Bile Salts and Calcium Absorption

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(Received 10 January 1966)

1. The study of the effect of bile salts on enhancing calcium absorption in the rachitic chick has been extended to bile salts not present in chick bile, e.g. glycine conjugates and bile alcohol sulphates. 2. Bile and bile salts cause an increase in calcium absorption from sparingly soluble calcium hydrogen phosphate when compared with a suspension of calcium hydrogen phosphate in saline. 3. If the bile ducts of normal rats are tied the absorption of calcium from calcium hydrogen phosphate decreases but can be restored by giving bile salts with the calcium salt. 4. Bile salts increase solubility in water of the sparingly soluble calcium salts, phytate and phosphate at pH values between 6 and 8. 5. Bile salts increase the solubility in lipid solvents of calcium in approximately the same proportion as they increase the absorption of calcium from the gut. 6. The physiological role of bile in calcium absorption and its mode of action are discussed.

It was reported previously (Webling & Holdsworth, 1965) that bile, owing to its bile salt content, enhances calcium absorption in the chick by: (i) aiding the intestinal absorption of vitamin D₃, thus indirectly increasing the absorption of calcium by the effect of the vitamin on the gut; (ii) increasing the aqueous solubility of calcium from sparingly soluble calcium hydrogen phosphate; (iii) directly increasing the absorption of calcium from water-soluble forms.

It was also reported that a synthetic anionic detergent, sodium lauryl sulphate, had effects on calcium absorption similar to the direct effects of taurine-conjugated bile salts, and it was shown that the increases in calcium absorption that occurred with these bile salts and lauryl sulphate closely paralleled their abilities to form calcium complexes soluble in both aqueous and lipid solvent phases.

The results of some further studies on effects of taurine-conjugated bile salts, and of some other bile salts characteristic of a wide range of vertebrates, on calcium absorption in rachitic chicks and non-rachitic rats are now reported, as well as further studies on calcium solubility under the influence of bile salts.

EXPERIMENTAL

Chicks and rats

Chicks. Three-week-old rachitic chicks were used in most of the calcium absorption experiments. For the 3 weeks

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these chicks had been given the vitamin D-deficient diet described by Sallis & Holdsworth (1962).

Rats. Non-rachitic rats were used in one experiment to test the effects of bile salts on calcium absorption in vertebrates other than birds. They were male Wistar hooded *Rattus norvegicus*, obtained from the Waite Agricultural Institute, University of Adelaide. They had been reared on a normal laboratory rat diet and showed no clinical signs of rickets. The percentage of ash in the tibia, determined by the same method as used for chick tibia, was 50.0 (s.e.m. ± 4.0) (28 animals). The weight of the rats at the beginning of the experiment was 150 ± 2 g. During the 3 days of the experiment they were given their normal diet and water at all times except for 2 hr. before tying their bile ducts and 1 hr. before dosing with calcium.

Methods of tying bile ducts

Chicks. The bile ducts of certain of the chicks were tied as described in detail by Webling & Holdsworth (1965).

Rats. Under ether anaesthesia and fully aseptic conditions, the common bile duct at its entry into the duodenum was cut between ligatures through a 2 cm. subcostal transverse incision and the abdominal wall closed, without drainage, with Michel clips. The rats were allowed to recover on their normal dietary regime before being used for calcium absorption studies. The effectiveness of the occlusion of the bile ducts was checked after killing the animals during the calcium absorption experiment, but in any case the occlusive effect could be seen in the icterus of the plasma samples taken for calcium estimations.

Measurement of calcium absorption

Chicks. Calcium absorption in chicks was measured either by the closed ileal loop method or by the oral dosing method. Both methods have been described in detail by

Webbing & Holdworth (1965). After administration of the ^{45}Ca -labelled salt, together with the various test substances, the radioactivity in the blood plasma was measured after 20 and 60 min. and in the tibia after 60 min.

Rats. Calcium absorption in rats was measured by a technique similar to the oral dosing method used for chicks. A suspension of $^{45}\text{CaHPO}_4$ in 0.15M-NaCl, with or without bile salts, was introduced into the oesophagus by a metal cannula. However, only one blood sample was taken instead of two as in the chick. At 45 min. after dosing, each rat was anaesthetized with ether and a blood sample taken from the heart. Each rat was then killed by a blow on the head and a tibia removed. The radioactivities of the plasma and the bone were determined as for the corresponding samples in the chick experiments.

Preparation of ^{45}Ca -labelled compounds

^{45}Ca , obtained from The Radiochemical Centre (Amersham, Bucks.) as $^{45}\text{CaCl}_2$, was used as a tracer in all experiments. The specific activity of the solutions containing ^{45}Ca decreased with time and no attempt was made to use the same amount in each experiment. Therefore the radioactivity counts, which are taken to reflect calcium uptake and deposition, are only consistent within each experiment and cannot be compared directly between experiments, although ratios to the control levels may be compared.

Calcium was used in different experiments in soluble and sparingly soluble forms. The soluble form was $^{45}\text{CaCl}_2$, diluted with non-radioactive CaCl_2 to give the required specific activity and concentration shown with the experimental results. The sparingly soluble forms were: (i) $^{45}\text{CaHPO}_4$, prepared as described by Webbing & Holdworth (1965) at a specific activity of approx. $0.2\mu\text{C}/\text{mg.}$ of CaHPO_4 ; (ii) [^{45}Ca]calcium phytate, prepared from $^{45}\text{CaCl}_2$ by precipitation with potassium phytate at pH 7; the precipitate was washed twice with water at pH 7 and dried for 7 hr. at 100° , and the specific activity was approx. $0.1\mu\text{C}/\text{mg.}$ of the final product.

Dosing solutions used in calcium absorption experiments

Calcium chloride solutions. The ileal loop method in chicks was used. In two experiments the dose was 0.5 ml./bird of a solution containing $^{45}\text{CaCl}_2$ (0.2M), bile salt (0.02M) and NaCl (0.15M); the final pH was 6.5. In one experiment the dose was 1.0 ml./bird of a solution containing $^{45}\text{CaCl}_2$ (0.01M; $4\mu\text{C}$) in 0.15M-NaCl, with or without bile salt (0.02M).

Calcium phosphate suspension. $^{45}\text{CaHPO}_4$ was triturated in a mortar with 0.5% (w/v) gum tragacanth solution at pH 7 and was magnetically stirred continuously before being drawn up in the dosing syringe.

(a) Ileal loop method in chicks. The $^{45}\text{CaHPO}_4$ suspension (9 mg. in 0.25 ml.) was taken into the syringe together with 0.25 ml. of 0.15M-NaCl or 0.25 ml. of 0.02M-bile salt solution. The mixture was introduced into the prepared ileal loop.

(b) Oral dosing method in chicks and rats. The $^{45}\text{CaHPO}_4$ suspension (15 mg. in 0.5 ml.) was taken into the syringe together with 0.5 ml. of 0.15M-NaCl, 0.5 ml. of 0.007M-

sodium lauryl sulphate, or 0.5 ml. of 0.1M-bile salt. The mixture was introduced into the proventriculus (chick) or oesophagus (rat) and this was followed by 0.5 ml. of water to rinse out the syringe and tube.

Test substances used in dosing solutions and solubility experiments

Chick bile was obtained from the gall bladders of 3-week-old rachitic chicks and stored at -15° until used.

Rabbit bile was obtained from the gall bladder of a healthy laboratory rabbit immediately after being killed and was stored at -15° until used.

Sodium lauryl sulphate (technical grade) was obtained from British Drug Houses Ltd. (Poole, Dorset).

Chick bile salts were prepared by the method of Gordon, Kuksis & Beveridge (1963) from chick bile. Taurocholate was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Glycocholate was obtained from Calbiochem (Los Angeles, Calif., U.S.A.). Taurochenodeoxycholate and glycochenodeoxycholate were obtained from Mann Research Laboratories (New York, N.Y., U.S.A.). Scymnol sulphate and ranol sulphate were a generous gift from Professor G. A. D. Haslewood, University of London. These bile salts were used at a final concentration of 0.02M in most experiments.

In each experiment, solutions of 0.15M-NaCl were used in place of the test substance solutions, as controls. These are designated 'saline' below.

Solubility studies on calcium salts under the influence of bile salts and lauryl sulphate

Aqueous solubility of calcium from sparingly soluble salts under the influence of bile, bile salts and lauryl sulphate.

(i) Calcium phytate: effects of bile and bile salts. [^{45}Ca]Calcium phytate (5 mg.) was weighed into stoppered centrifuge tubes and shaken with 2 ml. of saline, bile or bile salts for 1 hr. at 40° . Chick and rabbit bile at 1:5 dilution and taurocholate and taurochenodeoxycholate at 0.02M (final concn.) were tested. In this experiment the pH of the solutions was adjusted to pH 7 before addition to the phytate, but no later reading or further adjustment of pH was made. After shaking, the tubes were centrifuged at 2000 g for 15 min. and 0.100 ml. of the supernatant was taken for scintillation counting. The results were compared with a standard solution prepared by dissolving 5 mg. of [^{45}Ca]calcium phytate in 2 ml. of *N*-acetic acid, and the solubility of calcium in the various solutions was calculated as mg. of Ca/100 ml.

(ii) Calcium hydrogen phosphate: effects of bile salts and lauryl sulphate, and of pH. $^{45}\text{CaHPO}_4$ (10 mg.) was weighed into each of the appropriate number of stoppered 5 ml. centrifuge tubes and 4 ml. of saline (control) or saline containing bile salt (0.02M) or sodium lauryl sulphate (0.02M) was added. To attempt to simulate the conditions in the gut, estimations were made in the range pH 6–8. The solutions were adjusted to pH 5.5, the $^{45}\text{CaHPO}_4$ was added, the tubes were shaken briefly and centrifuged for 10 min. at 2000 g, and, without removing the CaHPO_4 , the pH was adjusted to about pH 6 by using a fine combined electrode dipped into the tube contents and adding minimal volumes of *N*-NaOH on the tip of a pointed glass rod. The

tubes were then stoppered and shaken for 2 hr. at 40° and centrifuged for 10 min., and 0.100 ml. of the supernatant was taken for scintillation counting. The pH was recorded and adjusted to about 0.5 pH unit higher with *N*-NaOH. This process was repeated until the pH of the solutions approached pH 8. This experiment was repeated once. Each experiment gave three or four readings at different pH values in the range pH 6–8.

Results from scintillation counting were compared with a standard solution prepared by dissolving 10 mg. of $^{45}\text{CaHPO}_4$ in 4 ml. of *N*-acetic acid and the solubility in the different solutions was calculated as mg. of Ca/100 ml.

Solubility of calcium complexes in butanol-benzene.

(i) Relative solubilities in lipid solvents of the calcium complexes formed with bile salts or lauryl sulphate. The solutions were those used in the ileal loop experiments and contained $^{45}\text{CaCl}_2$ (0.2 M) in 0.15 M-NaCl and the various substances used in absorption experiments. The solutions were shaken to distribute any precipitate and 1 ml. was taken and shaken with 1 ml. of butan-1-ol-benzene (1:1, v/v) for 15 min. The mixture was centrifuged for 15 min. at 2000 *g* and 0.100 ml. of the upper organic phase taken for scintillation counting.

Some of the original solution was centrifuged to remove any water-insoluble calcium complex and 1 ml. of the supernatant shaken with 1 ml. of butanol-benzene as above. In this way the lipid solubilities of any calcium complex formed from both water-soluble and water-insoluble forms were determined in the first extraction. Only the lipid solubilities of the water-soluble complexes were determined in the second extraction. The results were all expressed relative to the lipid solubilities of the complex formed by chick bile salt (assigned value 1.00).

(ii) Butanol-benzene/saline partition coefficients of calcium complexes of bile salts. (a) Preparation of [^{45}Ca]-calcium bile salts and [^{45}Ca]-calcium lauryl sulphate in saline. Aqueous 0.15 M-NaCl solutions (2 ml.) at pH 7, containing CaCl_2 (0.01 M) (with ^{45}Ca at 1 $\mu\text{C}/\text{mg.}$ of Ca), were prepared with bile salt (as the sodium salt) (0.02 M), or without bile salt as a control. The solutions were centrifuged at 2000 *g* for 15 min. and 1 ml. of the supernatant was taken for determination of the partition coefficients as described below. Taurocholate, taurochenodeoxycholate, taurodeoxycholate, tauroolithocholate, glycocho-

late, glycochenodeoxycholate and lauryl sulphate were used. At these concentrations, tauroolithocholate formed a heavy precipitate and lauryl sulphate a light precipitate, both of which were removed during the centrifugation. The supernatant solutions of these two substances were included in the rest of the experiment, but are not of course at the same concentrations as the rest of the solutions.

(b) Determination of partition coefficients. Portions (1 ml.) of the above aqueous solutions, after centrifugation, were each shaken in stoppered centrifuge tubes with 1 ml. of butanol-benzene (1:1, v/v) at 40° for 2 hr. After centrifugation for 15 min. at 2000 *g*, 0.100 ml. of each layer in each tube was taken for scintillation counting, and the partition coefficients were calculated from the results.

Radioactivity determinations

The radioactivities of calcium solutions and of plasma were determined by scintillation counting, and that of bone samples by end-window counting at infinite thickness of bone ash, as described by Webling & Holdsworth (1965).

RESULTS

Effects of glycine-conjugated bile salts on the absorption of soluble calcium from ileal loops of rachitic chicks. When a 0.2 M solution of calcium chloride was used (Table 1) glycochenodeoxycholate produced a heavy precipitate in the dosing solution, which, however, was shaken up and injected into the gut segment. The glycocholate dosing solution was clear. Taurochenodeoxycholate produced its previously reported enhancing effect on calcium absorption and glycocholate was also effective. Glycochenodeoxycholate appeared to depress slightly the calcium absorption compared with the saline control, although the differences were not significant.

When calcium chloride was used at a concentration (0.01 M) equivalent to the 0.02 M-glycochenodeoxycholate (Table 2) no precipitate was formed.

Table 1. *Effect of glycine-conjugated bile salts on the absorption of soluble calcium from ileal loops*

Ileal loops of rachitic-chick intestine were established *in vivo*. Each loop contained 0.5 ml. of solution containing CaCl_2 (0.2 M) and bile salt (0.02 M). Blood samples were taken at 20 and 60 min. Tibiae were taken at 60 min., ashed and the ^{45}Ca was counted on 1 cm.² planchets at infinite thickness. The results are the means $\pm$ S.E.M. for four birds. The mean increase in tibial radioactivity compared with the control birds, in this and subsequent Tables, serves to compare results between different experiments.

Test substance	Plasma		Tibiae (counts/min. at 60 min.)	Mean increase in tibial radioactivity
	(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)		
Saline	496 $\pm$ 65	354 $\pm$ 73	553 $\pm$ 61	(1.0)
Taurochenodeoxycholate	1134 $\pm$ 201*	921 $\pm$ 119*	1191 $\pm$ 199*	2.2
Glycochenodeoxycholate	384 $\pm$ 71	293 $\pm$ 78	519 $\pm$ 76	0.9
Glycocholate	924 $\pm$ 102*	493 $\pm$ 63	812 $\pm$ 93*	1.5

* Significantly greater than saline control ($P < 0.1$).

In these circumstances glycochenodeoxycholate was significantly better than taurochenodeoxycholate in enhancing calcium absorption from calcium chloride solution.

Effect of ranol sulphate and scymnol sulphate on the absorption of soluble calcium from ileal loops of rachitic chicks. Both these bile alcohol sulphates, as examples of lower-vertebrate bile salts, were effective in enhancing calcium absorption, though less so than chick bile salts and taurochenodeoxycholate with the particular conditions used in this experiment (Table 3).

Effects of glycine-conjugated bile salts and lauryl sulphate on the absorption of calcium from calcium hydrogen phosphate by oral administration in rachitic chicks. Lauryl sulphate and glycochenodeoxycholate significantly increased calcium absorption from $^{45}\text{CaHPO}_4$, and taurochenodeoxycholate showed its previously demonstrated effect (Webbing & Holdsworth, 1965). As shown in Table 4, glycocholate gave rise to a significant increase only in the tibial radioactivity.

Effect of the normal biliary secretion, bile salts and lauryl sulphate on the absorption of calcium from

calcium hydrogen phosphate in rats. In normal rats with tied bile ducts all bile salts, with the exception of glycocholate, significantly increased the absorption of calcium from $^{45}\text{CaHPO}_4$ given by mouth. Table 5 shows that there was a great difference in the amount of ^{45}Ca absorbed in the group of rats in which the bile ducts were intact compared with those with tied-off bile ducts, suggesting that the normal biliary secretion in rats is a potent influence in absorption of calcium from calcium hydrogen phosphate.

Effect of bile salts on the absorption of calcium from orally given calcium hydrogen phosphate in vitamin D₃-treated chicks. In vitamin D₃-treated chicks with tied bile ducts, chick bile salts given with $^{45}\text{CaHPO}_4$ caused an earlier increase in plasma radioactivity than in a similar group given no bile salts (Table 6). The latter group showed a later rise in plasma radioactivity, however, and the tibial radioactivities in the two vitamin D₃-treated groups were not significantly different, although both were significantly above that of a control rachitic group given $^{45}\text{CaHPO}_4$ without bile salts.

Effect of bile salts on the absorption of calcium

Table 2. *Effect of glycine-conjugated bile salts on the absorption of soluble calcium from ileal loops*

This experiment was similar to that in Table 1 but with the CaCl_2 concentration decreased to 0.01 M (bile salt concentration still 0.02 M). The results are the means $\pm$ S.E.M. for four birds.

Test substance	Plasma		ibiae (counts/min. at 60 min.)	Mean increase in tibial radioactivity
	(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)		
Saline	2148 $\pm$ 321	1635 $\pm$ 205	897 $\pm$ 99	(1.0)
Taurochenodeoxycholate	6688 $\pm$ 753*	5005 $\pm$ 821*	1876 $\pm$ 401*	2.1
Glycochenodeoxycholate	10264 $\pm$ 1762*	9563 $\pm$ 831*	2521 $\pm$ 483*	2.8

* Significantly different from saline control ($P < 0.1$). Taurochenodeoxycholate and glycochenodeoxycholate were also significantly different from one another ($P < 0.1$).

Table 3. *Effects of scymnol sulphate and ranol sulphate on the absorption of soluble calcium from ileal loops*

Experimental details were the same as those of Table 1. The results are the means $\pm$ S.E.M. for four birds.

Test substance	Plasma		Tibiae (counts/min. at 60 min.)	Mean increase in tibial radioactivity
	(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)		
Saline	894 $\pm$ 219	147 $\pm$ 54	260 $\pm$ 34	(1.0)
Chick bile salts	1800 $\pm$ 280*	678 $\pm$ 135*	656 $\pm$ 73*	2.5
Taurochenodeoxycholate	1926 $\pm$ 135*	807 $\pm$ 86*	681 $\pm$ 75*†	2.6
Scymnol sulphate	1326 $\pm$ 360	855 $\pm$ 171*	401 $\pm$ 32*†	1.5
Ranol sulphate	1545 $\pm$ 600*	708 $\pm$ 103*	502 $\pm$ 78*†	1.9

* Significantly greater than control saline group ($P < 0.05$).

† Amount of ^{45}Ca accumulated by the tibiae of chicks receiving bile alcohol sulphates was significantly less than those receiving taurochenodeoxycholate ($P < 0.1$).

Table 4. *Effects of glycine-conjugated bile salts and lauryl sulphate 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, or 0.1 M-bile acid solution, by tube into the crop. This was immediately followed by 0.5 ml. of water. Blood samples were taken at 20 and 60 min. and a tibia at 60 min. The results are the means $\pm$ s.e.m. for four birds.

Test substance	Plasma		Tibiae
	(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	(counts/min. at 60 min.)
Saline	117 $\pm$ 22	130 $\pm$ 21	95 $\pm$ 10
Glycocholate	189 $\pm$ 43	194 $\pm$ 30	132 $\pm$ 11*
Glycochenodeoxycholate	208 $\pm$ 18*	216 $\pm$ 21*	142 $\pm$ 17*
Taurochenodeoxycholate	233 $\pm$ 28*	277 $\pm$ 22*	147 $\pm$ 15*
Lauryl sulphate	253 $\pm$ 27*	261 $\pm$ 31*	156 $\pm$ 17*

* Significantly greater than control group ($P < 0.1$).

Table 5. *Effect of biliary secretion and of bile salts on the absorption of calcium presented as calcium hydrogen phosphate in rats*

Normal rats were used and all except the first group (which were sham-operated) had their bile ducts tied 48 hr. before the experiment. Solutions given by stomach tube were as described in Table 4. A blood sample and tibiae were taken at 45 min. The results are the means $\pm$ s.e.m. for groups of four rats.

Group	Plasma	Tibiae
	(counts/min./ml. at 45 min.)	(counts/min. at 45 min.)
Bile ducts patent: CaHPO_4 in saline	1132 $\pm$ 203	1043 $\pm$ 211
Bile ducts tied: CaHPO_4 in saline	399 $\pm$ 45*	175 $\pm$ 95*
Bile ducts tied: CaHPO_4 + taurocholate	599 $\pm$ 84†	374 $\pm$ 92
Bile ducts tied: CaHPO_4 + taurochenodeoxycholate	667 $\pm$ 91†	512 $\pm$ 42†
Bile ducts tied: CaHPO_4 + glycocholate	506 $\pm$ 63	273 $\pm$ 41
Bile ducts tied: CaHPO_4 + glycochenodeoxycholate	823 $\pm$ 103†	633 $\pm$ 111†
Bile ducts tied: CaHPO_4 + lauryl sulphate	1464 $\pm$ 223†	788 $\pm$ 97†

* Significantly less than group with patent bile ducts ($P < 0.02$).

† Significantly greater than saline control group ($P < 0.1$).

Table 6. *Effect of bile salts on the absorption of calcium presented as calcium hydrogen phosphate (oral method) in vitamin D₃-treated chicks*

The experimental procedure was that described in Table 4. Chicks that received vitamin D₃ had 200 i.u. 16 hr. before the experiment. The results are the means $\pm$ s.e.m. for four birds.

Group	Plasma		Tibiae
	(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	(counts/min. at 60 min.)
Rachitic: CaHPO_4 in saline	617 $\pm$ 101	738 $\pm$ 75	309 $\pm$ 83
Vitamin D ₃ -treated: CaHPO_4 in saline	856 $\pm$ 74	2812 $\pm$ 200*	1093 $\pm$ 107*†
Vitamin D ₃ -treated: CaHPO_4 + bile salts	1624 $\pm$ 111*	1228 $\pm$ 130*	846 $\pm$ 82*†

* Significantly greater than rachitic saline group ($P < 0.1$).

† Vitamin D₃-treated groups not significantly different from one another ($P < 0.1$).

Table 7. *Effect of bile salts on the absorption of calcium presented as calcium hydrogen phosphate from ileal loops in rachitic and vitamin D₃-treated chicks*

Ileal loops of washed-out chick intestine were established *in vivo*. $^{45}\text{CaHPO}_4$ (9 mg.) suspended in 0.5 ml. of 0.5% gum tragacanth solution was injected into the loops with or without chick bile salts (0.02 M). Chicks that received vitamin D₃ had 200 i.u. 16 hr. before the experiment. The results are the means $\pm$ S.E.M. for four birds.

Group	Plasma		Tibiae (counts/min. at 60 min.)
	(counts/min./ml. at 20 min.)	(counts/min./ml. at 60 min.)	
Rachitic: CaHPO_4 in saline	74 $\pm$ 12	98 $\pm$ 10	32 $\pm$ 11
Rachitic: CaHPO_4 + bile salts	91 $\pm$ 5	105 $\pm$ 5	42 $\pm$ 12
Vitamin D ₃ -treated: CaHPO_4 in saline	98 $\pm$ 9	154 $\pm$ 13†	65 $\pm$ 14†
Vitamin D ₃ -treated: CaHPO_4 + bile salts	163 $\pm$ 20*	150 $\pm$ 12†	62 $\pm$ 10†

* Significantly greater than saline control group ($P < 0.1$).

† Significantly greater than rachitic control groups ($P < 0.1$).

Table 8. *Effect of chick and rabbit bile and some bile salts on the solubility of calcium from calcium phytate*

Portions (5 mg.) of [^{45}Ca]calcium phytate were shaken with 2 ml. of saline, chick bile, rabbit bile, taurocholate and taurochenodeoxycholate in saline in the concentrations shown for 1 hr. at 40°. The solutions were added at pH 7 but no adjustment or later check was made on this pH. After centrifugation, 0-100 ml. of the supernatant was taken for scintillation counting and the results were compared with a standard solution of 5 mg. of [^{45}Ca]calcium phytate in 2 ml. of N-acetic acid, and the solubility of calcium in the various solutions was calculated.

Solution	Solubility of Ca (mg./100 ml.)
Saline	5
Chick bile (diluted 1:5)	9
Rabbit bile (diluted 1:5)	10
Sodium taurocholate (0.02 M)	8
Sodium taurochenodeoxycholate (0.02 M)	7

from calcium hydrogen phosphate from ileal loops in vitamin D₃-treated chicks. The absorption of $^{45}\text{CaHPO}_4$ from washed tied-off ileal loops *in vivo* with rachitic chicks was extremely small (Table 7) and bile salts did not significantly improve absorption.

With both vitamin D₃-treated groups calcium absorption was increased after 60 min. but after 20 min. only in the vitamin D₃-treated group given bile salts. The tibial radioactivities in the two vitamin D₃-treated groups were not significantly different.

Aqueous solubility of calcium from calcium phytate under the influence of bile and bile salts. Chick bile, rabbit bile, taurochenodeoxycholate

and taurocholate increased the solubility of calcium from calcium phytate compared with a saline control (Table 8).

Aqueous solubility of calcium from calcium hydrogen phosphate under the influence of bile salts and lauryl sulphate, at various pH values. The solubility of calcium hydrogen phosphate in saline falls rapidly as the pH of the solution increases from 6 to 8. Some bile salts, notably taurocholate and glycochenodeoxycholate, and also lauryl sulphate, were able to maintain higher concentrations of calcium in solution at pH values above 7.2, whereas others, such as glycocholate and taurochenodeoxycholate, were poorly effective above pH 7.2. The experimental values were plotted to give Figs. 1(a) and 1(b).

Relative butanol-benzene solubilities of various calcium complexes as used in ileal loop dosing solutions. In a previous paper (Webling & Holdsworth, 1965) we reported values for the relative butanol-benzene solubilities of the different substances tested for enhancement of calcium absorption from calcium solution.

Table 9 shows the relative butanol-benzene solubilities (compared with that of the calcium complex formed with chick bile salts) of most of the substances reported as tested for enhancement of calcium absorption from chick ileal loops in both the previous and this present paper, and these are compared with the different mean increases in tibial radioactivity taken from the several chick experiments *in vivo*. As noted in the previous paper, after removal of water-insoluble precipitates the relative butanol-benzene solubilities correspond in a general way to the abilities of these substances to enhance absorption from calcium chloride solutions in ileal loops.

Fig. 1. Effects of bile salts and lauryl sulphate on the solubility of CaHPO_4 at various pH values. (a) ●, Saline; △, taurocholate; ▲, taurochenodeoxycholate; ○, lauryl sulphate. (b) ●, Saline; ○, glycocholate; ▲, Glycochenodeoxycholate.

Table 9. *Relative butanol-benzene solubilities of calcium complexes*

The solutions used for experiments with ileal loops were examined by taking 1 ml. of solution or 1 ml. of a supernatant obtained by centrifugation and shaken for 15 min. with 1 ml. of butan-1-ol-benzene (1:1, v/v). The mixture was then centrifuged and 0.1 ml. counted by scintillation counting.

Test substance	Solution before centrifugation		Supernatant after centrifugation		Mean increase in tibial radioactivity
	^{45}Ca counts/min./ml. in butanol-benzene	Relative butanol-benzene solubility	^{45}Ca counts/min./ml. in butanol-benzene	Relative butanol-benzene solubility	
Saline	21	0.003	16	0.002	(1.0)
Zephiran (0.2%)	22	0.003	25	0.003	No increase
Tween 80 (0.2%)	27	0.003	31	0.004	No increase
Chick bile acids (hydrolysed bile salts) (saturated)*	1680	0.21	113	0.02	No increase
Glycochenodeoxycholate (0.02 M)*	8542	1.09	1005	0.14	No increase (Ca 0.2 M); 2.8 (Ca 0.01 M)
Taurocholate (0.02 M)	1832	0.23	1889	0.25	1.4
Glycocholate (0.02 M)	2595	0.33	2633	0.35	1.5
Tauroolithocholate (0.02 M)*	8732	1.11	5721	0.77	1.2
Taurodeoxycholate (0.02 M)	6471	0.83	6433	0.87	2.0
Taurochenodeoxycholate (0.02 M)	7184	0.92	7370	0.99	2.3
Chick bile salts (0.02 M)	7831	1.00	7413	1.00	2.5
Koala bile salts (0.02 M)	7926	1.01	8020	1.07	2.2
Lauryl sulphate (0.02 M)*	15046	1.92	8971	1.20	2.0
Lauryl sulphate (0.007 M)	10016	1.28	9073	1.25	2.9

* Precipitate formed.

Table 10 shows corresponding values for the relative butanol-benzene solubilities for the dosing solutions used in the bile alcohol sulphate experiment (Table 3), and these again show a corre-

spondence to relative increases in calcium absorption.

Butanol-benzene/saline partition coefficients of some calcium-bile salt complexes. The partition

Table 10. *Relative butanol-benzene solubilities of scymnol sulphate and ranol sulphate*

The method was that described in Table 9 and the solutions were those used in the experiment reported in Table 3.

Test substance	⁴⁵ Ca counts/ min./ml. in butanol-benzene	Relative butanol-benzene solubility	Mean increase in tibial radioactivity
Saline	31	0.001	(1.0)
Chick bile salts	29075	1.0	2.5
Taurochenodeoxycholate	25128	0.86	2.6
Scymnol sulphate	8285	0.28	1.5
Ranol sulphate	12104	0.41	1.9

Table 11. *Butanol-benzene/saline partition coefficients of calcium-bile salt complexes*

Portions (1 ml.) of ⁴⁵CaCl₂ (0.01 M) in saline or in 0.02 M-sodium salt of the bile acid (or sodium lauryl sulphate) were shaken with 1 ml. of butan-1-ol-benzene (1:1, v/v) at 40° for 2 hr. The mixture was centrifuged and 0.1 ml. of each layer taken for scintillation counting. At this concentration tauroolithocholate and lauryl sulphate gave precipitates and these were removed by centrifugation. Thus these partition coefficients are not under the same conditions but they are included for comparison.

Substance	Butanol- benzene/ saline partition coefficient	Mean increase in tibial radioactivity
Saline	0.0001	(1.0)
Taurocholate	0.01	1.3
Glycocholate	0.02	1.5
Taurochenodeoxycholate	0.04	2.3
Taurodeoxycholate	0.04	2.1
Glycochenodeoxycholate	0.26	2.8*
Tauroolithocholate	0.30	1.2
Lauryl sulphate	1.3	2.9

* Result obtained in Table 2.

coefficients of some calcium-bile salt complexes again show some correspondence to increases in calcium absorption from ileal loops (Table 11).

DISCUSSION

The results of experiments with calcium chloride solutions in chick ileal loops show that the enhancing action of bile salts on calcium absorption is not confined to the taurine-conjugated bile salts that are secreted in the bile of birds, but that some members of the other two classes of bile salts, bile alcohol sulphates (characteristic of lower vertebrates) and glycine conjugates (which occur only in higher mammals), are also capable of this effect. Both taurine conjugates (Webling & Holdsworth, 1965), and now glycine conjugates, have been

shown to increase calcium absorption from the sparingly soluble calcium hydrogen phosphate in both rachitic chicks and normal rats, and the normal biliary secretion appears to be responsible for a considerable portion of the calcium absorption in both chicks (Webling & Holdsworth, 1965) and rats. From these results it appears that the action of bile salts on calcium absorption may be of importance in the total calcium uptake of many if not most vertebrates. The most striking finding, obtained with normal rats, was that if the bile duct was occluded then absorption of calcium from the sparingly soluble calcium hydrogen phosphate was about one-third of the absorption of the sham-operated group (Table 5). There was no vitamin D deficiency in this experiment.

Bile salts caused an earlier effect on calcium absorption from calcium hydrogen phosphate with vitamin D₃-treated chicks, although the bone radioactivities of the two groups after 60 min. were not significantly different whether or not bile salts had been present. It is suggested that this effect is due to the provision of a more soluble form of calcium, as was shown to occur under the influence of bile salts in a previous paper (Webling & Holdsworth, 1965) and also in the present work. The apparent limitation of total absorption (from the values for tibial radioactivity) in vitamin D₃-treated birds, whether or not bile salts were present, is difficult to explain from these results, and further experiments over a longer time of observation, with recording of total calcium balance, would be desirable. This phenomenon may be concerned with the manifest failure of bile salts to prevent rickets, although they enhance calcium absorption. It seems possible that the actions of vitamin D and bile salts in calcium absorption could both be necessary for optimum calcium intake, particularly with poorly soluble dietary forms of calcium such as calcium hydrogen phosphate. The apparent increase in aqueous solubility of calcium from calcium phytate in the presence of bile and some bile salts suggests that this action of bile salts may be important with other common sparingly soluble dietary forms of calcium. Coates & Holdsworth

(1961) stressed the need to study these more sparingly soluble forms, and showed that phosphate prevented the absorption of calcium in duodenal loops *in vivo*. Similarly, in the present work we found that calcium hydrogen phosphate was very poorly absorbed from ileal loops even in the presence of bile and vitamin D₃. Evidently passage through the intestinal tract changes the calcium salt, as might be expected, but an excess of phosphate would probably render the calcium unavailable.

There is a close parallel between the ability of bile salts (and lauryl sulphate) to increase the absorption of soluble calcium from the gut, and the relative butanol-benzene solubilities or benzene-butanol/saline partition coefficients of the calcium salts of the respective substances. It is possible that this ability to make calcium lipid-soluble is important in the mechanism of action of the bile salts.

Schachter (1963) proposed that calcium is absorbed from the gut by a two-step process: (1) passive diffusion; (2) a more deeply situated active energy-dependent process that is confined to the upper small gut. Harrison & Harrison (1965) as a result of their experiments in rats have proposed that vitamin D acts by modifying the cell wall, making it more permeable to calcium and so enhancing step (1) above. Bile salts could be conceived as also enhancing step (1) by forming a calcium complex more rapidly diffusible through the lipid cell wall.

An attempt was made to explain the mechanism of action of vitamin D by suggesting that the

vitamin, or a product arising from it, could inhibit a metabolically operated pump that normally keeps calcium out of the cell (Holdsworth, 1965). Inhibition by vitamin D, bile salts or lauryl sulphate of a pump that was keeping calcium out of mucosal cells would lead to an increased translocation of calcium from mucosa to serosa. It is still possible that the action of bile salts or lauryl sulphate depends on the lipid-solubility of their calcium salts to enable them to penetrate the cell or mitochondrial membrane.

Our gratitude is due to the Trustees of the 'X' Benefaction of the University of Adelaide for the financial support of D. D'A. W., to Professor G. A. D. Haslewood for gifts of bile acids and for helpful discussions, to Dr G. F. Shanks for help with the preparation of some bile acids, and to Miss G. Schwietzke and Miss H. L. Hanns for technical assistance.

REFERENCES

- Coates, M. E. & Holdsworth, E. S. (1961). *Brit. J. Nutr.* **15**, 131.
Gordon, B. A., Kuksis, A. & Beveridge, J. M. (1963). *Canad. J. Biochem. Physiol.* **41**, 77.
Harrison, H. C. & Harrison, H. E. (1965). *Amer. J. Physiol.* **208**, 370.
Holdsworth, E. S. (1965). *Biochem. J.* **96**, 475.
Sallis, J. D. & Holdsworth, E. S. (1962). *Amer. J. Physiol.* **203**, 497.
Schachter, D. (1963). In *The Transfer of Calcium and Strontium across Biological Membranes*, p. 197. Ed. by Wasserman, R. H. New York: Academic Press Inc.
Webling, D. D'A. & Holdsworth, E. S. (1965). *Biochem. J.* **97**, 408.