The metabolism and biological activity of esterified vitamin D in the rat

By D. R. FRASER and E. KODICEK

*Dunn Nutritional Laboratory, University of Cambridge
and Medical Research Council, Cambridge*

1. [1-³H]Cholecalciferol and [1-³H]cholecalciferol palmitate in amounts equivalent to 2 µg cholecalciferol were injected into rachitic rats as aqueous preparations (intravenously) or in arachis oil (intramuscularly).

2. The radioactive faecal metabolites collected for up to 17 days were fractionated according to their polarity. The same pattern of excreted radioactivity was seen with intravenous and intramuscular cholecalciferol and intravenous cholecalciferol palmitate. Intramuscular cholecalciferol palmitate produced a higher proportion of the most polar metabolites in faeces.

3. Parenterally administered cholecalciferol palmitate in a rickets-healing biological assay had activity equivalent to unesterified cholecalciferol.

4. Vitamin D ester synthesized in vivo is considered to be eventually metabolized as vitamin D alcohol and to be available to vitamin D-requiring processes.

Evidence has been presented that fatty acid esters of vitamin D are formed in the rat by enzymes with low specificity for the vitamin (Fraser & Kodicek, 1968c) and that these esters remain in tissues while vitamin D alcohol is metabolized and excreted (Fraser & Kodicek, 1968b). However, it is not apparent whether the esterified vitamin is available to the vitamin D-requiring processes in the animal. To investigate this point an examination was made of the metabolic fate and utilization of parenterally administered synthetic vitamin D ester. In one experiment the faecal radioactivity was measured after parenteral injection of [1-³H]cholecalciferol palmitate. Another experiment compared the biological activities of free and esterified vitamin D.

EXPERIMENTAL

Materials. Radioactive vitamin D was obtained by saponification of [1-³H]cholecalciferol 3,5-dinitrobenzoate and subsequent purification of the product as described by Wilson, Lawson & Kodicek (1967). Unlabelled crystalline cholecalciferol (Koch-Light Laboratories Ltd, Colnbrook, Bucks.) was used to dilute the labelled form to appropriate specific activity. The palmitic acid ester of [1-³H]cholecalciferol (specific radioactivity 141 mc/m-mole) was prepared (Fraser & Kodicek, 1968a) and was bound to bovine plasma albumin in aqueous 0·9 % NaCl (Fraser & Kodicek, 1968c). Free [1-³H]cholecalciferol was similarly bound to albumin. In metabolic studies the concentration of cholecalciferol in these preparations was 2 µg (80 i.u.) per 0·1 ml. For biological assay, unlabelled cholecalciferol and cholecalciferol palmitate were bound to albumin (0·25 %, w/v, in 0·9 % aqueous NaCl) to give a cholecalciferol to albumin weight ratio of 1:500. Three concentrations of both the ester and free vitamin of 0·25,

0·5 and 1 μg vitamin D/0·2 ml were prepared. A low level of [1-³H]cholecalciferol was added to the starting material in the preparation of the esterified and non-esterified vitamin so that each had the same specific radioactivity. This enabled accurate estimation of vitamin D concentration in each preparation. Radioactivity was measured by liquid scintillation spectrometry with a counting efficiency for tritium of 20% (Fraser & Kodicek, 1968*a*).

Diethyl ether was shaken with saturated aqueous ferrous sulphate, washed with water, dried with solid NaOH and distilled and collected over sodium. Anhydrous ethanol was prepared by refluxing and then distilling 95% (v/v) ethanol over magnesium with iodine as catalyst.

Animals and treatment. Rachitic, vitamin D-deficient hooded rats were raised as previously described (Fraser & Kodicek, 1968*a*).

Table 1. *Experimental design for comparison of the metabolism of* [1-³H]*cholecalciferol and* [1-³H]*cholecalciferol palmitate (specific radioactivity 141 mc/m-mole)*

(Three rachitic rats/group)

Group no.	Vitamin D preparation	Quantity of cholecalciferol	Type of injection
1	[1-³H]cholecalciferol bound on albumin in 0·9% NaCl solution	2 μg/0·1 ml	Intravenous
2	[1-³H]cholecalciferol in sterile arachis oil	2 μg/0·1 ml	Intramuscular
3	[1-³H]cholecalciferol palmitate bound on albumin in 0·9% NaCl solution	2 μg/0·1 ml	Intravenous
4	[1-³H]cholecalciferol palmitate in sterile arachis oil	2 μg/0·1 ml	Intramuscular

In the metabolic study, rachitic rats weighing 70–80 g, were divided into four groups of three animals each and were fitted with faecal collection cups as described by Barnes, Fiala & Kwong (1963). [1-³H]Cholecalciferol and [1-³H]cholecalciferol palmitate (specific radioactivity 141 mc/m-mole) were administered according to the scheme in Table 1. Faeces were collected daily and were stored at −15°.

The biological assay was set up and vitamin D activity was measured by a method based on that of Bourdillon, Bruce, Fischmann & Webster (1931). Fifty-four rachitic rats of both sexes, weighing from 60 to 80 g, were divided into three groups, each containing three subdivisions of six rats. Grouping was designed to give an even distribution of litter-mates and body-weights. The mean weight range of the nine subdivisions was 64–72 g. International standard cholecalciferol in arachis oil, and albumin-bound cholecalciferol and cholecalciferol palmitate were administered according to the scheme in Table 2. Radiographs of the right tibial proximal metaphysis of each rat were made at 7 and 14 days. The degree of healing was assessed visually on the radiographs and was given a subjective score from 0 to 12 in comparison with a standard scale of Bourdillon *et al.* (1931).

Extraction of faecal radioactivity. The daily faeces collected from each rat were combined into two periods over 17 days of the metabolic study (see Fig. 1); the

samples were lyophilised and powdered. Each sample was extracted on a Soxhlet apparatus, first with anhydrous diethyl ether for 6 h and then with 100% ethanol for the same time. The residue was shaken for 24 h with two changes of 50 ml ethanol-water (1:1, v/v) and was then filtered. The three extracts were each reduced in volume to 10 ml and radioactivity was measured in triplicate 0·1 ml aliquots. It was necessary to add 1·5 ml ethanol to the most polar extracts to facilitate solution in the scintillator liquid. Quenching was calculated with the use of n-[1,2-^{3}H$_2$]hexadecane as an internal standard.

Table 2. *Experimental design for the biological assay of cholecalciferol and cholecalciferol palmitate*

Group no.	Vitamin D preparation	Route of administration	Subgroup*	Dose as cholecalciferol (μg)
1	Cholecalciferol in arachis oil	Oral	1	0·25
			2	0·5
			3	1
2	Cholecalciferol in aqueous albumin solution	Intraperitoneal	4	0·25
			5	0·5
			6	1
3	Cholecalciferol palmitate in aqueous albumin solution	Intraperitoneal	7	0·25
			8	0·5
			9	1

* Each subgroup contained six rats.

RESULTS AND DISCUSSION

Metabolic studies

When vitamin D is given intramuscularly in oil, it is released very slowly and, compared with oral administration, is inefficient in the healing of rickets (Brown & Sturtevant, 1949). Oil depots of 0·5 mg cholecalciferol in chickens were absorbed slowly over 7 months (Bots, 1957) and the level of vitamin D in the blood of sheep given a single intramuscular injection of 25 mg cholecalciferol in oil was elevated for 5 months afterwards (Quaterman, Dalgarno & Adam, 1964). In this present experiment an oily injection of free and esterified [1-^{3}H]cholecalciferol enabled the metabolism of very small amounts that were continuously released to be compared with the metabolism of a relatively large single dose given intravenously.

The three extraction solvents fractionated the vitamin D metabolites in faeces according to their polarity (Kodicek, 1958). Hence an approximate measure of the relative metabolism of esterified and free vitamin D was obtained. The mean percentages of radioactivity in each extract are illustrated in Fig. 1. More radioactivity was excreted in the 7 days after intravenous injection of the free vitamin (36% of dose) than of the ester (27% of dose). Only 2·3% and 0·2% respectively of the dose were found in faeces over 7 days after intramuscular injection of the two forms of vitamin D. However, despite differences in amount, there was little difference in the proportion of faecal radioactivity in the three extracted fractions between intramuscular and
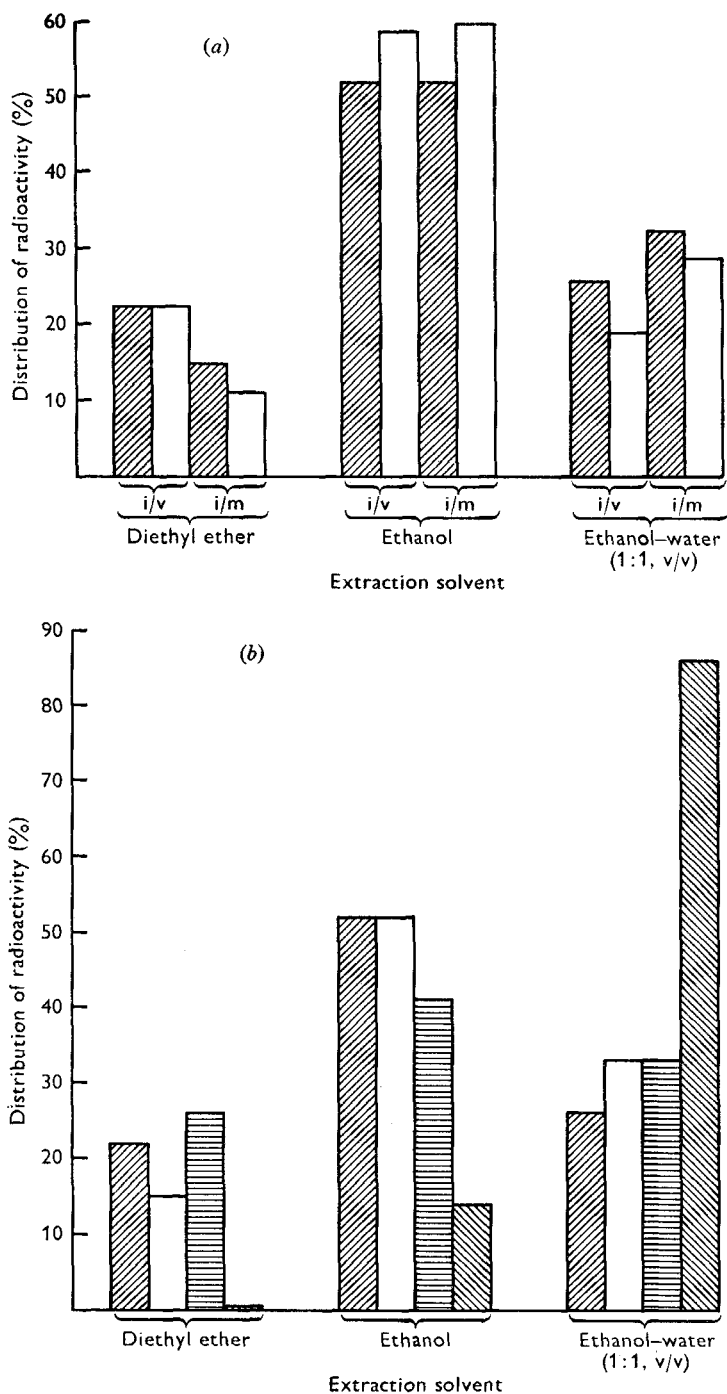
Fig. 1. A comparison of radioactive fractions extracted by diethyl ether, ethanol and ethanol–water (1:1, v/v) from faeces of rats. (a) 0–7 day (▨) and 7–17 day (☐) pooled collections after 2 μg [1-³H]cholecalciferol given intravenously on bovine serum albumin (i/v) or given intramuscularly in arachis oil (i/m). Three rachitic rats were used for each method of administration. (b) 0–7 day pooled collections after administration of 2 μg [1-³H]cholecalciferol, intravenously (▨) or intramuscularly (☐) or 2 μg [1-³H]cholecalciferol palmitate, intravenously (▤) or intramuscularly (◫). Three rachitic rats were used for each method of administration.

intravenous vitamin D alcohol (Fig. 1*a*). Even between the two time intervals of 0–7 and 7–17 days after injection the composition did not change.

Intravenous administration of cholecalciferol and cholecalciferol palmitate gave a similar pattern of faecal excretion products (Fig. 1*b*). Thus it would appear that vitamin D ester and vitamin D alcohol are metabolized in a similar fashion. This suggests that the ester is hydrolysed and that the resulting free alcohol then undergoes its usual metabolic changes. It is now considered that this metabolism occurs regardless of the dose since the small amount of cholecalciferol gradually released from the intramuscular oil depot was handled in the same way as the single intravenous injection. After 17 days approximately 90% of the original radioactivity was found at the site of intramuscular injection, so in effect a dose of 0·2 μg (8 i.u.) had been given over this time. On this basis, the radioactivity in faeces from these rats as a proportion of the dose (23% of 0·2 μg) was similar to that of the intravenously injected rats (26% of 2 μg).

In contrast to these results the excretion pattern after intramuscular injection of cholecalciferol palmitate was different. Nearly all the radioactivity was in the most polar fraction and none could be extracted with diethyl ether (Fig. 1*b*). It is possible that the small amount of palmitate released from the depot over the period of 7 days is metabolized more slowly and gives more polar metabolites than a similar quantity of cholecalciferol alcohol.

Biological assay

The mean values of healing for the six rats at each dose level were plotted against the dose level in Fig. 2. The 95% fiducial limits from the regression lines fell within 73% to 137% of these mean values. At both 7 and 14 days the activities of the three vitamin D preparations appeared similar. This confirmed that esterified vitamin D had high biological activity. However, on close examination some differences are seen between the two graphs. Although there is no significant difference between the
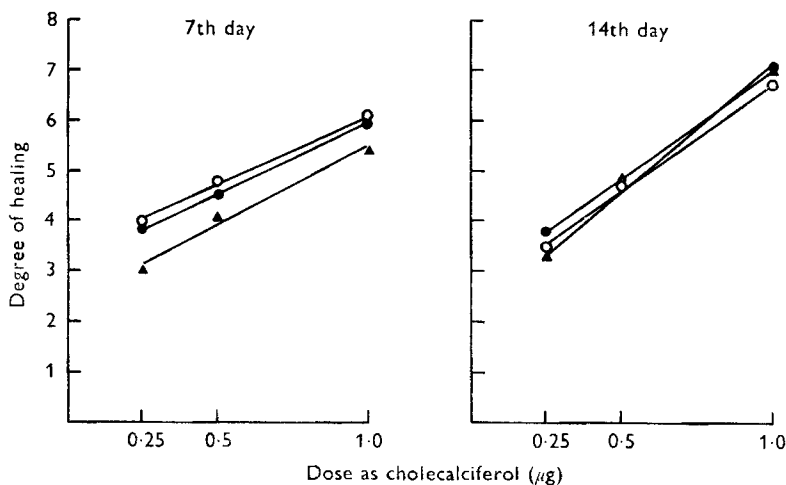

Fig. 2. Comparison of the healing of rachitic lesions in rats by cholecalciferol given orally (○————○) or intraperitoneally (●————●) or cholecalciferol palmitate given intraperitoneally (▲————▲). Each point represents the mean score of healing for six rats.

biological activity of the three vitamin D preparations, at 7 days there is a tendency for the palmitate at each dose level to have less activity than the free cholecalciferol. At 14 days all activities were the same. This may indicate that the vitamin continues to be released from its ester and is therefore more efficient at healing rickets than one dose of the free vitamin. Unfortunately the rats were not maintained for more than 14 days and the long-term effect of the single dose was not determined.

Others have found that esterified vitamin D has diminished biological activity when given orally (Bailey, 1943; Norman, Lund & DeLuca, 1964). This may be explained by reduced absorption of the vitamin from the intestine either as the ester or in the free form after hydrolysis. However, when vitamin D ester is administered parenterally it is as active as the free vitamin. It is concluded therefore that vitamin D ester, synthesized in vivo, can be of functional significance.

REFERENCES

Bailey, B. E. (1943). *J. Fish. Res. Bd Can.* **6**, 103.
Barnes, R. H., Fiala, G. & Kwong, E. (1963). *Fedn Proc. Fedn Am. Socs exp. Biol.* **22**, 125.
Bots, J. P. L. (1957). *Recl. Trav. chim. Pays-Bas Belge* **76**, 209.
Bourdillon, R. B., Bruce, H. M., Fischmann, C. & Webster, T. A. (1931). *Spec. Rep. Ser. med. Res. Coun.* no. 158.
Brown, R. A. & Sturtevant, M. (1949). *Vitams Horm.* **7**, 189.
Fraser, D. R. & Kodicek, E. (1968a). *Biochem. J.* **106**, 485.
Fraser, D. R. & Kodicek, E. (1968b). *Biochem. J.* **106**, 491.
Fraser, D. R. & Kodicek, E. (1968c). *Biochem. J.* **109**, 457.
Kodicek, E. (1958). *Proc. int. Congr. Biochem.* iv. *Vienna* p. 198.
Norman, A. W., Lund, J. & DeLuca, H. F. (1964). *Archs Biochem. Biophys.* **108**, 12.
Quaterman, J., Dalgarno, A. C. & Adam, A. (1964). *Br. J. Nutr.* **18**, 79.
Wilson, P. W., Lawson, D. E. M. & Kodicek, E. (1967). *Biochem. J.* **103**, 165.