Investigations on Metabolites of Vitamin D in Rat Bile

SEPARATION AND PARTIAL IDENTIFICATION OF A MAJOR METABOLITE

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(Received 18 July 1969)

1. Young rats with cannulated bile ducts were given 0.34 mg. of [1α - ^3H]cholecalciferol or 0.54 mg. of [^{14}C]ergocalciferol by intravenous infusion. Of the radioactivity in the dose of [1α - ^3H]cholecalciferol 31% was recovered in bile within 24 hr. 2. The metabolites in bile were separated by gradient-elution column chromatography on silicic acid into five components, all more polar than cholecalciferol or 25-hydroxycholecalciferol. [^{14}C]Ergocalciferol gave a similar pattern of metabolites in bile. 3. The three most polar metabolites were shown to be ionic. The major component has been identified as a glucuronide conjugate, which was not identical with synthetic cholecalciferol glucuronide.

That vitamin D is metabolized by the rat to a number of more polar breakdown products was first demonstrated by Kodicek (1960). With the availability in recent years of specifically tritiated vitamin D, particularly [1α - ^3H]cholecalciferol (Callow, Kodicek & Thompson, 1966) and [$1,2$ - $^3\text{H}_2$]cholecalciferol (Neville & DeLuca, 1966), extensive studies of the distribution of the vitamin and its metabolites have been undertaken, and have led to the conclusions that an oral dose of vitamin D is absorbed from the intestine into lymph (Schachter, Finkelstein & Kowarski, 1964; Fraser & Kodicek, 1968) and is eventually excreted in the faeces (Kodicek, 1960; Callow *et al.* 1966), presumably via the bile.

Characterization of the metabolites in plasma and tissues has been attempted by DeLuca and co-workers, who have recently reported the identification of one metabolite, obtained from porcine plasma, as 25-hydroxycholecalciferol (Blunt, DeLuca & Schnoes, 1968). A more polar metabolite occurring in chick intestinal nuclei has been described (Lawson, Wilson & Kodicek, 1969*a,b*; Haussler, Myrtle & Norman, 1968). Callow *et al.* (1966) investigated the metabolites in rat faeces, but did not identify any of the polar metabolites.

The present investigations were undertaken to confirm the biliary route as the main excretory pathway for vitamin D, and to attempt some separation and characterization of the metabolites in bile. In a preliminary communication (Bell & Kodicek, 1967) the separation of the metabolites of [1α - ^3H]cholecalciferol in bile and the partial identification of the major radioactive component

of bile as a glucuronide conjugate were reported. A detailed account of this work is presented here.

MATERIALS AND METHODS

[1α - ^3H]Cholecalciferol 3,5-dinitrobenzoate was prepared by the method of Callow *et al.* (1966), and was saponified by the method of Havinga & Bots (1954). The free cholecalciferol was purified by t.l.c. by the method of Wilson, Lawson & Kodicek (1967). [^{14}C]Ergocalciferol was prepared by irradiation of [^{14}C]ergosterol obtained biosynthetically from yeast grown on [^{14}C]acetate (Kodicek, 1959), and was purified in a similar manner. In view of the known instability of vitamin D in aqueous dispersion, and of its stabilization by adsorption on proteins (Chen, Terepka, Lane & Marsh, 1965), material for intravenous injection was given as the albumin-bound complex. The albumin-bound form of the vitamin was prepared by slow injection of a solution of the vitamin in ethanol (0.2 ml.) into an aqueous solution of bovine serum albumin (5 ml.; vitamin/albumin ratio 1:500) in an ice bath at -5° . Solvents were removed by freeze-drying, and the albumin was redissolved in 0.9% NaCl at 0° to give the required concentration of the vitamin. Crystalline cholecalciferol was obtained from Peboe Ltd., Northolt, Middx. Peroxide-free diethyl ether (May and Baker Ltd., Dagenham, Essex) was used throughout. All other solvents and chemicals were of A.R. grade and were used as received. Diazomethane, in ether solution, was prepared from Diazald (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) by the method of De Boer & Backer (1954). Bovine liver β -glucuronidase, of activity 220 000 units/g., was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6. Saccharo-1,4-lactone was obtained from Calbiochem Ltd., London W.1.

T.l.c. was performed with 20 cm.-long plates spread with a layer 0.25 mm. thick of silica gel G (E. Merck A.-G., Darmstadt, Germany) and activated at 110° for 2 hr.

before use. Plates were developed to a 15 cm. solvent front; for determination of radioactivity successive 0.5 cm. or 1 cm. bands of adsorbent were scraped into counting vials and the radioactivity was measured by scintillation counting. Solvent systems used were chloroform (system 1), chloroform-methanol (9:1, v/v) (system 2), or butan-1-ol-ethanol-water (4:2:1, by vol.) (system 3). Column chromatography was carried out on columns (50 cm. $\times$ 1.5 cm.) of silicic acid (Hopkin and Williams Ltd., Chadwell Heath, Essex), packed as a slurry in the least polar solvent used for elution. As some components of mixtures were frequently insoluble in the least polar eluting solvent used, samples (10^4 – 10^5 d.p.m.) were applied to columns by dissolving in the minimum volume of methanol (approx. 1 ml.) and adding sufficient dry silicic acid (approx. 5 g.) to absorb the solution completely. The free-flowing powder so formed was packed on to the column and elution was commenced in the normal way. In some cases the silicic acid was deactivated with water (10%, w/w) before use. Elution commenced with light petroleum (b.p. 60–80°)–diethyl ether (3:1, v/v) (500 ml.) followed by a linear gradient (400 ml.) to diethyl ether, a linear gradient (700 ml.) from diethyl ether to methanol, and a linear gradient (300 ml.) to aq. 70% (v/v) methanol. Where stepwise elution was used, the usual sequence was: light petroleum (b.p. 60–80°)–diethyl ether (3:1, v/v) (600 ml.); diethyl ether (200 ml.); diethyl ether-methanol (9:1, v/v) (200 ml.); diethyl ether-methanol (3:1, v/v) (200 ml.); diethyl ether-methanol (1:1, v/v) (200 ml.); methanol (200 ml.); methanol-water (4:1, v/v) (200 ml.). In certain cases, where separation of the less polar metabolites was not of interest, elution commenced with diethyl ether. The eluate was collected in 10 ml. fractions.

Radioactivity was measured by scintillation counting of samples in a Packard Tri-Carb model 3375 liquid scintillation spectrometer. Counting efficiency was approx. 50% for unquenched ^3H and 85% for unquenched ^{14}C . Quenching was assessed by automatic external standardization by using a standard calibration curve. Solutions for counting were transferred to counting vials, blown to dryness in a stream of air and dissolved in methanol (2 ml.) to which was added 10 ml. of a counting medium consisting of 2,5-diphenyloxazole (PPO) (4 g.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) (0.1 g.) in toluene (1000 ml.). The presence of methanol gave rise to quenching, but was necessary to ensure solution of the more polar metabolites. Samples of silica gel removed from thin-layer chromatograms were counted in the same medium.

Melting points were determined by using capillary tubes in a Gallenkamp and Co. Ltd. melting-point apparatus, and are corrected. The u.v. spectra were recorded for ethanol or methanol solutions on a Unicam SP.700 or SP.800 recording spectrophotometer. The i.r. spectra were recorded for micro KBr discs on a Unicam SP.200 recording spectrophotometer. The n.m.r. spectra were obtained with a Perkin-Elmer n.m.r. spectrometer operating at 60 MHz, with tetramethylsilane as internal reference. Elemental analyses and measurements of optical rotation were performed by the Microanalytical Department, National Institute for Medical Research.

EXPERIMENTAL

Male and female hooded rats were fed for 3–4 weeks from weaning on the rachitogenic diet 2965 of Steenbock &

Black (1925) as modified by Numerof, Sassaman, Rodgers & Schaefer (1955), but with a vitamin D supplement. Their bile ducts were cannulated under anaesthesia and the rats were given [1α - ^3H]cholecalciferol or [^{14}C]ergocalciferol by intravenous infusion and the metabolites extracted. By this means the complications from bacterial action in the intestine, which arise when faecal metabolites of vitamin D are studied, can be entirely avoided.

Bile collection. A young female rat (90 g.), cannulated in the jugular vein and bile duct, was given albumin-bound [1α - ^3H]cholecalciferol (0.34 mg., 141 mc/m-mole) in 0.9% NaCl (1 ml.) by continuous slow infusion into the jugular vein over 18 hr. In the first 2 hr. 16% of the dose was injected; the rate of infusion was then decreased by one-third for the remaining 16 hr. The animal was kept in a restraining cage and allowed access to food and to water containing 0.9% of NaCl and 3% of glucose. Bile was collected at 4 hr. intervals for 24 hr. from the commencement of the infusion. Each 4 hr. bile collection was diluted to a total volume of 100 ml. with acetone-ethanol (1:1, v/v), the radioactivity in each extract being measured by scintillation counting of duplicate samples. Equal volumes of each extract were combined to give an extract representative of the total 24 hr. bile, samples of which were analysed by column chromatography. Samples of the 4–8 hr. and 20–24 hr. bile were similarly investigated.

A young male rat (83 g.), similarly cannulated, was given albumin-bound [^{14}C]ergocalciferol (0.54 mg., 0.1 mc/m-mole) in 0.9% NaCl (0.45 ml.) by intravenous injection, and bile was collected for 24 hr. under conditions similar to those described above.

A sample (6×10^4 d.p.m.) of the 24 hr. bile extract from the rat given [1α - ^3H]cholecalciferol was refluxed with 6 M-HCl (2 ml.) for 1 hr. in an atmosphere of N_2 . Evaporation of the solution to dryness in a stream of N_2 was followed by column chromatography of the residue.

A further sample (6×10^4 d.p.m.) of the 24 hr. bile extract from the rat given [1α - ^3H]cholecalciferol, in methanol (2 ml.), was applied to a 15 cm. $\times$ 1 cm. column of Amberlite IRA-400 anion-exchange resin (acetate form) in methanol. The material eluted from the column with methanol (50 ml.) was rechromatographed on a silicic acid column in the usual way.

Separation of metabolite D from bile. Part of the 24 hr. bile extract from the rat given (1α - ^3H)cholecalciferol was chromatographed on a column of silicic acid, stepwise elution being used to resolve five metabolites. The material eluted with methanol (metabolite D) was rechromatographed by using gradient elution, and the fractions containing the metabolite were pooled. Analysis of a small portion of this pooled extract by the same technique showed that a single symmetrical peak of radioactivity was present.

Distribution studies with metabolite D. Samples of the metabolite (10^4 d.p.m.) in water (0.2 ml.) were added to each of the following two-phase systems, each comprising 10 ml. of each phase: chloroform-water; chloroform-2 M-HCl; chloroform-0.3 M-pyridinium sulphate; butan-1-ol-water. After vigorous shaking, the phases were allowed to separate, and samples were withdrawn from each phase for counting.

Treatment of metabolite D with diazomethane. The metabolite (10^4 d.p.m.) in methanol was treated with an excess of an ethereal solution of diazomethane at 0°. After 1 hr.

solvents and excess of diazomethane were removed in a stream of N_2 , and the residue was examined by t.l.c. in solvent systems 2 and 3.

Treatment of metabolite D with diazomethane and with acetic anhydride. The product from treatment of the metabolite with diazomethane, in pyridine (0.5 ml.), was treated with acetic anhydride (0.2 ml.) for 12 hr. at room temperature. Reagents were removed in a stream of N_2 and the residue, in methanol, was examined by t.l.c. in systems 2 and 3.

Acid hydrolysis of metabolite D. The metabolite (10^4 d.p.m.) was refluxed in 6M-HCl (1 ml.) for 1 hr. Evaporation of the solution to dryness was followed by chromatography of the residue in systems 2 and 3.

Treatment of metabolite D with periodate. The metabolite (10^4 d.p.m.) in methanol (1 ml.) was treated with $NaIO_4$ (100 mg.) in 0.25M- H_2SO_4 (1 ml.) for 1 hr. at room temperature. The solution was extracted with ethyl acetate, the organic extract being washed with water and blown to dryness in a stream of N_2 . The residue was subjected to t.l.c. in solvent systems 2 and 3.

Enzyme hydrolysis of metabolite D. Portions of the metabolite (10^4 d.p.m.) in water (0.2 ml.) were incubated at 37° for various times with 2% bovine liver β -glucuronidase (0.1 ml.) in acetate buffer, pH 5.05 (0.5 ml.). The enzyme was inactivated by heating at 100° for 2 min., or by the addition of 0.01M-saccharo-1,4-lactone (0.1 ml.). The hydrolyses were terminated by placing the reaction tubes in an ice bath. Water (2 ml.) and chloroform (3 ml.) were added, the mixture was shaken vigorously and samples of each phase were withdrawn for measurement of radioactivity.

Cholecalciferol β -D-glucopyranosiduronate. Methyl 2,3,4-tri-O-acetyl-1 α -bromo-1-deoxy-D-glucopyranuronate was obtained from glucuronolactone via methyl tetra-O-acetyl- β -D-glucopyranosiduronate by the method of Bollenback, Long, Benjamin & Lindquist (1955). The tetra-acetyl ester had m.p. 176 – 178° and the bromo ester had m.p. 103 – 104° [Bollenback *et al.* (1955) give m.p. 176 – 178° and 106 – 107° respectively]. Coupling of the bromo ester with cholecalciferol was performed by the method of Becker (1965) for steroid glucuronides. Fresh Ag_2CO_3 was prepared by mixing 2M- $AgNO_3$ (5 ml.) with 3M- Na_2CO_3 (5 ml.) and washing the precipitate successively with water, ethanol, dry acetone and dry benzene. The dry precipitate was then suspended in dry benzene (50 ml.). Part of this suspension (25 ml.), together with cholecalciferol (1 g.) in dry benzene (50 ml.), was placed in a flask equipped for distillation that had previously been oven-dried, and that was closed with $CaCl_2$ drying tubes. Benzene was slowly distilled off, and a solution of the bromo ester (3 g.) in dry benzene (50 ml.) was added at the same rate. The remainder of the suspension of Ag_2CO_3 was added to the reaction mixture in small portions over a 30 min. period, at the end of which the reaction mixture was cooled and filtered. The residue was washed with chloroform, and filtrate and washings were combined and evaporated to dryness under reduced pressure. The residual oil was dissolved in the minimum volume of hot ethanol and kept at 0° to give crystals of methyl (cholecalciferyl 2,3,4-tri-O-acetyl- β -D-glucopyranosiduronate (1.8 g.); m.p. 121 – 123° ; $[\alpha]_D^{25} + 6.8^\circ$ (c 1 in $CHCl_3$) (Found: C, 66.6; H, 8.4. $C_{40}H_{60}O_{10}$, H_2O requires C, 66.8; H, 8.7%); ν_{max} . 1220 (acetate), 1746 (C=O) and 2950 cm^{-1} ; λ_{max} . 266.5 nm . ($E_{1\%}^{1\text{cm}}$. 238);

$\zeta(CDCl_3)$:AB system, A at 3.74, B at 3.89 (J_{AB} 12 Hz, two protons, =CH-CH=), 6.22 [singlet, nine protons, $(CO_2\cdot CH_3)_3$], 7.97 (singlet, three protons, CH_3CO_2-), 9.04 (doublet, J_{AB} 5 Hz, three protons, CH_3-CH), 9.11 (doublet, J_{AB} 6 Hz, six protons, $CH_3-CH-CH_3$) and 9.44 (singlet, three protons, CH_3-C) p.p.m. The compound had R_F 0.56 on t.l.c. on silica gel in chloroform, compared with R_F 0.41 for cholecalciferol.

The methyl triacetyl ester (400 mg.) was dissolved in hot ethanol (5 ml.) and refluxed with 4M-NaOH (0.5 ml.) for 1 hr. A brown precipitate formed, which was filtered off, washed with a little ethanol and recrystallized from aq. ethanol to give sodium (cholecalciferyl β -D-glucopyranosiduronate (130 mg.); m.p. 152 – 153° (decomp.); λ_{max} . 265 nm . ($E_{1\%}^{1\text{cm}}$. 251); ν_{max} . 1050, 1610 (C=O), 2950 and 3440 (OH) cm^{-1} . Satisfactory analytical values could not be obtained for this sample. The β -configuration was confirmed by enzymic hydrolysis of the compound with β -glucuronidase and identification of the liberated cholecalciferol by t.l.c. The compound had R_F 0.41 on t.l.c. on silica gel in butanol-1-ol-ethanol-water (4:2:1, by vol.) and R_F 0.30 in chloroform-methanol-acetic acid (8:1:1, by vol.).

Co-chromatography of metabolite D with authentic cholecalciferyl glucuronide. (a) A solution of the metabolite (2.5×10^4 d.p.m.) and authentic sodium cholecalciferyl β -D-glucuronide (5 mg.) in water (10 ml.), 1M-pyridinium sulphate (10 ml.) and saturated NaCl soln. (10 ml.) was extracted with chloroform ($3 \times 20\text{ ml.}$). The combined chloroform extracts were evaporated to dryness under reduced pressure, and the residue was dissolved in methanol for column chromatography with gradient elution. Samples (5 ml.) of each fraction were taken for measurement of radioactivity; the remainder of each fraction was evaporated to dryness in a stream of N_2 and the residue was redissolved in ethanol (5 ml.) for measurement of the extinction at 265 nm .

(b) The metabolite (5×10^4 d.p.m.) and authentic sodium cholecalciferyl β -D-glucuronide (5 mg.) were together converted into the pyridinium salts as described in (a) above. The product, in methanol (1 ml.) was treated with an excess of ethereal diazomethane at 0° for 1 hr. Solvents were removed in a stream of dry N_2 , and the residue was acetylated by treatment with dry pyridine (0.5 ml.) and acetic anhydride (0.2 ml.) for 12 hr. at room temperature. Solvents were again removed in a stream of N_2 , and the residue was dissolved in methanol (1 ml.). This solution was chromatographed on a silicic acid column, the radioactivity and extinction at 265 nm . of each fraction being determined as in (a) above.

Chromatography of products from hydrolysis of metabolite D with β -glucuronidase. Metabolite D (3×10^5 d.p.m.) in water (0.2 ml.) was incubated with β -glucuronidase (4400 units, 20 mg./ml.) (0.1 ml.) in 0.1M-sodium acetate buffer, pH 5.05 (0.5 ml.), for 8 hr. at 37° . On completion of the incubation, water (3 ml.) was added and the solution was extracted with small volumes of chloroform. The combined chloroform extracts were evaporated to dryness under reduced pressure, and the residue, together with authentic cholecalciferol (5 mg.), was dissolved in methanol (1 ml.). This solution was chromatographed on a column of silicic acid, the radioactivity and extinction at 265 nm . of each fraction being measured in the usual way.

Control incubation of [1α - ^3H]cholecalciferol with β -glucuronidase. [1α - ^3H]Cholecalciferol (2×10^4 d.p.m.,

141mc/m-mole) in ethanol (0.05ml.) was added to an incubation mixture consisting of β -glucuronidase (4400 units, 20mg./ml.) (0.1ml.), water (0.2ml.) and 0.1M-sodium acetate buffer, pH5.05 (0.5ml.), which was then maintained at 37° for 8hr. On completion of the incubation, acetone-ethanol (1:1, v/v) (10ml.) was added, the mixture was filtered to remove precipitated protein and solvents were removed under reduced pressure. The residue was analysed by t.l.c. A sample of the original [1α - 3 H]cholecalciferol was similarly chromatographed.

RESULTS

After intravenous administration of albumin-bound [1α - 3 H]cholecalciferol at a rate that simulated the rate of uptake of a similar oral dose into lymph (Fraser & Kodicek, 1968), it was found that 31% of the radioactivity was excreted into the bile within 24hr. of commencing the injection, the maximum excretion occurring between 8 and 12hr. The time-course of biliary excretion of radioactivity is shown in Table 1. Gradient-elution column chromatography of the 24-hr. bile extract gave the pattern shown in Fig. 1(a). Although separation of the metabolites was incomplete with this technique, the absence of vitamin D ester, previtamin D and vitamin D, which are normally eluted with light petroleum-diethyl ether (3:1, v/v), was clearly demonstrated. The other striking feature of this chromatogram was the large portion of radioactivity present as highly polar metabolites, a feature shown more clearly when stepwise elution was used. These metabolites have been denoted as A, B, C, D and E in order of increasing polarity (Fig. 1a). The validity of the stepwise system was confirmed by rechromatography of the individual components in a gradient-elution system, when each component was found to give rise to a single peak of radioactivity. The chromatogram was also substantially unchanged when deactivated silicic acid was used, conditions under which 25-hydroxycholecalciferol is eluted with high concentrations of diethyl ether

in light petroleum. 25-Hydroxycholecalciferol was not identical with any of the metabolites found. The distribution of radioactivity between the different components for the 24hr.-bile extract, derived from stepwise-elution chromatograms, is shown in Table 2. The major component was that eluted with methanol, metabolite D, which accounted for 34% of the recovered radioactivity. That the radioactivity distribution did not vary significantly with time was shown by chromatography of portions of the 4-8hr. and 20-24hr. collections of bile, each of which gave a distribution pattern similar to the other and to the pattern for the total 24-hr. bile collection (Table 2).

Only a small amount of bile was collected from the rat given [14 C]ergocalciferol. This animal developed obstructive jaundice, but, despite this, 12.8% of the radioactivity of the administered vitamin was recovered in the bile within 24hr. Column chromatography of this material gave the pattern shown in Fig. 1(b), identical with that obtained after administration of [1α - 3 H]cholecalciferol, with three large peaks corresponding in position to metabolites A, C and D obtained previously. No metabolites less polar than that corresponding to metabolite A were found.

After acid hydrolysis of a sample of the bile extract from the rat given [1α - 3 H]cholecalciferol, it was found by stepwise elution chromatography that the peaks due to metabolites D and E had almost disappeared, that metabolite A was increased

Table 1. *Excretion of radioactivity into the bile of a young rat after intravenous infusion of 0.34mg. of [1α - 3 H]cholecalciferol*

Results are expressed as percentages of administered radioactivity.

Time interval (hr.)	Radioactivity excreted (%)
0-4	2.6
4-8	5.7
8-12	7.6
12-16	6.7
16-20	4.9
20-24	3.4
Total	30.9

Fig. 1. Separation of metabolites of (a) [1α - 3 H]cholecalciferol and (b) [14 C]ergocalciferol in rat bile by silicic acid column chromatography. The composition of the eluting solvent is shown by the broken line.

Table 2. *Radioactivity distribution in bile extracts*

Results are expressed as percentages of total biliary radioactivity recovered.

Metabolite	Bile collection Eluent	Distribution of radioactivity (%)			
		0-24 hr.	4-8 hr.	20-24 hr.	0-24 hr. after acid hydrolysis
Non-polar products	Ether	5	4	6	33
A	Ether-methanol (9:1)	8	9	8	20
B	Ether-methanol (3:1)	12	13	9	15
C	Ether-methanol (1:1)	26	25	21	20
D	Methanol	34	35	40	9
E	Methanol-water (4:1)	15	14	16	3

Table 3. *Effect of various reagents on the t.l.c. mobility of metabolite D*

R_F ranges quoted are those over which each 1 cm. band of adsorbent contained more than 5% of the total radioactivity recovered from the plate. Solvent systems were: (1) chloroform; (2) chloroform-methanol (9:1, v/v).

Reagent	R_F range	
	System 1	System 2
None	—	0.20-0.47
Diazomethane	0.0-0.27	0.60-0.87
Diazomethane, acetic anhydride	0.73-0.93	0.67-0.87
HCl	0.67-0.93	0.73-0.93
NaIO ₄	0.27-0.67	0.73-0.93

and that a large non-polar radioactive peak was present. The radioactivity distribution after acid hydrolysis may be compared in Table 2 with that for the bile extract before hydrolysis. In a further experiment, a sample of the bile extract was chromatographed on an anion-exchange resin. Only 26.2% of the radioactivity of the original sample was eluted from the resin with methanol. Silicic acid column chromatography of this eluate showed that the amount of non-polar material and of metabolites A and B was unchanged, but that the eluate contained only 2.5% of metabolite C, 1.1% of metabolite D and 0.7% of metabolite E, thus indicating the ionic nature of metabolites C, D and E.

Confirmation of the ionic nature of metabolite D was obtained from distribution studies. The distribution coefficient for chloroform-water was 0.06, but for chloroform-2M-hydrochloric acid and for chloroform-0.3M-pyridinium sulphate it was 0.69 and 0.60 respectively, and for butan-1-ol-water it was 1.35.

The effects of a number of reagents on the chromatographic mobility of metabolite D are shown

Table 4. *Chloroform-extractable radioactivity from metabolite D after incubation with β -glucuronidase*

The metabolite and bovine liver β -glucuronidase (400 units) were incubated in acetate buffer, pH 5.05, at 37°. The inhibitor was 0.01 M-saccharo-1,4-lactone.

Enzyme	Incubation time (hr.)	Chloroform- extractable radioactivity (%)
Boiled	8	13
Active	2	44
Active	8	62
Active	24	72
Inhibited	8	24

in Table 3. The mobility was increased by methylation and by acetylation, by acid hydrolysis, or by treatment with glycol-splitting agents such as periodate. These observations were indicative of a conjugate, and the only common conjugating agent having properties concordant with the above reactions is glucuronic acid. Accordingly the metabolite was incubated with β -glucuronidase with the results shown in Table 4. There was an increase in lipid-soluble radioactivity with the active enzyme over the value for controls, and this increase was largely prevented by the presence of saccharo-1,4-lactone, a specific inhibitor of β -glucuronidase.

Cholecalciferol β -D-glucuronide was synthesized by the method used by Becker (1965) for the synthesis of glucuronides of androsterone and its isomers. The final product had maximum u.v. absorption at 265 nm., indicating that the calciferol entity was intact, and was hydrolysed by β -glucuronidase to the free vitamin, confirming the β -linkage. Further confirmation was obtained by i.r. spectroscopy, which showed the presence of a strong hydroxyl band and of a carboxylic carbonyl group, and which compared well with the spectra obtained for cholesterol β -D-glucopyranosiduronate

Fig. 2. Silicic acid co-chromatography of cholecalciferol glucuronide (▲) and metabolite D (●): (a) untreated and (b) after methylation and acetylation. In (a) the composition of the eluting solvent is shown by the broken line; in (b) the eluting solvent was diethyl ether.

and its methyl triacetyl ester (Nagayama, Saito & Idler, 1966). Satisfactory analytical values could not be obtained for the glucuronide, a problem that has also arisen in studies on other steroid glucuronides (Elce, Carpenter & Kellie, 1967). More satisfactory values were obtained for the methyl triacetyl ester; in particular the n.m.r. spectrum of the protected glucuronide clearly showed the spectrum of cholecalciferol with that for the glucuronic acid residue superimposed.

The authentic glucuronide, together with a sample of metabolite D, was converted into the pyridinium salt by the method of McKenna & Norymberski (1960), and was then examined by column chromatography. By using gradient elution, there was a discrepancy of four fractions between the peak due to the metabolite, as measured by the distribution of radioactivity, and that due to cholecalciferol glucuronide, as measured by u.v. absorption. Metabolite D was slightly more polar than cholecalciferol glucuronide, as shown in Fig. 2(a). Conclusive proof of non-identity was provided by co-chromatography of the metabolite and cholecalciferol glucuronide after methylation and acetylation (Fig. 2b). As expected, the synthetic glucuronide gave a single product, whereas metabolite D gave two peaks, neither of which was in register with that due to the methyl triacetyl ester of cholecalciferol glucuronide.

The nature of the aglycone fragment of the metabolite was further investigated by column chromatography of the products of β -glucuronidase

Fig. 3. Silicic acid co-chromatography of products of β -glucuronidase hydrolysis of metabolite D (—) and cholecalciferol (●). The composition of the eluting solvent is shown by the broken line. Fractions 130–140 represent unhydrolysed metabolite D.

hydrolysis of metabolite D, together with authentic cholecalciferol. This demonstrated the absence of free cholecalciferol from the hydrolysis products of the metabolite, the radioactive hydrolysis products being present as two components (fractions 34–38 and 79–82) more polar than the vitamin itself, the more polar of the two components corresponding in chromatographic behaviour to metabolite A as shown in Fig. 3.

The possibility that decomposition of the aglycone might have occurred during enzymic hydrolysis was investigated by incubation of [1α - 3 H]cholecalciferol with β -glucuronidase under the same conditions as were used for metabolite D. Under these conditions 75% of the vitamin was recovered unchanged.

DISCUSSION

The results reported here confirm that the main route of excretion of metabolites of vitamin D in the rat is via the bile, a result that could be expected in view of the finding of Kodicek (1960) that only 2.1% of the radioactivity from an oral dose of [14 C]ergocalciferol was present in the urine of rachitic rats, compared with 79.3% in the faeces and intestinal contents. Similar results have been reported by Callow *et al.* (1966).

The chromatographic systems reported here are capable of separating the entire spectrum of metabolites. The striking feature of the distribution of radioactivity in bile is the absence of free vitamin D and 25-hydroxycholecalciferol, and the presence of ionic metabolites. These observations are in keeping with results reported by Avioli, Lee, McDonald, Lund & DeLuca (1967) of the examination of bile from human subjects with bile fistulas. They report that 3–6% of an intravenous dose of [G - 3 H]cholecalciferol was excreted in bile within

48hr. of dosing, and that only 8.7% of the biliary radioactivity was extractable with chloroform. They also confirm that the biliary excretion of free vitamin D is negligible. That [^{14}C]ergocalciferol gives a similar pattern of metabolites in bile to [$1\alpha\text{-}^3\text{H}$]cholecalciferol provides evidence that the products obtained with [$1\alpha\text{-}^3\text{H}$]cholecalciferol are derived from the vitamin and are not substances labelled by exchange.

The identification of metabolite D as a glucuronide conjugate rests on the evidence that it contains an α -glycol and a carboxylic acid function, and on the fact that the metabolite is hydrolysed specifically by β -glucuronidase. In the chromatographic systems used, the metabolite appeared homogeneous; the presence of two components after methylation and acetylation might be explained by the incomplete reaction of some additional functional group, probably hydroxyl, on the aglycone, but no explanation can at present be offered for the production of two components by enzymic hydrolysis of the metabolite.

The nature of the aglycone is at present unknown, but its identity with cholecalciferol can be excluded. In keeping with this, Avioli *et al.* (1967) found 40% of the biliary radioactivity in man to be made chloroform-extractable after β -glucuronidase hydrolysis, but only one-twentieth was tentatively identified as cholecalciferol.

The biliary excretion pattern for vitamin D metabolites, with a high proportion of polar components and the virtual absence of cholecalciferol, may indicate a similar behaviour to the biliary excretion of foreign compounds studied in the rat by Millburn, Smith & Williams (1967). They concluded that the excretion is dependent on molecular weight and on the presence of a strongly polar anionic group. The minimum molecular weight below which little biliary excretion occurred was found to be about 325 ± 50 . It is possible that the principal biliary metabolites of vitamin D will satisfy these same criteria.

We are indebted to Dr D. R. Fraser for performing the bile-duct cannulations.

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