The Effect of Bile, Bile Acids and Detergents on Calcium Absorption in the Chick

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1. Bile from rachitic or normal chicks causes an immediate increase in the intestinal absorption of soluble calcium in rachitic and vitamin D₃-treated chicks as tested *in vivo* by intestinal-loop and oral-dosing methods. 2. This effect is apparently solely due to the taurine-conjugated bile acids present in the bile and is independent of the action of vitamin D. 3. Chick bile and bile acids can increase the solubility and the absorption of calcium presented as sparingly soluble calcium hydrogen phosphate. 4. In addition, bile is necessary to some extent at least for the intestinal absorption of vitamin D₃ in the chick and this would indirectly enhance the absorption of calcium. 5. Thus bile is capable of a threefold action in the absorption of calcium in the chick. It is suggested that the direct action on sparingly soluble forms of calcium is of considerable physiological importance since most of the calcium in the normal bird's diet would be in this form. 6. Bile acids enhance the absorption of calcium in all regions of the small intestine of the chick. 7. Of a range of bile acids and detergents tested for enhancement of calcium absorption, various taurine-conjugated bile acids and sodium lauryl sulphate, an anionic detergent, are effective. A non-ionic detergent (Tween 80) and a cationic detergent (Zephiran) are without effect. 8. The ability of a substance to increase directly the intestinal absorption of soluble calcium appears to depend to some extent on an anionic detergent action, i.e. the ability to form a salt or complex soluble to some extent in both aqueous and lipid phases. 9. In chicks the immediate deposition of calcium (⁴⁵Ca) in the bones closely reflects any increase in plasma calcium radioactivity regardless of the cause of the increase and regardless of the vitamin D₃ status. Although sodium lauryl sulphate can increase markedly the calcium absorption from the gut and the immediate deposition in the bones it has no significant effect on rickets. 10. Some of the implications of these findings are discussed.

It has been believed for some time that bile is concerned in the absorption of calcium from the intestine. Pavlov (1905) reported that osteoporosis and general weakness occurred in dogs with biliary fistulae after a few months, although there was no apparent thickening of the chondrocostal junctions (as in rickets). Among others, Looser (1907) and later Wisner & Whipple (1922) confirmed these findings in dogs. Seidel (1910) reported two cases of middle-aged women who had had complete biliary fistulae for some time and who showed marked osteoporosis at autopsy. Buchbinder & Kern (1927) showed that dogs with ligated bile ducts had a low serum calcium concentration, and reported three human cases with jaundice, due to various causes, in whom the serum calcium concentration was low but which returned to normal

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with recovery from the jaundice. Beznak (1931) showed that the addition of sodium taurocholate to a diet containing fat and calcium increased calcium absorption from the intestine of dogs, and claimed that bile acids play an important role in calcium metabolism. Greaves & Schmidt (1932), as a result of their experiments on dogs, claimed that bile enhances calcium absorption only as a result of an effect on the absorption of vitamin D. Taylor, Weld & Sykes (1935) agreed with this conclusion for dogs and could find no antirachitic effect in ox bile by feeding it for 10 weeks to fowls on a rachitogenic diet, nor did it appear to enhance the action of a suboptimum dose of irradiated ergosterol in another group of fowls. However, irradiated ergosterol contains only vitamin D₂, which is poorly antirachitic in birds. Klinke (1928) and later Verzar & McDougall (1936) suggested that calcium, fatty acids and bile acids together form a soluble

diffusible complex in the intestine that could be absorbed readily as such and broken down within the body, liberating free calcium.

It seems certain that bile is concerned in the absorption of vitamin D. Heyman (1937) found that the vitamin disappeared from the serum of dogs when the bile duct was ligated. Work by Schachter, Finkelstein & Kowarski (1964) has shown in rats that tritiated vitamin D₃ is absorbed by means of the lymphatics and that sodium taurocholate increases the amount absorbed.

An effect of bile on calcium absorption, independent of any action on vitamin D, was suggested by the work of Lengemann & Dobbins (1958), who found that an intraperitoneal injection of sodium taurocholate improved the absorption of ⁴⁵Ca given by mouth to normal rats. The giving of the bile salt by mouth was not as effective as parenterally, and no effect was obtained when the bile salt and calcium were given orally to rats with ligated bile ducts. Ligation of the bile ducts, however, prevented the effect usually produced by the parenteral injection of taurocholate. Vaughan & Filer (1960), adding bile (from an unstated source) directly to closed ileal and duodenal segments of rat intestine *in vivo*, were unable to confirm this reported effect of bile on calcium absorption.

While investigating the absorption of ⁴⁵Ca from the small intestine of rachitic chicks *in vivo*, Coates & Holdsworth (1961) found that bile from rachitic chicks, which therefore presumably contained negligible amounts of vitamin D₃, would markedly increase the immediate absorption of calcium.

These conflicting reports suggested that a more thorough investigation of the role of bile in calcium absorption was needed.

MATERIALS AND METHODS

Birds and diet. White Leghorn cockerels (1 day old) were obtained from a local poultry farm and were raised under artificial light on a diet deficient only in vitamin D for 3–4 weeks, as described by Sallis & Holdsworth (1962). In addition to this rachitogenic diet, selected groups of chicks were given supplements of substances as described below.

Vitamin D₃. This was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. To study the effect of vitamin D₃ the rachitic chicks were given the vitamin in one of the following ways.

(a) Short-term treatment. Two methods were used: (1) a single dose of 200 i.u. in 0.5 ml. of maize oil was given orally, by tube into the crop 16 hr. before an experiment; (2) a single dose of 200 i.u. in 0.25 ml. of propylene glycol was injected intramuscularly into a pectoral muscle 16 hr. before an experiment (this method was used in some experiments involving chicks with tied bile ducts).

(b) Long-term treatment. In this method 100 i.u. in 0.25 ml. of maize oil was given orally, by tube into the crop, twice a week until the chicks were killed, 24 days from hatching, i.e. there were seven doses in all, the last dose

being given on the twenty-second day. This method was used in determining the bone ash and plasma calcium, and for a source of bile from vitamin D₃-treated chicks (normal 3-week bile).

Sodium lauryl sulphate. This was L.R.-grade reagent from British Drug Houses Ltd., Poole, Dorset. To study the effect of this substance on rickets, a group of 20 chicks was given the rachitogenic diet with the addition of 0.1% of finely powdered sodium lauryl sulphate for 24 days from hatching. As controls, two other groups of chicks from the same hatching were raised for the same period: one group of 20 chicks on the rachitogenic diet alone, and the other group of 10 chicks on the rachitogenic diet plus 100 i.u. of vitamin D₃ twice a week, as described above.

Assessment of rickets. The degree of rickets was assessed by comparing the percentages of ash in dried fat-free tibiae and the plasma calcium concentrations in the test groups of chicks with those of chicks raised on the rachitogenic diet with a supplement of vitamin D₃.

Determination of bone ash percentage. This was done as in the preparation of tibiae for the counting of ⁴⁵Ca (see below) by weighing the fat-free tibiae before and after incineration.

Plasma calcium concentration. This was estimated by titration with 1.3 mM-EDTA, essentially by the method of Baron & Bell (1957), but with 1 ml. of N-NaOH in place of the glycine buffer, a modification due to G. B. Storer (personal communication).

Bile. (a) Chick bile. The chicks were kept without food for 2 hr. from 9.00 a.m. and killed by wringing their necks, and the abdomen was opened through an incision over the gall bladder. While the cystic duct was pinched between thumb and forefinger, the bile was aspirated from the gall bladder into a bottle through an 18-gauge hypodermic needle. The bile was stored at -15° until used.

Chick bile was obtained from the following sources: (i) from 3–4-week-old rachitic chicks (this was designated 'rachitic bile'); (ii) from 3-week-old chicks reared on the rachitogenic diet but with a supplement of 100 i.u. of vitamin D₃ given orally twice a week (this was designated 'normal 3-week bile'); (iii) from 8-week-old chicks reared on a commercial poultry farm (this was designated 'normal 8-week bile').

(b) Ox bile. This was obtained fresh from the Adelaide Metropolitan Abattoirs and kept at -15° until used.

Preparation of conjugated bile acids from ox bile and from bile samples from rachitic and normal chicks. This was done according to the method of Gordon, Kuksis & Beveridge (1963), except that the equivalent Amberlite resins CG-120 and CG-400 (100–200 mesh) were used.

Assay of bile acids. The bile acid content of various samples of chick bile was estimated according to the method of Singer & Fitschen (1961) as trihydroxy and dihydroxy acids, by using the volumes specified for human gall-bladder bile. Attempts were made to use taurocholic acid and taurochenodeoxycholic acid as standards in this method but sufficiently pure samples could not be prepared. Repeatedly recrystallized cholic acid and deoxycholic acid, which gave single spots on thin-layer chromatography, were finally used as standards, as in the original method.

Hydrolysis of taurine-conjugated bile acids of chicken bile. The taurine conjugates from 5 ml. original volume of bile from rachitic, normal 3-week and normal 8-week biles were each refluxed for 18 hr. with 0.5 g. of KOH. The pH was then adjusted to pH 4 with N-HCl, when the bile salts were

precipitated as a gum. After standing overnight, the gum was separated, washed with N-HCl , dried and dissolved in 5 ml. of 95% (v/v) ethanol.

Sources of single bile acids. Cholic acid and sodium taurocholate were from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; chenodeoxycholic acid and lithocholic acid were from L. Light and Co. Ltd.; deoxycholic acid was from Sigma Chemical Co., St Louis, Mo., U.S.A.; koala bile salts were obtained by courtesy of Professor G. A. D. Haslewood (these consist mainly of tauro-3 α -hydroxy-7-oxo-cholanic acid according to G. A. D. Haslewood, personal communication). Taurochenodeoxycholate, taurodeoxycholate and tauroolithocholate were prepared according to the method of Norman (1955), and their identities and purities assessed by thin-layer chromatography. All so-called 'single' bile acids showed very small traces of bile acids other than the named acid on thin-layer chromatography, but no attempt was made to achieve absolute purity, except for cholic acid and deoxycholic acid, which were repeatedly crystallized from 99% (v/v) ethanol until they showed a single spot on thin-layer chromatography. These were used as standards in the assay of bile acids in bile.

Thin-layer chromatography of bile acids. This was done on 20 cm. $\times$ 20 cm. plates coated with Kieselgel G nach Stahl (E. Merck A.-G., Darmstadt, Germany). For taurine conjugates the following solvent system was used: isoamyl acetate (commercial mixture of 3- and 2-methylbutyl acetate)-propionic acid-propan-1-ol-water (2:3:4:1, by vol.).

For unconjugated bile acids, the solvent systems S10, S13 and S15 of Eneroth (1963) were used. The spots were identified by spraying with SbCl_3 in acetic acid (20:20, w/v) or with the anisaldehyde spray of Kritchevsky, Martak & Rothblat (1963), and heating for 10 min. at 120°.

Method of tying bile ducts and forming bile fistulae in chicks. Under ether anaesthesia and semiseptic conditions, a small longitudinal incision was made in the anterior abdominal wall over the gall bladder. The two common bile ducts were cut between ligatures just before their entry into the duodenum. The contents of the gall bladder were aspirated through an 18-gauge hypodermic needle, and one end of a 2.5 cm. length of fine polythene tubing (1 mm. bore) was tied into the gall bladder with a purse-string suture, and the other end then led out obliquely through the abdominal incision and tied in place with the abdominal wall closing sutures. The tip of a polythene glove was tied firmly over the external end of the polythene tube and protected by a pad of cotton-wool stuck to the skin with compound tincture of benzoin. The chicks were allowed to recover for 48 hr. on their previous dietary regime before being used for calcium-absorption experiments. The glove tip containing bile was emptied at the end of 24 hr. and replaced to prevent excessive back pressure, leakage and infection of the biliary tree.

Radioactive calcium. The $^{45}\text{CaCl}_2$ obtained from The Radiochemical Centre, Amersham, Bucks., was used throughout as a tracer for calcium. The original solution was diluted with unlabelled CaCl_2 to give a specific activity of approx. $2\mu\text{C}/\text{mg.}$ of calcium. The calcium was usually administered as CaCl_2 at a pH of 6.5, either in a dose of 4 mg. of Ca^{2+} containing 8 μC per bird (ileal-loop method), or in a dose of 2 mg. of Ca^{2+} containing 2 μC (oral-dosing method).

[^{45}Ca] Calcium phosphate was prepared from $^{45}\text{CaCl}_2$ as mainly CaHPO_4 by precipitation with Na_2HPO_4 (A.R.

grade; British Drug Houses Ltd.) at pH 8. The precipitate was washed twice with 0.01 M- Na_2HPO_4 and then with 95% (v/v) ethanol, and dried for 8 hr. at 110°. The specific activity was approx. 0.2 $\mu\text{C}/\text{mg.}$ of CaHPO_4 . This was used to determine the ability of bile and bile acids to increase the solubility and the absorption of calcium presented in an insoluble form.

Methods of estimating intestinal absorption of calcium. Two general methods of giving the isotope with the substances to be tested were used, both *in vivo*.

(a) Closed-ileal-loop method. The test solutions were given into a closed segment of the ileum between the attachments of the yolk sac and the blind ends of the caeca (about 12.5 cm.) by the method described by Sallis & Holdsworth (1962). Blood samples (0.5 ml.) were taken into heparin-treated syringes by cardiac puncture at 20 and 60 min., and the birds were killed at 60 min. and one tibia was removed and cleaned for determination of bone deposition of ^{45}Ca .

In one experiment the absorption of calcium was studied at other portions of the intestine. Closed loops were formed, as described by Sallis & Holdsworth (1962), in the duodenum, the jejunum and ileum. These loops were all approx. 10–12 cm. long. The junction between the jejunum and ileum was considered to be at the attachment of the yolk sac.

The test solutions were given in a total volume of 0.5 ml. per bird, made up by adding the equivalent of 0.3 ml. of 0.9% NaCl per bird to the equivalent of 0.1 ml. per bird each of calcium solution and of the substance to be tested, all in aqueous solution, with the final pH adjusted to 6.5.

The substances tested were included in the following concentrations and amounts per bird:

Bile: rachitic and normal (3-week and 8-week) chick bile, and ox bile, 0.1 ml. of original bile in 0.5 ml.

Taurine conjugates of bile acids from rachitic and normal (3-week and 8-week) chick bile: equivalent to 0.1 ml. of original bile in 0.5 ml.

Koala bile acids: 0.02 M (final concn.).

Taurine conjugates of bile acids from ox bile: 0.02 M (final concn.).

Hydrolysed bile acids from rachitic chick bile: 0.1 ml. of a saturated solution at room temperature in 0.5 ml. (solubility in water was very slight; see single acids below).

Chenodeoxycholic acid and cholic acid: 0.1 ml. of saturated solutions at room temperatures in 0.05 ml. (solubilities of these are very slight; less than 0.05 g./100 ml. of water at 20°).

Taurocholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid and tauroolithocholic acid: 0.02 M (final concn.).

Sodium lauryl sulphate: 0.02 M and 0.007 M (final concn.).

Zephiran (alkylbenzyltrimethylammonium chlorides) (Bayer Pharma Pty. Ltd., Sydney, N.S.W., Australia): 0.2% (final concn.).

Tween 80 [polyoxymethylene (20) sorbitan mono-oleate (L. Light and Co. Ltd.)]: 0.2% (final concn.).

It was noted that, at these concentrations, marked precipitation of (presumably) the calcium salt occurred with tauroolithocholic acid, chick hydrolysed bile acids, cholic acid and chenodeoxycholic acid, and some precipitation occurred with the higher concentration of sodium lauryl sulphate. No attempt was made to separate the precipitates; the solutions were shaken before injection so as to include a proportion of the precipitate.

(b) Oral-dosing method. The test solutions for this method

were usually given by tube into the crop in a total volume of 1 ml. containing the 2 mg. of calcium and the substance to be tested in the following concentrations or amounts:

Bile: rachitic chick bile, 0.9 ml.

Sodium lauryl sulphate: 0.003 M (final concn.) in aq. 0.9% NaCl.

Blood samples were taken at 20 and 60 min. and the tibia at 60 min.

With the above methods, solutions of equivalent volume containing the calcium dose in aq. 0.9% NaCl were used as control (this solution is designated 'saline' below).

(c) Oral-dosing method with calcium phosphate. The $^{45}\text{CaHPO}_4$ prepared as above was triturated in a mortar with aq. 0.5% gum tragacanth solution at pH 7 to give a dose of 15 mg. (approx. $3\mu\text{C}$ of ^{45}Ca) of CaHPO_4 in 0.5 ml. per bird. The suspension was magnetically stirred continuously to maintain an even distribution. Just before administration the 0.5 ml. dose of CaHPO_4 suspension was drawn up in the administering syringe with 0.5 ml. of a saline control, bile or bile acid solution at pH 7 in the concentrations given below, and the mixture given by polythene tube into the crop. This was immediately followed by 0.5 ml. of distilled water given with the same syringe and tube.

The test substances and concentrations used in this experiment were:

Rachitic bile: undiluted.

Taurochenodeoxycholate: 0.1 M in 0.9% NaCl.

Taurocholate: 0.1 M in 0.9% NaCl.

Food and water were allowed throughout the experiment. Blood samples were taken at 20 and 60 min. and a tibia at 60 min., as in the other experiments.

Determination of ^{45}Ca . (a) In blood plasma. After centrifugation of the heparin-treated blood, 0.250 ml. of plasma was treated with 0.250 ml. of N-HClO_4 and centrifuged, and 0.100 ml. of the supernatant counted by scintillation counting.

(b) In tibiae. These were extracted with 95% (v/v) ethanol and then diethyl ether in a Soxhlet extractor, each solvent for 24 hr., dried *in vacuo* in a desiccator and weighed. They were then incinerated at 700° to constant weight, ground to a powder and counted at infinite thickness in a 1cm^2 planchet by end-window counting, by using an Ekco type N 620 shielded end-window Geiger-Müller tube and correcting for background radiation.

The specific activity of the ^{45}Ca solutions decreased with time and no attempt was made to administer the same amount of radioactivity in each experiment. Therefore the radioactivities, which are taken to reflect calcium absorption, are only consistent within each experiment and cannot be compared between experiments.

Sodium lauryl [^{35}S]sulphate. This was obtained from The Radiochemical Centre. The original material contained 44 mc/m-mole and was diluted with 10 times its weight of carrier sodium lauryl sulphate. The final concentration used in ileal loops was 0.007 M.

Determination of ^{35}S . (a) In plasma. This was determined essentially by the same method as used for ^{45}Ca in plasma, by scintillation counting.

(b) In tibiae. The tibiae were broken into a crucible, dissolved in 5 ml. of conc. HNO_3 , evaporated to dryness and incinerated at 700° for 4 hr. The residues were then counted at infinite thickness in a 1cm^2 planchet by end-window counting and corrected for background radiation.

Scintillation counting. Samples (0.100 ml.) in 5 ml. of

Bray's Phosphor (Bray, 1960) were counted for 5 min. in an Ekco M 664A counter and corrected for background radiation.

Solubility of various calcium complexes in butanol-benzene. These were determined as follows: 2 ml. of aq. 0.9% NaCl solutions containing 4 mg. of calcium (as the chloride)/ml., and with specific activities of about $1\mu\text{C}/\text{ml}.$, and containing the various substances used in testing for calcium absorption in the same concentrations and at the same pH, i.e. 6.5, as used in the ileal-loop solutions, were spun for 5 min. at 2000g in a bench centrifuge to remove gross precipitates. Then 1 ml. portions of the supernatants were shaken for 15 min. with 1 ml. of butan-1-ol-benzene (1:1, v/v) and centrifuged for 15 min. at 2000g. Samples (0.100 ml.) of the organic supernatants were counted in a scintillation counter. Corrections were made for quenching with an internal standard, and for background radiation.

Determination of the solubility of calcium as calcium phosphate with various bile acids. A 10 mg. sample of $^{45}\text{CaHPO}_4$, prepared as described above, was shaken with 2 ml. of 0.9% NaCl or with 0.9% NaCl made 0.02 M with respect to the sodium salts of various bile acids for 1 hr. at 37° . The undissolved calcium phosphate was centrifuged down at 2000g for 15 min. and 0.1 ml. of the clear supernatant counted by scintillation counting. A standard solution of the calcium phosphate in *N*-acetic acid was also counted and the solvent effect of the various substances on the calcium in this form was calculated.

RESULTS

Effect of whole bile on calcium absorption. Whole bile from rachitic chickens and from normal (3-week and 8-week) chicks each caused an immediate and approximately equal increase in calcium absorption from ileal loops (Table 1) in rachitic chicks; this also occurred when 0.9 ml. of rachitic bile was given orally with calcium, yet did not occur when 0.9 ml. of rachitic bile was given orally 16 hr. before the calcium (Table 2). Bone counts reflected the plasma counts in each case.

Effect of ox bile on calcium absorption from ileal loops. Although there was a difference in the means, suggesting that some enhancing effect of ox bile on calcium absorption had occurred, these differences were not significant (Table 3). These results are included to show the difficulties in using a physiological substance from a widely different source in an experimental animal.

Effect of whole bile on calcium absorption after vitamin D_3 treatment. Whole rachitic bile appeared to enhance the increased absorption of calcium seen 16 hr. after an oral dose of vitamin D_2 , both from ileal loops and by oral administration (Tables 4 and 5), although the difference between the means was of doubtful significance ($P = 0.1$). Bone counts reflected the plasma counts in each case.

Relation between bile secretion and vitamin D_3 effect. Vitamin D_3 given intramuscularly 16 hr. before the experiment caused an equal increase in calcium absorption (oral-testing method) in birds with and

Table 1. *Effect of rachitic and normal (3-week and 8-week) biles on calcium absorption from ileal loops*

Ileal loops of rachitic-chick intestine were established *in vivo*. Each loop received 4 mg. of ^{45}Ca (approx. $8\mu\text{C}$) in a total volume of 0.5 ml. Bile was added, 0.1 ml. per loop. Blood samples were taken at 20 and 60 min. Tibiae were taken at 60 min. and ashed, and the ^{45}Ca was counted in 1 cm.² planchets at infinite thickness. The results are given as mean values $\pm$ S.E.M.

Test substance	No. of birds	Plasma		Tibiae (counts/min. at 60 min.)
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Saline	5	1076 $\pm$ 84	931 $\pm$ 40	179 $\pm$ 12
Rachitic bile	5	2952 $\pm$ 209*	1187 $\pm$ 70	326 $\pm$ 40*
Normal 3-week bile	5	2783 $\pm$ 180*	1105 $\pm$ 80	321 $\pm$ 44*
Normal 8-week bile	5	2699 $\pm$ 215*	1011 $\pm$ 240	309 $\pm$ 35*

* Significantly different from saline control ($P = 0.05$).

Table 2. *Effects of whole rachitic bile on calcium absorption (oral method)*

A 1 ml. sample of solution containing 2 mg. of ^{45}Ca (approx. $2\mu\text{C}$) and either 0.9% NaCl or 0.9 ml. of rachitic chick bile was given by tube into the crop of rachitic chicks. Blood samples were taken at 20 and 60 min. and a tibia at 60 min. The results are mean values $\pm$ S.E.M.

Test substance	No. of birds	Plasma		Tibiae (counts/min. at 60 min.)
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Saline	5	924 $\pm$ 212	1172 $\pm$ 328	88 $\pm$ 8
Rachitic bile (16 hr.)	5	904 $\pm$ 236	1132 $\pm$ 316	85 $\pm$ 10
Rachitic bile (<i>statim</i>)	5	2288 $\pm$ 728*	2464 $\pm$ 232*	196 $\pm$ 38*

* Significantly different from saline control ($P = 0.05$).

Table 3. *Effect of ox bile on calcium absorption from ileal loops*

Ileal loops were established in rachitic chicks *in vivo* as described in Table 1 with the exception that 0.1 ml. of ox bile was used in place of chick bile. The results are mean values $\pm$ S.E.M.

Test substance	No. of birds	Plasma		Tibiae (counts/min. at 60 min.)
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Saline	5	1756 $\pm$ 600	1184 $\pm$ 880	252 $\pm$ 140
Ox bile	5	2148 $\pm$ 800*	1376 $\pm$ 760*	329 $\pm$ 176*

* Not significantly different from the saline control.

without tied bile ducts. The difference between vitamin D₃-treated and non-treated birds, both with tied ducts, was greater than the differences obtained in the presence of bile (Table 6). Bone counts again reflected the plasma counts in each case.

Effect of vitamin D₃ orally in the absence of bile (oral-testing method). Chicks with tied bile ducts failed to show any significant increase in calcium absorption 16 hr. after an oral dose of vitamin D₃, whereas another group with tied ducts, but given 1 ml. of rachitic bile at the same time as the dose of

vitamin D₃, showed an increase in calcium absorption, not as great as, but approaching, the usual response to vitamin D₃ in rachitic chicks. Bone counts reflected the plasma counts in each case (Table 7).

Apparent physiological function of bile in absorption of calcium. When 0.9 ml. of whole rachitic bile plus calcium was given orally to chicks with tied bile ducts there was an immediate increase in calcium absorption compared with that in a control group with tied ducts but not given bile (Table 8). Bone counts reflected the plasma counts in each case.

Table 4. *Effect of whole rachitic bile on calcium absorption after vitamin D₃ treatment*

The absorption of calcium from ileal loops of chick intestine *in vivo* was studied as described in Table 1. Chicks given vitamin D₃ received 200 i.u. orally 16 hr. before the experiment. The results are mean values $\pm$ S.E.M.

Group and test substance	No. of birds	Plasma		Tibiae
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	(counts/min. at 60 min.)
Rachitic; + saline	4	1053 $\pm$ 178	902 $\pm$ 304	73 $\pm$ 12
Rachitic; + bile	4	2248 $\pm$ 293*	1876 $\pm$ 401	181 $\pm$ 35*
Vitamin D ₃ (16 hr.); + saline	4	3597 $\pm$ 399*	1982 $\pm$ 601	258 $\pm$ 39*†
Vitamin D ₃ (16 hr.); + bile	4	4006 $\pm$ 382*	3173 $\pm$ 411	342 $\pm$ 41*†

* Significantly different from saline control ($P = 0.05$).

† Significantly different from one another ($P = 0.1$).

Table 5. *Effect of whole rachitic bile on calcium absorption after vitamin D₃ treatment*

The effect of an oral dose of bile on calcium absorption was studied as described in Table 2. Chicks given vitamin D₃ received 200 i.u. orally 16 hr. before the experiment. The results are mean values $\pm$ S.E.M.

Group and test substance	No. of birds	Plasma		Tibiae
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	(counts/min. at 60 min.)
Rachitic; + saline	5	1126 $\pm$ 89	1463 $\pm$ 115	326 $\pm$ 72
Rachitic; + bile	5	2540 $\pm$ 345*	1636 $\pm$ 104	560 $\pm$ 98*
Vitamin D ₃ (16 hr.); + saline	5	4948 $\pm$ 547	4100 $\pm$ 198	1313 $\pm$ 141
Vitamin D ₃ (16 hr.); + bile	5	6438 $\pm$ 793†	4424 $\pm$ 513	1512 $\pm$ 101

* Significantly different from saline control ($P = 0.05$).

† Significantly different from vitamin D₃-treated saline control ($P = 0.1$).

Table 6. *Independence of the vitamin D₃ effect on calcium absorption from that of bile*

The effect of different treatments on the absorption of calcium given orally was studied by the method described in Table 2. Two groups of rachitic chicks had their bile ducts ligated 48 hr. before the experiment. One of these groups and another non-operated group were given 200 i.u. of vitamin D₃ intramuscularly 16 hr. before the experiment. The results are mean values $\pm$ S.E.M.

Group and state of bile ducts	No. of birds	Plasma		Tibiae
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	(counts/min. at 60 min.)
Rachitic; tied bile ducts	3	420 $\pm$ 182	800 $\pm$ 405	67 $\pm$ 10
Vitamin D ₃ (16 hr.); tied bile ducts	3	8228 $\pm$ 1545*	6888 $\pm$ 1076*	928 $\pm$ 101*
Vitamin D ₃ (16 hr.); patent bile ducts	4	7445 $\pm$ 839*	6327 $\pm$ 761*	853 $\pm$ 83*

* Significantly different from rachitic control group ($P = 0.05$).

Isolation and characterization of chick bile acids. The bile acids were prepared from 50 ml. of rachitic chick bile, from 5 ml. of normal (3-week) chick bile and from 5 ml. of normal (8-week) chick bile. Thin-layer chromatographic examination of the initial ethanolic extract of bile in the method of Gordon

et al. (1963) and of the effluents from the cation-exchange column and both effluents from the anion-exchange column showed no apparent difference between normal and rachitic bile acids, and also that the bile acids present in both consisted almost completely of the taurine conjugates of

Table 7. *Effect of vitamin D₃ orally in the absence of bile (oral-dosing method)*

Calcium was given orally and its absorption studied as described in Table 2. Two groups of rachitic chicks had their bile ducts ligated 48 hr. before the experiment. One of these groups was given 200 i.u. of vitamin D₃ in maize oil orally 16 hr. before the experiment. The other operated group was given the vitamin D₃ together with 1 ml. of rachitic chick bile orally 16 hr. before the experiment. Control groups of non-operated rachitic and vitamin D₃-treated chicks were included. The results are mean values $\pm$ s.e.m.

Group and state of bile ducts	No. of birds	Plasma		Tibiae (counts/min. at 60 min.)
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Rachitic; patent bile ducts	5	1020 $\pm$ 85	1137 $\pm$ 102	113 $\pm$ 13
Vitamin D ₃ (16 hr.); patent bile ducts	5	6020 $\pm$ 306*	4913 $\pm$ 308*	397 $\pm$ 42*
Vitamin D ₃ (16 hr.); tied bile ducts	4	1104 $\pm$ 113†	1125 $\pm$ 201†	104 $\pm$ 21†
Vitamin D ₃ (16 hr.); + 1 ml. of bile; tied bile ducts	4	4109 $\pm$ 463*	3872 $\pm$ 402*	321 $\pm$ 58*

* Significantly different from rachitic control ($P = 0.05$).

† Not significantly different from rachitic control.

Table 8. *Apparent physiological function of bile in absorption of calcium*

The absorption of calcium given orally was studied as described in Table 2. Two groups of rachitic chicks had their bile ducts ligated 48 hr. before the experiment. One of these groups was given calcium in saline, and the other group was given calcium mixed with 0.9 ml. of bile. A non-operated group of rachitic chicks was given calcium in saline. The results are mean values $\pm$ s.e.m.

Group	No. of birds	Plasma		Tibiae (counts/min. at 60 min.)
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Patent bile ducts; + saline	4	1019 $\pm$ 242	1307 $\pm$ 419	407 $\pm$ 95
Tied bile ducts; + saline	3	588 $\pm$ 101*	624 $\pm$ 132*	76 $\pm$ 31*
Tied bile ducts; + bile	3	1620 $\pm$ 176	2424 $\pm$ 194	348 $\pm$ 32

* Significantly less than either of the other groups ($P = 0.05$).

Table 9. *Concentration of bile acids in chick bile*

The concentrations of dihydroxy and trihydroxy bile acids were determined in whole bile samples from rachitic and vitamin D₃-dosed (normal 3-week) chicks and one pooled sample from commercially reared (normal 8-week) chicks by the method of Singer & Fitschen (1961). Total bile acids were calculated as the sum of dihydroxy and trihydroxy acids. Results are given as mean values $\pm$ s.e.m. Differences between bile groups are not significant.

Bile	No. of samples	Dihydroxy acids (mg./ml.)	Trihydroxy acids (mg./ml.)	Total acids (mg./ml.)	Dihydroxy acid Trihydroxy acid ratio
Rachitic	5	93 $\pm$ 9	19 $\pm$ 3	112 $\pm$ 12	4.9 $\pm$ 0.3
Normal (3-week)	5	98 $\pm$ 7	20 $\pm$ 2	118 $\pm$ 7	5.0 $\pm$ 0.3
Normal (8-week)	1	88	18	106	4.8
	(pooled)				

cholic acid and chenodeoxycholic acid, mainly the latter, with trace amounts of other taurine conjugates, no glycine conjugates and negligible quantities of unconjugated bile acids. These findings agree with the findings of Anderson, Haslewood & Wootton (1957).

Thin-layer chromatography of the hydrolysates

of the taurine conjugates from both rachitic and normal chick biles confirmed that the main acids present were cholic acid and chenodeoxycholic acid. A small amount of an acid, probably 3 α -hydroxy-7-oxocholanic acid, also appeared to be present; and a trace of another acid, as yet unidentified, which was probably a monosubstituted acid, showed

Table 10. *Effect of the taurine conjugates of bile acids prepared from chick bile on calcium absorption*

Calcium absorption was studied in ileal loops *in vivo* as described in Table 1. The taurine conjugates isolated from chick bile were added to the loops in amounts equivalent to 0.1 ml. of original bile. The results are mean values $\pm$ S.E.M.

Test substance	No. of birds	Plasma		Tibiae (counts/min. at 60 min.)
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Saline	4	1341 $\pm$ 103	1432 $\pm$ 205	98 $\pm$ 12
Rachitic taurine conjugates	4	3243 $\pm$ 195	2402 $\pm$ 283	201 $\pm$ 43
Normal (8-week) taurine conjugates	4	3061 $\pm$ 287	2411 $\pm$ 262	198 $\pm$ 45
Whole rachitic bile	4	2978 $\pm$ 221	2409 $\pm$ 217	217 $\pm$ 53

Table 11. *Effect of bile acids and vitamin D₃ on calcium absorption at various levels of the small intestine*

Loops from the duodenal, jejunal or ileal portions of the small intestine of the chick *in vivo* were tested for calcium absorption, each under three sets of circumstances. These were: (i) Rachitic birds; calcium in saline solution. (ii) Rachitic birds; calcium in 0.02 M solution of taurine-conjugated bile salts from chick bile. (iii) Birds treated with 200 i.u. of vitamin D₃ 16 hr. before; calcium in saline solution. Each loop received 4 mg. of ⁴⁵Ca (approx. 8 μ C) in a total volume of 0.5 ml. Results are given as mean values $\pm$ S.E.M.

Groups	Intestinal site	No. of birds	Plasma		Tibiae (counts/min. at 60 min.)
			(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Rachitic; Ca in 0.9% saline	Duodenum	3	3936 $\pm$ 300	4038 $\pm$ 382	327 $\pm$ 41
	Jejunum	3	3964 $\pm$ 252	4524 $\pm$ 410	272 $\pm$ 38
	Ileum	3	2986 $\pm$ 408	3596 $\pm$ 371	258 $\pm$ 39
Rachitic; Ca in 0.02 M chick bile acid	Duodenum	3	5846 $\pm$ 490	7998 $\pm$ 590	398 $\pm$ 29
	Jejunum	3	7986 $\pm$ 653	5872 $\pm$ 571	430 $\pm$ 38
	Ileum	3	6300 $\pm$ 700	5232 $\pm$ 501	403 $\pm$ 36
Vitamin D ₃ (16 hr.); Ca in 0.9% saline	Duodenum	3	10830 $\pm$ 972	11694 $\pm$ 1104	450 $\pm$ 50
	Jejunum	3	18240 $\pm$ 1217	16980 $\pm$ 1583	577 $\pm$ 52
	Ileum	3	12888 $\pm$ 891	14856 $\pm$ 997	530 $\pm$ 49

up on the chromatograms. Wiggins (1955) showed that chick bile (7-day-old and adult) consisted of the taurine conjugates of chenodeoxycholic acid (80%), cholic acid (13%) and 3 α -hydroxy-7-oxo-cholanic acid (5%).

Concentration of bile acids in chick bile. Table 9 shows that there was no significant difference between the concentrations and ratios of dihydroxy and trihydroxy bile acids in five samples each of rachitic and normal (3-week) bile and in one pooled sample of normal (8-week) bile estimated according to the method of Singer & Fitschen (1961).

Effect of chick-bile taurine conjugates on calcium absorption. Taurine conjugates from rachitic bile and from normal (8-week) bile in equivalent doses to whole bile caused immediate and approximately equal increases in absorption of calcium by the ileal-loop method (Table 10). Bone counts reflected the plasma counts in each case.

Site of absorption of calcium. It has been previously found that calcium is absorbed at all levels

of the small intestine and that vitamin D₃ increases absorption in the three portions tested (Coates & Holdsworth, 1961; Sallis & Holdsworth, 1962). The results in Table 11 confirm these findings and show also that bile increases absorption at all levels in the small intestine.

Comparison of the effects of various bile acids on calcium absorption. Ileal loops *in vivo* were used for testing the effect of various bile acids on calcium absorption. The synthesized taurine-conjugated bile acids were used at a final concentration of 0.02 M. The hydrolysed chick bile acids gave a mixture that was poorly soluble in water and gave a precipitate on the addition of calcium. This precipitate was dispersed in the liquid by ultrasonic vibration and the suspension used. Table 12 shows the results obtained and clearly indicates that the taurine-conjugated bile acids can account for the effect of bile on calcium absorption. Koala bile, which is largely tauro-3 α -hydroxy-7-oxocholanic acid, is also very effective. The trihydroxy bile

Table 12. *Effect of various bile acids on calcium absorption (ileal-loop method)*

Calcium absorption was studied in ileal loops *in vivo* as described in Table 1. Bile acids were used in a final concentration of 0.02M. Since all acids were not tested at the same time two experiments are shown. Results are mean values $\pm$ S.E.M.

	Test substance	No. of birds	Plasma		Tibiae (counts/min. at 60 min.)
			(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Expt. I	Saline	4	4284 $\pm$ 318	2728 $\pm$ 164	1010 $\pm$ 95
	Hydrolysed chick bile acids	3	3768 $\pm$ 412	2096 $\pm$ 241	810 $\pm$ 109
	Tauroolithocholate	3	7940 $\pm$ 976	4572 $\pm$ 722	1550 $\pm$ 113
	Taurocholate	3	6724 $\pm$ 782	3812 $\pm$ 341	1390 $\pm$ 199
	Koala bile acids	3	8876 $\pm$ 1778	6543 $\pm$ 1343	2190 $\pm$ 278
	Chick bile acids	3	9872 $\pm$ 1754	7863 $\pm$ 411	3090 $\pm$ 201
Expt. II	Saline	4	3287 $\pm$ 243	2563 $\pm$ 207	987 $\pm$ 78
	Taurocholate	4	5862 $\pm$ 511	3419 $\pm$ 311	1276 $\pm$ 104
	Taurochenodeoxycholate	4	6983 $\pm$ 519	4926 $\pm$ 306	2072 $\pm$ 220
	Taurodeoxycholate	4	6100 $\pm$ 434	3527 $\pm$ 247	1982 $\pm$ 109
	Tauroolithocholate	4	5898 $\pm$ 511	3512 $\pm$ 287	1980 $\pm$ 92

Table 13. *Effect of detergents on calcium absorption from ileal loops*

Calcium absorption was studied in ileal loops *in vivo* as described in Table 1. The concentrations of substances added to the loops are shown in column 1. The results are mean values $\pm$ S.E.M.

Test substance	No. of birds	Plasma		Tibiae (counts/min. at 60 min.)
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Saline	4	3870 $\pm$ 451	2963 $\pm$ 752	321 $\pm$ 41
Chick taurine conjugates (0.02M)	4	11032 $\pm$ 981	5816 $\pm$ 619	677 $\pm$ 72
Zephiran (0.2%)	4	3761 $\pm$ 506	3047 $\pm$ 476	308 $\pm$ 43
Tween 80 (0.2%)	4	4074 $\pm$ 532	3128 $\pm$ 572	304 $\pm$ 54
Sodium lauryl sulphate (0.2%)	4	12175 $\pm$ 1089	8686 $\pm$ 1173	937 $\pm$ 105

acid also increased calcium absorption but apparently to a less extent than the other acids, although this difference was not statistically significant. The hydrolysed chick bile acids slightly depressed calcium absorption below that of the saline (control) group. This may have been due to the removal of some of the calcium from solution as an insoluble salt.

Effect of different detergents on calcium absorption.

As it was thought that the effects seen with bile might be due to its detergent action, three differently acting detergents were examined for their effect on calcium absorption by using ileal loops of intestine *in vivo*. The substances were tested at a final concentration of 0.2% and the results are shown in Table 13, together with a group that were given the taurine-conjugated bile acids from chick bile. A cationic detergent, alkylbenzyltrimethylammonium chloride (Zephiran), and a non-ionic detergent, polyoxyethylene sorbitan mono-oleate (Tween 80), had no significant effect on calcium absorption in the concentration used. Sodium lauryl sulphate

had a very marked effect, and under the conditions of this experiment was more effective than chick bile salts. This is shown particularly in the amount of ^{45}Ca found in bone.

Effect of sodium lauryl sulphate on rickets. Table 14 shows the results of giving 0.1% sodium lauryl sulphate in the rachitogenic diet to a group of chicks for 24 days. The sodium lauryl sulphate exerted no significant effect on the rickets over the period of time studied, according to the bone ash content and serum calcium; the sodium lauryl sulphate-treated birds still had clinical signs of rickets at the end of the testing period, similar to the control group.

Absorption of sodium lauryl sulphate with and without calcium. By using sodium lauryl [^{35}S]-sulphate, an attempt was made to determine the mode of action of sodium lauryl sulphate on the absorption of calcium from ileal loops by assessing the plasma counts and bone counts of ^{35}S with and without calcium added to the testing solutions. The results are shown in Table 15. The presence of

calcium inhibited the absorption of lauryl sulphate to some extent, as assessed by the plasma counts, but the amount of lauryl sulphate detected in the tibiae after 1 hr. was the same with or without calcium, suggesting that calcium was not carried and held in the bones by lauryl sulphate. The ^{35}S

found in the bone may be SO_4^{2-} produced by hydrolysis of the lauryl sulphate, but this aspect has not been investigated.

Effect of sodium lauryl sulphate on calcium absorption after vitamin D₃ treatment. Sodium lauryl sulphate appeared to enhance the effect of vitamin D₃ on calcium absorption as tested by the oral method (Table 16), but the difference between the means was not significant.

Solubility of calcium complexes in butanol-benzene and comparison with the effect on absorption of calcium. As reviewed by Schanker (1961), lipid-solubility is considered an important factor in the absorption of many substances from the intestine. No attempt was made to determine the partition coefficients of the various calcium complexes between butanol-benzene and water. Instead, the relative amount of ^{45}Ca made soluble in the organic phase by the different substances in the amounts used in chick experiments was determined. The approximate effectiveness for calcium absorption of each substance is placed alongside that compound for comparison. This effectiveness was assessed from the concentration of ^{45}Ca in the plasma at 20 min. as compared with the saline control. The results are shown in Table 17. There is a close correlation between the ability to increase calcium

Table 14. *Effect of sodium lauryl sulphate on rickets after 24 days*

Three groups of 1-day-old chicks were placed on the rachitogenic diet. The control group received no further supplement, the second group had 0.1% (w/w) of sodium lauryl sulphate added to the diet, and the third group received 100 i.u. of vitamin D₃ orally twice a week. Plasma calcium and tibia bone ash were determined after 24 days. The results are mean values $\pm$ S.E.M.

Diet	No. of birds	Plasma calcium (m-equiv./l.)	Tibia ash (% of bone)
Rachitogenic	20	3.4 ± 0.5	27 ± 2
Rachitogenic; + sodium lauryl sulphate (0.1%)	20	3.8 ± 0.5	28 ± 3
Rachitogenic; + vitamin D ₃ (100 i.u. twice/week)	10	4.8 ± 0.7	35 ± 4

Table 15. *Absorption of sodium lauryl sulphate with and without calcium*

The absorption of lauryl [^{35}S]sulphate was studied by placing 0.5 ml. of 0.007 M-sodium lauryl sulphate (4 mc/m-mole) into ileal loops of rachitic chicks (*in vivo*). One group also contained 4 mg. of CaCl_2 in each loop. The results are mean values $\pm$ S.E.M.

Test substance	No. of birds	^{35}S radioactivity		
		Plasma		Tibiae
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	(counts/min. at 60 min.)
Sodium lauryl [^{35}S]sulphate	5	3876 ± 395	3268 ± 310	108 ± 11
Sodium lauryl [^{35}S]sulphate + calcium	5	$2784 \pm 301^*$	$2372 \pm 286^*$	$109 \pm 14^*$

* Not significantly different from group without calcium.

Table 16. *Effect of sodium lauryl sulphate on the absorption of calcium after vitamin D₃ treatment*

The absorption of calcium given orally was studied as described in Table 2. The calcium was in saline or in 0.02 M-sodium lauryl sulphate. Vitamin D₃-treated chicks had received 200 i.u. of vitamin D₃ in maize oil 16 hr. before the experiment. The results are mean values $\pm$ S.E.M.

Group and test substance	No. of birds	Plasma		Tibiae
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	(counts/min. at 60 min.)
Rachitic; + saline	4	1850 ± 207	1791 ± 231	241 ± 35
Rachitic; + sodium lauryl sulphate (0.02 M)	5	6520 ± 792	4768 ± 353	1803 ± 279
Vitamin D ₃ (16 hr.); + saline	5	6340 ± 835	5016 ± 619	1761 ± 292
Vitamin D ₃ (16 hr.); + sodium lauryl sulphate (0.02 M)	5	8562 ± 987	5980 ± 703	2102 ± 143

Table 17. *Effect of bile, bile acids and detergents in rendering calcium lipid-soluble*

Portions (2ml.) of aq. 0.9% saline containing 8mg. of Ca as CaCl_2 (with approx. $2\mu\text{C}$ of ^{45}Ca) and containing the various substances in the same concentrations as used in the ileal-loop chick experiments were spun at 2000g for 5 min. A 1ml. portion of supernatant was shaken for 15 min. with 1ml. of butanol-benzene (1:1, v/v) mixture. After centrifugation at 2000g for 15 min., 0.100ml. of the organic phase was counted by scintillation counting and corrected for quenching and background radiation. Column 3 shows the relative solubilities of any calcium complexes present as compared with rachitic-chick bile acids, which are assigned a value of 1.00. For comparison column 4 shows the approximate increase in absorption of calcium brought about by the various substances.

Test substance	^{45}Ca (counts/min./ml. of organic supernatant)	Relative solubility in butanol-benzene	Approx. increase in calcium absorption <i>in vivo</i>
Saline (0.9%)	0	0	1.0x
Zephiran (0.2%)	120	0.004	No increase
Tween 80 (0.2%)	144	0.005	No increase
Hydrolysed chick bile acids (saturated)	502	0.018	No increase
Taurocholate (0.02M)	8620	0.31	1.5x
Taurolithocholate (0.02M)	24684	0.87	1.5x
Taurodeoxycholate (0.02M)	24872	0.90	1.5x
Taurochenodeoxycholate (0.02M)	26749	0.95	2.0x
Rachitic-chick bile acids (0.02M)	27976	1.00	2.0x
Koala bile acids (0.02M)	32215	1.15	2.0x
Sodium lauryl sulphate (0.006M)	42835	1.53	2.5x

Table 18. *Effect of bile, bile acids and lauryl sulphate on the solubility of calcium in calcium phosphate*

The $^{45}\text{CaHPO}_4$ (10mg.) was equilibrated for 1 hr. at 37° with 2ml. of the indicated solutions at pH7. After centrifugation at 2000g for 15 min., 0.1ml. of supernatant was counted by scintillation counting. By reference to a standard solution of the calcium phosphate in *N*-acetic acid the solubility of calcium in the various solutions was calculated.

Aqueous solvent	Solubility of calcium (mg./100ml.)
Sodium lauryl sulphate	15.0
Chick bile (1:5 dilution)	11.3
Taurochenodeoxycholate (0.02M)	7.6
Chick bile acids (0.02M)	6.9
Taurocholate (0.02M)	6.6
Saline (0.9%)	3.2

absorption and the ability to increase the lipid-solubility of calcium.

Effect of bile and bile acids on the solubility of calcium as calcium phosphate. The solubility of calcium presented as calcium phosphate was increased by chick bile and bile acid solutions compared with that in saline, as shown in Table 18.

Effect of bile and bile acids on the absorption of calcium presented as calcium phosphate. Rachitic bile, taurochenodeoxycholate and taurocholate all markedly increased the absorption of calcium given orally as calcium hydrogen phosphate to chicks that had had their bile ducts tied 48 hr. previously.

Bone counts reflected the plasma counts in each case (Table 19).

DISCUSSION

In previous work with closed ileal loops (Sallis & Holdsworth, 1962) it was shown that the amount of calcium absorbed by the gut was reflected in the radioactive ^{45}Ca found in plasma and in the tibia taken at 60 min. Therefore we have used plasma ^{45}Ca and tibia ^{45}Ca as a measure of calcium absorption when investigating the effect of bile on this process. It seems clear from our results that, in the chick, bile plays several important roles in calcium metabolism. We have confirmed that bile is important for the absorption of vitamin D_3 from the intestine of chicks (Table 7), and this may be one physiological role for bile, although the need for this dietary source of the vitamin might be by-passed by the biosynthesis of vitamin D_3 in the presence of adequate sunlight. This role of bile is indirect in that it is necessary for the absorption of vitamin D_3 , which in turn has a marked effect on the absorption of soluble calcium from the gut. It is necessary to emphasize that much of the evidence on the increased absorption of calcium brought about by vitamin D has been obtained with soluble salts of calcium. When the calcium was present as insoluble phosphate no absorption could be measured (Coates & Holdsworth, 1961). Yet, at the pH of the small intestine and in the presence of phosphate, fatty acid and other ions forming insoluble calcium salts, the concentration of soluble

Table 19. *Effect of bile and bile acids on the absorption of calcium presented as calcium hydrogen phosphate (oral method)*

All birds had had their bile ducts tied 48 hr. previously. Each bird was given 15 mg. of $^{45}\text{CaHPO}_4$ (approx. $3\mu\text{C}$ of ^{45}Ca) suspended in 0.5 ml. of 0.5% gum tragacanth solution, mixed just previously with 0.5 ml. of saline, rachitic-chick bile, or 0.1 M-bile acid solution, by tube into the crop. (Final concentrations of bile acids with CaHPO_4 suspension were 0.05 M, about half that of bile and CaHPO_4 suspension.) This was immediately followed by 0.5 ml. of distilled water. Blood samples were taken at 20 and 60 min. and a tibia at 60 min. Results are mean values $\pm$ S.E.M.

Test substance	No. of birds	Plasma		Tibiae (counts/min. at 60 min.)
		(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Saline	4	293 $\pm$ 120	538 $\pm$ 99	277 $\pm$ 59
Rachitic bile	4	2060 $\pm$ 460	4310 $\pm$ 730	1408 $\pm$ 360
Taurochenodeoxycholate	4	880 $\pm$ 180	1684 $\pm$ 240	635 $\pm$ 79
Taurocholate	4	864 $\pm$ 189	1696 $\pm$ 307	641 $\pm$ 109

calcium must be very small. We have shown that bile and bile acids increase the solubility and the absorption of calcium presented as the sparingly soluble calcium hydrogen phosphate (Tables 18 and 19), and this is a further and direct effect of bile on calcium absorption. Such an effect of bile was suggested by Klinke (1928), who showed that sodium deoxycholate increased the solubility of calcium carbonate, phosphate and oleate. He postulated that calcium was absorbed as a complex with long-chain fatty acids and bile salt. The effect of increasing the amount of calcium available for absorption may be a very important mode of action of bile.

A third important effect of bile has been demonstrated in the present paper and that is that bile can immediately increase the intestinal absorption of soluble calcium in chicks. This action is independent of the action of vitamin D_3 , as shown by the following considerations:

(a) Rachitic and normal chick bile have approximately equal activities.

(b) Bile acts immediately in increasing calcium absorption (i.e. within 20 min.) whereas vitamin D_3 has not been shown to act in less than 4 hr., even with massive doses (Sallis & Holdsworth, 1962).

(c) Bile from rachitic chicks is immediately effective, yet has no appreciable content of vitamin D_3 , as shown by its lack of effect when given 16 hr. before testing for calcium absorption (Table 2).

(d) An equal increase in calcium absorption was produced by a parenteral dose of vitamin D_3 in chicks with or without tied bile ducts.

(e) No significant difference, qualitative or quantitative, could be detected between the bile acid patterns of rachitic and normal chick bile.

This immediate effect of bile on calcium absorption was exclusively due to its content of bile salts. The taurine conjugates of bile acids isolated from

chick bile or single taurine-conjugated bile acids prepared by partial synthesis had marked effects on calcium absorption in the chick. Taurochenodeoxycholate, the main bile salt of chick bile, had an effect on calcium absorption equal to that of chick bile.

If a chick was deprived of both bile and vitamin D_3 the absorption of calcium was very small, as shown in Table 8 (particularly the bone-ash data). Administration of bile with the calcium greatly improved the absorption. Vitamin D_3 -deficient chicks at 4 weeks of age contain approx. 28% of ash in the bone and therefore these chicks have obviously been absorbing significant amounts of calcium. It seems likely that bile has helped to bring about this absorption in rachitic chicks. When the ^{45}Ca was mixed with rachitic bile and given by mouth to rachitic chicks the amount of calcium absorbed was greater than if the ^{45}Ca was in saline (Table 2). This is surprising since these chicks would presumably have adequate supplies of endogenous bile. However, mixing the ^{45}Ca with bile before giving to the bird may be more effective than depending on mixing in the intestine, particularly since, in birds, the bile enters at the distal end of the duodenum. Russell, Taylor & Chichester (1934) reported that there was a significant increase in the size of gall bladders of rachitic compared with normal chicks at $7\frac{1}{2}$ weeks of age. This was not due to any difference in the lipid content of the diet. Unfortunately, these investigators did not determine whether the total volume of bile produced was different in the two groups. If rachitic chicks produce more bile than normal chicks it would suggest that this was a compensatory mechanism to improve calcium absorption in the absence of vitamin D_3 .

It is difficult to extrapolate from the physiological role of bile in the chick to the role of bile in other

species because of the different bile acid patterns in most mammals, where glycine conjugates of the bile acids tend to replace the conjugates with taurine to a variable extent. Human bile, however, contains considerable amounts of taurochenodeoxycholate (Wiggins, 1955), which was the most effective bile salt found in chicks. We have not adequately tested the ability of glycine conjugates to increase calcium absorption, but preliminary experiments suggest that they are much less effective than taurine conjugates. Some of the difficulties experienced by other investigators in demonstrating a role of bile salts in calcium absorption may be due to the employment of taurocholate, which we found less effective in the chick, and also to the fact that to demonstrate an unequivocal effect of bile on calcium absorption when using soluble calcium salts it is usually necessary to use vitamin D₃-deficient animals.

A remarkable and unexpected finding was that the non-physiological substance, sodium lauryl sulphate, was even better than bile in increasing calcium absorption. This substance improved absorption even in those chicks that had previously received vitamin D₃. The investigation of the way in which sodium lauryl sulphate affects calcium transport may be useful in attempts to understand the mechanism of calcium absorption. A cationic and a non-ionic detergent were without effect on calcium transport.

Deposition of calcium in bone. Our results show that the calcium depositions in the tibia reflected the plasma concentrations of calcium, regardless of the cause of the height of the plasma concentration and regardless of the vitamin D₃ status of the chicks. Many investigators, notably Migicovsky & Emslie (1949, 1950) and Keane, Collins & Gillis (1956), have shown, with parenteral administration of ⁴⁵Ca, that vitamin D₃ is without apparent effect on the deposition of the ⁴⁵Ca in bone once it is in the circulation. Bile salts, sodium lauryl sulphate and (see Sallis & Holdsworth, 1962) dihydrotachysterol have all been shown to improve calcium absorption, and this calcium can be deposited in bone. However, these substances are unable to prevent rickets in the chick. Indeed, the deposition of ⁴⁵Ca in bone may bear no relationship to the process of bone accretion and may be largely an exchange of ⁴⁵Ca for ⁴⁰Ca in bone. Vitamin D₃ increases calcium absorption and also brings about normal calcification of bone, and must therefore have effects on calcium metabolism at some site(s) in addition to that in the intestine.

Mechanism of action of bile salts and sodium lauryl sulphate. The intestinal absorption of calcium in the chick appears to be enhanced by substances having an ability to form compounds with a favourable partition between an aqueous

and a lipid phase. Thus the taurine conjugates of bile acids were effective in the following order: dihydroxy acids > monohydroxy acids > trihydroxy acid. This order appears to be a balance between water and lipid solubility, e.g. the calcium salts of the monohydroxy taurine conjugates were highly lipid-soluble but poorly water-soluble, that of the trihydroxy taurine conjugate was poorly lipid-soluble but comparatively highly water-soluble, and those of the dihydroxy acid taurine conjugates were soluble in both phases. Unconjugated bile acids, though capable of forming a highly lipid-soluble complex with calcium, are very sparingly water-soluble.

It has been known since the work of Overton (1902) that the lipid-solubility of a substance considerably affects its ability to penetrate cell membranes. Other reports, notably from Höber & Höber (1937), Schanker, Tocco, Brodie & Hogben (1958) and Hogben, Tocco, Brodie & Schanker (1959), have confirmed and amplified this concept and shown that the rates of absorption of drugs from the intestine of rats in general parallel the lipid-solubilities of these drugs, particularly for substances having a molecular weight greater than 100. Another factor that has been shown to influence the rate of absorption of a substance from the intestine is its state of ionization. The results shown in Table 17 support the idea that lipid-solubility is important in calcium transport but also show that it is important to have a favourable balance of solubility between aqueous and lipid phases.

The concept of the transport of ions as lipid-soluble complexes is not currently a favoured one. Particularly in the instance of Na⁺ it is postulated that the ion is transported by an 'ion pump'. However, it is often necessary at some stage of a theory of ion transport to invoke a 'carrier' molecule, often of unspecified composition. Such a 'carrier' molecule would probably be anionic and also capable of association with the lipophilic cell membranes, i.e. a situation comparable with the bile salt-calcium interaction shown above. The simple physicochemical view of the effect of lipid-solubility on calcium transport is difficult to reconcile with the view that calcium is transported by an 'active' process, as suggested by Schachter & Rosen (1959) and Sallis & Holdsworth (1962). An alternative explanation of the effect of vitamin D₃, lauryl sulphate and bile salts on calcium transport is that these substances inhibit a metabolically operated pump that normally returns calcium to the lumen (Holdsworth, 1965).

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