COMPOSITION OF BONE.

IV. PRIMARY CALCIFICATION.*

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The chemistry of the calcium phosphates together with its bearing on calcification was discussed in the preceding communication (1). The evidence indicated that the composition of bone when first deposited might be different from the composition of old bone. In inorganic aqueous solutions the primary precipitate of $CaHPO_4$ with a Ca : P ratio of 1.29 undergoes transformation, in many cases, to a solid phase in which Ca : P approximates 1.94. Similarly, although in bone the ratio Ca : P also approximates 1.94, it seemed possible that this ratio might be different in freshly deposited bone.

This secondary reaction, which precipitates of calcium phosphate undergo, is completed in about 8 days in inorganic solutions as Holt, La Mer, and Chown (2) have shown. If a similar reaction occurs *in vivo* its detection would require that analysis be made on bone that is not more than a few days old. We have utilized for such analysis the fresh calcification deposited in ricketic rats whose rickets had just begun to heal. The analytical procedures employed were those described in the first paper of this series (3).

Normal Rat Bone.

Sixteen specimens of bone from normal rats were analyzed with the micro technique. Calcium, phosphate, and CO_2 were determined in duplicate on each specimen. The analytical results are given in Table I. It is seen from this table that the ratio residual

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TABLE I.

Normal rat.

Age.	Bones.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100$.	$\frac{\text{Residual Ca}}{\text{P}}$.	Deviation.
days		*per cent*	*per cent*	*per cent*			
1	All.	13.7	6.4	1.23			
		13.2	6.5	1.32	9	1.89	−0.10
5	Leg.	15.8	7.2	1.62			
		15.9	7.5	1.61	9	1.95	−0.04
5	Rib.	11.5	5.4	1.24			
		12.1	5.4	1.31	10	1.96	−0.03
5	Head.	14.6	6.7	1.62			
		14.5	6.7	1.54	10	1.97	−0.02
13	Leg.	12.4	5.7	1.12			
		12.4	5.7	1.16	8	2.00	+0.01
15	"	11.4	5.6	1.36			
		12.3	5.7	1.22	10	1.88	−0.11
15	Rib.	11.6	5.3	1.14			
		11.6	5.2	1.03	9	2.00	+0.01
20	Leg.	13.4	6.4	1.30			
		13.2	6.2	1.28	9	1.92	−0.07
20	Rib.	11.5	5.1	1.06			
		11.7	5.1	1.09	8	2.08	+0.09
38	Leg.	13.9	5.8	1.61			
		13.9	5.9	1.54	10	2.12	+0.13
38	Rib.	13.2	6.1	1.18			
		13.0	5.9	1.20	8	2.00	+0.01
46	Leg.	18.8	7.8	2.57			
		18.6	7.8	2.49	12	2.10	+0.11
63	Shafts.	21.8	9.3	3.20			
		21.7	9.2	3.19	13	2.03	+0.04
5 mos.	Leg.	21.0	8.9	3.62			
		21.7	8.8	3.99	16	2.01	+0.02
Adult 1.	"	24.1	10.3	4.05			
		23.9	10.2	4.16	16	1.97	−0.02
" 2.	"	23.5	10.1	3.79			
		23.4	10.2	3.86	15	1.96	−0.03
						Mean = 1.99	*a.d.* = ±0.05 *A.D.* = ±0.01

Residual Ca: P = 1.99 ± 0.01.

Ca : P obtained with the micro technique has a mean value of 1.99 for normal rat bone. The average deviation from the mean, *a.d.*, is ±0.05, and *A.D.* = ±0.01. The mean value of 1.99 ±0.01 obtained with the micro technique is in agreement with similar results obtained by others using macro methods.

The values in the column entitled $\frac{\text{"carbonate Ca}}{\text{total Ca}} \times 100$" were obtained by means of the expression

$$\frac{\text{Per cent } CO_2 \times 91}{\text{Per cent Ca}} = \frac{\text{carbonate Ca}}{\text{total Ca}} \times 100$$

The per cent of CO_2 multiplied by 0.91 gives the per cent of calcium assumed to be present as bound to carbonate in $CaCO_3$; *i.e.*, this gives the per cent of carbonate calcium. Dividing per cent of carbonate calcium by per cent of total calcium and multiplying by 100 gives the values in this column.

It is seen from this column that the bones of very young rats contain less $CaCO_3$ in proportion to calcium phosphate than do bones of older rats. In young rats carbonate Ca constitutes only 8 to 10 per cent of the total Ca, while in adult rats it constitutes about 15 per cent. This means that in the bones of rats the proportion of $CaCO_3$ to the other calcium salts increases markedly with age. Transition values were obtained in rats 6 and 9 weeks old.

This difference in the carbonate content of the bones of young and old rats is not due to experimental error. If the CO_2 values in young rats were too low, or if the values of total Ca were too high, then these errors would be reflected in the ratio residual Ca : P. All the bones, from rats varying in age from 1 day to maturity, gave the same value within the experimental deviation for the ratio residual Ca : P; the change in carbonate content with increasing age is, therefore, a real one.

The analyses and ratios given in Table I are selected values. Some of these bones were analyzed in duplicate and some in triplicate; in one case five analyses were made of the same bone. All of these analyses were performed as described previously—the calcium, phosphorus, and CO_2 in each case were determined at least in duplicate. The ratios obtained in all of these analyses

TABLE II.

Normal Rat.

Summary of all ratios.

Age.	Bones.	Ratio.	Deviation.
days			
1 and 2	All.	1.89	−0.12
5	Leg.	1.95	−0.06
5	Rib.	1.96	−0.05
5	Head.	1.97	−0.04
		1.97	−0.04
		1.90	−0.11
13	Leg.	2.10	+0.09
		2.00	−0.01
15	"	1.88	−0.13
15	Rib.	2.12	+0.11
		2.00	−0.01
		2.02	+0.01
20	Leg.	1.94	−0.07
		2.05	+0.04
		1.92	−0.09
20	Rib.	2.08	+0.07
38	Leg.	1.93	−0.08
		2.12	+0.11
		2.12	+0.11
38	Rib.	2.21	+0.20
		2.02	+0.01
		2.00	−0.01
46	Leg.	2.14	+0.13
		2.10	+0.09
63	Shafts.	2.03	+0.02
5 mos.	Leg.	2.01	0.00
Adult 1.	"	1.98	−0.03
		1.99	−0.02
		2.00	−0.01
		1.97	−0.04
		2.04	+0.03
" 2.	"	2.04	+0.03
		2.03	+0.02
		1.96	−0.05
		1.98	−0.03
		Mean = 2.01	*a.d.* = ±0.06 *A.D.* = ±0.01

Residual Ca: P = 2.01 ± 0.01.

are given in Table II. These ratios have all been given here and have all been included in calculating the mean and the *A.D.*, for the sake of comparison with the ratios given in Table VI. The value 2.21 for example is obviously erroneous (two subsequent analyses of the same bone gave ratios of 2.02 and 2.00); it has nevertheless been included. The mean obtained from *all* the analyses of normal rat bone is 2.01 ±0.01.

TABLE III.

Primary Calcification (Rat).

Healing induced with cod liver oil concentrate.

Bone.	No. of samples analyzed.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100.$	$\frac{\text{Residual Ca}}{\text{P}}$.
		per cent	*per cent*	*per cent*		
Fresh calcification (lines).	4	7.8	3.1			
		7.5	3.0			
		7.9	2.9			
		7.6	2.9			
		7.7	2.8	0.89		
			2.9	0.93	11	2.38
			2.9	0.91		
Epiphyses and diaphyses.	3	10.1				
		9.9	4.2	1.46		
		10.2	4.1	1.43	13	2.10
		10.1	4.2	1.45		
Old bone (shafts).	1	15.6	6.4	2.78		
		15.5	6.3	2.61	16	2.05
		15.6	6.4	2.70		

Primary Calcification.

Rickets was produced in rats by feeding Steenbock's Ration 2965.[1] Severe rickets was obtained in 4 weeks; sections from the upper end of the tibia showed a wide layer of uncalcified cartilage and a wide metaphysis. Twenty-five of these rats were fed cod liver oil concentrate for 8 days. Autopsy showed that the rickets had begun to heal; there was a line of fresh calcification in the provisional zone of cartilage.

[1] For the composition of Ration 2965 see Steenbock, H., and Black, A., *J. Biol. Chem.*, 1925, lxiv, 263.

All the rats were killed, and the upper end of each tibia was removed and sectioned. Each section contained the center of ossification, the epiphyseal cartilage, the line of fresh calcification, the metaphysis, and a little of the shaft. Each section was cut in three places at right angles to the axis of the shaft; the different portions of the sections were combined in three groups and labelled "shafts," "epiphyses and diaphyses," and "lines." Each of these three portions of the upper end of the tibia was then analyzed by means of the micro technique. The results are given in Table III.

In each case calcium, phosphate, and CO_2 were determined at least in duplicate. In the case of the new bone from the lines there were four analyses for calcium and six analyses for phosphate. Separate samples were digested for the additional analyses; they do not represent merely aliquots of the same solution. It is seen that the shafts gave a ratio of 2.05; this is within the normal range. The epiphyses and diaphyses gave a ratio of 2.10; this is somewhat high, probably because there was some fresh calcification present in addition to the older bone. The fresh calcification of the lines gave a decidedly high ratio of 2.38.

A similar study was carried out on another set of ricketic rats except that irradiated yeast was employed as the antiricketic agent. In these rats the healing of the rickets was not confined to the test line, as there was also considerable fresh calcification in the metaphysis itself. The sections of the tibia were therefore divided into three groups somewhat different from those in the preceding set of rats; these groups were "lines," "metaphyses," and "shafts." The results are given in Table IV.

Table IV shows that the old bone from the shafts gave a ratio of 2.05; this is within the normal range. The metaphyses gave a high ratio of 2.25 and the lines gave a moderately high ratio of 2.16. In analyzing a normal bone it sometimes happens that two errors occur in which a high calcium value and a low phosphorus value are obtained simultaneously. This would result in a high Ca : P ratio. To rule out this possibility ten analyses were made for calcium and twelve for phosphorus. A number of samples of the material were digested and analyzed separately; the value 2.16 is calculated from the mean values so obtained.

A third study was made in which the healing of the rickets was induced by butter. To the ricketic diet was added 1 per cent but-

ter; this mixture was fed for 4 weeks. At the end of this period autopsy showed that there was fresh calcification in the metaphysis but not in the provisional zone. The metaphyses were removed as described above, and analyzed. The shafts were also analyzed. The results are given in Table V.

TABLE IV.

Primary Calcification (Rat).

Healing induced with irradiated yeast.

Bone.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100.$	$\frac{\text{Residual Ca}}{\text{P}}$.
	per cent	*per cent*	*per cent*		
Fresh calcification (lines).	12.8	4.9			
	12.3	4.9			
	12.3	5.4			
	12.2	5.2			
	12.2	5.3			
	12.6	5.2			
	12.4	5.2			
	12.3	5.2			
	12.4	5.1			
	12.6	5.1			
	12.4	5.1	1.67		
		5.1	1.51	12	2.16
		5.1	1.59		
Fresh calcification (metaphyses).	11.7				
	12.2	4.6			
	11.9	4.6			
	12.2	4.8	1.64		
	12.1	4.9	1.48	12	2.25
	12.0	4.7	1.56		
Old bone (shafts).	20.4	8.5			
	20.7	8.5			
	20.7	8.5	3.43		
	20.8	8.7	3.41	15	2.05
	20.7	8.6	3.42		

The old bone in the shafts gave a normal ratio of 1.94; the new bone in the metaphyses gave a high ratio of 2.14. To exclude the possibility of this high ratio being due to experimental error, repeated analyses were made for calcium and phosphorus. The ratio in the last column was calculated from the mean values.

In another group of ricketic rats healing was induced by irradiated cholesterol. The deposition of fresh calcification occurred in the provisional zone of cartilage; the lines were removed and analyzed. The results are given in Table VI. It is seen that this gave a high Ca : P ratio of 2.24. For comparison the leg shafts of a number of normal rats of the same age, *i.e.* 63 days old, were removed and analyzed. A normal ratio of 2.03 was obtained.

TABLE V.

Primary Calcification (Rat).

Healing induced with butter.

Bone.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100.$	$\frac{\text{Residual Ca}}{\text{P}}$.
	per cent	*per cent*	*per cent*		
Fresh calcification (metaphyses).	10.0	4.4			
	10.1	4.2			
	10.4	4.2			
	10.1	4.2			
	10.2	4.2			
	10.2	4.6			
	10.2	4.5			
		4.4			
		4.4			
		4.3	1.10		
		4.3	1.11	10	2.14
		4.3	1.11		
Old bone (shafts).	20.3	8.9			
	19.8	8.5			
	20.1	9.0	3.32		
		8.7	3.26	15	1.94
		8.8	3.29		

DISCUSSION.

In interpreting the results of analyses of bones it is customary to assume that all the CO_2 is present as $CaCO_3$ and that all the Mg is present as $Mg_3(PO_4)_2$. The carbonate calcium is deducted from the total calcium, and the phosphate equivalent to the magnesium is deducted from the total phosphate. It has been repeatedly found that the residual calcium and residual phosphate so obtained give a ratio of Ca : P which approximates that calcu-

lated from the formula $Ca_3(PO_4)_2$. When analyzed with our micro technique, normal rat bone gives a ratio for residual Ca : P which approximates 1.94.[2] The Ca : P ratios which we have obtained for normal rat bone by means of the micro technique are therefore in agreement with the results of previous investigators.

Proportion of $CaCO_3$.—Our analyses show that the proportion of $CaCO_3$ to calcium phosphate in bone increases with the age of

TABLE VI.

Primary Calcification (Rat).

Healing induced with irradiated cholesterol.

Bone.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100$.	$\frac{\text{Residual Ca}}{\text{P}}$.
	per cent	*per cent*	*per cent*		
Fresh calcification (lines).	9.9	3.8			
	9.9	3.8			
	9.3	3.6			
	9.0	3.6			
	9.0	3.6			
	9.2	3.9			
	9.1	3.8			
	9.2	3.8			
	9.2	3.8			
	9.3	3.7			
		3.6	1.12		
		3.8	1.16	11	2.24
		3.7	1.14		
Old bone (shafts of normal rats 63 days old).		9.3			
	21.8	9.3	3.20		
	21.7	9.2	3.19	13	2.03
	21.8	9.3	3.20		

the animal. There are no recent analyses of unashed bones with which we may compare these results.

This question has apparently not been studied within the last 35 years; we have found no recent analyses, either of ashed or of unashed bone, which have bearing on this point. In 1855 Frémy (4) concluded that in humans there is, with increasing age, a relative increase of $CaCO_3$ with respect to

[2] The effect of the omission of the magnesium analysis is discussed in the first paper (3).

calcium phosphate. Von Recklinghausen (5) in 1858 concluded from his own analyses and from the work of Bibra (1844), of Stark (1845), of Heintz (1849), and of Frémy, that there was no observable difference in composition between the bones of young and those of old individuals. In 1860 Milne Edwards (6) reported that his own analyses agreed with those of Bibra and of Frémy in showing that the proportion of carbonate in the bones of young animals was smaller than in adult bone. Zalesky (7) in 1866 reviewed the literature and concluded that the bones of children of various ages have the same composition as those of adults, not only as a whole, but also as regards the proportion of individual constituents. He pointed out that the methods of determining CO_2 employed by previous investigators were defective. In 1872 Wildt (8) analyzed[3] the ashed bones of rabbits of different ages and found an increase in the proportion of CO_2 with increasing age.

Weiske (10) in 1889 analyzed the ashed bones of birds and found an increase in CaO and CO_2 with advancing age, while the P_2O_5 remained constant. Graffenberger (11) in 1891 analyzed the ashed bones of rabbits and also found an increase of the ratio calcium carbonate: calcium phosphate with increasing age.

The only analyses of unashed bone performed during the last 30 years with which we are acquainted are the few analyses of Gassmann (12) and those of Howland, Marriott, and Kramer (13). The results of the latter investigators show that the ratio of calcium phosphate to calcium carbonate is not necessarily constant; this proportion is different in ricketic and normal bones, and is different in normal human bone as compared with normal rat bone. The effect of age on this ratio was not studied.

Our findings are in agreement with those of Howland, Marriott, and Kramer as regards the increase in the proportion of $CaCO_3$ in rickets. From our tables it is seen that for normal rats approximately 2 months old, carbonate Ca:total Ca is about 13 per cent; for ricketic rats of the same age it is 15 and 16 per cent, the same as in normal adult rats.

Since the proportions of the inorganic constituents vary also with age, as we have just demonstrated, it is incorrect to assign a definite formula to bone. The use of the famous formulas $3Ca_3(PO_4)_2 \cdot CaCO_3$ of Hoppe-Seyler (14) and $\left[Ca\begin{pmatrix} OPO_3Ca \\ > Ca \\ OPO_3Ca \end{pmatrix}_3 \right] CO_3$ of Werner (15) should therefore be discontinued as incorrect and misleading.[4]

[3] The data of Wildt and of Weiske are also given by Forbes and Keith (9).

[4] De Jong has attempted to determine the nature of the "compound"

Primary Calcification.—The results of our analyses of fresh calcification are summarized in Table VII. It is seen that in freshly deposited bone salts the proportion of $CaCO_3$ is low, carbonate Ca : total Ca being about 11 per cent, while in the older bones of these same animals carbonate Ca : total Ca has a value of 15 to 16 per cent. This shows the same phenomenon as do the data in Table I; *i.e.*, that new bone contains a smaller proportion of $CaCO_3$ to total calcium than does older bone.

TABLE VII.

Primary Calcification (Rat).

Specimen.	Antiricketic factor.	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100$.	$\frac{\text{Residual Ca}}{\text{P}}$.	Deviation.
Test lines.	Cod liver oil concentrate.	11	2.38	+0.15
Metaphyses.	Butter.	10	2.14	−0.09
Test lines.	Irradiated cholesterol.	11	2.24	+0.01
" "	Irradiated yeast.	12	2.16	−0.07
Metaphyses.	" "	12	2.25	+0.02
		11	Mean = 2.23	*a.d.* = ±0.07 *A.D.* = ±0.03

Primary calcification, residual Ca: P = 2.23 ± 0.03.
Old bone " " " = 1.99 ± 0.01.

The ratio residual Ca : P obtained for primary calcification is 2.23 ±0.03; for older bones analyzed with the same technique this ratio has the value 1.99 ±0.01. At the present time there

in bone by means of x-ray analysis on the basis that "The ratio $\frac{CaCO_3}{Ca_3(PO_4)_2}$ being found about the same everywhere it may readily be suspected that bones are built up chiefly by one compound." He employs the formula $3Ca_3(PO_4)_2 \cdot CaX_2$ (*Proc. Acad. Sc. Amsterdam*, 1926, xxix, 870).

appear to be several ways of interpreting this high Ca : P ratio in primary calcification. They are: (1) experimental error, (2) presence of an acid-soluble organic calcium compound, (3) presence of inorganic calcium in cartilage, and (4) presence of an insoluble basic calcium salt.

The results do not appear to be ascribable to experimental error. Five different specimens were analyzed. The analyses were made repeatedly, on separate samples of each specimen, thus ruling out the simultaneous occurrence of an accidental high calcium value and of an accidental low phosphorus value. In the second place, the ratios for fresh calcification are all high; if the deviations were due to experimental error, some ratios should be below normal. In the third place, the occurrence of ratios as high as 2.20 is quite rare when due to experimental error; only one such ratio was obtained in forty-four analyses, as is seen from Table VI of Paper I of this series. Primary calcification gave a ratio of 2.23 $\pm$0.03 as compared with 1.99 $\pm$0.01 for normal rat bone. When all the ratios obtained in analyses of normal rat bone were averaged, including even those shown by subsequent analyses to be too high, a mean value of 2.01 $\pm$0.01 was obtained. When 2.23 $\pm$0.03 is compared with 2.01 $\pm$ 0.01 it is seen that the accidental occurrence of high ratios does not account for the value obtained for primary calcification.

It is possible that the material analyzed for primary calcification contains an acid-soluble organic calcium compound. However, until the presence of such a compound is demonstrated it is simpler to assume that the apparent excess of calcium is present as inorganic calcium.

The cartilage itself may contain some inorganic calcium aside from the calcium due to the presence of bone salts. Thus even if the ratio of Ca : P in freshly deposited bone salts has a normal value of 1.94, the presence of additional inorganic calcium in the cartilage itself would increase the value of the ratio. At the present time, however, the simplest interpretation of the experimental findings is that the high Ca : P ratio is due to the presence of basic calcium in the freshly deposited bone salts.

From the considerations discussed in the preceding communication we had expected that primary calcification would consist of $CaHPO_4$, and would be found to give *low* ratios. Analysis,

however, gave *high* ratios. On reconsidering the chemistry of the calcium phosphates we noted that $CaHPO_4$ is obtained as a precipitate only from acid solution. Cameron and Seidell (16) found that the precipitate was $CaHPO_4$ when the supernatant solution was acid, and that when the solution was alkaline the precipitate always contained a larger proportion of lime than is required for $Ca_3(PO_4)_2$. Cameron and Bell (17) advanced the view that the solid phases were solid solutions whose composition varied from that of $CaHPO_4$ to that of pure $Ca(OH)_2$ and that the solid phase which is usually given as $Ca_3(PO_4)_2$ may be a mixture of $CaHPO_4$ and $Ca(OH)_2$. They suggested that there might be "only one series extending over the whole range in which the solids are dicalcium phosphate and lime."

Serum has an alkaline reaction; the tissue fluid from which the bone salts are deposited may likewise be alkaline. If this is the case, then a simultaneous deposition of $CaHPO_4$ and basic calcium might be expected on the basis of analogy to inorganic systems. This would account for the apparent significance of the ion product $[Ca^{++}] \times [HPO_4=]$ in clinical and experimental calcification as discussed in the preceding paper, and also for the presence of high Ca : P ratios in primary calcification.

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SUMMARY.

1. When analyzed with the micro technique, unashed normal rat bone gave a value of 1.99 $\pm$0.01 for the ratio residual Ca:P; this is in agreement with the results of analyses of large amounts of bone by macro methods.

2. The proportion of carbonate in normal rat bone increases with age. The ratio carbonate Ca : total Ca is about 8 to 10 per

cent in the bones of young rats and 15 to 16 per cent in those of adult rats.

3. The proportion of carbonate in ricketic rat bone is greater than in normal rat bone of the same age.

4. The proportion of carbonate in primary calcification is less than in the older bones of the same animals.

5. The ratio residual Ca : P in primary calcification has a high value of 2.23 ±0.03. This high ratio is interpreted as indicating the presence of a basic calcium salt in freshly deposited bone salts.

6. The composition of the primary calcification appears to be independent of the antiricketic reagent; high Ca : P ratios were obtained regardless of whether the fresh calcification was induced by cod liver oil concentrate, butter, irradiated cholesterol, or irradiated yeast.

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