CCLXXXIII. THE MINERAL CONSTITUENTS OF BONE.

I. METHODS OF ANALYSIS.

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A KNOWLEDGE of the composition of the mineral constituents of bone is important for two reasons. First, the most popular view of the mechanism of ossification supposes that in bone formation a salt is precipitated from its saturated solution. The desirability of knowing exactly what salt is thus deposited has been considered by Shear and Kramer [1928, 1] and Roseberry *et al.* [1931]. Secondly, work on calcium metabolism frequently suggests that calcium is being laid down in, or removed from, bone [*e.g.* Greenwald, 1926; Greenwald and Gross, 1926], but it is impossible to detect these bone changes in the presence of the large amount of calcium stored there. If, however, bone salt were first deposited in some specific form which slowly changed to the salt of adult bone, or if some labile form of calcium were removed first, such changes might be detected.

Examination of the mineral constituents of bones of many species by physical methods [Taylor and Sheard, 1929; Roseberry *et al.*, 1931], or by chemical methods [Shear and Kramer, 1928, 1, 2; Morgulis, 1931], has established the fact that they consist largely of calcium, phosphate and carbonate in such proportions that the calcium may be assumed to be mainly carbonate and tertiary phosphate, and that they have the crystalline structure of the apatite series. By different chemical methods, Shear and Kramer [1928, 1] and Morgulis [1931] concluded that small quantities of $\text{Ca}(\text{OH})_2$ are present in addition to the carbonate and phosphate. These workers assumed that all the carbonate was bound to calcium, and they calculated the ratio to the phosphate for the calcium not thus bound, *i.e.* the residual Ca/P. They found this ratio to average 1.99 (Shear and Kramer) and 2.04 (Morgulis), whilst for tertiary phosphate the theoretical figure would be 1.94.

In the examination of the ash from large numbers of young rats, Burns [1929] found a ratio for the total Ca/P ranging from 1.99 to 2.10, which approximated rather to that of the residual Ca/P than to the total Ca/P as found by the aforementioned workers. Hammett [1925] gave proportions of calcium and phosphate in the bone ash from young rats which showed a total Ca/P ratio of 1.89 to 2.04. It seemed therefore possible that the bones of young animals either contained little or no carbonate, or that there was present a form of phosphorus, which appeared as phosphate in the ash, but was not estimated as such by the methods of Shear and Kramer or of Morgulis. An attempt was therefore made to locate this phosphorus in the bone and to determine the influence of age on the composition of the bone salt.

Before analysis, the bones were dried at 100–110° and extracted with alcohol for 8 hours and ether for 16 hours, the epiphyses being previously torn off and treated separately. The same sample was used for carbon dioxide, calcium and phosphorus estimations; the solution for the last two was prepared during the estimation of the first, which was done by one of the following methods.

Estimation of carbon dioxide. 1. *Manometric method.* This method will estimate 0.13–0.63 ml. of CO_2 corresponding to 0.015–0.13 g. of bone according to its degree of calcification. A Barcroft blood-gas manometer was used; 2 ml. of boiled distilled water were placed in each bottle and the weighed sample of bone in one. 0.45 ml. of 80 % trichloroacetic acid was placed in each stopper, and the estimation was carried out exactly as for the determination of the O_2 content of blood.

The Barcroft bottle was previously calibrated using a standard solution of sodium or guanidine carbonate, so that the constant included an approximate correction for CO_2 remaining in solution. 1 ml. of 0.17 % sodium carbonate or of 0.36 % guanidine carbonate is a convenient quantity. Seven calibrations of 4 estimations each gave a maximum variation of less than 3 % in each group.

The following analyses were carried out on a sample of ground bone to check the accuracy of the method:

CO_2 % 2.53, 2.51, 2.51, 2.62; Ca % 22.15, 21.95, 22.30, 22.75; % Ca as carbonate 10.40, 10.40, 10.20, 10.45. Maximum variation 2.5 %.

2. *Titration method.* When larger amounts of bone were available for analysis the method used was a modification of that described by Van Slyke [1918]. The modifications consisted of (a) the use of 80 % trichloroacetic acid to liberate the carbon dioxide, instead of the hydrochloric acid used by Van Slyke. Trichloroacetic acid is non-volatile and does not dissolve out protein when dissolving the salts of bone. (b) After the removal of the washed tube containing the bone solution from the flask containing the mixture of baryta and barium carbonate, the latter was not filtered off, but the baryta was determined directly by simple titration with 0.1 *N* potassium hydrogen phthalate with phenolphthalein as indicator. The acid potassium phthalate was without effect on the barium carbonate. This direct titration diminished the possibility of contamination with atmospheric carbon dioxide.

To avoid the formation of a thick protein precipitate which prevented the further action of acid on the bone, 2 ml. of boiled distilled water were added to the bone powder, which was mixed in the tube to form a smooth paste. Two or three drops of octyl alcohol were added to prevent foaming. Pure sodium and guanidine carbonates were used to test the method. Under a pressure lower than about 60 mm., the tap grease was not satisfactory and the vacuum was not preserved long enough to enable the reaction to go to completion in the bone. A pressure of 60–70 mm. was therefore used. The results of twelve estimations of carbonate in standard sodium carbonate solution gave an average error of -1.5 % and a range of error of -0.5 to -3.0 %. On a similar batch on dry guanidine carbonate, the error ranged from -3.0 % to $+2.0$ % with an average of -1 %. Figures with an error of -5 % to -8 % were secured when a pressure of 110 mm. was used. To minimise the picking up of atmospheric CO_2 by the baryta while it was being run into the conical flask, the baryta burette was fitted with a long tip which ran down to the bottom of the flask.

Compared with the manometric method the titration method showed the following percentage variations in a series of 10 duplicate analyses: -0.8 , -5 , -1.5 , -4.8 , $+0.8$, $+2.4$, $+1.7$, $+2.2$, $+3.75$, $+12.4$. (This last high value did not agree with the Ca estimation on the same sample.)

Estimation of calcium. The extract of bone was made up to 2 to 10 % of trichloroacetic acid according as the amount of bone used ranged from 0.01 to 0.1 g., and the volume chosen was such that 2–3 ml. of filtrate contained 0.6 to 0.8 mg. of Ca. Calcium was estimated as described by Burns [1933], and in this concentration the range of error was ± 1 %.

Estimation of phosphate. Two methods were used. In the first a solution containing 2–5 mg. of phosphorus was brought to the boil and to each 100 ml. of the solution were added 60 ml. of an ammonium molybdate solution prepared according to the method of Cole [1926]. The solution was boiled for a few minutes and then allowed to stand on a hot bath till the precipitate had settled and the solution was clear and colourless. The filtered precipitate, washed free from acid with boiling water, was dissolved in 0.5 *N* NaOH. The ammonia was not boiled off, and the NaOH was titrated with 0.5 *N* HCl. With phenolphthalein as indicator it was found that 1 ml. of 0.5 *N* NaOH was equivalent to 0.675 mg. P. This method gave an accuracy of $\pm 1\%$.

The second method used was the colorimetric one of Fiske and Subbarow [1925]. The acidity of the trichloroacetic acid solutions was not found to interfere in this estimation, and in only one case did opalescence occur on the addition of ammonium molybdate. This method was used for the estimation of 0.04 to 2 mg. of P. The forms of phosphate estimated by these methods included the inorganic phosphate and any labile organic phosphate. Total phosphate in the trichloroacetic acid extract was estimated by the method described by Baldwin and Needham [1933]. For the colorimetric method, an accuracy of $\pm 1\%$ was found in 80% of a large series of estimations on standard phosphates. The remaining 20% had an error not exceeding $\pm 2\%$.

The possible presence of phosphorus which was not extractable by cold trichloroacetic acid, but which would give phosphate on ashing, was tested by drying and ashing the washed residue from six samples after the trichloroacetic acid extraction had taken place. Dry ashing was done in the presence of sodium carbonate to prevent loss of phosphate. The subsequent estimation of phosphorus was done by the modification of their method suggested by Fiske and Subbarow [1925] for use in the presence of silica, since some silica was always picked up from the crucible. The six samples tested varied in weight from 0.2 to 0.5 g. and contained from 12 to 50 mg. of phosphorus. They included all the bone tissues, dense and cancellous bone, calcified and uncalcified cartilage and marrow. In only one of the six samples was as much as 0.6% of the original phosphorus left. This seemed to preclude the possibility of the presence in bone of a form of phosphorus not extractable by acid.

Sampling. A whole bone is often too large for analysis and half a bone may be unrepresentative, *e.g.* the upper half of a femur contained 14.4% and the lower half 10.4% of Ca. A whole bone, ground up in a coffee-mill, gave a powder which was not homogeneous. Moreover, if the whole bone is used for analysis differences in the individual tissues are overlooked; and the only information gained is the proportion of the whole structure which is calcified. Therefore, in order to follow the actual chemical changes which occur as bone is laid down, the following tissues have been examined separately; cartilage, calcified cartilage, cancellous bone, dense bone surrounding it, bone from the middle of the shaft and marrow. It is essential to use the whole of the cancellous bone, since it is not homogeneous in structure and composition, and different parts vary in degree of ossification by as much as 50%. When the quantity is inconveniently large, it is possible to make a representative mixture by grinding the tissue in a mortar. Cancellous bone may vary in quantity and composition under different conditions, so that it is necessary to know how far the specimens, removed by hacking them out with a penknife, are representative in either respect. Corresponding bones from the same animal were compared. Of samples from 14 pairs of small bones (diaphysis < 6 cm.) 5 differed in weight by 0–10%, 5 by 10–20% and 4 by 20–30%. A difference in weight of less than 30% could not therefore

be considered certainly significant. Two kid's femora, 12 cm. long, from which the epiphyses were not removed, were cut down the middle and the knee cancellous scraped out. Its weight in the two bones differed by 17%. In the same 14 samples, the CO_2 values of 9 pairs differed by 5%, of 4 by 5–10% and of 1 by 10.5%. A difference of over 10% in the carbon dioxide would therefore probably be significant.

For purposes of direct comparison with the above analytical methods, those used by Shear and Kramer [1928, 1] and by Morgulis [1931] were also tried.

A sample of powder from an adult bone shaft was analysed as above, while a second sample was extracted with hydrochloric acid and examined as by Shear and Kramer, except that the ammonium molybdate precipitate was centrifuged off instead of being removed by filtration. The calcium figures for the two samples were 24.38 and 24.42% whilst the phosphorus figures were 11.53 and 11.48% respectively. In the centrifuged solution, a faint precipitate appeared during the development of the blue colour. Therefore in the next sample, a fairly well calcified epiphysis, the ammonium molybdate precipitate was filtered off through ash-free filter-paper. In this sample, the total P estimated by Baldwin and Needham's method on the hydrochloric acid extract was 4.68% whilst the inorganic P as estimated by Shear and Kramer's method was only 4.36%. Large numbers of analyses have shown that in such bone the inorganic P determined by our method does not differ from the total P by more than 3%. In this sample therefore the % P as given by Shear and Kramer's method was low.

Two samples of fetal epiphyses of approximately the same age were examined by the two methods respectively. By the trichloroacetic method the first sample was found to contain Ca 0.51%; inorganic P 0.36%; total P 0.52%. (Cartilage has been found to contain a certain amount of non-inorganic phosphorus.) The other sample extracted with hot hydrochloric acid contained Ca 0.64%; total P 0.80% and inorganic P, 0.19, 0.22 and 0.28% according to the dilution of the solution in which the ammonium molybdate precipitation was carried out. Clearly the protein precipitate had carried down varying quantities of phosphate. In a further sample with total phosphate 1.45%, Kramer's method gave only 0.45 and 0.95% P according to dilution. The cancellous bone from a femur of a rabbit suffering from bone deformities was analysed by our method and gave Ca/P 2.18; residual Ca/P 1.78. The cancellous bone from the other femur was examined by Shear and Kramer's method. The total P was 3.14% and the total Ca/P was 2.20, residual Ca/P being 1.80, but by Shear and Kramer's method the P was only 2.33%, the Ca/P was 2.96 and the residual Ca/P was 2.43. A further 0.33% of P was found by washing filter-papers from four estimations free from soluble phosphate, igniting the filter papers and estimating the phosphorus in the ash. A considerable amount of the protein precipitate was washed through the filter-paper, and some phosphorus may have been lost in ignition, as sodium carbonate was not used, but the protein precipitate had obviously carried down appreciable amounts of phosphate [*cf.* Fiske and Subbarow, 1925; Goldblatt, 1935]. The method which involves filtering off the ammonium molybdate precipitate is therefore satisfactory for highly calcified bone in which little protein passes into solution but is increasingly unsatisfactory as the ratio of protein to phosphate in the solution increases, since more and more phosphate is carried down with the protein precipitate and the Ca/P ratio becomes correspondingly too high.

The method used by Morgulis consisted of removing the organic matter of the bone by heating it with 3% KOH in glycerol. The bone ash was left as a white salt which was washed with water and alcohol to remove the alkaline reagent.

Analyses for calcium, carbonate and phosphate were then made on the dry ash. This method was applied to a salt supplied as "pure calcium phosphate precipitated by ammonia" and to samples of different bones. In order that the results obtained by the two methods might be compared, it was necessary that they should be applied to ground and mixed samples. Neither powdered calcium phosphate nor the ash left after leaching out bone powder with the alkaline glycerol could be picked out of the reagent as described by Morgulis for pieces of bone. So the glycerol was decanted off into a beaker, and to it were added the water and alcohol washings from the salt. The glycerol and wash-waters were filtered through ash-free filter-paper, on which the washed salt was collected, as it was desired to determine the total calcium, phosphate and carbonate in the salt and not only the ratio. (If the alkaline glycerol was decanted directly on to the filter-paper, filtration took many days.)

The glycerol filtrate and washings were made just acid to phenolphthalein with hydrochloric acid, dried and ignited gently to a black ash in a silica dish. On this ash total phosphate and calcium were estimated, allowance being made for the probable presence of silica. Blank estimations on the reagent similarly heated in the silica dish gave only minute traces of calcium or phosphate (< 0.1 mg.). Before this method was applied to the "precipitated calcium phosphate" the latter was examined directly to determine the salt present. Considerable discussion has taken place previously, reviewed by Shear and Kramer [1928, 2], as to whether the salt precipitated by alkali from a solution containing calcium and phosphate is a specific compound, the tertiary salt, in equilibrium with the solution, whether it is a mixture of lime and secondary phosphate, with the latter in equilibrium with the solution, whether the secondary phosphate first formed subsequently reacts with the solution round it to give the tertiary salt, or whether in fact the tertiary salt is an actual chemical entity. Roseberry *et al.* [1931] have produced X-ray evidence that the tertiary salt can exist as a member of the apatite series but it is not clear from their work whether the samples they examined were prepared by simple precipitation. Four samples of the precipitated salt were examined by the above method in simple acid solution. They contained respectively Ca 33.5, P 18.6; Ca 34.4, P 18.8; Ca 34.8, P 18.9; Ca 34.2, P 18.8%; mean Ca/P 1.82. All the samples contained 0.2% CO_2 and traces of magnesium and chloride. They were free from ammonia and only lost 0.5 to 1% of moisture when heated at 100° for 4 hours, but lost 6–8% of their weight when heated in the blast furnace, total calcium and phosphate being unchanged. The heated salt contained 72.9% of its phosphate as orthophosphate and 27.1% as pyrophosphate. This suggested that 27.1% of the phosphate in the original salt was present as secondary phosphate but did not prove the chemical composition of the original mixture. Equal weights of secondary phosphate and lime, of di-acid phosphate and lime and of secondary phosphate and calcium carbonate were mixed intimately and samples heated gently and strongly, the products being examined for total and orthophosphate. In every mixture, strong heating produced salts containing more than 60% of the phosphate in the ortho-form, whilst gentle heat produced only 30–40% in that form. The di-acid salt gave some extremely insoluble metaphosphate and a mixture of pyro- and ortho-salts. These results could only be explained on the assumption that the pyro- or meta-salts first formed reacted on further heating with lime to give tertiary calcium phosphate, and that lime from calcium carbonate could be used for this purpose.

This is in agreement with the X-ray evidence of Roseberry *et al.* [1931] that this salt is a real chemical entity, but it does not prove that this salt is precipitated by alkali from solutions containing calcium and phosphate. An attempt to study the question as to whether tricalcium phosphate exists as such in the precipitate produced by alkali from a solution containing calcium and phosphate, and therefore possibly in bone at some stage of ossification, was made by studying the effect of gentle heat on such precipitates and on mixtures of lime and secondary phosphate. For this purpose the following were used: (1) secondary calcium phosphate; (2) a commercial precipitated calcium phosphate; (3) a mixture of equal weights of lime and secondary phosphate; (4) an air-dried freshly prepared calcium phosphate (Ca/P 1.88) precipitated from a solution just alkaline to phenol red; (5) a freshly prepared secondary calcium phosphate (Ca/P 1.44) made by precipitating calcium phosphate from a solution acid to methyl red and drying between filter-papers; (6) the secondary calcium phosphate from (5) dried in the air overnight on a glass plate; (7) a mixture of the secondary phosphate (5) with lime (Ca/P 5.3); (8) this mixture dried overnight on a glass plate. 0.2 g. of each of these substances was heated over a low burner regulated so that secondary phosphate was converted into pyrophosphate in 1 min. When 0.2 g. of the mixtures was heated on this burner for varying times, varying proportions of ortho- and pyro-salts were found.

From the results of these experiments the following facts may be deduced:

(a) The brief heating over the low flame sufficed to convert 99% of the dry secondary phosphate (CaHPO_4) into pyrophosphate. Heating the air-dried secondary calcium phosphate converted 97% of the phosphate into pyrophosphate. This air-dried compound contained 39% water. (Here, as in all the water contents of these mixtures, the water was measured by the loss of weight on ignition to constant weight, and includes adherent moisture, water of crystallisation and the water lost from the molecule in converting secondary phosphate into pyrophosphate.) The Ca/P ratio was 1.44. This would be compatible with the presence of about 95% of the crystalline $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ and 5% $\text{Ca}(\text{OH})_2$, or of a mixture of 79.2% of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ and 20.8% $\text{Ca}_3(\text{PO}_4)_2$ with about 23% of the phosphate in the condition in which it would not be converted into pyrophosphate by heat. The precipitate from the phosphate solution made just acid to methyl red was therefore mainly the secondary salt mixed with small quantities of lime.

(b) In a mixture of equal weights of lime and secondary phosphate free from water of crystallisation (water content of mixture 10%), 12% of the phosphate was found as orthophosphate after 1 minute's heating, and 23.6% after 30 min. Since 99% of this phosphate heated alone was converted into pyrophosphate in 1 min., the orthophosphate found after heating the mixture 1 min. would not probably be unchanged secondary phosphate. However, the relatively rapid production of tertiary orthophosphate in the first minute and the slow subsequent production suggest two reactions. In the first stage while water is present, the process is practically one of heating a strong solution of lime and secondary phosphate, when presumably the reaction

occurs. Later, when all the phosphate has been changed into tertiary orthophosphate or into pyrophosphate, the slow reaction (1) (p. 2389) produces further orthophosphate.

(c) After being heated 1 min., calcium phosphate precipitated from an alkaline solution showed in one case 60.9 and in the other 79.3% of the total

phosphate as orthophosphate. Further heating for 30 min. only increased the proportion to 66.4 and 87.4 % respectively although ignition in the blast furnace brought the figures up to 82.5 and 94.6 %. The figures of 60.9 and 79.3 % may be compared with the 12.0 % of orthophosphate produced by heating a known mixture of lime and secondary phosphate for one minute. The precipitated calcium phosphate contained 7 and 15 % of water respectively, so that whilst some of the 60.9 and 79.3 % of the orthophosphate might be produced by heating the wet salt by the rapid reaction (2), it does not seem probable that this could account for the production of 60 % of tertiary phosphate in a mixture of water content 7 %, whilst only producing 12 % of orthophosphate in a mixture of water content 10 %. It seems therefore improbable that the precipitated salt consists merely of a mixture of lime and secondary phosphate, but seems much more probable that from 50 to 70 % of the phosphate found as orthophosphate after 1 minute's heating was in the form of tricalcium phosphate before the heating began. This is also supported by the water content of the mixtures. Lime, Ca(OH)_2 , contains 23 % of water even when free from adherent moisture, whilst crystalline secondary phosphate gives up on heating 26.1 % of water. It is difficult to see how any mixture of these, dried at a temperature not exceeding 15°, could give a mixture of water content 15 %.

It was not possible by this method to determine whether the tertiary phosphate was formed in contact with the solution or in the process of drying, since heating secondary phosphate with excess of wet lime caused such rapid formation of the tertiary salt. The mixture of lime and wet secondary phosphate, thoroughly ground together and air-dried, showed however a water content of only 17.5 %, from which it seemed probable that these salts in the presence of moisture had reacted to form some tricalcium phosphate. It was therefore fairly certain from these studies that the precipitated calcium phosphate to be examined by Morgulis's method was a mixture of tricalcium phosphate, secondary calcium phosphate and lime.

Samples of this precipitated calcium phosphate were treated with the alkaline glycerol, and the salt and extract were analysed as already described. Table I shows the results. In Exp. 3, part of the salt was not used for analysis, but was again treated with the glycerol reagent. Ignited salts were also sub-

Table I. *Ca/P of initial salt 1.82. CO_2 , 0.22%.*

Exp.	Sample (g.)	Treatment	Ca of extract mg.	P of extract mg.	Soluble P in extract as % total P	CO_2 in final salt (%)	Residual Ca/P in final salt
1	—	Alkaline glycerol	0.30	14.3	—	0.63	1.96
2	0.990	„	7.6	21.6	9.5	0.60	1.99
3	1.012	„	2.5	25.8	13.0	0.55	2.08
4	0.365 of salt from Exp. 3	„	0.36	1.1	1.4	0.67	2.11
5	0.598 of ignited salt	„	0.5	0.5	0.3	0.22	1.82
6	0.602	Boiling water	4.37	6.4	5.8	—	—
6a	Salt from above	Hot aqueous alkali	—	10.4	9.4	0.69	2.07
7	0.989	4 successive quantities of cold alkali	0.08	14.6	8.0	0.40	1.98
8	0.559 of ignited salt	2 successive lots of hot aqueous alkali	—	0.8	0.7	0.22	1.79
9	0.305 of CaHPO_4 (Ca/P=1.26)	Hot aqueous 0.5 N NaOH for 10 min.	—	26.0	37.7	—	2.02

mitted to the treatment. Further samples were treated with hot or cold aqueous alkali, and salt and solution were analysed. A sample of salt containing 96 % secondary calcium phosphate and 4 % di-acid calcium phosphate was also heated with 0.5 *N* aqueous NaOH.

It may be seen that after the salt had been strongly heated, treatment with either aqueous alkali or alkaline glycerol extracted little phosphorus and left the CO₂ content unchanged, and the ratio Ca/P for the final salt was very close to that for the initial salt. Where however the unheated salt was used, varying quantities of phosphate were removed in the extract, and the ratio Ca/P for the final salt always exceeded 1.94, that for tertiary phosphate. (Small quantities of the salt were occasionally washed through the filter-papers; when this did not happen the calcium of the alkaline extract was negligible. To avoid the tedious re-filtering of these solutions, this calcium was assumed to be present in the form of the salt on the filter, and the amount of phosphate combined in this form was subtracted from the total phosphate in the extract to determine the amount present in the soluble form.)

If it be assumed that in the presence of hot alkali the salts of the mixture react according to the equations (2) (p. 2390) and

then it would be expected that the resultant salt would be tricalcium phosphate, while one-third of the phosphate of that portion of the secondary salt which could not combine with lime would be found in the extract. A mixture of 84 % tertiary calcium phosphate and 16 % secondary calcium phosphate would have a Ca/P ratio of 1.82, and the phosphate of the secondary salt would be 17.95 % of the total. It might therefore be expected that 5.95 % of the total phosphorus would appear in the extract. Actually the phosphate in the extract always exceeded this and the Ca/P of the final salt indicated the presence in it of lime. This lime might be produced by the hydrolysis of either the tertiary or secondary salt, but its presence seems to be independent of the presence of lime in the original salt, since it was found when secondary calcium phosphate, free from lime, was treated with hot alkali. It would seem also more probable that it is the secondary salt which reacts in this way with the alkali, since the mixture of tertiary phosphate and pyrophosphate (Exps. 5 and 8) produced by ignition, and the mixture of tertiary phosphate and lime produced by treatment with alkaline glycerol (Exp. 6), reacted negligibly with the further alkaline treatment.

These experiments indicate very clearly that the composition of the salt produced by this treatment may differ very markedly from that of the original salt, although the method may be applied to phosphates known to be the tertiary phosphate or pyrophosphate. It would seem to be generally inapplicable to mixtures of unknown composition which might contain secondary or similar phosphates. Moreover, in these experiments some carbon dioxide was picked up by the salt.

Various samples of bone were examined directly and after leaching with alkaline glycerol. Table II shows the results, the figures marked *E* being secured by direct analysis, those marked *M* being the figures for analysis of the salt produced by the leaching out process. The carbonate is expressed as the percentage of the total calcium with which it could combine. In ten out of the fourteen bone samples used, the carbonate content of the salt as prepared by leaching out was from 15 to 25 % higher than was indicated by the direct determination. Bones 11 to 14 were not ground up before analysis and could be lifted out of the solution and washed whole as described by Morgulis, only the traces

Table II.

Bone	...	1	2	3	4	5	6	7	8	9	10	11	12	13	14*
CO ₂ as % Ca, <i>E</i>	...	13.0	13.0	13.4	12.6	11.5	11.6	11.3	10.3	10.8	8.4	—	9.5*	10.0*	9.8*
CO ₂ as % Ca, <i>M</i>	...	15.1	15.0	15.2	12.7	13.6	14.4	13.9	10.9	14.0	10.2	12.6*	9.4	9.6	10.0
P lost in extract (%)		2.0	3.1	3.1	4.7	4.0	5.4	3.8	6.8	8.8	6.0	1.4	5.8	4.9	4.3
Residual Ca/P <i>M</i>		1.90	1.89	1.88	2.03	1.93	1.92	1.92	1.95	1.95	1.89	1.96	1.99	1.89	1.88
Residual Ca/P <i>M</i> corrected for CO ₂ picked up		1.95	1.94	1.92	2.03	1.98	1.98	1.99	1.97	2.02	1.94	1.96	1.99	1.89	1.88
Residual Ca/P <i>E</i>		1.88	1.90	1.87	1.87	1.89	1.88	1.89	1.87	—	—	—	—	—	—
Residual Ca/P <i>M</i> corrected for CO ₂ picked up and P lost		1.91	1.87	1.88	1.90	1.90	1.86	1.91	1.84	1.86	1.83	1.94	1.88	1.79	1.80

* Salt picked out of solution and washed whole.

falling into the solution being washed on to the filter-paper. Exact duplicates of the samples could not be secured, but similar parts of bones from the same animal were examined directly and the carbon dioxide contents agreed as closely as for two estimations made on such material by the direct method. No duplicate for bone 11 was available, but its carbon dioxide content was not high for the age of the old cat from which it was taken. These results seemed to indicate that the extra carbon dioxide was picked up by the salt during the exposure to air necessitated by the very slow process of collecting the salt on the filter-paper. If the alkaline glycerol had reacted with the bone phosphate as with the mixture of phosphates to give some free lime, this lime exposed when moist to a slow filtration would pick up carbon dioxide. The true residual Ca/P ratio for the leached out salt is therefore shown in Table II in the Ca/P *M*, corrected for CO₂. This residual Ca/P is deduced by using the CO₂ content of the salt as found by direct analysis and indicates more accurately the amount of lime produced by the alkaline treatment. If this corrected residual Ca/P is compared with the residual Ca/P found by direct analysis, it is seen to be in every case higher than the latter, although in bones 2 and 3 the difference is not outside the experimental error. When the residual Ca/P of the salt is corrected for the phosphate lost in the extract as well as for the CO₂ picked up, the resulting figures are found to lie within the experimental error of the figures found by direct examination and are not all ranged on one side of these figures; of the eight samples completely examined by both methods, five are slightly above and three slightly below the figures for direct analysis. Samples 1-4 and 12 were from the bones of two old cats and samples 5-8 from a young adult cat. Samples 9, 10, 11, 13 and 14 were from the bones of young rapidly-growing kittens. The figures for the dense bones of the old animals, samples 1, 2, 3 and 12, would indicate empirically that the leaching out method leaves a salt which can safely be used as a sample of the bone salt, but the figures for the cancellous bone of an old animal (sample 4), and all the bones of the younger animals, indicated that enough phosphate may be lost in the extract appreciably to affect the Ca/P ratio. Examination of further six samples of bone treated with aqueous or glycerol alkali, showed the removal of 2 to 10% (average 7%) of the phosphate in the alkaline extract. Morgulis tested the leaching out method on a salt of Ca/P ratio 1.92 and on a bone for which his figures indicate a residual Ca/P ratio of 1.93 by direct analysis and 1.96 by analysis of the leached out salt. For these salts, the method would give results closely similar to those by direct analysis, but samples 4 to 11, 13 and 14 indicate that bone salt may not always have this highly specific composition, and that the method is not therefore generally applicable, the residual Ca/P thus found being frequently appreciably too high.

The actual value of the residual Ca/P ratio in indicating the probable composition of bone salts may be questioned. The ratio is derived from three separate estimations—for calcium, phosphate and carbon dioxide, the range of error of which is such that a certain proportion of the figures for the Ca/P ratio must have an error of $\pm 4\%$. Thus a figure which ought to be 1.88 might be 1.96 or 1.80, but a residual Ca/P ratio of 1.88 would indicate the presence of about 10% of the calcium as secondary phosphate, whilst a ratio of 1.80 would mean that more than 20% of the calcium was in this form. Individual figures have therefore little value in indicating the form of combination of the calcium and phosphate. In a total of 18 samples done in duplicate, only 1 pair differed by as much as 2.5%. The eight samples shown in Table II have values for residual Ca/P by both methods which agree within $\pm 2\%$, when the necessary corrections for CO_2 picked up and phosphate lost in the extract are made. The average of a large number of estimations will therefore give some information as to the composition of bone salt. There is, according to Kramer and Howland [1926], Hammett [1925] and Morgulis [1931], and from certain figures secured in the course of this work, an amount of magnesium in bone salt equivalent to about 1–3% of the calcium. If this magnesium is ignored in the calculation as here and in the papers of Shear and Kramer [1928, 1, 2] and Morgulis [1931], and if this magnesium is entirely combined with phosphate, a residual Ca/P of 1.88 to 1.92 would be compatible with the presence of all the calcium as the tertiary phosphate. On the other hand, if all the magnesium is present as carbonate, a residual Ca/P as low as 1.94 would not preclude the possible presence of small quantities of lime. The mean residual Ca/P ratio of 270 samples of bone from cats, dogs, rabbits, goats and sheep of varying ages was 1.85. This figure was also the mode. Table III shows how these figures were distributed through the total range of 1.68 to 1.97.

Table III.

Residual Ca/P	<1.76	1.76–1.80	1.80–1.84	1.84–1.88	1.88–1.92	1.92–1.96	1.96–1.97
% of samples	4.1	11.8	21.2	32.3	22.3	7.3	0.8

The range of 1.88 to 1.92 for the residual Ca/P, which in the presence of the magnesium would indicate that all the calcium phosphate was tertiary, was found in 22.2% of the cases. This range was exceeded in 8.1% of the samples, but 69.2% of the figures fell below it. This seems to indicate the probable existence in some samples of bone of small quantities of a phosphate other than tertiary calcium phosphate in the form of apatite, but, in view of the difficulties already discussed in the accurate determination of the residual Ca/P, it would seem that the existence of such a phosphate could only be regarded as proved if this phosphate were actually separated.

An attempt to study the question by igniting 14 samples of bone and examining the ash for pyrophosphate led to inconclusive results. In only two samples did the total phosphate exceed the orthophosphate by more than 3%, and these figures, 4.5 and 4.7%, were of doubtful significance. Since, however, precipitated calcium phosphate containing at least 20% secondary phosphate, when mixed with CaCO_3 in the proportions of bone and heated as strongly as is necessary to ignite bone, only showed 0.3 to 4% pyrophosphate, the failure to find appreciable amounts of pyrophosphate in the ash resulting from the ignited bone must be regarded as inconclusive evidence as to the presence of secondary phosphate in the original bone salt. Attempts are being made to discover the degree of heating which will convert secondary phosphate into pyrophosphate in the presence of organic material, whilst causing the minimum production of tertiary phosphate

from the carbonate and the pyrophosphate. It is not impossible that some of the phosphate contained in bone is combined with alkali metals. Three kitten bones contained 0.18 to 0.22 % of potassium on the wet bone, equivalent to about 0.6 % of the salt. No analyses for sodium were made. Compounds of phosphate with organic substances are also possible, although if present they must be labile to heat or acid.

The variation in the composition of bone salt with age, species and diet is being investigated in greater detail.

SUMMARY.

1. Chemical evidence is adduced to support the view that the phosphate produced when calcium phosphate is precipitated from an alkaline solution and allowed to reach equilibrium with the solution actually contains tricalcium phosphate as well as lime and secondary phosphate.

2. Analysis of bone phosphate by a method which involves filtering off the precipitate produced by ammonium molybdate gives results which are too low. The resulting Ca/P ratio is therefore too high.

3. The salt produced by heating bone with alkaline glycerol is not accurately representative of the original bone salt. In all cases some phosphate is lost in the extract, though this amount may in some cases be extremely small.

4. Analysis of salt mixtures shows that the leaching out method is applicable to tertiary calcium phosphate or calcium pyrophosphate but not to secondary salts or mixtures containing these.

5. Methods are described for the analysis of the calcium and phosphate of bone with an average accuracy of $\pm 1.5\%$, and of the carbonate with an accuracy of $\pm 3\%$.

6. In 270 analyses of bone, 70 % of the figures for calcium, carbonate and phosphate were such as to indicate the probable presence in bone of a phosphate not tertiary calcium or magnesium phosphate.

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