

The absorption and secretion of calcium in the gastrointestinal tract of germ-free and conventional chicks

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1. The uptake, transport and secretion of calcium into the gastrointestinal tract of germ-free (GF) and conventional (CV) chicks was determined by a variety of in vivo and in vitro techniques using ^{45}Ca as a marker following a standard fast-feed regimen.
2. Following an oral dose of Ca in conscious chicks, the transit time of digesta in the GF birds was appreciably faster than in the CV controls.
3. Although the proportion of the Ca dose absorbed was similar in both groups of chicks, the accumulation of Ca in the intestinal wall was much greater in CV birds. Distribution of the labelled Ca in plasma and bone was similar in both groups.
4. Following intravenous Ca injection in conscious chicks the Ca secretion into the intestinal lumen was similar in both groups, but the amount appearing in the gut wall was appreciably greater in CV chicks.
5. There were regional differences in the ability of the gut to absorb Ca. There was a tendency for Ca uptake to be greater in the GF chicks but only in the proximal ileum did this approach significance.
6. When the Ca was confined to an intestinal loop the uptake/g tissue was similar in the proximal ileum of both groups but significantly greater in the distal ileum of the GF group. Uptake/10 mm gut was similar in both groups at both the proximal and the distal site.
7. It is suggested that the absence of a microbial load does not affect the ability of the enterocyte to absorb Ca but the subsequent removal of the absorbed load from the intestinal wall is appreciably slower in the CV group.

The growth rate of chicks maintained in a germ-free (GF) environment is faster than that of their conventional (CV) counterparts (Coates *et al.* 1963) and since there is close correlation between growth rate and calcium uptake (Adams & Norman, 1970) the requirement for this mineral might be expected to be greater in the GF chick. Absorption of Ca and magnesium, as estimated by balance studies, has been shown to be greater in the GF rat compared to CV controls (Reddy *et al.* 1969) while uptake of Ca and its incorporation into bone was found to be greater in GF chicks when compared to CV birds (Edwards & Boyd, 1963). In addition to the increased growth rate in the GF environment, the absence of a microflora causes marked thinning of the gut, due to a reduction in the lamina propria (Gordon & Bruckner-Kardoss, 1961), and a reduction in cell turnover and migration rate (Rolls *et al.* 1978). Such differences in morphology might be expected to increase the absorptive capacity of the GF gut.

The present study was undertaken to assess the effects of the microflora on the uptake of Ca by the enterocytes, the site of uptake and the fate of an oral and a parenteral Ca load.

A preliminary report of part of this work has already been published (Palmer & Rolls, 1978).

MATERIALS AND METHODS

Chicks. Rhode Island Red \times Light Sussex chicks were used. Eggs from a specified pathogen free flock maintained at the Institute were incubated for 18 d in a commercial incubator and then decontaminated with peracetic acid (Harrison, 1969). Those destined for the

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production of GF chicks were transferred to Gustafsson stainless-steel isolators to hatch, while the rest were returned to the incubator and the chicks subsequently hatched were reared as CV controls. After hatching, the GF chicks were divided into groups of four and housed in stainless-steel cages with mesh floors within the isolators while the CV controls were housed in similar cages in a room where the environmental conditions could be matched to those of the isolators. As far as possible males and females were distributed evenly among the groups. Sterility checks on the chicks within the isolators were made at intervals (Fuller, 1968).

Diets. The chicks were fed a practical mash (SCM) the composition of which was (g/kg) maize meal 350, wheat meal 300, middlings 85, white fish meal 100, dried skim milk 75, dried grass meal 30, dried brewer's yeast 30, limestone flour 15, sodium chloride 4.697, hydrated manganese sulphate 0.3; maize oil 10 and potassium iodide 3 mg/kg. Cholecalciferol was dissolved in the maize oil to provide 16 $\mu\text{g}/\text{kg}$ diet. 'A500 Rovimix' (Roche Products Welwyn Garden City, Herts.) was added to give 2 mg retinol/kg. The diet was packed in plastic bags which were evacuated and sterilized by 5 Mrad γ radiation.

Reagents. $^{45}\text{CaCl}_2$ was obtained from the Radiochemical Centre, Amersham, Bucks. All other reagents used were of Analar grade and were obtained from British Drug Houses, Poole, Dorset.

Plan of experiments

All investigations were made on comparable groups of GF and CV chicks from the same batch of eggs.

Since transit times of digesta are known to increase following a period of fasting, a pre-experimental feeding regimen was adopted for all experiments. The chicks were fasted for 16 h and then allowed access to food for exactly 1 h before the experiment began.

Expt 1. This was designed to compare transit time and uptake of Ca at various sites within the gastrointestinal tract and accumulation of Ca into bone at graded intervals up to 1 h in conscious chicks. Each chick was intubated into the proventriculus and a dose of 0.1 μCi ^{45}Ca in 40 mM- CaCl_2 was administered in a total volume of 2 ml, giving a total Ca load of 3.5 mg. Two birds from each group were killed by intravenous injection of sodium pentobarbitone at intervals of from 5 min to 1 h after dosing. The gastrointestinal tract was rapidly tied off to isolate sections as follows: (1) proventriculus, (2) duodenum, (3) proximal ileum (pancreatic duct to yolk-sac stump), (4) distal ileum 1 (yolk-sac stump to limit of caeca), (5) distal ileum 2 (to the ileocaecal junction). The gut was removed and each segment was washed through with isotonic saline (8.5 g NaCl/l) and the washings collected for counting. Each segment was opened lengthways, rinsed in saline and blotted dry. The right tibiotarsus was removed and scraped clean of muscle. Washings, gut tissue and bone were ashed and the ash dissolved in 2.0 M-hydrochloric acid and the ^{45}Ca content measured by liquid-scintillation counting using a Packard Tri-carb liquid-scintillation spectrophotometer (Model 3003) using the internal standard technique.

Expt 2. In an attempt to assess the amount of Ca secreted into the gut, the procedure adopted in Expt 1 was repeated replacing the oral dose of Ca with an intravenous injection of 0.1 μCi (3.5 mg Ca) in a total injection volume of 0.5 ml. Gut washings and tissues were treated as described previously.

Expt 3. Since differences in transit time may mask differences in absorptive capacity at the various sites, Expt 3 was designed to assess *in vitro* the potential of the enterocytes for Ca uptake. The gut was removed from anaesthetized chicks and divided as in Expt 1. Each segment was everted and rings, 3 mm in width, were cut from the mid part of each segment. The rings were incubated in a modified oxygenated Krebs saline (Spencer *et al* 1978) containing 0.1 μCi $^{45}\text{Ca}/20$ ml saline. Rings were removed at intervals of from 1 to 15 min

from the incubation medium, rinsed briefly, blotted dry, weighed and then dissolved in 5 M-HCl for 24 h before ^{45}Ca was determined.

Expt 4. In vivo uptake of Ca from intestinal loops was studied. Chicks were anaesthetized with sodium phenobarbitone (100 g/l) at a dose of 20 mg/kg body-weight. An incision was made in the abdominal wall to expose the intestine and the position of the yolk-sac stump was located. Loops were made of either proximal or distal ileum. The loops were washed through with oxygenated Krebs saline before being filled with saline containing $20\ \mu\text{Ci}\ ^{45}\text{Ca}/\text{l}$. The loops were replaced in the abdominal cavity and left *in situ* for periods of from 5 to 60 min. The loops were then emptied, washed through with saline and the saline together with washings was dried and ashed as described previously. The loops were weighed, the length measured and the ^{45}Ca content determined as previously.

Statistical analysis. The data from Expts 3 and 4 were subjected to analysis of variance. In Expt 3 the data for each site were treated separately and each of the sixteen chicks was considered to be an experimental unit with repeated observations being made at seven times. The analysis followed the suggestion of Rowell & Walters (1976).

In Expt 4 each chick gave only one result so the data were regarded as independent. However, reliability of the data increased as uptake increased so the results were transformed into logarithms to give homogeneous variances. The logarithms were analysed by analysis of variance and time and environment effects were assessed using the variation between chicks within times and environments.

RESULTS

Expt 1 (Fig. 1) shows the luminal ^{45}Ca content and the accumulation of ^{45}Ca within tissues for each of the segments of the gastrointestinal tract following an oral dose of labelled Ca. The rate of appearance in the various segments indicated that the transit time was appreciably shorter in the GF compared with the CV birds. The total uptake of Ca from all segments is shown in Fig. 2. This was calculated by addition of the Ca remaining in the lumen of all sections of the gut minus the Ca secretion into the lumen determined after intravenous dosing in Expt 2. Uptake was very rapid and within 5 min of the dose being given approximately four-fifths had disappeared from the lumen. There was no difference between the GF and CV birds in either the rate of uptake or the amount of the dose absorbed. At the longer time intervals less Ca appeared to be absorbed by the CV birds.

Although the pattern of accumulation of the labelled Ca in the gut tissues was similar in both groups, both exhibiting a peak at 5 min and a rather smaller peak after 30 min (Fig. 3), the magnitude of the accumulation was different in the two groups. The CV birds accumulated twice as much Ca in the gut wall at the two peaks compared with the GF birds although the amount left 60 min after dosing was similar. The deposition of the labelled Ca in bone was similar in both groups.

Following an intravenous dose of Ca (Expt 2) the pattern and amount of secretion into the gut lumen was similar in both groups (Fig. 4). Analysis of the gut tissue showed that the amount of the dose appearing in the CV gut was approximately twice that of the GF gut within the first 5 min after the dose was given but the amount held in the gut wall diminished gradually and by 60 min following dosing the Ca content of the CV gut was only a little greater than that of the GF chicks.

Table 1 shows the ^{45}Ca uptake by isolated rings of intestine (Expt 3) and Fig. 5 gives an example of the data for the distal ileum with the fitted line. Considering the results for each site and time separately, only duodenum at 4 min, proximal ileum at 15 min and distal ileum at 1 min showed significant differences between germ-free and conventional chicks ($P < 0.05$, $P < 0.05$ and $P < 0.01$ respectively). It is noticeable however, that values for germ-free chicks were generally slightly greater than values for conventional birds, but such

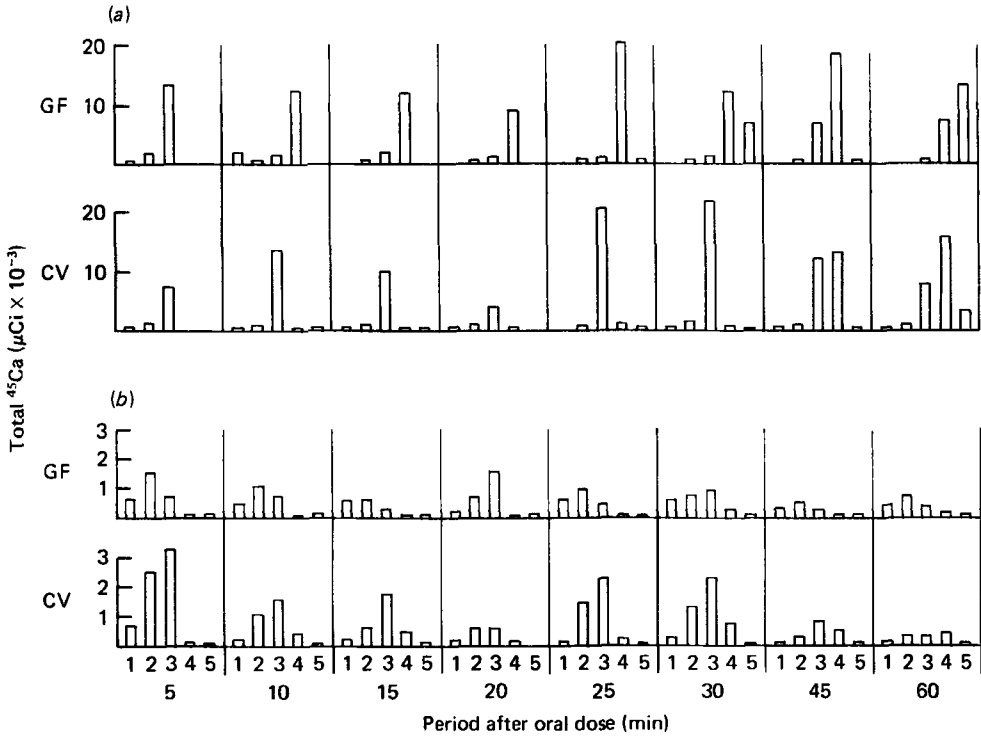


Fig. 1. Expt 1. Total ^{45}Ca ($\mu\text{Ci} \times 10^{-3}$) found in (a) luminal contents and (b) intestinal tissues of the following sections of the gut of groups of two germ-free (GF) and conventional (CV) chicks at intervals after oral administrations. (1) Proventriculus, (2) duodenum, (3) proximal ileum, (4) distal ileum 1 and (5) distal ileum 2.

consistency might be expected since the same sixteen birds were involved in each comparison. Analysis of variance as outlined in Materials and Methods, showed no significant differences between environments. In contrast, highly significant linear ($P < 0.001$) and quadratic ($P < 0.01$) effects were evident at the first four sites, at distal ileum 2 only the linear effect was significant ($P < 0.001$). There was also a strong suggestion of an interaction between the linear effect and environment for the proximal ileum ($P = 0.051$) indicative of more rapid uptake by the germ-free isolated rings. There were some regional differences in uptake, the sites of greatest uptake being the proventriculus and the distal ileum 2.

Table 2 gives the ^{45}Ca uptake in tied loops of proximal and distal intestine (Expt 4). These results indicate that initially in the proximal ileum there was a marked uptake but little subsequent increase with time, whilst in the distal ileum after the initial rapid uptake there was a substantial increase between 30 and 60 minutes ($P < 0.01$). When uptake/g tissue was considered there was no difference between the groups in uptake of Ca in the proximal ileum but in the distal ileum the uptake in the GF birds was always greater and the mean significantly so ($P < 0.001$). Uptake/10 mm gut failed to reveal any difference in uptake either in the proximal or the distal ileum. The lengths of the segments used were very comparable in the two groups but as shown in Table 2 the weight/unit length was significantly less in the GF chicks.

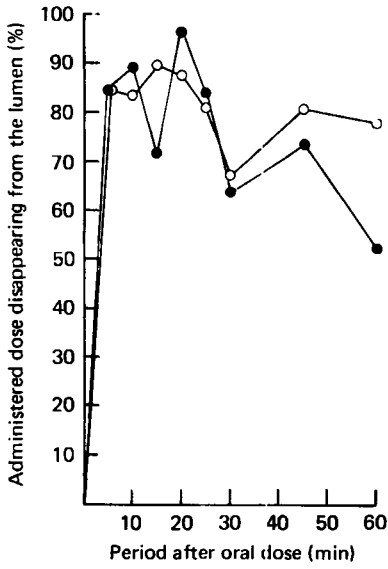


Fig. 2.

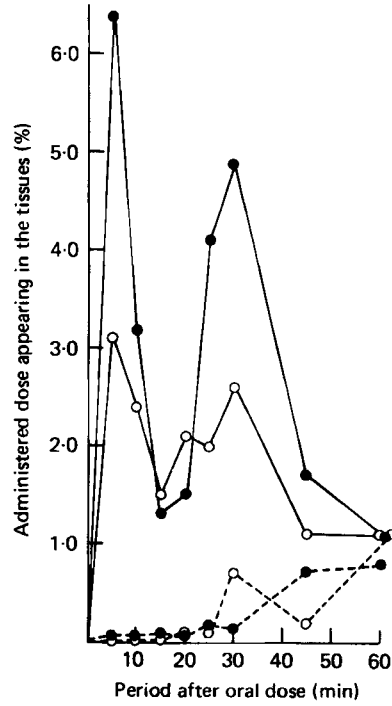


Fig. 3.

Fig. 2. Expt 1. Rate of disappearance of an oral ^{45}Ca load expressed as a % of the total dose given in germ-free (○—○) and conventional (●—●) chicks.

Fig. 3. Expt 1. Rate of appearance of ^{45}Ca in the gut wall (—) and bone (---) in germ-free (○) and conventional (●) chicks expressed as percentage of the oral dose given.

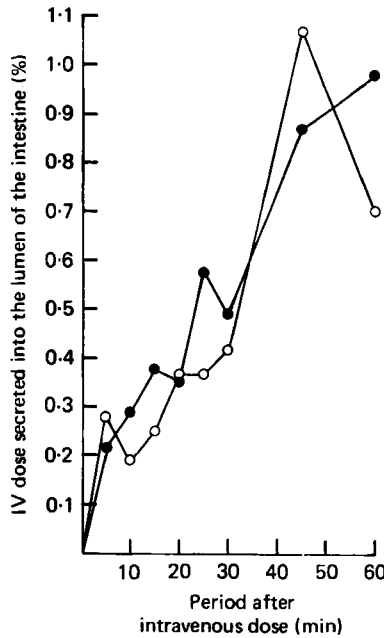


Fig. 4. Expt 2. Secretion of ^{45}Ca into the lumen of the gut following intravenous (IV) dosing in GF and CV chicks expressed as percentage total dose given.

Table 1. Expt 3. Calcium uptake ($^{45}\text{Ca } \mu\text{Ci} \times 10^{-6}/\text{mg tissue}$) by isolated rings of germ-free (GF) and conventional (CV) chick intestine taken from various sites after different periods of incubation in a modified Krebs saline containing $0.1 \mu\text{Ci } ^{45}\text{Ca}/20 \text{ ml saline}$ (Values given are means with their standard errors for eight samples)

Time of incubation (min)	Proventriculus			Duodenum			Proximal ileum			Distal ileum 1			Distal ileum 2							
	GF	CV		GF	CV		GF	CV		GF	CV		GF	CV						
	Mean	SE	CV	Mean	SE	CV	Mean	SE	CV	Mean	SE	CV	Mean	SE	CV					
1	1.26	0.091	1.19	0.048	0.73	0.086	0.65	0.043	0.89	0.056	0.83	0.057	1.06	0.066	0.77	0.070	1.16	0.121	1.13	0.146
2	1.57	0.080	1.46	0.089	0.97	0.071	0.83	0.081	1.11	0.085	1.03	0.096	1.22	0.109	1.04	0.080	1.32	0.161	1.20	0.144
3	1.76	0.091	1.73	0.093	1.42	0.040	1.12	0.126	1.17	0.087	1.11	0.134	1.28	0.120	1.09	0.090	1.38	0.108	1.38	0.114
4	2.06	0.109	1.88	0.061	1.18	0.062	0.96	0.060	1.29	0.075	1.01	0.054	1.34	0.160	1.14	0.048	1.56	0.163	1.56	0.122
5	2.03	0.194	1.96	0.061	1.21	0.108	1.20	0.135	1.46	0.093	1.25	0.081	1.52	0.130	1.38	0.246	1.63	0.158	1.66	0.161
10	2.60	0.099	2.61	0.107	1.52	0.112	1.40	0.188	1.64	0.131	1.45	0.102	1.65	0.178	1.79	0.195	1.87	0.132	2.22	0.322
15	3.11	0.201	2.65	0.398	1.57	0.130	1.49	0.149	1.89	0.144	1.45	0.095	1.95	0.109	1.64	0.264	2.38	0.237	2.53	0.479

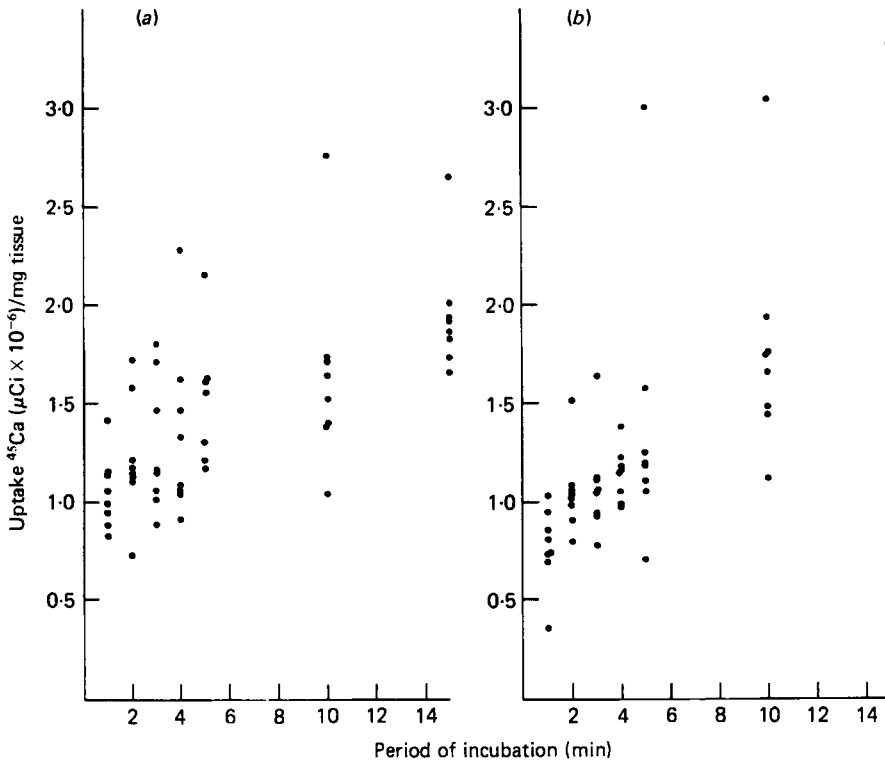


Fig. 5. Expt 3. ^{45}Ca uptake ($\mu\text{Ci} \times 10^{-6}$)/mg wet weight tissue by rings of distal ileum 1 in (a) germ-free and (b) conventional chicks at intervals of incubation in modified Krebs saline containing $0.1 \mu\text{Ci}$ ^{45}Ca /20 ml saline.

DISCUSSION

Comparing the Ca uptake by chicks in the GF and CV environments no difference between the groups was demonstrated. The enterocyte uptake mechanism, although initially probably a passive process (Spencer *et al.* 1978), must occur very rapidly since within 5 min of a dose of labelled Ca being given either orally in conscious birds (Expt 1) or into loops of intestine in anaesthetized chicks (Expt 4), some four-fifths of the dose had been absorbed. When the time course of the uptake was studied in an *in vitro* preparation (Expt 3) the rapidity of the uptake process was confirmed and saturation, or near saturation of the uptake mechanism is apparent within 5–10 min incubation with the label. The amount of ^{45}Ca disappearing from the lumen at 45 and 60 min after dosing (Expt 1) was less than that at the earlier times which is probably a reflection of resecretion of unlabelled Ca into the lumen of the gut.

The accumulation of Ca within the intestinal wall exhibited a double peak, the maximum value occurring 5 min after dosing and a second smaller peak 30 min after dosing (Expt 1). The peak values in the gut wall of the CV chicks were twice those of the GF chicks. This pattern of appearance of Ca in the gut wall could be explained by very rapid uptake of Ca by the enterocytes and slow removal of the absorbed dose into the blood stream to form the first peak, while the second peak might reflect the redistribution of Ca in the blood returning to the gut, since the peak occurring at 30 min was also seen following intravenous dosing (Expt 2). GF chicks have a thinner gut wall than their CV counterparts mainly due

Table 2. *Expt 4. Calcium uptake ($^{45}\text{Ca } \mu\text{Ci} \times 10^{-3}$) by tied loops of proximal and distal ileum in germ-free (GF) and conventional (CV) chicks at intervals after introduction of Krebs bicarbonate saline containing $2.0 \mu\text{Ci } ^{45}\text{Ca}/100 \text{ ml}$ saline into the loop*
(Values given are mean logarithms of groups of six chicks. Antilogarithms are given in parentheses.)

	Mean (\pm SE) intestinal weight (g/100 mm)	Time of incubation (min)			Pooled SE	Mean	Difference (GF-CV)	SE of difference
		5	20	30				
Proximal	GF	0.88 \pm 0.02**	0.844 (2.33)	0.836† (2.31)	0.865 (2.38)	0.804 (2.23)	0.033 ^{NS}	0.0847
	CV	1.09 \pm 0.04	0.730 (2.08)	1.052 (2.86)	0.799 (2.22)	0.635 (1.85)		
	CV	0.65 \pm 0.02***	0.899 (2.46)	0.861 (2.37)	0.849† (2.34)	1.497‡ (4.47)	0.586 (1.80)	0.441***
Distal	GF	0.82 \pm 0.02	0.319 (1.38)	0.562 (1.75)	0.450† (1.57)	1.015‡ (2.76)		
	CV							
	CV							
Uptake $^{45}\text{Ca } \mu\text{Ci} \times 10^{-3}/10 \text{ mm gut}$								
Proximal	GF		-1.660 (0.190)	-1.575† (0.207)	-1.599 (0.202)	-1.590 (0.204)	-0.180 ^{NS}	0.0975
	CV		-1.404 (0.246)	-1.273 (0.280)	-1.447 (0.235)	-1.581 (0.206)		
Distal	GF		-1.769 (0.171)	-1.892 (0.151)	-1.945† (0.143)	-1.247† (0.287)	0.213 ^{NS}	0.1194
	CV		-2.152 (0.116)	-1.946 (0.143)	-2.152‡ (0.116)	-1.459† (0.232)		

Significance of difference between GF and CV: NS, not significant, $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

† One missing value.

‡ Two missing values.

to a reduction in the lamina propria (Gordon & Bruckner-Kardoss, 1961) thus the enterocyte-capillary distance would be smaller and a more rapid removal of absorbed nutrients from the gut wall might be expected to occur. Other factors which might explain the faster removal of Ca from the gut wall could be an increased rate of synthesis of calcium-binding protein (CaBP) or local alterations in blood flow.

Although the rate of disappearance of the labelled Ca from the gut lumen was similar in both groups, its transport away from the gut wall was more rapid in the GF chicks (Expt 1), but since the levels of Ca in the blood were similar in both groups it seems likely that there might have been a more rapid excretion of the dose in the GF group. Gustafsson & Norman (1962) suggested that as GF rats frequently develop urinary calculi, indicating an increased excretion of Ca, the whole Ca equilibrium is disturbed in the absence of the microflora. There has been some evidence to suggest that Ca deposition in bone is greater in the GF state (Edwards & Boyd, 1963) but these findings were not confirmed in the present study: the incorporation of the labelled Ca into bone over a period of 1 h being similar in both groups (GF 0.8×10^{-3} , CV 1.1×10^{-3} μCi).

There was no difference between the groups as to the amount of Ca appearing in the secretions of the gut in the conscious chicks and since most of this Ca is derived from the pancreatic secretions this would suggest that pancreatic function is similar in the two groups.

Measurement by Ford (1971) of transit times through the entire intestine failed to show any difference between the GF and CV chicks whereas we found a very striking difference in the transit of an oral dose of Ca, the transit time in the GF group being appreciably shorter than in the CV controls. The lack of agreement between the two findings may result from the different experimental conditions. Loss of muscle tone has been reported in many species and seems to be characteristic of the GF state. This characteristic may account for the difference in intestinal transit time.

All parts of the gut appear to have the ability to absorb Ca but the experiment using isolated rings of intestine (Expt 3) demonstrates that there are regional differences in uptake ability; the proventriculus and distal ileum having a greater uptake than the duodenum. There was a trend for uptake to be greater in the GF chicks compared with the CV controls and in the proximal ileum the interaction between the linear effect and the environment approached significance suggesting a greater uptake in the GF chicks.

Measurements of Ca uptake by loops in anaesthetized chicks (Expt 4) showed that there was no difference in uptake in the proximal ileum whether expressed in relation to unit weight or unit length but in the distal ileum uptake/g tissue was significantly greater in the GF chicks, while uptake expressed/10 mm approached significance $0.1 > P > 0.05$. This is somewhat surprising since Ca uptake is more efficient at acid pH and Ford (1974) showed that the pH of the distal ileum was lower in CV birds. The thinning of the intestine is much more apparent at the distal region of the intestine and the difference in uptake probably reflects the reduced enterocyte-capillary distance rather than any real difference in the ability of the enterocyte to absorb Ca.

Although the uptake of Ca was generally greater in the GF birds, and significantly greater in the distal ileum of the GF birds, there was no evidence that the overall Ca economy was affected. Deposition in bone and plasma Ca concentration were similar in both environments suggesting either that the extra absorption was quantitatively insignificant or that excretion was more rapid.

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