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THE METABOLISM OF SOME THYROID HORMONES BY LIMB-BONE RUDIMENTS CULTIVATED *IN VITRO*

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In previous experiments it was shown that thyroxine (T_4) (Fell & Mellanby, 1955) and triiodothyronine (T_3) (Fell & Mellanby, 1956) added to the culture medium affected the growth and development *in vitro* of the cartilaginous limb-bone rudiments of 6-day chick embryos. Qualitatively the two hormones had similar effects. Both accelerated the histological development of the five rudiments investigated, the femur, tibia, humerus, ulna and radius; they also produced a constant differential effect on the growth in length of the various rudiments, whereby growth was inhibited in different degrees in the tibia, femur, humerus and ulna in that order, but stimulated in the radius. Quantitatively, T_3 was roughly four times as potent as T_4 in retarding the growth of the femur and tibia.

These results were thought to indicate that T_4 and T_3 had a direct action on the explants. Gross & Pitt-Rivers (1953), however, suggested that triiodothyronine might be the active form of the thyroid hormone at the peripheral level, resulting from a partial de-iodination of thyroxine. It was thought that in a simple system such as that provided by organ cultures *in vitro* it would probably be relatively easy to detect de-iodination or other chemical transformations taking place in the iodothyronine molecules. It was decided therefore to grow embryonic bones in the presence of ^{131}I-labelled thyroxine and triiodothyronine and examine both the bones and culture medium after incubation to see whether any transformation had occurred in the iodothyronine molecule. T_3 and the iodothyroacetic acids were thought to be likely metabolites of T_4.

Two main groups of experiments were made. In the first the growth of the bone rudiments in medium to which triiodothyroacetic acid (TA_3) or tetraiodothyroacetic acid (TA_4) had been added, was studied, and the effect of TA_3 on the explants was directly compared with that of T_3. Control experi-

ments were also made with potassium iodide. In the second group, radioactive T_4 or T_3 was introduced into the medium, and subsequently the break-down products of the hormones present in the rudiments, in the medium on which the bones were grown and in 'blank' medium incubated without explants, were identified by chromatographic analysis.

METHODS

Material. The femora, tibiae, humeri, ulnae and radii of 6–8-day chick embryos were cultivated. The 6-day rudiments were entirely cartilaginous, but the degree of development varied somewhat in different chicks; thus in some embryos all the rudiments consisted of early, small-celled cartilage, whereas in others cellular hypertrophy had already begun in the shafts of the femur and humerus, and sometimes also in the tibia, ulna and radius. In the 7-day embryos hypertrophy and early periosteal ossification were usually present in all the five rudiments studied and in the 8-day bones the hypertrophic cartilage was at an advanced stage of differentiation.

Experiments of group 1

Culture technique. The explants, which were all 6-day rudiments, were grown in watch glasses 4 cm in diameter, enclosed in a moist chamber, by the technique previously described (Fell & Robison, 1929; Fell & Mellanby, 1955). The culture medium consisted of three parts of cock plasma and one part of embryo extract; the extract was made by thoroughly mincing and grinding a 13-day embryo and mixing the minced tissue with an equal volume of Tyrode solution supplemented with 1% glucose. In addition, a more dilute extract was prepared by mixing one part of embryo mince with two parts of Tyrode solution not supplemented with extra glucose.

The substances to be tested were introduced into the medium as follows. TA_3, T_3 and KI were weighed into a sterile tube and sterilized with a few drops of absolute ethanol, which was then evaporated off; the compounds were dissolved in Tyrode solution and added to the plasma immediately before use to give a concentration of approximately 16 μg/100 ml. of the final culture medium, the same quantity of Tyrode solution alone being added to the control medium. TA_4 was dissolved in ethanol and added to the plasma so that the final medium contained 16 μg TA_4/100 ml. and 0·2% ethanol; the same amount of ethanol was introduced into the controls.

The newly dissected rudiments were washed several times in Tyrode solution, and the dilute embryo extract was then substituted for the saline. The explants were transferred to the clot with a pipette and the surplus extract was removed with a very fine pipette; one of each pair of rudiments was placed on medium containing the agent to be tested and the other on control medium. Every 2 days the rudiments were removed from the old clot, washed in Tyrode solution, followed by dilute embryo extract, and transferred to fresh medium; they were grown for 8 days.

Measurement of growth in length. The bones were drawn with the aid of a camera lucida when first explanted and thereafter at 2-day intervals; their lengths were measured in the manner previously described (Fell & Mellanby, 1955).

Two experiments were made with each of the three compounds mentioned above. Three embryos were used for every experiment, so that each compound was tested on six paired femora, tibiae, humeri, ulnae and radii. The average growth rate of the six experimental and control explants was calculated for each rudiment and each compound.

Histological methods. The explants from one experiment with TA_3 and one with TA_3 and T_3 were fixed for 20–30 min in 3% acetic Zenker's fluid followed by 45 min in Zenker's fluid without acetic acid; they were embedded in paraffin wax and serially sectioned. The sections were stained with Delafield's haematoxylin and chromotrop.

Experiments of group 2

Culture technique. The rudiments were explanted in large watch-glasses 6 cm in diameter enclosed in 10 × 1·5 cm Petri dishes; the dishes were carpeted with cotton-wool saturated with 20 ml. of sterile distilled water. Each watch-glass contained 1·5 ml. plasma and 0·5 ml. embryo extract. In some experiments (see Table 1) the concentrated embryo extract described above was used, but in others the extract was much more dilute (1 part embryo mince: 4 parts Tyrode solution supplemented with 1% glucose).

Synthetic T_4 and T_3 labelled in the 3′, 5′ or 3′ positions were used. Biosynthetically labelled hormones were also prepared by alkaline hydrolysis of the thyroid glands of mice after injection of ^{131}I and chromatographic separation. The labelled T_4 and T_3 were dissolved in a few drops of 0·1 N-NaOH and were then taken up in carrier solutions of T_4 and T_3. The solutions were added to the plasma shortly before use, as described above, to give concentrations of approximately 16 μg/100 ml. of medium.

In four experiments (see Table 1) 6–8 femora and tibiae from 6-, 7- or 8-day chick embryos were explanted in each watch-glass on medium containing labelled T_4 or T_3. In one experiment (no. 183) the bones were grown for 6 days in the presence of unlabelled T_4 by the method used for group 1, and were then transferred to large watch-glasses containing T_4, six bones being placed in each vessel; in another experiment (no. 204) the rudiments were grown for 4 days in the presence of unlabelled T_3 before being transplanted to medium containing the labelled T_3. A 'blank' clot containing the labelled hormone but without explants was prepared in each experiment.

After 18–22 hr growth in labelled medium the cultures were harvested aseptically. With a medium-sized pipette the explants with the fluid immediately surrounding them were collected in one tube, the plasma: embryo-extract clot on which they had grown was placed in another and the 'blank' clot in a third. The material was analysed within 24 hr of harvesting.

Analysis of radioactive products in (a) bones and (b) medium. After incubation the bones were separated from the medium, finely chopped in 0·2 ml. methanol-ammonia (3:1) and mechanically shaken for 15 min. The resulting gelatinous solution, the medium separated from the bones, the control medium and a sample of the original ^{131}I-labelled hormone were analysed by ascending chromatography in butanol:dioxan:2N-NH_3 (4:1:5). Thyroxine, triiodothyronine and iodide were added as markers. The radioactivity on the chromatograms was detected by scanning with a recording strip counter and quantitative results were obtained from the record by measuring the area under each peak. Iodide was visualized by staining with 0·1% palladium chloride; the iodothyronines were detected with diazotized sulphanilic acid as previously described (Gross & Leblond, 1951).

RESULTS

The behaviour of 6-day rudiments grown in the presence of TA_3, TA_4 and KI

The effect of TA_3. In each of two experiments, the femora, tibiae, humeri, ulnae and radii were removed from three 6-day embryos; one of each pair of bones was cultivated for 8 days on medium containing 16 μg/100 ml. medium and the other on control medium.

During the first 2 days in culture there was little or no difference between the experimental and control explants, but by the 4th day the ends of all the TA_3-treated rudiments were larger, and in the femora, tibiae and humeri (but not in the ulnae and radii) the shafts were shorter than in the corresponding controls. These differences became more conspicuous during the next 2 days, when the TA_3-treated bones appeared thick and clumsy as compared with their more slender and shapely controls.

The average growth rates of the rudiments were estimated from the serial camera lucida drawings mentioned in Methods. The results showed that TA_3 produced a similar type of differential effect (Fig. 1*A*) on the growth in length of the various rudiments to that previously observed with T_4 and T_3 (Fell & Mellanby, 1955, 1956).

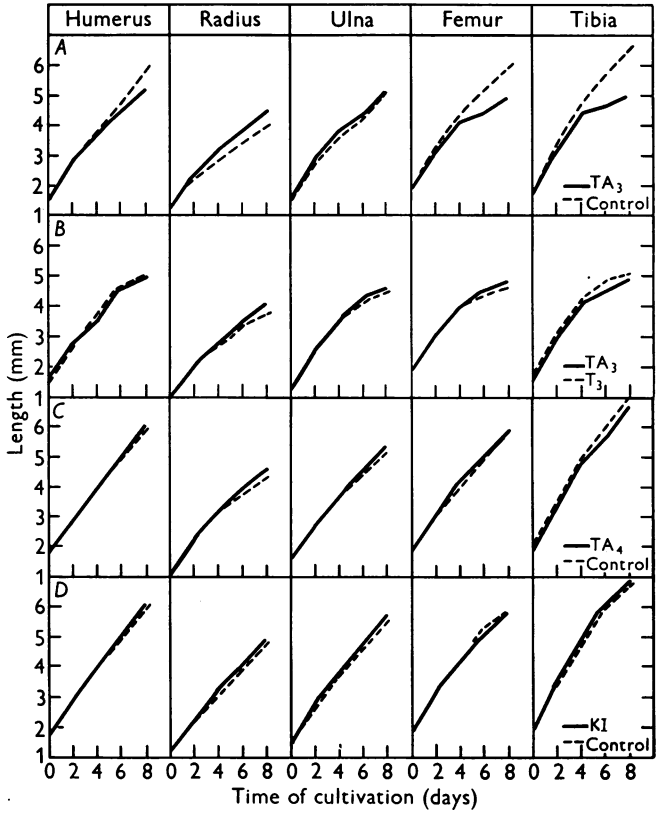

Fig. 1. Graphs showing the average growth in length of paired limb-bone rudiments during cultivation in normal medium and when exposed to various chemical compounds. Each graph represents six pairs of rudiments; the concentration of all the compounds tested was approximately 16 μg/ 100 ml. of medium. *A*, TA_3; the growth of the tibia, femur and humerus has been diminished in that order of degree, there is no significant effect on the ulna and the growth of the radius has been increased. *B*, TA_3 and T_3; the effects of the two agents are similar. *C*, TA_4; little or no effect. *D*, KI; no effect.

A comparison of the histological structure of the TA_3-treated and control explants showed that TA_3 produced the same type of effect as that already described for T_4 and T_3. In all five rudiments the hypertrophy of the cartilage cells had spread precociously into the proliferative zones of flattened cells; in the three wing-bones these zones were greatly reduced as compared with those

of the corresponding controls, and in the two leg-bones they were almost obliterated so that the hypertrophic cartilage extended almost to the borders of the epiphyses. In the hypertrophic cartilage of the TA_3-treated rudiments, especially the femur, tibia and humerus, the intercellular partitions were narrower and more cells were degenerate than in the controls, but in the epiphyses of all five rudiments the cells were larger and the matrix more plentiful in the TA_3-treated explants. Periosteal ossification was proceeding actively in both series.

Comparison of the effects of TA_3 and T_3. Two experiments were made to compare the effects of TA_3 and T_3 in the same concentration (16 μg/100 ml. of medium), on the five limb-bone rudiments. One of each pair of bones was grown on medium containing TA_3 and the other in medium to which T_3 had been added.

No significant differences between the two series could be detected in gross anatomy, histological structure or growth in length (Fig. 1*B*); both sets of bones showed the changes described in the preceding section on the effects of TA_3 and in an earlier paper (Fell & Mellanby, 1956) on the action of T_3.

The behaviour of the explants in the presence of TA_4. Two experiments, exactly similar to those with TA_3, were made with TA_4. In a concentration of 16 μg/100 ml., TA_4 had no appreciable effect on either the gross structure or growth in length (Fig. 1*C*) of the rudiments; the explants were not examined histologically.

The behaviour of the explants in the presence of KI. Two control experiments were made to see whether potassium iodide (16 μg/100 ml. of medium) would affect the growth and development of the five rudiments. There was no significant difference in either the growth rate (Fig. 1*D*) or gross morphology of the two series. This result confirmed unpublished observations of the late Sir Edward Mellanby.

The metabolism of T_4 and T_3 by bones and medium

Thyroxine. In all experiments thyroxine and iodide were the only radioactive compounds detected in the bones or medium after incubation with ^{131}I-labelled T_4. The relative amounts of iodide and T_4 found in the different fractions and controls are shown in Table 1. Some spontaneous de-iodination of T_4 always occurred during storage. Although partial de-iodination by the medium was observed, it was considerably less than that produced by the bones. The proportion of the original thyroxine de-iodinated by the bones varied in each experiment, but could not be correlated with factors such as age of the embryo or length of culture period.

Triiodothyronine. T_3 and iodide were the only compounds detected in the bones or medium after incubation with ^{131}I-labelled T_3 (Table 1). T_3 underwent less spontaneous de-iodination than T_4, so that control values were always

lower. De-iodination by the medium was only slight but a considerable amount occurred in the bones. The results of the two T_3 experiments were very similar despite the different culture periods, and although synthetically labelled T_3 was used in one experiment (no. 178) and biosynthetically labelled T_3 in the other (no. 204), this did not appear to have any effect on the results.

TABLE 1. Experiments with labelled compounds (marked *)

Expt. no.	Compound	Age of embryo (days)	Culture period	Exposure to T_4* or T_3* (hr)	Culture medium	Iodide formed (%)			
						Bones	Medium from bones	Medium alone	Iodothyronine alone
173	T_4	7	23 hr	23	Plasma + T_4 + concentrated embryo extract	69	40	38	17
176	T_4	7	20 hr	20	Plasma + T_4* + dilute embryo extract	33	22	20	11
183	T_4	6	7 days	22	For 6 days, plasma + T_4 + concentrated embryo extract				
					7th day: (*a*) plasma + T_4* + concentrated embryo extract;	(*a*) 37	24	22	—
					(*b*) plasma + T_4* + dilute embryo extract	(*b*) 45	22	24	—
191	T_4 (syn)	8	21 hr	21	Plasma + T_4* + concentrated embryo extract	52	40	41	40
178	T_3	7	19½	19½	Plasma + T_3* + dilute embryo extract	38	9	6	6
204	T_3 (syn)	6	5 days	23	For 4 days, plasma + T_3 + concentrated embryo extract; 5th day, plasma + T_3* + concentrated embryo extract	39	15	10	6

DISCUSSION

The results described above show that under the conditions of these experiments, the effect of TA_3 on the five limb-bone rudiments studied is quantitatively and qualitatively indistinguishable from that of T_3; earlier experiments (Fell & Mellanby, 1956) demonstrated that T_3 is roughly four times as potent as T_4 in reducing the growth rate of the femur and tibia in culture. In the same concentration TA_4 had no effect on the explants; whether this compound was indeed incapable of affecting the bones or whether it was rapidly broken down by the culture medium is not known.

The analytical results indicate that when embryonic bones are grown in the presence of T_4 or T_3, simultaneous partial de-iodination of the hormones takes place. T_3 was never detected in experiments in which T_4 had been used, although de-iodination had occurred. The de-iodination *in vitro* of T_4 by brain and muscle preparations without the production of significant amounts of T_3 has previously been described (Tata, 1957; Tata, Rall & Rawson, 1957); the present experiments substantiate the view that in many biological systems T_4 undergoes de-iodination without the production of detectable amounts of T_3. In our experiments iodide probably represents one of the end products of complete break-down of the hormones.

These results, therefore, provide no evidence for the hypothesis that T_4 owes

its biological activity to a process of partial de-iodination, nor has the degradation of the alanine side-chain of the iodothyronine to the corresponding acetic acids been demonstrated.

SUMMARY

1. The investigation was undertaken to see whether limb-bone rudiments grown in culture in the presence of thyroxine (T_4) or triiodothyronine (T_3) produced any detectable transformation of the iodothyronine molecule.

2. T_3 and the iodothyroacetic acids were thought to be likely metabolites of T_4; the effects of these agents and also of potassium iodide on the growth of the explanted humeri, ulnae, radii, femora and tibiae of 6-day chick embryos were therefore studied, all the compounds being added to the culture medium in a concentration of 16 μg/100 ml.

3. Triiodothyroacetic acid (TA_3) diminished the growth of the tibia, femur and humerus in that order of degree, had little or no effect on the ulna and increased the growth of the radius; its effects were indistinguishable from those of T_3.

4. Tetra-iodothyroacetic acid (TA_4) had no appreciable effect on the rudiments.

5. In control experiments potassium iodide had no effect on the explants.

6. To investigate the effect of the limb-bones on T_4 and T_3, tibiae and femora from 6–8-day embryos were grown in medium containing ^{131}I-labelled T_4 and T_3; the break-down products of the hormones present in the explants, in the medium on which the rudiments had grown and in 'blank' medium incubated without explants were examined by chromatographic analysis.

7. T_4: at the end of the incubation period T_4 and iodide were the only radioactive compounds detected. T_3: only labelled T_3 and iodide were found.

8. The medium caused some de-iodination of T_4 but less than that produced by the explants; there was considerable de-iodination of T_3 by the rudiments, but very little by the medium.

9. These results support the view that in many biological systems T_4 is de-iodinated without the production of detectable amounts of T_3, TA_3 or TA_4.

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