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The Metabolism of Cholecalciferol in the Liver of Japanese Quail (*Coturnix coturnix japonica*) with Particular Reference to the Effects of Oestrogen

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1. Studies were carried out *in vitro* with the livers of Japanese quail that had been fed from hatching on diets supplying their full requirements for vitamin D. 2. 25-Hydroxycholecalciferol was the major metabolite when liver homogenates of egg-laying female and oestrogen-treated quail of both sexes were incubated with [^{3}H]cholecalciferol. 2. Very little 25-hydroxycholecalciferol was generated from liver homogenates of adult male and immature quail. Instead the cholecalciferol was converted into one or more compounds less polar than 25-hydroxycholecalciferol and into a number of highly polar metabolites, some of which were water-soluble. 4. Oestrogen not only stimulated the 25-hydroxylation of cholecalciferol but also protected both cholecalciferol and 25-hydroxycholecalciferol from degradation by the enzymic pathways active in immature and male birds. 5. These actions of oestrogen may be of physiological significance in relation to the high requirements of laying birds for 1,25-dihydroxycholecalciferol to support the intense metabolism of calcium associated with egg-shell calcification.

The most profound changes occur in the liver metabolism of female birds at the onset of the reproductive period, associated with the synthesis of lipids and proteins destined for the egg-yolk. These changes are induced by oestrogens and persist throughout the laying period (Hillyard *et al.*, 1956). Equally profound changes occur in the calcium metabolism of birds at this time in anticipation of the increased requirements of calcium for egg-shell formation (Taylor, 1965). One aspect of the altered calcium metabolism is an increase in the absorption of calcium from the gut associated with an increase in calcium-binding protein in the intestinal mucosa under the influence of the active metabolite of vitamin D_3, 1,25-dihydroxycholecalciferol (Corradino, 1973; Corradino *et al.*, 1976). The first step in the pathway of cholecalciferol metabolism involves its hydroxylation in the liver to 25-hydroxycholecalciferol (Ponchon *et al.*, 1969), and one of the objects of the present work was to investigate the possibility that oestrogens may increase 25-hydroxylation in the liver. Another part of this investigation was concerned with the normal metabolism of cholecalciferol in the liver of immature and adult birds of both sexes.

Materials and Methods

Birds and their treatment

The Japanese quail were hatched and reared in our own animal house and fed on commercial turkey-starter crumbs containing (per kg) 10 g of Ca, 8 g of P and 50 μg (2000 i.u.) of cholecalciferol. They were killed by decapitation.

Chemicals

Glucose 6-phosphate dehydrogenase (type XI), glucose 6-phosphate, L-malic acid and $NADP^+$ were purchased from the Sigma Chemical Co. (London, S.W.6, U.K.), silica gel GF_{254} (type 60) was from Merck A.G. (Darmstadt, West Germany), scintillants were from Koch–Light Laboratories (Colnbrook, Bucks., U.K.), [1α,2α-^{3}H]cholecalciferol (4.6–12.6 Ci/mol) and 25-hydroxy[26,27-^{3}H]cholecalciferol were from The Radiochemical Centre (Amersham, Bucks., U.K.) and oestradiol-17β dipropionate was a gift from CIBA (Horsham, Surrey, U.K.).

Conditions for studying the metabolism of cholecalciferol in vitro

The liver was excised as quickly as possible, cut into several pieces and washed in ice-cold sucrose (300 mM). Approx. 1 g of tissue was blotted and placed in a pre-weighed glass homogenizer tube, which was then re-weighed. The tube was returned to the ice bucket, and 9 ml of ice-cold sucrose (300 mM) was added/g of liver. Homogenization was carried out by hand (12 strokes) on ice with a loose-fitting pre-cooled Teflon pestle. The homogenate (2 ml) was added to an ice cold 100 ml conical flask containing 8 ml of potassium phoshate buffer, pH 7.4, in which was dissolved an

NADPH-generating system. The final incubation mixture (10ml) contained KH_2PO_4 (0.2 M), Mg^{2+} (4mM), L-malate (7.5mM), glucose 6-phosphate (3.5mM), $NADP^+$ (0.3mM) and glucose 6-phosphate dehydrogenase (6 units). The reaction was initiated by the addition of 30pmol of [^{3}H]cholecalciferol in 50 μl of ethanol. The flasks were incubated in air in a shaking water bath (80 oscillations/min) at 37°C for 2h. These incubation conditions were established as optimal for this tissue in a series of preliminary experiments: when greater amounts of homogenates were used the t.l.c. plates used for separating the steroids were overloaded with lipid and separation was unsatisfactory.

Extraction, separation and counting of the radioactivity of labelled steroids

At the end of the incubation period the mixture was poured into a 100ml polypropylene centrifuge tube and extracted with a total volume of 34ml of chloroform/methanol (1:2, v/v), according to the procedure of Bligh & Dyer (1959). The chloroform-soluble residue from this extraction was taken up in a small volume of choroform for t.l.c. on 100mm × 200mm glass plates coated with silica gel 0.75 mm in thickness. The lipid extract was applied in a narrow band across the bottom of the plate, and enough non-radioactive cholecalciferol to be visible under u.v. light was added as a marker. The plates were run in the dark at 4°C with, routinely, benzene/acetone (9:1, v/v) as solvent, although several other solvent systems were employed to characterize and identify the metabolites in particular studies. At the end of the run the cholecalciferol band, about 10mm in depth, was scraped into a scintillation vial and successive 5 mm bands were removed between the cholecalciferol band and the origin. A similar band was also removed above the cholecalciferol region. Standards containing [^{3}H]cholecalciferol and 25-hydroxy[^{3}H]-cholecalciferol were run in the presence of lipid material extracted from 2ml of a 10% (w/v) liver homogenate. The sterols were solubilized in 10ml of scintillation fluid [8g of 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD)/litre of toluene], the silica was allowed to settle for 1 h and the ^{3}H radioactivities of the samples were then counted in a Philips liquid-scintillation counter with automatic quench correction.

After the lipid-soluble components of the incubation mixture had been extracted with chloroform, radioactivity was determined on a sample (1 ml) of the aqueous phase by liquid-scintillation counting after the addition of 10ml of a Triton X-100-based scintillation fluid [500ml of Triton X-100, 4g of 2,5-diphenyloxazole (POP), 500mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl-POPOP) and 1 litre of toluene].

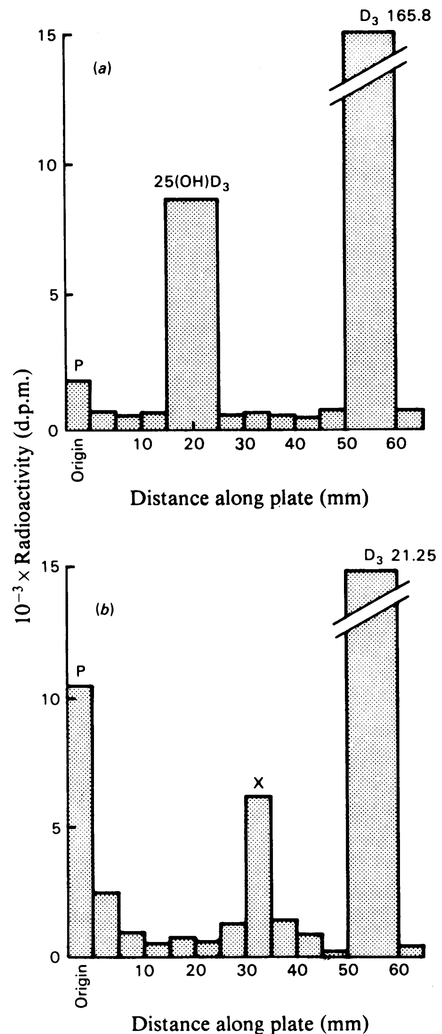

Fig. 1. *Typical t.l.c. profiles obtained by incubating [^{3}H]-cholecalciferol with liver from (a) a laying female quail and (b) an immature quail 3 weeks of age*

Peak P represents the radioactivity remaining at the origin and peak X the unknown metabolite running between the 25-hydroxycholecalciferol [25(OH)D_3] and cholecalciferol (D_3) peaks in the liver extracts from immature (and mature male) birds.

Results

Metabolism of [^{3}H]cholecalciferol in liver homogenates from mature and immature birds of both sexes

A typical t.l.c. profile for the tritiated lipid-soluble material extracted from the liver of a mature (laying) female is shown in Fig. 1(*a*); mature cocks and immature birds of both sexes (3–4 weeks of age) gave

Table 1. *Metabolism of cholecalciferol in the livers of male and female quail*
Liver homogenates of adult male, laying female and immature female quail were incubated with [^{3}H]cholecalciferol, and the radioactivities of the various fractions isolated by t.l.c. and of the water-soluble products were measured as described in the Materials and Methods section. Values are expressed as mean percentages of the total radioactivity recovered (±S.E.M.) for four birds per group.

	Radioactivity recovered (%)		
	Adult male	Immature female	Laying female
Unchanged cholecalciferol	49.4±9.6	46.9±8.2	91.0±1.0
Peak X*	9.8±1.0	11.0±1.6	—
25-Hydroxycholecalciferol	—	—	4.6±0.2
Peak P (polar metabolites at origin)*	13.5±0.9	12.5±1.3	1.0±0.1
Water-soluble products	27.3±1.1	29.6±2.8	3.4±0.7

* See Fig. 1(*b*).

Table 2. *Effect of oestrogen on the metabolism of cholecalciferol in the liver of male quail*
Mature male birds were injected with oestradiol-17β dipropionate (10μg/g body wt.). Then 3 days later homogenates of treated and control birds were incubated with [^{3}H]cholecalciferol, and the radioactivities of the various fractions isolated by t.l.c. and of the water-soluble products were measured as described in the Materials and Methods section. Values are expressed as mean percentages of the total radioactivity recovered (±S.E.M.) for eight birds per group.

	Radioactivity recovered (%)	
	Control	Oestrogen-treated
Unchanged cholecalciferol	39.2±7.4	91.5±6.3
Peak X*	11.1±0.8	—
25-Hydroxycholecalciferol	—	5.5±0.2
Peak P (polar metabolites at origin)*	4.5±0.9	1.0±0.2
Water-soluble products	35.2±2.1	2.0±0.3

* See Fig. 1(*b*).

profiles similar to the one shown in Fig. 1(*b*). Significant amounts of 25-hydroxycholecalciferol were observed only in the lipids extracted from the laying female. In male birds and immature females the major products consisted of one or more compounds less polar than 25-hydroxycholecalciferol (peak X), together with one or more polar metabolites (peak P) that remained at the origin. The radioactivity remaining in the aqueous phase was also high and that of the unchanged cholecalciferol low compared with the results obtained with laying birds. Peak X material was not formed by the livers of laying birds and peak P was quite small. Table 1 shows the percentage distribution of the total radioactivity recovered from incubations of adult cock, immature female and laying female birds.

Identification of 25-hydroxycholecalciferol and partial characterization of peak X

The biologically produced 25-hydroxycholecalciferol was rechromatographed on the same plate as standard 25-hydroxy[^{3}H]cholecalciferol in three different solvent systems, ethyl acetate / n-heptane (1:1, v/v), benzene/acetone (9:1, v/v) and chloroform, and the mobilities of both peaks were identical in all systems. Furthermore, when the 25-hydroxycholecalciferol produced by liver homogenates of laying hens was acetylated with a mixture of acetic anhydride (0.1 ml) and pyridine (0.2 ml) in the dark at room temperature for 15 h, the resulting 3β-acetate co-chromatographed with authentic 25-hydroxycholecalciferol 3β-acetate with benzene/acetone (9:1, v/v) as solvent.

The mobility of peak X was only slightly greater than that of 25-hydroxycholecalciferol, and the possibility that 25-hydroxycholecalciferol was one of the components of peak X had to be considered. Accordingly, an incubation with mature cock liver was carried out and the lipid extract divided into two portions, to one of which standard 25-hydroxy[^{3}H]-cholecalciferol was added. The two extracts were chromatographed on the same plate, and, whereas only one peak (peak X) appeared between the origin and the cholecalciferol peak with the control extract, two distinct peaks were apparent with the extract to which standard 25-hydroxycholecalciferol had been added.

Peak X material was saponified by refluxing with 5 ml of 10% (w/v) KOH in methanol in the presence of α-tocopherol (0.5 mg) for 0.5 h in an atmosphere of N_2, and the products were extracted with diethyl ether. Chromatography of the extract gave two peaks, both of which were more polar than peak X itself. One peak was slightly more polar than standard 25-hydroxy[^{3}H]cholecalciferol and the other remained at the origin. These observations suggest that the peak X compound contains at least two ester groups either

on the same or on different molecules: if on the same molecule, one of the groups would need to be somewhat resistant to hydrolysis.

When peak X material was acetylated with acetic anhydride in pyridine, the polarity of the product was very greatly diminished, demonstrating that the peak X compound possessed a primary or secondary hydroxy group.

Effect of oestrogen on the activity of cholecalciferol 25-hydroxylase

Mature male quail were given a single intramuscular injection of a solution of oestradiol-17β dipropionate in ethyl oleate (10μg/g body wt.), and the metabolism of cholecalciferol in their livers was studied 3 days later. Control birds were injected with the solvent. The results are given in Table 2, and show that the effect of the oestrogen treatment was to change the metabolism of cholecalciferol from the male pattern to that of the laying female. Similar results were obtained when immature female quail were injected with oestrogen.

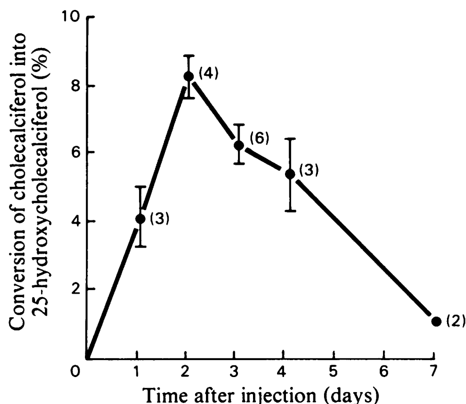

Fig. 2. *Time course study showing the effect of a single injection of* 10μg *of oestradiol dipropionate/g body wt. on the activity of cholecalciferol 25-hydroxylase in liver homogenates of immature female quail incubated for* 2h *with* 30*pmol of* [^{3}*H*]*cholecalciferol*

For experimental details see the text. Numbers of observations are given in parentheses.

Time-course study of the effect of oestrogen on cholecalciferol metabolism in the liver

Immature female quail (21–28 days of age) were given a single intramuscular injection of oestradiol-17β dipropionate (10μg/g body wt.) and the activity of cholecalciferol 25-hydroxylase in their livers was studied over the following 1–7-day period. The results of this experiment are shown in Fig. 2. Even after 1 day the activity of the enzyme was greatly increased, and the maximum conversion of cholecalciferol into 25-hydroxycholecalciferol was observed after 2 days; thereafter the rate of conversion slowly declined until, by day 7, it had almost returned to the control value.

Metabolism of 25-hydroxy[^{3}H]cholecalciferol in the liver of control and oestrogen-treated adult male birds

When homogenates from the livers of control and oestrogen-treated males were incubated with 25-hydroxy[^{3}H]cholecalciferol under the same conditions as those described previously, a band corresponding to peak X did not appear on the plates. Instead, two peaks more polar than 25-hydroxycholecalciferol appeared; one remained at the origin and the other (peak Y) was located half-way between the origin and 25-hydroxycholecalciferol. About one-third of the total radioactivity recovered from the control homogenates was in the form of water-soluble products, one-third as polar lipid-soluble compounds and the remaining third as unchanged 25-hydroxycholecalciferol. Only small amounts of polar metabolites, lipid-soluble or water-soluble, were produced by the livers of oestrogen-treated birds, and 86% of the 25- hydroxy[^{3}H]cholecalciferol was recovered unchanged (Table 3).

Table 3. *Effect of oestrogen on the metabolism of* 25-*hydroxycholecalciferol in the liver of adult male quail*

Birds were injected with oestradiol-17β dipropionate (10μg/g body wt.). Then 3 days later liver homogenates from treated and control birds were incubated with 30 pmol of 25-hydroxy[26,27-^{3}H]cholecalciferol (12.9 Ci/mmol) under the same conditions as those described in the Materials and Methods section for the [^{3}H]cholecalciferol incubations, and the radioactivities of the various fractions isolated by t.l.c. and of the water-soluble products were measured. A single incubation was carried out with control homogenate that had been heated at 100°C for 10 min. The values are expressed as mean percentages of the total radioactivities recovered (±S.E.M.) for four birds per treatment.

	Radioactivity recovered (%)		
	Control	Oestrogen-treated	Boiled
Unchanged 25-hydroxycholecalciferol	32.6±2.9	86.3±2.2	93.8
Peak Y	7.2±0.6	1.2±0.1	1.0
Polar metabolites at origin	26.2±1.0	4.7±0.1	1.2
Water-soluble products	34.0±1.8	7.8±0.8	4.0

Discussion

Other workers who have studied the metabolism of cholecalciferol in avian liver have used tissue from young chicks of the domestic fowl, and all of the experiments reported have been with birds fed on a vitamin D-deficient diet from hatching or with deficient birds injected with various amounts of vitamin D (Tucker *et al.*, 1973; Bhattacharyya & DeLuca, 1974). Our experiments were carried out with Japanese quail fed from hatching on a normal stock diet, and it may therefore be assumed that, unlike birds used in other studies, their livers were physiologically normal with respect to cholecalciferol; this, we suggest, may be one of the factors responsible for the low production of 25-hydroxycholecalciferol that we observed in the livers of the immature and adult male birds.

Tucker *et al.* (1973) reported that the livers of rachitic birds injected with 14i.u. of cholecalciferol/day for 17 days did not show a decrease in 25-hydroxylase activity compared with uninjected controls, and Bhattacharyya & DeLuca (1974) made similar observations with rachitic chicks given a single injection of 10i.u. of cholecalciferol. However, when the latter authors increased the dose of vitamin D to 50i.u. the activity of the 25-hydroxylase was decreased to 17% of that observed in the vitamin D-deficient (control) chicks after 1 day, and they interpreted their results in terms of a physiological requirement to conserve the vitamin when supplies are abundant. Our results do not support this conclusion: the conversion of cholecalciferol into the 25-hydroxy derivative was certainly low in the liver homogenates of the immature and adult male birds, but destruction of the vitamin was extremely high, namely about 50–60% compared with less than 10% for the laying female and the oestrogen-treated birds.

The experiments performed with 25-hydroxy[^{3}H]-cholecalciferol as substrate showed that homogenates of liver tissue from adult male birds were very active in converting this compound into polar metabolites, and it is possible that any 25-hydroxycholecalciferol produced from [^{3}H]cholecalciferol by the livers of immature and adult males was further metabolized during the 2h incubation period.

The nature of the peak X compound produced in the experiments with liver tissue from immature and adult male birds is not known, but it is unlikely to represent a degradation product of 25-hydroxycholecalciferol, for it did not appear on the t.l.c. plates prepared from extracts of liver homogenates of male birds incubated with labelled 25-hydroxycholecalciferol. It seems more probable that both it and the more polar compounds formed during the incubations with cholecalciferol and with 25-hydroxycholecalciferol are metabolites on alternative pathways of cholecalciferol metabolism leading ultimately to the production of the abundant water-soluble metabolites observed in the homogenates of livers of immature and adult male birds. The latter may be inactive products, analogous to bile acids, that are excreted in the bile.

Experiments performed *in vivo* and studies with livers perfused with [^{3}H]cholecalciferol and 25-hydroxycholecalciferol are clearly needed in order to explore further the metabolism of vitamin D in the livers of immature and adult male birds given diets adequate with respect to the vitamin.

In recent years, research on the metabolism of vitamin D has tended to concentrate on the hydroxylation of 25-hydroxycholecalciferol in the kidney, and interest in the role of the liver has waned. This is because it is recognized that production of the calcium-regulating hormone 1,25-dihydroxycholecalciferol is modulated by a number of ionic and hormonal mechanisms that influence the activity of the kidney 25-hydroxycholecalciferol 1-hydroxylase, whereas there is no general agreement at present as to whether or not the production of 25-hydroxycholecalciferol by the liver is regulated at all (for review see Holick & Clark, 1978), in spite of the work of Bhattacharyya & DeLuca (1974) referred to above. Among the hormones that regulate the 1-hydroxylase in avian kidney are the oestrogens, and Tanaka *et al.* (1976) and Baksi & Kenny (1978) showed that pharmacological doses of oestrogen caused dramatic increases in 1-hydroxylase activity in the kidneys of chickens. The former authors reported that simultaneous administration of androgen was necessary in order to elicit this response, but we have not been able to confirm this observation (S. H. Sedrani & T. G. Taylor, unpublished work).

The results of the experiments reported in the present paper clearly indicate that production of 25-hydroxycholecalciferol by the liver is greatly increased by oestrogen treatment, but it is not possible to say whether this is brought about by an increase in the activity of the 25-hydroxylase or by a decrease in the activity of enzymes responsible for the degradation of cholecalciferol and 25-hydroxycholecalciferol or by both mechanisms operating together. If the same changes in the metabolism of cholecalciferol in response to oestrogen that occur in liver homogenates *in vitro* occur also *in vivo*, they may well be of physiological significance in laying birds, both in conserving vitamin D and in maintaining the plasma concentration of 25-hydroxycholecalciferol.

We conclude from these experiments that, when the dietary intake of vitamin D is high relative to requirements, i.e. in immature and adult male birds fed on a normal stock diet, degradation of the vitamin represents the major pathway followed in the liver. In laying birds, in which requirements for 1,25-dihydroxycholecalciferol are high in relation to the need to absorb large amounts of calcium from the gut

for the calcification of the egg-shell, degradation of cholecalciferol in the liver is suppressed under the influence of oestrogen. The effect of oestrogen on the activity of the 25-hydroxylase must remain an open question, but, by analogy with the known action of oestrogen in other systems, it would not be surprising if it resulted in a increase in enzyme protein synthesis.

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