Brit. J. Nutr. (1961), 15, 149

The effect of a rachitogenic factor on calcium metabolism in chicks

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(*Received* 3 *August* 1960)

Raw pig's liver has been shown by Coates & Harrison (1957) to have rachitogenic properties for chicks. The rachitogenic activity was heat-labile and water soluble, but attempts to isolate and characterize the responsible factor have so far been unsuccessful. Meanwhile the studies reported here were undertaken to throw some light on its mode of action. Rachitogenic activity might depend on an interference with either the absorption of calcium or its deposition in bone; both these possibilities were investigated.

EXPERIMENTAL AND RESULTS

The management of the birds, determination of Ca, counting methods and surgical procedures were as described in a previous paper (Coates & Holdsworth, 1961).

Preparation of rachitogenic factor

Fresh pig's liver was homogenized in a Waring Blendor, spread on stainless-steel trays and dried in a current of air at 40°. It was milled finely and extracted with a mixture of 2 parts light petroleum (b.p. 40°–60°), 2 parts diethyl ether and 1 part absolute ethanol. This extraction procedure has been shown to remove any vitamin D_3 present in the liver, leaving most of the rachitogenic activity in the residue. The extracted liver powder was spread on trays and allowed to dry at room temperature.

The dried extracted liver powder was added to the Olsson rachitogenic diet at the expense of whole diet, to provide the equivalent of 20 g fresh liver in 100 g diet (usually about 6% of the dried product). This quantity counteracts the antirachitic effect of about 3 i.u. vitamin D_3 (Coates & Harrison, 1957).

Determination of bone ash

The birds were killed by breaking their necks; the left tibia was then removed from each and cleaned of soft tissue by scraping with a scalpel. The bones were defatted by extraction in a Soxhlet apparatus for 24 h with ethanol and with diethyl ether for a further 24 h. They were dried in a desiccator, weighed and incinerated at about 700°. The resulting ash was weighed, the weight of ash being then expressed as a percentage of the weight of the defatted bone. For determination of ^{45}Ca, the ash was dissolved

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in conc. HCl diluted 1:1, any debris being removed by centrifuging. The calcium oxalate precipitated from a portion of the solution by adding 4% ammonium-oxalate solution was washed once with hot ammonia solution (1 part ammonia of sp.gr. 0·88 to 99 parts water), dried in a desiccator and spread on 25 mm diameter metal planchets, and the activity was counted at infinite thickness.

Effect of rachitogenic factor on uptake of Ca from the duodenum

Two experiments were done by the duodenal-loop technique on groups of chicks that had received the basal diet alone or with 23 i.u. vitamin D_3/100 g or with a supplement of dried extracted liver equivalent to 20 g raw liver/100 g diet. In the first experiment the chicks had been on these treatments from 1 day old to 4 weeks and three birds were used from each. Calcium chloride containing 4 mg labelled Ca was placed in duodenal loops and allowed to remain for 2 h. At intervals during this time samples of blood were taken, and the radioactivity of the plasma was determined. At the end of the experiment the duodenal loops were removed and ashed, and the residual Ca was determined by the titration method. At the same time one tibia from each chick was removed for determination of radioactivity and bone ash. The values for all samples are presented in Table 1, and the plasma radioactivity is plotted in Fig. 1. The birds given vitamin D_3 absorbed a significantly greater amount ($P < 0.01$) of Ca than did those given the unsupplemented diet. The chicks that had received a supplement of dried liver were more rachitic than those on the basal diet, as indicated by the tarso-metatarsal distances and percentages of bone ash. They appeared to absorb even less Ca than the negative controls, but the difference was not statistically

Table 1. *Effect of vitamin D_3 and a rachitogenic factor on absorption of calcium from duodenal loops in 4-week-old chicks*

(4 mg Ca as ^{45}Ca-labelled $CaCl_2$ were placed in washed-out duodenal loops, and the Ca absorbed was determined after 2 h. The ^{45}Ca in bone was determined by counting the activity of the ash at infinite thickness. Each value is for one chick.)

Supplement/100 g diet	T.M.T.* (mm)	Ca absorbed (mg)	Bone ash (%)	Activity in bone ash (counts/min)
None	2·125	0·82	34·8	529
	2·5	0·82	35·1	818
	2·5	1·10	35·3	992
Mean	2·375	0·91	35·1	780
23 i.u. vitamin D_3	0·875	2·85	46·1	958
	1·0	1·67	43·3	724
	0·875	2·11	43·9	1076
Mean	0·917	2·21	44·4	919
Dried liver (≡ 20 g raw)	2·875	0·49	33·6	190
	2·625	0·58	32·4	613
	3·5	0·61	32·7	847
Mean	3·0	0·56	32·9	550

* Tarso-metatarsal distance.

significant. The different effects of these three treatments on absorption of Ca from duodenal loops were reflected in the levels of radioactivity found in plasma and tibias.

In a second experiment, chicks were given the basal diet with or without 5 i.u. vitamin D_3/100 g up to the age of 4 weeks. Four birds from each group were then given a supplement of dried liver ($\equiv$ 20 g raw liver/100 g diet) for a further 5 days, when their uptake of Ca from duodenal loops was compared with that of four similar birds from each group that had received no liver supplement. Three chicks that had

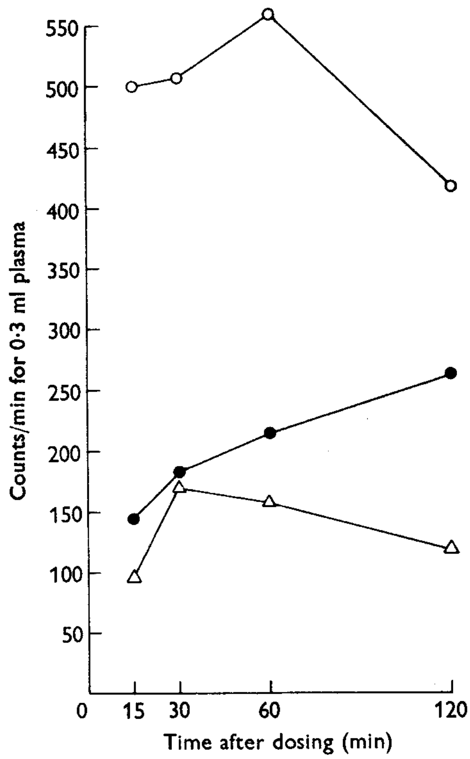

Fig. 1. Levels of ^{45}Ca in plasma of normal and rachitic chicks given $^{45}CaCl_2$ placed in duodenal loops. Each point is the mean value for groups of three birds. ○—○, basal diet + 23 i.u. vitamin D_3/100 g; ●—●, basal diet; △—△, basal diet + dried liver ($\equiv$ 20 g raw/100 g).

received an optimal quantity of vitamin D_3 from hatching were also included. The results are given in Table 2; as expected, the birds given vitamin D_3 absorbed significantly ($P < 0·01$) more Ca than any of the rest. Although the apparent differences between the other four treatments did not reach statistical significance, there was a strong indication that the liver supplement had interfered with the ability of the duodenum to absorb Ca, in both presence and absence of vitamin D_3.

Effect of the rachitogenic factor on deposition of ^{45}Ca in bone

The disappearance from plasma of injected ^{45}Ca and its deposition in bone was compared in groups of chicks given the Olsson diet with or without vitamin D_3 or rachitogenic factor. Calcium chloride containing 1 mg labelled Ca in 0·25 ml solution was given intravenously into the jugular vein of each bird, and blood samples were taken from the wing vein at intervals after injection. The birds were then killed, and one tibia was removed and ashed. Two of the experiments lasted for 2 h and one for

Table 2. *Effect of supplements of dried pig's liver on absorption of calcium from duodenal loops of 4-week-old chicks*

(4 mg Ca as ^{45}Ca-labelled $CaCl_2$ were placed in washed-out duodenal loops, and the residual ^{45}Ca was determined after 2 h. Each value is for one chick)

Supplement/100 g diet: During 4 weeks	Supplement/100 g diet: During last 5 days	Ca absorbed (mg)	Mean
None	None	0·9, 0·9, 0·8, 0·6	0·80
	Dried liver (≡ 20 g raw liver)	0·9, 0·6, 0·4, 0·6	0·63
5 i.u. vitamin D_3	5 i.u. vitamin D_3	0·8, 1·4, 0·9, 1·0	1·03
	5 i.u. vitamin D_3 and dried liver (≡ 20 g raw liver)	1·0, 0·8, 1·0, 0·7	0·88
23 i.u. vitamin D_3	23 i.u. vitamin D_3	1·3, 1·9, 1·7	1·63

Table 3. *Mean values for the effect of vitamin D_3 or a rachitogenic factor on the deposition of injected ^{45}Ca in the tibias of 4-week-old chicks*

(1 mg $^{45}CaCl_2$ (0·5 μc) was injected into the jugular vein of the birds and blood samples were taken from the wings. Birds were killed after either 30 min or 2 h, and the tibias were taken for bone ash and radioactivity measurements. The activity of the calcium oxalate from the bone ash was counted at infinite thickness)

Supplement/100 g diet	Time of expt	No. of birds per group	Bone ash (%)	Wt of Ca oxalate (g)	Activity of Ca oxalate (counts/min)	Counts/min × wt of Ca oxalate
23 i.u. D_3	2 h	4	43·8	0·577	494	285
None	2 h	4	35·0	0·398	843	336
Dried liver (≡ 20 g raw liver)	2 h	4	33·3	0·359	867	311
23 i.u. D_3	2 h	3	46·5	0·517	358	189
None	2 h	3	37·0	0·367	568	206
Dried liver (≡ 20 g raw liver)	2 h	3	36·0	0·338	615	207
23 i.u. D_3	30 min	3	45·5	0·470	340	160
None	30 min	3	37·5	0·349	521	184
Dried liver (≡ 20 g raw liver)	30 min	3	34·7	0·350	697	243

30 min. The levels of radioactivity found in the plasma are plotted in Fig. 2; values referring to its appearance in bone are given in Table 3.

As expected, in all experiments the percentage bone ash of the birds given the rachitogenic factor was lower, and that of the chicks given vitamin D_3 was considerably higher, than in the corresponding groups receiving only the basal diet. The radioactivity in the bones of the birds given vitamin D_3 was little more than half tha in the other two groups. However, direct comparison of the counts/min in samples from each experimental group is valueless, since the birds that had received vitamin D_3 were heavier and consequently had much larger bones than those given either of the other two treatments. On multiplying the counts/min at infinite thickness of each sample by the total weight of calcium oxalate from the tibia, an arbitrary value is obtained that seems to us to offer a more valid basis for comparison. Inspection of

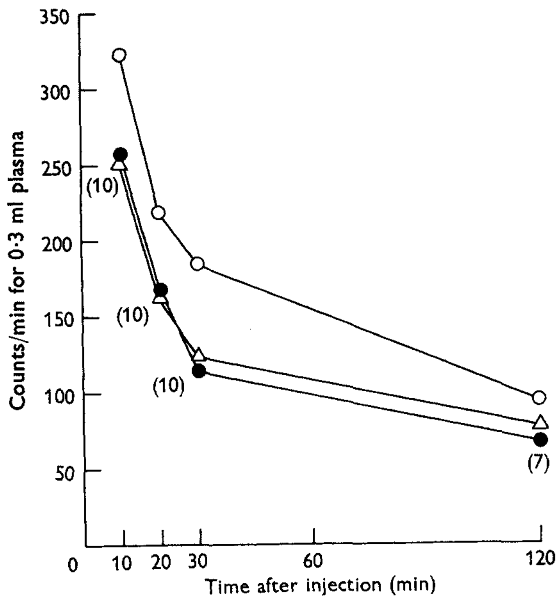

Fig. 2. Levels of ^{45}Ca in plasma of normal and rachitic chicks given $^{45}CaCl_2$ intravenously. Figures in parentheses are the number of chicks represented by each point. ○—○, basal diet+23 i.u. vitamin D_3/100 g; ●—●, basal diet; △—△, basal diet+dried liver (≡ 20 g raw liver/100 g).

these values shows that in 2 h relatively less Ca was deposited in the bones of the normal birds than in those of either of the rachitic groups. There was no difference between the values for the negative controls and those for the birds given the rachitogenic factor. In the one experiment that was terminated after 30 min a similar difference in Ca deposition was observed, but on this occasion the value for the chicks given the basal diet alone was intermediate between that for the normal birds and that for those given the rachitogenic factor.

From Fig. 2 it is apparent that the plasma radioactivity decreased more rapidly in the two groups of rachitic birds than in those with adequate vitamin D_3. This finding is in accord with that of a greater deposition of ^{45}Ca in the bones, but might also be the result of a higher rate of excretion of ^{45}Ca by the rachitic birds. To test the latter possibility, groups of four normal and four rachitic chicks were each given intravenously 1 mg labelled Ca as chloride. The two groups were placed in cages with wire-screen floors, and their droppings were collected on filter-paper throughout 2 h. The birds were then killed, and the kidneys and ureters from each group were combined with the droppings and ashed. The radioactivity was determined in a sample of calcium oxalate precipitated from the total ash, with the results:

	Total weight of oxalate (g)	Counts/min at infinite thickness	Counts/min × weight of oxalate (g)
Normal birds	0·2955	282	83·3
Deficient birds	0·1489	449	66·9

When adjustment is made for the difference in weight of ash from the two groups, it is clear that the deficient chicks excreted rather less ^{45}Ca than the normal ones. It can, therefore, safely be concluded that the more rapid disappearance of ^{45}Ca from the plasma of rachitic chicks was the result of a greater deposition in the skeleton. Except for the experiment lasting 30 min, there was no observable difference between the groups given the rachitogenic factor and those given the basal diet alone, and on no occasion was there any indication that the rachitogenic factor interfered with deposition of ^{45}Ca in the bones.

DISCUSSION

In a previous paper Coates & Holdsworth (1961) have shown that vitamin D_3 increased the rate at which rachitic birds absorbed Ca from a duodenal loop. The results obtained here confirm and extend these observations. Birds given a diet devoid of vitamin D_3 for the first 4 weeks of life absorbed significantly less Ca than their hatchmates that had received ample vitamin D_3. When a supplement of dried pig's-liver was included in the rachitogenic diet, there was a strong indication that uptake of Ca was even further depressed, and this effect was observed whether the liver was given from hatching or only during the last 5 days of the 4-week experiment. Although the apparent differences between uptake of Ca in birds receiving the liver and their corresponding controls were not statistically significant at the $P = 0{\cdot}05$ level, there is a strong suggestion that the rachitogenic properties of the pig's liver depend on its interference with absorption of Ca and that this effect can be reversed by small amounts of vitamin D_3. A depressed absorption of Ca might occur if some reaction between the pig's liver and the Ca in the gut rendered the Ca less available to the animal. However, this explanation is unlikely, since the effect was obtained in washed-out loops of intestine from which all but minute traces of food, and hence of the liver preparation, had been removed.

It has often been suggested that vitamin D has a direct effect on the calcification of bone. That this is unlikely was shown by Migicovsky & Emslie (1950) when they found similar amounts of ^{45}Ca deposited in bone after intramuscular injection of

$^{45}CaCl_2$ into both rachitic and normal chicks. Our results substantiate these findings, since by measuring the decrease in plasma ^{45}Ca with time after an intravenous injection of $^{45}CaCl_2$ the rate of removal of Ca, presumably into the skeleton, could be followed. Rachitic chicks and rachitic chicks that had been given the rachitogenic liver extract both removed ^{45}Ca from the plasma more rapidly than did normal birds. It was shown that this was not due to more rapid excretion of ^{45}Ca and that the bones of the rachitic birds contained more ^{45}Ca than those of normal birds. It therefore appears that calcification of rachitic bone does not involve the direct effect of vitamin D_3, although the reservation must be made that the rachitic chicks were probably not completely deficient of vitamin D_3, and the minute amount of vitamin remaining might have had some function in calcification besides its primary effect on absorption. The rachitogenic factor also did not interfere with the ability of the bone to calcify, and it therefore seems that the rachitogenic effect of the pig's liver was due mainly to its effect on the mechanism of transport of Ca through the intestinal wall. Further investigation of this phenomenon awaits purification of the active material.

SUMMARY

1. The absorption of calcium was measured in tied-off duodenal loops in normal chicks and in chicks given a dietary supplement of pig's liver.

2. The pig's liver reduced the amount of Ca absorbed in both normal and rachitic chicks.

3. Deficiency of vitamin D_3 in chicks did not interfere with the deposition in the skeleton of intravenously injected ^{45}Ca.

4. In chicks given pig's liver the ability to deposit ^{45}Ca in the bone was similarly unimpaired. It is concluded that the rachitogenic effect of the pig's liver depended mainly on its interference with the uptake of Ca from the gut.

We are pleased to record our thanks to our colleague Dr R. F. Glascock for advice and assistance with the counting methods.

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Printed in Great Britain