Effect of cholestyramine on the absorption of vitamin D₃ and calcium

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SUMMARY The absorption of 10 µg doses of vitamin D₃-³H in rats was found to be decreased by the addition of cholestyramine to the diet in an amount sufficient to cause steatorrhoea. Studies *in vitro* suggested that the effect of cholestyramine was due to its known ability to bind bile salts and thus disrupt micelles containing vitamin D.

The absorption of 1 mg ⁴⁵calcium chloride was similar in both control and cholestyramine-fed rats, whether estimated by the whole body counting or the faecal excretion technique.

These findings show that the administration of cholestyramine causes a reduction in the absorption of vitamin D₃-³H but not of radiocalcium.

Cholestyramine is an anion exchange resin which binds bile acids in the intestinal lumen, thus impairing their absorption. This property has led to its use in the treatment of hypercholesterolaemia (Hashim and Van Itallie, 1965; Howard, Brusco, and Furman, 1966) and the pruritus of partial biliary obstruction (Carey and Williams, 1961; Datta and Sherlock, 1963). In addition, cholestyramine has been shown to induce steatorrhoea in both rats (Harkins, Hagerman, and Sarett, 1965) and man (Hashim, Bergen, and Van Itallie, 1961).

Vitamin D deficiency after therapy with cholestyramine has not been reported, although malabsorption of this fat-soluble vitamin might be expected to accompany cholestyramine-induced steatorrhoea. This, if sufficiently marked to cause vitamin D deficiency, might eventually lead to an impairment of calcium absorption. On the contrary, Briscoe and Ragen (1963) have observed increased calcium absorption in three patients treated with cholestyramine, and there have also been reports of calcification in the biliary tree of patients receiving this drug on a long-term basis (Schaffner, Klion, and Latuff, 1965; Wells, Kneppshield, and Davis, 1968).

In an attempt to clarify this apparent paradox the effect of cholestyramine on the absorption of both radioactive vitamin D and calcium has been measured

in rats. The results of these studies show that cholestyramine markedly interferes with the absorption of vitamin D₃-³H but not with that of radiocalcium. In addition, some studies *in vitro* were undertaken to determine the mechanism of the interaction between cholestyramine, bile acids, and vitamin D.

METHODS

PREPARATION AND PURIFICATION OF VITAMIN D₃-³H Crystalline vitamin D₃² was labelled with tritium by random exchange³ and then purified and characterized as previously described (Thompson, Lewis, and Booth, 1966). The batch of vitamin D₃-³H used in these experiments had a specific activity of 62.8 µc per mg, and a biological activity of approximately 40 iu per µg when assayed in rachitic rats (Bourdillon, Bruce, Fischmann, and Webster, 1931). The stock solution of vitamin D₃-³H was stored at 4°C and repurified at approximately weekly intervals by thin-layer chromatography. The labelled vitamin was chromatographed on plates of Kieselgel H⁴ containing Rhodamine 6G with chloroform as the solvent, and its position located with an ultra-violet lamp. The vitamin was then eluted with chloroform and its specific activity determined.

ABSORPTION OF VITAMIN D₃ Eleven male Wistar rats, each weighing approximately 200 g, were divided into two groups. All rats received a standard diet containing 3% fat and 5% fibre⁵, but in one group cholestyramine (Merck, Sharp, and Dohme) was mixed in with the

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diet in a proportion of 4% of dry weight, equivalent to 2% of wet weight. After nine days of equilibration on this regime the rats were put in metabolic cages and their vitamin D absorption was determined as follows.

Doses of 10 μ g (400 iu) of vitamin D₃-³H in chloroform were dried in a rotary evaporator and then redissolved in 0.2 ml propylene glycol in ethanol 1:1, v/v. Calibrated doses were administered via an intragastric tube to non-fasting rats. Faeces were collected in individual containers for five days after the vitamin D₃ administration and then weighed and homogenized in 100 ml water. Approximately 5 g was then weighed and refluxed for two hours in 100 ml 2:1 chloroform-methanol, v/v. The faecal extract was cooled and filtered into separating funnels. The flask was rinsed with 10 ml 2:1 chloroform-methanol, v/v, and the phases were separated with 22 ml water (Folch, Lees, and Sloan Stanley, 1957). Centrifugation was employed to speed separation. The chloroform extract was dried down in a rotary evaporator and then redissolved in 5 ml toluene, of which 0.5 ml was added to 10 ml of a scintillation fluid containing butyl PBD (2-(4-butylphenyl)-5-(4-biphenyl)-1, 3,4-oxdiazole) 6 g/l.¹ Radioactivity in these samples was assayed in a Beckman model 250 B liquid scintillation counter. Quenching was corrected by means of an external standard.

Samples of 0.5 ml of both urine and of the methanol phase of the faecal extract were counted in 10 ml of a scintillation fluid containing butyl PBD 8 g, naphthalene 80 g, ethoxyethanol 400 ml, and toluene 600 ml. Faecal fat determinations were carried out by the method of van de Kamer, ten Bokkel Huinink, and Weyers (1949).

STUDIES IN VITRO Approximately 40 μ g of vitamin D₃-³H in chloroform was dried down, either alone or together with appropriate quantities of glyceryl-1-monooleate² and linoleic acid.³ In some experiments linoleic acid-1-¹⁴C⁴ was used to label the fatty acid. The dried lipid was gradually dissolved in 8 ml of 40 mM sodium taurocholate⁵ with heating and manual agitation of the flask. In some experiments sodium taurocholate-24-¹⁴C⁶ was also added. Finally 8 ml of a saline-phosphate buffer (0.15 M sodium chloride: 0.1 M sodium dihydrogen phosphate, 5:1 v/v) was added to dilute the final concentration of taurocholate to 20 mM, and the pH was adjusted to 6.7 with 1 N sodium hydroxide. By this means micellar solutions were prepared to contain 6.5 μ M of vitamin D₃-³H in either 20 mM taurocholate alone, or with added 4.8 μ M glyceryl monooleate plus 9.6 μ M of linoleic acid per millilitre.

Weighed amounts of cholestyramine were placed in stoppered test tubes and wetted with 0.5 ml of the saline-phosphate buffer. Of each test solution, 5 ml was carefully pipetted in triplicate into the tubes containing 25, 50, and 100 mg of cholestyramine and 0.1 ml was taken immediately for radioassay. After 15 minutes of vigorous

shaking at 37°C in a water bath, the tubes were centrifuged at 3,000 $\times$ g for five minutes and a further 0.1 ml of the supernatant was then taken for counting. There was no change in pH during the entire procedure. The difference in radioactivity (total DPM) in the solution before mixing and after centrifugation was taken as the amount of vitamin D₃-³H or linoleic acid-1-¹⁴C or sodium taurocholate-24-C⁴ removed from solution by the action of cholestyramine. In the double isotope experiments ³H and ¹⁴C were counted in separate channels and appropriate corrections made for the spillover of ¹⁴C into the ³H channel.

Of the mixed micellar solution, 1 ml taken before the addition of cholestyramine and 1 ml of the supernatant taken after centrifugation were each added to 20 ml 2:1 v/v chloroform-methanol in separating funnels. The phases were separated with 4 ml of water. The chloroform phase was dried down, and then chromatographed together with a vitamin D₃ marker, as described above. For radioassay, 1 cm strips of the chromatograph were scraped off the plate into counting vials containing 10 ml scintillation fluid.

RADIOCALCIUM ABSORPTION Radioactive calcium (⁴⁷Ca), freshly prepared by the Radio-chemical Centre, was mixed with 1 mg of nonradioactive calcium chloride. Absorption was measured both by means of the faecal excretion of radioactivity and by whole body counting.

Two similar studies were carried out. The first consisted of the 11 rats previously given vitamin D₃-³H, six of whom had continued to receive 4% cholestyramine during the previous three weeks. The second group consisted of 12 fresh rats who received 2.5 μ g (100 iu) of unlabelled vitamin D₃ by gastric tube before the experiment was started, so as to avoid any possible bias that might result from malabsorption of vitamin D in cholestyramine-treated animals. Six of the second group of rats subsequently received 4% cholestyramine in their diet for three days. All 12 were then placed in individual metabolic cages and starved overnight until two hours before the administration of calcium, at which time they resumed their diet. The results within each of the two control and two cholestyramine subgroups were found to be very similar and were therefore pooled.

Alternate control and treated animals were given 0.3 ml of the labelled calcium chloride containing 1 to 3 μ C by stomach tube at 15-minute intervals. Half an hour after dosage, each rat was placed in a polythene bag in a beaker and lowered into a large ring counter. Radiation from ⁴⁷scandium, the main daughter product of ⁴⁷Ca, was excluded by a lead shield. Counting proceeded until 10,000 counts had accumulated above the background. Exactly 24 hours later the animal was killed and the entire intestinal tract removed. The carcass was then recounted, with appropriate correction for the 24-hour decay in ⁴⁷Ca activity.

Control doses, urine, faeces, and the small and large bowel of each rat were placed in separate counting vials and counted in an automatic gamma counter (Nuclear Enterprises, Edinburgh, Scotland). Samples from control and treated animals were alternated to minimize the error due to isotopic decay.

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RESULTS

ABSORPTION OF VITAMIN D₃-³H The mean body weight, food intake, and faecal weight of the control and cholestyramine-treated rats were all very similar (Table I). However, the faecal fat excretion of the cholestyramine group (2.86 ± 0.29 m-equiv/day) was more than double that of the controls (1.23 ± 0.05 m-equiv/day; $P < 0.001$).

TABLE I

EFFECT OF CHOLESTYRAMINE ON FAECAL FAT EXCRETION

Group	No. of Rats	Body Weight (g)	Food Intake (g/day)	Faecal Weight (g/day)	Faecal Fat (m-equiv/day)
Control	5	190 ± 23^1	47 ± 2	33 ± 7	1.23 ± 0.05
Cholestyramine-treated	6	201 ± 19	47 ± 2	37 ± 5	2.86 ± 0.29

¹Results are mean $\pm$ 1 SD

The effect of cholestyramine on the absorption of vitamin D₃-³H is shown in Table II. The faecal excretion of radioactivity was measured during the five days following the oral dose of vitamin D₃-³H. Absorption was calculated as the dose of radioactivity given minus the chloroform-soluble radioactivity excreted in the faeces, expressed as a percentage of the administered dose. The absorption of vitamin D₃-³H in the control group was $64.7 \pm 4.1\%$ whereas it was reduced to $28.1 \pm 14.7\%$ in the cholestyramine-treated animals ($P < 0.001$). In addition there was also a difference between the two groups regarding the methanol-soluble faecal radioactivity, which amounted to $7.4 \pm 2.7\%$ in the controls but was only $1.9 \pm 1.0\%$ in the cholestyramine group ($P < 0.01$; see Table II).

TABLE II

EFFECT OF CHOLESTYRAMINE ON ABSORPTION OF 10 μ g VITAMIN D₃-³H

Group	No. of Rats	Faecal Radioactivity (% of Dose ¹)		Absorption (%)
		Chloroform Fraction	Methanol Fraction	
Control	5	35.3 ± 4.1^1	7.4 ± 2.7	64.7 ± 4.1
Cholestyramine treated	6	71.0 ± 14.7	1.9 ± 1.0	28.1 ± 14.7

¹Results are mean $\pm$ 1 SD

Urinary radioactivity never exceeded 1.2% of the administered dose during the subsequent three days in either group.

FIG. 1. Amount of vitamin D₃-³H removed from taurocholate and mixed micellar solutions by 25, 50, and 100 mg cholestyramine. This is calculated as the percentage difference between the radioactivity initially present minus the residual radioactivity after the addition of cholestyramine.

EFFECT OF CHOLESTYRAMINE ON VITAMIN D₃ Figure 1 illustrates the comparative effects of 25, 50, and 100 mg of cholestyramine in removing vitamin D₃-³H from solution in mixed micelles and taurocholate micelles.

Centrifugation of solutions of vitamin D₃-³H in either taurocholate or mixed micelles caused no change in radioactivity. However, the addition of 25, 50, and 100 mg of cholestyramine to the mixed micellar solution resulted in the disappearance from the supernatant solution respectively of 41.2%, 70.4%, and 72.2% of the radioactivity initially present. In contrast 100% of the vitamin D₃-³H in the 20 mm taurocholate solution was removed by 100 mg of cholestyramine. This amount of cholestyramine was equivalent to 2% w/v, which was the same concentration as was used in the studies *in vivo*.

In another experiment equal amounts of vitamin D₃-³H were dissolved in the following media: 20 mm taurocholate, taurocholate plus 4.8 μ m/ml glyceryl monooleate, taurocholate plus 9.6 μ m/ml linoleic acid and mixed micelles. Cholestyramine 100 mg was then added to each of these solutions, as previously described, and the radioactivity remaining in each supernatant was determined. The results are shown in Figure 2. It is evident that less vitamin D was removed from the solutions containing monoolein (39.4%) or mixed micelles (48.9%)

FIG. 2. Amount of vitamin D₃-³H removed from various micellar solutions by cholestyramine 100 mg. TC = taurocholate 20 mM, LA = linoleic acid, MO = monoolein.

than from those containing fatty acid (83.2%) or taurocholate alone (100%).

It was shown on thin-layer chromatography that 83% of the radioactivity present in the mixed micellar solution before the addition of cholestyramine had the same *R_f* as vitamin D₃. After admixture with cholestyramine and centrifugation, 75% of the residual radioactivity was located in this region.

In a subsequent study linoleic acid-1-¹⁴C was used to label the fatty acid component of the mixed micellar solution. In this instance it was found that 62.4% of the vitamin D₃-³H and 71.1% of the linoleic acid-1-¹⁴C were removed from solution by 100 mg of cholestyramine.

EFFECT OF CHOLESTYRAMINE ON TAUROCHOLATE-¹⁴C In order to investigate the varying susceptibility to cholestyramine of vitamin D₃-³H when incubated in different forms of micellar solution the following experiment was performed.

Sodium taurocholate-24-C¹⁴ was added to the taurocholate component of pure bile salt micelles, monoglyceride micelles, fatty acid micelles, and mixed micelles. The results (Table III) showed that the amount of taurocholate-¹⁴C removed from each micellar solution by 100 mg cholestyramine was similar, irrespective of the type of micelle, and ranged from 70.3% for taurocholate alone to 63.8% for linoleic acid micelles.

RADIOCALCIUM ABSORPTION The absorption of ⁴⁷Ca in the two groups of rats is shown in Table IV.

TABLE III

EFFECT OF CHOLESTYRAMINE ON REMOVING 20 mM SODIUM TAUROCHOLATE-24-¹⁴C FROM DIFFERENT MICELLAR SOLUTIONS

Type of Micelle	Taurocholate- ¹⁴ C Removed (%)
Taurocholate (TC)	70.3
Fatty acid (TC + FA)	63.8
Monoglyceride (TC + MO)	66.3
Mixed (TC + FA + MO)	67.0

Using the whole body counting method absorption was 50.2 ± 13.8% in the control group and 48.4 ± 6.2% in the cholestyramine-treated group.

Measurement of the faecal excretion of ⁴⁷Ca showed the absorption to be 44.8 ± 13.1% in controls and 43.2 ± 14.4% in the cholestyramine groups. These results suggest that radio-calcium absorption in the two groups was very similar. However, when the control and cholestyramine results were pooled and the two methods of estimating calcium absorption compared, no significant correlation between them could be shown (*r* = 0.32, *P* > 0.05).

In both groups of rats less than 1% of administered radioactivity was recovered from urine within 24 hours.

TABLE IV

EFFECT OF CHOLESTYRAMINE ON ABSORPTION OF ⁴⁷CALCIUM

Group	No. of Rats	Weight (g)	Absorption of 1 mg of ⁴⁷ Calcium (% of dose ¹)	
			Whole Body Method	Faecal Method
Control	11	238 ± 66	50.2 ± 13.8	44.8 ± 13.1
Cholestyramine treated	12	235 ± 62	48.4 ± 6.2	43.2 ± 14.4

DISCUSSION

These studies clearly show that the administration of cholestyramine to rats in a dose sufficient to produce steatorrhoea leads to marked malabsorption of vitamin D₃. Harkins *et al* (1965) found that the degree of steatorrhoea induced was proportional to the dose of cholestyramine given. In the present study cholestyramine was administered as 4% of the dry weight of diet, since this was considered to be the minimal amount required to induce consistent steatorrhoea.

A strictly physiological dose of vitamin D is difficult to determine, but is probably of the order of 0.25 μg or 10 iu daily (Carlsson and Lindquist, 1955). In the present study, owing to the limited

specific activity of the vitamin D₃-³H, the minimal dose which could be used was 10 µg. However, Schachter, Finkelstein, and Kowarski (1964) found no significant difference in the percentage absorption of 40 µg compared with 1 mg of vitamin D in intact rats, which suggests that vitamin D absorption is not dose-dependent within this range.

The radioactivity recovered in the chloroform phase of faecal extracts following administration of vitamin D₃-³H probably represented both unabsorbed vitamin D₃ and biologically inactive metabolites formed by the action of colonic bacteria (Avioli, Lee, McDonald, Lund, and DeLuca, 1967). In contrast, methanol-soluble radioactivity was probably largely due to biliary metabolites, formed by conjugation in the liver. In the cholestyramine-treated rats an increased faecal excretion of chloroform-soluble radioactivity was accompanied by reduced amounts of methanol-soluble radioactivity, suggesting that the amount of radioactivity excreted in bile was dependent upon the amount of vitamin absorbed. As has been shown previously (Thompson *et al.*, 1966), the urinary excretion of radioactivity after administration of vitamin D₃-³H was minimal.

Bile salts are necessary for the absorption of vitamin D (Schachter *et al.*, 1964), and the malabsorption of vitamin D₃-³H in the cholestyramine-treated rats presumably resulted from binding of bile salts by the resin and their subsequent excretion in the faeces (Carey and Williams, 1961; Tennent, Segel, Zanetti, Kuron, Ott, and Wolf, 1960). Overall depletion of bile salts due to interruption of the enterohepatic circulation (Weiner and Lack, 1968) would tend to impair micelle formation, but local binding of bile salts in vitamin D₃ containing micelles is probably also an important factor. This latter effect of cholestyramine was illustrated by the studies *in vitro* which showed that over 60% of the vitamin D in mixed micelles was removed from solution by the resin. When the vitamin D was dissolved in taurocholate micelles it appeared to be even more susceptible to the action of cholestyramine, which caused its total disappearance from solution (Fig. 1). Although Gallo, Bailey, and Sheffner (1965) found that unionized drugs such as digoxin may be non-specifically bound to cholestyramine, it seems unlikely that the resin would bind vitamin D₃ to a sufficient extent to explain the present findings.

The fatty acid component of mixed micelles was removed from solution by cholestyramine to a similar extent as vitamin D, although whether this was due to direct binding of ionized fatty acid was not determined. Vitamin D appeared to be least susceptible to the effect of cholestyramine when dissolved in a micellar solution of glyceryl mono-

oleate. This protective effect of monoglyceride for vitamin D was also evident when using mixed micelles. Since the amount of taurocholate removed from solution by cholestyramine was fairly constant, whatever the type of micelle, it seems probable that the beneficial effect of monoglyceride was related to its ability to remain in micellar solution even after the resin had markedly reduced the concentration of taurocholate. Hofmann (1963) has shown that monooleate is solubilized on almost a mole for mole basis by taurodeoxycholate, and in the present study the residual concentration of taurocholate was in the region of 5 to 7 mM. This concentration would theoretically have been able to maintain in micellar form virtually all of the monooleate originally present and consequentially much of the vitamin D.

Malabsorption of fat-soluble vitamins due to cholestyramine therapy has not been proven in man although Visintine, Michaels, Fukayama, Conklin, and Kinsell (1961) reported the development of vitamin K deficiency in a patient on long-term cholestyramine therapy. However, the patient described had primary biliary cirrhosis so that vitamin K malabsorption could not be ascribed solely to the resin, although the work of Whiteside, Harkins, Fluckiger, and Sarett (1965) suggested that it would be a contributory factor. The present studies suggest the possibility that vitamin D deficiency could arise during long-term cholestyramine therapy: regular parenteral administration of vitamin D, and probably the other fat-soluble vitamins, would seem advisable in patients receiving the resin in amounts sufficient to induce steatorrhoea. This problem is particularly relevant in patients with chronic biliary obstruction, in whom vitamin D absorption is often impaired, even before the administration of cholestyramine (Thompson *et al.*, 1966).

The effect of long-term administration of cholestyramine on calcium absorption has previously been investigated by Briscoe and Ragan (1963). These authors found an increase in the absorption of calcium, given with a meal, in the three human subjects whom they studied. Their findings are somewhat difficult to reconcile with the existing experimental evidence, since Webbing and Holdsworth (1966) and Lengemann and Dobbins (1958), both working with rats, showed that bile salts enhanced the absorption of calcium. This effect could be abolished by ligating the common bile duct and it seems reasonable to assume that cholestyramine might act in an analogous manner. However, this was not borne out by the results of the present study, which showed that administration of cholestyramine over periods of three days to three weeks had no effect on radiocalcium absorption. There

were wide individual variations in radiocalcium absorption in both control and cholestyramine-treated rats, as has been found by others (Hertoghe, Phinney, and Rubin, 1967), but there were no significant differences in mean absorption between the two groups, irrespective of whether absorption was calculated from total body uptake or from the faecal excretion of ^{47}Ca .

Hertoghe *et al* (1967) found good agreement between the mean absorption of ^{47}Ca estimated by the whole body counting and faecal excretion methods, although they did not analyse their data statistically. They used young rats of 80 to 100 g, on account of their reproducibly high absorption and retention of radiocalcium. However, when the pooled results obtained by each method during the present study were compared, no significant correlation between them could be shown. The individual results obtained by the whole body method were usually higher than those obtained by the faecal excretion method, with less variation about each mean. These findings suggest that the whole body counting method was possibly the more reliable.

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