 29 μc/μg. The stock solution in 50% propylene glycol was diluted with water to the desired concentration. Cell-free extracts of tissues of larvae reared on control diet of wheat flour and on diets supplemented with thyroxine (2 μg/g of diet) were tested for activity to deiodinate thyroxine. Larvae maintained on control and thyroxine-supplemented diets for 20-25 days were picked and allowed to starve for about 3 hours. After cleaning thoroughly with a camel hair brush, they were ground in previously chilled mortar and homogenized in ice-cold isotonic potassium chloride in Potter-Elvehjem glass homogenizer equipped with Teflon pestle (Arthur H. Thomas and Co.). The homogenate was filtered through cotton wool and used as the enzyme source. *Analysis of larval excreta.* *Corcyra* larvae were reared on thyroxine-supplemented diets(2). Thyroxine was included at concentration of 2 μg/g of diet. After most of diet had been consumed, fecal pellets were separated from diet particles and cleaned well with camel hair brush. The excreta (500 mg) were then extracted twice with 5 ml portions of ethanol-2N ammonia (1:1) mixture. After centrifuging, the supernatant was concentrated *in vacuo*. The residue was dissolved in suitable volume of ethanol ammonia mixture and aliquots were taken up for chromatography. *Paper chromatography* was carried out on Whatman No. 1 filter paper. In each experiment, at least 2 of the following systems were employed. 1. n-butyl alcohol: acetic acid:water 78:10:12 or 78:5:17. 2. *idem* : dioxane : 2N ammonia 4:1:5. 3. *idem* ethyl alcohol : *idem* 5:1:2. In chromatographic experiments with radioactive materials, the samples were usually supplemented with suitable amounts of carrier substances. The positions of these substances were located by application of the ceric sulphate-arsenious acid reagent of Bowden *et al.*(7) as modified by Dragunova and Langer(8). Radioautographs of the chromatograms were made on Ilford-Ilfex X-ray films. All radioactive measurements were made in a well type scintillation detector (DS-S-5) attached to precision rate-meter (Nuclear Chicago Corp.). Measurements were corrected for background and decay. Distribution of radioactivity on chromatogram strips was determined by cutting each strip into segments of 0.5 cm width and directly counting individual segments. The activity corresponding to each spot was expressed as percent of total activity in the strip. The incubation medium for examina-

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tion of deiodinase activity of larval homogenates comprised 2 ml of appropriate enzyme solution, 0.1 ml of substrate solution and buffer to make up the volume to 3 ml. Blank and control tubes received respectively 2 ml of isotonic KCl and boiled (10-15 minutes) larval homogenates in place of the enzyme solution. Incubations were carried out at 37°C for 5 or 16 hours with occasional shaking.

Analysis of incubation media. Inorganic iodide, which was the major radioactive metabolite, was estimated as follows: 0.5 ml aliquots of various incubation mixtures were withdrawn into tubes containing 1 ml of 20% trichloroacetic acid (TCA). The contents were filtered, the precipitate washed twice with 0.5 ml of 5% TCA solution, and filtrate and washings pooled. TCA was then removed by repeated extraction with ether until pH came down to about 3-4. Ether extract was washed once with dilute thiosulphate solution and the washings added to original solution. 100 mg of deactivated charcoal(9) was then added to the solution (pH 3-4) and the tube shaken vigorously. At the end of 30 minutes, the contents were centrifuged, and the supernatant transferred carefully to another tube and radioactivity measured. This represents the inorganic iodide fraction. In experiments where a search for other possible metabolites was made, chromatographic analysis was carried out to get both quantitative and qualitative assessment of the compounds. Further, the method served as a check on the value of inorganic iodide fraction as obtained by procedure outlined above.

Results. Nature of excretory metabolites of thyroxine. Chromatographic analysis of excreta of larvae fed thyroxine and comparison with authentic samples established the presence of thyroxine and iodide in the excreta. Control experiments carried out with excreta of larvae fed control diet of wheat flour alone, showed no distinct iodine-containing compounds. The fact that iodide was the major identifiable metabolite suggested the presence of a deiodinating mechanism in the larvae. Further, the chromatograms often showed faint spots corresponding to monoiodotyrosine and diiodotyrosine, thus suggesting that a fission at the ether linkage is another aspect of metabolism of thyroxine in the larvae.

In vitro deiodination of thyroxine by larval homogenates. 10% (w/v) homogenates of larval tissues were employed. Thyroxine was included in mixtures at concentrations ranging from 10^{-6} M to 10^{-8} M. The average results of replicate experiments presented in Table I demonstrate conclusively that tissues of thyroxine-fed larvae are capable of forming iodide from thyroxine, whereas, larvae reared on normal diets lack this ability. In many replicate experiments the extent of deiodination was about 10%, under conditions employed. Deiodination of thyroxine was studied at pH values of 7.2 and 8.6 (phosphate buffer) and 9.5 (carbonate-phosphate buffer). The results presented in Table I indicate that the enzymic deiodination of thyroxine occurred over a wide range of pH.

Chromatographic analysis of the incubation media. Chromatographic analysis of both control and experimental incubation media of

TABLE I. Deiodination of Thyroxine by Larval Homogenates. The test system contained 2 ml of larval homogenate, 0.1 ml of radioactive thyroxine (conc. 1.24×10^{-7} M and activity 1.5×10^5 cpm) and 0.9 ml of appropriate buffer solution. Incubation period 5 hr at 37°C. Iodide was determined by charcoal treatment procedure as described in text.

Nature of homogenate	Exp. system	% iodide formed* at pH value of 7.4	8.6	9.3
Tissue of larvae fed control diet	Blank	2.4 (2.0– 2.7)†		
	Boiled enzyme	2.5 (2.1– 2.7)		
	Test	2.7 (2.6– 2.9)		
Tissue of larvae fed diet containing thyroxine	Blank	2.9 (2.6– 3.2)	2.6 (2.3– 2.8)	1.9 (1.4– 2.3)
	Boiled enzyme	5.9 (5.1– 6.4)	5.4 (5.1– 5.6)	5.8 (5.3– 6.2)
	Test	14.8 (13.7–16.0)	15.3 (14.8–16.0)	17.0 (16.6–17.3)

* Results are expressed as $\frac{\text{Radioactivity in inorganic iodide fraction} \times 100}{\text{Total activity added to incubation medium}}$.

† Values in parentheses represent range.

homogenates of larvae fed control diet revealed feeble activity (less than 2%) in areas corresponding to iodide. These results confirm the absence of deiodinase system in control larvae. The results of a typical experiment with the homogenates of thyroxine-fed larvae at pH 8.6 are represented in Fig. 1. In the control as well as blank, little or no deiodinase activity was observed. The extent of deiodination in the experimental averaged from the replicate experiments was 10.9%. These results are in close agreement qualitatively and quantitatively with those obtained by the charcoal treatment procedure.

Besides thyroxine and iodide, another radioactive area having an R_f of 0.5 in both butanol : acetic acid : water and butanol : dioxane : ammonia solvent systems, was noticed. This spot accounted for less than 10% of total activity of the chromatogram and further, the activity of this spot was comparatively greater in chromatograms of short-term incubation mixtures. Detailed analysis of time-course curves of the degradation of thyroxine might reveal the significance of this factor in deiodination of thyroxine.

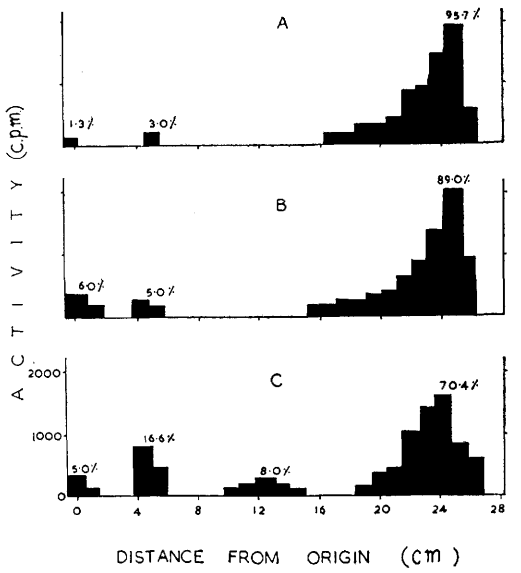

FIG. 1. Deiodination of I^{131}-labeled thyroxine by 10% homogenate of thyroxine-fed larva. Distribution of radioactivity in chromatogram. Substrate conc. 2.48×10^{-7} M; incubation period 5 hr at 37°C in phosphate buffer pH 8.6; solvent system—butanol : acetic acid : water, 78 : 5 : 17. Values expressed as % of total activity in the entire chromatogram. A—Blank; B—Boiled enzyme control; C—Test. Shaded areas in C correspond to origin, iodide, unknown, and thyroxine.

Discussion. The possibility of iodide being formed in *in vitro* experiments as a consequence of chemical interaction between thyroxine and some compounds in tissues, has been stressed by Michel(10). Such a possibility could however be eliminated since no iodide could be detected in boiled enzyme blank either by charcoal adsorption procedure (Table I) or by chromatographic method (Fig. 1). The consistent presence of iodide in chromatograms developed with acidic or alkaline solvents further establishes that it is not an artifact formed during chromatography.

Two-dimensional chromatography of the incubation mixtures failed to reveal formation of triiodothyronine in most experiments. However, as experienced by Tata *et al.*(11), a spot corresponding to triiodothyronine also showed up occasionally in our experiments. Thus, presence of triiodothyronine in the incubation media was not a consistent feature. In the light of these observations, it is difficult to judge the importance of the deiodinating mechanism in conferring biological activity to thyroxine molecule.

Attempts to detect acetic or propionic acid analogs of thyroxine in the incubation mixtures led to results which were not unequivocal, inasmuch as authentic samples of these compounds added to the larval homogenates tended to streak along or coincide with thyroxine spot on the chromatograms.

The observation that enzyme activity is exhibited only by larvae fed thyroxine and not by larvae grown normally, suggests the possibility that the enzyme is elaborated only under stress imposed by thyroxine, presumably as a detoxication mechanism. Thus, synthesis of the enzyme may represent an adaptive response on the part of the insect organism to the thyroxine. Further work in progress, on purification of the enzyme as well as a study of enzyme levels at different periods of thyroxine feeding, might throw more light on this interesting phenomenon.

Summary. The major excretory metabolite of thyroxine in the insect *Corcyra cephalonica*

St. is iodide, though monoiodotyrosine and diiodotyrosine are present in small amounts. *In vitro* experiments with homogenates of larval tissue have shown that an enzyme system capable of forming iodide from thyroxine is present only in tissues of larvae fed thyroxine-supplemented diets and not in those of larvae fed control diet alone. Activity of the enzyme is destroyed by boiling for 10-15 minutes.

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Isolation and Identification of Testosterone in Systemic Blood from Normal Human Male Adults.*† (25316)

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It is well recognized that testosterone and Δ^4-androstene-3,17-dione are the predominant steroid hormones of the human testis(1,2). These androgens have also been found in canine spermatic vein blood(3) and in dogs, spermatic vein concentration of testosterone and Δ^4-androstene-3,17-dione increases markedly after human chorionic gonadotropin (HCG) administration(4). Heretofore, neither testosterone nor Δ^4-androstene-3,17-dione have been reported in systemic blood of man. In the present communication, isolation of testosterone from systemic human blood after administration of HCG is described.

Materials and methods. Chemicals, unless otherwise specified, were reagent grade and all solvents used were freshly distilled. Evaporation was done at +40°C under nitrogen. Blood was obtained from 3 normal human male subjects 2 hours after intramuscular injection of 1000 units HCG.‡ Heparin was used as anticoagulant and a plasma pool of 400 ml was processed. The plasma was extracted 4 times with 500 ml ethylacetate/ether (1:1 v/v) each time. The combined extracts were evaporated to dryness and the residue suspended in 25 ml 70% methanol (MeOH) and stored at −15°C for 15 hours. The MeOH solution was then centrifuged 15 minutes at 2000 rpm in a refrigerated centrifuge. The supernatant was carefully removed, the precipitate consisting of lipid material(5) was washed once with 5 ml ice cold 70% MeOH, and the MeOH extracts were combined. Last traces of lipids were removed from the combined 70% MeOH solution by extraction with 30 ml n-hexane. The hexane was discarded and the MeOH solution was evaporated to drynes. The dry residue was transferred to a paper strip with chloroform-MeOH (1:1 v/v) and on another strip 50 γ synthetic

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