The Effects of Additions of Calcium Carbonate to the Diet of Breeding Mice

1. Effects on Reproduction and on the Heart and Thymus Weights of the Weanlings

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Within recent years several experiments on laboratory animals at this Institute have indicated that additions of calcium carbonate to the diet may have deleterious effects. Thus the effects of pyridoxin deficiency induced in suckling rats by excess of aneurin were accentuated when calcium carbonate was also added to the diet (Richards, 1945); when the amount of added $CaCO_3$ was increased, there was also a retardation in the rate of growth of young rats after weaning (Richards, 1949*a*). Simpson (1947) found that additions of $CaCO_3$ to the diet of adult rats increased iodine excretion and diminished the iodine content and concentration in the thyroid. More recently Howie & Porter (1950) found that a slight modification of the B diet of Sherman gave better reproductive results in mice than another modification in which one of the changes was a 1% addition of $CaCO_3$.

These observations led to the present experiments, of which the initial object was to test the effects on reproduction in mice of including different levels of $CaCO_3$ in the original slightly modified Sherman B diet and at the same time to assess the relative importance of the various changes made in this diet by Howie & Porter (1950). The results of the first experiment indicated that any differences resulting from the other modifications were of minor importance compared with those due to the additions of $CaCO_3$. A second experiment was therefore designed to investigate means by which the deleterious effects of these additions could be counteracted. As the work developed, observations were made indicating that it would be of interest to record the weights of various organs of the young mice and their mothers. These aspects are best considered separately, and the present paper is accordingly presented in two parts: the first deals with reproductive effects and the second with effects on organs.

1. EFFECTS ON REPRODUCTION

Two experiments are reported. Materials and methods were similar for both and are described together, but designs and results are given separately for each experiment.

MATERIALS AND METHODS

Both experiments were done with white 'Swiss' mice of the Institute's stock colony. To supply the breeding females for each experiment sufficient litters containing four females were bred at the same time. They were bred on stock diet (diet 61), which consisted of Rowett Institute stock cubes (diet 1 of Howie & Porter, 1950) with a daily supplement of fresh whole milk given *ad lib.* to the mated pairs of adult mice. Four female mice were allotted to each group in Exp. 1 and eight in Exp. 2 with randomization of litter-mates. Each breeding female was reared on diet 61 to the age of 7 weeks and was then given the appropriate experimental diet a week before being mated with a male of the same age. The males were reared in the same way as the females; mating of litter-mate brothers and sisters was avoided. The method of continuous monogamous mating was used, the effect of the diets on reproduction being judged by the number and weight of the young reared in a given period, as described by Bruce & Emmens (1948). In Exp. 1 the males were removed from the breeding cages after 80 days, and the females were maintained on the diets long enough to rear any litters that had been conceived. In Exp. 2 sufficient time was allowed for the rearing of only two litters. The observations were ended by killing the litters on weaning at 21 days of age. Post-mortem examinations were made as detailed in Part 2 of this paper.

EXPERIMENT 1

The object of Exp. 1 was to test on reproduction in mice the effect of adding three levels of $CaCO_3$ to the slightly modified Sherman B diet used by Howie & Porter (1950)—their diet 2— and to three further modifications of it. Diet 2 consisted of: ground whole wheat 66, dried whole milk 33 and NaCl 1 %. Diet 5—the modified diet that gave poorer reproductive results than diet 2 in their experiments—contained: ground whole wheat 60, casein (lactic, unextracted, Glaxo Laboratories Ltd.) 5, dried whole milk 33, $CaCO_3$ 1 and NaCl 1 %. The most likely causes of the poorer results with diet 5 seemed to be either the addition of $CaCO_3$ or a deficiency of the vitamin B complex. Two factors might have contributed to such a deficiency, namely the reduction in the proportion of wheat and the increased requirement for vitamin B resulting from the addition of protein as casein. These possibilities were therefore considered in planning the experiment.

Design

A factorial design of sixteen squares was drawn up, as shown in Table 1, each square representing an experimental diet. Each of the four rows was built upon a different basal diet. In the first row the basal diet was diet 2. In the second row the basal diet (diet 70) was formed from diet 2 by substituting 6 g maize starch for 6 g wheat, thus reducing the vitamin B content without increasing the protein. In the third row the basal diet (diet 74) was derived from diet 2 by substituting 6 g casein for 6 g wheat, thus reducing the vitamin B content but this time increasing the protein. Rows 1, 2 and 3 were completed by the addition of $CaCO_3$ at the rate of 0·5, 1·0 and 2·0 g/100 g of the respective basal diets. In the fourth row 5 g casein were added to 100 g of diet 2 to form diet 78, and the same additions of $CaCO_3$ were made to 105 g of this basal diet. The number in each square of the plan in Table 1 indicates the number of the diet and is used also to designate the group of experimental animals fed on the diet.

Table 1. *Exp.* 1. *Plan of dietary groups to show factorial design*

(The numbers within the table refer to diets. They are also used in the text to designate the corresponding groups of experimental animals)

Row no.	Composition of basal diet for each row, i.e. the diets for column 1: Constituent	Parts	Amount of added $CaCO_3$ (g/100 g basal diet in rows 1–3; g/105 g in row 4): 0 (column 1)	0·5 (column 2)	1·0 (column 3)	2·0 (column 4)
1	Ground whole wheat	66	2	67	68	69
	Dried whole milk	33				
	NaCl	1				
	Total	100				
2	Ground whole wheat	60	70	71	72	73
	Maize starch	6				
	Dried whole milk	33				
	NaCl	1				
	Total	100				
3	Ground whole wheat	60	74	75	76	77
	Casein	6				
	Dried whole milk	33				
	NaCl	1				
	Total	100				
4	Ground whole wheat	66	78	79	80	81
	Casein	5				
	Dried whole milk	33				
	NaCl	1				
	Total	105				

Results

First litters. A summary of the reproductive results for the first litters born to each breeding female is given in Table 2. In the upper part of the table figures are given for the separate groups according to the plan shown in Table 1, and in the lower part the results are summarized in columns according to the $CaCO_3$ additions.

The results for the separate groups show that the effect of the $CaCO_3$ additions was marked. Taking the diets in order of the increasing amounts of added $CaCO_3$ (columns 1–4), the numbers of young weaned were, in row 1: 20, 31, 22 and 16; in row 2: 20, 33, 10 and 10; and in row 3: 14, 27, 29 and 8. (See below for row 4, which is treated separately.) Naturally the total weights weaned depended to a large extent on the numbers weaned, so that for these three rows the total weight weaned was highest in column 2, with a marked falling off in columns 3 and 4. Considering the weight per litter weaned, thus eliminating the effect of litters dying before weaning, the same gradation is apparent in the results, the highest weight being found in column 2, with a marked falling off in columns 3 and 4. In row 4, column 2 shows a lower number weaned and total weight weaned than does column 1, owing to the loss of a complete litter, but the weight per litter weaned is as high as in column 1, and there is the same marked falling off in columns 3 and 4 as in the first three rows. Differences between the rows did not reach conventional levels of statistical significance, and the results may therefore be summarized in columns according to the $CaCO_3$ additions. This is done in the lower part of Table 2. Analysis of these results showed that at the highest level of Ca (column 4) there was a significant decrease in the total weight weaned ($P \ll 0.001$) and in the total number weaned ($P \ll 0.001$), and a significant increase in both the number and proportion of deaths ($P < 0.01$). Although the differences in the numbers born at the different $CaCO_3$ levels were not statistically significant, it may be noted that there was some decrease at the two higher levels (columns 3 and 4 of Table 2, lower part). In all the measurements the best results were obtained in column 2, i.e. with a $CaCO_3$ addition of 0·5 g/100 g (or 105 g for row 4) of basal diet.

Table 2. *Exp. 1. Reproductive results for first litters. Each dietary group comprised four females continuously mated*

(See Table 1 for factorial design of experiment)

Statistic	Row no.	Additions (g/100 g in rows 1–3; g/105 g in row 4) of $CaCO_3$ to basal ration: 0	0·5	1·0	2·0
No. weaned/no. born	1	20/29	31/34	22/31	16/26
	2	20/30	33/33	10/29	10/18
	3	14/33	27/29	29/29	8/31
	4	28/33	24/33	12/26	5/30
No. of litters weaned/no. of litters born	1	4/4	4/4	4/4	4/4
	2	4/4	4/4	2/4	2/3*
	3	3/4	4/4	4/4	2/4
	4	4/4	3/4	2/4	2/4
Total weight weaned (g)	1	122·3	187·3	150·7	91·3
	2	157·7	237·6	84·7	56·8
	3	116·0	215·5	161·9	58·3
	4	211·0	159·2	75·8	26·2
Weight/litter weaned (g)	1	30·6	46·8	37·7	22·8
	2	39·4	59·4	42·4	28·4
	3	38·7	53·9	40·5	29·2
	4	52·8	53·1	37·9	13·1
Totals for rows 1–4:					
Born (no.)	—	125	129	115	105
Weaned (no.)	—	82	115	73	39
Deaths (no.)	—	43	14	42	66
Proportion of deaths (%)	—	34·4	10·9	36·5	62·8
Litters born (no.)	—	16	16	16	15*
Failures to rear litters (no.)	—	1	1	4	5
Total wt. weaned (g)	—	607·0	799·6	473·1	232·6
Wt./litter weaned (g)	—	40·5	53·3	39·4	23·3

* One mated female of experimental group no. 73 failed to conceive because the male was infertile.

Second and subsequent litters. Results are not given separately for the second and subsequent litters, which showed greater variability. In general, the tendency in the later litters on all diets was towards improvement, as has been found in previous work at this Institute with borderline deficiency diets (Richards, 1949*b*), but a few animals that suffered from vaginal prolapse failed to produce any litters after the first. An investigation of these cases indicated that the prolapses should be attributed to genetic rather than dietary causes. Adjustments were therefore made by assuming that the measurements for these animals were equal to the means for their dietary groups.

The complete reproductive results for all litters produced, up to four litters in a few animals, are summarized in Table 3, which gives the figures obtained in the experiments, and in Table 4, which gives the treatment means adjusted for litter-mate effects and for vaginal prolapses. There was a significant decrease in the total weight of animals weaned on diets containing the highest level of $CaCO_3$ ($P=0·001$) and in the total number of animals weaned ($P<0·01$). The average number of deaths on diets with this level of $CaCO_3$ was significantly higher than on diets with lower levels of $CaCO_3$ ($P=0·01$), and the highest proportion of deaths also occurred with the highest

Table 3. *Exp.* 1. *Summary of the reproductive results for the whole period of the experiment (continuous mating for* 80 *days), arranged in columns according to the* $CaCO_3$ *additions, sixteen females being tested for each level of* $CaCO_3$

Statistic	Additions (g/100 g in rows 1–3; g/105 g in row 4) of $CaCO_3$ to basal ration			
	0	0·5	1·0	2·0
Total young born (no.)	313	307	290	274
Total young weaned (no.)	247	274	217	154
No. of deaths:				
Total	66	33	73	120
In 1st week of life	23	14	48	53
In 2nd week of life	11	2	4	2
In 3rd week of life	32	17	21	65
Total proportion of deaths (%)	21·1	10·7	25·2	43·8
Litters born (no.)	42	37	42	42
Litters reared (no.)	38	36	35	33
Failures to rear litters (no.)	4	1	7	9
Total weight weaned (g)	2065·0	2082·8	1739·1	1144·8
Weight weaned/litter (g)	54·3	57·9	49·7	34·7

Table 4. *Exp.* 1. *Treatment means for reproductive data after adjustment for litter differences and vaginal prolapses*

Mean per mated female	Additions (g/100 g in rows 1–3; g/105 g in row 4) of $CaCO_3$ to basal ration			
	0	0·5	1·0	2·0
Total young born (no.)	19·6	19·2	18·2	17·1
Total young weaned (no.)	16·4	17·8	14·6	9·6
Total deaths (no.)	4·1	2·3	3·8	7·5
Total proportion of deaths (%)	0·21	0·12	0·22	0·48
Litters born (no.)	2·6	2·3	2·6	2·6
Total weight weaned (g)	138·2	135·7	116·8	71·6

$CaCO_3$ level ($P<0·001$). The chief danger periods in the rearing of the young mice were the 1st and 3rd weeks of life, relatively few deaths occurring in the 2nd week. In both the danger periods the number of deaths in column 4 was about four times that in column 2. There was no significant effect on the total number of litters or total number of animals born.

EXPERIMENT 2

The significant effects on reproduction resulting from the $CaCO_3$ supplements used in Exp. 1 directed attention to the Ca content of the diets and the possible influence of the calcium:phosphorus ratio. From direct chemical analysis of the individual constituents the Ca and P contents of the diets and their Ca:P ratios were calculated.

The Ca contents of the diets in the four columns of Table 1, taken in order of increasing amounts of added $CaCO_3$, averaged 0·34, 0·54, 0·73 and 1·11 %, and the Ca:P ratios 0·70, 1·1, 1·5 and 2·3. The best results for most measurements were found with the diets quoted in column 2 of Table 1, i.e. with the diets whose Ca content averaged 0·54 % and whose Ca:P ratio was about 1·1, whereas in column 4, which showed the values worst in all respects, the corresponding figures were roughly twice these. Exp. 2 was therefore planned so as to test the effect of reducing the higher Ca:P ratios with a phosphorus supplement. Further, post-mortem examination of the weanlings of Exp. 1 (see Part 2 of this paper) had suggested the presence of anaemia, and this was confirmed by haemoglobin determinations (Greig, 1952). The possibility of an iron deficiency was therefore also tested in Exp. 2.

Design

In Exp. 2 the basal diet chosen was diet 78 of Exp. 1 (Table 1). The breeding mice were divided into eight groups, each containing eight animals. The groups were arranged in four pairs, one group of each pair having per 105 g basal diet a 0·5 g

Table 5. *Exp. 2. Plan showing the calcium and phosphorus contents and the* Ca:P *ratios of the diets*

		$CaCO_3$ (g) added to 105 g basal diet							
		0·5 (column 1)				2·0 (column 2)			
Row no.	Diet	Group no.	Ca (%)	P (%)	Ca:P	Group no.	Ca (%)	P (%)	Ca:P
1	Basal diet no. 78 Ground whole wheat 66 Casein 5 Dried whole milk 33 NaCl 1 Total 105	79	0·55	0·48	1·15	81	1·10	0·48	2·29
2	Diet no. 78 + Fe (10 p.p.m.)	89	0·55	0·48	1·15	90	1·10	0·48	2·29
3	Diet no. 78 + 2·87 g sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	91	0·54	1·00	0·54	92	1·07	0·98	1·09
4	Diet no. 78 + Fe (10 p.p.m.) + 2·87 g sodium dihydrogen phosphate	93	0·54	1·00	0·54	94	1·07	0·98	1·09

$CaCO_3$ supplement and the other 2·0 g (Table 5). These two levels were chosen because in Exp. 1 they had produced respectively the best and worst results. The first pair, with no addition to the basal diet except $CaCO_3$, thus received the same treatment as groups 79 and 81 of Exp. 1. For the second pair, i.e. groups 89 and 90, the iron content of the diet was increased from 35 p.p.m. (determined spectrographically) to 45 p.p.m. by adding 5·624 mg ferric citrate ($C_3H_4OH(COO)_3Fe.3H_2O$), equivalent to 1·050 mg Fe, to 105 g basal diet. For the third pair, i.e. groups 91 and 92, 2·87 g sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) were added per 105 g basal diet, to make the Ca:P ratio of the high-Ca group (group 92) approximately equal to that of group 79. For the fourth pair, i.e. groups 93 and 94, ferric citrate and sodium dihydrogen phosphate were added together in the same amounts as for the two preceding pairs of groups. The plan of the experiment, showing the Ca and P contents of the diets and their Ca:P ratios, is given in Table 5.

Table 6. *Exp. 2. Total weight weaned and weight/litter weaned for first and second litters*

(See Table 5 for explanation of dietary groups. The nature of the dietary supplements other than Ca is indicated in parentheses below the number of the group)

Lower Ca groups				Higher Ca groups			
No. of group	No. of litter	Total weight weaned (g)	Weight/litter weaned (g)	No. of group	No. of litter	Total weight weaned (g)	Weight/litter weaned (g)
79	1	480·4	60·1	81	1	302·0	50·3
(nil)	2	390·3	65·1	(nil)	2	365·9	52·3
89	1	466·9	66·7	90	1	321·6	64·3
(Fe)	2	510·5	72·9	(Fe)	2	578·8	72·4
91	1	464·7	58·1	92	1	365·9	61·0
(P)	2	458·5	65·5	(P)	2	422·6	60·4
93	1	459·2	57·4	94	1	459·8	57·5
(Fe+P)	2	610·0	76·3	(Fe+P)	2	564·9	80·7

Each group contained eight females continuously mated.

Results

Number born. The addition of Fe, P or the higher level of Ca had no significant effect on the number of young born, either in the first or second litters. Combining the figures for both litters, the average numbers born per litter in the four lower Ca groups, taken in the order of column 1 in Table 5, were 8·8, 8·5, 7·1 and 9·1; and in the corresponding higher Ca groups 7·6, 9·2, 7·7 and 8·1. The total numbers born were 518 for thirty-two mice in the lower Ca groups and 491 in the higher Ca groups with one animal fewer. One mated female in group 81 failed to produce a litter within the time limit of the experiment and was therefore discarded. It was later proved that the male was infertile.

Number and weight weaned. The number weaned and total weight weaned were considerably affected by a number of failures to rear first litters. There were six of these failures among the thirty-one females in the higher Ca groups and only one among the thirty-two females in the lower Ca groups, but the mice that failed with their first litters all succeeded in rearing some of the young in their next litters, so that the total number of deaths did not eventually differ greatly in the two sets of groups, averaging 1·6 per litter born in the lower Ca groups and 1·8 in the higher Ca groups. This failure to rear first litters on the higher Ca diets, followed by an improved performance with their second litters, was also observed in another experiment (unpublished) done at the same time. In the unpublished experiment, seven of eighteen mice on diets containing the higher level of $CaCO_3$ failed to rear their first litters, but five of the seven succeeded in rearing part of their second litters. The incidence of failures to rear first litters on the higher level of Ca is not significant in the conventional statistical sense, but nevertheless appears to be of enough interest to warrant repetition of the experiment with greater numbers. This ability to rear some young in their second litters, although they had failed to do so in first litters, is commonly observed with small laboratory animals under unfavourable dietary conditions. It may be due to the mother's power of adaptation to those dietary conditions, or possibly to her own lessened requirement for some dietary essential.

Because of the number of complete-litter failures it seems best to consider the weights weaned both as total weight weaned and weight per litter weaned. These have been collected in Table 6, in which the results for first and second litters are given separately. Considering the results for the first litters only, the influence of the complete failures is reflected in the total weights weaned. For each of the first three pairs of groups the total weight weaned in the higher Ca group (nos. 81, 90 or 92) was much below that in the corresponding lower Ca group (nos. 79, 89 or 91). For the last pair (nos. 93 *v.* 94), in which there were no complete failures, the weight weaned was the same in both groups. The diet had thus been improved by the addition of both Fe and phosphate. Considering the mean weaning weights of the first litters that were reared (Table 6), the depressing effect of the higher $CaCO_3$ level is still apparent in the first pair of groups (nos. 79 *v.* 81), but this effect has been eliminated by the addition of Fe (groups nos. 89, 90) and also by the addition of phosphate (groups nos. 91, 92) and of Fe and phosphate together (groups nos. 93, 94). For the second litters the weaning weights per litter show an increase in all groups except one, no. 92, where there is almost no change, the improvement being particularly marked in groups nos. 93 and 94, which received supplements of both Fe and phosphate.

2. EFFECTS ON ORGAN WEIGHTS

Marked differences in the weights and general condition of the young mice in the different dietary groups of Exp. 1 led to post-mortem examination of some of the weanlings when they were killed at 21 days of age. This revealed the frequent occurrence of pale speckled livers, enlarged hearts and small thymus glands, and it soon became evident that these conditions were more pronounced on the diets with the higher additions of $CaCO_3$. As an example of these findings, Pl. 1, 1 shows the marked differences in the colour of the livers between a litter of weanlings from group no. 78 (no added $CaCO_3$) and one from group no. 80 (added $CaCO_3 = 1{\cdot}0$ g/105 g of diet 78). Differences in heart and thymus weights of these two litters were also marked, as can be seen from figures given in the description of Pl. 1, 1. For comparison there are included the mean heart and thymus weights for fifteen normal litters on various stock diets outwith the present experiments.

The remaining litters of Exp. 1, which included some of the second and all subsequent litters, were therefore examined more systematically, weights being recorded for thymus, heart, liver, spleen and kidneys of representative mice from each litter.

EXPERIMENT 1

Heart and thymus weights. As the investigation of organ weights was not begun from the start of the experiment, the results are incomplete and are therefore not recorded in detail, but sufficient evidence is available to show that there was a significant effect of $CaCO_3$ in increasing the heart weights of the weanlings. The heart weights of the mothers were not significantly affected. Although the effect of $CaCO_3$ on the thymus weights of the weanlings was not found to be statistically significant when all the results were analysed, the figures for heart and thymus weights of the groups in row 4 (Table 1), in which most litters were available, show the tendency for thymus weights to decrease as heart weights increase with increasing amounts of added $CaCO_3$. The figures are:

Organ weights (mg/100g body-weight) of animals in row 4 of Table 1

	Column 1	Column 2	Column 3	Column 4
Heart	906	959	1418	2133
Thymus	333	329	231	158

Other organs. Analysis of the weights (mg/100g body-weight) of liver, spleen and kidneys of the young mice and their mothers did not reveal any significant effects of calcium or other dietary factors on these organs.

EXPERIMENT 2

Methods

In Exp. 2, post-mortem examinations were made of weanlings from all litters. As a rule four mice (if possible two males and two females) were examined from each litter. Heart weights were recorded for both first and second litters and also for the mothers, and the hearts, together with the median lobes of the livers, were stored in 4 % (w/v) formol-saline (10 parts commercial formalin in 90 parts normal saline) for histological examination (see Greig, 1952). In view of the possibility that the heart enlargement found in these experiments might be due, in part at least, to oedema of the heart muscle, estimations were made of the dry-matter content of the hearts from second litters not required for histology. Since these had been stored in isotonic formol-saline, any effect of the fixative on the weight of dry matter would be negligible, particularly for comparative purposes.

Results

Heart weights of weanlings

Absolute heart weights. In general, the weights of organs have little significance as absolute values and must be related to body-weights, but in this experiment the absolute increases in heart weights for weanlings of the same age in the higher Ca groups were very striking. The mean heart weights and corresponding body-weights for the first litters of all groups are given in Table 7. The table shows the number of litters and also the total number of weanlings included in each group average. When the pairs of groups have almost the same mean body-weight, the effect of the higher level of Ca in increasing heart weight is clearly observed, e.g. groups nos. 79 *v.* 81, 89 *v.* 90, and 93 *v.* 94, the increase being especially marked in the first and third of these pairs of groups. In groups nos. 91 *v.* 92 the effect is even more striking, since the higher Ca group, in spite of having a lower body-weight, yet has a considerably higher heart weight. Diminution of heart weight by Fe is seen by comparing groups nos. 81 with 90, 92 with 94, and 91 with 93. Both Ca and Fe effects were significant ($P < 0.001$).

Pl. 1, 2 illustrates this result. The mouse on the left, which may be regarded as a normal weanling, represents a litter from group 89, receiving added Fe; the heart weights and body-weights of the litter averaged 60 mg and 10 g respectively. The animal on the right came from group no. 92, the high Ca plus phosphate group; the heart weights of this litter averaged 131 mg and the body-weights 6·6 g. We have occasionally had a weanling with a heart heavier than that of its mother, although her body-weight was from five to six times as great as that of her offspring.

Table 7. *Exp. 2. Mean heart weights and corresponding mean body-weights of weanlings from first litters.*

(The figures in the table are group averages calculated from the litter means. The nature of the dietary supplements other than Ca is indicated in parentheses after the number of the group: quantities of the supplements as shown in Table 5)

Lower Ca groups				Higher Ca groups			
No. of group	Mean heart weight (mg)	Mean body-weight (g)	No. of litters included in mean*	No. of group	Mean heart weight (mg)	Mean body-weight (g)	No. of litters included in mean*
79 (nil)	57·9	7·78	8 (34)	81 (nil)	87·4	7·82	6 (23)
89 (Fe)	68·4	9·18	7 (28)	90 (Fe)	75·3	9·17	5 (20)
91 (P)	72·5	9·36	8 (34)	92 (P)	85·9	8·79	6 (26)
93 (Fe+P)	51·8	8·08	8 (28)	94 (Fe+P)	69·9	8·15	8 (30)

* Figure in parentheses gives the number of young.

Table 8. *Exp. 2. Mean relative heart weights of weanlings (first and second litters) and mothers; and mean relative thymus weights of weanlings (second litters)*

(The nature of the dietary supplements other than Ca is indicated in parentheses after the number of the group: quantities of the supplements as shown in Table 5)

No. of group	Mean heart weight (mg/100 g body-weight)*: First litters	Second litters	Mothers	Mean thymus weight (mg/100 g body-weight) second litters*
		Lower Ca groups		
79 (nil)	754 (8)	790 (6)	492 (8)	358 (6)
89 (Fe)	745 (7)	636 (7)	487 (8)	423 (7)
91 (P)	784 (8)	690 (7)	450 (8)	399 (7)
93 (Fe+P)	645 (8)	621 (8)	462 (8)	439 (8)
		Higher Ca groups		
81 (nil)	1154 (6)	1022 (6)	524 (7)	283 (6)
90 (Fe)	843 (5)	783 (5)	484 (8)	323 (8)
92 (P)	993 (6)	954 (5)	531 (8)	275 (7)
94 (Fe+P)	871 (8)	753 (7)	530 (8)	401 (7)

* Figure in parentheses gives the number of litters or mothers included in the mean.

Relative heart weights. The effects of the dietary supplements on the ratio of heart weight to body-weight are shown in Table 8. By comparing the values for each pair of diets (groups nos. 79 *v.* 81, 89 *v.* 90, 91 *v.* 92, and 93 *v.* 94) it is seen that the higher level of Ca increased the relative heart weight. This effect is significant ($P < 0.001$). The effect of Fe in decreasing relative heart weight, both at the lower and the higher

Ca level, is also significant ($P < 0.001$), and is evident from a comparison of the relevant groups (nos. 79 *v.* 89, 91 *v.* 93, 81 *v.* 90, and 92 *v.* 94). The effect of the phosphate addition on relative heart weight was not statistically significant. Groups nos. 92 and 93, with added phosphate, showed both in first and second litters slight reductions in relative heart weight compared with groups nos. 81 and 89, the corresponding

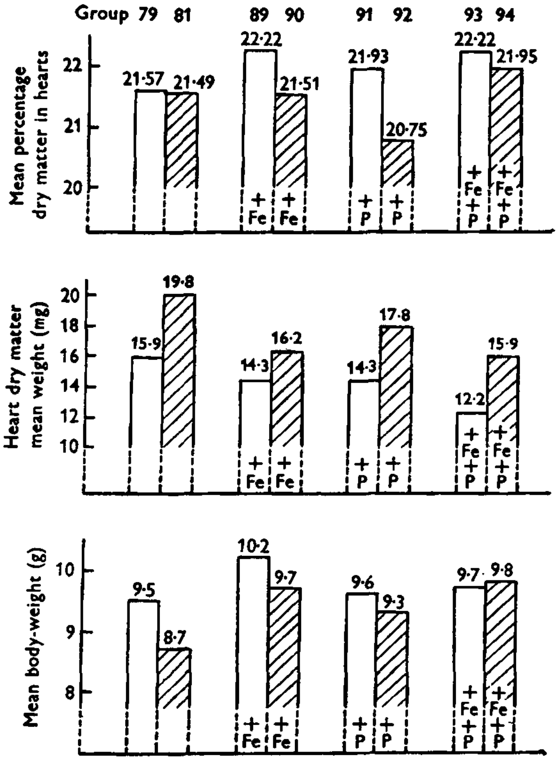


Fig. 1. Exp. 2. Diagram showing mean percentage dry matter in hearts, mean absolute weight of heart dry matter and mean body-weight of second litters. The weight of heart dry matter is greater in the higher Ca group of each pair (shaded columns), in spite of decreased percentage dry matter in heart and diminished body-weight in most animals. For dietary groups see Table 5.

groups without phosphate; but when groups nos. 91 and 94, with added phosphate, are compared with the corresponding groups nos. 79 and 90, the reduction is seen only in the second litters. It may be noted that in all groups except group no. 79 the relative heart weights of second litters were less than those of first litters, that is, they showed an improvement.

Heart dry matter. The percentage of dry matter in the heart was significantly lowered by the higher level of Ca ($P < 0.001$) and significantly increased by Fe ($P < 0.001$), whereas phosphate had no significant effect (Fig. 1). Examination of the results showed, however, that the increased moisture content of the hearts in the higher Ca groups was by no means sufficient to account for the increased weights observed. The weights of dry matter present in the hearts, along with the percentage dry-matter contents and the average body-weights of the different groups, are shown diagrammatically in Fig. 1. The increased absolute weight of dry matter in the hearts of the higher Ca groups is clear.

Relation between heart weights and blood haemoglobin. We have found that a highly significant correlation exists between the heart enlargement in the young mice and the degree of anaemia, as shown by the blood-haemoglobin estimations described by Greig (1952). The closeness of this relationship can be seen from the scatter diagram

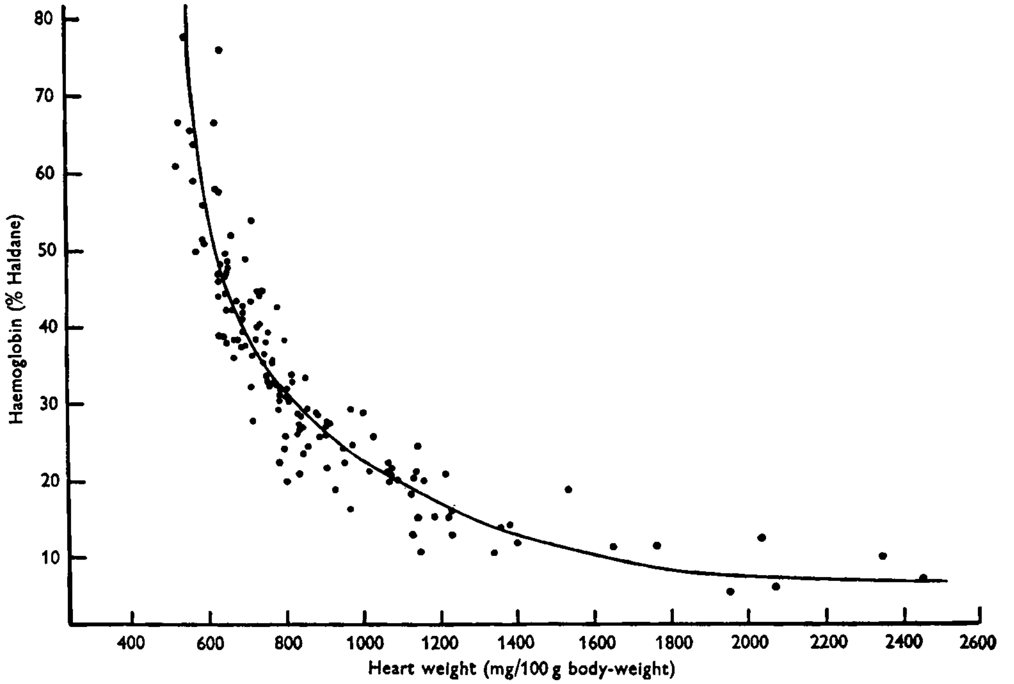


Fig. 2. Exp. 2. Scatter diagram, showing the relationship between blood haemoglobin and heart weight relative to body-weight, for 141 litters irrespective of diet. Each point represents the mean value for one litter.

in Fig. 2, in which Hb is plotted against heart weight expressed in mg/100 g body-weight (H.W. %) for all litters in Exp. 2, irrespective of their group, and for thirty-three litters from the unpublished experiment mentioned in part 1 of this paper. Statistical analysis showed that the relationship between log H.W. % and log Hb was inverse and linear. For the 141 litters observed the correlation coefficient r was -0.936 ± 0.010, the regression equations for the prediction of one variable from the other being:

$$\log \text{H.W. } \% = 3.780 - 0.580 \log \text{Hb} \quad \pm 0.018,$$

and

$$\log \text{Hb} = 5.955 - 1.533 \log \text{H.W. } \% \pm 0.049,$$

where Hb is expressed as a percentage of the Haldane standard (100 % = 14.8 g haemoglobin/100 ml. blood), and H.W. % = mg heart/100 g body-weight.

Heart weights of mothers

The heart weights of the mothers were also affected by the Ca level of the diet. The higher level of Ca increased absolute heart weight ($P < 0.05$), increased heart weight relative to body-weight ($P<0.001$) and decreased the percentage of heart dry matter ($P<0.05$). The mean relative heart weights are shown in Table 8. Comparison of the groups shows that the addition of Fe or phosphate did not cause a statistically significant reduction in the heart weights of the mothers. It is possible that the effects would have been more pronounced had the animals been reared on the experimental diets from weaning.

Thymus weights

Thymus weights of second litters were significantly affected both by the Ca and Fe additions, but in the opposite direction from the heart weights. Thus the absolute weight of the thymus and its weight relative to body-weight were both diminished by the higher level of Ca ($P<0.001$) and increased by the addition of Fe ($P<0.001$). Again the addition of P had no significant effect. The mean weights of the thymus relative to body-weight are shown in Table 8. The figures show clearly both the diminution in weight in the higher Ca groups and the increase in the groups receiving Fe.

DISCUSSION

In these mouse experiments the adverse effects of $CaCO_3$ on reproduction were seen mainly in a reduction of the numbers weaned, caused by complete or partial failure to rear litters, and in a reduction of the weaning weights of the surviving litters. The number of deaths in the 1st week of life was high. Doubtless some of these deaths, in all groups, must be attributed to causes other than diet, since deaths in the first few days of life are not unusual with small laboratory animals even on normal diets; it is also possible that with the system of continuous monogamous mating the presence of the male may have been an additional disturbing factor. The figures given for Exp. 1 indicate, however, that diet must have been the major cause of these early deaths, since the mortality on the two higher levels of $CaCO_3$ was much greater than on the two lower levels. In the 3rd week the mortality was greatest on the highest level of $CaCO_3$, and it seems justifiable to attribute almost all the deaths during this period to dietary causes. The mother's milk may have been deficient in quality or quantity, or possibly some defect in the young animal may have prevented its proper utilization. This could account both for the large number of deaths during this period and for the decreased weaning weights of the survivors. The most likely explanation of these findings would appear to be an induced Fe deficiency in the mothers, which may have led to a reduced Fe content of the milk, always very low, or to a reduced iron reserve in the young animal at birth.

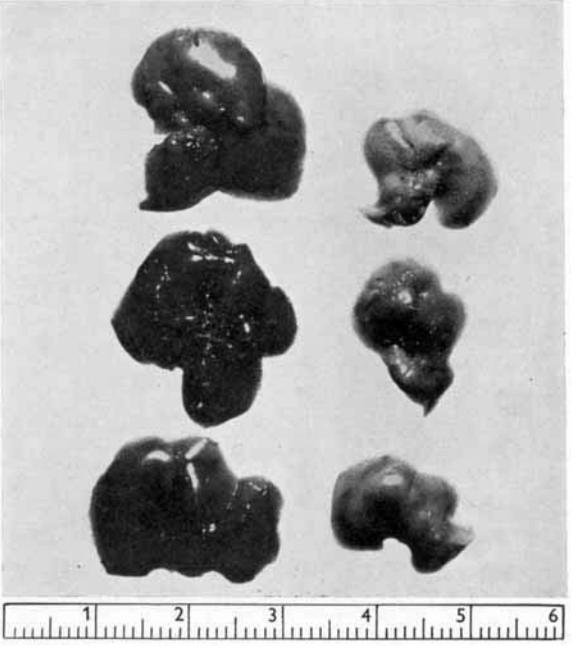
Although the adverse effects of the $CaCO_3$ supplements in Exp. 1 were more apparent on the rearing of the young than on the fertility or fecundity of the mothers, there was, nevertheless, a tendency towards decrease in the numbers born at the two higher levels. The number of animals in each group of that experiment was small, and it seems possible that with larger numbers the decrease in the birth rate might have reached significance. In this connexion it may be noted that in the experiments of Howie & Porter (1950), with twenty-two breeding females in each group, the animals on their diet 2 were not only markedly superior in the number and weight of young weaned to those on their diet 5, but also showed a marked increase in the number of young born. One of the main differences between these two diets was that diet 5 contained 1 % of added $CaCO_3$. The differences in our experiments could perhaps also have been increased by giving the experimental diets to the breeding females at weaning instead of at 7 weeks of age.

As to the mode of action of $CaCO_3$ in impairing reproduction, our results suggest that a dietary Ca:Fe relationship was involved. Excess of Ca seemed to reduce in some way the amount of available Fe, and improvement was effected by increasing the proportion of Fe to Ca, as was shown by the improved weaning weights of the Fe groups, particularly in the second litters. This point is more fully discussed in the second paper of this series (Greig, 1952). To what extent the Ca:P ratio was involved is less clear. Reduction of this ratio by the addition of phosphate did effect some improvement in the average weaning weights of the litters, but to a smaller extent than did the addition of Fe. The effect of P on the organ weights of the weanlings was also much less marked than that of Fe. Thus our results indicate that the Ca:Fe relationship was more important than the Ca:P ratio.

The reproductive findings in these experiments are of interest in view of a recent paper by Hignett (1950), who considers that calcium-phosphorus imbalance in the diet is one cause of herd infertility in cattle. He contends that the $CaO:P_2O_5$ ratio should not be much wider than 1:1, with a sufficient intake of P, and mentions instances of herds in which breeding efficiency was markedly improved by a narrowing of the Ca:P ratio. Shaw (1950) also reported infertility in cows in a district with a hard-water supply, where heavy liming was practised, and stated that a general improvement in the conception rate corresponded to decreased liming.

Our experiments also showed that hypertrophy of the heart and atrophy of the thymus gland were produced in weanling mice by additions of $CaCO_3$ to their mothers' diet. Similar changes were reported by Schmidt (1928) in young mice on a low-iron diet of milk and rice and suffering from a direct iron-deficiency anaemia. He found that the abnormalities could be completely prevented by administering iron in the diet. Since in our experiments the heart and thymus changes could also be prevented by supplementing the mothers' diet with Fe, there seems little doubt that they were associated with an iron deficiency, produced indirectly by the $CaCO_3$ additions to the diet. Thus the heart hypertrophy and thymus atrophy would seem to result from the same essential cause as the impairment in reproduction, namely an imbalance in the dietary Ca:Fe relationship.

The finding that the principal cause of the increase in heart size was an increase in its dry-matter content indicates that a compensatory hypertrophy of the heart muscle resulted from the anaemia. The atrophy of the thymus may have been a consequence of the general under-development frequently observed in the young mice. It is conceivable, however, that the cause of the atrophy may have been purely physical, and that 'accidental involution' resulted from compression of the gland by the enlarged heart. On the other hand, it is known that pyridoxin deficiency causes anæmia in dogs and swine, that it leads to thymus atrophy in young rats and that the effects of



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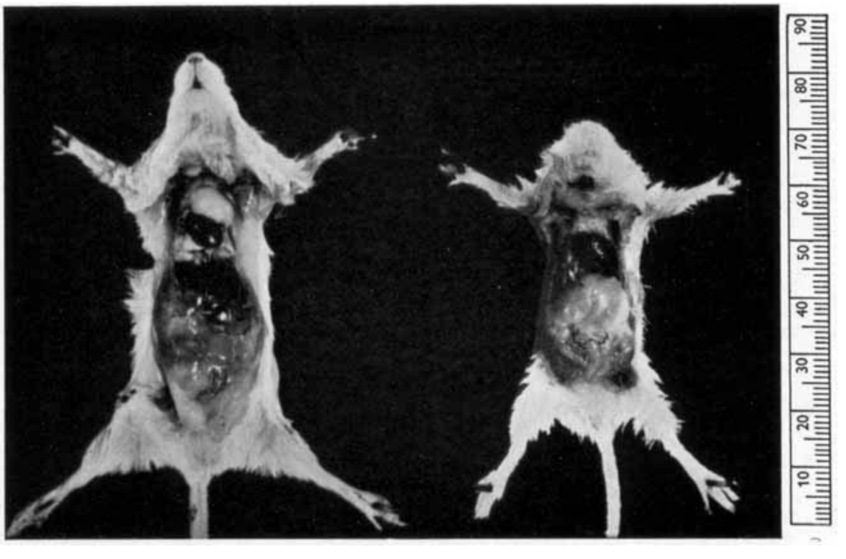

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the deficiency are intensified by additions of $CaCO_3$ to the diet. There may therefore be some more fundamental relationship between the thymus atrophy and the anaemia induced by the $CaCO_3$ additions to the diet.

SUMMARY

1. In a breeding test on mice, using the method of continuous monogamous mating, three levels of calcium carbonate were added to a slightly modified B diet of Sherman and to three further modifications of it. On all the diets the highest addition of calcium carbonate, which gave a Ca intake of 1·1 % and a Ca:P ratio of approximately 2·3, significantly lowered the number and total weight of young weaned and increased the number and proportion of deaths. There was also some decrease in the number of births.

2. When supplements of ferric citrate and sodium dihydrogen phosphate were added to the diet, a number of mothers in the high Ca groups failed to rear their first litters, but in the litters that were reared the weaning weights seemed to be favourably affected by the addition of Fe or by a reduction of the Ca:P ratio.

3. Both absolutely and relatively to body-weight the heart weights of the weanlings were significantly increased by Ca and decreased by Fe, whereas thymus weights were decreased by Ca and increased by Fe. The high Ca level also caused a significant increase in the absolute and relative heart weights of the mothers.

4. Although the percentage of dry matter in the hearts of the weanlings was significantly lowered by the higher level of Ca, there was a marked increase in the absolute weight of heart dry matter at this level, indicating the presence of a cardiac hypertrophy.

5. A highly significant relationship was found between heart weight and blood haemoglobin in the weanling mice, an increase in heart weight accompanying a decrease in Hb. The relationship between log heart weight, expressed in mg/100 g body-weight, and log Hb was linear.

We are indebted to Mr H. M. Quenouille, Statistics Department, University of Aberdeen, for statistical analysis of our data, to Dr R. L. Mitchell, Macaulay Institute for Soil Research, Aberdeen, for spectrographic determinations of iron in the foodstuffs, and to Mr J. Davidson, of this Institute, for their chemical analysis. We wish also to thank Miss Isabel Knowles for assistance in the care and feeding of the animals.

EXPLANATION OF PLATE

1. Exp. 1. Livers of weanling mice showing the effect on liver colour of adding $CaCO_3$ to the mother's diet. (Scale in cm.) Left, livers from group no. 78 (no added $CaCO_3$); right, livers from group no. 80 (1·0 g $CaCO_3$ added to 105 g of diet 78). Mean heart and thymus weights (mg/100 g body-weight) for these two litters and for fifteen normal litters on various stock diets were:

	Heart	Thymus
Litter from group no. 78	952	310
Litter from group no. 80	1987	179
Normal litters	645	471

2. Exp. 2. The weanling mouse on the left from group no. 89, receiving added Fe, shows normal heart, thymus and liver. That on the right from group no. 92 shows the enlarged heart, small thymus and pale liver characteristic of weanlings from mothers receiving excess of $CaCO_3$ in the diet. (Scale in cm.)

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The Effects of Additions of Calcium Carbonate to the Diet of Breeding Mice

2. Haematology and Histopathology

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Richards & Greig (1952) reported pale livers and enlarged flabby hearts in weanling mice whose mothers' diets contained 1–2 % of added calcium carbonate. It seemed likely that the calcium carbonate had induced an unexpected anaemia in the young.

The work reported in the present paper was undertaken to investigate this possibility and to examine the pathology of the condition. The presence of anaemia was confirmed by haemoglobin estimations, and fuller examination of the blood indicated that it was probably of the iron-deficiency type. A further experiment showed that the anaemia could be prevented by feeding an iron supplement, but not by balancing the additional dietary calcium with sufficient phosphorus to restore the original Ca:P ratio of the diet.

METHODS

The two reproduction experiments reported by Richards & Greig (1952) provided the material for this work. The designs of both experiments have already been described in detail by these authors, and need only brief mention here.

Experiment 1 (Richards & Greig, 1952).

Design. Sixteen litters of four female mice, stratified by weight, were allocated to four Latin squares with litter-mates distributed orthogonally. The other component variables of the squares were the calcium carbonate level of the diet (in vertical columns), and the levels of wheat and of casein in the diet factor (in horizontal rows).