Vitamin D₃ and absorption of calcium in the chick

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Although it has been known for many years that vitamin D is implicated in the metabolism of calcium, the exact biochemical mechanism of its action is still not clear. There have been reports that yeast (Braude, Kon & White, 1943), some grasses and other leafy vegetable matter (Ewer & Bartrum, 1948; Grant, 1951; Weits, 1952; Raoul, Marnay, Le Boulch, Prelot, Guerillot-Vinet, Bazier & Baron, 1957) and pig's liver (Coates & Harrison, 1957) have rachitogenic effects in certain species. It is difficult to study the mechanism on which these rachitogenic properties depend until the role of vitamin D in preventing rickets is better understood. For this reason the experiments reported here were undertaken.

EXPERIMENTAL AND RESULTS

Management of the chicks

Rhode Island Red × Light Sussex cockerels from a commercial flock were received on the day of hatching and placed in electrically heated tier brooders in a laboratory from which sunlight was virtually excluded. As the chicks used in these experiments were usually also the negative and positive controls in routine assays of vitamin D₃ by the Olsson (1941) technique, the procedure described below was adopted. For the first 6 days they received a diet having the percentage composition: ground maize 30, ground wheat 36, defatted soya-bean meal 13·5, dried skim milk 10, dried yeast 3, artificially dried grass 6, sodium chloride 1, wood charcoal 0·5. To prevent perosis, $MnSO_4.4H_2O$ was added to the diet at the rate of 17 p.p.m. At 6 days the birds were weighed, the extremely light and heavy ones were culled and those left distributed into test groups. From then on they received a diet consisting of 94·9 parts of the above mixture, 4·1 parts of ground bone meal and 1 part of arachis oil. When a supplement of vitamin D₃ (cholecalciferol) was necessary, it was dissolved in the arachis oil. At 4 weeks of age the left hock joint of each bird was radiographed and the degree of rickets was assessed by measurement of the tarso-metatarsal distance (T.M.T.). The degree of rickets shown by the birds given no vitamin D₃ varied from time to time, possibly depending on the amount of sunlight to which their dams had been exposed, so that the mean T.M.T. values ranged from about 2·5 to 4·0 mm in the course of this work. Similarly the amount of vitamin D₃ necessary for the complete prevention of rickets also varied; on some occasions a content of 14 i.u./100 g diet sufficed and at

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other times 23 i.u. were necessary to give normal bone formation, as indicated by a T.M.T. value of about 1·0 mm.

Although batches of about twenty to fifty rachitic and normal chicks were available at any one time, the amount of labour involved in the experiments on Ca absorption limited the size of the experimental groups to two to five birds. In an attempt to minimize variations within these groups, birds were selected having T.M.T. values within a limited range of the mean for the whole batch. The birds were 4–5½ weeks old at the time of experiment.

Calcium chloride

The $^{45}CaCl_2$ was obtained from the Radiochemical Centre, Amersham. On arrival its specific activity was of the order of 2 $\mu c/mg$. It was diluted with unlabelled $CaCl_2$ solution to give a specific activity of about 0·2 $\mu c/mg$, but as each solution usually lasted for 6–12 months the specific activity was considerably below this value in some experiments. The activity of standard solutions was counted at the same time as that of the experimental material, and the results were calculated as mg Ca. In perfusion experiments $^{45}CaCl_2$ of high specific activity (600 $\mu c/mg$) was occasionally used. Unlabelled $CaCl_2$ was prepared by dissolving appropriate amounts of $CaCO_3$ of A.R. quality in 2N-HCl, evaporating to dryness, making to volume and adjusting to pH 6·0 with sodium hydroxide. Throughout the text the dosage is given in mg Ca, which was invariably administered as chloride.

Chemical determination of Ca

Duodenal loops in which the Ca was to be measured were dried on a water-bath and then ashed at not more than 600° in platinum crucibles: 2 ml conc. HCl diluted 1:1 were added to dissolve the ash and the solution was evaporated to dryness. The residue was taken up in water containing one drop of the diluted HCl, and any debris was removed by centrifuging. The supernatant liquid was diluted to about 10 ml with water, and 10 ml 4% (w/v) ammonium-oxalate solution were added. After standing for 2 h the precipitated calcium oxalate was washed three times with hot ammonia solution (1 part ammonia of sp.gr. 0·88 to 99 parts water). Occasionally at this stage the calcium oxalate was dried and weighed, but more usually it was dissolved in 10 ml 2N-H_2SO_4, the solution being then warmed to 60° and titrated with 0·02N-$KMnO_4$.

Counting methods

Calcium oxalate, prepared as described above, was spread on 25 mm metal planchets and counted at infinite thickness. Blood was collected from the wing vein for determination of radioactivity. Either serum or plasma was prepared, spread on metal planchets, dried under a 250 W infrared lamp and counted directly. Counts were made in a G.E.C. EMH 2 mica end-window counter.

Surgical procedure

The birds were anaesthetized with ether, and an incision was made in the left side of the abdominal wall through which the duodenal loop was brought to the outside. A ligature was tied just below the gizzard and another about 1 cm beyond the entry

of the bile duct. The duodenum was severed below the upper and above the lower ligature, and the segment thus formed was washed through with warm saline to remove any traces of food. The lower end of the loop was then tied off. The required dose of Ca, usually about 1 ml solution, was delivered through the upper end of the loop from a tuberculin syringe fitted with a blunt-ended needle surrounded by a ligature, which was pulled tight as the needle was withdrawn. On some occasions lengths of small intestine other than the duodenum were used. Thus a known amount of Ca could be placed in a blind loop of intestine which still had its blood supply and absorptive mechanism intact. The loop was replaced and the abdomen closed, and the birds were
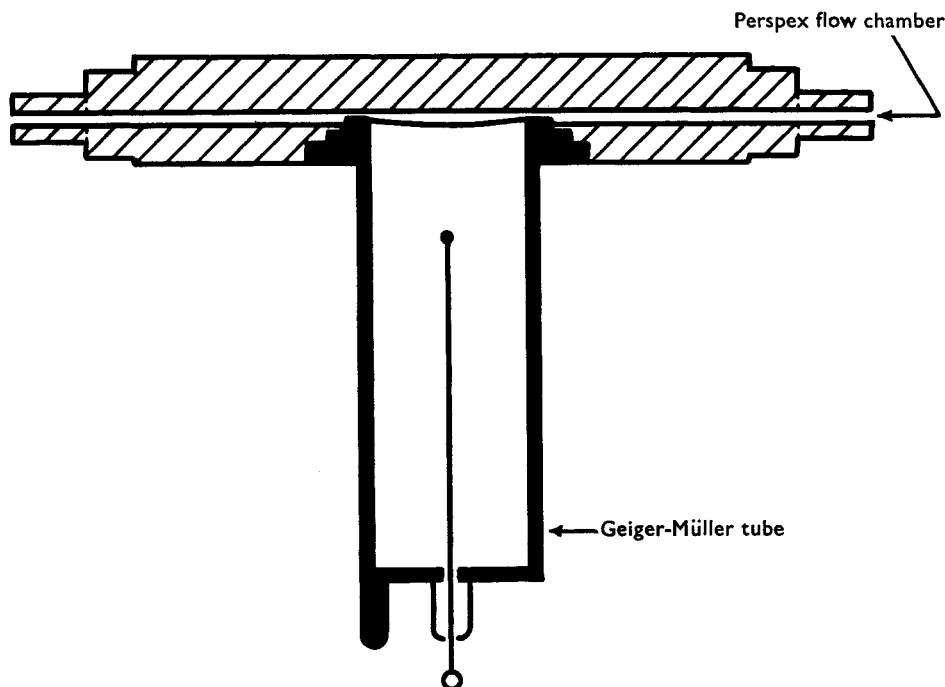

Fig. 1. Flowing electrode for continuous recording of ^{45}Ca in perfusion fluids (actual size).

allowed to live for 2 or 3 h. They were then anaesthetized again, and the entire loop was removed for determination of Ca. Occasionally in some birds the loops were much distended with fluid at the end of the experimental period. The cause could not be ascertained, but the distension appeared to have little effect on the experimental results, since in any one group uptake of Ca was not consistently different between birds showing it and those with loops of normal appearance.

Perfusion experiments

In these experiments oxygenated Krebs–Ringer–bicarbonate–glucose buffer (Umbreit, Burris & Stauffer, 1949) was circulated through surviving isolated loops of duodenum or through the duodenum in live birds anaesthetized with urethane. The perfusion apparatus has already been described elsewhere (Holdsworth & Coates,

1961). The surgical procedure described above was followed as far as the washing-out of the duodenum, which was then connected to the inner circulation system by glass cannulas at each end. The lower ligature was arranged so as to exclude the entrance of the bile duct from the length to be perfused. If the perfusion was required to be done in vivo, the duodenum was replaced as far as possible in the abdominal cavity and the incision covered with a pad of moist cotton-wool. If a surviving isolated preparation was required, the duodenal loop, with the pancreas attached, was removed from the bird after the inner circulation had been connected. It was rinsed rapidly in warm saline and suspended in the outer vessel through which oxygenated Krebs–Ringer–bicarbonate–glucose was already being circulated. Perfusions were allowed to proceed for up to 1 h.

In all the perfusion experiments $^{45}CaCl_2$ was used; in order to record continuously the radioactivity in either the inner or the outer circulating fluids a flowing electrode, shown in Fig. 1, was constructed. It consisted of a mica end-window counter to which was fixed a Perspex chamber 2 mm deep. Perspex tubing was sealed in at either side so that the circulating fluid could be passed through the chamber. The output of the electrode led into a rate-meter from which the c.p.m. were continuously recorded on a pen recorder. The counting efficiency of such a device is low, so that higher activities of ^{45}Ca were needed than in the loop experiments. The instrument was calibrated with Ca solutions of known specific activity.

Preliminary experiments

Site of absorption of $CaCl_2$ in the small intestine. Two experiments were done to determine the optimal site in the small intestine for the study of Ca absorption. In the first, normal birds fasted for 1–2 h were anaesthetized with ether, and ligatures were placed round the small intestine so as to divide it into three roughly equal loops as described on p. 133, but on these occasions the intestine was not washed out with saline. Into each loop were placed 9 mg labelled ^{45}Ca, the abdomen was closed, and the birds were allowed to live for 3 h. Each loop was then ashed and the Ca determined by precipitation as oxalate and also by counting the activity of the oxalate. The residual amount of Ca calculated from these two methods of determination is shown in Table 1.

In the first two birds there was good agreement at the three sites between the two methods of Ca estimation. These birds were operated on in the morning and had therefore been fed only for a short period before the experiment began. In the birds used later in the day there was good agreement for the duodenal loop, but lower down the intestine there was evidence of considerable amounts of unlabelled Ca, presumably because the fasting period was not long enough to ensure that the intestine was empty of food taken in during the morning.

In the second experiment the whole small intestine was washed with about 100 ml warm saline before being separated into the three loops. Normal and rachitic birds were used; some were given 4 mg and some 9 mg labelled Ca into each loop, and the experiment lasted 2 h. After ashing the loops, Ca was determined by titration and by counting as oxalate. The results are shown in Table 2. On these occasions the results

obtained by titration were consistently slightly higher than those found radiometrically, even when allowance was made for the small blank values found in the untreated loops. There was no indication that $CaCl_2$ was better absorbed from any particular section, so the duodenal loop was chosen for future experiments, being easily accessible and well defined. Further, in this area there was less chance of complications arising from the possible re-excretion of Ca into the small intestine.

Table 1. *Site of absorption of calcium in normal chicks*

(9 mg Ca as [45]Ca-labelled $CaCl_2$ were placed in each of three equal loops of small intestine, and the residual Ca was determined after 3 h. The control bird had no [45]Ca added to the loops)

| | | Ca left in loop | |
| | | Calculated from weight of oxalate (mg) | Assessed by counting [45]Ca (mg) |
Bird no.	Site		
Control	Duodenum	0·4	—
	Middle loop	3·5	—
	Lowest loop	10·3	—
1	Duodenum	5·6	5·4
	Middle loop	4·6	4·9
	Lowest loop	8·4	7·2
2	Duodenum	5·1	5·0
	Middle loop	4·9	5·2
	Lowest loop	4·7	4·6
3	Duodenum	4·6	4·6
	Middle loop	6·9	5·7
	Lowest loop	14·8	4·0
4	Duodenum	3·9	4·1
	Middle loop	5·9	5·7
	Lowest loop	19·2	2·3

Determination of pH of chick gut contents. Since the solubility and hence probably the absorption of Ca might be expected to be affected by pH, a survey was made of the pH of the gut contents throughout the length of the alimentary tract of chicks given the experimental diets. Under ether anaesthesia the alimentary tract was exposed and immediately divided with forceps into sections consisting of the crop, proventriculus, gizzard, duodenum, middle segment of small intestine, lowest segment of small intestine and caeca. The whole gut was then removed, the contents of each section were squeezed out, and pooled samples from two normal or four rachitic chicks were centrifuged at 2000 *g*. As insufficient fluid could be squeezed from the proventriculi, they were washed through with a few drops of distilled water. The pH of the supernatant liquids was determined with a hanging drop glass electrode (Doran Instruments, Stroud). A sample of the experimental diet was shaken with water and centrifuged, and the pH of the supernatant liquid was measured. The findings are given in Table 3.

There was little difference between the pH of the gut contents of normal and rachitic birds, and the use of $CaCl_2$ solutions at pH 6·0 in these experiments on duodenal absorption did not seriously change the natural pH of that area.

Uptake of Ca from the intestine of normal and rachitic chicks. The uptake of Ca by the chick was determined either by measuring the loss of Ca in a given time from a dose placed directly in tied-off duodenal loops or by the appearance of radioactivity in the plasma after a dose of $^{45}CaCl_2$.

Table 2. *Site of absorption of calcium in normal and rachitic chicks*

(4 or 9 mg Ca as ^{45}Ca-labelled $CaCl_2$ were placed in washed-out loops of small intestine, and the residual Ca was determined after 2 h)

Description of chick	Site	Ca left in loop	
		Assessed by titration (mg)	Assessed by counting ^{45}Ca (mg)
	After 4 mg dose of Ca		
Normal	Duodenum	2·8	1·8
	Middle loop	2·6	2·0
	Lowest loop	2·7	2·0
	Duodenum	2·2	1·2
	Middle loop	2·8	2·3
	Lowest loop	3·4	2·6
Rachitic	Duodenum	3·9	2·9
	Middle loop	2·3	1·7
	Lowest loop	3·5	2·6
	Duodenum	3·5	2·7
	Middle loop	2·8	2·2
	Lowest loop	2·8	2·3
	After 9 mg dose of Ca		
Normal	Duodenum	5·0	3·5
	Middle loop	7·5	5·8
	Lowest loop	8·2	6·6
	Duodenum	5·4	3·8
	Middle loop	5·5	4·4
	Lowest loop	5·2	4·4
Rachitic	Duodenum	7·4	6·0
	Middle loop	8·0	7·0
	Lowest loop	6·3	5·1
	Duodenum	7·0	6·3
	Middle loop	7·7	7·2
	Lowest loop	7·3	6·7
	After no added Ca		
Normal	Duodenum	0·4	—
	Middle loop	0·3	—
	Lowest loop	0·3	—
Rachitic	Duodenum	0·5	—
	Middle loop	0·2	—
	Lowest loop	0·2	—

In the experiments on duodenal loops, either 4 mg or 9 mg Ca as chloride were put into the duodenum of normal and rachitic birds, which were then killed 2 or 3 h later. The results of all these experiments are presented in Table 4. There was considerable variation between experiments in the absolute amounts of Ca taken up by the birds, but we were unable to relate these differences to any particular part of the experimental

technique. In every instance, however, much less Ca was taken up by the rachitic chicks than by the normal controls; by analysis of variance this difference was found highly significant ($P \ll 0.001$) at both levels of Ca given.

In the experiment illustrated in Fig. 2, radioactivity was measured in the blood collected at intervals after administration of 2 mg labelled Ca as chloride in 1 ml water by mouth to normal and rachitic chicks. Each point represents the counts/min in a

Table 3. *pH of the contents of alimentary tracts of normal and rachitic chicks*

Site	Normal*	Rachitic†
Crop	4·56	4·55
Proventriculus	3·66	2·05
Gizzard	2·91	2·37
Duodenum	6·55	6·55
Small intestine (middle section)	6·92	6·86
Small intestine (lowest section)	7·83	7·96
Caeca	5·25	4·82
Diet	6·20	

* Pooled contents from two birds.　　† Pooled contents from four birds.

Table 4. *Uptake of calcium introduced as CaCl₂ into washed-out duodenal loops of normal and rachitic chicks (see p. 133)*

Expt no.	Ca absorbed in 2 h from a dose of 4 mg Ca (mg) Normal chicks	Rachitic chicks	Expt no.	Ca absorbed in 2 or 3 h from a dose of 9 mg Ca (mg) Normal chicks	Rachitic chicks
1	3·0	1·0	8 (3 h)	5·9	2·1
	1·9	0·9		5·5	4·3
	2·3	1·1			2·1
2	2·1	0·6			2·3
	1·7	Lost	9 (3 h)	3·0	1·4
	2·3	1·4		3·8	1·0
				3·7	0·6
3	1·8	1·0			
	0·8	0·4	10 (3 h)	3·2	0·3
	2·1	0·8		4·2	0·3
4	1·7	0·9	11 (3 h)	3·7	2·6
	1·5	0·6		4·1	3·1
	1·4	0·6		4·1	1·5
	1·4	0·5		5·2	2·0
5	1·3	0·9	12 (2 h)	5·5	3·0
	1·9	0·9		5·2	2·7
	1·7	0·8	13 (2 h)	3·4	0·7
	—	0·6		3·3	0·6
6	2·4	0·6		4·0	0·0
	2·0	0·5	14 (2 h)	2·2	0·7
	1·8	0·6		3·2	0·2
7	2·2	1·1	15 (2 h)	3·0	1·6
	2·8	1·3		3·5	1·3
Mean	1·91	0·81	Mean	3·99	1·56

pooled sample of plasma prepared from equal volumes of blood from three chicks, and it is clear that once again absorption of ^{45}Ca was much greater in the normal than in the rachitic birds. Results were not always so clear-cut, however, and on some occasions normal birds failed to absorb ^{45}Ca to any appreciable extent. This failure occurred more frequently, though not consistently, in birds fasted for long periods, a phenomenon that had been observed in previous (unpublished) work on the absorp-
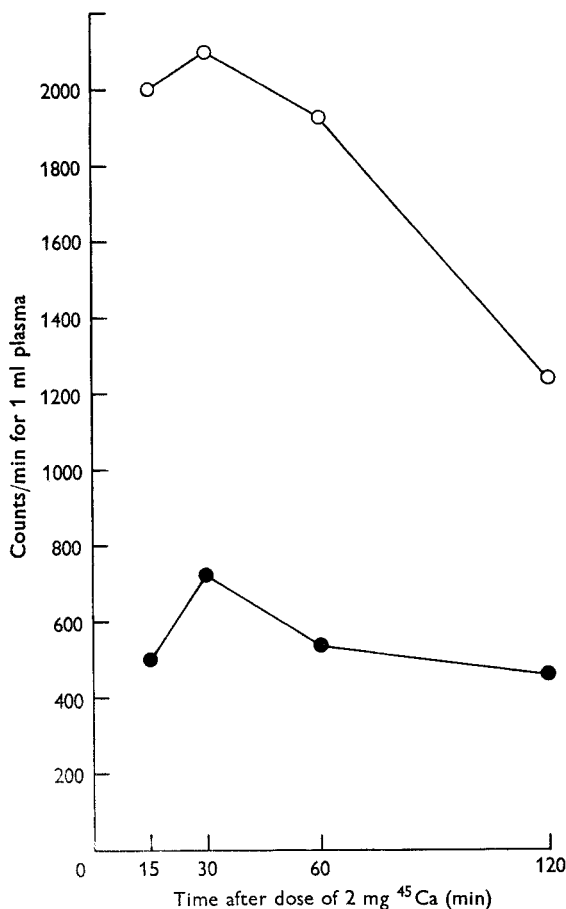
Fig. 2

Fig. 3

Fig. 2. Appearance in blood of ^{45}Ca given by mouth as ^{45}CaCl$_2$ to normal and rachitic chicks. Pooled samples from three birds. O—O, basal diet + 23 i.u. vitamin D$_3$/100 g; ●—●, basal diet.
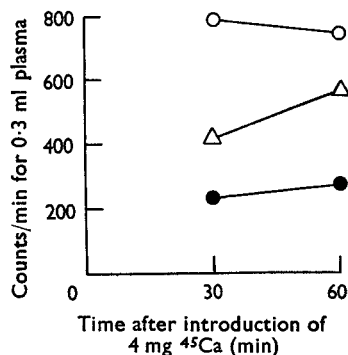
Fig. 3. Appearance in blood of ^{45}Ca placed in duodenal loops as ^{45}CaCl$_2$ in normal and rachitic chicks. Mean values for two birds. O—O, basal diet + 14 i.u. vitamin D$_3$/100 g; △—△, basal diet + 5 i.u. vitamin D$_3$/100 g; ●—●, basal diet.

tion of ^{32}P-labelled phosphate by chicks. Since no satisfactory explanation could be found for these occasional inconsistencies, in later experiments dosing by mouth was abandoned. Instead, the dose of 2 mg labelled Ca as chloride was placed directly into washed-out duodenal loops as already described, and blood was collected at intervals.

Fig. 3 illustrates an experiment done by this technique, from which it can be seen that plasma levels of ^{45}Ca in chicks that had received only 5 i.u. vitamin D_3/100 g diet were intermediate between those of birds given 14 i.u. vitamin D_3/100 g diet and of birds given no vitamin D_3.

Effect of a single dose of vitamin D_3 on absorption of Ca by rachitic chicks

Vitamin D_3 given simultaneously with $CaCl_2$. The absorption of 9 mg Ca as chloride from duodenal loops of rachitic chicks was studied in the presence and absence of 100 i.u. vitamin D_3. After 1 ml of an aqueous solution of $CaCl_2$ had been drawn up into a syringe, and then 0·1 ml arachis oil containing 100 i.u. vitamin D_3, the mixture was expelled into the duodenal loop. Since the oil was not emulsified, it is possible that the vitamin D_3 was not available to the chick by this method of administration, and a further treatment was included in which 0·1 ml bile was added to the mixture in the syringe to aid emulsification of the oil. Controls received the $CaCl_2$ solution

Table 5. *Effect of simultaneous administration of vitamin D_3 on the absorption of calcium from duodenal loops in rachitic chicks*

(9 mg Ca as $CaCl_2$, together with the vitamin, were placed in washed-out duodenal loops, and the residual Ca was determined after 3 h. Each value is for one chick)

Substance given with Ca	Ca absorbed (mg)
None	3·36
	Lost
0·1 ml bile	2·92
	3·73
100 i.u. vitamin D_3 in arachis oil	2·32
	2·34
100 i.u. vitamin D_3 in arachis oil + 0·1 ml bile	3·01
	3·05
100 i.u. vitamin D_3 injected intravenously	2·61
	3·00

alone or containing 0·1 ml bile. The bile had been collected from the gall bladders of freshly killed 4-week-old rachitic chicks. There were two chicks on each treatment, and another two birds were given an intravenous injection of 100 i.u. vitamin D_3, in the form of a commercial 'water-soluble' preparation (presented by Phillips-Roxane, Amsterdam), immediately before the $CaCl_2$ solution was delivered into the duodenum. The Ca remaining in the loops after 3 h was determined by the titration method; the amounts absorbed are shown in Table 5. There was no indication that the vitamin D_3 given simultaneously, either directly into the loop with or without bile or by intravenous injection, had any effect on the uptake of $CaCl_2$ from the gut.

Vitamin D_3 given several hours before $CaCl_2$. Two experiments were done by the duodenal-loop technique in which a dose of 100 i.u. vitamin D_3 in arachis oil was given by mouth to groups of from two to five rachitic birds 16 and 4 h before the operation. The uptake of Ca from a dose of 4 mg as chloride by these birds was compared with

that by untreated controls and their hatchmates that had received adequate vitamin D_3 in the diet. From the results, shown in Table 6, it is clear that administration of vitamin D_3 4 h beforehand had a negligible effect on Ca absorption, whereas when the same dose of vitamin D_3 was given 16 h before, the Ca absorption was very close to that of normal birds.

Table 6. *Effect of administration of vitamin D_3 several hours previously on the absorption of calcium from duodenal loops in rachitic birds*

(4 mg Ca as $CaCl_2$ were placed in washed-out duodenal loops, and the residual Ca was determined after 2 h)

	Ca absorbed (mg)	
Substance given	Expt 1	Expt 2
None	1·03 (2)	0·65 (3)
100 i.u. vitamin D_3 simultaneously with Ca	1·16 (3)	—
100 i.u. vitamin D_3 4 h before Ca	1·28 (3)	0·70 (5)
100 i.u. vitamin D_3 16 h before Ca	1·75 (3)	1·46 (5)
None (normal birds)	2·06 (3)	1·5 (4)

Figures in parentheses are the number of chicks in each group.
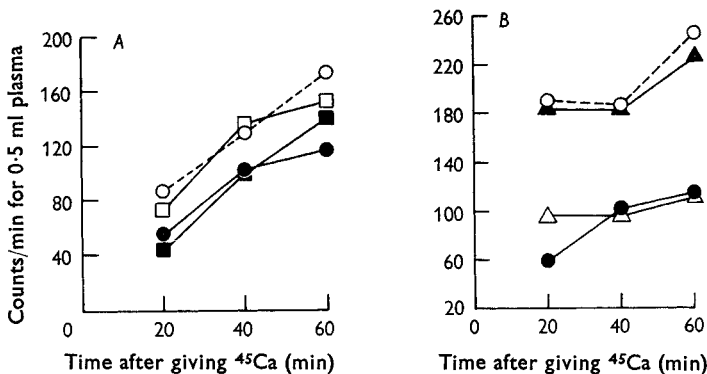

Fig. 4. Effect of a single dose of vitamin D_3, given by mouth or intramuscular injection, on absorption of ^{45}Ca by rachitic chicks. (A) Mean values for four birds; (B) mean values for two birds. O – – – O, normal birds; ●—●, no vitamin D_3; ▲—▲, 100 i.u. vitamin D_3 by mouth 16 h before test; △—△, 100 i.u. vitamin D_3 by intramuscular injection 16 h before test; □—□, 100 i.u. vitamin D_3 by mouth 8 h before test; ■—■, 100 i.u. vitamin D_3 by mouth 3 h before test.

In two further experiments the absorption of Ca was followed by measurement of radioactivity in the plasma at intervals after an oral dose of 2 mg ^{45}Ca as chloride in 1 ml saline. Groups of two or four rachitic chicks were given 100 i.u. vitamin D_3 in arachis oil by mouth 3, 8 or 16 h before the $^{45}CaCl_2$, and their uptake of ^{45}Ca was compared with that of untreated rachitic or normal chicks. In one experiment the vitamin D_3 was also given by intramuscular injection 16 h before the dose of ^{45}Ca. Fig. 4 illustrates the results of these experiments, which were in agreement with the findings by the duodenal-loop technique; vitamin D_3 given to rachitic chicks 3 h before the dose of $^{45}CaCl_2$ did not improve uptake of ^{45}Ca, whereas absorption was

normal 16 h after the same dose of vitamin D_3. Further, the effect of the vitamin D_3 was appreciable after 8 h. If the vitamin was given intramuscularly, it did not improve the uptake of ^{45}Ca even after 16 h.

Effect of phosphate and citrate ions on the uptake of $CaCl_2$

In the experiments reported so far, Ca was given as chloride for the sake of convenience, but it may well be that under normal feeding conditions the chloride is not the form in which Ca is absorbed. It was considered advisable, therefore, to investigate the fate of $CaCl_2$ given into duodenal loops when phosphate or citrate ions or both were also present.

In the first experiment, groups of three normal or rachitic chicks received 4 mg Ca as chloride into duodenal loops with or without 28 mg disodium hydrogen phosphate (Na_2HPO_4), thus providing more PO_4^{3-} than would be necessary to combine completely with 4 mg Ca. On this occasion 25 mg glucose were given with the phosphate, since glucose is known to be implicated in the transport of some nutrients across the gut wall. To prevent precipitation of calcium phosphate before the mixture was delivered into the gut, 0·5 ml of a solution of 0·4M-Na_2HPO_4 at pH 6·9, containing 25 mg glucose, was first placed in the loop of gut and then 1 ml of a solution containing 4 mg Ca as chloride delivered from a second syringe. Birds not given phosphate received 0·5 ml water with glucose and then the $CaCl_2$ solution. A similar technique was used in a second experiment, when 9 mg Ca were given with 14 mg Na_2HPO_4 or 22 mg

Table 7. *Effect of phosphate and citrate ions on absorption of calcium from duodenal loops in normal and rachitic chicks*

(9 or 4 mg Ca as $CaCl_2$, together with various substances, were placed in washed-out duodenal loops, and the residual Ca was determined after 2 h. Each value is for one chick)

Substance given	Ca absorbed (mg)	
	Normal chicks	Rachitic chicks
4 mg Ca + 25 mg glucose	2·4, 2·0, 1·8 } 2·1	0·6, 0·5, 0·6 } 0·6
4 mg Ca + 28 mg disodium hydrogen phosphate + 25 mg glucose	−0·3, −0·1, 0 } −0·1	0, −0·2, −0·2 } −0·1
9 mg Ca	3·0, 3·5 } 3·3	1·6, 1·3 } 1·5
9 mg Ca + 14 mg disodium hydrogen phosphate	—	0·7, 0·7 } 0·7
9 mg Ca + 22 mg sodium citrate	—	1·4, 1·3 } 1·4
9 mg Ca + 14 mg disodium hydrogen phosphate + 22 mg sodium citrate	—	0·6, 0·5, 0·1 } 0·4

sodium citrate ($C_6H_5O_7Na_3.2H_2O$) or both to rachitic chicks. In each experiment the Ca remaining in the loops after 2 h was determined by the titration method, and the results are summarized in Table 7. The presence of excess PO_4^{3-} completely inhibited

uptake of Ca in both normal and rachitic chicks. In the second experiment a smaller quantity of PO_4^{3-} markedly depressed uptake of Ca in rachitic chicks, and the depression was not counteracted by the presence of citrate. Citrate alone had no effect on absorption of Ca by rachitic birds.

Effect of bile on absorption of $CaCl_2$

Our earlier experiments showed that it was necessary for some hours to elapse before an oral dose of vitamin D_3 exerted any effect on Ca absorption in rachitic chicks. One explanation might be that during this time the vitamin D_3 is absorbed, converted into a physiologically active form and re-excreted into the gut, possibly in the bile. To test this possibility, the effect of bile from normal or vitamin D_3-depleted chicks on the absorption of Ca was investigated. Bile was withdrawn by a syringe from the gall-bladders of freshly killed normal 4-week-old chicks or of their hatchmates that had received no vitamin D_3 throughout their lives. By the duodenal-loop technique, groups of three rachitic chicks were given 9 mg Ca as chloride with or without 0·1 ml bile from normal or depleted chicks. As controls, a group of three normal

Table 8. *Effect of bile on absorption of calcium from duodenal loops in rachitic chicks*

(9 mg Ca as $CaCl_2$, together with bile from normal or rachitic birds, were placed in washed-out duodenal loops, and the residual Ca was determined after 2 h. Each value is for one chick)

Substance given with $CaCl_2$	Ca absorbed (mg)	
	Normal chicks	Rachitic chicks
Expt 1		
None	3·4 ⎫ 3·3 ⎬ 3·6 4·0 ⎭	0·7 ⎫ 0·6 ⎬ 0·4 0·0 ⎭
0·1 ml bile from normal chicks	—	1·9 ⎫ 2·4 ⎬ 1·9 1·3 ⎭
0·1 ml bile from chicks deprived of vitamin D_3	—	2·9 ⎫ 3·2 ⎬ 2·9 2·7 ⎭
Expt 2		
None	3·2 ⎫ 2·7 2·2 ⎭	0·7 ⎫ 0·5 0·2 ⎭
0·5 ml bile from chicks deprived of vitamin D_3	—	3·2 ⎫ 3·7 4·1 ⎭

chicks received the $CaCl_2$ alone. In a further experiment groups of two rachitic chicks were given 9 mg Ca as chloride with and without 0·5 ml bile from depleted birds. In Table 8 are given the amounts of Ca absorbed in 2 h by each chick. In both experiments the presence of bile markedly increased absorption of Ca by rachitic birds. Bile from depleted birds was just as effective as that from normal chicks.

Investigation of diffusible Ca in the duodenums of normal
and rachitic chicks

In the Olsson diet (see p. 131) the greater part of the Ca is contributed as phosphate by the bone meal. Since under the conditions of our experiments phosphate depressed the absorption of Ca, it seemed of interest to determine whether the amount of diffusible Ca differed in the gut of normal and rachitic chicks under natural feeding conditions. Groups of three normal or rachitic chicks, or rachitic chicks that had been given a dose of 200 i.u. vitamin D$_3$ in arachis oil 14 h previously, were taken for experiment, after being allowed to eat at will for about 5 h. The duodenal loops were removed under ether anaesthesia and the contents squeezed out and pooled from each

Table 9. *Diffusible calcium in duodenal contents of three*
normal or rachitic chicks (see above)

Substance added to basal diet	Volume of supernatant liquid from duodenal contents	Total Ca (mg/ml)	Diffusible Ca (mg/ml)
None	1·0	3·7	1·9
Single oral dose of 200 i.u. vitamin D$_3$ 14 h before experiment	0·9	2·7	2·0
23 i.u. vitamin D$_3$/100 g	1·5	1·4	1·2

experimental group. They were centrifuged at 10000 *g*, and 0·1 ml of the clear liquid was taken for determination of total Ca. The remaining supernatant liquid was ultrafiltered through ¼ in. Visking tubing in the apparatus described by Gregory (1954), and the Ca in 0·4 ml of the ultrafiltrate was determined. The results are given in Table 9. In the sample from normal chicks there was only half as much Ca as in that from rachitic birds, possibly because the rate of absorption of Ca was more rapid in the normal birds. Further, the Ca from the normal gut was almost all diffusible, whereas in rachitic birds only half of it was. The supernatant liquid from rachitic birds that had received a single dose of vitamin D$_3$ contained less total Ca than that from the untreated birds, but the amount of diffusible Ca was unaffected.

Perfusion experiments

We attempted to study the continuous passage of Ca across the gut wall of the chick by circulating [45]Ca through the duodenum and recording either its disappearance from the inner fluid when the duodenum was perfused *in situ* or its appearance in the outer fluid during perfusion of a completely isolated preparation. The perfusion fluid was Krebs–Ringer–bicarbonate–glucose buffer, but Ca was omitted from the original fluid in the inner circulation and [45]CaCl$_2$ was added as required. For experiments on isolated duodenums the flowing electrode, connected to a recorder, was incorporated in the outer circulation, and some preliminary trials were made on duodenums from normal and rachitic chicks. A few minutes after addition of [45]Ca to the inner circulation, there was evidence of radioactivity in the outer fluid, which increased at a

steady rate throughout 45 min, when the experiments were terminated. The rate of increase in radioactivity, indicating the rate of passage of ^{45}Ca across the wall of the duodenum, was not sensibly different whether small amounts (0·02–4 mg) of ^{45}Ca of high specific activity or larger amounts (50–100 mg) of ^{45}Ca of low specific activity were used in the inner fluid. The rate of passage of Ca was not apparently influenced by the presence or absence of PO_4^{3-} from the buffer in the inner circulation, even though a precipitate of calcium phosphate occurred as soon as the $^{45}CaCl_2$ was added to buffer containing K_2HPO_4; addition of 20 mg sodium citrate during the experiment solubilized the precipitate, but did not alter the course of Ca absorption. The experimental conditions finally chosen were: 40 ml Krebs–Ringer–bicarbonate–glucose buffer for the outer fluid and 20 ml of the buffer without $CaCl_2$ for the inner. At the beginning of the experiment, which lasted 45 min, 100 mg $^{45}CaCl_2$ (specific activity 0·9 $\mu c/mg$) were added. On the first occasion the duodenums from one rachitic chick and one normal chick were perfused consecutively. Inspection of the recordings showed that more radioactivity had passed through the gut of the rachitic than of the normal bird. On another occasion perfusions were made on duodenums from two normal chicks, two rachitic chicks and two rachitic chicks that had received a dietary supplement of dried pig's liver containing the rachitogenic factor described by Coates & Harrison (1957). From the recordings made during 45 min the radioactivity in the outer fluid appeared least in duodenums from birds given the rachitogenic factor, slightly more in preparations from rachitic birds and greatest in those from the normal chicks, but the differences were small. Samples taken from the outer bath at the end of each perfusion were plated out, and their activity was counted directly. By comparison with the standard solution of $^{45}CaCl_2$ the total amount of labelled Ca in the outer perfusate was calculated, with the following results:

Duodenums from:	normal birds	0·78 and 0·70 mg
	rachitic birds	0·55 and 0·76 mg
	rachitic birds given rachitogenic factor	0·55 and 0·66 mg

Thus by this technique no great difference could be demonstrated in the passage of soluble Ca across the wall of the gut of normal or rachitic chicks.

Another approach was tried in which the duodenum was not removed but the perfusion carried out *in situ* while the bird was under light urethane anaesthesia. For this purpose only the inner circulation was necessary, and the flowing electrode was incorporated in it. As perfusing fluid, 25 ml of the buffer without Ca were used, and 1 mg $^{45}CaCl_2$ (0·5 $\mu c/mg$) was added at the beginning of the experiment. As soon as it had become uniformly mixed into the inner fluid a steady value was registered on the recorder, which did not alter throughout 60 min perfusion of either a normal or a rachitic chick duodenum. In both preparations, however, the volume of circulating fluid fell noticeably. A sample of blood was taken from one of these birds, and radioactivity equivalent to at least 1 μg $^{45}Ca/ml$ was detected in the plasma, which suggested that water was being absorbed during the perfusion, carrying with it Ca in the same concentration as was present in the circulating fluid. In order to assess the volume of

water absorbed, the experiment was repeated with 1 mg creatinine/ml included in the buffer, as suggested by Sheff & Smyth (1955). After addition of the $^{45}CaCl_2$ solution a sample of the perfusing fluid was withdrawn and the concentration of creatinine was measured. From it could be calculated the exact volume of fluid present at the beginning of the perfusion time. After 45 min perfusion was stopped, and the perfusion fluid was drained from the system, which was then washed through with saline to make a total volume of 100 ml. On the assumption that the creatinine concentration remains constant throughout the perfusion time, the final volume of fluid remaining at the end of the experiment could be calculated from the total creatinine washed out of the perfusion system. As on the previous occasion, the radioactivity circulating through the gut maintained a constant value in both normal and rachitic birds throughout the perfusion. Out of the original 25 ml perfusion fluid the volume absorbed by the normal chick was 4·4 ml and by the rachitic chick 5·0 ml, showing once again no appreciable difference in the amounts of Ca transported across the gut walls of the two kinds of bird.

<div align="center">DISCUSSION</div>

Many of the investigations so far made into the function of vitamin D have been with rats. Rats do not become rachitic from the simple exclusion of vitamin D from the diet, but must also be given a diet with a grossly altered Ca:P ratio. Such animals show healing of the rachitic bone when transferred to a normal rat diet. We have preferred to use chicks in this study, because a clear-cut deficiency can be produced in them on a diet essentially free from vitamin D without manipulation of the Ca:P ratio. The absorption of Ca in a given time was followed by measuring the difference between original and residual Ca in a loop of intestine that had been washed out and into which 4 or 9 mg of Ca as chloride had been injected. There did not appear to be any region in the small intestine with a clearly higher degree of Ca absorption than any others. Three portions of the small intestine from the gizzard to the caeca absorbed similar amounts of Ca, although the lower part of the gut often contained considerable amounts of Ca either from residual food or possibly as a result of re-excretion into the gut. For most of our studies we used the duodenal loop, since this structure has a readily reproducible length of uniform size and there is little re-excretion of Ca into this region of the intestine.

The pH of the intestinal contents rose rapidly after they had left the gizzard and was high enough all through the small intestine for Ca to remain insoluble as the phosphate unless prevented by some unknown agency. Indeed, we found that addition of phosphate in amounts sufficient to precipitate all the Ca would prevent its uptake and that addition of citrate as a solubilizing agent did not reverse the effect. The problem of the chemical state of Ca at the time of absorption has not been studied carefully, but we found that under ordinary feeding conditions most of the soluble Ca in the intestine of normal chicks was diffusible, i.e. was not attached to large molecules. In the absence of any knowledge of the state of Ca in the gut, we studied the absorption of Ca as the chloride. The amount absorbed depended on the vitamin D_3 status of the animal. Our results furnish direct proof that vitamin D increases absorption of Ca

from the intestine, a view put forward by Harris (1932) nearly 30 years ago and since substantiated by the results of balance-type experiments, such as used by Nicolaysen (1937 a, b) with rats and by tracer studies with chicks (Keane, Collins & Gillis, 1956).

We found that chicks receiving 14 or 23 i.u. vitamin D_3/100 g diet absorbed at least twice as much Ca as those receiving no vitamin. Further, the increased absorption could be observed with a single dose of 100 i.u. vitamin D_3 ($= 2 \cdot 5 \mu g$) given by mouth to rachitic chicks 16 h before the absorption experiment. The increased absorption was also shown indirectly by the greater amount of ^{45}Ca in the blood stream of chicks that had received vitamin D_3, thus confirming the work of Keane et al. (1956).

Since the effect of the vitamin appears to be primarily through the gut, it seems worth emphasizing that simultaneous administration of Ca and vitamin D_3 was not effective. Intravenous injection of an aqueous dispersion of vitamin D_3 just before the absorption experiment also showed no increased uptake. Thus it appears that a few hours must elapse after oral administration of the vitamin before increased absorption of Ca can occur; during this time possibly some change may take place in the cells of the intestinal mucosa, or alternatively the vitamin D_3 itself may have to be changed into a metabolically active form. These possibilities appear worth exploring.

If vitamin D_3 is changed to a more active form, it might be excreted into the gut in the bile, in which event bile from chicks receiving the vitamin would be active in promoting absorption of Ca. In an early experiment (see Table 5) 0·1 ml bile from chicks deprived of vitamin D_3 had little or no effect on absorption of Ca from duodenal loops. In later work, however (see Table 8), when the uptake of Ca by rachitic chicks was extremely low, addition of 0·1 ml bile from either normal or depleted birds markedly increased the amount absorbed from duodenal loops in 2 h. An even greater effect was observed when the volume of bile was increased to 0·5 ml. Although the chicks used in our experiments were sufficiently depleted of vitamin D_3 to show clinical signs of rickets, it is unlikely that they were devoid of all traces of the vitamin. If in fact the active form of vitamin D_3 is excreted into the gut with the bile, it is possible that a volume of 0·1 ml (the average content of the gall bladder of one 4-week-old rachitic chick) might still have contained sufficient of the active material to exert an effect on the uptake of Ca. Further work on the nature of this material in bile responsible for these effects is in progress.

In our studies of the isolated gut by the method of Fisher & Parsons (1949) we could find no significant difference between the rates of passage of Ca across rachitic and normal guts. Similarly, when the small intestine was perfused in situ with 20 ml of an isotonic solution containing 1 mg ^{45}Ca, the Ca concentration remained constant, Ca being absorbed along with the water; no marked difference was observed between the amounts of water absorbed by rachitic and normal guts. Presumably under the conditions prevailing in the perfused intestine Ca is transported through the wall by simple diffusion without the aid of vitamin D; hence these systems are unsuitable for studying its mechanism of action in Ca absorption.

SUMMARY

1. The uptake of calcium from tied-off loops of small intestine was measured in normal and rachitic chicks. The duodenum proved the most convenient site for this study.

2. The rate of absorption of Ca from washed duodenal loops in vivo was at least twice as great in chicks given vitamin D₃ as in rachitic controls. The effect was observed whether vitamin D₃ was given in the diet from hatching or as a single dose by mouth of 100 i.u. to rachitic birds 16 h before the experiment. A single oral dose had no effect on Ca absorption if given less than 8 h before experiment.

3. Normal chick bile increased Ca absorption from duodenal loops. On two out of three occasions bile from rachitic chicks similarly increased the uptake.

4. Perfused preparations of chick duodenum, either in vivo or completely isolated, showed no difference between Ca uptake of normal and rachitic birds.

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