Enzyme Studies on the Esterification of Vitamin D in Rat Tissues

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1. The mechanism of vitamin D esterification in the rat was studied with liver, small-intestinal mucosa, pancreatic juice and blood plasma as enzyme sources and [1-³H]cholecalciferol, [U-¹⁴C]ergocalciferol and [4-¹⁴C]cholesterol as substrates. 2. No esterification of vitamin D could be detected with liver preparations nor with homogenates or acetone-dried powder extracts of intestinal mucosa. 3. Pancreatic juice esterified [1-³H]cholecalciferol with oleic acid, and specificity studies indicated that a cholesterol-esterifying enzyme was using vitamin D as substrate. 4. Plasma cholesterol-esterifying enzyme also esterified vitamin D. 5. The specificity of the esterification reaction is discussed in relation to (a) the molecular structure of the substrates and (b) their availability, in a micellar solution, to the enzyme. 6. It is concluded that cholesterol-esterifying enzymes esterify vitamin D *in vivo* during absorption from the small intestine and while it is transported in blood.

After the oral administration to rats of vitamin D as [1-³H]cholecalciferol, some was found esterified with fatty acids in liver, kidney, intestinal tissue, plasma and intestinal lymph (Fraser & Kodicek, 1968*a,b*). A number of studies have indicated that esterification takes place during transport of vitamin D from the intestinal lumen to the intestinal lymph (Schachter, Finkelstein & Kowarski, 1964; Bell, 1966; Blomstrand & Forsgren, 1967; Fraser & Kodicek, 1968*b*). No other site of synthesis has yet been described and it was therefore decided to investigate the general enzymology of vitamin D esterification; this could be mediated by either an enzyme or enzymes specific for vitamin D or one that esterifies some other monohydroxylic compound such as cholesterol or retinol.

At least three different enzyme mechanisms appear to be present in the rat for the esterification of cholesterol. The first is a direct union of free fatty acid and cholesterol with no external energy source. Such an enzyme occurs in intestinal tissue and pancreatic juice (Swell, Byron & Treadwell, 1950). A blood-plasma enzyme transfers a fatty acid from the β -position of lecithin to esterify it with cholesterol, again without the introduction of external energy (Glomset, 1962). A third mechanism requires activated fatty acids that are esterified with cholesterol under the influence of a fatty acyl-CoA-cholesterol acyltransferase; this mechanism is found in liver (Goodman, Deykin & Shiratori, 1964) and adrenal gland (Longcope & Williams, 1963).

Retinol is esterified by intestinal and pancreatic enzymes in a similar way to cholesterol; however,

separate and specific enzymes are claimed for these two substrates (Murthy & Ganguly, 1962). An enzyme in liver esterifies retinol, but, in contrast with cholesterol esterification in that organ, requires no activation of the fatty acids (Futterman & Andrews, 1964).

Liver, small-intestinal mucosa, pancreatic juice and plasma were chosen as material for assay of 'vitamin D-esterifying activity'. The conditions *in vitro* were based on published methods for cholesterol and retinol esterification so that any action on vitamin D by these enzymes would be likely to be observed. No vitamin D esters could be found by using preparations of liver or small intestine as enzyme sources *in vitro*, but pancreatic juice did synthesize cholecalciferol esters, and the specificity of the reaction was studied. Plasma also esterified some cholecalciferol, and this reaction as well as that in pancreatic juice is considered to be catalysed by cholesterol-esterifying enzymes.

EXPERIMENTAL

Materials. Substrate vitamin D was either [1-³H]cholecalciferol or [U-¹⁴C]ergocalciferol prepared from their 3,5-dinitrobenzoate esters by the method of Wilson, Lawson & Kodicek (1967). Unlabelled crystalline cholecalciferol (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) and ergocalciferol (Glaxo Laboratories Ltd., Greenford, Middx.) were used to dilute the labelled forms to appropriate specific radioactivities. [4-¹⁴C]Cholesterol (24.8 mc/m-mole) (The Radiochemical Centre, Amersham, Bucks.) was diluted with unlabelled cholesterol that had been recrystallized once from

acetone and twice from ethanol. Radioactivity was measured by liquid-scintillation spectrometry with a counting efficiency for ^3H of 20% and for ^{14}C of 58%.

Oleic acid, ATP and CoA were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.), and sodium taurocholate (A grade) was from Calbiochem (Los Angeles, Calif., U.S.A.), sodium deoxycholate from Koch-Light Laboratories Ltd., *p*-chloromercuribenzoic acid from British Drug Houses Ltd. (Poole, Dorset) and bovine plasma albumin from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex). Retinol was prepared immediately before use from retinyl acetate (Roche Products Ltd., London, W. 1) by saponification in the presence of 0.25% pyrogallol. Free retinol was extracted with diethyl ether, its purity checked by thin-layer chromatography and its concentration measured by ultraviolet spectrophotometry.

Precholecalciferol was prepared from cholecalciferol by creating equilibrium conditions for the previtamin D $\rightleftharpoons$ vitamin D reaction at which 20% of previtamin is present (Velluz, Amiard & Petit, 1949). Cholecalciferol, dissolved in benzene, was heated for 16 hr. at 60°. The mixture was applied to a preparative thin-layer chromatography plate and developed in chloroform, on which cholecalciferol (R_F 0.37) separated from precholecalciferol (R_F 0.48). The latter was eluted with diethyl ether and collected at 0°. The eluted material was checked by repeating the chromatography, when most ran as previtamin D. A small region of cholecalciferol could be observed, but this may have formed during the chromatography. Labelled precholecalciferol was prepared in the same way from [^3H]cholecalciferol and contained 91% of the radioactivity as precholecalciferol. The preparations were stored at -20° before use.

Rachitic vitamin D-deficient hooded rats were raised as previously described (Fraser & Kodicek, 1968a), and these or vitamin D-supplemented rats on the rachitogenic diet supplied all tissues for enzymic assay.

All other materials were as described by Fraser & Kodicek (1968a,b).

Presentation of substrate. Vitamin D is unstable in an aqueous environment (Chen, Terepka, Lane & Marsh, 1965) unless protected by high concentrations of organic solvents or adsorbed on protein molecules. For short-term experiments *in vitro* it was decided either to emulsify vitamin D directly in water or to present it bound to plasma albumin. The procedure of Hernandez & Chaikoff (1957) for the emulsification of cholesterol was used to emulsify vitamin D, oleic acid and taurocholate in buffer without any organic solvent in the final solution. An alternative method was the addition of vitamin D and oleic acid in ethanol to buffered taurocholate to give an ethanol concentration of 5–10%; the suspension was vigorously agitated for 30 sec. on a vibrating rubber disk to produce a clear or opalescent solution.

To prepare a stable aqueous solution of vitamin D a method was devised to bind it to plasma albumin with the minimum of destruction. Aq. 4% (w/v) bovine plasma albumin, pH 7.3 (5 ml.), was placed in a 150 ml. round-bottomed ground-glass-necked flask provided with a magnetic stirrer and set up in a CaCl_2 -ice freezing mixture. The temperature was maintained at -4° to -5°, and 0.2 ml. of ethanol containing 250 μg . of [^3H]cholecalciferol was run in over a 10 min. period from a micrometer syringe pipette, the tip of which dipped beneath the surface of the albumin solution. The water and ethanol were then removed by freeze-drying, and the albumin was redissolved in 10 ml. of

water at 0° to give a clear solution containing 20 mg. of bovine plasma albumin and 25 μg . of cholecalciferol/ml. This was stored at -15°, and the stability was confirmed by extracting the vitamin with 25 vol. of acetone-ethanol (1:1, v/v) and chromatographing the extract on thin layers with chloroform. Only one radioactive spot was found and this ran adjacent to marker vitamin D (R_F 0.35). Aqueous solutions of [^3H]cholesterol were also prepared by the above methods.

Studies of esterification in vitro

Liver. In general 4 g. of liver was homogenized with 12 ml. of 0.44 M-sucrose at 4° in a Potter-Elvehjem homogenizer. The disrupted tissue was fractionated by differential centrifugation at 4° into debris, mitochondria, microsome and soluble-supernatant fractions, and in some experiments nuclei were purified from the debris fraction (Hogeboom, 1955). Pellets were resuspended in 5 ml. of 0.44 M-sucrose. The interval between killing and the beginning of incubation was 2–3 hr.

Incubation mixtures contained: 1.4 ml. of 0.2 M-sodium phosphate buffer, pH 7.3; 0.1 ml. of 0.15 M-NaF; 0.2 ml. of 75 mM-ATP, pH 7.3; 0.2 ml. of 0.6 mM-CoA; 2.5 μg . of [^3H]cholecalciferol or [^3H]cholesterol on albumin (0.1 ml.); 2 ml. of liver cell fraction. The total volume was 4 ml. Flasks were shaken at 37° for 2 hr., after which the contents were extracted with chloroform-methanol (2:1, v/v). Extracts were chromatographed by a method of Deykin & Goodman (1962), and radioactivity was measured in 'esterified' and 'non-esterified' substrate fractions.

Intestinal mucosa. Methods of assaying esterifying activity were modified from procedures published previously (Mahadevan, Murthy, Krishnamurthy & Ganguly, 1961; Murthy, Mahadevan, Seshadri Sastry & Ganguly, 1961b; Murthy & Ganguly, 1962; Lossow, Migliorini, Brot & Chaikoff, 1964). Small intestine was freed from pancreatic tissue and mesenteric adipose tissue and placed in 500 ml. of aq. 0.9% NaCl soln. at 4°. The lumen was washed through with 20–30 ml. of NaCl soln., the intestine was slit open and blotted on a porcelain slab, and the mucosa was scraped off with the edge of a microscope slide. Some mucoid material was removed by washing the scrapings in 150 ml. of NaCl soln., and the cells were recovered by centrifuging at 1000g for 5 min. The pellet (1 g.) was homogenized in a Potter-Elvehjem homogenizer with 10 ml. of 0.15 M-KCl.

Acetone-dried powders were also prepared from the mucosa of groups of 16 rats. In one procedure the washed mucosal scraping was homogenized in 120 ml. of water in a Waring Blendor for 30 sec. at 0°. The homogenate was stirred rapidly into 1 l. of acetone at -15°, filtered and washed with acetone and peroxide-free diethyl ether in a Buchner funnel, and finally dried in a desiccator at low pressure. Other batches of mucosal powder were prepared by the method of Mahadevan *et al.* (1961) and both types of powder were extracted with water at 0° as described by those authors.

Incubation mixtures contained: 1.3 ml. of 0.14 M-barbital-acetate buffer, pH 6.4; 0.1 ml. of aq. 80 mM-sodium taurocholate; 0.1 ml. of 40 μM -oleic acid (in ethanol or as albumin-stabilized aq. potassium oleate); 0.1 ml. of 20 μM -[^3H]cholecalciferol (approx. 280 000 counts/min.); 0.4 ml. of enzyme preparation. The total volume was 2 ml. After a

2 hr. incubation at 37° the contents of each flask were added to 50 ml. of acetone-ethanol (1:1, v/v) and shaken for 4 hr. After being filtered and dried under reduced pressure, each extract was fractionated into 'free' and 'esterified' vitamin D by standard thin-layer chromatography with chloroform as ascending solvent (Fraser & Kodicek, 1968a).

When a soluble protein extract was assayed for activity a simpler extraction procedure was adopted in which 20 μ l. of incubation mixture was removed in a disposable pipette and added to 20 μ l. of acetone-ethanol on a spotting tile. This entire mixture was applied with 10 μ g. each of standard cholecalciferol and cholecalciferol palmitate to micro-thin-layer plates (Vahouny, Borja & Weersing, 1963), which were developed with hexane-diethyl ether-acetic acid (20:4:1, by vol.). The micro-plates were stained with iodine vapour to locate free and esterified fractions and destained by heating at 50°, and the silica gel was scraped quantitatively into two vials for measurement of the radioactivity in each area by liquid-scintillation spectrometry.

Pancreatic juice. Bile and pancreatic juice were collected separately in tubes at 0° from cannulae implanted in the hepatopancreatic duct. The bile-collecting cannula was inserted where the duct ran free in the lesser omentum between the porta hepatis and the pancreas, and the second cannula collected pancreatic juice from the point where the duct entered the duodenal wall. The rat was restrained after surgery in a cage described by Bollman (1948) and was supplied with a commercial cube diet and water containing 5% glucose. Pancreatic juice, a clear colourless liquid, was stored in 1 ml. volumes at -15°. The mean daily output over 16 days was 9 ml.

The assay of esterifying activity was similar to that described by Shah, Lossow & Chaikoff (1965), except that 1 ml. volumes were used in 12 cm. $\times$ 1.5 cm. test tubes closed with aluminium foil. Incubation was performed with continuous shaking of 135 oscillations/min. in a water bath at 37°. The incubation mixture contained: 0.86 ml. of 0.1M-sodium phosphate buffer, pH 6.2; 0.05 ml. of 80 mM-sodium taurocholate; 0.4 μ mole of cholecalciferol or cholesterol plus 0.8 μ mole of oleic acid in 0.02 ml. of ethanol; 0.7 μ mole of [3 H]cholecalciferol (45 000 counts/min.) or 1.5 μ moles of [14 C]cholesterol (43 000 counts/min.) in 0.02 ml. of ethanol. Dispersion of the substrate was aided by agitation on a vibrating rubber disk for 30 sec. Pancreatic juice (0.05 ml.) was added 15 sec. before the tube was placed in the water bath. At the end of the incubation, 1 ml. of acetone-ethanol was added to each tube and approx. 0.1 ml. of this mixture was directly chromatographed on micro-thin-layer plates, and radioactivity in the free sterol and ester fractions was estimated.

Plasma. Rat blood was collected into heparinized syringes by cardiac puncture and plasma was obtained immediately by centrifuging at 4°. Incubation flasks contained 2 ml. of plasma, to which was added [3 H]cholecalciferol or [14 C]cholesterol as albumin-bound substrate. A blank flask contained plasma heated at 60° for 30 min. Incubation was carried out with shaking at 37° for 24 hr. by a method for the determination of cholesterol esterification by plasma (Glomset, Parker, Tjaden & Williams, 1962). At the end of the incubation period the plasma was extracted with 5 ml. of acetone-ethanol, and the extract was filtered and dried under reduced pressure. By means of floridin-earth chromatography (Fraser & Kodicek, 1968a) ester fractions were obtained from the extract.

RESULTS

Esterification studies with tissues

Esterification studies with liver. The overall results indicated that vitamin D was not esterified by liver under the conditions provided. A small amount of radioactivity in ester fractions from incubations was sometimes higher than from corresponding fractions from boiled-enzyme controls. However, this radioactivity was so low (0.4% of total) that its identity as ester could not be established. The control experiments with [14 C]cholesterol showed clearly that esterification of this substrate took place in a 2 hr. incubation, when 50–70% of the radioactivity was found in the ester fraction.

To exclude the possibility that unfavourable conditions were the cause of the failure to synthesize vitamin D esters a range of variables was introduced: incubation time, the presence or absence of Mg^{2+} , concentration of protein, and concentration and method of presentation of substrates. None of these succeeded in revealing esterifying activity. Kidney homogenates were also used in one experiment and again no evidence of cholecalciferol esterification was found.

It is concluded that there is no positive proof that vitamin D is esterified by liver or kidney, and that this supports the suggestion that the small amount of vitamin D ester found in these sites *in vivo* is probably synthesized elsewhere (Fraser & Kodicek, 1968b).

Esterification studies with small-intestinal mucosa. In preliminary experiments with whole-mucosal homogenates, no esterification of vitamin D could be measured, and with [14 C]cholesterol under the same conditions only 2–3% of the recovered radioactivity was esterified. Acetone-dried powders of mucosa, prepared by both methods described above, were assayed in a similar manner to that described by Murthy & Ganguly (1962); however, in no case was more than 2.7% of cholesterol ester formed. It was concluded that the poor esterification of cholesterol by these preparations was due either to the presence of an active hydrolytic enzyme or to the virtual absence of the esterifying enzyme. Since the pH optimum for cholesterol-ester hydrolysis is reported to be pH 8.6 (Murthy & Ganguly, 1962), the pH of 6.4 used in this study should not be favourable for this reverse reaction. Inactivation of the enzyme was again unlikely as a 10% homogenate of rat pancreas in water in the same assay system esterified as much as 30–40% of added cholesterol. Hence the mucosal cholesterol-esterifying enzyme was considered to be present in very low amounts.

Variation in the activity of intestinal cholesterol-esterifying enzyme was observed by Niefert & Deuel (1949), who reported that activity was present only

when rats had been given a diet containing 1% of lanolin or 1% of cholesterol. Murthy, Mahadevan & Ganguly (1961*a*), by feeding a diet with 1% of cholesterol to young rats for 5 weeks, showed that mucosal acetone-dried powders from rats on the high-cholesterol diet had a 2.5-fold greater cholesterol-esterifying activity and a fourfold greater retinol-esterifying activity than control rats. Since the rachitogenic diet of Steenbock & Black (1925) contains no cholesterol, the rats in the present experiments had not received dietary cholesterol from weaning. Therefore weanling male or female rats were raised for 5 weeks on the 1% cholesterol diet of Murthy *et al.* (1961*a*) supplemented with vitamins A, D, E and K; over this period the rats increased in weight from 35 g. to 200 g. Acetone-dried powder of intestinal mucosa was prepared and extracted by the method of Mahadevan *et al.* (1961), and assayed for cholesterol-esterifying activity.

In several assays, these extracts only esterified 3.1–3.9% of the [$4\text{-}^{14}\text{C}$]cholesterol in 2 hr. In the similar experiments by Murthy *et al.* (1961*a*) the specific activity of the enzyme was increased from 52.4 to 130.6 μmoles of cholesterol esterified/mg. of protein/hr., whereas the present enzyme extract had a specific activity of only 0.4–1.7 μmoles of cholesterol esterified/mg. of protein/hr.

It was concluded that the hooded piebald rats of this Laboratory have a very low intestinal cholesterol-esterifying activity and it was not possible to test *in vitro* the specificity of its action for vitamin D. If, however, this enzyme were responsible for the vitamin D ester appearing in thoracic-duct lymph the much smaller amounts found in the lymph of our rats (Fraser & Kodicek, 1968*b*) compared with American reports on albino rats (Schachter *et al.* 1964; Bell, 1966) could be explained by a difference in this enzyme's activity between the two strains of rats.

Esterification studies with pancreatic juice

Because the pancreas had high cholesterol-esterifying activity and because the reaction mechanisms of the pancreatic and intestinal enzymes were very similar (Murthy & Ganguly, 1962), we compared the specificity of cholesterol esterification catalysed by pancreatic juice with a similar reaction for vitamin D. The methods used for defining the specificity of one enzyme towards two substrates were based on criteria listed by Dixon & Webb (1960), the main ones being: (1) failure to separate the two activities by all available fractionation procedures; (2) maintenance of a constant ratio between the two activities as the enzyme undergoes inactivation; (3) the 'mixed-substrate phenomenon': equal quantities of two substrates added together produce a lower total rate of reaction than the sum of the rates measured separately.

Preliminary experiments, with cholesterol as substrate, revealed that maximum esterification occurred by 30 min. and that the rate in the first 5 min. was much faster than subsequently (Fig. 1*a*). There are three possible explanations for this non-linearity of reaction: the enzyme is inactivated during incubation, or a secondary reverse reaction comes into play as ester concentration rises, or the micellar state of the substrate changes.

Proteolytic inactivation of the enzyme may have decreased its effective concentration, although the enzyme is said to be protected by taurocholate (Vahouny, Weersing & Treadwell, 1965). However, the addition of soya-bean trypsin inhibitor (50 $\mu\text{g.}$ /assay) did not prevent the tailing off of the reaction.

The second possibility, that the ester was being hydrolysed as well as synthesized, seemed more likely. When incubation was continued for 2 hr. the amount of ester sometimes dropped (Fig. 1*a*, curves i and ii) and the use of more enzyme and less substrate decreased the overall synthetic reaction (Fig. 1*a*, curve iii). This, along with a marked decline in specific activity as the protein concentration increased (Fig. 1*b*), suggested that the hydrolytic cholesterol esterase was active. Although the pancreatic hydrolysing enzyme has a pH optimum of 8.6, its activity is only halved at pH 6.2 (Murthy & Ganguly, 1962). To overcome this effect, it was considered that, if the enzyme concentration were decreased to give a lower reaction rate, linear esterification could be observed before ester hydrolysis became significant. This proved to be the case, for when pancreatic juice was diluted 1:3 with 0.1 M-phosphate buffer, pH 6.2 (giving 0.27 mg. of protein/assay), a linear rate of esterification was measured over 5–30 min. (Fig. 1*c*).

Because cholesterol and oleic acid are almost insoluble in water and are presented to the enzyme as a micellar dispersion, it is difficult to know the exact substrate concentration. Also the preferred substrate state is found to vary for different enzymes acting on lipids. The cholesterol-esterifying enzyme requires a micellar substrate (Vahouny, Weersing & Treadwell, 1964), in contrast with pancreatic lipase, which acts on the water-oil interface of triglyceride emulsions (Desnuelle & Röver, 1961). Hence, when the substrate concentration is given, it is a measure of the total in the assay rather than that which is always exposed to the enzyme. Nevertheless, when the cholesterol concentration was raised to 0.6 $\mu\text{mole/ml.}$ with a constant oleic acid concentration of 1.6 $\mu\text{moles/ml.}$, a linear increase in activity was obtained (Fig. 1*d*). This agrees with the results of Murthy & Ganguly (1962), who found that the activity increased linearly with up to 1 μmole of cholesterol/ml. The rate of increase was much greater with dilute (0.27 mg. of

Fig. 1. (a) Time-course of cholesterol-oleic acid esterification with pancreatic juice: (i) and (ii), $0.6 \mu\text{mole}$ of cholesterol and 0.05 ml. of pancreatic juice; (iii), $0.3 \mu\text{mole}$ of cholesterol and 0.1 ml. of pancreatic juice. (b) Effect of concentration of pancreatic juice on esterification ($0.3 \mu\text{mole}$ of cholesterol/assay). (c) Time-course of esterification: 0.05 ml. of pancreatic juice diluted 1:3 with 0.1 M -phosphate buffer, pH 6.2 ($0.6 \mu\text{mole}$ /assay). (d) Substrate-concentration curves for cholesterol. Oleic acid was constant at $1.6 \mu\text{moles/ml.}$: (i) 0.05 ml. of whole pancreatic juice; (ii) 0.05 ml. of pancreatic juice diluted 1:3 with 0.1 M -phosphate buffer, pH 6.2.

protein/assay) rather than whole (1.08 mg. of protein/assay) pancreatic juice.

It was not possible to have cholesterol concentrations above $0.6 \mu\text{mole/ml.}$ unless the ethanol concentration was also increased. Thus a balance had to be reached between the maximum amount of substrate and the minimum amount of ethanol to produce an efficient reaction rate. As a standard procedure, giving repeatable results, $0.4 \mu\text{mole}$ of

cholesterol and 4% of ethanol in 1 ml. of incubation mixtures were used.

Blank enzyme reactions with pancreatic juice previously heated at 60° for 30 min. gave no radioactivity in the ester fraction.

Comparison of cholesterol and cholecalciferol esterification by pancreatic juice. Cholesterol and cholecalciferol ($0.4 \mu\text{mole}$ of each) were incubated separately as substrates in the esterification assay. Vitamin D

radioactivity was provided by 0.7 μ mole of [$1\text{-}^3\text{H}$]cholecalciferol (45 000 counts/min.). After 15 min. 16% of the cholesterol was esterified, and about 2% of the cholecalciferol radioactivity chromatographed as ester. This was concentrated by florisil-earth chromatography and was run with standard vitamin D stearate, oleate, linoleate and linolenate on a 20 cm. $\times$ 5 cm. silver nitrate-impregnated plate, as described for the first dimension of ester separation by Fraser & Kodicek (1968a). The esters were observed under ultraviolet light after being sprayed with dichlorofluorescein (0.2%, w/v, in ethanol), and radioactivity in the spots was measured after elution with diethyl ether. Over 90% of this radioactivity was in the monounsaturated vitamin D ester, thus confirming that vitamin D oleate had been synthesized.

To identify the enzyme that was esterifying vitamin D, an attempt was made to fractionate whole, freeze-dried, pancreatic juice by DEAE-cellulose column chromatography (Marchis-Mouren, Charles, Ben Abdeljlil & Desnuelle, 1961). Although good resolution into about 12 peaks of protein was achieved, the cholesterol-esterifying activity was not associated with any of these peaks. It was decided therefore to examine the specificity of esterification indirectly with unfractionated pancreatic juice.

Mixed-substrate esterification. If cholecalciferol and cholesterol are esterified by the same enzyme then the rate of reaction for either substrate should be less when both are present, because of competition for the one active enzyme centre. A 15 min.

incubation was performed in duplicate with 0.2 μ mole of cholesterol and of cholecalciferol/ml., separately and together. This short time-interval decreased the effect of the hydrolytic reaction by keeping the ester concentration at a low value. Cholesterol esterification was hardly altered by the presence of cholecalciferol and the esterification rate of the latter fell by 19% with cholesterol present (Table 1). Ideally, both rates should have dropped if the same enzyme were involved, but as the cholesterol reaction was over 10 times faster, competition by cholecalciferol may not have been observed.

A similar experiment was carried out on 0.2 μ mole of retinol/ml. incubated with cholesterol and with cholecalciferol. Retinol esterification was not measured, but the fact that it occurred was confirmed visually by retinol fluorescence in ultraviolet light on the micro-thin-layer plates (free retinol R_F 0.1; ester R_F 0.6). Cholesterol and cholecalciferol esterification rates rose by 16% and 17% respectively in the presence of retinol. The cause of this rise could not be determined; however, as there appears to be competition for esterification in the presence of cholesterol but not in the presence of retinol, the involvement of the cholesterol-esterifying enzyme is favoured in the synthesis of vitamin D ester. Moreover, the equal stimulatory effect of retinol on both cholesterol and cholecalciferol esterification suggests that again the same enzyme mechanism was affected.

Cofactor requirements. Studies on bile-salt requirements supported the concept that the cholesterol-esterifying enzyme was acting on cholecalciferol.

Table 1. *Comparison of reaction rates of esterification with pancreatic juice and mixed substrates*

The reaction rate (μ moles of substrate esterified/mg. of protein/hr.) was calculated on 15 min. assays. Expt. 1: cholesterol and cholecalciferol as mixed substrates; Expt. 2: cholesterol and cholecalciferol as mixed substrates with retinol. Oleic acid concentration was 0.8 μ mole/assay; substrate concentration was 0.2 μ mole of each substrate/assay.

	Substrate	Cholesterol esterification			Cholecalciferol esterification		
		Reaction rate	Mean	Change (%)	Reaction rate	Mean	Change (%)
Expt. 1	Cholesterol	468 } 471 }	470	0			
	Cholecalciferol				47.6 } 40.0 }	43.8	0
	Cholesterol + cholecalciferol	501 } 456 }	479	+ 2	35.6 } 35.6 }	35.6	- 19
Expt. 2	Cholesterol	546 } 534 }	540	0			
	Cholecalciferol				35.6 } 32.7 }	34.2	0
	Cholesterol + retinol	629 } 618 }	624	+ 16			
	Cholecalciferol + retinol				38.9 } 41.1 }	40.0	+ 17

When taurocholate was replaced by deoxycholate, the cholesterol and cholecalciferol reaction rates declined by 78% and 32% respectively (Table 2). The absence of bile salt gave similar decreases in the reaction rates. Bile salts are not required for the esterification of retinol by pancreas acetone-dried powder extracts (Pollard & Bieri, 1960) or small-intestine slices (Olson, 1964); indeed, their presence inhibits the reaction. Hence the opposite effect found with both cholesterol and cholecalciferol makes it less likely that the retinol enzyme is involved.

Inhibitor studies. Heavy-metal ions and *p*-chloromercuribenzoic acid are potent inhibitors of the pancreatic cholesterol-esterifying enzyme (Hernandez & Chaikoff, 1957; Murthy & Ganguly, 1962). The effect of these inhibitors on the present pancreatic-juice enzyme is given in Table 3. With 10 μ M-*p*-chloromercuribenzoic acid and 10 μ M-Cu²⁺ (as cupric acetate), instead of inhibition there was activation of both cholesterol and cholecalciferol esterification. When the concentration of *p*-chloromercuribenzoic acid was raised to 0.1 mM, the degree of activation of the cholesterol reaction was de-

creased, and cholecalciferol esterification was inhibited by 14%. Both reactions approached the control value when 1 mM-cysteine was added with 0.1 mM-*p*-chloromercuribenzoic acid. Increasing the Cu²⁺ ion concentration to 0.1 mM caused inhibition of cholesterol and cholecalciferol esterification by 13% and 59% respectively.

Activation of the reaction at the low inhibitor concentration is interpreted as a greater inhibition of hydrolysis than of esterification. With increased concentration, both reactions were inhibited, although cholecalciferol esterification was more sensitive to the effect of the inhibitors.

Relative esterification rates. A careful measure was made of the relative esterification rates of cholesterol, cholecalciferol and precholecalciferol by pancreatic juice. Precholecalciferol was included as it was known to be present in equilibrium with cholecalciferol in the previous assays. It also has biological activity, explainable by its conversion into cholecalciferol (Hanewald, Rappoldt & Roborgh, 1961), and, as it is the immediate precursor of cholecalciferol (Velluz, Amiard & Goffinet, 1955), it

Table 2. *Effect of bile salts on esterification by pancreatic juice*

The reaction rate (μ moles of substrate esterified/mg. of protein/hr.) was calculated on 30 min. assays. Oleic acid concentration was 0.8 μ mole/assay, the substrate concentration 0.4 μ mole/assay, and the bile-salt concentration 4 μ moles/assay.

Substrate	Bile salt	Reaction rate	Change (%)
Cholesterol	Taurocholate	825	0
	Deoxycholate	182	-78
	None	142	-83
Cholecalciferol	Taurocholate	72	0
	Deoxycholate	49	-32
	None	48	-33

Table 4. *Comparative rates of esterification by pancreatic juice for cholesterol, cholecalciferol and precholecalciferol*

Reaction rate (μ moles of substrate esterified/mg. of protein/hr.) was calculated on 15 min. assays. Oleic acid concentration was 0.8 μ mole/assay; substrate concentration was 0.4 μ mole/assay.

Substrate	Reaction rate	Mean	Rel. reaction rate
Cholesterol	1244	1258	100
	1273		
Cholecalciferol	103	108	9
	113		
Precholecalciferol	315	332	26
	348		

Table 3. *Inhibition of pancreatic-juice esterifying enzyme*

The reaction rate (μ moles of substrate esterified/mg. of protein/hr.) was calculated on 30 min. assays. Oleic acid concentration was 0.8 μ mole/assay, and substrate concentration 0.4 μ mole/assay. PCMB, *p*-chloromercuribenzoic acid.

Substrate for esterification	Inhibitor	Cholesterol		Cholecalciferol	
		Reaction rate	Change (%)	Reaction rate	Change (%)
...	None	662	0	98	0
	10 μ M-PCMB	793	+20	116	+18
	0.1 mM-PCMB	735	+11	84	-14
	0.1 mM-PCMB + 1 mM-cysteine	713	+8	95	-3
	10 μ M-Cu ²⁺	698	+5	109	+11
	0.1 mM-Cu ²⁺	578	-13	40	-59

Table 5. *Comparative rates of esterification of cholecalciferol and ergocalciferol catalysed by pancreatic juice in vitro*

Reaction rate (m μ moles of substrate esterified/mg. of protein/hr.) was calculated on 15 min. assays. Oleic acid concentration was 0.8 μ mole/assay.

	Assay 1		Assay 2	
	Reaction rate	Rel. reaction rate	Reaction rate	Rel. reaction rate
Cholecalciferol	184	5.3	254	5.2
Ergocalciferol	35	1	49	1

Table 6. *Recovery of vitamin D after administration of ergocalciferol in vivo*

[U-¹⁴C]Ergocalciferol (sp. radioactivity 0.10 mc/m-mole) was given to rats on a rachitogenic diet supplemented with cholecalciferol: (1) oral administration of 1.4 mg. of [U-¹⁴C]ergocalciferol; (2) intravenous injection of 0.54 mg. of [U-¹⁴C]ergocalciferol.

	(1)		(2)	
	% of dose recovered	% of recovered vitamin D found as ester	% of dose recovered	% of recovered vitamin D found as ester
Liver	10.4	4.8	25.9	25.3
Kidneys	0.36	0	0.8	0
Plasma	—	12.5	—	13.7

would be formed *in vivo* in skin under normal conditions. A 15 min. incubation enabled a determination of the relative reaction rates to be made without allowing the previtamin D $\rightleftharpoons$ vitamin D equilibrium time to shift to the right. At 40° only 2% of vitamin D is formed from previtamin D in 24 min. (Hanewald *et al.* 1961). The comparative activities (Table 4) show that precholecalciferol is esterified at a rate about three times that of cholecalciferol and about a quarter that of cholesterol. When the esterification of cholecalciferol and ergocalciferol was compared it was observed that the rate for [1-³H]-cholecalciferol was five times that of [U-¹⁴C]ergocalciferol (Table 5). The lowered specificity of the pancreatic-juice enzyme for ergocalciferol *in vitro* was not reflected in the whole animal, where esterified forms of ergocalciferol and cholecalciferol were found in comparable quantities (Table 6).

Esterification studies with blood plasma

As a preliminary to studies *in vitro*, an experiment was performed *in vivo* to see whether vitamin D esterification occurred without intestinal transport. If vitamin D were given parenterally to rats with hepatic-duct cannulae, then the intestinal enzyme would not be exposed to the vitamin. A rat weighing 90 g., prepared with a cannulated bile duct, was infused via a jugular vein cannula with 0.34 mg. of [1-³H]cholecalciferol (sp. radioactivity 141 mc/

Table 7. *Recovery of vitamin D after intravenous infusion of 0.34 mg. of [1-³H]cholecalciferol (sp. radioactivity 141 mc/m-mole) into a rat with bile-duct cannulation*

Tissue	% of dose	% of recovered vitamin D found as ester
Liver	7.0	3.3
Kidneys	1.7	4.6
Small intestine	0.5	0
Left tibia, femur and patella	0.1	0
Thyroid gland	0.004	0
Plasma	1.6	7.0

m-mole) bound to bovine plasma albumin (50 mg./ml.) in 1 ml. of aq. 0.9% sodium chloride solution. To simulate absorption of vitamin D from the intestine, continuous infusion with a slow-injection apparatus was carried out over 18 hr. The rate of infusion was faster for the first 0.2 ml., to give a peak at 2–3 hr. resembling the absorption curve from intestine (Fraser & Kodicek, 1968b). The rat was killed 24 hr. after the start of the infusion and tissues were collected for the estimation of free and esterified vitamin D by thin-layer chromatography. Esterified vitamin D was found in liver, kidney and plasma, but not in small intestine, bone or thyroid (Table 7). The absence of ester from intestine

Table 8. *Esterification of [4-¹⁴C]cholesterol and [1-³H]cholecalciferol by rat plasma in vitro*

Substrate	Substrate added (μ moles)	Enzyme	% of added substrate esterified in 24 hr.
Cholesterol	3.5	Plasma (2 ml.)	26
Cholecalciferol	4.4	Plasma (2 ml.)	2.2
	17.3	Plasma (2 ml.)	1.0
	17.3	Plasma (2 ml.)	1.9
	4.4	Heated plasma (2 ml.) (60° for 30 min.)	0

Table 9. *Comparison of the fatty acid compositions of [4-¹⁴C]cholesterol and [1-³H]cholecalciferol esters synthesized by rat plasma in vitro*

Two chromatograms (i and ii) were run on each sample.

Degree of fatty acid unsaturation	Composition of fatty acids (%)					
	Cholesterol esters			Vitamin D esters		
	(i)	(ii)	Mean	(i)	(ii)	Mean
Saturated	10	9	10	16	12	14
Monounsaturated	7	7	7	5	6	6
Diunsaturated	31	28	29	23	25	24
Triunsaturated	0.2	2	1	5	3	4
Tetraunsaturated	52	54	53	50	54	52

indicated that this had not been the site of synthesis, and, as the concentration of ester was higher in plasma than in liver and kidney, it was possible that it had been synthesized in circulating blood.

Experiments *in vitro* revealed that both cholecalciferol and cholesterol were esterified by plasma (Table 8). The identity of the vitamin D ester was confirmed by thin-layer chromatography with chloroform, when 98% of the radioactivity ran opposite standard vitamin D ester. The degrees of unsaturation of the esterified fatty acids of the esters were compared. Silver nitrate-impregnated thin-layer plates were prepared, spotted with the radioactive esters plus standard cholesterol or vitamin D stearate, oleate, linoleate and linolenate esters, and developed with diethyl ether-hexane (1:4, v/v). The separated esters were observed under ultraviolet light after being sprayed with dichlorofluorescein (0.2% in ethanol) and their radioactivity was measured after elution with diethyl ether. Much of the natural plasma cholesterol ester was the tetraunsaturated arachidonate, and cholecalciferol radioactivity in this spot was assumed to be the tetraunsaturated vitamin D ester. The synthesized vitamin D esters were similar in composition to those of cholesterol with most of the radioactivity in the di- and tetra-unsaturated esters (Table 9).

Another rat was prepared as described above and blood was collected 48 hr. after the beginning of an

Table 10. *Composition of whole-blood vitamin D esters after intravenous infusion of 0.2 mg. of [1-³H]-cholecalciferol (sp. radioactivity 141 mc/m-mole) into a rat*

Blood was collected 48 hr. after the start of the infusion, which lasted 16 hr. Two chromatograms (i and ii) were run on each sample.

Degree of fatty acid unsaturation	Composition of fatty acids in vitamin D ester (%)		
	(i)	(ii)	Mean
Saturated	52	49	51
Monounsaturated	6	7	7
Diunsaturated	13	13	13
Triunsaturated	29	31	30
Tetraunsaturated			

intravenous infusion of 0.2 mg. of [1-³H]cholecalciferol. Esterified vitamin D was 2.9% of the total vitamin D extracted, and its composition, when compared with vitamin D esters synthesized by plasma *in vitro*, showed a decrease in the proportion of polyunsaturated esters and a corresponding increase in the saturated esters (Table 10).

DISCUSSION

The failure to synthesize vitamin D esters *in vitro* with preparations from liver and kidney is

insufficient evidence to say that these organs do not esterify vitamin D. However, the findings that vitamin D ester can be formed by an intestinal enzyme and by plasma provide explanations for the presence of ester in liver and kidney. Thus, unless a complex situation exists where synthesis, hydrolysis and transport of ester are all taking place in liver, the small amount of esterified vitamin D at that site was probably carried there along with vitamin D alcohol. It may be that vitamin D, rather than being esterified, was catabolized by liver enzymes to material insoluble in chloroform. Indeed, recovery of radioactivity in chloroform after incubation of [^3H]cholecalciferol with liver was never more than 50%. This loss was not investigated, but another cause for it might be inability fully to extract vitamin D, which in an aqueous environment is tightly bound to protein (Chalk & Kodicek, 1961; Chen & Lane, 1965).

Similarly the failure to synthesize vitamin D esters with mucosal preparations throws some doubt on the ability of the intestine to esterify the vitamin. Nevertheless, the finding of esters in intestinal lymph and the esterification of vitamin D *in vitro* by a pancreatic cholesterol-esterifying enzyme, the reported properties of which are indistinguishable from a mucosal enzyme, support the idea of vitamin D esterification by the intestine *in vivo*. Moreover, the activity of the mucosal cholesterol-esterifying enzyme was very low *in vitro* whereas the liver cholesterol ester synthetase, which has a quite different mechanism, was found to be very active. This would mean that this fatty acyl-CoA-cholesterol acyltransferase in liver was far more specific for its substrate than the intestinal esterifying enzymes were.

Pancreatic cholesterol-esterifying enzyme is known to have varying activities for other sterols. From published results (Table 11) the essential substrate requirements of the reaction can be defined as (1) β -configuration of the $\text{C}_{(3)}$ hydroxyl group, (2) A/B-*trans*-decalin configuration, and (3) saturation of the $\text{C}_{(7)}$ - $\text{C}_{(8)}$ carbon-carbon bond. Certain side-chain modifications such as a $\text{C}_{(24)}$ ethyl group, as in β -sitostanol and β -sitosterol, or a $\text{C}_{(24)}$ ethyl group and a $\text{C}_{(22)}$ - $\text{C}_{(23)}$ *trans* double bond, as in stigmasterol, depress but do not abolish esterification.

Apart from the configuration of the hydroxy group the integrity of the B ring appears to be the most important factor for esterification. The presence of a $\text{C}_{(5)}$ - $\text{C}_{(6)}$ double bond is not essential, but a $\text{C}_{(6)}$ - $\text{C}_{(7)}$ double bond, a 7β -hydroxy group (Korzenovsky, Rust & Diller, 1955) or alteration in the size of the B ring (e.g. in B-norecholesterol), abolishes activity.

The experiments described in the present paper suggest that cholecalciferol is esterified by a chole-

Table 11. *Comparison of pancreatic cholesterol-esterifying enzyme activities for different substrates*

Values were taken from the literature and are related to an activity for cholesterol of 100. Oleic acid was co-substrate in all experiments. References: (1) Swell, Field & Treadwell (1954) (pig pancreas); (2) Hernandez & Chaikoff (1957) (pig pancreas); (3) Korzenovsky, Diller, Marshall & Auda (1960) (pig pancreas); (4) Murthy & Ganguly (1962) (rat pancreas).

Substrate	References	Rel. activities			
		(1)	(2)	(3)	(4)
Cholestanol		148	109	135	—
Cholesterol		100	100	100	100
β -Sitostanol		—	—	92	—
β -Sitosterol		41	39	69	35
Stigmasterol		15	22	—	4
Ergosterol		12	3	—	—
7-Dehydrocholesterol		—	0	—	—
Epicholesterol		—	0	5	—
Epicholestanol		—	—	5	—
Coprostanol		—	—	7	—
B-Norecholesterol		—	—	0	—
Cholesteryl chloride		—	0	0	—
Oestradiol		—	0	—	—
Diethylstilboestrol		—	0	—	—

sterol-esterifying enzyme. There is a competitive effect when the two substrates are together, the same cofactor is required and enzyme inhibitors produce similar changes in activity. Yet cholecalciferol has no B ring and has a 3-hydroxy group which, although defined from the parent sterol as 3β , is physically in the 3α -position. On these grounds it should not be esterified at all by the enzyme. However, the use of molecular models has demonstrated that there are spacial similarities between vitamin D and cholesterol, which suggest that these substances are not as unlike as their formulae indicate.

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