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Aqueous solubilisation of vitamin D_3 in normal man

M RAUTUREAU AND J C RAMBAUD
with the technical assistance of Mrs A Bisalli, and M Rongier

From the Unité de Recherches sur la Physiopathologie de la Digestion, INSERM U. 54, Hôpital Saint-Lazare, Paris, France

SUMMARY Jejunal aqueous solubilisation of vitamin D_3 was assessed in eight normal subjects, after ingestion of a standard liquid test meal. Percentages and total concentrations of vitamin D_3 in the aqueous phase were significantly higher in the first post-prandial 30 minutes than during the following two hours, as were bile salts, total lipids, and free fatty acids. As shown by partial correlation analysis, a statistically significant relationship was found between aqueous concentrations of free fatty acids and of vitamin D_3 in the jejunal content during all the $2\frac{1}{2}$ hours of the study. From these data it is concluded that, in healthy man, vitamin D_3 is solubilised *in vivo* in mixed micelles only, and is governed in the aqueous phase.

Intestinal absorption of vitamin D_3 includes three steps: the intraluminal aqueous phase, the enterocyte phase, and the entry into mesenteric lymph. Most studies have been devoted to the second and the third steps, but information regarding the first step is not available. Some indirect evidence has been reported[1] that dietary fats may enhance vitamin D_3 absorption and one of the possible mechanisms of this would be the formation of mixed micelles in the intestinal lumen, which would increase the aqueous solubility of vitamin D_3.[2]

Vitamin D_3, like cholesterol, belongs to the class I insoluble non-swelling polar lipids, which are poorly solubilised in bile salt micelle solutions.[3] However, the actual aqueous solubilisation, *in vivo*, of vitamin D_3 has not been studied in man. The purpose of the present study was to determine, in healthy subjects receiving a standard homogenised liquid test meal, whether aqueous solubilisation of vitamin D_3 in the jejunal lumen depends primarily on bile salt or lipid in the aqueous phase.

Methods

SUBJECTS AND EXPERIMENTAL PROCEDURE

Eight subjects (21–64 years old, six males and two females) were studied who were without gastrointestinal, pancreatic, hepatic, or bile duct disease. Their daily diet contained 60–100 g fat. All were inpatients, and all gave informed consent. The evening before the study, a radio-opaque double lumen tube was passed and the subject fasted until the next morning. The tips of the tube were then positioned under fluoroscopic control in the stomach and in the first jejunal loop respectively.

A standard test meal consisting of 30 g corn oil, 25 g milk protein powder, 50 g D-glucose, 12 μCi = 0·78 nmol synthetic (1α 2α (n) 3H) vitamin D_3 (^{3}H–D_3) (98% purity, Amersham, England) 20 nmol unlabelled vitamin D_3 and adjusted to a total volume of 400 ml with water, was emulsified in a blender and quickly infused into the stomach. Jejunal contents were collected by siphonage every two minutes. An aliquot of each two minute sample was heated at 70°C for 10 minutes to inactive lipase, and then maintained at 37°C. When vitamin D_3 is heated to 80°C, 78% remains in the same molecular form, and 22% is isomerised to previtamin D_3.[4] The 15 two minute inactivated aliquots obtained in each $\frac{1}{2}$ hour period were pooled. Collections were continued for $2\frac{1}{2}$ hours. Samples of each $\frac{1}{2}$ hour period were centrifuged at 100 000 *g* for 18 hours at 37°C to separate the aqueous phase. The entire aqueous phase was recovered by aspiration through the side wall of the polycarbonate centrifuge tube, near the sediment. Other samples of each $\frac{1}{2}$ hour period, taken after thorough mixing, were not centrifuged and represented the total phase of the jejunal content Assays of bile salts, free fatty acids, total lipids, and ^{3}H–D_3 were performed in the aqueous and total phases.

TECHNIQUES

Lipids were extracted using the system of Blanken-

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Table *Concentrations of bile salts, lipids, free fatty acids, and vitamin 3H-D3 in total and aqueous phases*

Periods of sampling (min)	Bile salts (mmol/l)		Lipids (g/l)		Fatty acids (mmol/l)		Vitamin ^{3}H-D_3 (mmol/l)	
	Total	Aqueous	Total	Aqueous	Total free	Aqueous	Total	Aqueous
1 0–30	16·18±3·08*	14·90±3·27*	8·32±1·7	4·75±1·09*	13·84±2·42†	8·08±1·81	13·22±2·64	8·67±1·82*
2 30–60	7·77±1·12	6·45±1·06	4·34±1·51	1·9 ±0·59	7·95±2·78	3·12±1·01	6·73±1·95	3·63±1·07
3 60–90	5·88±1·09	4·97±0·89	2·18±0·7	0·75±0·18	4·37±1·14	1·33±0·30	3·21±0·75	1·74±0·43
4 90–120	6·67±0·94	5·45±1·06	4·41±2·11	1·15±0·54	7·16±2·94	1·87±0·89	6·33±3·0	2·37±0·95
5 120–150	6·6 ±0·63	5·15±0·41	6·02±3·03	1·33±0·53	8·67±3·87	2·21±0·91	9·8 ±6·07	2·86±1·29
Mean n=40	8·62±2·06	7·38±2·08	5·05±0·89	1·98±0·36	8·40±1·26	3·32±0·61	7·86±1·53	3·85±0·64
Analysis of variance	F=7·69 P<0·001	F=8·44 P<0·001	F=2·02 NS	F=10·68 P<0·001	F=3·02 P<0·05	F=12·91 P<0·001	F=2·10 NS	F=12·43 P<0·001

Newman-Keuls test *> 1, 2, 3, 4, 5 P<0·01.
†> 1, 3 P<0·05.
Results are given as mean±SEM (n=13).

horn and Ahrens[5] and measured gravimetrically. Free fatty acids were estimated by Dole's technique[6]; Bile salts were separated by thin layer chromatography after successive passages in three solvent systems.[7 8 9] The four main fractions isolated: taurocholic acid (TC), taurochenodeoxycholic acid (TCD)+taurodeoxycholic acid (TDC), glycocholic acid (GC); glycochenodeoxycholic acid (GCD)+glycodeoxycholic acid (GDC) were eluted with methanol and estimated fluorimetrically[10] with 3α-hydroxysteroid-dehydrogenase[11] by comparison with a standard sample obtained in the same technical conditions. In two subjects, monoglycerides were separated by thin-layer chromatography[9] and glycerol was measured fluorimetrically.[12]

Tritiated vitamin D_3 radioactivity was measured by liquid scintillation counting in Bray's fluid using a Tricarb-Packard spectrometer.

Oil phase and sediment volumes were neglected for the calculation of bile salt, lipid, free fatty acid, and vitamin D_3 concentration in the aqueous phase. All results were expressed as mean±SEM. Interrelationships between the different variables were assessed by partial correlation analysis. Analysis of variance, which takes into account the block effect, was carried out to study differences between the periods, and the Newman-Keuls test was used for comparisons of the means.

Results

The concentrations of bile salts, total lipids, free fatty acids, and ^{3}H–D_3 in the total and aqueous phases are shown in the Table. For all periods mixed, the bile salt concentration in the total phase was 8·62±0·92 mmol/l (n=40). The values for TC, TCD+TDC, GC, GCD+GDC, were 0·92±0·1, 1·47±0·15, 2·65±0·28, 3·58±0·43 mmol/l respectively.

The percentage of bile salts present in the aqueous phase was 83·7±2·3%. This percentage was 87·9±3·8 for TC, 83·8±2·5 for TCD±TDC, 87·8±1·8 for GC, and 78·3±2·9 for GCD+GCD.

Analysis of variance with the block method followed by the Newman-Keuls test showed that bile salt concentration in the total phase was significantly higher in the first 30 minute period than in the following ones, which did not differ significantly from each other (Table). The concentrations of total lipids and ^{3}H-D^3 and of free fatty acids in the total phase showed small variations during the whole study, although statistical analysis showed significant differences between two periods for free fatty acids (Table).

In the aqueous phase, significant differences were seen in the five periods, between bile salt concentrations and between free fatty acid and vitamin D_3 concentrations. The concentrations of these three compounds were significantly higher in the first period than during the other ones, which did not

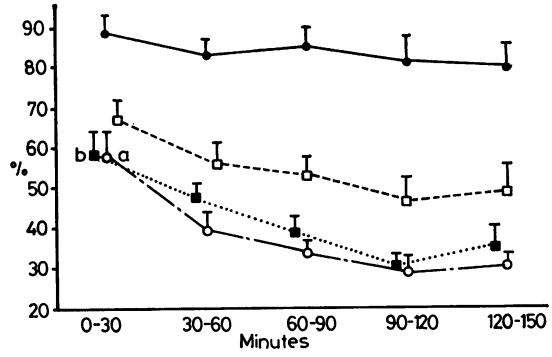

Fig. 1 *Percentages of bile salts, lipids, free fatty acids, and vitamin ^{3}H-D_3 present in the aqueous phase. Bile salt ●. Lipids ■. Free fatty acids ○. Vitamin ^{3}H-D_3 □. Vertical bars = means±SEM (n=13): means significantly higher (by Newman-Keuls test) are shown as: a (1>2, 3, 4, 5, P< 0·01). b (1>2, 3, 5, P< 0·05).*

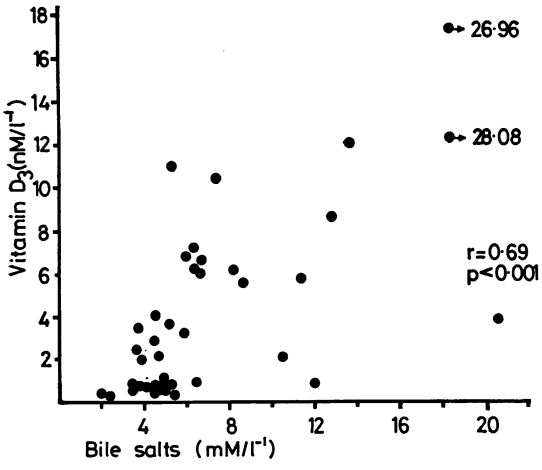

Fig. 2 *Correlation between concentrations in the aqueous phase of bile salts and of vitamin ^{3}H-D_3.*

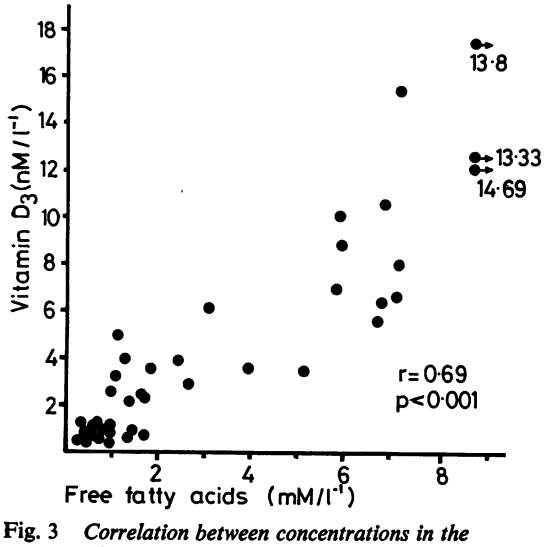

Fig. 3 *Correlation between concentrations in the aqueous phase of free fatty acids and of vitamin ^{3}H-D_3.*

differ significantly from each other. The percentages of bile salts and ^{3}H–D_3 present in the aqueous phase did not differ significantly between the periods, whereas the percentages of total lipids and free fatty acids followed the same pattern as in their respective aqueous concentrations, and were significantly higher in the first period than during the following ones (Fig. 1).

The composition of the aqueous and total phases expressed as percentages of the molar sum of free fatty acids (FFA) monoglycerides (MG), diglycerides (DG), and triglycerides (TG) was determined in two subjects. In the total phase, the mean values for FFA, MG, DG, TG were respectively 70·3±2·4, 16·7±1·4, 8·6±1·2, 4·4±0·9%, and in the aqueous phase: 76·2±1·8, 18·2±1·4, 4·5±1, 1·1±0·4%.

For all periods mixed, significant correlations were found between aqueous bile salt concentrations and aqueous free fatty acid, lipid, or ^{3}H–D_3 concentrations (Fig. 2). There was also a significant correlation between aqueous free fatty acid (Fig. 3) monoglyceride (r=0·66, P<0·05) or total lipid concentrations and aqueous ^{3}H–D_3 concentrations. Calculation of partial correlation coefficients (Fig. 4) showed that, for a constant concentration of aqueous free fatty acids or lipids, aqueous concentration of bile salts and ^{3}H–D_3 were not correlated, whereas, for a constant concentration of aqueous bile salts, there was a statistically significant correlation between aqueous concentration of ^{3}H–D_3 and aqueous free fatty acids or total lipid concentrations.

Discussion

Indirect evidence suggests that dietary fats enhance vitamin D absorption in man.[1] The means of this enhancement are complex. Thompson *et al.*[2] and Hollander *et al.*[13] have studied, with conflicting results, the effects of aqueous swelling lipids on the entry of vitamin D_3 into the small intestinal mucosa by the method of intraluminal infusion: decreased entry of vitamin D_3 was found in one study[13] while

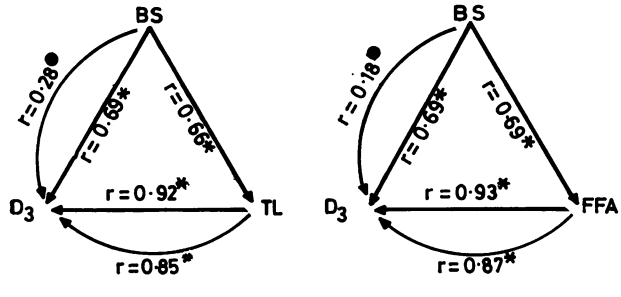

Fig. 4 *Correlations between aqueous bile salts (BS), aqueous vitamin D_3 (D_3) aqueous free fatty acids (FFA, right), or aqueous lipids (TL, left). Straight lines: correlations, curved lines: partial correlations.* ● *NS.* *P< 0·001.

no significant effect compared with simple taurocholate micelles was observed in the other.[2] Moreover, Thompson *et al.*[2] have found that swelling lipids increased transport of vitamin D_3 out of the intestinal wall into the lymph. Our results show that dietary lipids also govern the aqueous solubilisation of vitamin D_3. With the test meal we used, the aqueous phase of the intestinal content contained mixed micelles, which were chiefly composed of free fatty acids, monoglycerides, and bile salts. Biliary lecithin was probably also present in a significant amount during the first 30 minute period, but was thereafter hydrolysed in lysolecithin, which is readily absorbed.[14,15] Monoglycerides and 'fatty acids soaps' (combination of anion and protonated free fatty acid) behave as insoluble swelling amphiphiles which expand the size of the micelle when added to bile salt solutions; this micelle expansion is necessary to solubilise vitamin D_3.[3]

There was a significant partial correlation between aqueous free fatty acids and vitamin D_3, but not between aqueous bile salt and vitamin D_3. A partial correlation between monoglycerides and vitamin D_3 could not be determined, as monoglycerides were assayed in only two subjects. There was, however, a significant correlation between aqueous monoglycerides and vitamin D_3. Moreover, as aqueous free fatty acid concentrations were more than three times those of monoglycerides, their role in the aqueous solubilisation of vitamin D_3 is probably more important. These findings offer an explanation of why, in normal subjects, appreciable quantities of vitamin D_3 are absorbed when fed with triglycerides, hydrolysis of which produces free fatty acids and monoglycerides, whereas, when vitamin D_3 only is fed, it is scarcely absorbed.[16] Similar conclusions have been drawn for cholesterol, another insoluble non-swelling polar lipid.[3,17]

We have found that the mean percentage of total bile salts present in the aqueous phase, is close to that published by Simmons *et al.*,[18] but is lower than that reported by other authors.[18a–21] In order to minimise the errors produced by the ultracentrifugation,[22,23] it was performed at a high speed, over a prolonged period of time, and with polycarbonate tubes, and the aqueous phase was recovered *in toto* to avoid concentration gradients. The other factor which determines the concentration of bile salts in the aqueous phase is the amount of bile salts present in the total phase.

The fact that aqueous solubilisation of vitamin D_3, an absolute prerequisite for its absorption, is dependent upon mixed micelle formation has pathophysiological implications. In pancreatic insufficiency, the usual malabsorption of vitamin D_3 demonstrated by tritiated vitamin D_3 oral test[24,25]; may be explained by the impaired hydrolysis of triglycerides into monoglycerides and free fatty acids. In adult coeliac disease, osteomalacia and malabsorption of vitamin D_3 may be observed in spite of minimal steatorrhoea.[25] This could be explained by the low ability of the ileum—which is usually normal in these cases of coeliac disease—to absorb vitamin D_3, whereas the fatty acid absorption rate, although lower than in the jejunum, is still noticeable. However, another possibility is that the secretory state prevailing in the proximal small intestine[26] and the delayed contraction of the gallbladder[27] lower the intraluminal bile salt concentration. This, in turn, would produce a decrease in aqueous lipids leading to vitamin D_3 malabsorption.

References

[1]Knudson A, Floody RJ. Fat as a factor in the healing of rickets with vitamin D. *J Nutr* 1940; **20:** 317.

[2]Thompson GR, Ockner RK, Isselbacher KJ. Effect of mixed micellar lipid on the absorption of cholesterol and vitamin D_3 into lymph. *J Clin Invest* 1969; **48:** 87–95.

[3]Carey MC, Small DM. Micelle formation by bile salts. *Arch Intern Med* 1972; **130:** 506–27.

[4]Hanewald KH, Rappoldt MP, Roborgh. The antirachitic activity of previtamin D_3. *Recueil* 1961: **80:** 1003–14.

[5]Blankenhorn DH, Ahrens EH. Extraction, isolation and identification of hydrolytic products of triglyceride digestion in man. *J Biol Chem* 1955; **212:** 69–81.

[6]Dole VP. A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *J Clin Invest* 1956; **35:** 150–4.

[7]Gänshirt JT, Koss FW, Morianz K. Untersuchungen zur quantitativen Auswertung der Dünn-schichtchromatographie. *Arzneim Forsch* 1960; **10:** 943–7.

[8]Kritschevsky D, Martak DS, Rothblat GH. Detection of bile acids in thin-layer chromatography. *Anal Biochem* 1963; **5:** 388–92.

[9]Pie A, Giner A. Solvents for thin-layer chromatography of blood serum lipids. *Nature* 1966; **212:** 402–3.

[10]Murphy GM, Billing BH, Baron DN. A fluorimetric and enzymatic method for the estimation of serum total bile acids. *J Clin Pathol* 1970; **23:** 594–8.

[11]Iawata T, Yamasaki K. Enzymatic determination and thin-layer chromatograph of bile acids in blood. *J Biochem* 1969; **56:** 424–31.

[12]Sardesai VM, Manning JA. The determination of triglycerides in plasma and tissues. *Clin Chem* 1968; **14:** 156–61.

[13]Hollander D, Muralidhara S, Zimmerman Z. Vitamin D_3 intestinal absorption *in vivo*: influence of fatty acids, bile salts and perfusate pH on absorption. *Gut* 1978; **19:** 267–72.

[14]Borgström B, Dalquist A, Lundh G, Sjovall J. Studies of intestinal digestion and absorption in the human. *J Clin Invest* 1957; **36:** 1521–36.

[15]Nilsson A, Borgström B. Absorption and metabolism

of lecithin and lysolecithin by intestinal slices. *Biochem Biophys Acta* 1967; **137:** 240–54.

[16]Borgström B. Bile salts. Their physiological functions in the gastrointestinal tract. *Acta Med Scand* 1974; **196:** 1–10.

[17]Simmonds WJ, Hofmann AF, Theodor E. Absorption of cholesterol from a micellar solution: intestinal perfusion studies in man. *J Clin Invest* 1967; **46:** 874–90.

[18]Simmons F, Bouchier IAD. Intraluminal bile salt concentrations and fat digestion after cholecystectomy. *S Afr Med J* 1972; **46:** 2089–92.

[18a]Hofmann AF, Borgström B. The intraluminal phase of fat digestion in man: the lipid content of the micellar and oil phases of intestinal content obtained during fat digestion and absorption. *J Clin Invest* 1964; **2:** 247–57.

[19]Badley BWD, Murphy GM, Bouchier IAD, Sherlock S. Diminished micellar lipid in patients with chronic non alcoholic liver disease and steatorrhea. *Gastroenterology* 1970; **58:** 781–9.

[20]Modai M, Theodor E. Intestinal contents in patients with viral hepatitis after a lipid meal. *Gastroenterology* 1970; **58:** 379–87.

[21]Miettinen TA, Siurala M. Bile salts, sterols, sterols esters, glycerides and fatty acids in micellar and oil phases of intestinal contents during fat digestion in man. *Z Klin Chemie Klin Biochemie* 1971; **9:** 47–52.

[22]Porter HP, Saunders DR. Isolation of the aqueous phase of a fatty meal. *Gastroenterology* 1971; **60:** 997–1107.

[23]Lee KY. Artefacts in ultracentrifugal estimation of aqueous fatty acids concentration. *J Lipid Res* 1972; **13:** 745–9.

[24]Thompson GR, Lewis B, and Booth CC. Absorption of vitamin D_3-^3H in control subjects and patients with intestinal malabsorption. *J Clin Invest* 1966; **45:** 94–102.

[25]Prost A, Rambaud JC, Miravet L *et al.* Les ostéomalacies révélatrices de la maladie coeliaque de l'adulte. A propos de sept observations. *Nouv Presse Méd* 1972; **20:** 1329–36.

[26]Schmid WC, Philips SF, Summerskill WHJ. Jejunal secretion of electrolytes and water in non-tropical sprue. *J Lab Clin Med* 1969; **73:** 772–83.

[27]Dimagno EP, Go VLW, Summerskill WHJ. Impaired cholecytokinin pancreozymin secretion, intraluminal dilution and maldigestion of fat in sprue. *Gastroenterology* 1972; **63:** 25–32.