Differences in Calcification between the Humerus and Femur of Young Rats receiving High Calcium, Low Calcium and Stock Diets

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The calcification of bones in both normal and pathological conditions, especially in rickets, is frequently described in terms of ash content in preference to histological structure. The methods used in the determination of bone ash vary little from one laboratory to another as regards the procedures for the extraction of the fat and the ashing of the bones, but the choice of the particular bone or bones to be examined is a matter of individual preference. Chick & Roscoe [1926] ashed femur, tibia and fibula together in the determination of the ratio mineral ash/organic residue as a rachitogenic index. Kon & Henry [1935], in carrying out prophylactic estimations of vitamin D, determined the percentage ash from one femur and one humerus together for each rat. Other workers have chosen one bone only as sufficiently representative, e.g. the tibia [Schultzer, 1931] or the femur [Bethke, Kick & Wilder, 1923]. In any of these procedures the final value for each rat is obtained from a single determination or from duplicate determinations made on right and left bones. Bethke *et al.* [1923] ashed separately the humeri and femora of each rat and obtained an average value from separate analyses of bones from different parts of the body.

In this laboratory it has been customary to follow Bethke's technique, and after many determinations it became apparent that these two bones from rats receiving a rickets-producing diet showed a difference in the degree of calcification, and that this difference increased with an increase in the severity of the rickets. Coward, Kassner & Waller [1938] have published a series of bone ash values from rats receiving a diet deficient in calcium. These values were all much lower than normal even with ample supplements of vitamin D. Their results show that in all groups the percentage ash was at a level usually associated with rickets but that there was little difference between humerus and femur. To study this point further a detailed analysis of all bone ash values which had been obtained in this laboratory was undertaken.

COLLECTION OF DATA

Data have been obtained over a period of several years, and include results already published. The percentage ash in the dry fat-free humerus was

chosen arbitrarily as the basal value, and the results have been arranged in order of magnitude with respect to this datum, irrespective of sex of the rat, and of the dose of vitamin D given. The difference (d) between the percentage ash in the humerus and femur was related to the percentage ash in the humerus (H) by means of the expression $(d \times 100)/H$.

EXPERIMENTAL

All the animals were albino rats from the inbred colony of Wistar strain, maintained on a stock diet [Coward, 1938]. The young rats were weaned at 23 days, at an average weight of 40–45 g.; the test period was 5 or 6 weeks, except in one early experiment of only 4½ weeks; four rats out of the total of 38 on stock diet were fully grown. At the end of the test period, both humeri and both femora were dissected out and the ash content determined on the dry fat-free bones, the right pair being taken from each rat. In two early experiments both humeri and both femora were ashed together, but this was not the usual procedure.

RESULTS

A. The influence of diet

Evidence was available from three different types of diet:

(a) Rachitogenic diet of the high Ca and low P type, Steenbock 2965 [Steenbock & Black, 1925]: 194 rats.

(b) Low Ca diet, the 'poorer class' diet described by Coward *et al.* [1938]: 109 rats.

(c) Stock diet as used in the rat colony: 38 rats.

The results, which for convenience have been grouped in ranges of four according to the percentage ash of the humerus, are shown in Table 1 and represented graphically in Fig. 1. The rats receiving stock diet, being so few in number, have been included in a single group in the table. In severe rickets the difference in calcification between humerus and femur is clearly shown. This difference decreases as the state of calcification improves. On the other hand, there is no difference between the two bones of rats receiving either stock diet or low Ca diet.

All the evidence available related to differences between humerus and femur from rats which had received the rachitogenic diet + vitamin D, so an experiment was carried out to determine the effect of the unsupplemented diet. It has been shown [Bourdillon, Bruce, Fischmann & Webster, 1931;

greatly in excess of the prophylactic requirement for young rats of our colony; group 3 received the rachitogenic diet only.

As comparatively few figures are available in the literature for individual responses in bone calcification, full protocols as well as the average values for this test are presented in Table 2. The regularity of response in these three groups, where maximum effects are being measured, is the outstanding feature of this table. The range of variation in the percentage ash of the humerus in all three groups is lower than that usually found among rats receiving the rachitogenic diet with a supplement of vitamin D insufficient to give complete calcification. Particularly is this the case for group 3, for under these conditions of diet rickets develops with regularity.

Fig. 1. The relation between the ash content of the humerus and the difference in calcification of humerus and femur of rats receiving three types of diet. ●● Rachitogenic diet. x—x Low calcium diet. [o] Stock diet. Small figures give the number of animals.

Bacharach, 1940; Coward & Kassner, 1941] that the variation between litters is greater than the variation within the litter for many types of biological response. In the figures so far quoted no account has been taken of litter-mates nor indeed of any variable other than the percentage ash in the dry fat-free humerus. Therefore in this experiment comparisons were made of litter-mates subjected to the different conditions.

In the rachitic bones from group 3 in which the calcification is very poor (28.11% ash in the humerus), the difference between humerus and femur is striking and is clearly significant. On a complete diet there is a difference in calcification between the humerus and femur, $t_4 = 2.10$, $P =$ very slightly > 0.05 for group 1. The calcification in group 2 is less than that in group 1, and the difference between the humerus and femur, though small, is significant, $t_4 = 3.55$, $P =$ much < 0.01 . The inability of vitamin D alone to correct the faulty calcification brought about by a rachitogenic diet of the high Ca and low P type has often been recognized and is generally attributed to the limiting effect of the P [Hume, Pickersgill & Gaffikin, 1932].

It should also be noted that when the ash is calculated on the basis of mg./100 g. body weight

Table 1. The absolute and percentage differences in ash content of humerus and femur of rats receiving three types of diet and the standard deviations of those differences

Range of % ash in the humerus	Mean difference 'd' between the % ash in the humerus and femur			Standard deviation of 'd'			Mean difference 'd' expressed as % (d x 100)/H			Standard deviation of (d x 100)/H		
	Rachitogenic diet	Low Ca diet	Stock diet	Rachitogenic diet	Low Ca diet	Stock diet	Rachitogenic diet	Low Ca diet	Stock diet	Rachitogenic diet	Low Ca diet	Stock diet
20-23	(3.04)*	—	—	—	—	—	(13.5)*	—	—	—	—	—
24-27	6.22	—	—	1.38	—	—	23.0	—	—	5.08	—	—
28-31	6.29	—	—	1.77	—	—	20.9	—	—	5.85	—	—
32-35	5.77	—	—	2.09	—	—	17.0	—	—	6.17	—	—
36-39	5.06	-0.09	—	2.39	1.69	—	13.4	-0.3	—	6.32	4.41	—
40-43	3.49	-0.24	—	2.40	2.06	—	8.4	-0.5	—	5.81	4.93	—
44-47	2.25	+0.15	—	2.88	1.26	—	4.9	0.3	—	6.35	2.70	—
48-51	1.09	+0.58	—	1.33	1.42	—	2.2	1.2	—	2.69	2.81	—
52-55	(0.63)†	+1.47	—	—	1.14	—	(1.2)†	2.7	—	—	2.06	—
54-63	—	—	0.11	—	—	0.79	—	—	0.2	—	—	1.36

* Two rats only. † One rat only.
The number of animals in each group varies from 11 to 61 and is given in Fig. 1.

39 rats were divided into three litter-mate groups of six bucks and seven does. Group 1 received stock diet; group 2 received the rachitogenic diet with 80 i.u. vitamin D₂ per week, this being a dose

the calcification in the group receiving the rachitogenic diet alone is very much less (54.4 mg. ash/100 g.) than that of the rats receiving either stock diet (130.8 mg. ash/100 g.) or the rachitogenic

Table 2. *Full protocols of individual responses as regards bone ash and body weight of rats receiving stock diet or rachitogenic diet with and without added vitamin D₂*

Group	Diet	Litter	Sex	% ash in humerus <i>H</i>	Difference in % ash between humerus and femur <i>d</i>	Difference expressed as % (<i>d</i> × 100)/ <i>H</i>	Body weight		Increase in wt. g.	Total ash from both bones expressed as mg./100 g. body weight
							At start g.	At end g.		
1	Stock	9700	♀	56.40	-0.24	-0.4	42	111	69	141.4
		9702	♂	57.28	0.70	1.2	45	173	128	120.3
			♀	55.91	-0.72	-1.3	46	124	78	145.1
			♂	57.14	-0.23	-0.4	46	138	92	139.6
		9715	♀	56.52	-0.06	-0.1	46	155	109	119.5
			♂	59.06	1.23	2.1	44	144	100	141.0
			♀	54.50	0.74	1.4	48	153	105	121.4
		9720	♂	57.85	1.18	2.0	42	131	89	129.1
			♀	57.50	1.49	2.6	46	158	112	125.5
			♂	58.33	0.04	0.1	48	175	127	122.7
		9728	♀	58.65	0.78	1.3	46	127	81	134.3
		9744	♂	57.41	2.58	4.5	48	180	132	122.7
			♀	57.14	-0.45	-0.8	41	128	87	138.2
			♂							
Average				57.21	0.54	0.94	45.2	145.9	100.7	130.8
S.D.				1.20	0.93	1.63	—	—	—	9.36
2	Rachitogenic plus excess vitamin D ₂	9700	♀	51.33	1.80	3.5	46	77	31	118.7
		9702	♂	49.78	0.66	1.3	49	83	34	122.8
			♀	51.45	0.87	1.7	44	84	40	124.9
			♂	50.63	0.40	0.8	50	98	48	119.1
		9715	♀	50.13	0.86	1.7	50	107	57	105.6
			♂	49.75	1.67	3.4	43	71	28	113.0
			♀	49.83	4.55	9.1	45	70	25	109.6
		9720	♂	49.34	2.65	5.4	47	66	19	127.9
			♀	49.06	0.55	1.1	47	96	49	105.0
			♂	52.73	0.63	1.2	45	83	38	121.2
		9728	♀	51.92	1.19	2.3	44	59	15	144.7
		9744	♂	48.64	1.12	2.3	49	70	21	126.9
			♀	48.40	-0.69	-1.4	41	64	23	122.7
			♂							
Average				50.23	1.25	2.49	46.2	79.1	32.9	120.2
S.D.				1.31	1.27	2.55	—	—	—	10.62
3	Rachitogenic	9700	♀	27.37	8.91	32.6	43	54	11	48.3
		9702	♂	28.50	6.91	24.2	50	68	18	52.2
			♀	27.57	4.42	16.0	43	56	13	56.4
			♂	27.16	6.83	25.1	45	53	8	60.6
		9715	♀	28.15	6.86	24.4	45	53	8	57.0
			♂	28.75	5.58	19.4	44	52	8	57.9
			♀	27.92	6.95	24.9	48	63	15	54.9
		9720	♂	29.08	5.74	19.7	46	56	10	55.5
			♀	29.25	7.31	25.0	48	63	15	51.6
			♂	27.64	5.17	18.7	46	61	15	53.1
		9728	♀	29.43	7.21	24.5	46	59	13	57.1
		9744	♂	28.21	8.92	31.6	49	65	16	51.1
			♀	26.39	6.99	26.5	41	51	10	51.8
			♂							
Average				28.11	6.75	24.05	45.7	58.0	12.3	54.4
S.D.				0.89	1.30	4.77	—	—	—	3.41

diet + vitamin D (120.2 mg. ash/100 g.). The increase in body-weight is considerably less for rats on the rachitogenic diet than for rats on stock diet, even with excess of vitamin D. On the rachitogenic diet alone, this increase was very small from the start, and during the last 2 weeks a slight decrease took place.

B. *Distribution of calcification in the bone*

In the rachitic bone the metaphyses are greatly affected while the changes in the shaft are smaller and proceed at a slower rate during the development of the lesions [Hess, Berliner & Weinstock, 1931]. This suggested that the relatively small total ash content of the femur of a rachitic rat might be due entirely to the relatively large metaphyses. The weights and ash contents of the ends and shaft of the bones were therefore determined separately.

Left bones representative of each of the three diets (stock diet, low Ca diet, rachitogenic diet) were taken. The division of each bone into three, a shaft and two ends, was chosen arbitrarily but the cuts were always made as nearly as possible in the same places—in the femur just below the lesser trochanter and just above the condyles, in the humerus at the surgical neck and just below the supratrochlear fossa. For each bone the shaft was weighed alone and the two ends together. The results are shown in Table 3. Calcification is distri-

whole femur was thus not entirely due to the fact that its metaphyses were relatively large, though it was partly due to this factor since the percentage difference was larger for the whole bone (6.68) than for either the shaft (4.60) or ends (4.27).

The fall in the ash content of the bones of the rachitic rats was most marked in the femur and particularly in the ends of the femur. This is perhaps due to the strain placed upon this bone because the weight of the rat is carried mostly on its hind legs.

In view of the many sources of possible error both in cutting dry fat-free bones and in ashing very small pieces of bone it should be emphasized that the figures in Table 3 represent only a general state of affairs. When the percentage ash in the whole left bone was calculated from that of the two parts and compared with the percentage ash previously determined for the corresponding right bone, the agreement in nearly all cases was good, showing that the figures in the table are representative of the bone both as regards relative proportion of parts and as regards their calcification.

These results suggest that, as is the practice in both the X-ray and the line test techniques, one part of one bone only might, with advantage, be used in the assay of vitamin D by the bone ash method. The difference in ash content between a severely rachitic bone and one showing the maximum calcification brought about by vitamin D alone, is 19.89 % for the humerus alone, 26.06 %

Table 3. *Ash content of ends as compared with whole bone and comparison between these portions in femur and humerus*

Group	No. of animals	Diet	Weight of ends Total weight × 100		% ash in whole bone (calculated)		Difference in % ash in humerus and femur		
			Humerus	Femur	Humerus	Femur	Shaft	Ends	Whole bone
1	8	Rachitogenic	51	61	29.40	22.72	4.60	4.27	6.68
2	6	Rachitogenic + vitamin D	47	49	49.82	48.23	2.66	-0.27	1.59
3	10	Low Ca	46	45	46.70	45.61	2.87	-0.88	1.09
4	7	Stock	42	41	57.85	56.19	2.75	0.36	1.66

buted in approximately similar proportions between shaft and ends in both humerus and femur for groups 2, 3 and 4; that is to say for rachitogenic diet + vitamin D₂, low Ca diet and stock diet, the humerus and femur show about the same degree of calcification, although the level varies with the diet, and so do the ends of both humerus and femur. In contrast to this, these bones from rats which have received the unsupplemented rachitogenic diet differ from one another both as regards the distribution of calcification between shaft and ends and also as regards absolute values for ash contents. The figures for rachitic rats show a difference between the two bones even when the shafts and ends are considered separately. The relatively low ash content of the

for the femur alone, 22.98 % for the average of the humerus and femur and 28.31 % for the ends of the femur alone. These figures suggest that by using for the assay only the ends of the femur, which show the large response to vitamin D, an increase in the range of calcification is secured and hence a steeper curve of response and one which might be more sensitive for low doses. But further work is required, since the data here reported are insufficient to establish the point.

C. *Influence of sex*

In the young female rat ossification proceeds more rapidly than in the male [Cox & Imboden, 1936]. Donaldson [1924] gives figures showing that

Table 4. *The influence of sex on bone ash content*

Diet	Range of % ash in the humerus	No. of animals		Average value of <i>d</i>		Average value of (<i>d</i> × 100)/ <i>H</i>	
		♂	♀	♂	♀	♂	♀
Rachitogenic	24-27	18	9	6.29	6.07	23.4	22.4
	28-31	35	26	6.49	6.02	21.7	19.9
	32-35	20	15	5.89	5.60	17.4	16.4
	36-39	12	8	4.38	6.07	11.7	16.0
	40-43	10	12	3.16	3.77	7.6	9.1
	44-47	3	8	4.58	1.38	9.7	3.0
	48-51	7	8	1.09	1.09	2.2	2.2
Low Ca	36-39	10	5	0.12	-0.50	0.3	-1.4
	40-43	8	13	-0.43	-0.12	-1.1	-0.2
	44-47	20	12	0.38	-0.24	0.8	-0.5
	48-51	17	8	0.82	0.08	1.6	0.2
	52-55	6	10	1.34	1.55	2.5	2.9
Stock	54-62	20	18	0.17	0.05	0.3	0.1

at both 100 and 150 days of age the humerus and femur of female rats have a higher percentage of ash than those of male rats. The figures given in Table 1 have been analysed in Table 4 according to the sex of the animal. The differences in ash content between the rachitic humerus and femur is shown equally by both sexes.

SUMMARY

1. When low calcification in rats, as measured by the ash content of the humerus, is due to shortage of vitamin D, the femur shows an even lower calcification. When, however, it is due to a deficiency of either Ca or P without a simultaneous shortage of vitamin D in the diet, no difference in calcification

between the two bones is observed, neither is a difference found in the bones of rats receiving a stock diet.

2. In the femur both the relatively large metaphysis and the extremely low calcification of this part make the ash content of the whole bone lower than that of the humerus when there is a deficiency of vitamin D in the diet.

3. The differences between the rachitic humerus and femur are not influenced by the sex of the animal.

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The Preparation of Deoxycholic Acid from Cholic Acid

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Deoxycholic acid (3:12-dihydroxycholanolic acid) has been made from cholic acid in small yield by hydrogenation of the derived 3:12-dihydroxycholonic acid [Boedecker & Volk, 1922], but this method is of

academic interest only. The present work, as already stated [Haslewood, 1942], aims at the production of deoxycholic acid by a convenient method from the readily available cholic acid, to supplement the