DIFFRACTION STUDIES ON IMMATURE CHICK BONES

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Russell J. Krasy

1955

This is to certify that the

thesis entitled

Diffraction Studies on

Immature Chick Femurs

presented by

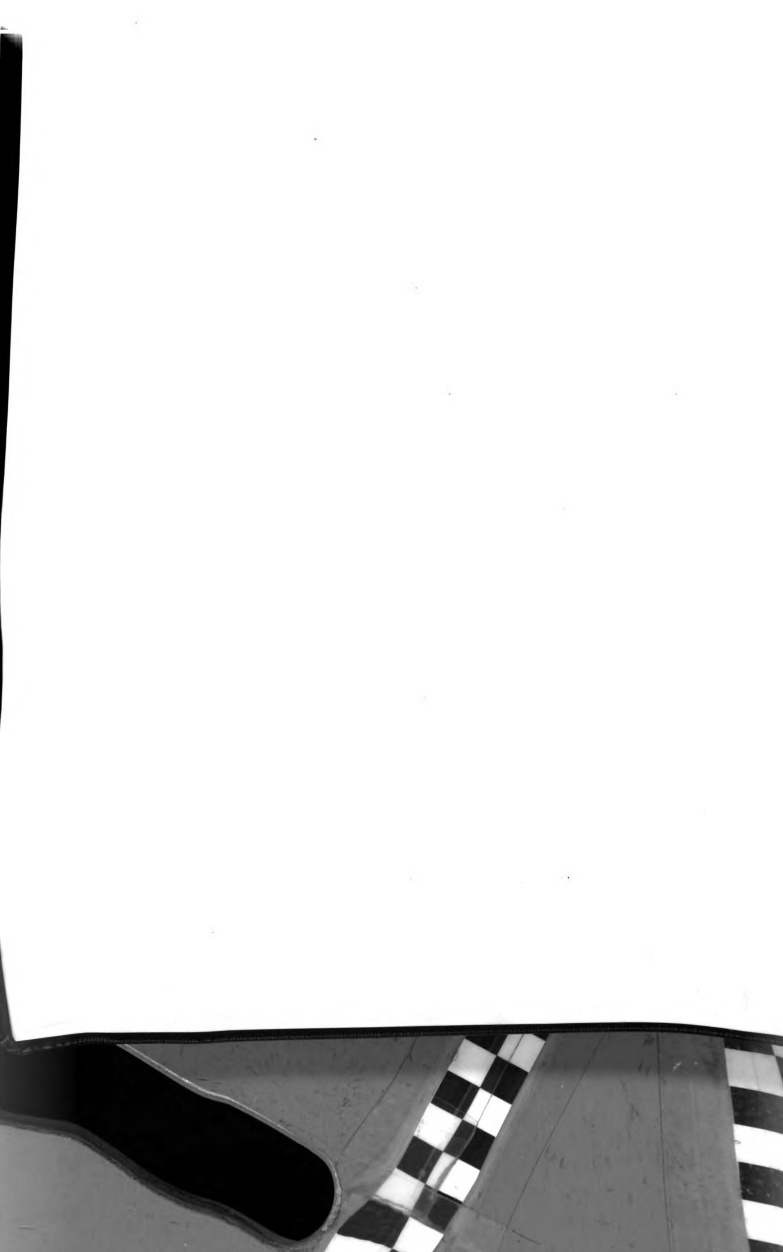
Russell Kraay

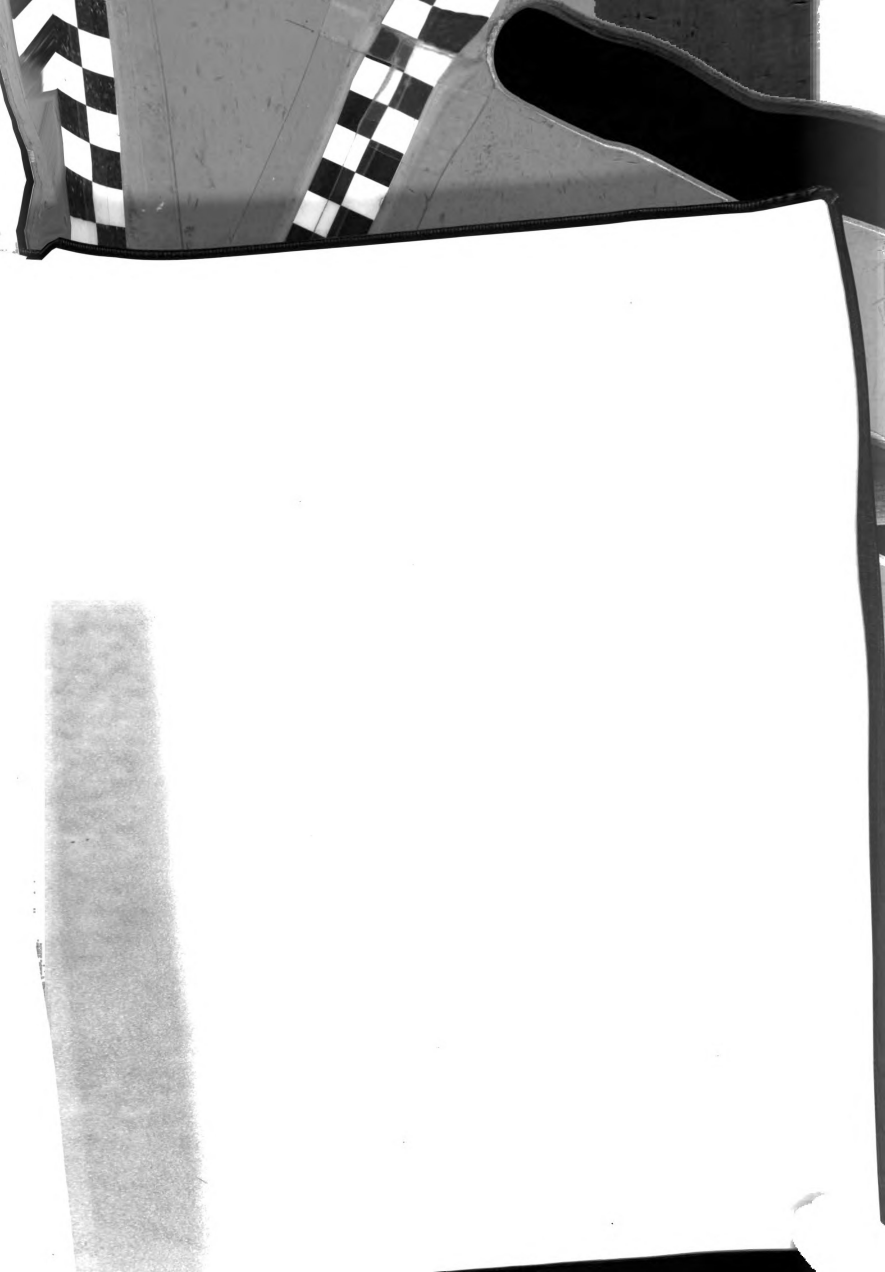
has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology

Lester F Wolf
Major professor

Date June 17, 1955





DIFFRACTION STUDIES ON IMMATURE CHICK BONES

By

Russell James Kraay

AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology and Pharmacology

Year 1955

Approved Lester F. Whitzel

6-17-55

Russell J. Kraay

Immature chick femurs were studied using electron and x-ray diffraction in an attempt to answer the following questions regarding the process of calcification and the nature of the bone mineral: (1) are crystalline precursors present before the formation of bone apatite, (2) are there detectable changes in the preosseous cartilage prior to ossification, (3) is the first bone mineral actually crystalline or does it become crystalline upon dehydration, (4) is there any change in apatite as bone matures, (5) is bone mineral prepared by ethylene diamine extraction altered in ways which allow conclusions to be drawn with respect to the original bone?

Dry, heat ashed, and ethylenediamine extracted embryonic chick femurs were finely powdered, transfered, and examined by electron microscopy and diffraction. In the second experiment, embryonic chick femurs from six to 21 days incubation were studied by x-ray diffraction either in the wet, dry, heat ashed, or ED ashed state. Nickel filtered copper K-alpha radiation was used with a powder camera having an effective film diameter of 14.32 cm.

By means of electron microscopy, a thin crystalline species was observed. Its diffraction pattern was always hexagonal, with a calculated "a" axis of 5.29 Å (assuming its innermost spots to be from the 100 plane). Since the crystal was also seen on grids other than those containing bone preparations, it must be regarded as a contaminant. No other crystal species ever gave rise to a diffraction pattern.

The x-ray diffraction patterns from dry preosseous femurs showed the presence of crystalline sodium and potassium chloride in the approximate ratio of one to three. After bone apatite was detected (from the nine day femurs) no crystalline NaCl could be found and the amount of crystalline KCl was markedly reduced. The possible involvement of various binding mechanisms is discussed.

No lines were visible on x-ray diffraction pattern from young wet bone, due to the high background. However, in the absence of distinguishable apatite lines, it is possible that the first mineral laid down in normal hydrated bone is non-crystalline.

The study of dry embryonic chick bone shows no detectable change in lattice parameters from the time it is first seen.

Ash from twenty one day femurs after extraction with ethylene diamine, gave a diffration pattern nearly indistinguishable from that of dry bone, the only difference being the loss of two broad bands, presumably due to the organic portion of dry bone. This indicated that in the process of ED extraction, there had been no detectable shift in lattice parameters, and no induced crystal growth.

Heating ED extracted bone produced shifts in lattice parameters as compared with similarly heated dry bone. The "c" axis remained unchanged, but the "a" axis was decreased from 9.42Å for heated dry bone to 9.36Å for heated ED ashed bone. The calculated value for heated dry bone corresponds closely to literature values for hydroxylapatite, whereas, the value for heated ED ashed bone corresponds more closely to values given for carbonate apatite.

DIFFRACTION STUDIES ON IMMATURE CHICK BONES

By

Russell James Kraay

A THESIS

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology and Pharmacology

Year 1955

Approved Lester F Wolbach

6-17-55

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to Dr. L. F. Wolterink, whose constant inspiration and keen insight have contributed greatly to this work.

He is also indebted to Dr. B. V. Alfredson, Head, Department of Physiology and Pharmacology, and to the other members of the department where this work was carried out in part.

Grateful acknowledgment is due to Mr. D. E. Van Farowe, Chief Chemist, and to the office personnel of the Division of Occupational Health, Michigan Department of Health, where the x-ray diffraction experiments were carried out. Also to Mr. H. W. Thomas, Draftsman, for his technical assistance.

The author also wishes to thank Dr. H. M. Bendler, Electron Microscopist, Physics Department, and Mr. D. A. Libby, Department of Poultry Husbandry.

Acknowledgment is also made to the Atomic Energy Commission, Division of Biology and Medicine, for supplying the powder camera used in the x-ray diffraction work.

Russell James Kraay

candidate for the degree of

Doctor of Philosophy

Final examination, June 17, 1955, 10:00 A. M., Room 213A, Giltner Hall

Dissertation: Diffraction Studies on Immature Chick Bones

Outline of Studies

Major subject: Physiology
Minor subjects: Endocrinology, Biochemistry

Biographical Items

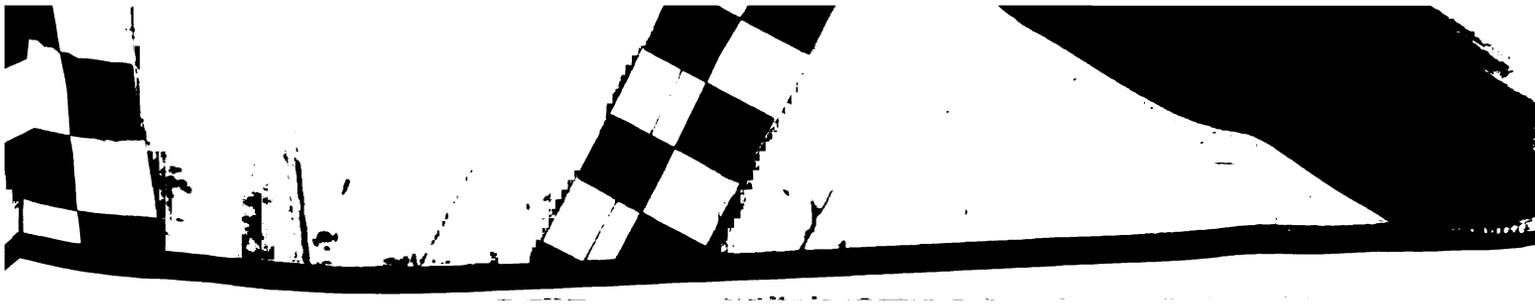
Born, April 24, 1926, Munster, Indiana

Undergraduate Studies, Hope College, 1943-4, Cont. 1946-9

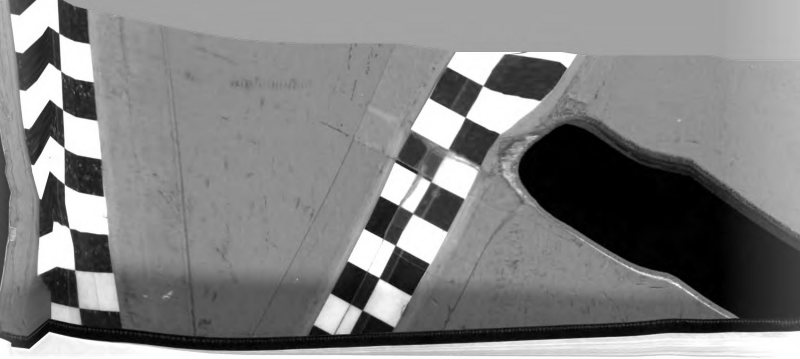
Graduate Studies, Michigan State University, 1950-1955

Experience: U. S. Navy, 1944-1946, Control Chemist, 1950, DePree Chemical Company, Holland, Michigan, Radiochemical Technician, Michigan State University, 1950-1951, Instructor in Physiology, Michigan State University, 1951-1952, Graduate Assistant, Michigan State University, 1952-1954, Industrial Hygiene Chemist, Michigan Department of Health, 1954-1955.

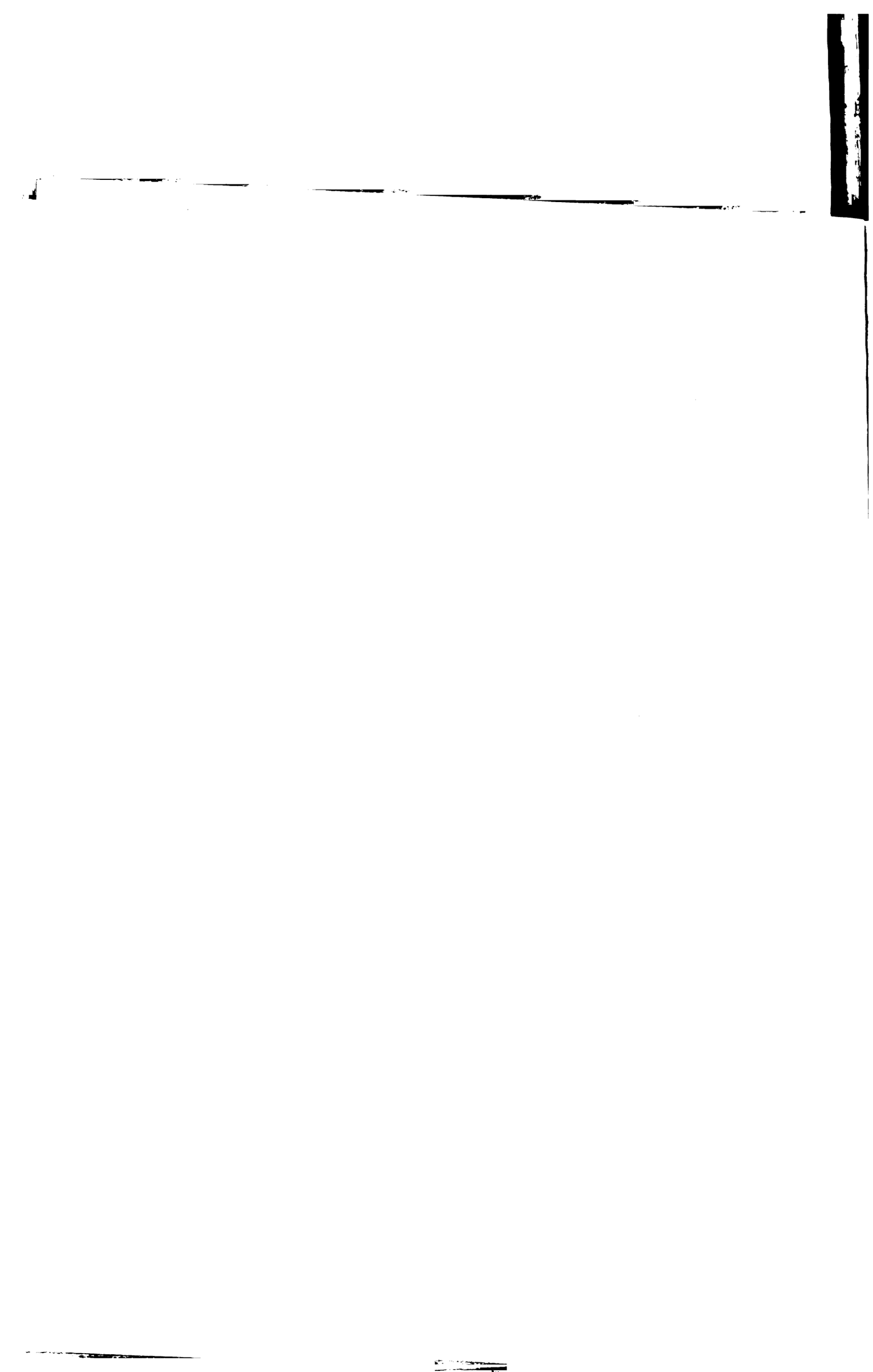
TABLE OF CONTENTS



LIST OF TABLES



LIST OF FIGURES



I. INTRODUCTION AND LITERATURE SURVEY

A. The Nature of Apatites

Crystallographic investigation into the nature of the bone mineral began with the x-ray diffraction work of DeJong (1926) in which he recognized the similarity between the diffraction patterns from bone and those from the apatite minerals. Earlier, however, Berzelius (1845), Hoppe - Seyler (1862) and Werner (1907), in attempting to combine analytical data for bone into the chemical formula for a single molecule, had called attention to the apatite nature of the bone salt.

Mineralogically, the apatites are a large group of naturally occurring hexagonal crystals belonging to the tripyramidal class (Dana, 1932). The apatite most thoroughly studied is fluorapatite, whose unit cell consists of 10-Ca, 6-P, 24-O, and 2-F. Its formula is $3\left[Ca_3(PO_4)_2\right] \cdot CaF_2$, and the structural arrangement of the atoms in its lattice has been worked out by Mehmel (1931). The apatites form ionic, rather than molecular or metallic crystals, despite their generally low solubility.

Within the same general lattice, considerable substitution can occur, with only slight shifts in lattice parameters. Replacement of the F_2^{--} with Cl_2^{--} or $(OH)_2^{--}$ gives the corresponding chlorapatite or hydroxylapatite, with no significant detectable change in lattice parameters as determined by x-ray diffraction methods (Altschuler, Cisney, and Barlow, 1953).

Carbonate apatites are also known having the general formula $CaCO_3 \cdot [Ca_3(PO_4)_2]_n$. X-ray diffraction reveals slight but significant differences in lattice parameters as compared with fluoro- and hydroxylapatite. Altschuler, Cisney and Barlow give the lattice parameters of OH apatite as a = 9.413 Å, c = 6.875 Å whereas carbonate apatite parameters are given as a = 9.344 Å, c = 6.881 Å. Roseberry, Hastings and Morse (1931), studying bone and teeth by x-ray analysis, concluded that the diffraction patterns of bone and teeth are essentially those of dahlite, a mineral carbonate apatite. The problem of the position of carbonate in the apatite has been particularly studied by Gruner and McConnell (1937). It was recognized that the carbonate of calcified tissue might very well occupy the same position as that of the carbonates in the mineral apatites. From a study of francolite, they originally proposed a 1:1 substitution of certain CO_4 groups to replace PO_4 groups. Hendricks and Hill (1942) regard the replacement of 3 PO_4 groups by 4 CO_3 groups as much more likely. Investigators using, in addition to x-ray diffraction, crystal optics and mole refractivity, have not been able to rigorously prove the exact position of carbonate in large mineral crystals, since a number of equally plausible alternatives exist. Agreement is qualitative or semi-quantitative at best.

Additional substitutions which have been postulated are discussed by Neuman (1953). Heteroionic exchange of a sodium (Hodge, et al., 1943), strontium (Hodge, 1945) and uranyl ion (Neuman, et al., 1949) have been studied in bone. However, no careful crystallographic study has ever established the actual presence of any of these ions in the apatite lattice. Simply on the basis of ionic radii, some ions are

denied a position in the lattice. Potassium ion, for example, with an ionic radius of 1.33 Å could not replace calcium in apatite with an ionic radius of 0.99 Å without seriously disrupting the lattice structure.

Although this statement is commonly repeated by such authorities as McConnell, Hendricks, Armstrong, and Hodge, there are numerous examples in minerology where K is assumed to replace Ca in a hexagonal phosphate lattice. Thus:

TABLE I*

SOME SODIUM AND POTASSIUM SUBSTITUTED PHOSPHATES

Mineral	a	c	Author
$NaSrPO_4$	10.65	5.81	Klement
$KSrPO_4$	10.70	5.87	"
$NaCaPO_4$	10.53	5.76	"
$KCaPO_4$	10.60	5.84	"
$CaNaPO_4$	5.23	7.13	Bredig
$CaKPO_4$	5.58	7.60	"

* All values found in this Table were taken from Donnay and Nawacki (1954).

Numerous examples may also be found in the silicates. In view of the examples cited, the statement that potassium is denied a position in the lattice must be regarded as questionable. It should be pointed out that the substitutions of sodium and potassium reduce the Ca/P ratio and that they represent approaches toward secondary phosphates (H^+ substituted by monovalent cation). On the other hand, strontium, with a radius of 1.13 Å is apparently able to replace calcium. If

monovalent sodium (ionic radius 0.95 Å) replaces calcium, as it might well do on the basis of size, the question of the unsatisfied valence must be answered. As yet, biological studies regarding heteroionic exchange must wait for mineralogic evidence that any of these ions are actually ever part of the lattice.

B. Relationship of Bone to Mineral Apatites

Returning to the problem of bone, Armstrong (1950) reviews ten proposed formulations for the nature of the bone salt (See Table II). All start with what is regarded as the best analytical data. To this they have added a series of secondary observations: solubility, incongruent solubility, temperature curves, and diffraction.

The present status of bone is subject to the following limitations. First, since the composition of adult bone is not uniform, the problem has been to explain the variations within the framework of what is generally known about apatite structure. Second, the origin of the apatite lattice in calcified tissues is far from clear since it goes down only in selected biological situations, and under conditions such that it does not have a true solubility constant.

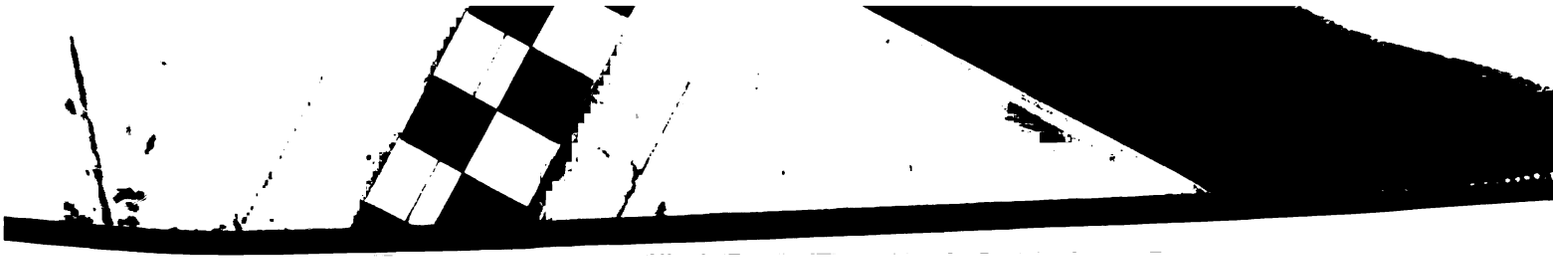
In view of the widespread occurrence and uses of the apatite phosphates, as fertilizers, there is a surprising lack of information regarding the mechanisms of precipitation so as to give the various ionic ratios encountered. The chief reason for this is that there are primary, secondary and tertiary orthophosphates, all of which form various types of mixed complexes depending on pH and the presence or absence of a variety of both cations and anions. The presence or absence of water in the final crystal lattice is also critical.

TABLE II*

CONCEPTION OF THE CONSTITUTION OF THE MINERAL PHASE OF CALCIFIED TISSUE

	Formulation	Proponents	Year & Ref.	Ca/PO_4
1.	Mixture of $Ca_3(PO_4)_2$ $Mg_3(PO_4)_2$, $CaCO_3$, CaF_2, etc.			
2.	$Ca_5H_2(PO_4)_2$	Berzelius	1845 (2)	2.5
3.	$Ca[(O,PO_3Ca)_2Ca]_3CO_3$	Hoppe-Seyler; Werner; Gassman Gassman	1862 (3) 1907 (4) 1937 (5)	1.67
4.	$Ca(OH)_2[Ca_3(PO_4)_2]_3$ + simple carbonates and bicarbonates	Klement	1929 (6)	1.67
5.	$CaCO_3[Ca_3(PO_4)_2]_n$ n = 2 or 3	Bogert and Hastings	1931 (15)	1.67-1.75
6.	$(OH)_2Ca_6 \cdot [(P,C)O_4]_6(Ca,C)_4]$	Gruner, McConnell, and Armstrong	1937 (13)	
7.	$Ca(OH)_2 \cdot [Ca_3(PO_4)_2]_3$	Hodge, et al.; Bale; Thewlis, Glock, and Murray	1938 (20) 1936 (21) 1939 (22)	1.67
8.	$CaCO_3 \cdot n[Ca_3(PO_4)_2]$ n = 1.86 - 3.33 m $CaHPO_4 \cdot n[Ca_3(PO_4)_2] \cdot CaCO_3$	Sobel, Rockenmacker, and Kramer	1945 (23)	
9.	$(Ca, Mg, Na)_9 \cdot (PO_4, CO_3)_6 \cdot (H_2O)_2$ (substituted hydrated tricalcium phosphate)	Hendricks and Hill	1947 (24)	
10.	$Ca_3(PO_4)_2 \cdot H_2(OH)_2$ + $CaCO_3$, $MgCO_3$, etc.	Dallemagne	1947 (25-31)	

* This Table appears as Table I, page 12, Armstrong (1950), slightly modified.



Although the apatites are classically described as anhydrous crystals, McConnell is convinced that water occupies a lattice position and Neuman and Neuman (1953) suggest the presence of hydronium ions. Both agree that water is lost between 300-500° C. as reported by Dallemagne (1952).

It is clear that in the case of bone, the problem is that of a true heterogeneous system, in the physical chemical sense, with numerous "impurities", and in which exchange and adsorption play highly significant roles. Co-precipitation has often been suggested but never proved. Therefore, the biologic situation cannot be described only in terms of simple analytical values for calcium and phosphorus. The significant components of this system are still not completely established. As a result, in a recent review (Neuman and Neuman, 1953) the following statement is made, "It is generally agreed that the apatite lattice of bone mineral approximates the structure of hydroxylapatite." This is a very small advance in the years since the observations of DeJong. Obviously, proof for the many hypotheses currently in print requires more rigorous and sustained investigation than is presently being pursued.

C. Views Concerning the Nature of Bone Mineral

In addition to its major constituent, basic calcium phosphate which is present to the extent of 80 - 85%, bone mineral contains 3 - 5% carbonate (Neuman and Neuman, 1953), with small amounts of citrate - 2%, sodium - 180 mEq/Kg, potassium - 19 mEq/Kg, magnesium - 1% and a trace of fluorine (Gabriel, 1894, Bergstrom, 1952, Huggins, 1937, McLean and Urist, 1955). The Ca:PO_4 ratio does not vary much in normal adult bone and is about 1.92-1.99 (Huggins, 1937). Leulier, Policard,

and Revol (1941) reported a calcium to phosphate ratio of 1.8 in the diaphyseal bone of chick embryos. Low calcium, high phosphate diet has been shown to decrease the $Ca:PO_4$ mole ratio (Sobel, Rockenmacher, Kramer, 1945). In spite of the variations encountered in bone composition, x-ray diffraction has shown only the presence of an apatite structure (Neuman and Neuman, 1953). However, it is impossible to rigorously prove the presence of many plausible minor crystal species in the presence of strong apatite patterns unless their concentration is considerably greater than might be expected.

The "formulation 4" from Armstrong's Table $Ca(OH)_2\ [Ca_3(PO_4)_2]_3$ proposed by Klement (1929), indicates that the basic calcium phosphate, hydroxylapatite, is the principal constituent of bone. This formula tacitly assumes that either carbonates or bicarbonates of Na, K and Ca are mixed in the bone mineral as separate species along with hydroxylapatite or that non-crystalline components exist.

The problem of carbonate in bone has been extensively discussed by Gruner and McConnell (1937), McConnell (1952), Hendricks and Hill (1942 and 1951), and Hendricks (1952). Both groups studied mineral carbonate apatite and attempted to relate their findings to bone. Their formulations (numbers 6 and 9 in Table II) again may be viewed as an effort to relate the analytical data to a molecular structure. Even though both groups agree that carbonate can be and probably is present in the apatite lattice, the exact position and total amount so incorporated is vigorously argued. McConnell, as a professional crystallographer, has not yet felt it necessary to abandon the attempt to fit analytical components into a unified crystal structure.

Hendricks, on the other hand, with crystallographic experience also dating back to the early twenties, is frankly willing to admit various types of association short of incorporation into an apatite lattice. Since they are in possession of the same experimental data, but draw divergent conclusions, it is obvious that present data are not sufficient for the solution of the problem.

There is a great deal of evidence in favor of hydroxylapatite as such. Among the foremost proponents of this concept are Thewlis, Glock and Murray (1939), Hodge and co-workers (French, et al., 1938) and Bale (1940). These workers have demonstrated that x-ray diffraction patterns obtained from "calcified tissue" and "synthetic hydroxylapatite" are indistinguishable. Unfortunately, doubt may be expressed both as to whether their "bone" was in the "normal" state and whether their synthetic hydroxylapatite was "pure", in the sense of being carbonate free. The general feeling among these investigators is that carbonates and other salts are occluded, adsorbed or interstitially crystallized. Hendricks and Hill (1951) quoted Brasseur and Dallemagne as saying that crystalline calcium carbonate of bone could not be detected in the amounts present, about 4 to 5 percent. However, Hendricks concluded that the methods used would detect even 1 percent carbonate (as calcite). In a very recent review, Posner (1955) claims to have shown the presence of calcite mixed with the phosphates of the inorganic portion of bone and teeth, and also in the mineral francolite by use of infrared spectroscopy. Dallemagne in 1952, primarily on the basis of dehydration studies, concluded that alpha (or hydrated) tricalcium phosphate is the principal bone salt. This may be represented

by the formula $[Ca_3(PO_4)_2]_3 \cdot H_2(OH)_2$ to show its similarity to $[Ca_3(PO_4)_2]_3 Ca(OH)_2$, given for hydroxylapatite. However, it is evident that α tricalcium phosphate has a lower $Ca:PO_4$ ratio than hydroxylapatite.

D. Techniques of Bone Preparation

A good deal of the confusion which exists regarding the nature of mineral phase of calcified tissue arises from the fact that untreated bone gives very poor diffraction patterns. Since the lines from untreated bone are broad and poorly defined (DeJong, 1926, Taylor and Sheard, 1929) a valuable clue is given regarding crystal size. At the same time, the poor resolution of the broad lines makes the pattern from untreated bone indistinguishable from those arising from a wide variety of mineral apatites (Roseberry, Hastings and Morse, 1931, and Bredig, 1933). Therefore, a number of techniques of bone preparation have been used in an effort to more rigorously establish the nature of the bone salt (Neuman and Neuman, 1953). Any manipulation may be criticized since even after simple removal from the intact organism and drying, bone can no longer be considered completely normal. Dry heat ashing, one of the most drastic techniques, produces an ash which gives a much sharper diffraction pattern than fresh bone (Klement & Trömmel, 1932). The procedure employed by Gabriel (1894) (boiling glycerol and KOH extraction of the organic matter) has been used extensively in bone preparation and has the advantage of a rather low temperature in an anhydrous medium. Even though some structural modifications in the bone salt undoubtedly occur, the diffraction pattern does not change (Dallemagne, 1952). A modification using ethylene

glycol and KOH has been used by Crowell, Hodge and Line (1934).

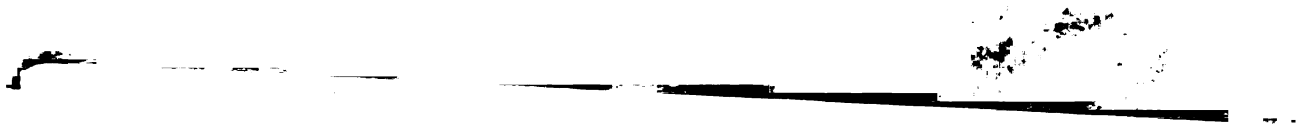
Boiling bone for 24 hours followed by tryptic digestion was employed by Bell, Chamber and Dawson (1947). This method causes structural modifications and loss of mineral elements (Dallemagne, 1952).

For preparation of bone and teeth for electron microscopy, Robinson and Bishop (1950) autoclaved fresh bone at 27 pounds for 2 hours and blended it in distilled water. Later, Robinson and Watson (1952) obtained an electron diffraction pattern from n-butyl-methacrylate embedded bone, which showed good agreement with x-ray diffraction data on bone treated by various other methods. Again the rigors of the embedding process may have altered the bone mineral considerably.

Recently ethylenediamine (ED) has been used by Arnold (1952) to study the amount of Ca^{45} combined with the organic matrix of bone at various times after deposition. This report did not give a chemical or x-ray diffraction analysis of the ED ashed material, so it is not possible to estimate an effect on the crystal lattice from the data reported.

E. Information Obtained From Behavior of Calcified Tissues Upon Heating

Taylor and Sheard (1929) showed that incinerated bones produced much sharper lines on a diffraction pattern, and attributed this to growth of the crystals of bone mineral. On the basis of the diffuse lines obtained from unheated bone they concluded that the bone crystals probably contained only a few hundred molecules. Klement and Trömmel (1932) also showed this sharpening of lines upon heating bone at 600° C.



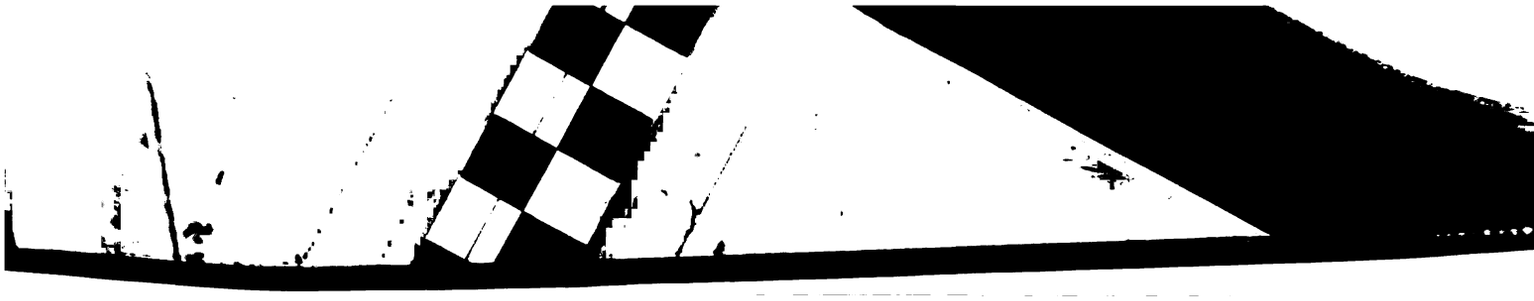
They also prepared a synthetic apatite with the material precipitating at pH 7.15, which when heated showed some β-$Ca_3(PO_4)_2$ lines.

Later, Hodge, LeFevre, and Bale (1938) conducted a thorough study of diffraction patterns of several calcium phosphates. They showed that precipitates containing a Ca:P mole ratio of 1.93 to 2.12 all exhibited a hydroxylapatite diffraction pattern before ignition, but after ignition at 900°C. for one hour, precipitates with Ca:P ratios of 1.93 and 1.98 gave a pattern of β-$Ca_3(PO_4)_2$, whereas, those with Ca:P ratios of 2.10 and 2.12 still gave a pattern of hydroxylapatite. They concluded that a reaction took place between the hydroxylapatite and excess adsorbed phosphate ions to give β-$Ca_3(PO_4)_2$ in the cases of Ca:P ratios of 1.93 and 1.98.

Sobel and co-workers (Hirschman, et al., 1947 and Sobel, et al., 1945 and 1949) were able to lower the Ca:PO_4 mole ratio of bone below 1.93 by diet and in each case varified the findings of Hodge, LeFevre and Bale on precipitated calcium phosphates. The pattern of ignited bone then can be used as a rough index of the Ca:P ratio. Neuman (1951), in discussing the work of Kunin, viewed the high phosphate bones of Sobel as a substitution of some of the calcium by hydronium ions which will dehydrate at about 400°C. When this occurs, the lattice configuration of apatite presumably collapses, giving β-tricalcium phosphate.

F. Chemical Properties of Bone Crystals Related to Size and Surface

Since the work of Taylor and Sheard (1929), in which they concluded that bone crystals probably contain only a few hundred molecules, much



work has been done on the estimation of crystal size. The earlier workers based their conclusions on the line broadening of the diffraction pattern, and estimated the crystals of bone salt at 10^{-5} to 10^{-6} cm (Logan, 1940). Determination of surface area by the gas adsorption technique (Woods, 1947 and Neuman, 1950) tend to confirm that the crystals are of this order of magnitude.

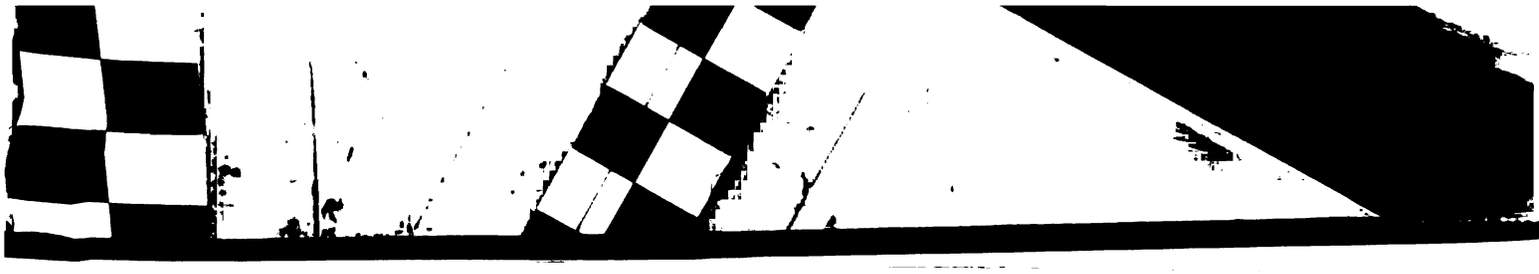
Confirmation of the size of bone crystals has come from the excellent work of Robinson (1951) and of Robinson and Watson (1952, 1953). By using the electron microscope they were able to see and measure bone crystals, and obtain the typical apatite pattern from them. They report the average dimensions for bone crystals as 500 x 250 x 100 Å, although in sectioned material from the outer cortex of the human rib, the average crystal is about half this size. This would give between 100 and 250 square meters of exchangeable surface area per gram of bone crystals. In terms of the entire skeleton of a 70 kg. man, this would exceed 100 acres (McLean and Urist, 1955). It is easy to see then why Neuman (1950) commented that "increasing numbers of investigations have been forced to use surface chemistry to explain results obtained in bone studies." This approach has strengthened the effort to explain the variation in the $Ca:PO_4$ ratio in bone and synthetic precipitates, (and indeed the presence of carbonate, citrate, Na, and other ions "foreign" to the apatite lattice) by assuming "adsorption" rather than incorporation into the lattice.

The fact that synthetic precipitates of "tricalcium phosphate hydrate" possess a $Ca:PO_4$ mole ratio of 1.50 rather than 1.67 (theoretical for hydroxylapatite) has caused two groups of investigators to invoke

an adsorption hypothesis, but each with a different interpretation. Dallemagne (1952) summarizes one position by pointing out that tricalcium phosphate hydrate with a $Ca:PO_4$ mole ratio of 1.50 may be thought of as a calcium deficient hydroxylapatite. The ratio could be increased by adsorption of calcium hydroxide, which might not be fixed in a crystal lattice unless brought to a high temperature. Hodge (1938) and co-workers have supported the alternative opinion that tricalcium phosphate is simply hydroxylapatite with sufficient PO_4 ions adsorbed to lower the $Ca:PO_4$ ratio to 1.50. Posner and Stephenson (1952) also support this view. Neuman and Neuman (1953) point out the limitations of the adsorption theory when considered in the light of surface area. It would require adsorption of one PO_4 for each 4 PO_4 ions in the crystal to give a $Ca:PO_4$ mole ratio of 1.33. Precipitates with a $Ca:PO_4$ ratio above 1.33 give only an apatite diffraction pattern (Arnold, 1950). Neuman and Neuman (1953) point out that because of the extremely small size of the crystals, one half to two-thirds of the unit cells are located in the surface. Postulation of an isomorphic substitution of hydronium ions for surface calciums would explain the existence of precipitates with $Ca:PO_4$ mole ratios of 1.4 to 1.8. Such a system would still retain a characteristic apatite lattice structure. This hypothesis approaches that of Dallemagne.

G. Chemistry of the Process of Calcification

"Knowledge of the composition of primary calcification, i.e., of the composition of bone salts immediately after deposition, would probably throw considerable light on the mechanism of calcification"

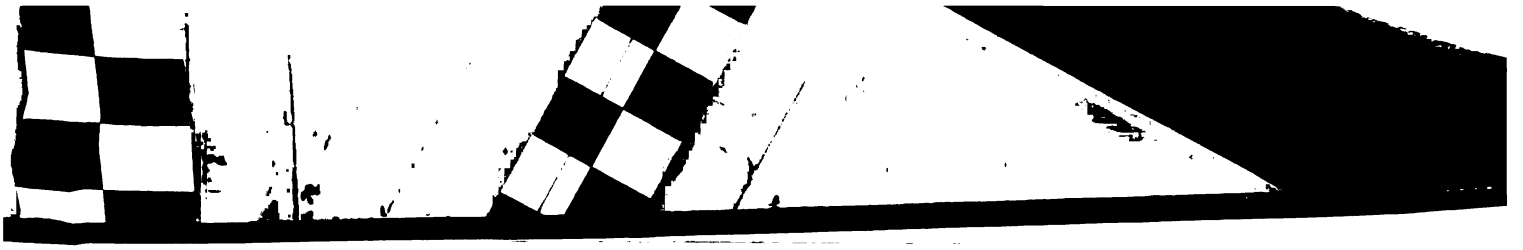


(Shear and Kramer, 1928a.).

The simplest structural unit of apatite contains 18 ions. It is inconceivable that this large a number of ions could spontaneously aggregate in the proper spacial configuration of the lattice. This has led investigators to postulate some inorganic intermediate in the process, which has gone undetected, or some organic complex or template which would serve as a nucleation center (Roseberry, Hastings and Morse, 1931). These two approaches have been reviewed in a number of papers (Huggins, 1937, Logan, 1940, and Neuman and Neuman, 1953).

An extensive study of calcium phosphate precipitates was carried out by Holt, LaMer, and Chown (1925) in an attempt to better understand the process of calcification. Shear and Kramer (1928a) developed the idea that $CaHPO_4$ was an intermediate in the formation of bone salt, even though it is not stable at the pH of body fluids. No $CaHPO_4$ has been detected in bone by x-ray diffraction techniques (Taylor and Sheard, 1929, and Roseberry, Hastings and Morse, 1931) even in "young" bone. Nor did Hirschman, Sobel, and Fankuchen (1953) find it in in vitro calcification studies of epiphyseal cartilage. However, $CaHPO_4$ could not be excluded from consideration on this basis alone since $CaHPO_4$ was converted to apatite when placed in the solutions employed for calcification in vitro. A number of points of evidence for and against the importance of $CaHPO_4$ in the process of calcification has been presented by Hodge (1950).

The recent work of Robinson and Watson (1953) showing the intimate relationship between the collagen fibers and bone crystals has given considerable impetus to the concept of some organic nucleation



center. This center would initiate a stepwise binding of the proper ions into the appropriate configuration for the complete crystal (Neuman and Neuman, 1953). The close association between the periodicity of the collagen fibers and the presence of bone crystals is suggestive. Whether periodic collagen side groups initiate the crystal lattice or merely prevent the growth of the crystal beyond a certain size is entirely within the realm of conjecture.

After some twenty-five years of concentrated effort we still read, "The calcification process is so poorly understood that no comprehensive hypothesis can be given" (Neuman and Neuman, 1953).

H. Objectives of Thesis

From the foregoing, it is evident that we do not really know the structure of bone, other than that finally some apatite lattice is involved. The biochemical relation of this general lattice to specific ions is obscure and the role, either active or passive, of the organic material is conjectural. How then, can one understand the initiation and development of this tissue when the significance and inter-relations of its component parts are unknown? The exact roles of vitamin D and of the parathyroid hormone and other agents in regulating exchange of radioactive calcium into and out of the system cannot finally be understood except in terms of the ionic anatomy which has been sketched above. There is yet no adequate explanation for the direct effects either of vitamin D or of parathormone on bone, although the "gross" histological picture is reasonably clear.

It is surprising that no work has been done on very young bone

which is just beginning to calcify. Nor has any serious attempt been made to study calcification of arteries by the physical methods initiated by DeJong. The study of forming bone has been done almost entirely on rachitic cartilage placed in various artificial calcifying media. There is no good chemical information other than total calcium and phosphorus, for any of these situations, or for the preosseous cartilage in which bone is about to form. In fact, for cartilages in general, Eichelberger (1952) was able to cite only two references to its ionic composition. And yet, would one expect to find a crystalline precursor for apatite, or an organic template, in mature bone in which growth has ceased and only the slow remodeling is going on?

No one has yet looked at the diffraction pattern of newly formed bone with the object of comparing it with older bone. Would one choose for such a study the growing bone of a weanling rat in which the actual new bone laid down in a day represents less than 1% of that already present?

What about the preosseous cartilage? Does it show any evidences at all of impending change?

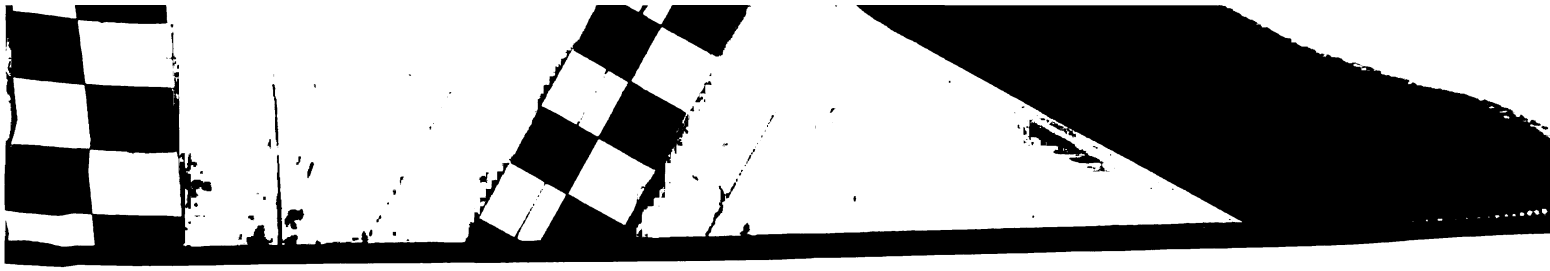
It was these questions which led to the selection of the embryonic chick femurs as the biologic material for this investigation.

Although general pessimism has been expressed concerning the adequacy of the various diffraction techniques, as currently employed, the data they provide are nevertheless clearly more sensitive than those resulting from routine chemical analyses. This will be demonstrated again in the data which follows. Further, electron diffraction with Selected Area Aperture, a technique which has scarcely been tried

in biology is an ideal method for circumventing the "averaging"or "weighting" effect inherent in standard x-ray diffraction.

This thesis, then, reports the data resulting from study, by diffraction methods, of the initial stage of bone formation. Search was made for crystalline "precursors" of apatite in a biologic situation where they should be relatively unobscured (if present at all) and using techniques to minimize interference by heavy apatite patterns. Further, specific study of the "apatite" patterns themselves was made to determine whether shifts could be detected between the time of initial lay down and later times when the lattice might be regarded as being more "mature". The heat conversion of apatite to whitlockite ($\beta Ca_3(PO_4)_2$) was also studied both on dried and on ethylene diamine extracted bone mineral in an effort to find lattice differences associated with removal of organic matter in the extraction procedure. Thus a serious effort was made to obtain direct crystallographic evidence for the presence of constituents of types which have often been regarded as necessary intheory but which existing data could neither prove nor disprove. This search was limited only by the instrumentation available.

Alternative approaches are conceivable but in general, they are less than satisfactory for the objectives of this study on two counts. First, many(very useful) animal experiments can be devised to show the importance of a variety of variables on such things as the rate of lay down of the "apatite" lattice, for example. Such experiments would prove only how fast "something" goes down but could not tell what that "something" was. In this investigation, we are concerned



with exactly what happens when ossification begins.

Second, alternate physical or chemical methods which would be useful in gross studies, in general, lack sufficient sensitivity for work on the micro scale required in this problem. Thus, even chemical analysis by diffraction often has a much greater sensitivity than conventional analytical methods, particularly when used on mixtures with interfering substances. The crystals being investigated are extremely small (less than the distance between the 640 Å spacing on collagen fibres, for example) and are surrounded by a formidable mixture, containing both electrolytes and non-electrolytes. This situation also rules out the usual techniques of crystal optics and molecular refractivity which have been used in studies of the large macro crystals of classical minerology.

The only logical alternative for the intimate study of the intimate study of the physiology of bone mineral formation involves the construction of more highly refined diffraction equipment. Although such work will undoubtedly be done in the next decade, it is obviously beyond the scope of this thesis.

II. EXPERIMENTAL METHODS

A. Electron Diffraction Studies

1. Preparation of Materials

Femurs were harvested from chick embryos and placed in two groups for further treatment. One group of bones was dried at 100° C. for 24 hours. A second group was ashed at 600° C. for 5 hours. Another group of femurs was taken from two-week old chicks. These were wet ashed with ethylenediamine for several hours (until the ash appeared nearly white, and the solvent in contact with the bone was colorless).

Ethylenediamine ashing was carried out by placing dry bones in the thimble of a continuous extraction apparatus with boiling ethylenediamine (ED) as the solvent. (B.P. 117° C.) In such a procedure, the temperature of the bone never exceeded the boiling point of ED. Extraction was usually complete in four hours, after which excess ED was removed by washing the ash with distilled water. The residue was air dried at 38° C. and the fragile white ash ground to pass a 200 mesh sieve. For electron microscopy all preparations were further reduced in size by placing the mortar ground material between two ground glass disks, one of which was rotated at about 500 revolutions per minute. The extremely fine powder thus produced was then transferred to an electron microscope specimen grid by directly dusting on to the surface of the Formvar film.[1] All grids were lightly shadowed with gold.

[1] Made from drying a 1% solution of polyvinyl formal in ethylene dichloride.

2. Instrumentation

The grids were studied and photographed in an RCA Electron Microscope EMU-2C at magnifications between 5,000 and 10,000. Additional photographic enlargement resulted in final magnification indicated on the figures. Diffraction patterns were obtained with a Selected Area Aperture which reduces the area contributing to the diffraction pattern to as little as one μ^2. Under favorable conditions, it was possible to observe and photograph patterns from single crystals.

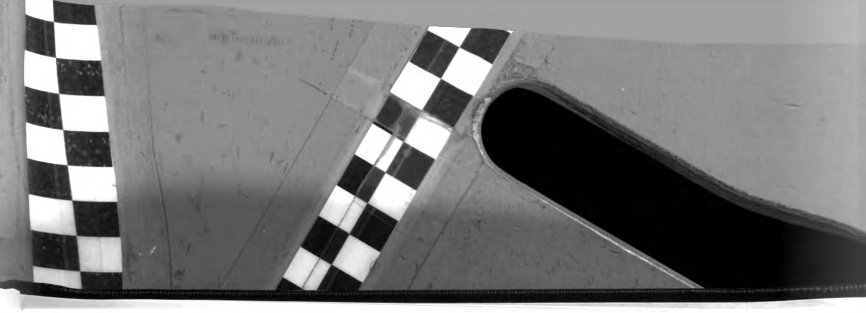
3. Mathematical Considerations

Determination of the interplanar spacings, which produce the diffraction pattern, may be done most accurately by comparison with an internal standard such as gold. Since the effective wave length of a 50 KV electron beam is very short, the diffraction angle, θ, is also very small. Consequently, cos θ is never less than .998 and may be taken as 1.000. This makes it possible to calculate an unknown interplanar spacing, d_X, from the simple relationship

$$d_x = \frac{\text{diameter Au ring} \cdot d_{Au}}{\text{diameter X ring}}$$

without introducing appreciable errors other than those of measurement. The value $d_{Au(100)} = 2.344$ Å. Since considerable ellipticity exists in the patterns, the diameter of the gold ring was measured at the same angle of rotation as a line connecting the spots being measured.

No other investigators in this field (or indeed in most electron diffraction work) have routinely used internal standards, nor are error estimates to be found in the literature. Since d values vary rather



widely in the literature, it is evident that technical factors have not been always adequately controlled. As an example of methods used in this study, a complete set of individual measurements and calculations from a single plate are found in Table XII in the appendix.

Considerable difficulty was experienced in locating the exact center of bright spots or of a relatively broad ring. An estimate of errors in interplanar spacings resulting from an error in linear measurement of 0.2 mm. and 0.5 mm. on a 2.7 X projection print is given in Table III. Since a measurement error of 0.5 mm. was easily possible, the difference between d values calculated from a single pattern may be explained solely on the basis of errors in linear measurements.

B. X-Ray Diffraction Studies

1. Preparation of Materials

As in the previous study, the biologic materials were chick embryos incubated at 38° C. from six days to 21 days; and also two week old chicks. The embryonic femurs were removed and then freed of adhering soft tissue by gentle movement on hard surface filter paper. This process also removed excess tissue fluid.

In studies where the material was run "wet", the femur or a small longitudinal section was placed in a glass capillary tube and sealed with a micro burner. Material run "dry" was first placed in a 100° C. oven for 24 hours. Material designated as ED ashed was obtained by the extraction method described previously.

The wet and dry embryonic femurs from six to eleven days were introduced directly into thin walled glass capillary tubes suitable for

TABLE III

CALCULATION OF ERRORS INVOLVED IN ESTIMATION OF SPOT POSITION ON AN ELECTRON DIFFRACTION PATTERN*

Region	Linear Error	Error in d Å
4.5 Å	0.2 mm.	4.50 ± .04
	0.5 mm.	± .09
2.6 Å	0.2 mm.	2.60 ± .02
	0.5 mm.	± .04
1.5 Å	0.2 mm.	1.500 ±.004
	0.5 mm.	±.010

*Calculations based on measurements of a 2.7 X projection print of the original pattern.

TABLE IV

CALCULATION OF ERRORS INVOLVED IN ESTIMATION OF LINE POSITION ON AN X-RAY DIFFRACTION PATTERN

Region	Linear Error	Error in d Å
8.0 Å	0.1 mm.	8.00 ± .05
6.0 Å	0.1 mm.	6.00 ± .03
4.0 Å	0.1 mm.	4.00 ± .02
3.0 Å	0.1 mm.	3.00 ± .01
2.5 Å	0.1 mm.	2.500 ± .005
2.0 Å	0.1 mm.	2.000 ± .003
1.5 Å	0.1 mm.	1.500 ± .002

x-ray diffraction.[1] All other bones were ground and introduced as a fine powder.

2. Technical Aspects

Capillary tubes containing the various bone preparations were placed in the cradle of the powder camera and oscillated through 20° at the rate of one complete oscillation per minute, the tube always remaining at the exact center of the camera. Exposures were made on Kodak No-Screen x-ray film usually for four hours. Nickel filtered copper K-alpha radiation was used with the tube being operated at 35 KV and 20 MA. The camera has an effective film diameter of 14.32 cm., so that with normal film shrinkage, the angle of diffraction, θ, may be obtained either by multiplying the distance (in cm.) between two corresponding lines by a factor of 2 or by multiplying the distance from the center of the undeviated beam to a line by a factor of 4. A graphic solution of Bragg's Equation, $n\lambda = 2\,d \sin\theta$, for each value of θ gave the desired interplanar spacings in Ångstroms (Parrish and Irwin, 1953). Test runs on standard materials[2] showed this calibration to be in excellent agreement with published values.[3]

Line positions were measured to the nearest millimeter and estimated

[1] Glaskapillaren für röntgenographische Aufnahmen nach Debye-Scherrer were obtained from Caine Sales Co., Chicago, Illinois, having a diameter of 0.5 mm and a wall thickness of 0.01 mm.

[2] Checks on calibration of powder camera made with silica, sodium chloride, and potassium chloride. Values for sodium chloride and potassium chloride are compared with ASTM (1950) values in Table XIV.

[3] All Figures reproduced in this thesis are slightly larger than the original film, by a factor of 1.08.

to the nearest 0.1 millimeter, with repeated measurements not varying more than ± 0.1 mm. for relatively sharp lines. Some difficulty was experienced in accurately determining the exact center of broad lines as they appear in the dry and ED ashed bone. A calculation of the error produced in the interplanar value by an error of 0.1 mm. in the linear measurement is given in Table IV.

Relative intensities were estimated by use of a convenient film comparator constructed by a method similar to that of Klug and Alexander (1954). A strip of film was wrapped in aluminum foil and exposed to a weak x-ray beam collimated with several narrow slits. The exposure time was varied so that a suitable range of film densities resulted. Visual matching of line densities with the standard allowed relative densities to be established.

Since it was noted that preosseous embryonic bone when dried contained a mixture of crystalline NaCl and KCl, a rough estimate of the relative amounts of each was thought to be of value. Therefore, an equal parts (by weight) mixture of NaCl and KCl was prepared and its diffraction pattern studied. It was observed that the 3.13 Å KCl line and the 2.81 Å NaCl line had about equal relative intensities. Since the intensity of the diffracted beam is directly proportional to the number of diffracting crystals, the relative amounts of NaCl and KCl can be estimated from the ratio of the relative intensities of their strongest lines. However, it must be recognized that this method provides only a very rough estimate of relative concentration because an error (which may occasionally be as high as 20%) may be involved in the estimation of the relative intensities. In addition, only a very

small part of the femur is in the path of the beam. There may be real local variations in the Na/K ratio.

Therefore, quantitative chemical analyses of sodium and potassium was carried out by flame photometric methods.

C. General Conventions Involving Diffraction Data

All measurements are reported after conversion to interplanar spacings in Ångstroms. Unit cell dimensions are easily calculated using the standard formula for hexagonal crystals. This reduces to $a = 2d$ for the 110 plane or $a = 4d$ for the 220 plane and $c = d$ for the 001 plane or $c = 2d$ for the 002 plane. These simplifications naturally do not apply to NaCl or KCl.

III. PRESENTATION OF DATA

A. Electron Diffraction Studies

Two distinct types of fragments were consistently seen on the grids when viewed in the electron microscope. One type consisted of rather dense fragments, which varied in size down to less than 0.1 μ. They were apparently too thick to allow passage of the electron beam or were insufficiently crystalline to give a diffraction pattern. Many of these particles may be seen in Figure I. In addition to these "dense" particles, thin well-formed crystals may also be seen in a variety of sizes and shapes up to several micra. The source of these thin crystals may not be bone, necessarily, since they have been seen on some grids which had not been "dusted" with the bone preparation. Good diffraction patterns have consistently been obtained from these crystals with the use of the Selected Area Aperture (See Figure 2). The average values for the six largest spacings are given in Table V. A complete summary of all measurements made is given in Table XIII in the appendix.

TABLE V

AVERAGE VALUES FOR SIX LARGEST SPACINGS OF CRYSTAL OBSERVED WITH CALCULATED VALUES FOR THE "a" AXIS

	100 d Å	110 d Å	200 d Å	120 + 210 d Å	300 d Å	220 d Å
Spacing	4.57	2.62	2.30	1.739	1.525	1.329
Calc. "a"	5.28	5.24	5.31	5.31	5.28	5.32

In all the patterns that were produced by the thin crystals, no

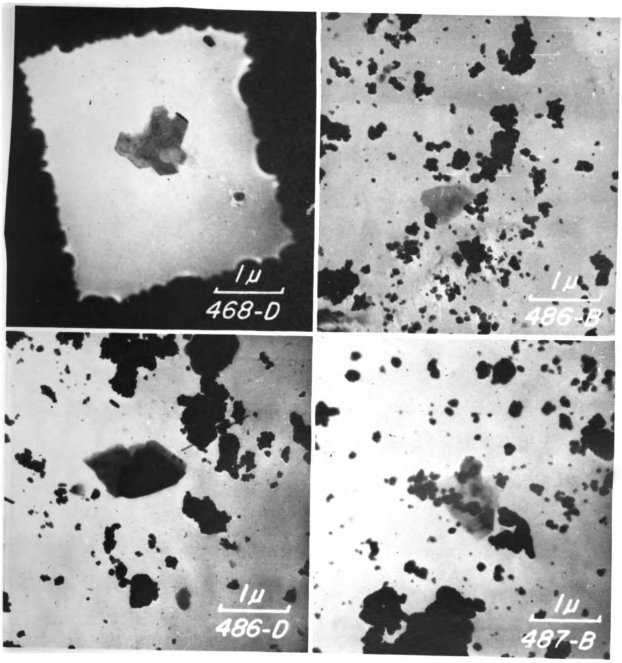


Fig. 1 *Electron Micrographs of Powdered Bone*

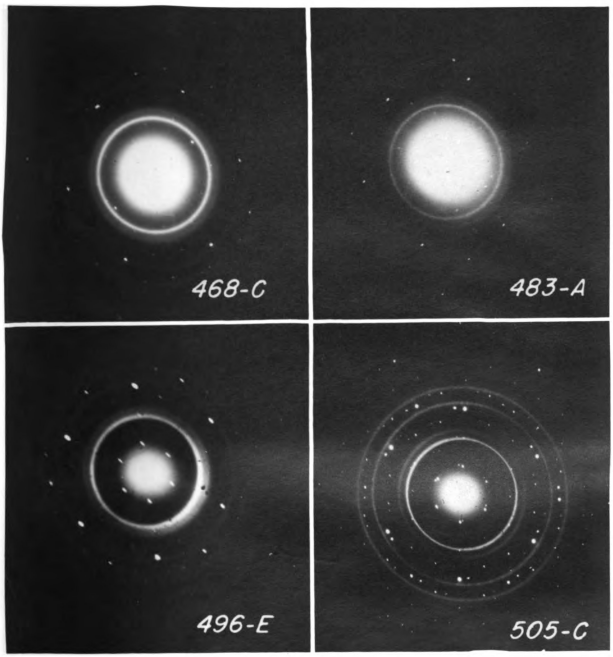
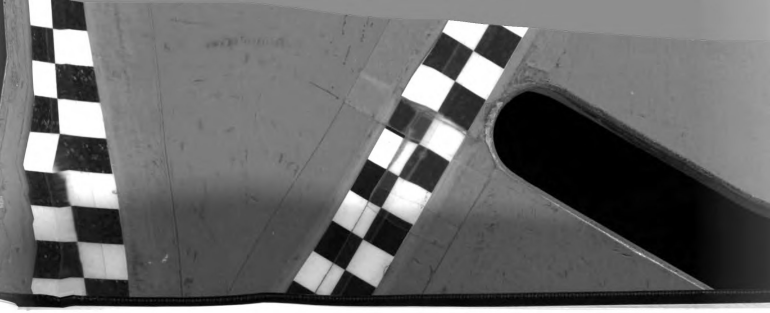


Fig. 2 *Electron Diffraction Patterns from Thin Crystals in Fig. 1*



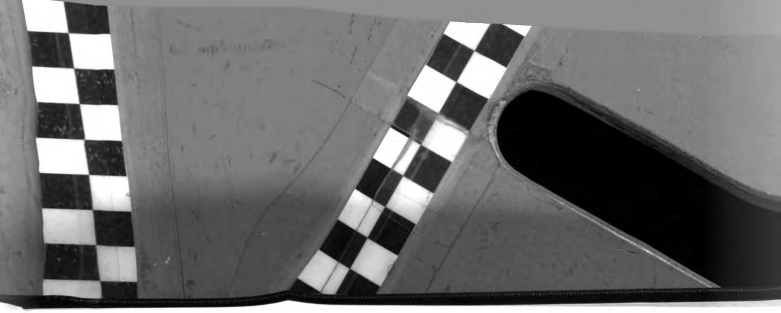
spots were ever seen other than those whose calculated spacings appear in Table V for the range covered by the Table. Additional spacings from greater diffraction angles all fit into the hexagonal pattern established by the larger spacings, and can be assigned diffracting planes consistent with those assumed for the given spacings. In some cases, several crystals were obviously contributing to the diffraction pattern, each giving its own characteristic hexagonal single crystal pattern (Figure 2).

Since considerable individual variation existed, no good estimate of relative intensity of diffraction spots was made. In general, however, as may be seen from Figure 2, the spots assigned to the (300) plane are the most intense with the 110 and 220 next in order. This is of little value for comparisons with x-ray diffraction data, since the x-ray values are based on powder patterns with random orientation of the crystals, which obviously is not the case here.

Even though the crystals here described must be regarded as a contaminant, identification would be of considerable interest. If we assume that the innermost spots visible in the diffraction pattern are due to the 100 plane of a hexagonal crystal, an "a" axis of 5.29 Å may be calculated.[1] Since the patterns observed were always hexagonal, it must be assumed that the "c" axis of the crystal was always parallel to

[1] Formula used to calculate spacings from hexagonal crystals.

$$d\,(hkl) = \frac{1}{\sqrt{\frac{4}{3}\left(\frac{h^2 + hk + k^2}{a^2}\right) + \left(\frac{l^2}{c^2}\right)}}$$



the electron beam. This makes it impossible to obtain a value for the "c" axis with present equipment.

Search of the available literature (Frevel and Rinn, 1953; American Society for Testing Materials, 1950; Donnay and Nowacki, 1954) reveals only very few possibilities. Among these, $CaNaPO_4$, a = 5.24, c = 7.14 fits the value calculated from the observed crystal pattern rather well and would seem likely to be present.

B. X-Ray Diffraction Studies

1. Data from Dry Embryonic Femurs of Six to Ten Days Incubation

Diffraction patterns were obtained from dry early embryonic femurs beginning with six days and continuing through ten days of incubation. A total of twenty-three patterns were measured and all lines present identified. Bones were obtained from two different settings of eggs (designated as series "a" and "b"). However, since no differences were observed between the two series, both series were averaged together. The complete tabulation of individual lines is found in Tables XV to XX in the appendix. The diffraction patterns suitable for reproduction are found in Figures 3 and 4, and Figures 9 and 13 in the appendix.

The interplanar spacings for the lines appearing on the diffraction patterns for six, seven, and eight day femurs were calculated and compared with values in the ASTM File (1950). It was found that they result from a mixture of NaCl and KCl. No additional crystalline components could be identified. Therefore, a diffraction pattern was made from an equal parts mixture of NaCl and KCl. The results were in ex-

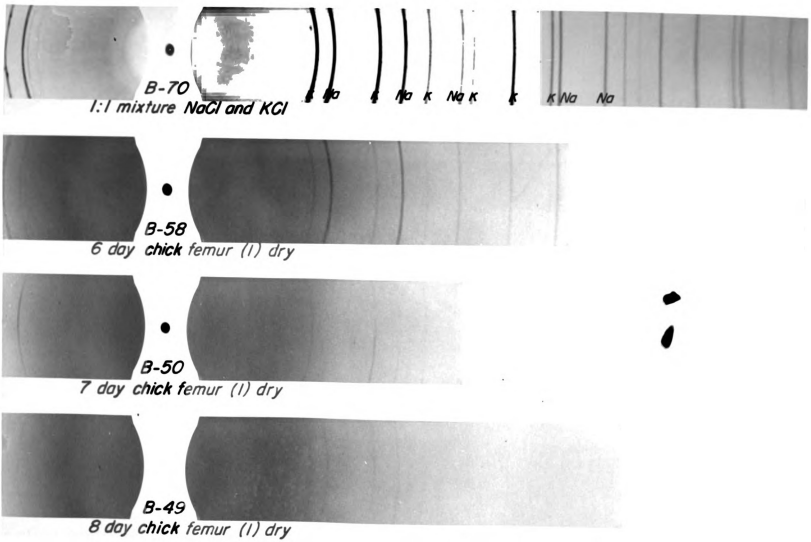


Fig. 3 X-Ray Diffraction Pattern from Preosseous Femurs Compared with Salt Mixture.

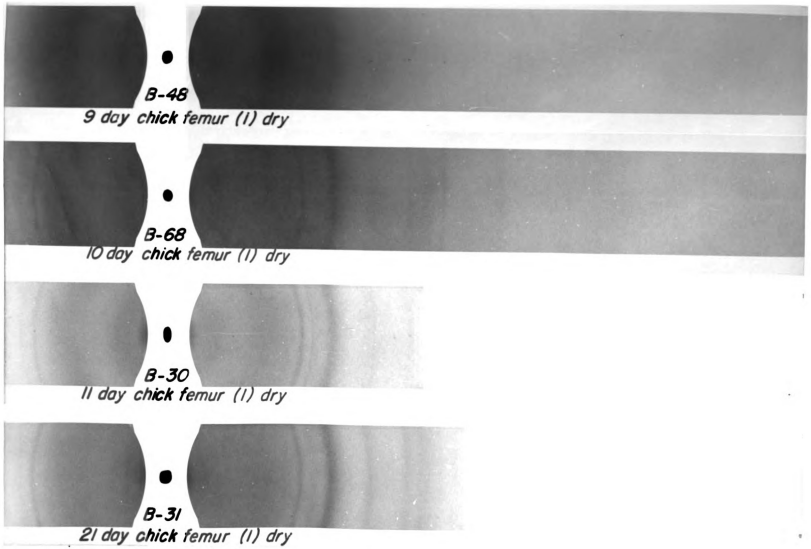


Fig. 4 X-Ray Diffraction Pattern from Embryonic Femurs.

TABLE VI

CRYSTALLINE COMPONENTS OF DRY EARLY EMBRYONIC CHICK FEMURS

Age (in days)	Series	Pattern Number	"Apatite"	NaCl	KCl
6	a	B-42*	-	30%	70%
		B-58	-	80%	20
7	a	B-43	-	30%	70%
		B-50	-	30%	70%
		B-56	-	20%	80%
	b	B-60	-	5%	95%
		B-62	-	0%	100%
		B-67	-	0%	100%
8	a	B-44	-	30%	70%
		B-49	-	50%	50%
		B-54	-	0%	0%
		B-57	-	5%	95%
	b	B-61	-	30%	70%
		B-64	-	10%	90%
9	a	B-45	+	-	-
		B-48	+	-	-
		B-55	+	-	+
	b	B-63	-	-	-
		B-66	+	-	-
10	a	B-46	+	-	-
		B-47	+	-	-
	b	B-65	+	-	-
		B-68	+	-	-
Average				25%	75%

- negative + present

*All "B" numbers in this and subsequent tables refer to the x-ray diffraction pattern from a certain bone preparation.

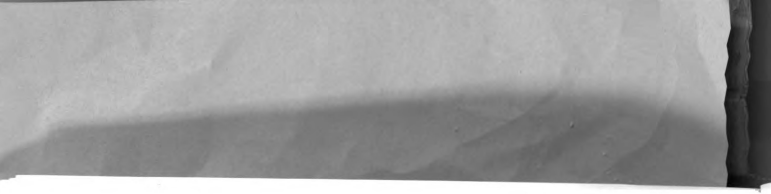
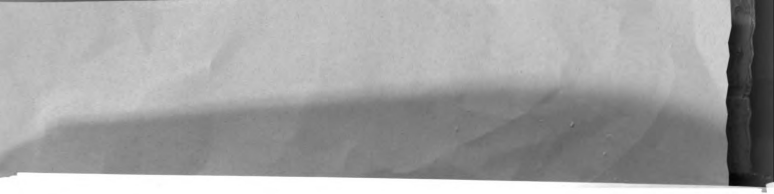


TABLE VII

AVERAGE VALUES OBTAINED FROM DRY BONES AT DIFFERENT AGES

9 day		10 day		11 & 21 day		Average*		Miller Index
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	hkl
3.86	20	3.83	10	3.81	5	3.83	10	111
3.43	50	3.43	50	3.42	40	3.43	50	002
3.08	20	3.10	15	3.10	20	3.10	20	210
2.80	100	2.80	100	2.80	100	2.80	100	121
2.270	10	2.269	10	2.250	20	2.265	15	310
1.986	5			2.056	5	2.021	5	113
1.938	10	1.940	10	1.930	10	1.937	10	222
1.833	10	1.834	10	1.823	10	1.830	10	123
1.710	10	1.710	10	1.709	10	1.710	10	141
		1.517	5			1.517	5	
1.447	5	1.440	5			1.444	5	
1.102	5					1.102	5	

* Intensities averaged to the nearest five.



cellent agreement with the ASTM values (Table XIV). Since the 3.13 Å line of KCl and the 2.81 Å line of NaCl had equal relative intensities, the relative amounts of crystalline NaCl and KCl present in each dry bone were estimated. The results appear in Table VI. As can be seen from the Table, all but one of the embryonic femurs from age six to eight days contained crystalline KCl and only three lacked detectable amounts of crystalline NaCl, as seen from the diffraction patterns. Apatite could not be detected in any femur of this age group.

After nine days of incubation, an osseous cylinder is usually seen when the femur is dried. The presence of "bone" is confirmed by x-ray diffraction as in all cases except B-63, the typical apatite pattern was observed. However, a remarkable event occurred concomitantly. There is a marked reduction in crystallizable sodium and potassium as evidenced by the complete absence of NaCl lines from all spectrograms and the presence of rather faint KCl lines in only one of the 23 plates studied. This indicates either an actual loss of these ions or their presence in a non-crystalline complex.

No crystalline calcium compounds, other than the typical "apatite", have ever been detected in bone. Further, there is no detectable change in lattice parameters at any time after the crystals are first laid down (See Table VII).

Chemical analyses for sodium and potassium found in embryonic femurs after seven to ten days of incubation are summarized in Table VIII.

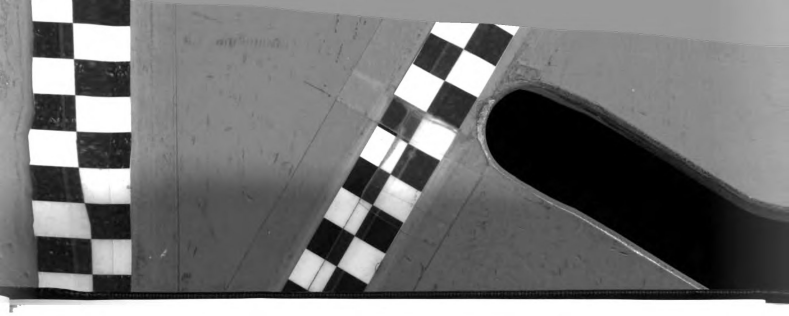


TABLE VIII

CHEMICAL ANALYSES ON EMBRYONIC FEMURS

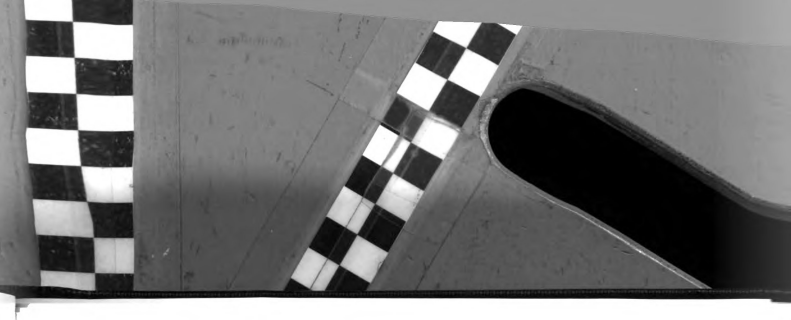
Bone	Day	No. Used	Total Wgt.*	Sodium		Potassium	
				γ/ mg.	mEq/100 gm.	γ/ mg.	mEq/100 gm.
-	7	6	0.4 mg	12	50	10	30
-	8	6	0.8	31	130	16	40
+	9	4	0.9	20	90	11	30
+	10	3	1.1	26	110	11	30

* Weighed dry on a balance with sensitivity ± 0.1 mg.
\+ Bone present - preosseous

Since the weight of the samples was so small, considerable error could have been introduced from weighing error alone. Only enough sample was available for one analysis per group. The difference observed cannot be regarded as significant, but the order of magnitude of the results is in agreement with the literature (Eichelberger, et al., 1952, and Everett, 1948).

2. X-ray Diffraction by "Wet" Femurs

The scattering of x-rays by the water present in femurs from six to eleven days was so great that it was impossible to detect any pattern at all, even though apatite was clearly present in dry bone after the eighth day. However, a longitudinal section of a wet 21-day femur showed a typical apatite pattern which was indistinguishable from dry bone (See Fig. 14). This observation has been reported by Reed and Reed (1942).



3. Comparison of Values from Dry and Ethylenediamine Ashed Twenty-One Day Femurs Ignited at High Temperatures

As has been previously reported (Klement and Trömmel, 1932) heating dry bone causes a marked sharpening of the apatite lines and also an increase in the number which appear on the diffraction pattern. This has generally been attributed to the removal of organic material and a growth of the bone crystals. This phenomenon was also observed (See Fig. 5). There does not seem to be an change in lattice parameters when the specimens were heated up to four hours at 500° C. or for two hours at 600° C. However, when heated for six hours at 600° C., there was a complete conversion to $\beta Ca_3(PO_4)_2$ (See Fig. 7, Table X). Heating at 700° C. for 6 hours produced the same pattern seen at 600° C. (Fig. 8).

Twenty-one day femurs which were ashed with ethylenediamine (ED) showed a pattern which was practically indistinguishable from dry 21 day bones. There was no increase in the number of lines, no shift in the position of those present, nor was a sharpening of the lines observed (See Fig. 5 and Table XXII). This indicates that there is no apparent alteration of the lattice parameters by the ED ashing procedure, nor has there been the crystal growth seen in heat ashing. The only observable difference was the loss of two broad bands seen at about 10 Å and 4.5 Å in all dry bones which are probably due to the organic components of the bone (Clark, 1931). These broad bands are also seen in diffraction patterns from dry preosseous femurs, from dry periosteum and from the epiphyseal cartilage of a 21 day femur. (Fig. 14).

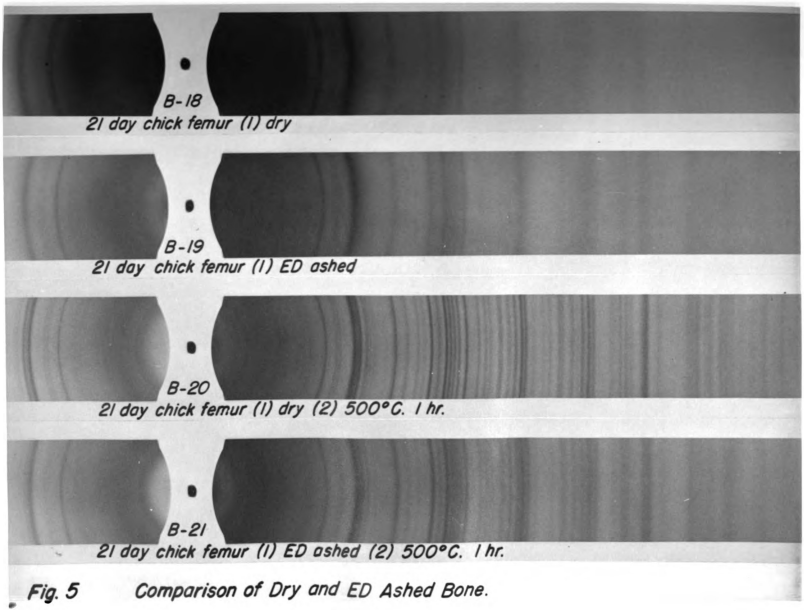


Fig. 5 Comparison of Dry and ED Ashed Bone.

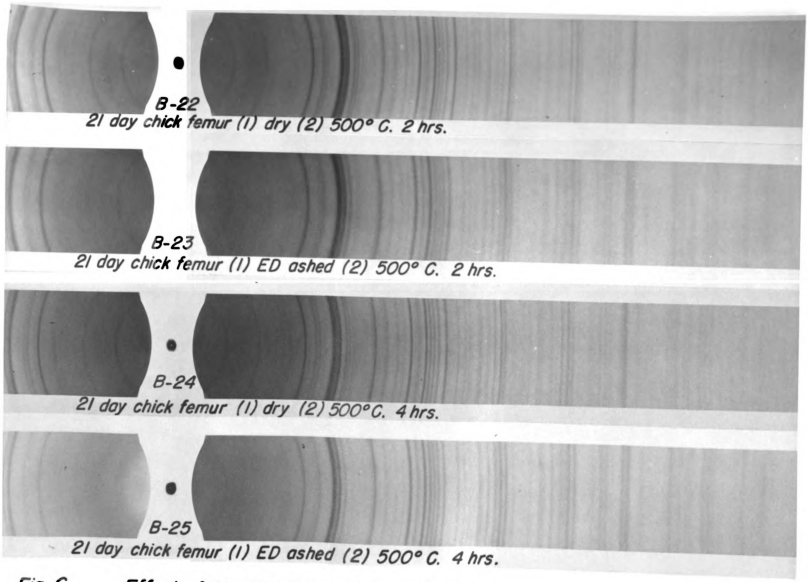
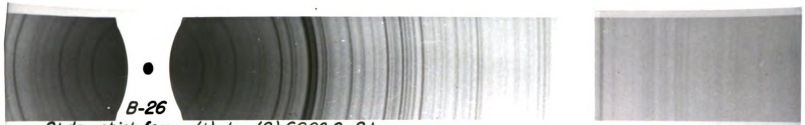


Fig. 6 *Effect of Heating Dry and ED Ashed Bone.*



B-26

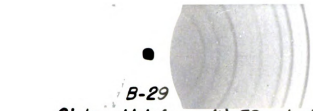
21 day chick femur (1) dry (2) 600° C. 2 hrs.

B-27

21 day chick femur (1) ED ashed (2) 600° C. 2 hrs.

B-28

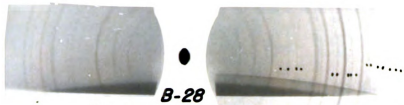
21 day chick femur (1) dry (2) 600°C. 6 hrs.



B-29

21 day chick femur (1) ED ashed (2) 600°C. 6 hrs.

Fig. 7 Conversion of Dry and ED Ashed Bone to Whitlockite.



B-28
21 day chick femur (1) dry (2) 600°C. 6 hrs.

B-29
21 day chick femur (1) ED ashed (2) 600°C. 6 hrs.

B-72
21 day chick femur (1) dry (2) 700°C. 6 hrs.

B-71
21 day chick femur (1) ED ashed (2) 700°C. 6 hrs.

Fig. 8 X-Ray Diffraction Patterns of Dry and ED Ashed Bone Converted to Whitlockite.

TABLE IX

OBSERVED VALUES FOR BONE APATITE AFTER HEATING COMPARED TO VALUES CALCULATED FOR BONE APATITE BASED ON LATTICE PARAMETERS

Ave. Dry		Ave. ED		P & F[1]			F & R[2]	Robinson[3]
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	hkl	d Å	d Å
9.28*	-	9.18*	-					
8.16	15	8.09	10			100	8.045	8.230
6.44*	10					001	6.959	6.854
5.19*	15	5.24*	5			101	5.263	5.269
4.74	5	4.73	2			110	4.645	4.737
4.33*	5	4.27*	2					
4.07	7	4.04	5	4.08	70	200	4.023	4.102
3.87	7	3.84	5	3.90	20	111	3.862	3.902
3.79	3							
3.51	2					201	3.483	3.522
3.42	40	3.43	30	3.44	80	002	3.481	3.439
3.32*	5							
3.17*	20	3.15*	5	3.18*	10			
3.08	15	3.07	10	3.08	20	210	3.041	3.099
2.99*	2							
2.822	100	2.797	100	2.82	100	121	2.786	2.826
2.769*	30					112	2.785	2.782
2.721	60	2.708	50	2.71	90	300	2.681	2.734
2.622*	15	2.618*	15	2.62*	50			
2.576*	15							
2.531	10	2.516	5	2.52	10	301	2.503	2.541
2.490*	2							
2.375	2					220	2.322	2.368
2.342	2					003	2.320	2.303
2.291	2	2.284	2			122	2.290	2.293
2.262	30	2.253	20	2.25	60	310	2.232	2.274
2.232	2					221	2.203	2.239
2.169*	5							
2.146	10	2.141	10	2.14	10	131	2.124	2.159
2.094*	2							
2.054	10	2.057	15	2.06	10	113	2.076	2.064
2.005	5			2.00	10	400	2.011	2.050
1.988*	5	1.989*	5			401	1.933	1.965
1.941	40	1.936	40	1.92	70	222	1.932	1.950
1.911*	5							
1.891	10	1.885	15	1.89	30	132	1.878	1.898
1.866*	5	1.860*	2					
1.834	40	1.835	40	1.83	70	123	1.844	1.843
1.809	20	1.800	20	1.80	40	231	1.784	1.815
1.782	20	1.776	20	1.77	40	410	1.756	1.789
1.754	20	1.750	20	1.75	40	004	1.740	1.720
1.710	30	1.714	30	1.75	40	141	1.702	1.733
1.642	15	1.640	10	1.64	20	223	1.641	1.647

*Values which do not fall in the range of calculated values based on lattice parameters. These values appear in Table X.

1 Prien and Frondel (1947). Observed

2 Frevel and Rinn (1953). Calculated from a = 9.29, c = 6.96, c/a = 0.749

3 Robinson (1951). Calculated from a = 9.47, c = 6.88, c/a = 0.727

After heating the dry and ED ashed bone for a period of one hour at 500° C., a large additional number of lines may be seen in both preparations. However, more lines are observed on the spectrogram of heated dry bone and they appear to be much sharper than those of the heated ED ashed bone. (See Fig. 5, and Tables XXI and XXII). Heating for periods of two and four hours at 500° C. further sharpens the lines in both cases but the difference between the dry and ED ashed bone is still apparent (Fig. 6).

Heating for two hours at 600° C. produced material which gave essentially the same patterns as at 500° C., however, two minor differences were noted in the ED preparation. The lines were somewhat sharper than at 500° C. for four hours, and there was a slight, but definite, shift of the strongest line at 2.79 Å (See Fig. 7). This shift is from a value of 2.824 Å (Table XXI, B-26) for heated dry bone to 2.790 Å (Table XXII, B-27) for the heated ED ashed bone. The difference of .034 Å is greater than the estimated error of measurement in this region but is less than the range of values in the literature given for bone "apatite" (See Table IX). A calculation of the "c" axis based on the 002 plane indicates little or no change in the "c" axis (6.84 Å - 6.83 Å) Using these values of "c", "a" calculated from 2.824 Å for the 121 plane is 9.47 for "dry", heated bone, and from 2.790 Å, "a" = 9.34 Å for ED ashed, heated bone.

Additional heating of both dry and ED ashed bone gave a material which is identified as $\beta Ca_3(PO_4)_2$ or whitlockite. The diffraction patterns appear in Figure 8. Calculated spacings from these four patterns are compared with observed data in the literature (See Table X).

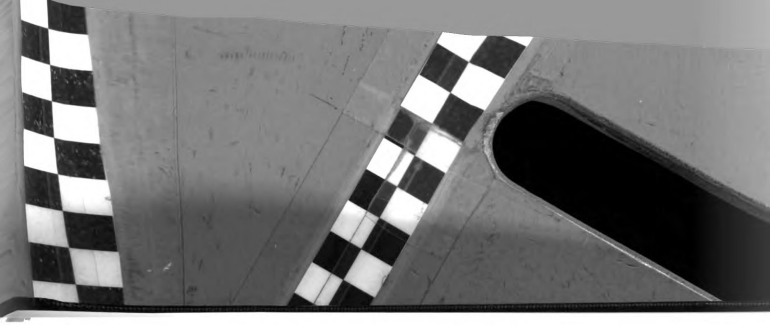


TABLE X

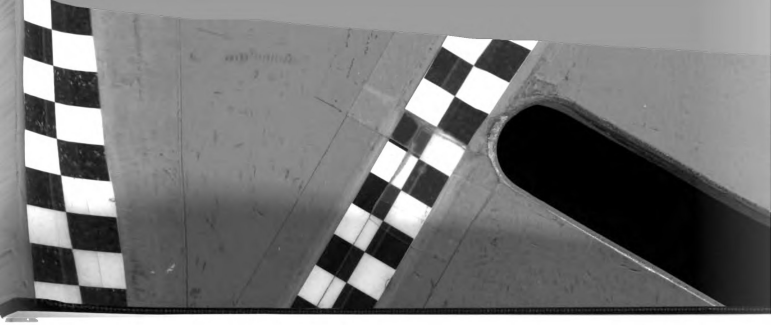
OBSERVED VALUES FOR WHITLOCKITE COMPARED TO VALUES IN LITERATURE

Literature[1]		Ashed Bone[2]		Heated Bone[3]	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
				9.28	-
8.17	10	8.06	20		
6.50	23	6.46	20	6.44	10
5.65	20				
5.23	47	5.18	40	5.19	15
		4.33	7	4.33	5
4.06	28	4.03	15		
		3.92	5	3.79	3
3.50	20	3.44	40		
3.42	48				
3.34	10	3.34	5	3.32	5
3.19	72	3.18	70	3.17	20
		3.10	2		
3.00	10	2.99	5	2.99	2
2.87	100	2.857	100		
2.76	25	2.737	10	2.769	30
2.69	18	2.667	2		
2.60	77	2.596	70	2.622	15
2.53	20	2.538	5	2.576	15
2.41	19	2.387	10	2.490	2
2.26	20	2.248	10		
2.19	18	2.181	10		
2.15	17	2.147	10	2.169	5
2.07	13	2.063	5	2.094	2
2.05	20				
2.01	15	2.019	10		
1.98	20	1.987	10	1.988	5
1.93	40	1.922	20	1.911	5
1.88	30	1.872	15		
1.86	30			1.866	5
1.82	18	1.813	2	1.809	20
1.77	30	1.763	2		
1.71	60	1.717	25		

[1] Average value from ASTM File #2436 d 3-0701, 2437 d 3-0702, 2435 d 1-0964, 2468 d 2-0783, 2409 d 3-0692, and Frien and Frondel (1947)

[2] Average values of B-28, B-29, B-71, and B-72. (See Table XX)

[3] Spacings from heated dry bones which cannot be assigned to apatite. (See Table IX).



The primary pattern seen in bones heated for several hours at 500° C. and at 600° C. is that of an apatite. This fact is readily observed when Table IX is consulted. In addition, the dry bone heated for only one hour at 500° C. showed an additional 18 lines which did not fall in the range of values for apatite. With the exception of only four, these lines may be assigned to $\beta Ca_3(PO_4)_2$ (See Table X). On the other hand, ED ashed bone when heated for one hour at 500° C. showed only seven lines that did not fall in the range of apatite; and of these, only three very weak lines could be assigned to $\beta Ca_3(PO_4)_2$.

The same observations were made also for heating periods of two, and four hours at 500° C., and for two hours at 600° C. It is clearly evident that $\beta Ca_3(PO_4)_2$ is much more readily produced by heating dry bone in a furnace than when ED ashed bone is heated.

There are several lines present on both the heated dry bone and heated ED ashed bone which cannot be assinged to either apatite or whitlockite. These lines represent interplanar spacings of 9.23 Å, 4.33 Å, 2.769 Å, and either 2.662 Å or 2.576 Å. They may be due to a contaminant, to some intermediate in the conversion of apatite to whitlockite, or to minerals containing Na, K, Mg or Cl. Quite evidently, the technique is sufficiently refined to detect, although not to identify, constituents other than apatite or whitlockite.

4. Values Obtained from Ashing Young Embryonic Femurs

Young femurs of eight to ten days were ashed at 600° C. for six hours in either porcelain or platinum crucibles. Extremely small quantities of ashed material were recovered after ashing, however,

enough was obtained for suitable x-ray diffraction patterns to be made. Spacings from these patterns appear in Table XXIV and the patterns in Figure 15. A mixture of NaCl and KCl when heated for six hours gave essentially the same x-ray diffraction as was seen in ashed eight to ten day femurs (See Fig. 15).

IV. DISCUSSION OF RESULTS

A. Electron Diffraction Studies

The techniques of electron diffraction impose rather severe limitations in choice of methods employed for preparation of biologic material. All specimens must be thoroughly dry and thin enough to permit penetration by the electron beam. Considerable heat is also generated by bombardment of the sample with electrons.

Early attempts to visualize bone mineral and tooth crystals usually employed fragmentation or thin shavings. By these methods, dry or ashed calcified material may be used without additional treatment. By grinding young teeth between watch glasses and dispersing in collodion, Boyle, Hillier, and Davidson (1946) were able to obtain electron diffraction patterns from enamel fragments which showed crystal orientation. A few years later, Robinson and Bishop (1950) used high pressure autoclaving and ultrasonic dispersion to prepare bone for electron microscopy. By this method they were able to make a reasonably accurate estimate of crystal size. Then Robinson and Watson (1952) showed bone crystals *in situ* on thin sections of n-butyl-methacrylate embedded human rib cortex. They also showed the typical apatite diffraction pattern from the thin section.

In each of these cases, the bone specimen was exposed to a large electron beam. (No Selected Area Aperture was used). The exact portion of the grid which gave rise to the diffraction pattern could not be

visualized and was certainly very large, since single crystal patterns did not appear. Instead, the image formed was the typical circular powder pattern, often showing orientation of the crystal population.

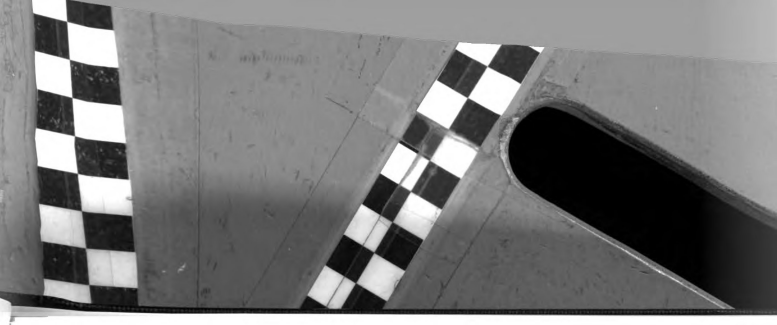
Using these methods, bone "apatite", which is stable under rather extreme conditions and present in high concentration, was the only crystalline material ever identified. The data are not sufficiently precise to allow conclusions as to the exact type of apatite. If some other crystalline component is present inbone to a much lesser extent, its contribution to the diffraction pattern must be so slight that it has remained undetected by the simpler techniques employed to date. Or, it is possible that the conditions used may have destroyed or altered other crystalline components which might conceivably be present.

In order to reduce the chance of these two possibilities in the present work, dry bone was ground to a fine powder and transferred directly to the film of the specimen grid. In addition, the size of the area contributing to the diffraction pattern was reduced by means of a Selected Area Aperture, so that the pattern observed was produced primarily by a single crystal or small group of crystals. Under these conditions, apatite was never seen but only the unknown "contaminant". When the grid was placed in the lower stage of the electron microscope (which would then expose a larger area of the grid to the electron beam) no pattern was ever seen. It is apparent that apatite, even when ground extremely fine, has a remarkable stopping power for electrons. The dense particles in Figure 1 must be apatite with its associated collagen. Even the finest of these, however, does not permit enough of the incident electrons to pass through to give diffraction patterns of sufficient

contrast to the surrounding beam to be photographed. Very short film exposures are used in electron diffraction (seconds to a minute or two) in contrast to the long exposures in x-ray work (4 hours to days). Increasing the exposure time merely weakens the contrast of spot or ring to background, since the background then becomes extremely black. If, in addition, the beam is reduced in size by the Selected Area Aperture, the number of electron diffracted (and thus available to initiate an image in the time of exposure) is very low.

One crystal, the "contaminant" frequently almost invisible in the conventional electron microscope field, gave very excellent single crystal diffraction patterns. It was never present in amounts exceeding about 5% of the total material on the grid (estimated from plates such as Figure 1) and was not visualized by lower stage diffraction procedures. It was remarkably transparent to electrons (as evidenced both by its lightness in the conventional field and its strong diffraction pattern a property which in itself is sufficient to differentiate it from the apatites.

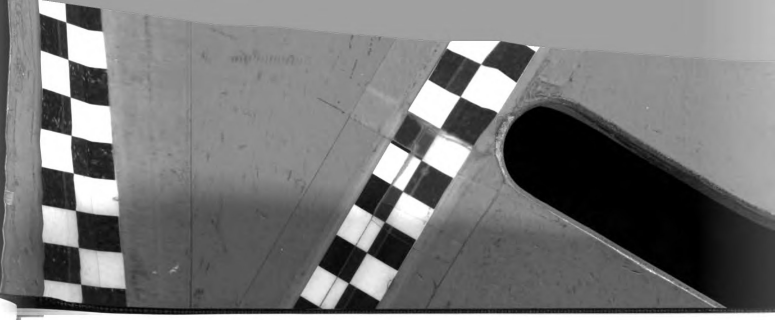
Identification of this material, which gave the diffraction patterns seen in Figure 2, would be of considerable interest. Apparently only the hk0 spacings are seen which makes positive identification impossible. Further difficulties arise from the fact that relative intensities such as reported in ASTM (1950) are not of much value, since they are dependent on random orientation of the crystals. Therefore, the only reasonable approach to the problem is to calculate a value for the "a" axis, having assumed that the crystal is hexagonal and that the innermost spots observed are due to the 100 plane. This



"a" value then may legitimately be compared with "a" values for hexagonal crystals.

There are surprisingly few crystalline materials listed in the literature whose "a" values correspond to the value a = 5.29 calculated for the crystals seen in Figure 1. Donnay and Nowacki (1954) and Frevel and Rinn (1953) have listed the "a" and "c" values for many hexagonal crystals. Some of those whose "a" values are closest to the calculated value are given in Table XXV. If an error has been made in the assumption that the innermost spacings observed are due to the 100 plane, and are due rather to the 200 plane, an additional number of compounds with an "a" axis of about 10.58 might be implicated. These also are given in Table XXV. However, this is unlikely because it would then be necessary to explain the systematic absence of all odd numbered spacings.

From Table XXV, it is obvious that the crystalline "contaminant" could be unequivocally identified if its "c" axis could be determined. In theory, this could be accomplished by tilting the "a" axis of the crystal away from the normal to the electron beam until hk1, hk2 or hk3 planes gave refractions which could be picked up and identified from the photographic plate. The simplest way to do this would involve the use of an internal standard and a good goniometer, operating, of course, inside the vacuum system and sufficiently well-constructed so that the crystal under examination could be visualized constantly during rotation and focusing. To construct a universal goniometer which would permit any minute crystal, no matter where located on the grid, to be rotated around one of its axes without leaving the focal



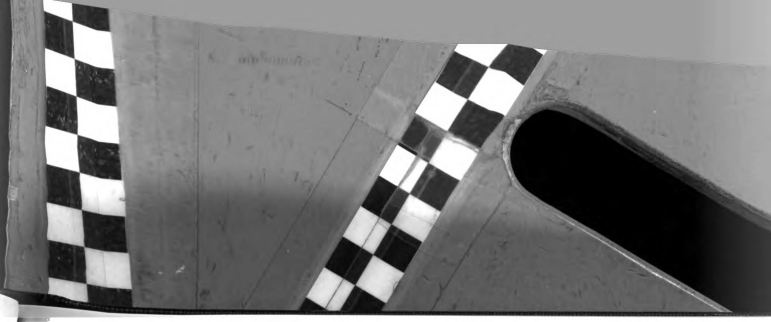
plane, would be difficult to say the least. An attempt was made by Dr. H. L. Bendlerto do this by insertion of wedges after an initial pattern was photographed. The same crystal could not be relocated, however, and the attempt was unsuccessful. In other words, this promising technique had to be abandoned for reason of inadequate instrumentation.

In viewing Figure 1, a striking similarity is seen between the micrographs shown there and micrograph of precipitated synthetic hydroxylapatite with recognizable crystals of secondary calcium phosphate also present. (Figure 12-B, Page 80, Hodge, 1950) Since bone cannot necessarily be regarded as the source of the thin crystals seen in Figure 1, the data presented cannot be directly applied to the bone problem. However, the method is useful and renewed efforts should be made to eliminate the contaminant and further studies should be conducted using preosseous embryonic femurs as a source of biologic material.

B. X-Ray Diffraction Studies

1. Discussion of Data Obtained from Dry Embryonic Femurs After Six to Ten Days Incubation.

Two general approaches have been made in the study of calcification, per se. One utilizes primarily the study of solubility products of materials present and of hypothetical intermediates in an effort to decide (usually by a process of subtraction) what actually "precipitates". Shear and Kramer (1928a), Logan and Taylor (1937), (1938), Huggins (1937), Logan (1940), Hodge (1950). Another group studies local factors such as enzyme systems or organic complexes ("metachromasia"),



which might be involved in calcification, Robinson, (1923), Sobel (1950), Gutman and Yu (1950), Marks and Shorr (1950). Although significant advances have been made from both approaches, elucidation of the actual sequence and spacial arrangement of the ions going down as apatite remains a problem, particularly since there is no apparent reason for them to stop "precipitating" when the crystal is only a few unit cells in size.

The study on six to ten day embryonic femurs was undertaken, therefore, to see if any information could be gained bearing on the mechanism of calcification. It is at about the ninth day of incubation that an osseous cylinder first begins to form around the shaft of the embryonic femur. By running diffraction patterns of wet embryonic femurs, an attempt was made to study the bone under conditions as nearly normal as possible. However, the large amount of scatter, presumably from the water present in these young femurs, made it impossible to see any diffraction lines.

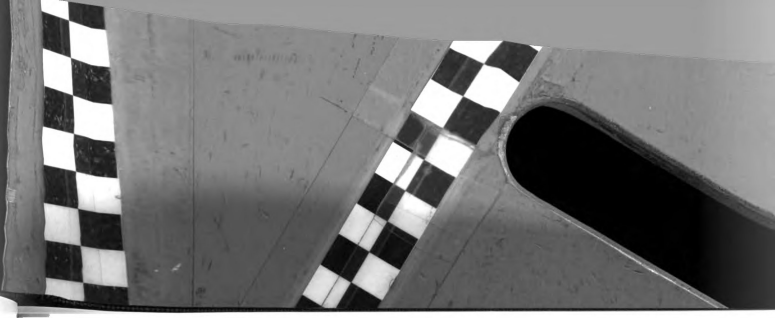
This is a considerable disappointment since it is difficult to believe that the "osseous" cylinder is not crystalline when first laid down, even though it is formed in the highly aqueous and only semi-solid "gel" which is its cartilaginous anlage. Nonetheless, diffraction lines were not observed although the method and instrumentation was adequate in the case of 21 day wet femurs.

The water content of puppy articular cartilage is 764 grams/kg (Eichelberger, 1952) and in the adult rat skeleton is 561 grams/kg (White and Rolf, 1955). Libby (pers. com.) reports up to 743 grams/kg in 21 day embryos. The 11 day femur could scarcely have contained more than 900 grams/kg. Consequently, the non-detectability of apatite

in 11 day wet "bones" must be due either to the masking effect of an extra 100-200 mg/kg (15 - 30% more H_2O) or actually to the lack of appreciable crystallinity in the bone first deposited, or, of course to the concurrent effect of both factors. In the absence of a definitive study of this point, it is well to point out the definite possibility that what first is laid down as bone may not be crystalline until water is removed, either physiologically as the bone "matures" or artificially as the bone, removed from the embryo, dries out.

The negative diffraction results should perhaps be expected, since hydrated crystals are usually (not just exceptionally) different in crystal habit, unit cell dimensions and all the other usual crystal characteristics. Further, materials such as the penicillins, are amorphous when hydrated and form regular crystals only when water is lost.

The potential significance of this point can scarcely be overemphasized since all the diffraction work on bones has been done either on mature bone, which has been physiologically dehydrated, or on younger bone which has been dried before examination, at the least in air, at the worst in a muffle furnace, and in the case of electron microscopy and diffraction by preliminary embedding in a non-aqueous plastic followed by examination in a vacuum system. The distinction (long ago drawn by the histologist and pathologist) between "calcification" of a tissue and "ossification" may have to be reviewed in modified form for the case of normal bone development. If this possibility were ever rigorously proved, the existing literature on the bone apatites would relate to secondary phases in bone development and not to the primary (first) processes.



Diffraction methods are available for the study of "non-crystalline" materials (radial distribution analysis). These have been developed largely in connection with research in polymers, most of which have, at best, a very low order of crystallinity at lower temperatures. Studies of collagen by small angle scattering are also pertinent in this connection.

The results obtained when the femurs were dried were also not anticipated. NaCl and KCl crystallized out of preosseous cartilage, but as soon as a detectable amount of bone apatite appeared, no NaCl was seen and a marked reduction occurred in crystallizable KCl. Chemical analyses showed that there was no significant decrease in the total amount of Na and K present. Consequently, it is assumed that after apatite started to form, both cations were present in a form which prevented them from crystallizing as the chlorides.

Even though considerable variation exists in the chemical analyses for Na and K of the femurs from 7 to 10 days, as might be expected from the possible error in weighing, the average values fit very well with those reported for preosseous articular cartilage (Eichelberger, et al., 1952) and cartilage (Everett, 1948). (See Table XI, Total Na and K) The potassium values are somewhat high as compared with literature values.

Some interesting calculations may be made regarding the amount of Na and K that might be free to crystallize upon drying, using the data, calculations, and assumptions of Eichelberger, et al.

They had previously established that a constant ratio of Na, K, and Cl is associated with the collagen present in tendon, and assumed

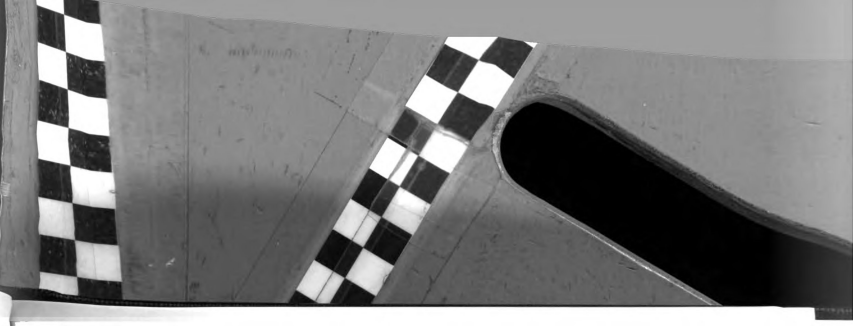


TABLE XI

CALCULATED AMOUNTS OF SODIUM, POTASSIUM, AND CHLORIDE ASSOCIATED WITH SEVERAL POSSIBLE BINDING MECHANISMS IN CARTILAGE[1]

	Eichelberger[2]	Everett[3]		
	Articular Cartilage	Cartilage	Preosseous Cartilage[4]	Ossifying Cartilage[5]
Sodium				
free Na	160	151*	57*	0
CS Na[6]	656	719	746	892
fibre Na	92	106	97	108
Total	908	976	900	1000
Potassium				
free	8	28	133*	-
cellular K	216	217	217**	300
Total	224	245	350	300
Chloride				
free	169	179	190*	0
fibre	95	109	100	111
Total	236	288	290	111

*Maximum **Minimum

[1]All values in table are mEq./kg solids

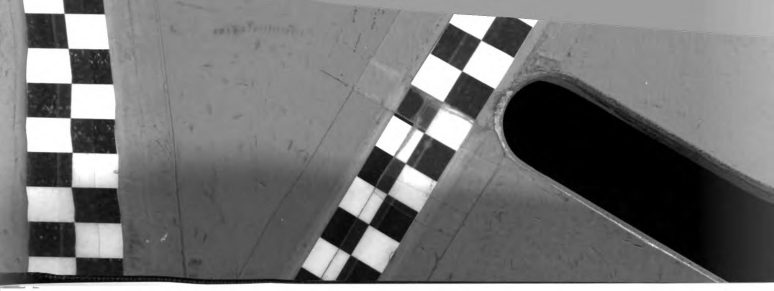
[2]Eichelberger et al., (1952)

[3]Everett,(1948)

[4]Average of seven and eight day femurs

[5]Average of nine and ten day femurs

[6]Sodium associated with chondroitin sulfate



the ratio to remain fixed in cartilage. For K and Cl, this leaves a certain amount "free". There then must have been enough "free" sodium to be associated with the excess free chloride. The remaining sodium must be bound and can be accounted for on the basis of chondroitin sulfate content. These calculations were also applied to the cartilage values of Everett, and the preosseous (7-8 day) and ossifying cartilage (9-10 day) of chick femurs. Since epiphyseal cartilage of 21 day chick femurs (similar to articular cartilage) did not show detectable amounts of crystalline NaCl and KCl by x-ray diffraction, the free Na and K as calculated from data of Eichelberger, et al. must not be the same fraction of Na, K and Cl which was found to crystallize when 7 and 8 day embryonic femurs were dried. This then suggests some binding other than by collagen or chondroitin sulfate. However, it cannot be definitely stated that the portion called "fibre" Na, K and Cl could not crystallize on drying and contribute to the diffraction pattern.

Several possibilities may be presented to explain this phenomenon of binding Na and K in bone. Boyd and Neuman (1951) showed equal binding of Na, Ca, and Ba ions by veal costal cartilage, and correlated this with the SO_4 content. On the basis of this, they felt chondroitin sulfate was the binding agent. Other workers have presented the idea that the sodium present in bone may well be "occluded, adsorbed or interstitially crystallized", (French, et al., 1938, Hodge, et al., 1943). This adsorption may be due to a hydration layer as proposed by Neuman (1952).

Neuman, et al. (1950) studying fluoride uptake by bone salt in vitro, assumed that the carbonate present in bone shared a monovalent

bond with calcium and that the second carbonate valance was occupied by sodium, i.e. - Ca - O - CO_2 - Na. Bergstrom (1954) also found that the changes observed following acidosis suggest that all of the Na and K in bone is present as Ca - O - CO_2 - Na(K).

It is also possible that heterionic exchange may be responsible for the binding - especially of sodium. Na and Ca both have approximately the same ionic radii (about .98 Å), therefore, sodium can and does replace Ca in the apatite. Potassium, on the other hand, with an ionic radius of 1.33 Å is usually considered too large to fit into the hole left vacant by a calcium ion (Hendricks, 1952, and Neuman and Neuman, 1953).

Citric acid may also be involved in the picture of Na and K binding. (Shear and Kramer, 1928b); however, this possibility does not seem to be as plausible as some of the others. Actually, very little is known about the position of citrate of bone (Armstrong, 1950). Dickens, (1941) determined the citrate content of embryonic chick bones and also computed the equivalent Ca to citrate ratio. The citrate content falls steadily from about 31.0 mg/100 gm embryo at 3-1/2 days to 9.4 mg/100 gm embryo at 21 days. The Ca: citrate ratio goes from 3.3:1 to 130:1 for the same periods. Between 8 and 12 days, there is very little change in citrate content, the period during which calcification first takes place. Whatever the mechanism of this binding of Na and K, which prevents their crystallization on drying when there is a demonstrable amount of apatite present, it must not be the only method by which Na and K are bound. As is seen from Table XI, dry preosseous cartilage contains about 90 mEq/100 gm sodium and 22 mEq/100 gm potassium

(Eichelberger, et al., 1952), whereas young bone contains much less of both Na and K. (11 mEq/100 gm Na; 3.4 mEq/100 gm of K. Bergstrom, 1952). Therefore, in the nine day embryonic femur, which has a small amount of apatite present, some of the sodium must be associated with the new mineral phase while most of it still remains bound by the cartilage. It is not clearly understood how the small amount of bone apatite present at nine days could bind sufficient quantities of Na and K to reduce the crystallizable portion below the limits of detection, since young bone contains only about one-tenth that of cartilage. However, some mechanism associated with the bone apatite must be responsible for the binding of the "crystallizable" portion because the cartilage present is probably binding Na and K as it did in the preosseous femur. This would tend to rule out binding of the crystallizable portion by chondroitin sulfate and favor some process of adsorption in the apatite lattice. This view is not inconsistent with that of Neuman and Bergstrom, since they assume that the carbonate is attached to the calcium of the apatite.

2. Studies on the Nature of Bone Mineral

In general, most investigators agree that the solid phase of bone may be regarded as a slightly impure basic calcium phosphate (Eisenberger, Lehrman and Turner, 1940). Differences in opinion arise regarding the exact position of the "foreign" ions, and whether or not they are important in the mineralization process.

Since rather extensive substitution into the apatite lattice, or adsorption of ions by apatite does not alter the lattice parameters appreciably, it is difficult or impossible to show the percentages of

substitution by x-ray diffraction. In addition, the diffraction pattern from fresh bone has very broad lines which makes accurate estimation of the line position even more difficult (Taylor and Sheard, 1929). The broad lines indicate that the crystal size in bone is extremely small, a finding which has recently been confirmed by electron microscopy (Robinson, 1951).

In order to sharpen the lines present on the diffraction pattern, so that more exact determination of lattice constants can be made, it has been customary to heat ash bone at high temperatures. It has usually been assumed that this procedure does only two things, eliminate scattering by water and organic matter and induce growth of the crystals.

A number of techniques have been more recently devised to rid the bone of its organic material so that the mineral phase could be better studied (Neuman and Neuman, 1953). In each case it can be shown that the ashed material resulting from the process is altered somewhat from the original mineral phase. The question is, however, which alterations are to be avoided and which are desirable. In other words, which portions of the bone composition are basic constituents and which have no bearing on the behavior of the system.

Ethylenediamine (ED) was used by Arnold (1952) to remove the organic portion of bone, and thus to obtain information regarding the relative amount of injected Ca^{45} organically combined. By using the method of ED ashing reported by Arnold, the organic material was removed from 21 day chick femurs. As shown earlier, the only difference between the diffraction patterns for dry bone and from ED ashed bone was the absence

of certain broad bands, probably due to the loss of collagen after ED extraction (Clark, 1931, Wyckoff and Corey, 1936). After one hour heating at 500° C., interesting differences begin to show on the diffraction patterns. Lines from the heated dry material are much sharper than those of the heated ED ashed material. The reason for this probably is due to the presence of organic matter in the dry material, which when heated to 500° C., burns and causes a local rise in temperature (Dallemagne, 1952). Even at this time, some whitlockite lines were identified. Whitlockite usually requires temperatures above 500° C. for its formation, although a few instances of low temperature formation have been reported (Trautz, 1955).

After additional time at 500° C. or when heated for two hours at 600° C., there is noticeable shift of the strongest line (2.79 Å) on the heated ED ashed bone when compared to the heated dry bone. By studying the diffraction patterns as they appear in Figures 5, 6 and 7, it can be seen that with heating dry bone, the broad line of dry bone gradually is resolved into three lines: 2.824 (100)[1], 2.770 (30), and 2.718 (60) (Table XXI, B-26). On heating ED ashed bone, this same broad band will resolve into sharp lines somewhat differently. The strongest line 2.790 (100) (Table XXII, B-27) appears at a position which had previously been the exact center of the broad band, whereas the strongest line of the heated dry bone appears at a spacing larger than the exact center of the broad band of the unheated bone.

Recent precise work by Perdak, W. as reported Posner (1955) gives

[1] Relative intensities

the lattice parameters of hydroxylapatite as a = 9.420 ± 0.001 Å; C = 6.885 ± 0.001 Å, whereas Altschuler, Cisney and Barlow (1953) give values of a = 9.413 ± .002, C = 6.875 ± .002 for hydroxylapatite. These precise values were both obtained from supposedly pure hydroxylapatite and yet the lattice parameters differ. Thus, precise values obtained in two different laboratories differ significantly. It is difficult to compare values from this thesis with those in the literature for this reason. When the diffraction lines are broad and diffuse as they are in untreated bone, considerably greater range is encountered in reported values (See Table IX). Obviously, further descriptive work is less important than theoretical analysis of the changes in the broad bands when treated in various ways.

In general, all the shifts in lines observed on ED extracted bone when heated indicate a decrease in the "a" axis, with little or no change in the "c" axis. By calculating the "a" axis from the (300), (200), and (310) planes for both dry and ED extracted bone when heated (See Table IX), a shift from a = 9.42 Å for the dry to a = 9.36 Å for the ED extracted bone was seen. Based on the (002) plane, only a slight change is noted in the "c" axis (6.84 Å - 6.83 Å). By using these c values to solve the hexagonal formula for "a", based on the (121) plane, "a" shifts from 9.45 Å to 9.33 Å. The whole question of line sharpening when bone preparations are heated must be studied from a crystallographic viewpoint to establish whether measurements of the center of broad lines give a true value for lattice parameters.

V. SUMMARY AND CONCLUSIONS

By means of electron microscopy and electron diffraction, young bone prepared by drying, heat ashing, and ethylenediamine ashing was studied. Two distinct types of fragments were observed. The dense fragments were assumed to be bone particles, although no diffraction patterns were ever obtained. Thin crystals of indeterminate source showed good electron diffraction patterns, always in a hexagonal form. Assuming the crystals to be hexagonal and assigning the innermost spots the hkl value of 100, an "a" axis of 5.29 Å may be calculated. Without being able to tilt the crystals so as to obtain additional spacings, the "c" axis cannot be determined. With better instrumentation, the crystal species could undoubtedly be established.

There is no evidence of any precursor crystalline material present in bone prepared under the described condition. However, the diffraction patterns from single crystals are evidence of the efficacy of the method.

X-ray diffraction patterns from dry preosseous embryonic femurs showed the presence of crystalline sodium and potassium chloride. When apatite was detected in the femurs, no crystalline NaCl was detected and KCl was found in only one sample. Since there was no significant change in total sodium and potassium over the seven to ten day period during which ossification occurs in the chick femur, some "binding" mechanism associated with the presence of apatite is indicated. Several methods of binding of ions are discussed, with their possible significance in binding "free" sodium and potassium in preosseous and ossifying

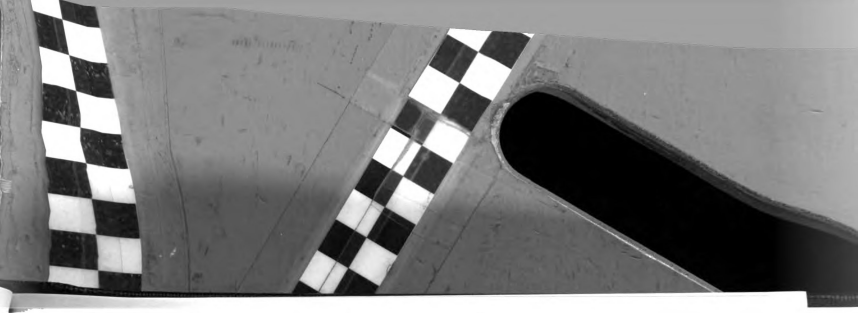
bone. No diffraction pattern was seen when young wet femurs were exposed to x-rays. This was probably due to scatter by the high water content. Since 21 day "wet" femurs produced good diffraction patterns, the possibility is suggested that bone as it is first laid down is non-crystalline or very poorly crystallized. The bone mineral might then crystallize upon "drying", either non-physiologically upon removal, or physiologically by maturation. This point bears further investigation.

Upon ashing at 600° C., embryonic femurs of eight to ten days showed a diffraction pattern similar to that of a mixture of NaCl and KCl heated at 600° C. No β-$Ca_3(PO_4)_2$ was seen in the diffraction pattern from the ashed femurs even though apatite was present in the dry samples of nine and ten day femurs. Since only a minute amount was recovered from each femur, this may be simply a lack of detection.

Twenty-one day embryonic chick femurs prepared by ethylenediamine ashing were shown to give a diffraction pattern indistinguishable from dry bone of the same age.

It was concluded that the process of ED extraction did not cause a significant change in lattice parameters and did not induce lattice growth. ED ashed bone would serve as an excellent preparation for further study on crystal size by electron microscopy.

Upon heating, dry bone produced a much sharper diffraction pattern and was more readily converted to β-$Ca_3(PO_4)_2$ than ED extracted bone. This can be explained on the basis of incineration of the organic material in dry bone which would cause a local rise in temperature above that of the furnace. The β-$Ca_3(PO_4)_2$ produced upon further heating was the same from both preparations.



Before conversion to whitlockite, x-ray diffraction patterns from ED extracted bone showed a shift in the "a" axis toward a smaller spacing as compared with the dry bone. The calculated value of a = 9.42 Å, c = 6.84 Å for heated dry bone corresponds closely to the values of hydroxylapatite, whereas the value of a = 9.36 Å for heated ED extracted bone corresponds more closely to carbonate apatite.

Addendum

Dr. H. M. Bendler, Department of Physics and Astronomy, suggests that the contaminant seen by electron microscopy and diffraction may be a species of clay mineral of the montmorillonite family, such as nontronite. Nontronite gives observed and computed spacings identical with those seen for the contaminant.

APPENDIX A

TABLES

TABLE XII

EXAMPLE OF INDIVIDUAL MEASUREMENTS FROM AN ELECTRON DIFFRACTION PATTERN[1]

Angle of Rotation[2]	Gold Diameter[3]	Crystal Diffraction Spots					
		Dia.[3]	d Å[4]	Dia.	d Å	Dia.	d Å
0°	16.1	8.3	4.60	16.5	2.30	24.5	1.548
60°	15.9	8.1	4.62	16.2	2.31	24.2	1.548
120°	15.2	7.9	4.53	15.7	2.28	23.4	1.530
30°	16.1	14.3	2.65	28.5	1.330		
90°	15.5	13.6	2.68	27.3	1.336		
150°	15.5	13.8	2.64	27.5	1.330		
20°	16.1	21.8	1.740				
40°	16.1	21.8	1.740				
80°	15.7	21.0	1.760				
100°	15.3	20.7	1.740				
140°	15.6	21.0	1.750				
160°	15.9	21.4	1.750				

[1] Pattern used for the example is #503-D appearing in Fig. 2. Measurements were made on a projection print, X 2.7.

[2] Angle of rotation is determined simply from a reference line drawn through a pair of points which then is designated as 0°.

[3] Linear measurements are given to the nearest 1/50th inch.

[4] Calculated from the relationship:

$$d_x = \frac{\text{diameter Au ring} \cdot d_{Au}}{\text{diameter x ring}}$$

where $d_{Au(100)} = 2.344$ Å

TABLE XIII

SUMMARY OF ELECTRON DIFFRACTION DATA*

		hkl	100	110	200	120 & 210	300	220
Age	Treatment	Plate	d Å	d Å	d Å	d Å	d Å	d Å
10 day	Heat Ashed	465-C					1.510	
"	"	465-E					1.509	
"	"	466-A		2.59			1.490	
"	"	466-C		2.61			1.519	
"	"	466-E	4.64				1.557	
"	"	467-A		2.68			1.538	
"	"	467-C		2.66			1.532	
"	"	467-E	4.57	2.65				
"	"	468-A	4.54	2.61				
"	"	468-C	4.52	2.58				
4 week	ED Ashed	491-A	4.61				1.541	1.327
19 day	Dry	495-A			2.29	1.735	1.522	
"	"	496-C	4.48	2.61	2.30	1.742	1.535	1.332
"	"	496-E	4.60	2.60	2.29	1.740	1.528	1.330
11 day	"	503-D	4.58	2.66	2.30	1.748	1.542	1.332
"	"	504-A	4.57	2.63	2.30	1.741	1.535	1.330
13 day	"	514-A	4.58				1.524	
"	"	514-C	4.50	2.59		1.710	1.500	1.320
"	"	515-E					1.522	
17 day	"	516-C	4.59	2.61				
"	"	516-E	4.56				1.527	1.331
Average			4.57	2.62	2.30	1.739	1.525	1.329

*All values appearing in this table are the average of three measurements with the exception of values for hkl 120 and 210. In this column each figure is the average of six measurements. No other spacings were ever observed above 1.3 Å.

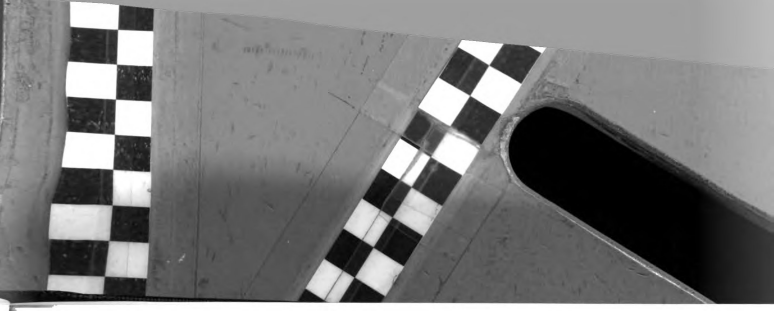


TABLE XIV

COMPARISON OF VALUES OBTAINED FROM A 1:1 MIXTURE OF NaCl AND KCl WITH THOSE FOUND IN THE ASTM FILE

NaCl + KCl		NaCl*		KCl	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
3.26	10	3.25	16		
3.13	100			3.12	100
2.817	100	2.81	100		
2.215	70			2.21	67
1.992	70	1.99	88		
1.812	30			1.81	20
1.703	10	1.70	5		
1.627	20	1.63	40		
1.573	10			1.57	7
1.405	40	1.41	33	1.40	17
1.293	2	1.29	4		
1.282	30			1.28	10
1.261	30	1.26	40		
1.151	20	1.15	30		
1.112	10			1.11	3
1.085	5	1.08	2		
1.050	15			1.05	7
.994	10	.99	18	.99	3

*Relative intensities for NaCl are given as an average of the values found on the three ASTM cards numbered 2548, 2550 and 2551.

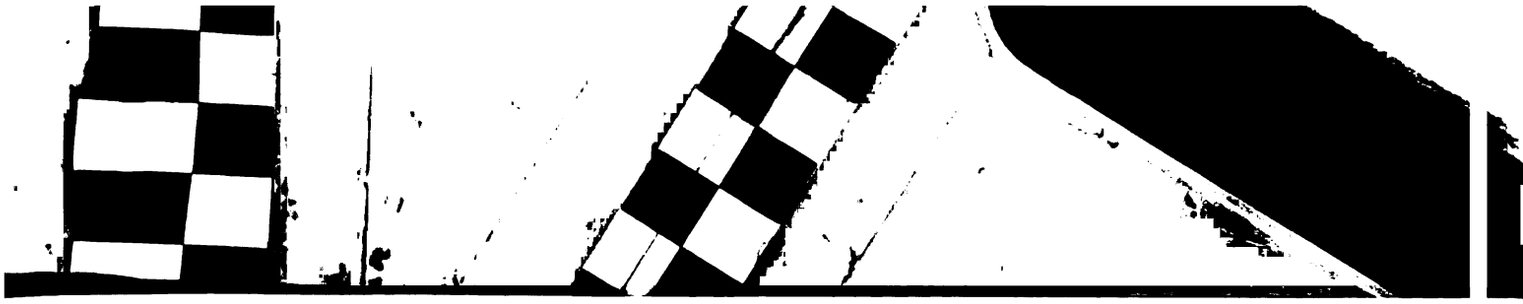


TABLE XV

VALUES OBTAINED FROM SIX DAY DRY EMBRYONIC FEMURS

B-42*		B-58	
d Å	I/I_1	d Å	I/I_1
3.13	100	3.13	30
2.817	40	2.810	100
2.215	70	2.215	20
1.989	20	1.989	80
1.805	40	1.810	5
1.625	5	1.625	30
1.565	10		
1.402	30	1.408	30
1.278	20	1.278	2
1.261	5	1.261	30
1.150	5	1.150	20
1.107	15		
1.044	15		
.992			

*All "B" numbers in this and subsequent Tables refer to the x-ray diffraction pattern from a certain bone preparation.

TABLE XVI

VALUES OBTAINED FROM SEVEN DAY DRY EMBRYONIC FEMURS

Series a								Series b			
B-43		B-50		B-56		B-60		B-62		B-67	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
3.13	100	3.12	100	3.13	100	3.13	100	3.12	100	3.12	100
2.817	50	2.811	40	2.817	30	2.817	5				
2.215	70	2.210	70	2.215	80	2.210	70	2.210	70	2.210	70
1.989	30	1.989	30	1.989	20	2.989	2				
1.805	30	1.805	10	1.810	40	1.810	10	1.805	20	1.805	20
1.628	5	1.625	5								
1.570	10			1.565	10	1.565	5	1.564	10	1.565	10
1.402	20	1.402	5	1.402	20	1.402	10	1.400	20	1.400	20
1.278	10	1.278	10	1.279	20			1.278	10	1.278	10
1.107	5										
1.044	5										

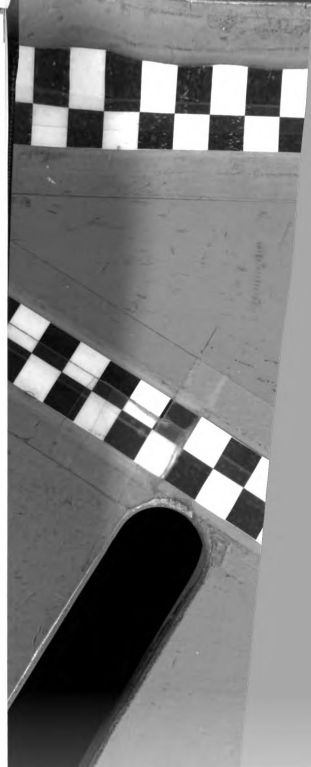


TABLE XVII

VALUES OBTAINED FROM EIGHT DAY DRY EMBRYONIC FEMURS

Series a						Series b					
B-44		B-49		B-54		B-57		B-61		B-64	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
3.14	100	3.12	100	No lines		3.13	100	3.13	100	3.13	100
2.817	50	2.817	100	appear on		2.817	5	2.817	40	2.817	5
2.223	70	2.210	60	this film		2.215	80	2.210	50	2.215	70
1.995	30	1.989	60			1.989	2	1.989	20	1.989	2
1.813	40	1.805	10			1.810	30			1.810	20
1.627	10	1.627	10								
1.573	10					1.567	10			1.570	10
1.405	20	1.402	10			1.403	20			1.402	20
1.282	15					1.279	10			1.279	10
1.262	5										
1.151	5										
1.108	5										
1.044	5										
.992	5										

TABLE XVIII

VALUES OBTAINED FROM NINE DAY DRY EMBRYONIC FEMURS

B-45		B-48		B-55		B-63		B-66	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
		3.86	20	3.84	(20)	Shows no			
3.43	50	3.42	50	3.42	(70)	apatite lines.		3.43	50
3.03	30	3.10	10	3.13	(30)	Only broad		3.12	20
2.79	100	2.80	100	2.80	(100)	collegan bands		2.80	100
		2.270	10	2.215	(20)	appear about			
1.986	5					10Å and 4.5Å			
1.938	5	1.938	10	1.938	(5)			1.940	10
1.833	5			1.833	(5)			1.833	10
1.708	10	1.710	10	1.710	(10)			1.710	10
1.447	2								
1.102	2								

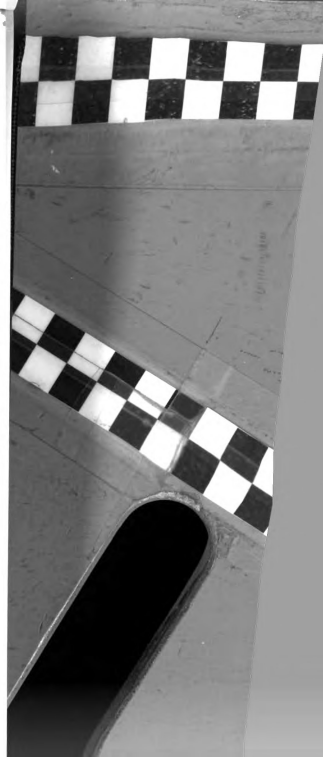
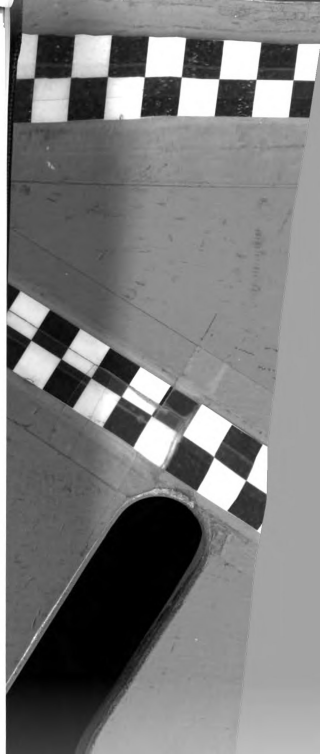


TABLE XIX

VALUES OBTAINED FROM TEN DAY DRY EMBRYONIC FEMURS

B-46		B-47		B-65		B-68	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
				3.83	10		
3.42	60	3.43	50	3.42	40	3.43	50
				3.10	15	3.10	15
2.79	100	2.80	100	2.80	100	2.80	100
		2.272	10	2.266	10	2.270	10
		1.938	10	1.940	10	1.940	10
1.838	5	1.833	10	1.833	10	1.833	10
1.710	10	1.710	10	1.710	10	1.710	10
1.517	5						
1.440	2					1.440	5



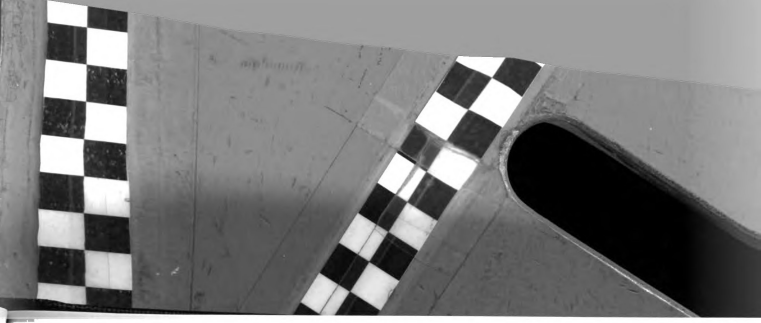


TABLE XX

VALUES OBTAINED FROM ELEVEN AND TWENTY-ONE DAY DRY FEMURS

B-30 (11 day)		B-31 (21 day)		B-35 (21 day)	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
		3.79		3.83	5
3.42	40	3.40	30	3.42	40
3.18	30	3.03	5	3.10	20
2.80	100	2.78	100	2.80	100
2.236	30	2.249	10	2.266	20
2.054	2			2.058	5
1.926	5	1.926	10	1.938	20
1.813	5			1.833	15
1.708	5			1.710	10

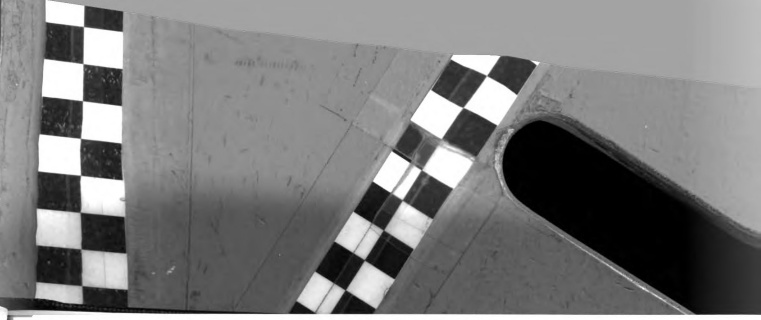


TABLE XXI

VALUES OBTAINED FROM DRY 21 DAY FEMURS WITH INCREASING HEATING, TIME AND TEMPERATURE

				500°C.				600°C.	
Dry		1 hour		2 hours		4 hours		2 hours	
B-18		B-20		B-22		B-22		B-26	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
		9.37	-					9.18	2
		8.19	10	8.12	20	8.19	15	8.12	20
		6.44	-	6.40	10	6.46	5	6.46	10
		5.18	10	5.18	20	5.20	15	5.20	20
		4.74	5	4.70	5	4.76	2	4.74	5
4.35	-	4.30	5	4.35	5	4.33	5	4.32	10
		4.05	10	4.07	5	4.10	5	4.07	10
		3.86	10	3.86	5	3.87	5	3.88	10
3.79	10			3.78	5			3.80	2
				3.51	2			3.50	2
3.41	50	3.42	30	3.42	40	3.43	40	3.42	40
		3.34	5	3.30	5	3.33	2	3.32	5
		3.17	15	3.16	15	3.18	20	3.17	20
		3.08	15	3.08	10	3.09	15	3.08	10
		2.98	2	2.99	2	2.99	2	2.98	5
2.80	100	2.817	100	2.817	100	2.830	100	2.824	100
		2.770	30	2.761	40	2.770	40	2.770	20
		2.724	60	2.718	60	2.724	70	2.718	50
		2.625	10	2.619	15	2.625	20	2.619	15
		2.578	5	2.572	10	2.584	15	2.572	20
		2.538	2	2.527	5	2.533	10	2.527	20
		2.490	2	2.484	2	2.495	2	2.490	5
		2.377	2	2.367	2	2.377	2	2.379	2
		2.344	2	2.335	2	2.348	2	2.339	2
		2.294	2			2.284	5	2.296	2
2.262	15	2.262	20	2.262	30	2.262	30	2.262	30
		2.236	2	2.228	2	2.232	2	2.231	5
		2.167	2			2.171	5	2.169	5
		2.144	10	2.148	10	2.147	10	2.144	10
				2.094	2				
		2.054	10	2.054	10	2.054	10	2.054	10
		2.006	5	2.002	2	2.006	2	2.006	5
		1.989	5	1.986	2	1.989	2	1.986	5
1.926	15	1.944	40	1.941	30	1.938	50	1.941	30
		1.913	5	1.910	5	1.911	5	1.910	5
		1.892	10	1.890	10	1.890	10		
		1.875	5	1.866	5	1.872	5	1.852	5

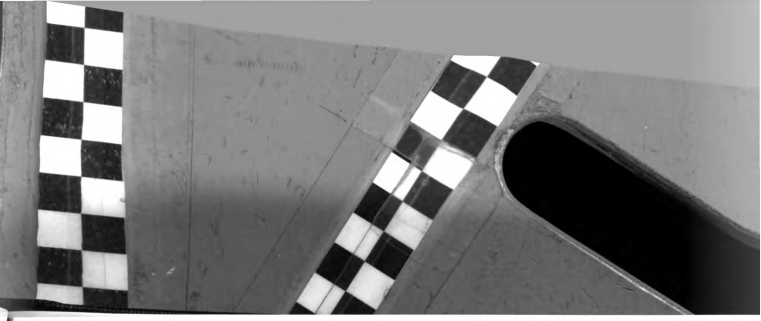


TABLE XXI (Cont'd)

VALUES OBTAINED FROM DRY 21 DAY FEMURS WITH INCREASING HEATING, TIME AND TEMPERATURE

		500°C.						600°C.	
Dry		1 hour		2 hours		4 hours		2 hours	
B-18		B-20		B-22		B-22		B-26	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
1.823	15	1.835	40	1.832	30	1.835	50	1.835	30
		1.808	20	1.808	20	1.811	30	1.808	20
		1.782	20	1.782	20	1.782	30	1.782	20
		1.754	20	1.754	20	1.754	30	1.754	20
1.700	15	1.710	30	1.708	30	1.708	40	1.710	30
		1.689	2	1.686	2	1.687	2	1.689	2
		1.666	2	1.664	2	1.664	2	1.666	2
		1.644	15	1.642	10	1.644	10	1.644	10
		1.606	10	1.606	10	1.606	10	1.606	5
		1.586	10	1.584	10	1.585	5	1.586	5
		1.573	2			1.571	2		
		1.546	15	1.546	10	1.544	5	1.548	10
		1.534	15	1.537	10	1.533	10		
		1.508	5			1.508	5	1.506	5
		1.501	5	1.499	5	1.499	5	1.495	5
		1.473	15	1.473	10	1.475	15	1.473	5
		1.450	20	1.448	20	1.450	25	1.450	20
		1.436	15	1.436	5	1.436	15	1.436	10

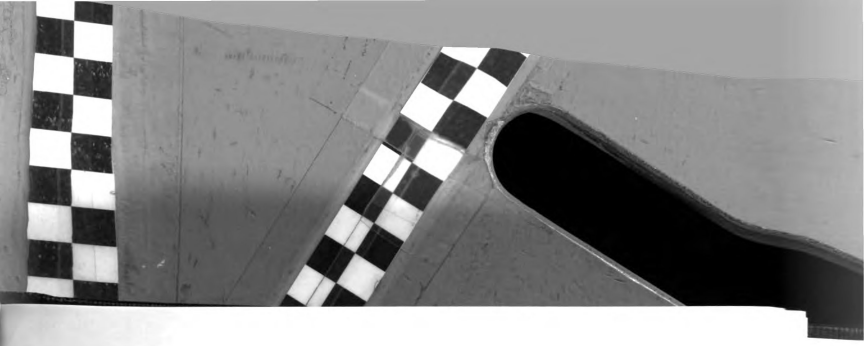


TABLE XXII

VALUES OBTAINED FROM ETHYLENEDIAMINE ASHED FEMURS WITH INCREASING HEATING, TIME AND TEMPERATURE

		500°						600°	
Unheated		1 hr.		2 hrs.		4 hrs.		2 hrs.	
B-19		B-21		B-23		B-25		B-27	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
		9.20	5	9.13	5	9.37	2	9.21	5
		8.02	5	8.12	15	8.25	5	7.96	10
				5.18	10	5.35	2	5.18	2
				4.72	5	4.78	2	4.68	2
		4.27	2						
		4.06	2	4.07	10	4.10	2	4.04	5
3.83	5	3.82	2	3.83	10	3.87	2	3.83	5
3.42	40	3.44	40	3.43	50	3.44	20	3.41	15
3.10	15	3.13	5	3.14	10	3.18	2	3.14	5
		3.07	10	3.06	15	3.08	5	3.05	10
2.797	100	2.797	100	2.797	100	2.804	100	2.790	100
		2.718	30	2.692	40	2.718	60	2.705	70
		2.620	15	2.613	15	2.625	10	2.613	15
		2.510	5	2.516	5	2.527	2	2.510	5
						2.293	2	2.275	2
2.265	20	2.262	25	2.249	25	2.258	15	2.244	20
2.132	5	2.143	10	2.143	10	2.140	5	2.136	10
2.058	5	2.058	10	2.054	10	2.061	2	2.054	5
		1.990	5	1.989	5	1.992	2	1.986	5
1.938	20	1.941	40	1.932	40	1.941	40	1.930	50
		1.890	15	1.881	15	1.890	10	1.881	10
								1.860	2
1.835	20	1.838	40	1.832	40	1.838	40	1.832	50
		1.803	15	1.795	20	1.805	20	1.797	30
		1.782	15	1.771	20	1.779	20	1.771	30
		1.754	15	1.744	20	1.754	20	1.746	30
1.712	20	1.717	25	1.712	30	1.715	20	1.712	30
						1.684	2		
		1.642	10	1.638	10	1.642	10	1.639	15
		1.602	5	1.602	10	1.608	5	1.602	10
		1.579	5	1.579	5	1.584	2	1.579	5
								1.561	2
1.531	5	1.534	10	1.529	10	1.534	10	1.534	5
1.502	5	1.498	15	1.495	10	1.502	10	1.497	10
		1.471	10	1.466	10	1.471	15	1.468	10
1.455	10	1.452	20	1.449	15	1.450	15	1.447	20
		1.425	10	1.424	10	1.430	15	1.427	20

TABLE XXIII

COMPARISON OF VALUES FROM HIGH TEMPERATURE INCINERATED DRY BONE AND ED ASHED BONE*

600°C. 6 hours				700°C. 7 hours			
B-28 (dry)		B-29 (ED ashed)		B-71 (ED ashed)		B-72 (dry)	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
8.07	15	8.02	20	8.07	20	8.07	20
6.47	15	6.40	20	6.48	20	6.48	20
5.22	30	5.15	40	5.18	50	5.18	40
4.35	2	4.30	5	4.35	10	4.35	10
4.04	5	3.99	10	4.04	20	4.04	20
3.90	5			3.92	5	3.92	5
3.47	50	3.42	40	3.44	40	3.43	40
3.33	10	3.32	5	3.35	5	3.33	5
3.19	80	3.18	70	3.18	70	3.18	70
3.08	2	3.12	5	3.09	2	3.10	2
3.00	5			2.99	5	2.99	5
2.859	100	2.851	100	2.859	100	2.859	100
2.738	5	2.731	10	2.738	10	2.738	10
2.673	2	2.661	2	2.667	2	2.667	2
2.654	2						
2.590	80	2.584	70	2.596	70	2.596	70
2.538	5			2.538	2	2.538	5
2.500	5	2.484	5	2.500	5	2.500	5
2.387	5	2.387	10	2.387	10	2.387	10
2.330	5						
				2.307	2		
2.249	5	2.240	10	2.249	10	2.249	10
2.187	5	2.179	10	2.179	15	2.179	10
2.144	5	2.147	10	2.148	10	2.148	10
2.065	5	2.058	10	2.065	5	2.062	5
2.023	5	2.012	10	2.019	10	2.019	10
1.989	5	1.989	10	1.986	10	1.986	10
1.920	10	1.920	20	1.923	25	1.923	25
1.870	10	1.869	15	1.875	15	1.872	15
1.816	2	1.811	2	1.813	2	1.813	2
		1.787	2	1.803	2	1.800	2
1.764	2	1.766	2	1.761	5	1.761	5
1.720	15	1.713	30	1.717	30	1.717	30
1.708	10			1.700	2	1.703	2
				1.672	2	1.672	2
				1.623	2	1.621	2
				1.592	5	1.592	5
1.542	2	1.542	2	1.542	20	1.542	20

*Values in this table are averaged and appear in Table X where they are compared with literature value.

TABLE XXIV

COMPARISON OF VALUES FROM HEAT ASHED EIGHT TO TEN DAY FEMURS WITH HEATED MIXTURE OF SODIUM AND POTASSIUM CHLORIDE

NaCl & KCl*		8 day*		8 day**		9 day**		10 day**	
B-73		B-69		B-53		B-52		B-51	
d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1	d Å	I/I_1
4.75	5	4.78	5	4.66	10	4.66	10	4.66	10
		4.36	5	4.36	5	4.36	5	4.36	5
				4.14	10	4.10	15		
3.95	60	3.98	60	3.90	100	3.90	100	3.90	100
3.57	20	3.55	10	3.57	50	3.57	50	3.57	50
		3.29	20						
3.17	5	3.19	10						
3.08	5	3.07	10						
2.852	100	2.852	60	2.817	50	2.817	50	2.852	70
2.798	100	2.777	100					2.797	70
		2.660	5	2.691	100	2.692	100	2.692	100
2.345	10	2.377	5	2.339	5	2.339	5	2.336	5
2.275	15	2.249	30						
2.203	5	2.200	5	2.207	5	2.210	5	2.215	5
2.120	10	2.128	5						
		2.087	5						
		2.028	5						
1.999	60	1.986	40	1.999	10	1.999	10	2.005	10
		1.947	10	1.960	10	1.960	10	1.960	10
				1.932	10	1.932	10	1.932	10
				1.862	2			1.850	2
1.779	10	1.798	5	1.779	2	1.779	2	1.782	2
		1.764	5						
1.621	20	1.672	2					1.623	2
		1.653	2						
1.602	20	1.610	2						
				1.555	2	1.555	2	1.555	2
1.497	20	1.492	5			1.490	2	1.502	2
1.395	15	1.390	5	1.393	2	1.395	2	1.400	2
				1.343	2	1.343	2	1.340	2

* Ashed in platinum crucible
** Ashed in porcelain crucible

TABLE XXV

LIST OF CRYSTALLINE MATERIALS WITH AN "a" AXIS NEAR 5.29A OR 10.58A*

Material	a	c	Reference
$KAlSiO_4$	5.17	8.67	Donnay & Nowacki
$Ca_2SiO_4 \cdot Ca_3(PO_4)_2$	5.21	6.90	" "
$BaAlO_4$	5.21	8.76	" "
$2Ca_2SiO_4 \cdot Ca_3(PO_4)_2$	5.22	6.91	Frevel & Rinn
$CaNaPO_4$	5.24	7.14	" "
$Pb(OH)_2$	5.26	14.7	Donnay & Nowacki
Micas, 3 layer structure			
$KR_3(OH,F)_2(Al,Si)_4O_{10}$	5.3	30.0	" "
$Sr_3(PO_4)_2$	5.38	19.8	" "
$2Ca_2SiO_4 \cdot Ca_3(PO_4)_2$	5.38	7.05	" "
Na_2SO_4	5.38	7.26	" "
$\beta Ca_3(PO_4)_2$ Whitlockite	10.31	37.00	Frevel & Rinn
$9Na_2SO_4 \cdot 2Na_2CO_3 \cdot KCl$	10.46	21.2	Donnay & Nowacki
$KNa_{22}(SO_4)_9(CO_3)_2Cl$	10.50	21.23	Frevel & Rinn
$Na_2Ce(Mn,Ta,Fe)H_2[(Si,P)O_4]_3$	10.53	45.5	Donnay & Nowacki
$NaCaPO_4$ (high temp.)	10.53	5.76	" "
$KCaPO_4$	10.60	5.84	" "
$(CH_3)_2CNOH$	10.61	7.02	" "
$NaSrPO_4$ (low temp.)	10.65	5.81	" "
$KSrPO_4$ (low temp.)	10.70	5.87	" "

*Crystalline metals not included in this list.

APPENDIX B

FIGURES

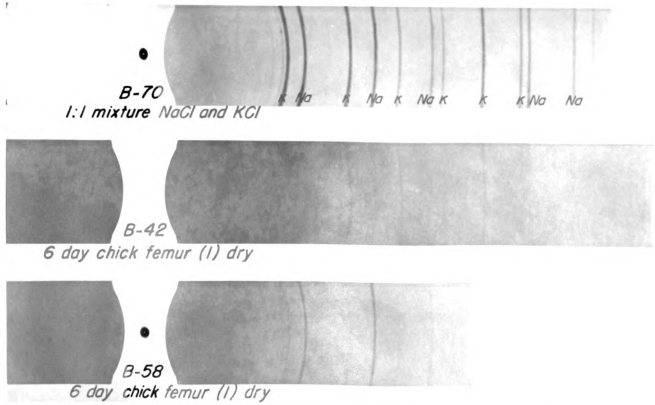


Fig. 9 X-Ray Diffraction Patterns of 6 Day Embryonic Femurs.

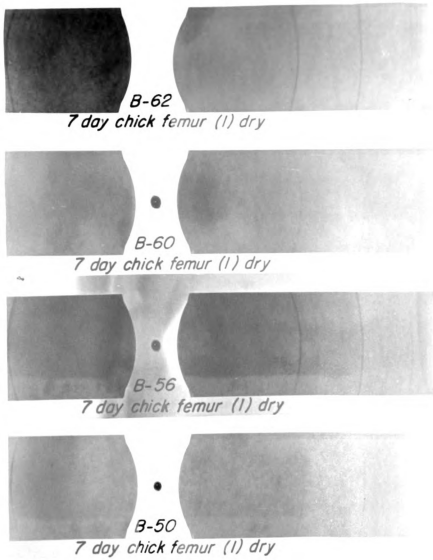


Fig. 10 X-Ray Diffraction Patterns of 7 Day Embryonic Femurs.

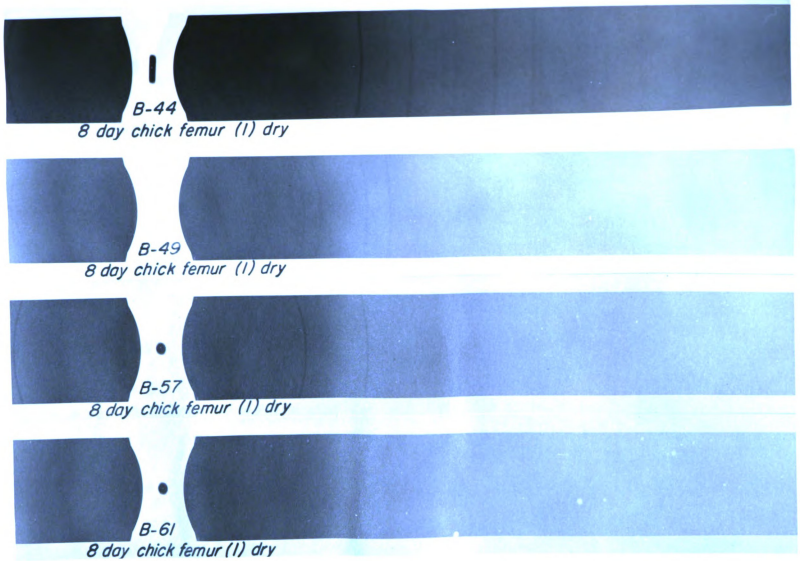


Fig. 11 X-Ray Diffraction Patterns of 8 Day Embryonic Femurs.

B-48
9 day chick femur (l) dry

B-55
9 day chick femur (l) dry

B-63
9 day chick femur (l) dry

B-66
9 day chick femur (l) dry

Fig. 12 X-Ray Diffraction Patterns of 9 Day Embryonic Femurs.

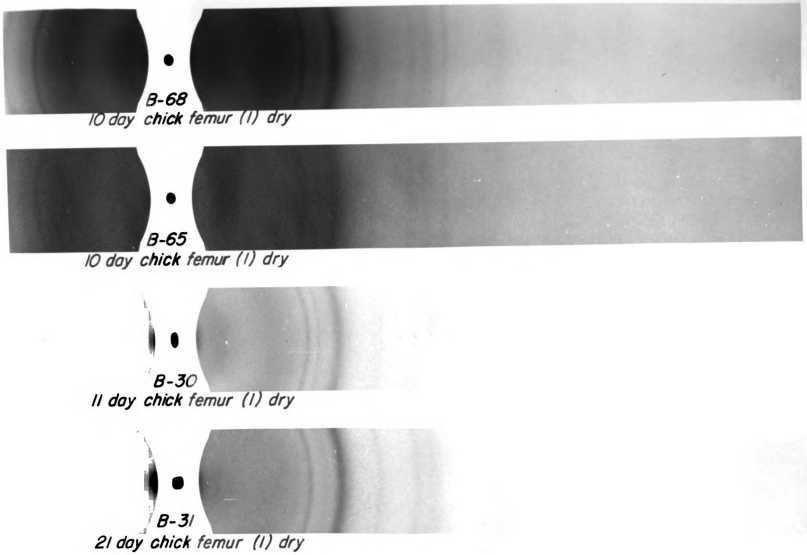


Fig. 13 Comparison of Diffraction Patterns from Embryonic Femurs of 10 to 21 Days.

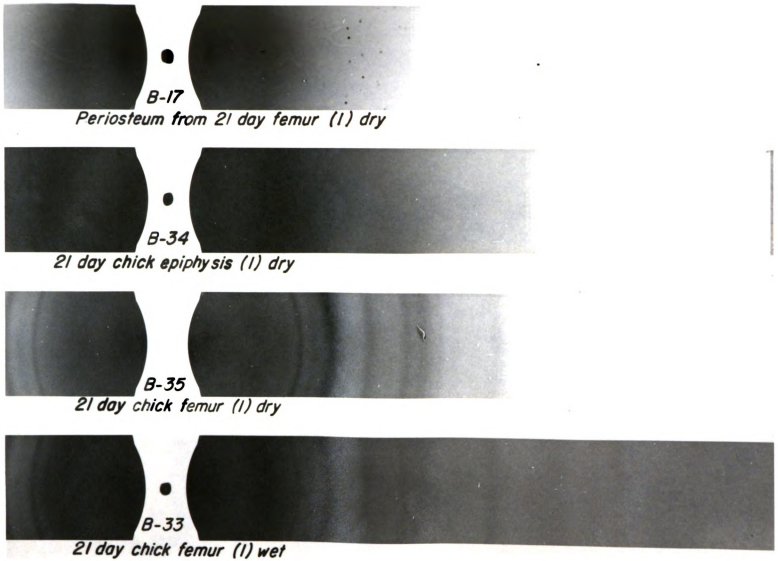


Fig. 14 Comparison of Diffraction Patterns of Various Parts of 21 Day Femur.

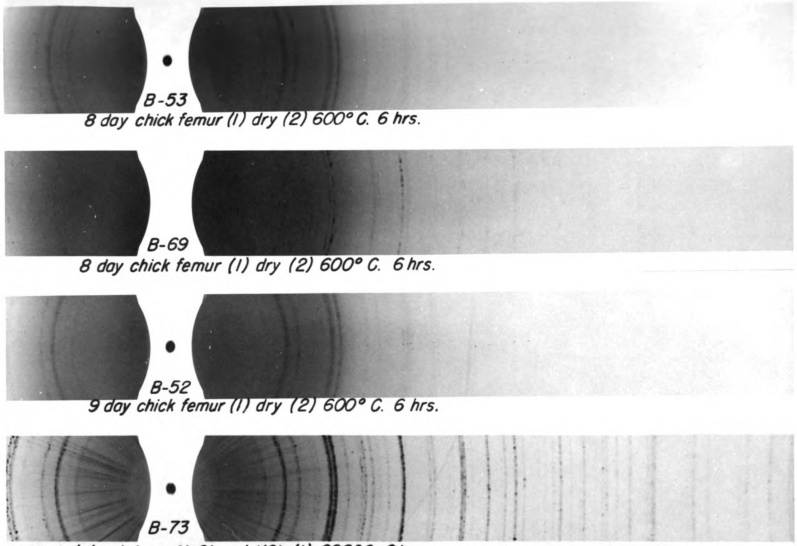


Fig. 15 *Comparison of Diffraction Patterns of Heat Ashed Preosseous Femurs with Heated Salt Mixture.*

LITERATURE CITED

Altschuler, Z. S., E. A. Cisney, and I. H. Barlow. X-Ray Evidence of the Nature of Carbonate-Apatite. Am. Mineral. 38: 328 (1953)

Armstrong, W. D. Composition and Crystal Structure of the Bone Salt. Trans. Macy Conference on Metabolic Interrelations. 2: 11-31 (1950)

Arnold, J. S. Calcium Metabolism of Growing and Mature Bone. Fed. Proc. 11: No. 1, Pt. I, page 5 (1952)

Arnold, P. W. The Nature of Precipitated Calcium Phosphates. Trans. Faraday Soc. 46: 1061-1072 (1950)

ASTM File, Alphabetical and Numerical Indexes of X-Ray Diffraction Patterns, 1950 Ed., American Society for Testing Materials, Philadelphia, 1950, 441 pp.

Bale, W. F. A Comparative Röntgen-Ray Diffraction Study of Several Natural Apatites and the Apatite-Like Constituent of Bone and Tooth Substance. Am. J. Roentgenol. Rad. Therapy. 43: 735-747 (1940) Seen in abstract only. C. A. 34: 6868^{6} (1940)

Bell, G. H., J. W. Chamber, and I. M. Dawson. The Mechanical and Structural Properties of Bone in Rats on a Rachitogenic Diet. J. Physiol. 106: 286-300 (1947)

Bergstrom, W. H. Bone as a Sodium and Potassium Reservoir. J. Clin. Invest. 31: 617 (1952)

Bergstrom, W. H. The Relationship of Sodium and Potassium to Carbonate in Bone. J. Biol. Chem. 206: 711-715 (1954)

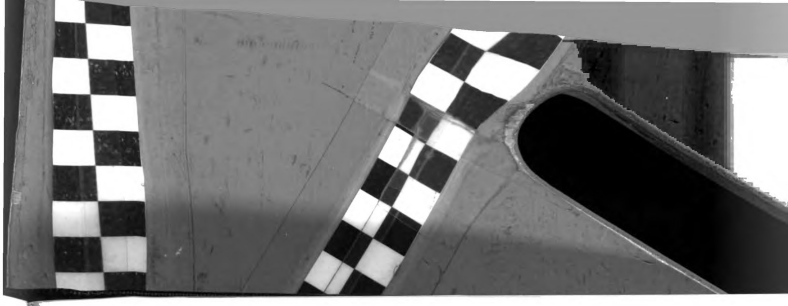
Berzelius, J. Uber basische phosphorsaure Kalkerde. Lieb. Ann. 53: 286-288 (1845)

Boyd, E. S. and W. F. Neuman. Surface Chemistry of Bone. V. Ion Binding Properties of Cartilage. J. Biol. Chem. 193: 243-251 (1951)

Boyle, P. E., J. Hillier, and N. R. Davidson. Preliminary Observations of the Enamel of Human and Guinea Pig Teeth Using Electron Microscope. J. Dent. Res. 25: 156 (1946)

Bredig, M. A. Zur Apatitstruktur der Anorganischen Knochen und Zahnsubstanz. Z. physiol. Chem. 216: 239-243 (1933)

Clark, J. H. A Study of Tendons, Bones, and other Forms of Connective Tissue By Means of X-Ray Diffraction Patterns. Am. J. Physiol., 98, 328-337 (1931)



Crowell, C. O., Jr., H. C. Hodge, and W. R. Line. Chemical Analysis of Tooth Samples Composed of Enamel, Dentine, and Cementum. J. Dent. Res. 14: 251-268 (1934)

Dana, E. S. A Textbook of Mineralogy, 4th ed., rev. by W. E. Ford. John Wiley and Sons, Inc., New York (1932) 851 pp.

Dallemagne, M. J. Some Recent Facts about the Properties of Tricalcium Phosphate and the Composition of the Bone Salt. Trans. Macy Conference on Metabolic Interrelations. 4: 154-168 (1952)

De Jong, W. F. La Substance Minerale Dans Les Os. Rec. trav. chim., 45: 445-448 (1926)

Dickens, F. Citric Acid Content of Animal Tissue, with Reference to its Occurrence in Bone and Tumour. Biochem. J. 35: 1011-1023 (1941)

Donnay, J. D. H. and W. Nowacki. Crystal Data, The Geological Society of America, New York, 1954. 719 pp.

Eichelberger, L., T. D. Brower, and M. Roma. The Distribution of Water, Electrolytes, Nitrogen and Chondroitin Sulfate in Hyaline Cartilages. Bk. II, pp. 560-577. Wolstenholme, G. E. W., Editor. Ciba Foundation Colloquia on Endocrinology, The Blakiston Company, New York, Vol. IV, 1952.

Eisenberger, S., A. Lehrman, and W. D. Turner. The Basic Calcium Phosphates and Related Systems, Some Theoretical and Practical Aspects. Chem. Rev. 26: 257-296 (1940)

Everett, M. R. Medical Biochemistry, Paul B. Hoeber, Inc., New York. 2nd Ed., 1948, 767 pp.

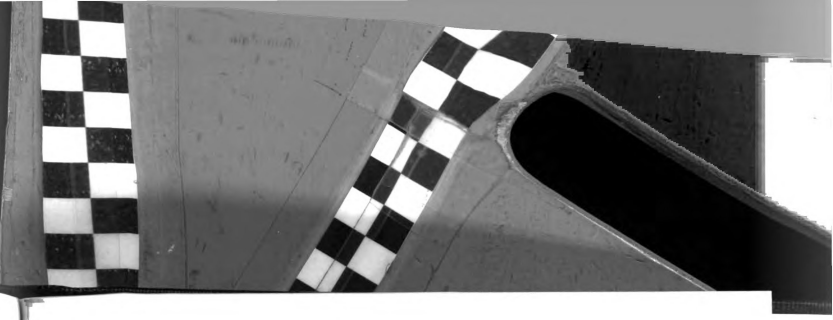
French, E. L., E. A. Welch, E. J. Simmons, M. L. LeFevre, and H. G. Hodge. Ca, P, and CO_2 Determination on all the Dentin from Sound and Carious Teeth. J. Dent. Res. 17: 401-410 (1938)

Frevel, L. K. and H. W. Rinn. Tabulated Diffraction Data for Hexagonal Isomorphs. Anal. Chem. 25: 1697-1717 (1953)

Gabriel, S. Chemische Untersuchungen über die Mineralstoffe der Knochen und Zähne. Z. physiol. Chem. 18: 257-303 (1894)

Gruner, J. W., D. McConnell, and W. D. Armstrong. The Relationship Between Crystal Structure and Chemical Composition of Enamel and Dentin. J. Biol. Chem. 121: 771-781 (1937)

Gutman, A. B. and T'Sai Fan Yu. A Concept of the Role of Enzymes in Endochondral Calcification. Trans. Macy Conference on Metabolic Interrelation. 2: 167-190 (1950)



Hendricks, S. B. Comments on the Crystal Chemistry of Bone. Trans. Macy Conference on Metabolic Interrelation. 4: 185-212 (1952)

Hendricks, S. B., and W. L. Hill. The Inorganic Constitution of Bone. Science 96: 255-257 (1942)

Hendricks, S. B., and W. L. Hill. The Nature of Bone and Phosphate Rock. Trans. Macy Conference on Metabolic Interrelations. 3: 173-189 (1951)

Hirschman, A., A. E. Sobel, B. Kramer, and I. Fankuchen. An X-Ray Diffraction Study of High Phosphate Bones. J. Biol. Chem. 171: 285-292 (1947)

Hirschman, A., A. E. Sobel, and I. Fankuchen. Calcification, X. An X-Ray Diffraction Study of Calcification in Vitro in Relation to Composition. J. Biol. Chem. 204: 13-18 (1953)

Hodge, H. C. Some Observations on the Dynamics of Calcification. Trans. Macy Conference on Metabolic Interrelations. 2: 73-112 (1950)

Hodge, H. C., M. L. LeFevre, and W. F. Bale. Chemical and X-Ray Diffraction Studies of Calcium Phosphates. Ind. & Eng. Chem. Anal. Ed. 10: 156-161 (1938)

Hodge, H. C., W. F. Koss, J. T. Ginn, M. Falkenheim, E. Gavett, R. C. Fowler, I. Thomas, J. F. Bonner, and G. Dessauer. The Nature of the Insoluble Sodium of Bone. J. Biol. Chem. 148: 321-332 (1943)

Hodge, H. C., E. Gavett, and I. Thomas. The Adsorption of Strontium at Forty Degrees by Enamel, Dentin, Bone, and Hydroxyapatite as Shown by the Radioactive Isotope. J. Biol. Chem. 163: 1-6 (1945)

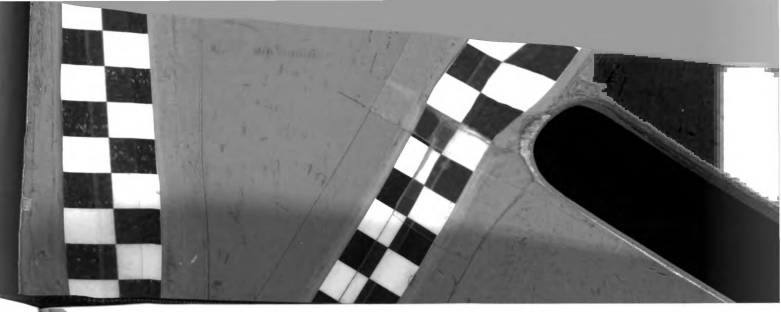
Holt, L. E., Jr., V. K. LaMer, and H. B. Chown. Studies in Calcification I. The Solubility Product of Secondary and Tertiary Calcium Phosphate Under Various Conditions. J. Biol. Chem. 64: 509-565 (1925)

Hoppe-Seyler, F. Untersuchungen über die Constitution des Zahnschmelzes. Arch. f. path. Anat. u. Physiol. 24: 13-32 (1862)

Huggins, C. The Composition of Bone and the Function of the Bone Cell. Physiol. Rev. 17: 119-143 (1937)

Klement, R. Die Zusammensetzung der Knochenstützsubstanz. Z. Physiol. Chem. 184: 132-142 (1929)

Klement, R. and G. Trömmel. Hydroxylapatit, der Hauptbestandteil der anorganischen Knochen und Zahnsubstanz. Z. Physiol. Chem. 213: 263-269 (1932)



Klug, H. P. and L. E. Alexander. X-Ray Diffraction Procedures, John Wiley and Son, New York, 1954, 716 pp.

Leulier, A., A. Policard, and L. Revol. La Teneur en Calcium et en Phosphore des Divers Constituants Histologiques der Os Longs Chez le Poulet; Ses Variations an Cours des Quatre Primiers Mois, Compt. rend. Soc. biol., 135: 1203-5 (1941)

Libby, D.A. written communication

Logan, M. A. Recent Advances in the Chemistry of Calcification. Physiol. Rev. 20: 522-560 (1940)

Logan, M. A., and H. L. Taylor. Solubility of Bone Salt. J. Biol. Chem. 119: 293-307 (1937)

Logan, M. A., and H. L. Taylor. Solubility of Bone Salt. II. Factors Affecting its Formation. J. Biol. Chem. 125: 377 (1938)

Marks, P.A., and E. Shorr. Factors Which Regulate the Deposition of Calcium and Strontium in Rachitic Cartilage in Vitro. Trans. Macy Conference on Metabolic Interrelations. 2: 191-202 (1950)

McConnell, D. The Crystal Chemistry of Francolite and Its Relationship to Calcified Animal Tissues. Trans. Macy Conference on Metabolic Interrelations. 4: 169-184 (1952)

McLean, F.C., and M. R. Urist. Bone: An Introduction to the Physiology of Skeletal Tissue. The University of Chicago Press, Chicago. 1955, 182 pp.

Mehmel, M. Beziehungen zwischen Kristallstruktur und Chemischen Formel des Apatits. Z. physik. Chem., Abt. (B) 15: 223-241 (1931)

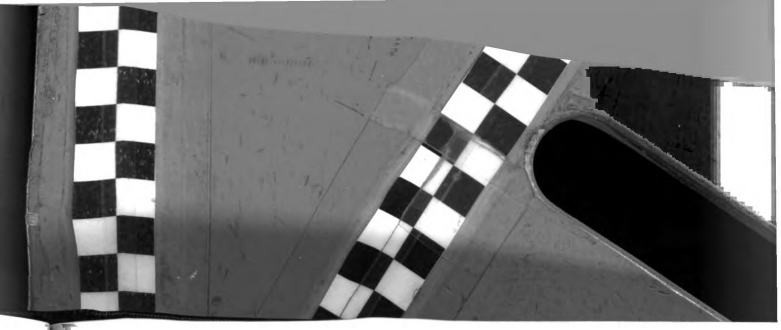
Neuman, W. F. Bone as a Problem in Surface Chemistry. Trans. Macy Conference on Metabolic Interrelations. 2: 32-72 (1950)

Neuman, W. F. Conference Discussion. Trans. Macy Conference on Metabolic Interrelations. 3: 184 (1951)

Neuman, W. F. Dehydration Studies with Synthetic Hydroxylapatite. Trans. Macy Conference on Metabolic Interrelations. 4: 213 (1952)

Neuman, W. F. and M. W. Neuman. The Nature of the Mineral Phase of Bone. Chem. Rev. 53: 1-46 (1953)

Neuman, W. F., M. W. Neuman, E. R. Main, and B. J. Mulryan. The Deposition of Uranium in Bone. V. Ion Exchange Studies. J. Biol. Chem. 179: 335-340 (1949)



Parrish, W., and B. W. Irwin. Data for X-Ray Analysis. Vol. I. Philips Technical Library. Irvington-on-Hudson, N. Y., 1953, 100 pp.

Posner, A. S. X-Ray Diffraction Studies of Tooth Structure. Norelco Reporter II, No. 2: 26-30 (1955)

Posner, A. S., and S. R. Stephenson. Crystallographic Investigation of Tricalcium Phosphate Hydrate. J. Dent. Res. 31:371-382 (1952)

Reed, C. I., and B. P. Reed. An Attempted Correlation of Mechanical Properties of Bone with Antirachitic Healing and with Molecular Structure as Determined by X-Ray Diffraction Techniques. Am. J. Physiol. 138: 34-41 (1942)

Robison, R. The Possible Significance of Hexosephosphoric Esters in Ossification. Biochem. J. 17: 286-293 (1923)

Robinson, R. A. Electron Micrography of Bone. Trans. Macy Conference on Metabolic Interrelations. 3: 271-289 (1951)

Robinson, R. A., and F. W. Bishop. Methods of Preparing Bone and Tooth Samples for Viewing in the Electron Microscope. Science. 111: 655-657 (1950)

Robinson, R. A., and M. L. Watson. Collagen Crystal Relationships in Bone as Seen in the Electron Microscope. Anat. Rec. 114: 383-409 (1952)

Robinson, R. A., and M. L. Watson. Electron Micrography of Bone. Trans. Macy Conference on Metabolic Interrelations. 5: 72-105 (1953)

Roseberry, H. H., A. B. Hastings, and J. K. Morse. X-Ray Analysis of Bone and Teeth. J. Biol. Chem. 90: 395-408 (1931)

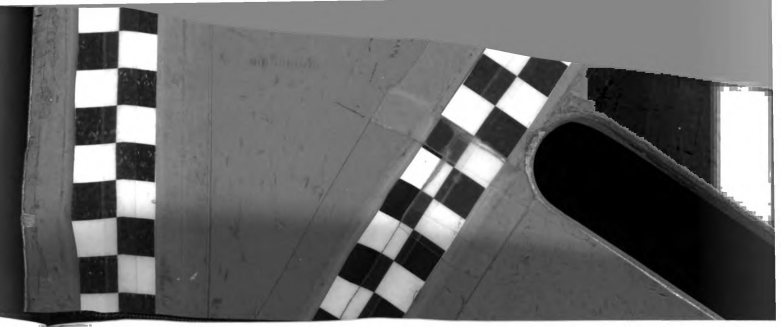
Shear, M. J., and B. Kramer. Composition of Bone. III. Physico-chemical Mechanism. J. Biol. Chem. 79: 125-146 (1928)

Shear, M. J., and B. Kramer. Composition of Bone. V. Some Properties of Calcium Citrate. J. Biol. Chem. 79: 161-176 (1928)

Sobel, A. E. The Local Factor in Calcification. Trans. Macy Conference on Metabolic Interrelations. 2: 113-143 (1950)

Sobel, A. E., M. Rockenmacher, and B. Kramer. Carbonate Content of Bone in Relation to the Composition of Blood and Diet. J. Biol. Chem. 158: 475-489 (1945)

Sobel, A. E., M. Rockenmacher, and B. Kramer. Composition of Bone in Relation to Blood and Diet. J. Biol. Chem. 159: 159-171 (1945)



Sobel, A. E., A. Hanok, H. A. Kirshner, and I. Fankuchen. Calcification of Teeth. III. X-Ray Diffraction Patterns in Relation to Changes in Composition. J. Biol. Chem. 179: 205-210 (1949)

Taylor, N. W., and C. Sheard. Microscopic and X-ray Investigation on the Calcification of Tissue. J. Biol. Chem. 81: 479-493 (1929)

Thewlis, J., G. E. Glock, and M. M. Murray. Chemical and X-Ray Analysis of Dental, Mineral and Synthetic Apatites. Trans. Faraday Soc. 35: 358-363 (1939)

Trautz, O. R. The Use of X-Ray Diffraction in Dental Research. Norelco Reporter II, No. 2: 19-22, 32 (1955)

Werner, A. Zur Konstitution basischer Salze und analog konstituierter Komplexsalze. Ber. 40: 4441-4449 (1907)

White, H. L., and D. Rolf. Whole Tissue Electrolyte Analyses in Normal and Adrenalectomized Rats. Am. J. Physiol. 180: 287-295 (1955)

Woods, N. V. Specific Surfaces of Bone, Apatite, Enamel, and Dentine. Science. 105: 531-532 (1947)

Wyckoff, R. W. G., and R. B. Corey. X-Ray Diffraction Patterns from Reprecipitated Connective Tissue. Proc. Soc. Exptl. Biol. Med. 34: 285-287 (1936)

Addendum

After this thesis was typed, the following two important references were received:

Miner, R. W., Ed. Recent Advances in the Study of the Structure, Composition, and Growth of Mineralized Tissues. Annals of the New York Academy of Sciences, Vol. 60, Art. 5, 541-806 (1955)

Spencer, M. C. and K. Uhler. The Structure, Composition, and Growth of Bone, 1930-1953, A Bibliography. Armed Forces Medical Library, Ref. Div., Washington, D. C. (1955)