J. steroid Biochem. Vol. 22, No. 2, pp. 187–193, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

THE DISTRIBUTION OF (5E)-(10S)-10,19-DIHYDROERCALCIOL AND ITS METABOLITES IN SERUM OF RATS

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(*Received* 11 *January* 1984)

Summary—The distribution of radioactivity in serum of male rats (275 g) after oral administration of tritium labelled (5E)-(10S)-10,19-dihydroercalciol (dihydrotachysterol$_2$) has been studied as a function of time and dose level. Nine distinct radioactive substances, chromatographing in the range between calciol and calcitriol, could be demonstrated. No attempt was made to establish chemical structures. After a single dose of 1.84 nmol, (5E)-(10S)-10,19-dihydroercalciol is very rapidly metabolized to more polar forms. The time course of appearance, ranging between 30 min and 1 day after administration, and the polarity of these substances indicated that they might be formed in sequence. The highest serum concentrations of the major substances occurred between 2 h and 10 h after administration, but compared with the dosage they were very low. In response to daily administration the concentrations of the major substances achieved steady-state levels within 1–4 days. The metabolism of (5E)-(10S)-10,19-dihydroercalciol was apparently not affected by its nutritional status at the dose level studied. After single administration of progressively increasing doses, ranging from 1.84 nmol to 1.84 μmol, the relative increments of the concentrations of the major substances rose in proportion to the relative increases of the dosage. The mechanisms responsible for the appearance of these substances in serum were found to be closely related. At dose levels up to 18.4 nmol feedback control was apparently absent.

INTRODUCTION

(5E)-(10S)-10,19-Dihydroercalciol [1] is used extensively in treatment of renal osteodystrophy [2] and hypoparathyroidism [3]. Unfortunately, the requirement for DHE is unpredictable in the individual patient and frequent monitoring of serum calcium concentrations is essential, for many patients show occasional hypercalcemic episodes. A factor in the elimination of these therapeutic problems could be the careful monitoring of blood levels of the biologically active forms of DHE. However, assays cannot be developed because a serious lack exists in the understanding of mode and sites of action of DHE. The present understanding of the metabolism and action of vitamins D is based on the numerous studies of the fate of isotopically labelled vitamin D sterols in experimental animals. It is obvious that, in order to elucidate the metabolism of DHE, a similar approach is chosen.

In our preceding report [4] the synthesis of tritium labelled DHE was described. It is the purpose of this study to follow the fate of this tracer in rats after single and daily, oral administration in different dosages by means of chromatographic fractionation of radioactivity in serum.

EXPERIMENTAL

Chemicals

[10S,(19)-^{3}H](5E)-(10S)-10,19-dihydroercalciol, sp. act.: 2.07 PBq/mol, was synthesized in our laboratory [4]. Crystalline DHE was a gift from Duphar b.v., Weesp, The Netherlands. Calcidiol, (24R)-hydroxycalcidiol and calcitriol were gifts from Hoffmann–LaRoche, Basel, Switzerland; their respective tritium labelled analogues were purchased from Radiochemical Centre, Amersham, England. Solvents for HPLC were obtained from Rathburn Chemicals Ltd, Walkerburn, Scotland. Stabilized standard rat serum was a product of Behringwerke A.G., Marburg, Western Germany. Calciol, and all other reagents and solvents were purchased from Merck, Darmstadt, Western Germany.

Animals

Male Wistar rats (Central Institute for Nutrition Research, Zeist, The Netherlands) were housed in

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The trivial names for the sterols used are: calciol, (5Z,7E)-(3S)-9,10-seco-5,7,10(19)-cholestatrien-3-ol; calcidiol, (5Z,7E)-(3S)-9,10-seco-5,7,10(19)-cholestatrien-3,25-diol; (24R)-hydroxycalcidiol, (5Z,7E)-(24R)-9,10-seco-5,7,10(19)-cholestatrien-3,24,25-triol; calcitriol, (5Z,7E)-(1S,3R)-9,10-seco-5,7,10(19)-cholestatrien-1,3,25-triol; (5E)-(10S)-10,19-dihydroercalciol: (5E,7E)-(3S,10S)-9,10-seco-5,7-ergostadien-3-ol; (5E)-(10S)-10,19-dihydrocalciol, (5E,7E)-(3S,10S)-9,10-seco-5,7-cholestadien-3-ol; ercalciol, (5Z,7E,22E)-(3S)-9,10-seco-5,7,10, (19), 22-ergostatetraen-3-ol.

Abbreviations used are: DHE, (5E)-(10S)-10,19-dihydroercalciol; [^{3}H]DHE, [10S(19)-^{3}H](5E)-(10S)-10,19-dihydroercalciol; HPLC, High Performance Liquid Chromatography; sp. act., specific radioactivity.

individual metabolism cages and were given food and water *ad libitum*. Weights ranged between 273 and 300 g. From birth and during the experiments they were fed a diet adequate in Ca (0.85%), P (0.60%) and calciol (2200 U/kg) (Hope Farms, Laboratory Animal Nutrition, Woerden, The Netherlands).

Animal experiments

Although dose equivalence between rat and man is not necessarily unity, the dose (1.84 nmol) used in this study was derived from extrapolation of a therapeutic dose (503 nmol), on the basis of the body mass of man (75 kg) and rat (275 g).

[^{3}H]DHE was dissolved in ethanol and subsequently diluted with unlabelled DHE to a sp. act. of 1.04 PBq/mol. Sum total of the concentrations of the compounds was 36.8 μmol/l. This stock solution was kept in a glass vial under dry nitrogen at -20°C. Throughout the animal experiments purity, sp. act. and exact concentration were determined daily as described in a previous report [4]. Just before oral administration the stock solution was diluted with 1 vol of distilled water. Thus, 100 μl contained 1.84 nmol of [^{3}H]DHE (1.09 MBq).

a. Single administration. Groups of 4 rats were given 1.84 nmol of [^{3}H]DHE by gastric tube, and killed 7.5, 15, 30, 60, 120, 240 and 600 min after dosing. At each point of time blood was collected by heart puncture. Groups of 3 rats, subject to the same procedures, were killed 1, 2, 4 and 8 d after dosing. During gavage the animals were kept under light ether anesthesia.

b. Daily administration. Over 7 days groups of 3 rats received 1.84 nmol of [^{3}H]DHE at 24 h intervals and blood was collected 24, 48, 72, 96, 144 and 192 h after the first administration.

c. Single administration of increasing doses. Groups of 3 rats were given either 1.84, 18.4, 184 or 1840 nmol of [^{3}H]DHE (1.90 MBq) in 100 μl of ethanol–water (1:1, v/v). Blood was collected 10 h after administration. The solutions of [^{3}H]DHE were prepared by addition of unlabelled DHE. The addition of H_2O yielded a relatively stable suspension at the 184 nmol level and caused precipitation of [^{3}H]DHE at 1840 nmol. The latter was stirred vigorously while being drawn into the gastric tube.

Extraction of serum

Serum (1 ml) was extracted successively twice with 1 ml of ethyl acetate–cyclohexane (1:1, v/v) and once with 1.4 ml of methanol–ethyl acetate–water (4:5:5, by vol). The combined supernatants were evaporated to dryness at 40°C under nitrogen and redissolved in 250 μl of HPLC eluent.

High Performance Liquid Chromatography (HPLC)

HPLC was performed with a Model 6000 A solvent delivery system. A Model 440 absorbance detector (254 nm) [Waters Associates, Milford, Massachusetts] was used for determination of the retention volumes of the principal metabolites of calciol.

Radioactivity in lipid extracts of sera of rats after administration of [^{3}H]DHE could be resolved successfully by using a Zorbax Sil 850 column (250 × 6.2 mm; Dupont de Nemours, Den Bosch, The Netherlands) eluted with *n*-hexane–propanol-2–water (85:15:0.05, by vol) at a flow rate of 1 ml/min, and introducing 150 μl of lipid extract solution via an automatic sample processor, Model WISP 710A (Waters Associates, Milford, Massachusetts). Fractions (0.5 ml) were collected directly in glass counting vials with a Multirac 2111 collector (LKB, Bromma, Sweden).

Ultraviolet spectrophotometry

The molecular absorptivities of calcitriol, ε 254 nm = 17.000, and DHE, ε 254 nm = 31.000, were measured in *n*-hexane–propanol-2 (85:15, v/v) with a Pye Unicam P 8800 UV/VIS spectrophotometry (Philips, Eindhoven, The Netherlands).

Measurement of radioactivity

After drying under nitrogen at 40°C residues of HPLC fractions were redissolved in 1 ml of ethanol and 10 ml of liquid scintillator (0.25% PPO and 0.025% POPOP in toluene) was added. Samples were assayed for 10 min in a Packard Tri-Carb Model 3380 liquid-scintillation counter (Packard Instruments Co., Downers Grove, IL) at 50% efficiency. The counting data were corrected for the experimental blank value (background). Readings of which the coefficient of variance was greater than 4% have not been included in the results to follow.

RESULTS

[10S(19)-^{3}H](5E)-(10S)-10,19-dihydroercalciol preserves its original purity and sp. act., corrected for natural radioactive decay, for at least 12 months when dissolved in ethanol and stored at -20°C under nitrogen. To investigate the chemical stability of [^{3}H]DHE in serum, solutions of [^{3}H]DHE (0.184 nmol) in stabilized rat serum (1 ml) were incubated at 37°C for 8 days, the maximal, experimental incubation time in the rat, and subsequently stored at 4°C for 2 weeks, the mean time required to process a serum sample. Neither noticeable changes in chromatographic [4] and spectrophotometric [5] properties, nor loss of radioactivity due to hydrogen exchange could be detected.

Figure 1A and 1B show typical chromatographic profiles of the extract of serum of rats that had received orally 1.84 nmol of [^{3}H]DHE (1.09 MBq) respectively at 4 and 24 h previously. At the 4-h time point the presence of 7 peaks of radioactivity could be established. At 24 h after administration the chromatograms showed two new, minor peaks, "d3" and "d4", but now peak "c1" had disappeared.

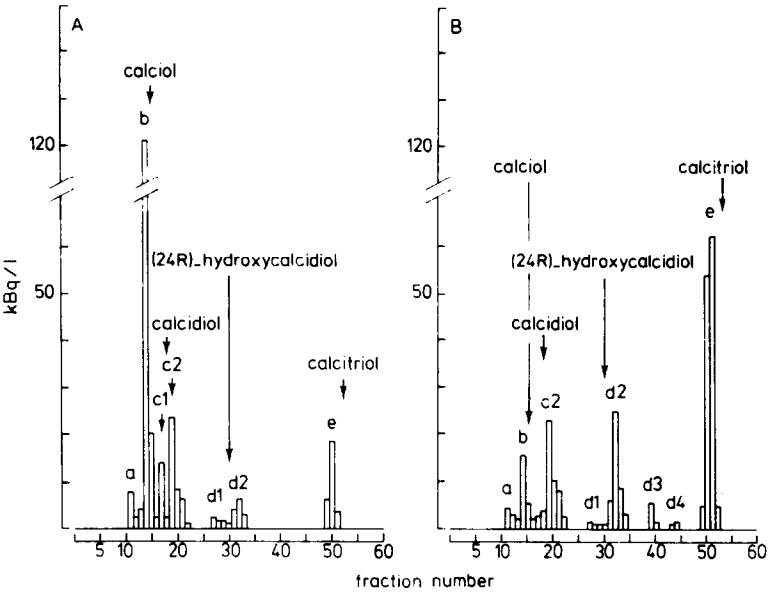

Fig. 1. Typical chromatographic distributions of radioactivity in lipid extracts of serum of rats after an oral dose of 1.84 nmol of [^{3}H]DHE. Blood was collected 4 h (A) and 24 h (B) after administration. Arrows indicate the retention positions of calciol, its principal metabolites and the [^{3}H]DHE derivatives "c1" and "c2". Conditions: see text; ordinate: kilobecquerel per liter of serum; abcissa: fraction numbers.

The data given in Figs 2, 3 and 4 have been corrected for analytical recovery after extraction and chromatography and have been expressed in nmol of tritium per liter of serum since it was presumed that the sp. act. of the material was unchanged. Arguments in support of this presumption will be given at the end of the results section and in the discussion section. To determine the individual analytical recoveries of [^{3}H]DHE and its metabolites a given spike of a rechromatographed peak of radioactivity was ad-

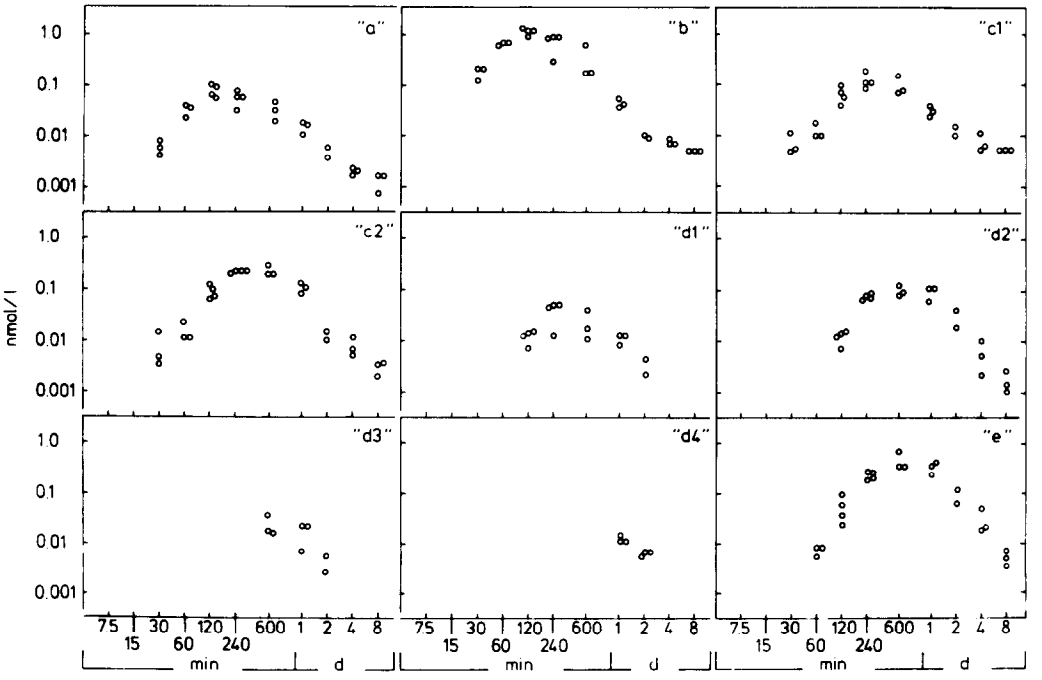

Fig. 2. Appearance and decay of radioactive substances in lipid extracts of serum of rats after an oral dose of 1.84 nmol of [^{3}H]DHE. Each point relates to a single animal. Marks (top corners) relate to the peaks in Fig. 1. For details see text; ordinate: concentration of ^{3}H in nanomole per liter of serum; abcissa: time between administration and bleeding in minute/day.

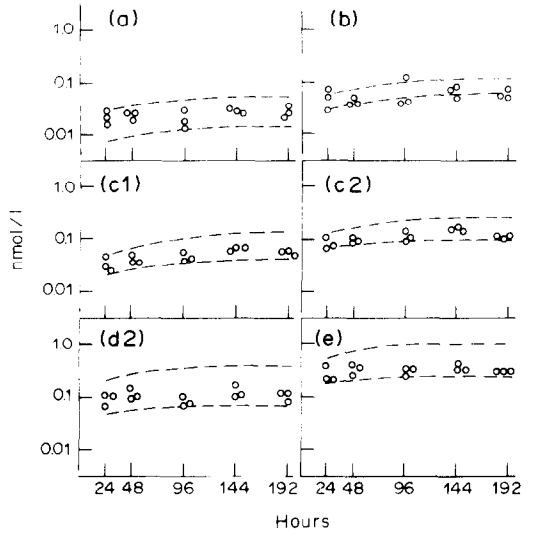

Fig. 3. Course of concentrations of radioactive substances in lipid extracts of serum of rats following daily oral doses of 1.84 nmol of [^{3}H]DHE. Each point relates to a single rat. Marks (top corners) relate to the peaks in Fig. 1. Dashed lines, constructed from the data given in Fig. 2, represent the 95% confidence limits of the expected courses. For details: see text; ordinate: concentration of ^{3}H in nanomole per liter of serum; abcissa: time between the first administration and bleeding in hour.

ded to serum and this was processed through the manipulations described. All [^{3}H]DHE samples reported in this study were analyzed on the same column without prior regeneration. After each 6 samples the column was tested with a reference preparation containing calciol, calcidiol, (24R)-hydroxycalcidiol, calcitriol and their radioactive analogues, but no significant changes in retention values, recoveries, resolution and background radiation could be observed.

Figure 2 shows the sequential changes in concentration of radioactive substances in serum lipid extracts of rats after oral administration of 1.84 nmol of [^{3}H]DHE. Each point relates to a single rat. This type of plot, showing the actual distribution instead of mean and variance, was preferred because of the small number of measurements at each point of observation. Unfortunately, the substances "b", "c1" and "c2" were poorly resolved and overlapped to an extent that made it impossible to determine whether peak "c1" had disappeared entirely 24 h after administration. Since no attempt was made to correct the respective serum concentrations for mutual carryover the radioactivity found in fractions 16 and 17 was considered to belong to substance "c1" at any point of time. Appreciable amounts of radioactivity could not be detected until 30 min after dosage. Peak "b" co-chromatographed exactly with authentic [^{3}H]DHE. The highest concentrations of the major substances were achieved between 4 and 10 h. At the 24-h time point "e" was the major circulating component. Three rats failed to metabolize [^{3}H]DHE to more polar material. Their lipid serum extracts contained merely large amounts of "a" and "b". Autopsy did not reveal any organic cause.

Except for the 30-min time point there existed a significant difference between the amount of radioactivity present in the serum lipid extracts before HPLC (the extractable radioactivity) and the amount of radioactivity recovered after HPLC. This difference which existed in spite of correction for HPLC-recovery per peak rose rapidly from 7.3% (SD = 2.5%; $n = 3$) at the 60-min time point to a plateau of 39.7% (SD = 9.7%; $n = 11$) after 24–192 h. The highest concentration of the mean extractable radioactivity occurred at the 4-h time point. However, expressed as percentage of the dose per ml of serum, it was low, viz 0.11%/ml. Mean extractable radioactivity recovered 24, 48, 96 and 192 h after administration had fallen to 54.1, 14.8, 2.2 and 1.4% respectively of the mean level after 4 h.

Figure 3 shows the increase of concentrations of [^{3}H]DHE and its major derivatives in lipid extracts of serum of rats in response to daily oral doses of 1.84 nmol of [^{3}H]DHE. Each point represents a single

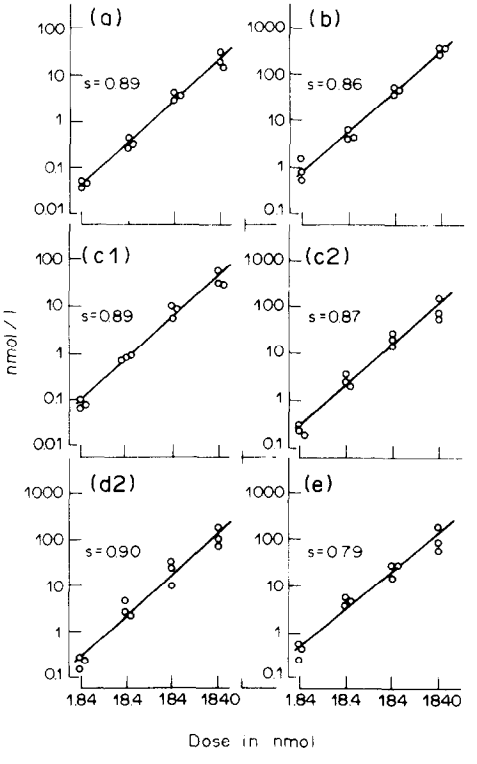

Fig. 4. Course of the concentrations of radioactive substances in lipid extracts of serum of rats after increasing oral doses of [^{3}H]DHE. Blood was collected 10 h after administration. Each point relates to a single animal. Marks (top corners) relate to the peaks in Fig. 1. "s" represents the slope of the regressions. For details: see text; ordinate: concentration of ^{3}H in nanomole per liter of serum; abcissa: dose in nanomole.

rat. Daily administration did not lead to the appearance of new circulating metabolites. Peak "a" and "b" did not show any increment in concentration beyond the first 24 h, the concentrations of the remaining substances achieved steady-state levels within 96 h. The increases of concentrations of all peaks of radioactivity ran within the 95% confidence limits (dashed lines in Fig. 3) of curves calculated from data obtained from the single dose experiment.

Figure 4 shows the course of the concentrations of [^{3}H]DHE and its major derivatives in lipid extracts of serum of rats following increasing oral doses of [^{3}H]DHE. Each point is a value of a single animal. Blood was collected 10 h after administration, assuming that at this point of time the coefficients of variance of the measurements would be minimal (see Fig. 2). At the highest dose level autopsy demonstrated without exception serious microhemorrhages in the liver.

After logarithmic transformation of the variables single regression analysis produced highly significant, positive correlations. The tangents of the slopes of the regressions are smaller than 1 implying that in a linear plot the courses of the serum concentrations are exponential functions of the dose convex with respect to the abcissa. The relative diminutions in the increments of the serum concentrations are directly proportional to the relative increase of the dose. The slopes of the regression lines "a", "b", "c1", "c2" and "d2" are not significantly different, but the tangent of the slope of regression "e" lies just below the lower 95% confidence limit of the distribution. New circulating radioactive substances could not be detected.

u.v. Chromatograms (254 nm) were recorded of all chromatographic runs. In view of the very low concentrations found it is obvious that at the migration position of the various radioactive substances u.v. absorbancies could not be observed, except at the highest dose level. At that level "e" occurred as a prominent, single peak. Granting that at 254 nm the molar extinction coefficient of "e" and authentic DHE are equal (this is true when the diene system in "e" is intact) it is possible to estimate the concentration of "e" in a serum sample from the comparison of its height in the chromatogram with the height of a standard amount of calcitriol. The differences between the serum concentrations of "e" computed in this way and the respective concentrations computed from the radioactive data ranged from -3.7 to $+6.3\%$. Since these differences were not significant in view of the methods used, it was likely that the sp. act. of "e" was equal to that of authentic [^{3}H]DHE. In order to estimate the reproducibility of both methods given spikes of tritiated and unlabelled calcitriol were added to serum. The recovery of radioactivity after extraction and HPLC was $75.9 \pm 5.9\%$ ($n = 6$). The coefficient of variance of the measurements of the peak height was 5.9% ($n = 6$) as well.

DISCUSSION

This study describes for the first time the fate of orally administered [^{3}H]DHE in serum of rats as a function of time and dose level. [^{3}H]DHE is converted rapidly to more polar substances. It is interesting that these substances are cleared rapidly from the blood. As a result repeated daily doses cause only a small increment of the concentrations and steady-state levels are reached within 24–96 h. The study indicates conditions leading to relatively high serum concentrations of some of the substances. However, structural analysis awaits more specific investigation because the chromatographic system used was not suitable for achieving purity requirements.

Since DHE, a 5,6-E analogue of ercalciol [5], shows great resemblances in chemical structure [6] and physiological action to (5E)-(10S)-10,19-dihydrocalciol, calciol and ercalciol [7] studies on the metabolisms of the last compounds were used for designing the present experiments and they can serve as a guide to the explanation of the results. Theoretically it cannot be excluded that the labels at C_{10} and C_{19} in [^{3}H]DHE are lost during *in vivo* metabolism. However, the metabolism of D vitamins takes place predominantly in the side chain attached to C_{17} [8]. The labels in [^{3}H]DHE were stable under some *in vitro* conditions and the observations on [^{3}H]DHE metabolite "e" did not prove any loss of sp. act. Yet, it is wise to consider the results of this study with some reserve [4].

The rate of intestinal absorption of vitamin D sterols depends entirely on the vehicle in which they are administered [9, 10, 11]. The therapeutic situation is approached most closely by giving sterol and food simultaneously. In practice, this method is irrelevant to time course studies in rats. The absorption of D vitamins is optimal when they are administered in vegetable oil [10, 11], but as in saline solutions stabilized by solubilizers, qualitative analysis of small amounts of [^{3}H]DHE in oily solutions is practically impossible. Analysis of ethanolic solutions is simple, but ethanol may cause a gastro-intestinal reflux or may be subject to rapid assimilation in the stomach or the duodenum, resulting in precipitation of the sterol. Despite these disadvantages, ethanol was chosen as vehicle for [^{3}H]DHE, since the feasibility of analysis was considered to be a prerequisite. Moreover, ethanolic solutions have been successfully used in studies of the intestinal absorption of D vitamins [11, 12, 13] as well as in studies of the metabolism of tritiated (5E)-(10S)-10,19-dihydrocalciol [14, 15, 16, 17].

Straight-phase HPLC of serum lipid extracts of rats that had received an oral dose of [^{3}H]DHE showed 9 distinct peaks of radioactivity. Basically the chromatographic patterns shown in Figs 1A and 1B resemble that presented in a previous report [4]. The latter, a pilot study which was solely meant to establish whether [^{3}H]DHE was suitable for tracer

experiments in rats, described 5 peaks of radioactivity namely "a", "b", "c2", "d2" and "e". Since blood samples were drawn exclusively at 24 and 48 h after administration it is obvious that peak "c1" could not be observed. The higher sp. act. of the dose used in the present study may account for the appearance of the smaller peaks "d1", "d3" and "d4". The absolute values and the proportions of the serum concentrations of the major radioactive substances found 24 and 48 h after administration after a single dose of [^{3}H]DHE are substantially different from those reported previously [4]. It is most likely that these conflicting results are associated with the other differences in experimental design, viz. the sex (growth rate) of the experimental animals [18, 19, 20], the batch of [^{3}H]DHE and the volume of the vehicle in which [^{3}H]DHE was administered.

The simultaneous appearance of the radioactive substances "b", "c1" and "c2" in serum of rats 30 min after a single dose of [^{3}H]DHE demonstrates that, in contrast with the metabolism of calciol, [^{3}H]DHE is rapidly metabolized to more polar products which are easily released from their sites of formation. It is important to note that several less obvious factors might be involved in the rapid formation of "c1" and "c2". It is most likely that, once absorbed, [^{3}H]DHE is transported to the blood via the mesenteric lymph [12, 13, 21]. After oral administration of radioactive calciol this is also the site of formation of not yet identified radioactive material chromatographing in the region of the monohydroxy metabolites of calciol [21]. [^{3}H]DHE may partly enter the portal blood [12, 13], thus being forced to pass the liver. In that case "c1" and "c2" can be metabolites, prematurely formed, or degradation products. Lastly, it cannot be excluded that "c1" and "c2" have been formed during digestion. With the exception of peak "a" the time course and polarity of the remaining substances suggest that they might originate from the previously formed derivatives.

The concentrations of radioactivity found in serum of rats after oral administration of 1.84 nmol of [^{3}H]DHE are much lower than have been found with an equivalent dose of radioactive calciol [9]. This result can be ascribed to poor intestinal absorption and/or rapid decay of radioactivity from serum. The latter might be attributed to loss of the radioactive label, rapid distribution among the tissues of the body and a fast rate of excretion. Rapid distribution between the tissues may account for the low serum concentrations found after a single dose of [^{3}H]DHE, but would result in a slow increase of the concentrations during daily administration. However, in response to daily administration the serum concentrations achieved steady-state levels within 24–96 h which suggests that the decay of serum radioactivity is associated with either peripheral catabolism or excretion.

Irrespective of loss of the labels, the difference between the extractable radioactivity and the HPLC-recoverable radioactivity suggests the presence of one or more very polar metabolites. Although studies with radioactive calciol [22, 23] showed that renal and fecal excretion account for only a small part of the decrease in serum radioactivity, a relatively high and proportionally steady concentration of very polar [^{3}H]DHE material might indicate that excretion quantitatively contributes to the disappearance of radioactivity from the circulation. Current studies with [^{3}H]DHE should resolve these questions.

After a single dose of [^{3}H]DHE proportionally negligible amounts of radioactivity are left in the circulation beyond the first day. On that account the daily increments of the serum concentrations in response to daily doses were expected to be very small. The means of the actual increments were within the 95% confidence limits of courses calculated from the data obtained in the single dose experiment. This finding implies that the metabolism of [^{3}H]DHE in rats is not affected by the DHE nutritional status at the dose level studied.

The proportion of a single dose of [^{3}H]DHE converted to its major radioactive derivatives in serum decreases with increase in dose. Although the respective log–log courses in Fig. 4 are significantly linear, they show a slight tendency to curving. In the first dose interval the tangents of the slopes are approx 1. Thus, at dose levels up to 18.4 nmol the serum concentrations of "c1", "c2", "d2" and "e" are directly proportional to the dose, indicating that in that range feed-back regulation of the respective serum concentrations is absent. The parallelism between the regressions "a", "b", "c1", "c2" and "d2" demonstrates that the mechanisms responsible for the appearance of these substances in serum are closely related.

Acknowledgement—This study was supported by a grant of the Dutch Kidney Foundation (Nederlandse Nierstichting).

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