THE NATURE OF THE MINERAL PHASE OF BONE[1]

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CONTENTS

I. INTRODUCTION

In nearly all of the higher forms of animal life, structural strength and rigidity are provided by the bony skeleton. The strength and rigidity of bone are derived from its composition and architecture, which is unique among living tissues. About one-third of its mass is in the form of mineral crystals, which are embedded

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in an extracellular matrix composed largely of a complex interwoven network of a tough fibrous protein, collagen. There is present also a poorly characterized interfibrillar "ground substance." Bone cells, attached to one another by protoplasmic processes, small blood vessels, and variable amounts of extracellular and intracellular fluid make up the rest of the organic matrix.

This review is concerned with the general problem of establishing the chemical nature and properties of the mineral crystals of bone. While this problem has been under investigation for over a century, very recently a number of new techniques, principally electron microscopy and tracer chemistry, have added a great deal to our understanding. With this newer knowledge an attempt has been made to present a unified concept of the problem. To do this it has been necessary, where critical data are lacking, to resort to speculation. It is the aim of this review to stimulate interest and research, not to predict the future. If, as new facts are learned, *all* of the speculation here presented proves false, the authors will be neither surprised nor discouraged.

Only certain phases of the subjects have been covered in detail. The reader is referred, therefore, to a number of excellent reviews (9, 65, 80, 81, 121, 139, 187, 241, 248, 262, 291, 325) for a more comprehensive bibliography.

II. THE CRYSTALS OF BONE

From x-ray diffraction evidence the crystalline nature of bone mineral was recognized long before (100) the crystals were demonstrated directly. The inability of the light microscope to visualize these crystals and the diffuseness of the x-ray diffraction patterns indicated that the size of the crystals was remarkably small. The first estimates of size, based on work with x-rays and necessarily tentative, were in good agreement, from 10^{-5} to 10^{-6} cm. (20, 257, 353). There were many claims that the crystals showed orientation with respect to the longitudinal direction of bone. These claims were based on x-ray diffraction (50, 51, 64, 115, 209, 258, 293, 295, 296, 308, 346, 351, 352, 353, 358) and optical methods (87, 99, 310, 353). Both of these methods are essentially statistical in nature and, while positive evidence of orientation is significant, negative results can always be explained as the average of random distribution of microelements which possess periodicity or orientation.

Recently, isotope-exchange studies (119) and measurements of the surface area of powdered bone by gas adsorption (374) resulted in estimates of the same order of magnitude. Much of this work is open to the criticism that, in many cases, the bone was subjected to rigorous ashing procedures which might result in crystal growth. In any event, the values may be regarded as maximal.

Crystals of such a size could be *visualized* only by the electron microscope under optimal conditions. The technical difficulties attending the preparation of specimens for such a study have resisted the efforts of investigators until very recently.

The first attempts (131, 132, 297) employed the surface replica technique, using etched and unetched samples of dentine and enamel. Although fine structure was evident in these early pictures, the crystals were not discernible. In 1946 (35), by combining electron microphotography and diffraction, orientation of

the crystallites in enamel could be easily demonstrated. In 1950, two groups of investigators (22, 194) described considerable ultramicroscopic detail in thin chips of compact bone, but the crystallites could not be identified with certainty. Another group reported replica studies in 1950 (321).

A novel approach was introduced by Robinson and Bishop (301), who were able to isolate crystals for study by pulverizing bone specimens. An extensive application of this method permitted Robinson (299, 300) to visualize crystals obtained from a variety of bone preparations. Whereas the clear crystal outlines of flat hexagonal tablets seen in many pictures were convincing evidence that the particles were indeed crystals of bone mineral, there remained the possibility that some fragmentation had occurred. Furthermore, the structural relationships between the organic phase and the mineral phase could only be surmised. Nonetheless, by measuring 1000 typical crystals in normal and shadow-cast specimens of autoclaved human bone, Robinson concluded that the *average* approximate dimensions of the crystals were 500 x 250 x 100 Å. These values were in excellent agreement with the estimates given earlier but have been modified slightly as improved procedures have been developed (see below).

In 1951 Engström and Zetterström (116) combined microdiffraction of x-rays and electron micrographs, using the replica technique to establish both orientation and size of the crystals. They estimated the crystals to be 200 Å. wide. Pease described decalcified sections of teeth (287) which showed "holes" with diameters less than 1000 Å.

To examine more closely the interrelationships between the organic and mineral phases of bone, Robinson and Watson developed a procedure for the preparation of sections of *intact* bone for electron-microscopic study (302). This procedure involves a minimum of manipulation and should not cause significant modification of the crystals. With this technique, beautiful pictures, as in figure 1, were obtained which demonstrated a definite orientation of the crystals with respect to the collagen fibers. The fibers themselves appeared to have a regular diameter of 800 Å. and possessed characteristic doublet-banding at regular intervals of 400–500 Å. in the direction of the length of the fiber. As in Gerould's replica pictures (132), the bands were in rough register with a suggestion of bridging between bands. Tabular crystals were seen to be lying between the doublets, with the long axes (350–400 Å.) in the direction of the fiber. The crystals were nearly as wide as they were long and had a thickness of 25–50 Å.! A comparison of electron microphotographs with electron-diffraction patterns demonstrated that the *c*-axes of the unit cells of the crystals were nearly parallel to the fiber axis.

In 1952, other applications of the electron microscope to the study of dental structures were reported (29, 319, 320, 322, 341) but crystals were seen in only one (341).

A limited number (161, 166, 368) of electron micrographs of synthetic precipitates of basic calcium phosphate have been made. These synthetic crystals (368), though usually larger in size, in many instances exhibit the same tabular form as the crystals found in bone.

These results with the electron microscope furnish visual confirmation of the

earlier claims of orientation and size based on x-ray diffraction and optical methods while adding a clear picture of the three-dimensional form and size. It is clear that the electron microscope will prove a very powerful tool in the study of the chemistry and biochemistry of the basic calcium phosphates. From the small number of studies thus far reported, it is already possible to represent the bone crystal provisionally as in figure 2. Two very important questions remain to be answered: Do the crystals vary in size and habit from species to species? Are newly formed crystals smaller in size than aged crystals?

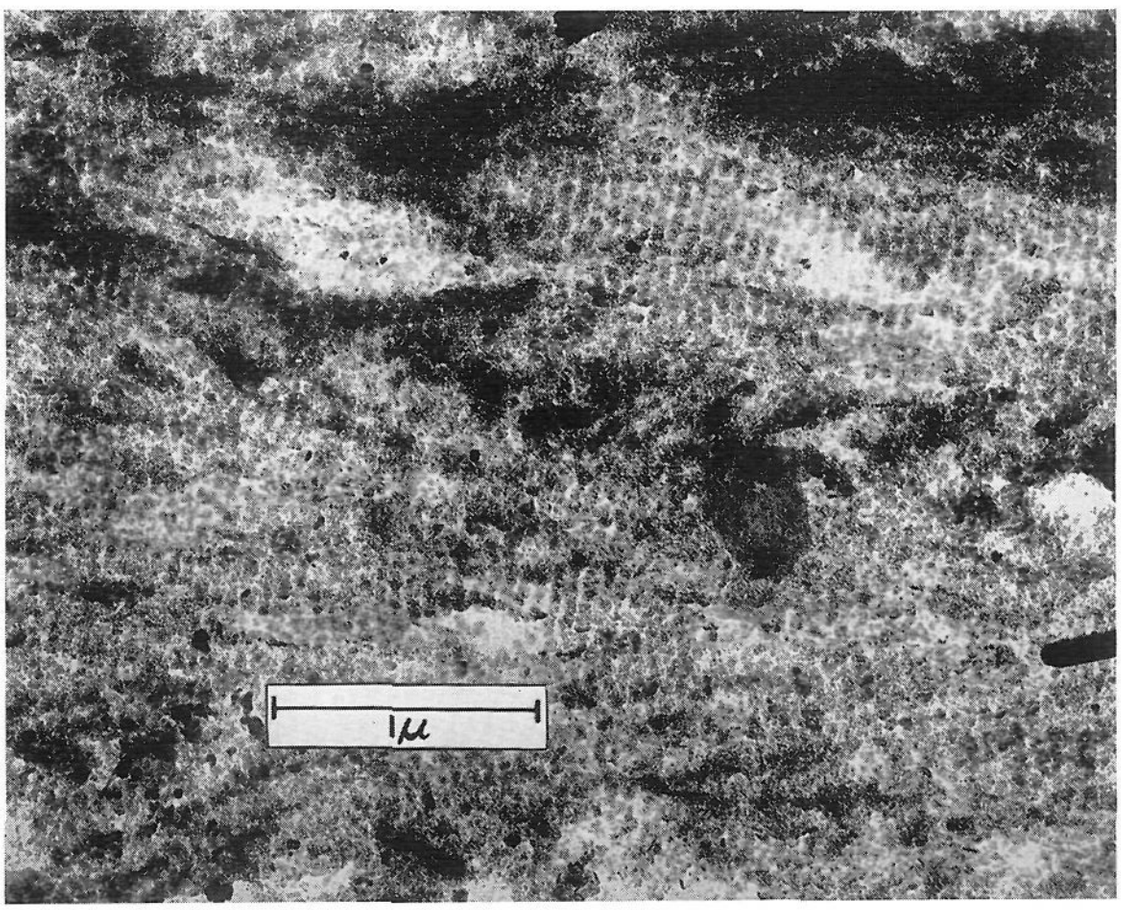

FIG. 1. An electron micrograph of a section of human rib (courtesy of R. A. Robinson and M. L. Watson). In this exposure the underlying collagen is barely visible, but the crystals are easily seen in regularly arranged bands corresponding to the main collagen striations. In this section, the longitudinal direction of the fibers is horizontal.

III. THE LATTICE

Whereas the size and shape of the crystals of bone are now known with some certainty, the chemical nature of these crystals is still poorly understood, after more than a century of investigation. A primary obstacle has been the variability in composition of the bones and teeth. In fact, there is no evidence that the crystals are comprised of a single compound, despite the proposal of at least ten "formulae" (10).

The principal constituents of bone mineral are calcium, phosphate, carbonate, and hydroxyl ions. Except for the presence of carbonate, the solid phase may be

regarded as a slightly impure, basic calcium phosphate (113). For this reason the crystal structure and chemical properties of the basic calcium phosphate system and the bone mineral are considered to be interchangeable in the subsequent discussion.

There is overwhelming evidence that any unignited preparation of basic calcium phosphate exhibits an x-ray diffraction pattern of the apatite lattice (15, 19, 26, 36, 38, 39, 43, 63, 70, 113, 115, 121, 126, 127, 130, 161, 165, 166, 172, 207, 243, 255, 256, 290, 307, 366, 367).

The apatite lattice is not a compound, but rather a space arrangement of atoms found in a number of minerals, of which fluorapatite may be considered the prototype. This mineral occurs naturally in macrocrystals of defined com-

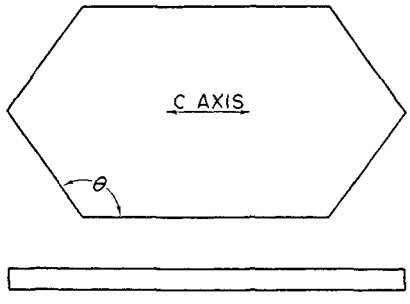

FIG. 2

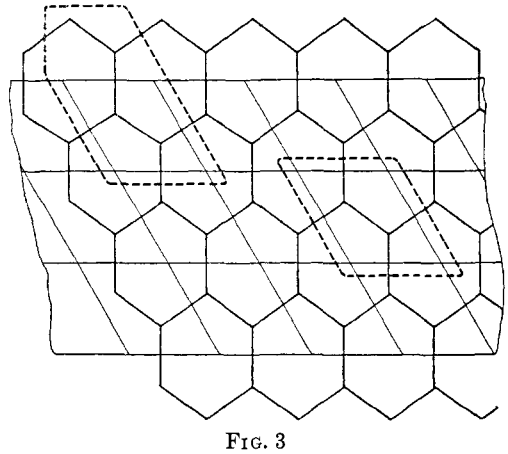

FIG. 3

FIG. 2. A diagrammatic representation of a hypothetical bone crystal. Approximate dimensions are: length, 350 Å.; width, 300 Å.; thickness, 25–50 Å. In synthetic crystals the angle θ averages 126°.

FIG. 3. A cross-section view of a crystal cut perpendicular to the c-axis. The hexagons represent columns of calcium atoms; the parallelepipeds, columns of hydroxyl groups. The dotted lines show the volumes represented by a surface unit cell and an interior unit cell depicted in figure 4. Other arrangements of the unit cells might be given, but they would not alter the quantitative considerations.

position; the three-dimensional arrangement of its atoms is well understood (252, 253, 267) and generally accepted (18, 26). The unit cell of fluorapatite may be represented as $Ca_{10}(PO_4)_6F_2$. The space arrangement of the unit cell is given in table 1.

Ordinarily, the bone mineral contains only traces of fluoride (241, 377). Because of this, it is generally agreed that the apatite lattice of the bone mineral approximates the structure of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, in which hydroxyl groups occupy the space positions of the fluoride ions in fluorapatite (36, 37, 40, 41, 42, 43, 70, 73, 78, 83, 84, 86, 113, 161, 228, 229, 245, 246, 260, 293, 294, 295, 340). Unfortunately, "hydroxyapatite" is a term which means many things to many men. This substance, unlike fluorapatite, rarely attains a

crystal size of even microscopic dimensions. There exists no reference material of defined composition and crystal habit. For these reasons, confusion and disagreement have persisted in the literature.

There is no doubt that the characteristic "apatite pattern" of x-ray diffraction can be given by almost any basic calcium phosphate precipitate exhibiting a molar calcium:phosphorus ratio[2] between the approximate limits of 1.33 to 2.0 (15, 19, 30, 31, 44, 123, 144, 161, 190, 214). In other words, any unignited calcium phosphate having a composition within 20 per cent of the theoretical value, 1.66, is hydroxyapatite! Materials with a calcium:phosphorus ratio less than 1.33 show the presence of secondary calcium phosphate dihydrate (15, 89, 91, 125, 126, 133, 254, 306) and those with a calcium:phosphorus ratio greater than 2.0 contain calcium hydroxide or other anions (15). In 1940, in an excellent and critical review of the literature covering phase-rule studies, titration curves,

TABLE 1

The structure of apatite (from Bale (18))

Atomic positions:

2 F (*a*) 00¼, 00¾
4 Ca_I (*f*) ⅓, ⅔, *u*; ⅔, ⅓, $\bar{u}$; ⅓, ⅔, ½ − *u*; ⅔, ⅓, *u* + ½
6 Ca_{II}, 6 P, 6 O_I, 6 O_{II} (*h*), *uv* ¼; $u\bar{v}$ ¾; $\bar{u}$, *u* − *v*, ¼; *v* − *u*, $\bar{u}$, ¼; *v*, *v* − *u*, ¾; *u* − *v*, *v*, ¾
12 O_{III} (*i*) *xyz*; $\bar{x}\bar{y}z$; *x* − *y*, *x*, *z*; $\bar{y}$, *x* − *y*, *z*; *x*, *y*, ½ − *z*; $\bar{x}$, $\bar{y}$, ½ + *z*; *x* − *y*, *x*, ½ + *z*; $\bar{y}$, *x* − *y*, ½ − *z*; *y*, *y* − *x*, ½ + *z*; *y* − *x*, $\bar{x}$, *z*; *y*, *y* − *x*, $\bar{z}$; *y* − *x*, *x*, ½ − *z*

	x/a	*y/a*	*z/c*
2 F	0	0	0.250
4 Ca_I	0.333	0.333	0
6 Ca_{II}	0.237	−0.015	0.250
6 P	0.416	0.361	0.250
6 O_I	0.333	0.500	0.250
6 O_{III}	0.600	0.467	0.250
12 O_{III}	0.333	0.250	0.062

solubilities, x-ray diffraction, etc., Eisenberger, Lehrman, and Turner (113) concluded that "at least for hydroxyl apatite, a substance with some small whole number ratio of atomic species does not exist but rather a crystal lattice common to a continuous series of solid solutions." While this statement describes the general situation it does not provide an explanation. Apparently some explanation is necessary because, although this statement was widely quoted for a time, agreement between investigators has become less and less with the passing years.

Two types of mechanisms have been proposed to account for the variability encountered in crystals of bone and hydroxyapatites: (*a*) Adsorption: Because of the minute size of the crystals, adsorption of calcium or phosphate ions may

[2] Throughout this discussion, *only molar ratios* will be used. The literature is confused by the use of such terms as weight ratios, CaO/P_2O_5, etc.

account for some variability in the calcium:phosphorus ratio (19, 20, 21, 93, 94, 133, 161, 166, 172, 190, 210, 222, 224, 290, 360). (*b*) Substitution: It has been proposed that water, hydrogen, hydronium, and other ions may, to a limited extent, substitute isomorphically within the lattice (15, 33, 34, 102, 148, 165, 172, 242, 243, 245, 246, 247, 349, 354, 360).

A. The adsorption hypothesis

The adsorption hypothesis is difficult to discuss, because the meaning of the term "adsorption" in this case has never been made clear by any of its proponents. It has been left to the reader to infer that, at the crystal surface, there are no limits to the calcium:phosphorus ratio. One group of investigators has suggested that calcium:phosphorus ratios above 1.5 are evidence for the presence of adsorbed calcium (74, 93, 94, 190), while others suggest that calcium:phosphorus ratios below 1.66 indicate adsorption of phosphate (19, 20, 21, 166, 178, 290). In 1950, Hendricks forcefully restated the adsorption idea and attributed the variations in calcium and phosphate content, the presence of carbon dioxide, sodium, citrate, proteinate, and the other trace minerals of bone to surface "adsorption" (162).

While it is true that the crystals of basic calcium phosphate preparations and of bone are extremely small and present a tremendous surface (269), the classical adsorption theory has definite limitations. How, for example, can apatite exhibit a calcium:phosphorus ratio of 1.33 by the adsorption of phosphate? To do this, each crystal would be required to adsorb one phosphate ion for every four in the crystal. The crystals are not small enough to permit this (368), and if one assumes a multilayer of adsorbed ions, what makes up the cation deficit? One cannot implicate hydrogen ions in this case, because a solid phase of adsorbed phosphoric acid could not be stable above pH 7. By the same reasoning, it is impossible to accept the proposal that high calcium:phosphorus ratios (approaching 2 (15, 144)) can be explained by adsorption of calcium. As a matter of fact, two apatite preparations having calcium:phosphorus ratios of 1.50 and 1.66 have been studied and the composition of the surfaces of the crystals did not account for the overall composition in either case (270). This analysis was performed by the use of isotopic exchange techniques based on the reasonable assumption that "adsorbed" ions would be exchangeable. Thus, only a small part of the observed variability of the calcium:phosphorus ratios of the basic calcium phosphate system can be attributed to adsorption in the classical sense. However, it must be emphasized that the presence in bone of ions "*foreign*" to the hydroxyapatite lattice, such as carbon dioxide, sodium, citrate, etc., may indeed be brought about by exchange (173, 269). This aspect is developed more fully in Section V,B.

B. Isomorphic substitution

The isomorphic substitution theory is not new (309) and, like the adsorption theory, has been extended to remarkable limits (242). In most cases, single specific substitutions have been considered and almost no attempt has been made to integrate the total collection of odd and ofttimes confusing facts into one com-

prehensive structural concept. The exception is an elegant paper by Arnold (15), who has courageously presented a variable three-dimensional structure to explain the x-ray and chemical data. Arnold's structures are probably unacceptable, since they presume the crystals to be elongated in one of the *a*-axes. The few observations made (368) indicate that in most crystals the *c*-axis and the long axis are parallel. However, his proposed structures indicate the possibility of isomorphic substitution of water for calcium within the unit cell and show how differences in the calcium content of the crystal may influence its size. There is no doubt that isomorphic substitution, both at the surface and within the crystal, is the principal reason why variable composition is so frequently encountered.

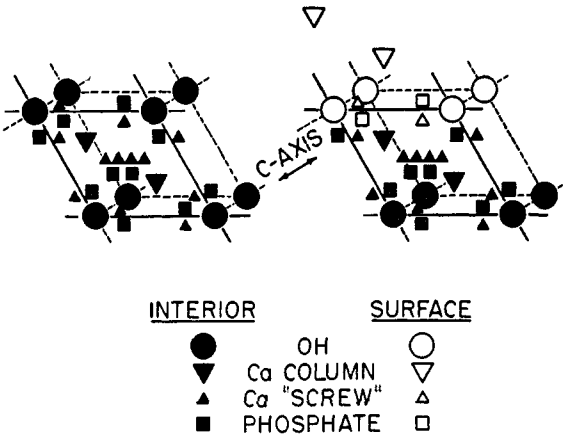

FIG. 4. A diagrammatic illustration of units cells from the crystal interior and surface. It can be seen that the compositions of the two unit cells differ markedly. Assuming no isomorphic substitution, the interior cell has the composition:

$$[Ca_{10}(PO_4)_6(OH)_2]^0_{interior}$$

while the surface cell has the composition:

$$[Ca_8(PO_4)_4OH]^{+++}_{interior} \text{ and } [Ca_4(PO_4)_2OH]^{+}_{surface}$$

C. The importance of surface substitutions

It is profitable to reëxamine the whole situation in the light of the crystal shape and size as shown by electron microscopy. In figure 3, a cross-section of a typical bone crystal has been drawn diagrammatically to indicate the crystallographic arrangement of the unit cells. Because the crystal tablets are only 2–4 unit cells thick, only one-third to one-half of the crystal is comprised of normal unit cells! One-half to two-thirds of the unit cells are located in the surface and possess one or more unshared sides. The importance of this is clearer in figure 4, where the composition of the surface cell[3] is shown to vary markedly from the

[3] It has been assumed that the hexagons of column calcium ions are nearly complete at the crystal surface. This is supported by the fact that the symmetry of the crystal is hexagonal and, as shown in the text, quantitative relationships are best satisfied by the resulting structure (table 2).

composition of the internal unit cell. One needs only to postulate an isomorphic substitution of hydronium ion for calcium ion in surface positions to explain the existence of precipitates with calcium:phosphorus ratios varying from 1.4 to 1.8. This structural concept explains how the basic calcium phosphate system can vary in composition while retaining a characteristic internal lattice structure as evidenced by x-ray diffraction.

At present, only one set of critical data is available with which to test quantitatively the proposed structure. The surface composition of a calcium phosphate preparation with a calcium:phosphorus ratio of 1.66 was measured by the isotopic exchange of both calcium and phosphate under conditions which were

TABLE 2

A calculation of the number of surface atoms of calcium and phosphorus per unit cell in the crystal surface

Given: Total surface area		67.8 m.²/g.
Dimensions of unit cell	c	6.88 Å.
	a	9.42 Å.
Exchangeable phosphorus		9.68 mg./g.
Exchangeable calcium		17.2 mg./g.

$$\text{Surface area per unit cell} = 6.88\ \text{Å.} \times 9.42\ \text{Å.} = 64.8 \times 10^{-20}\ \text{m.}^2$$

$$\text{Number of surface unit cells} = \frac{67.8\ \text{m.}^2/\text{g.}}{64.8 \times 10^{-20}\ \text{m.}^2/\text{unit cell}} = 1.04 \times 10^{20}\ \text{unit cells/g.}$$

$$\text{Number of calcium atoms in surface} = \frac{0.0172}{31} \times (6 \times 10^{23}) = 2.7 \times 10^{20}\ \text{calcium atoms/g.}$$

$$\text{Number of phosphorus atoms in surface} = \frac{0.00968}{31} \times (6 \times 10^{23}) = 1.9 \times 10^{20}\ \text{phosphorus atoms/g}$$

In terms of the surface structure here postulated, these results indicate a partial substitution of Ca^{++} by H_3O^+ at pH 7.0 and the surface unit cell composition, in nearest whole numbers, may be written:

$$[Ca_3^{++}(PO_4^{---})_2(H_3O^+)OH^-]^0$$

identical and which would not alter the surface composition significantly (270). The surface area was measured by adsorption of ethane gas (269). The dimensions of the unit cell were determined by x-ray diffraction (270). From these data, the number of calcium and phosphate ions present in each surface unit cell can be calculated, as in table 2. The results of these calculations lend strong support to the surface hypothesis.

The surface concept is also consistent with a number of other facts:

(*a*) The water content of preparations increases with decreasing calcium:phosphorus ratios.

(*b*) Most crystal preparations would possess a cationic excess in an aqueous medium and therefore would be positively charged, with a hydration shell con-

taining oriented but non-specific *boundary* anions. This accounts for the adsorption of anionic dyes and the hydration layer (*cf.* Section V,D).

(*c*) Preparations having low calcium:phosphorus ratios with maximum hydronium-ion substitution would approach electroneutrality, permitting the build-up of thicker crystals by O_{PO_4}—H_{H_2O}—O_{PO_4} bonds, as postulated by Arnold (15). This structure predicts that crystals formed with an excess of calcium and at alkaline pH's must be extremely small.

(*d*) Since the surface positions for calcium represent an extension of the underlying lattice, this proposal predicts specific limitations on cationic substitutions (see Section V,B).

(*e*) While some of the surface anion positions (screw axis) would retain specificity, a number of non-specific boundary anions are possible because of the net positive charge on the crystal (see Section V,B).

Several qualifications must be mentioned: This development has been based on the assumption that the crystal is three or four unit cells thick. This dimension is at the limit of definition of the electron microscope and is therefore a rough approximation. Such thin tablets cannot possibly give clearly defined x-ray diffraction patterns in unoriented specimens. Certainly the presence of a few adventitious crystals of larger size would dominate the patterns observed. Accordingly, critical x-ray diffraction experiments must be accompanied by electron micrographs to show the homogeneity of size of the crystals under investigation. Finally, *all* of the variability of composition seen in the basic calcium phosphate system cannot be attributed to surface variations alone. Even the tiny bone crystals do not possess sufficient surface to account for the variations observed. To determine the quantitative importance of the exposed surfaces, the entrapped surfaces, and the interior of the crystals separately is a monumental task which will require the combined application of physical chemistry, x-ray diffraction, electron microscopy, and crystallography.

The structural concept given here must be considered provisional, but it is neither unique nor new. It represents a fusion of the theories of adsorption and isomorphic substitution in terms of the new facts gained from electron microscopy. While the present concept is undoubtedly oversimplified, it suggests critical experiments and it does focus on an important point: namely, that the calcium:phosphorus ratio, the calcium:hydrogen ratio, and the ionic strength of the solution may determine the crystal composition and *size*! Under such circumstances, it is conceivable that precipitates may contain different crystals having different compositions and that inhomogeneities may occur within single crystals. Thus, even preparations having identical calcium:phosphorus ratios may vary in properties. In fact, with one possible exception (161), it is doubtful whether any investigator ever possessed a really pure sample of "hydroxyapatite" for study. The only significance of "hydroxyapatite" is its direct correspondence to fluorapatite, the prototype of the whole apatite series.

Another important aspect, not emphasized in the above treatment, is the lability of basic calcium phosphate precipitates. It has been demonstrated that the crystals undergo continuous and spontaneous recrystallization (271, 272,

273) in solutions of constant composition. In addition, many preparations undergo hydrolysis, becoming more basic (52, 53, 93, 96, 97, 125, 161, 200, 201, 203, 222, 224, 306). It appears that any manipulative procedure, even washing with water, can introduce marked alterations in the solid phase. This problem is especially important in the study of bone, where most measurements require isolation of the mineral phase. The common techniques have involved dry-ashing, ashing in ethylene glycol–potassium hydroxide (71), ashing in glycerol–potassium hydroxide (128), ashing in ethylenediamine (14), autoclaving (50), and acid-treatment to remove carbonate (76, 83, 92). Obviously, interpretations of a quantitative nature must be drawn with caution following such treatment of the mineral.

If one accepts the concept of a hydroxyapatite series with variations resulting from isomorphic substitution throughout the crystal, there still remains the problem of determining which member or members of the series most closely approximate the normal bone mineral. Recent information on this point is both voluminous and conclusive. Dallemagne and associates, in an exhaustive series of multi-disciplined experiments, have established that the calcium phosphate of *normal* bone does not correspond to "hydroxyapatite" (Ca:P = 1.66) but rather to a less alkaline, more highly hydrated member of the series exhibiting a calcium: phosphorus ratio approximating 1.5 (82). The evidence for this conclusion included chemical analyses (74), x-ray diffraction (39, 40, 41, 42, 83, 84, 93), refractive indices (79, 86, 88, 93), high-temperature calcination studies in the presence and in the absence of carbon dioxide (73, 78, 94), dehydration curves (82, 85), titrimetric results (79, 89, 91), and other adjunctive studies (76, 77). Dallemagne's conclusions have been confirmed (59, 60, 62) and are consistent with the overall composition of bone mineral as reported by others (172, 226, 238). It is unfortunate that Dallemagne did not recognize the continuity between basic calcium phosphates and chose to name the bone salt "α-tricalcium phosphate" (82). Otherwise, his principal conclusions are entirely acceptable.

A moment's consideration will show that this member of the apatite series, calcium:phosphorus = 1.5, is ideal from the standpoint of the animal's well-being. This particular ratio can undergo variations in *either* direction with minimal changes in the size and character of the crystals and permits the animal, under extremes of dietary stress, to lay down a satisfactory, functional bone salt.

In summary, normal bone salt is a compound of calcium, phosphate, and hydroxyl ions, and water which exhibits a calcium:phosphorus ratio of approximately 1.5 and which diffracts x-rays to give the characteristic apatite pattern. It represents but one small region of a series of similar compounds which may be termed the hydroxyapatite series, having a calcium:phosphorus ratio varying from 1.33 to 2.0. This series is characteristically indefinite, the transition from one end of the series to the other being gradual and mediated by isomorphic substitution of hydrogen ions and water for calcium in the prototype substance "hydroxyapatite." This material, the structure of whose unit cell may be written $Ca_{10}(PO_4)_6(OH)_2$, is analogous in structure to crystalline fluorapatite,

$Ca_{10}(PO_4)_6F_2$, in that hydroxyl groups occupy the space positions of fluoride ions.

In the subsequent discussion the bone crystal is pictured as consisting of: a surface hydration shell containing non-specific boundary anions in rapid equilibrium with the surrounding medium; an inner, crystal surface containing more or less specific cations and anions, also in equilibrium with the solution (or the hydration layer); and interior ions with a slow but measurable equilibration with the outer layers (recrystallization).

IV. SURFACE AREA OF THE CRYSTALS

The crystals of bone are so minute that they must necessarily possess a specific surface area ($m.^2/g.$) that is uniquely large. Indeed, Robinson (300) calculated the specific surface of crystals of autoclaved bone to be from 84 to 106 $m.^2/g.$, based on his average crystal dimensions and assuming a density of 3.0. Robinson also obtained similar values assuming 12 per cent of the calcium and phosphate (PO_4) ions to be in the crystalline surfaces. Very few measurements of the surface ions are available, but for fresh bone 8.6 per cent of the phosphate has been reported to be in the surface (271) and for glycol-ashed bone, values from 8.8 to 13.4 per cent have been given (269). The newer estimates of crystal size would correspond to over 200 $m.^2/g.$

Direct measurements of the surface area of bone crystals have not been successful with fresh bone because of interference by organic matter. However, several sets of data are available for steamed or glycol-ashed bone preparations. Although such pretreatment undoubtedly alters the mineral, there is no detectable increase in crystal size as shown by the electron microscope (300). These values agree in order of magnitude and are presented in table 3 for comparison.

There are three pertinent conclusions to be drawn from the results given in table 3. First, the magnitude of the measured surfaces lends strong support to the crystal dimensions proposed from electron-microscope studies. Second, with bone, there seems to be no correlation between the size of the aggregates and the surface area. Regardless of the apparent particle size, all of the surfaces of the individual crystals in ashed preparations are available for adsorption of gas. Third, enamel and the natural mineral francolite are markedly different from bone. These materials do show increasing surface with decreasing particle size, suggesting that they are composed of fused masses of individual crystals or aggregate crystals containing entrapped surfaces (166).

Hendricks and Hill (166) actually assumed that the ultimate crystals of francolite corresponded to a surface area of 60+ $m.^2/g.$ If this assumption proves to be valid, these investigators can explain the high carbon dioxide content of this mineral on the basis of exchange substitution on surfaces subsequently entrapped and have provided a strong argument against the existence of a true "carbonate apatite," $Ca_{10}(PO_4)_6CO_3$. They also suggest that the situation is similar in enamel, accounting for its low surface area by gas adsorption. However, since the largest surface area observed was 7.3 $m.^2/g.$, the extrapolation to 60 $m.^2/g.$ may be questioned (246).

The observed specific surface areas of ashed bone crystals provide the basis

for an interesting calculation. Taking 100 m.²/g. as an average value, the total surface area of the bone crystals in the skeleton of a man weighing 70 kg. exceeds 100 acres! Even if only a small percentage of these crystal surfaces are readily available, this calculation indicates that, *in vivo*, a few liters of circulating fluids flow over acres of active mineral surfaces. Under such circumstances, the conclusion is inescapable that the available bone and the plasma are in dynamic equilibrium.

V. CHEMICAL PROPERTIES RELATED TO SURFACE

It is the surface of the crystal that equilibrates with its surrounding fluid. Since the bone crystals are minute tablets and exhibit a specific surface area that is uniquely large, it is not surprising that the chemistry of bone mineral is essentially a problem in surface chemistry (269). In the past few years, bone

TABLE 3

A comparison of measurements of available surface area

PREPARATION	SIEVE SIZE	SURFACE AREA	REFERENCE
		m.²/g.	
Synthetic hydroxyapatite		67.8	(277)
		51.1	(374)
Dentine (fresh)	60+	2.4	(374)
Enamel (fresh)	60+	1.8	(374)
	60–120	2.1	(166)
	120–200	3.2	(166)
	200–325	3.7	(166)
	325+	6.8	(166)
Francolite	20–35	1.6	(166)
	100–200	3.2	(166)
	6 μ	7.3	(166)
Beef bone (steamed)	4–8	63.7	(166)
	16–30	66.2	(166)
	100–200	66.2	(166)
Rabbit bone (glycol-ashed)	60+	99	(374)
Veal bone (glycol-ashed)	40+	117	(277)

mineral has been shown to exhibit the following surface phenomena: (*a*) Its ions undergo exchange, isoionic and heteroionic, to the extent that they make up the crystalline surfaces. (*b*) The crystals are highly hydrated in an aqueous medium because of the presence of these exchangeable ions. (*c*) The composition of the crystals reflects the composition of the solution with which they are in contact, because of heteroionic exchange and isomorphic substitution in the surface and interior of the crystal. (*d*) The crystals of bone have demonstrated an ability to undergo uniquely rapid, spontaneous recrystallization in solutions of constant composition.

A. Isoionic exchange

Interest in ionic exchange processes has grown dramatically in the past few years, and there are now available a number of excellent textbooks covering

the theoretical and practical aspects of this subject. However, nearly all of the research to date is concerned with synthetic resins or the natural clays as the solid-phase exchangers (263). In dealing with synthetic resins, the physical chemist has been successful in deriving equations which accurately describe equilibrium conditions. It has been conclusively shown, for example, that cations are interchangeable on an equivalent basis, that the maximum exchange capacity is a fixed constant, and that the capacity is independent of pH provided the acidic group responsible for cation-binding is completely ionized and the cation does not undergo hydrolysis. This is a system capable of mathematical analysis (264).

However, ionic exchange with bone crystals represents a different situation. Rather than an accumulation of ionizable groups linked covalently to a solid having no finite solubility, the exchangeable ions of the bone crystal are linked principally by electrostatic forces to other exchangeable ions and the crystals themselves have a finite solubility. Synthetic resins are usually cation-exchangers or anion-exchangers. Bone crystals possess both exchangeable cations and exchangeable anions. The exchange capacity is not a fixed function of the mass of the solid phase, the capacity is not pH-independent, and other as yet ill-defined variables are operative.

Isoionic exchange may be defined as a process by which ions from the solution phase exchange with similar ions in the surfaces of the solid phase with no net change in the composition of the two phases. Such a process in bone mineral can be studied only through the use of labelled calcium or phosphate. Despite the availability of both radiocalcium and radiophosphate, very few studies have been made of the ionic exchange of bone mineral *in vitro*.

The earliest study involved the uptake of radiophosphate by enamel, dentine, and bone from ethylene glycol (233). The authors concluded that the results indicated adsorption although, in spite of their statement to the contrary, the results were also compatible with an exchange process. This was the first paper to show that isotope uptake was independent of particulate size as measured by sieving.

In 1940 (8), Armstrong compared the uptake of isotope by powdered dentine and enamel when placed in radioactive phosphate buffer. He concluded that the greater isotope incorporation by dentine was due to the smaller size of crystals in this tissue. Protein-binding of phosphate was negligible. It has been shown subsequently that organic phosphates do not exchange (136).

Except for two brief reports on exchange *in vitro* (23, 357), from 1941 to 1947, the only studies reported were those of Hodge and associates (118, 176, 179, 189), who compared bone, dentine, and enamel with synthetic hydroxyapatite with respect to affinity for radiophosphate. All of these results were expressed in terms of the adsorption isotherm of Langmuir. However, the adsorption isotherm can also be derived from exchange theory, and, in 1947, Falkenheim, Neuman, and Hodge (119) demonstrated conclusively by concomitant isotope and chemical analyses that ionic exchange rather than adsorption was responsible for the fixation of radiophosphate *in vitro*. Subsequently,

Hodge and associates reported similar findings, using radiocalcium (120, 362). In 1949, the application of exchange techniques was also helpful in demonstrating the mechanism of the skeletal fixation of uranyl ion (280).

Because of the growing importance of the general problem of ionic exchange between crystals and solutions (286), a comprehensive study of the variables affecting ionic exchange in the bone–buffer system should be made. A new technique, designed specifically for the study of the exchange properties of bone and the basic calcium phosphates, has been described (370). This procedure is more rapid and gives more reproducible results than methods previously reported (119) and has shown that—in addition to crystal size—the temperature, the solid-to-solution ratio, and the time of equilibration are important variables. There are also indications that the pH, the calcium:hydrogen ratio, and the nature of the bulk electrolyte of the solution are important in determining the final distribution of the isotope (270, 370). All of these findings, though at variance with some of the earlier results (119), are consistent with the concepts given in Section III. Except for the calculations presented earlier, however, they are too fragmentary to constitute a critical test of the structures proposed. With demonstration of the above variables and indications of many others, it is clear that published values for the percentage of exchangeable calcium and phosphorus of various preparations are of little more than qualitative interest, dependent on the conditions employed in the measurement. The final answers will be obtained only with preparations of known homogeneity in size and constitution and with sufficient control to minimize changes in the solid phase while in contact with the solution.

It is interesting, however, that glycol-ashed bone exhibited a greater percentage of exchangeable phosphorus than did powdered fresh bone under identical conditions (5, 271). How the presence of the organic phase reduced the exchange is not clear, but these facts suggest a very intimate association between the inorganic and the organic portions of bone.

In summary, the isoionic exchange of calcium and phosphate of bone mineral has been conclusively demonstrated and the importance of this process in the skeletal fixation of radioisotopes is well established (see Section VII,E). However, it is urgent that more information be obtained on the variables which affect exchange, including the importance of the organic portion of bone. With only one exception (table 2), published data on the percentages of surface ions are not sufficiently defined to permit a critical test of the theories regarding the nature of the apatite lattice and the structure of the bone crystal.

B. Heteroionic exchange

*Hetero*ionic exchange differs from *iso*ionic exchange in that an ion in the crystal surface is being displaced by a *different* ion from the solution. Among the heteroionic exchange reactions studied are the following: uranyl ion (280, 281), strontium ion (155, 177), sodium ion (180, 270), and hydronium ion (270) displacing surface calcium; carbonate ion (270) displacing surface phosphate, possibly also hydroxyl ion as bicarbonate; and fluoride ion (251, 282) displacing

hydroxyl and bicarbonate ions. Certainly all of the variables which affect isoionic exchange are operative here. In addition, several unexpected phenomena have been observed.

Some specificity is associated with substitutions involving the ions in the surfaces of hydroxyapatite crystals. For example, at least two monovalent ions (Na^+, H_3O^+) whose ionic radii (0.95 Å. and *ca.* 1.0 Å.) are similar to that of calcium ion (0.99 Å.) are able to displace calcium mole for mole. This necessarily must result in a lowered positive charge on the crystals and, as expected, these two cations reduce the number of boundary anions associated with the crystal surfaces (270). Potassium ion (radius = 1.33 Å.), on the other hand, exhibits an ionic radius one-third greater than that of calcium and does not undergo appreciable heteroionic exchange (164). Strontium ion (radius = 1.13 Å.) behaves normally (155, 177). That is, the ion is divalent, has an atomic radius of the same magnitude as that of calcium ion, and undergoes exchange with calcium ion on an equivalent basis. Barium ion, like potassium ion, appears to be too large (radius = 1.35 Å.) to compete effectively with calcium ion for the surface-combining site (270). Surprisingly, uranyl ion, which is even larger than barium ion, does displace surface calcium ions. In this case, however, 1 mole of uranyl ion displaces 2 moles of surface calcium ion, resulting in a lowered positive charge on the crystal and a corresponding loss of boundary anions measured as phosphate (280). In this connection, it would be interesting to reinvestigate the mechanism of skeletal fixation of both lead and radium ions, both of whose radii are too large for normal equivalent exchange. An early, but critical, investigation of the physicochemical relationships between lead (ionic radius = 1.32 Å.) and bone (17) showed that lead displaces less than 1 mole of calcium! However, this experiment involved unphysiological concentrations of lead, and the data can be extrapolated to low concentrations where one mole of lead displaces two moles of calcium.

Fluoride ion also behaves in an unusual fashion. Early work (282) demonstrated that fluoride ion competes with hydroxyl and/or bicarbonate ions for surface sites. Yet a subsequent study (250) cast doubt on the reversibility of the system. This situation has not yet been fully clarified. Unpublished results indicate that the exchange is affected by the pH in a complicated manner. At low pH, the reaction does not appear to be surface-limited; that is, the uptake is greater than can be explained in terms of surface alone and equilibrium is reached very slowly. Perhaps undissociated hydrogen fluoride is able to diffuse into the lattice, analogous to ion migration. This would account for the apparent irreversibility (250) and the magnitude of the exchange (282). However, more work is required before such a concept can be accepted.

C. The state of carbon dioxide in bone

Bone mineral contains approximately 5 per cent of carbon dioxide by weight. Carbon dioxide is therefore a major constituent, and its state in the solid has an important bearing on the foregoing discussions of the nature of the crystalline lattice. No study has presented proof that the carbon dioxide in bone occurs as carbonate, although this has been almost universally assumed. Indeed,

there is fragmentary evidence that some of the carbon dioxide may be present as bicarbonate ion (282). In terms of the structural concepts given earlier, isomorphic substitution of screw axis positions would probably require carbonate ions, but the boundary anion positions might well be occupied by bicarbonate. Because of the uncertainties involved, the indefinite term "carbon dioxide" seems preferable and will be used in the subsequent discussion.

The space position occupied by carbon dioxide in the bone crystal has been a subject of controversy for many years. Much of the argument has concerned data obtained from the study of such natural minerals as francolite and staffelite. Since the conditions of their formation were geological rather than physiological, these arguments may be entirely irrelevant. The important question is whether there exists real evidence that the carbon dioxide of bone resides *within* the crystal interior.

The principal and best-documented evidence that the carbon dioxide is surface-bound is based on differential rates of solubility. When bone preparations are placed in acid or water, carbon dioxide is invariably removed at a faster rate than is phosphate (62, 77, 83, 90, 92, 199, 205, 226). It must be admitted that this line of evidence is largely circumstantial. Even less conclusive is the fact that carbon dioxide is quantitatively lost when bone is heated (73). Perhaps more impressive is the recent observation that, in *young* rats, the skeletal carbon dioxide is in complete equilibrium with the carbon dioxide of the blood as demonstrated by C^{14} distribution (48). If carbon dioxide were buried within the lattice, such an equilibrium state requires that every crystal in the young rat undergo rapid and complete recrystallization. This is possible but unlikely.

In 1938, Klement (202) showed that apatite preparations incorporate carbon dioxide from solutions. His conclusion that the magnitude of the surface-fixation of carbon dioxide could not account for the carbon dioxide found in bone is not acceptable, since he ignored the importance of crystal *size*. This surface exchange has been confirmed (215, 216, 225), and it has been further shown that carbon dioxide is removed from solution well below the solubility product of calcium carbonate (222). Very recently, it has been demonstrated that carbon dioxide is reversibly bound by a synthetic apatite by a surface-limited process of heteroionic exchange of carbon dioxide for phosphate (270). This is shown graphically in figure 5, where the ratio of total carbon dioxide to total phosphorus in solution is plotted against the carbon dioxide taken up by the solid phase. It is especially interesting that when the apatite is equilibrated against a concentration of bicarbonate sufficient to give maximal uptake of carbon dioxide, nearly all exchangeable phosphate has been displaced (270). Apparently the anionic composition of the crystal surfaces depends not on the absolute concentration of bicarbonate or phosphate ions but rather on the concentration ratio of carbon dioxide to phosphorus in solution (270, 338). Finally, the magnitude of the surface substitution of carbonate for phosphate is sufficient to account for *all* of the carbon dioxide in bone (270) provided it is permissible to calculate the number of exchange sites from the percentage of exchangeable phosphate. This, at the moment, is a reasonable assumption.

It must be concluded that much of the carbon dioxide in bone is located

at the crystal surface, but, as yet, there is not adequate proof that *all* of it is so situated. There is no doubt that this available carbon dioxide is not present as a separate phase in the form of calcium carbonate, calcium bicarbonate, alkali bicarbonates, or other carbonates. Solubility experiments (222) ruled out such an explanation. This is also consistent with x-ray evidence (163, 244).

McConnell (245) has reviewed proposals regarding the substitution of carbon dioxide for phosphate isomorphically within the crystal. The extent, if any, to which this occurs in bone is one of the more important problems to be solved.

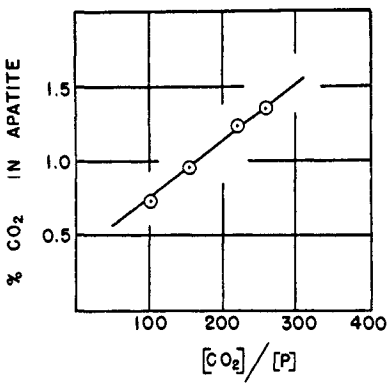

FIG. 5

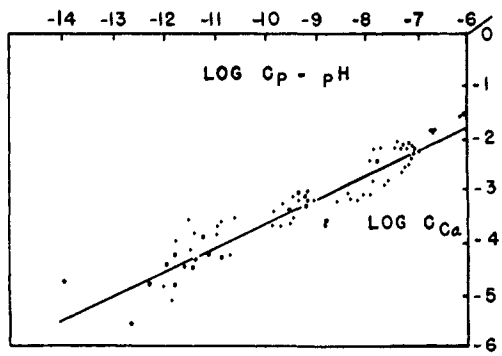

FIG. 6

FIG. 5. Data showing a 1:1 substitution of carbon dioxide for surface phosphate of hydroxyapatite, pH 7.2. These data have been analyzed by using the equation:

$$\text{Bone:}HPO_4 + CO_2 = \text{Bone:}CO_2 + HPO_4^{-}$$

from which it follows that the plot can be linear only if the substitution occurs on an equimolar basis.

FIG. 6. An empirical plot of the data of several investigators, showing a linear relation between log of total calcium dissolved and log of total phosphate − pH. Taken from Hodge (174).

D. The hydration layer

Synthetic organic exchange resins in an aqueous medium become highly hydrated, and the degree of this hydration correlates with the kind of exchangeable ion present (147, 265). The natural clays, too, have shown similar properties (193). It is not surprising, therefore, that the crystals of bone, which possess both exchangeable calcium and exchangeable phosphate, also become highly hydrated in an aqueous medium. There are three kinds of evidence that this water is truly water of hydration and not held by mechanical forces (277): (*a*) most of the water held resists centrifugal forces above 40,000 g; (*b*) it does not contain the electrolytes present in the bulk of the solution; and (*c*) hydroxyapatite crystals adsorb water from the gas phase in accordance with the Brunauer–Emmett–Teller theory (46, 47). The maximum amount of water held by synthetic hydroxyapatite is truly remarkable, being over 80 per cent of its weight.

It was found that (*a*) the crystals in fresh untreated bone were not as highly

hydrated as synthetic apatite and (*b*) nearly all of the water present in adult compact bone was water of hydration (277). These findings support two important conclusions, respectively: (*a*) the crystals are in such intimate contact (perhaps by actual bond formation) with the organic phase that much of the crystalline surface is unexposed, and (*b*) a large proportion of adult compact bone may be effectively isolated from the circulating fluids. Materials from the plasma can reach these isolated areas only by diffusion through a solid, and this is an extremely slow process (49).

The concept of the hydration shell requires considerable clarification. At the moment, it is not certain how much represents a partial hydration of ions vibrating slightly out of their normal lattice positions and how much represents an example of classical boundary phenomena where the surface exhibits a net positive charge surrounded by a diffuse shell of anions and oriented water molecules. In addition, the relation between the hydration layer and the organic matrix may be very important (5, 16, 277, 376).

E. Recrystallization[4]

Considerable evidence (171, 271, 272, 273, 334) has now accumulated to indicate that hydroxyapatite crystals, both synthetic and those from bone, undergo spontaneous recrystallization in an aqueous medium: (*a*) Isotopic calcium and phosphate added to the solution equilibrate slowly with the bulk of the mineral phase, as distinguished from the rapid, reversible exchange process. (*b*) There is no net transfer of calcium or phosphate between the solid and the solution. (*c*) Either isotope, once taken up by the mineral crystals, is not liberated by short-term exposure to non-isotopic buffer. (*d*) The rate of isotope incorporation is definitely temperature-dependent. (*e*) The process requires the presence of a water phase.

The rate of recrystallization is markedly influenced by the pretreatment of the crystals of bone. Glycol ashing greatly increases the half-time of the process (271), and newly deposited bone mineral recrystallizes much more rapidly than do crystals taken from established bone (57, 271).

Animal experiments have confirmed the occurrence of this process *in vivo* (171, 273, 334), and this concept is helpful in explaining, in part, the apparent irreversibility of isotope-fixation by the animal skeleton (see Section VII,E).

Recrystallization, as here defined, is a slow equilibration of the crystal interior with the solution phase. This process offers an explanation for the fact that some basic calcium phosphate preparations require years to attain equilibrium.

VI. THE SOLUBILITY OF BONE MINERAL

The solubility of bone mineral merits serious discussion because of its important physiological implications. Certainly, the blood levels of calcium, phos-

[4] Recrystallization may be a poor term in this case. The evidence does not support or deny the idea that the crystals dissolve and redeposit. The reaction could involve a slow exchange of the ions within the crystal with ions in the hydration shell. On the other hand, since the crystals are only about three unit cells thick, it is not inconceivable that the interior is exposed by the temporary removal of the outer layer of unit cells. A kinetic study might differentiate among these possibilities.

phate, bicarbonate, sodium, and other ions must be determined to some extent by the solubility of the bone mineral. Also, historically, the calcification process in bone formation has almost universally been assumed to involve an orderly precipitation of bone mineral within a highly organized organic matrix. This assumption has provided the basis for most theories of calcification, including the classical phosphatase theory of Robison (303). For precipitation to occur, it is necessary that some solubility product be exceeded, and it has been the search for this K_{sp} that has led many investigators to study the solubility behavior of bone and basic calcium phosphates.

It is difficult to evaluate all of these reports because the conditions employed were rarely comparable; nonetheless, as Hodge (175) has pointed out, one conclusion is inescapable,—no solubility product has ever been demonstrated.

With our present knowledge of the bone crystals, the composition of the crystalline lattice, and the ability of this lattice to mirror the composition of its fluid surroundings (215, 216) by ionic exchange, it is unreasonable to expect that the system will be governed by any simple ion product. In fact, the system must necessarily be so complex that it is doubtful that anything more than a few generalizations can be gleaned from existing data.

Unfortunately, much of the older work, however carefully done, must be regarded with suspicion. It has been demonstrated that the basic calcium phosphates show a marked tendency to become colloidally dispersed (98, 190, 270). Only ultracentrifugation can insure that analyses on supernatant solutions are not in error owing to the presence of suspended solids.

A. Time to reach equilibrium

Many investigators have found that considerable time is required before equilibrium can be reached (24, 30, 52, 54, 125, 161, 183, 205, 227, 309, 360, 372). Recently, investigators have found that only a few hours are needed (270) under special circumstances. The only known process by which these seemingly contradictory results can be explained is the recrystallization of the mineral phase (271). Equilibrium between *stable* crystals and water should indeed be quickly reached (161). However, the first solid to appear on the mixing of calcium and phosphate ions is an amorphous precipitate or, at least, a mass of tiny imperfect crystals, which on aging would result in changing surfaces and hence changing "solubilities" over extended periods. In the body, the situation must certainly be complex, with newly formed crystals and well-aged crystals present in the same system.

B. Solid-to-solution ratio

It has frequently been reported that the final equilibrium concentrations of both calcium and phosphate ions are dependent on the amount of solid added to a given volume of water. In general, the amount of material dissolved is found to increase with increasing solid phase (53, 98, 101, 142, 206, 214) with two exceptions (216, 224). From present information, it appears that this

phenomenon is an expression of changing the reactive surface of the solid phase rather than changing the mass of the solid.

C. Effect of pH

Increasing acidity invariably increases the solubility of the calcium phosphate system. In fact, Hodge has shown that the combined results of a number of investigators can be described by a linear plot (slope = 1) of the log of the calcium concentration *versus* pH (175). Whether this is a matter of solubility or, more probably, a reflection of the 1:1 heteroionic substitution of H_3O^+ for surface calcium is not established. The total phosphorus in solution also increases almost linearly with increasing hydrogen-ion concentration. These relations in general, and very approximately, indicate a constancy in two ratios:

$$[Ca^{++}]/a_{H+} \text{ and } [Ca^{++}]/[P_{total}]$$

By combining the two ratios the following relation is obtained (175):

$$2 \log Ca = \log P_{total} - pH + K$$

This relation does, indeed, describe many sets of data, as shown in figure 6, but it is empirically derived and there are many exceptions.

D. Effect of excess calcium or phosphate

In general, in the liquid in contact with a basic calcium phosphate there is observed an inverse relationship between calcium-ion concentration and phosphate-ion concentration (30, 216, 225, 270, 326). Furthermore, the addition of calcium ion reduces the pH value attained at equilibrium, while added phosphate ion results in elevated pH values (270). In terms of the structure of crystalline surfaces given earlier, this can be explained as follows: With lack of phosphate ion, calcium ion can saturate the surface positions only by displacing hydronium ions, giving a drop in pH. When phosphate ion is in excess all available calcium ion is required for the "screw axis" positions, leaving the surface hexagonal "column" positions to be filled by hydronium ions, which must be drawn from solution.

E. Equilibrium approached from "supersaturated" and "undersaturated" solutions

Almost invariably, dissolution experiments give results at variance with precipitation experiments (222). With the importance of the solid-to-solution ratio in mind, this is not surprising. To date, two investigators have successfully reached equilibrium from both supersaturation and undersaturation: Kuyper (215), by using large amounts of solid phase to minimize changes in surface composition; and Levinskas (221), in a slightly different manner, by adding critical amounts of a crystalline preparation to solutions having concentrations of calcium and phosphate ions only slightly in excess of equilibrium values.

Under the conditions cited above, the crystalline surface was the important factor in the solubility behavior of the basic calcium phosphate system. This fact, already suggested by Greenwald (142), points up the reason why agreement among investigators is exceedingly rare.

F. The importance of $CaHPO_4$

Since 1923 (186), there have been intermittent reports by puzzled investigators that their findings were more consistent with the constancy of the simple ion product $Ca \times P_{total}$ than with any other combination such as $[Ca]^3 \times [PO_4]^2$ or $[Ca]^{10} \times [PO_4]^6 \times [OH]^2$, etc. Evidence that this simple calcium–phosphorus product apparently governs the solubility of the basic calcium phosphate system is derived from such diverse disciplines as titration data (89, 372), solubility studies (184), clinical blood values in rachitic children (186), and calcification of rachitic rat cartilage *in vitro* (328). Yet solid $CaHPO_4$ is not stable above pH 6.2 (161, 174) because of its hydrolysis to a more basic compound.

The most reasonable explanation for this situation is the simplest. Since the basic calcium phosphate system is a solid phase of variable composition, *the principle of solubility product does not apply*. This is in accordance with, not a violation of, the laws of physical chemistry. Furthermore, since the simplest structural unit of the apatite contains eighteen ions, a solid phase of apatite could not possibly form spontaneously by a simultaneous collection of all the constituent ions. One is forced to conclude that basic calcium phosphate cannot be formed by *precipitation*. It can only form by *crystallization*, either by a stepwise addition of ions to a nucleation center or by a similar process involving the hydrolysis of secondary calcium phosphate. In a sense, almost any solution containing finite concentrations of calcium and phosphate ions can be considered to be metastable (30). That is, at least one crystal of hydroxyapatite would form, given sufficient time and a nucleation center. For practical purposes, however, the upper limit of stability is given by the solubility product of *secondary calcium phosphate*. If this is exceeded, precipitation of $CaHPO_4$ will occur. Indeed, there is evidence that the first precipitate formed when solutions of Ca^{++} and HPO_4^{--} are mixed is $CaHPO_4$ (125, 184, 225, 372). Undoubtedly, the appearance of $CaHPO_4$ is very transitory under certain conditions. When precipitation occurs above pH 6.2 in the presence of a sufficiently high calcium: phosphorus ratio in solution, there is an precipitous fall in pH indicating very rapid hydrolysis and, on isolation, the solid has already been converted to a hydroxyapatite (84, 125, 204, 372). The final product obtained is determined principally by the pH at which the initial precipitation begins and ends and by the relative proportions of calcium and phosphate present (91, 125, 161, 174, 183, 184, 192, 206, 254).

The significance of the K_{sp} of $CaHPO_4$ as an upper solubility limit *in vivo* and *in vitro* is thus easily recognized. It can also be easily seen why levels of calcium and phosphate approaching the K_{sp} of $CaHPO_4$ are most advantageous to the growing animal. While crystallization *can* occur at lower concentrations (142, 184, 223, 225, 326), the *rate* of crystallization depends primarily on collision frequency and would be maximal at the higher concentrations.

There has been an unfathomable resistance to this explanation of the solubility behavior of the basic calcium phosphate system. Even those investigators who have phrased this explanation in slightly different terms have continued obstinately to calculate weird solubility products in violation of their own conclusions (143, 222). Their "constants" and the "constants" of others which

varied over a range of 11 log units (175) are probably the best evidence that the hydroxyapatite system does not exhibit a K_{sp}.

G. The calcification process

At the outset, the term "calcification process" as used here must be defined. In this discussion, the term means only the formation of new bone crystals. It should not be construed to mean "bone formation," of which process calcification is only one phase. Nor should it be considered, as in the pathologists' usage, any calciferous deposit regardless of its composition.

Despite statements to the contrary (304), the calcification process is so poorly understood that no comprehensive hypothesis can be given. It is time, however, that speculations regarding the mechanisms involved take cognizance of what is known concerning solubility and the crystals. There exists no evidence that the calcification process involves precipitation. Indeed, there is much evidence against such a view. It has been shown above that the K_{sp} of $CaHPO_4$ must be exceeded for *precipitation* to occur, yet *calcification* occurs in individuals whose blood levels of calcium and phosphate are well below this critical product.[5] Furthermore, how precipitation can result in a highly organized, highly oriented pattern of mineral (see Section II) has never been explained. The evidence that (*a*) the characteristic size and shape of the bone crystals is determined by the composition of the solution, and (*b*) crystals can grow at calcium and phosphate levels well below the K_{sp} of $CaHPO_4$, has disposed of the need for a "precipitation" concept. It is more profitable to speculate on ways and means by which crystallization might be initiated. Such a "seeding" process could occur in at least two ways. The organic phase of osteoid or endochondral cartilage may bind either calcium or phosphate ions in the proper space relationships of the apatite lattice. A stepwise addition of ions to this surface or template would inevitably result in the growth of a complete, characteristic crystal, the orientation of which would correspond to the organic structure.

VII. BIOLOGICAL IMPLICATIONS

An attempt will be made to correlate bone metabolism, as observed in the intact animal, with the physicochemical properties of bone mineral as outlined in preceding sections. Special emphasis is given to newer data obtained with radioisotopes. It is hoped that this discussion will aid in differentiating between those events which occur passively in accordance with the laws of mass action and those which require the activity of cells.

A. General considerations

The principal gross biological and physicochemical factors which are known to control the equilibrium between blood and bone are illustrated diagrammatically in figure 7.

Though the *net* absorption from the gastrointestinal tract may be small

[5] Rarely do the total serum concentrations of calcium ion and inorganic phosphate ion exceed 10 and 5 mg%, respectively. At these concentrations, the thermodynamic product is approximately 10^{-7}, i.e., much less than the K_{sp} for $CaHPO_4$ (183).

indeed, the interchange of inorganic ions across the gut wall is very rapid. The intestine must be considered to be fully permeable in both directions to most ions. The direction and rate of net transfer are determined by the difference in concentrations of diffusible ion species between the blood and the contents of the intestine. Active processes for the absorption of calcium have not yet been demonstrated. Phosphate is involved in carbohydrate absorption, but no *direct* regulatory mechanisms for phosphate absorption have been shown.

In the absence of circulating colloidal calcium phosphate, the kidney glomerulus is nearly completely permeable to the inorganic phosphate of the plasma. Calcium, however, is only about 50 per cent filterable, because of the protein-bound fraction. Without going into detail, it is sufficient for present purposes to note that calcium and phosphate resorption and pH regulation are the result of special physiological tubular mechanisms and that, normally, most of the filtered calcium and phosphate is resorbed.

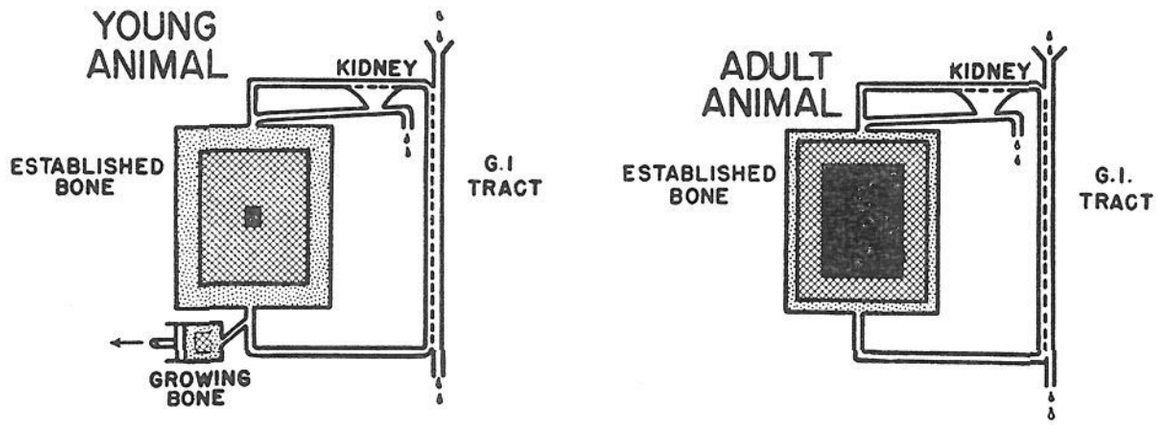

FIG. 7. A diagrammatic illustration of the general relationships governing the distribution of radioisotopes in the young and the adult animal. The dotted area represents the exchangeable fraction; the cross-hatched area, the recrystallizing portion; and the solid black area, the inert part of bone.

There are marked differences in skeletal activity between young growing animals and non-growing adults. There exists a growing body of evidence that, as the skeleton matures, a smaller and smaller fraction of the bone remains in equilibrium with the body fluids. The marked lack of hydration in adult compact bone as described above (see Section V,D) is one kind of evidence. Another indication is derived from long-term experiments *in vivo* with isotopes. As measured by the distribution of $C^{14}O_2$, the amount of available bone varies from nearly 100 per cent in young to 50 per cent in adult rats (48). The complete availability of the skeleton of the young rat was also demonstrated with radiophosphate (288). As measured by Na^{24}, the available fraction in adult dogs varied from 35 to 52 per cent (111, 112). Unpublished results from administration of Na^{24} to the adult human gave a value of approximately 30 per cent (211). Even the skeletal water does not exchange readily; some 20 per cent remained free of administered deuterium oxide after 30 hr. (112).

Besides these gross measurements, variations in reactivity have been shown at the histological level with radioisotopes (4, 114, 376). Here the correlation

is the same, with the most recent calcification showing the greatest reactivity and with established osteons of compacta appearing to be nearly inert.

There are many reasonable explanations for this phenomenon but none are established. It appears that newly formed crystals are very imperfect and recrystallize at an amazing rate (271, 376). Also, it is not unlikely that the crystal size of new crystals is smaller than that of aged crystals (376). This may be settled by electron microscopy, though it must be admitted that the problem approaches the limits of resolution of the instrument. Of not the least importance is the poor circulation of old established bone. In some areas, fluid interchange seems to be limited to the small canniculae interconnecting the lacunae of the resting osteocytes. Finally, the decreasing reactivity and the decreasing hydration of bone seem to be interrelated phenomena, but it is not known by what agent and by what means the hydration shell is displaced. Whatever the causative processes may be, the decrease in availability of the skeletal mineral with age is a new and important physiological concept (see Section VII,E). In this connection, a recent report (355) indicates that it is possible to determine the fraction of available bone by a mathematical analysis of specific activity–time curves of blood calcium following administration of Ca^{45}. If this proves feasible, the clinician will have an invaluable tool for the determination of the state of skeletal activity in human patients.

It must be emphasized that, in this generalized introduction, all details—histological and physicochemical—have been averaged out. The real situation is depressingly complex, with varying degrees of vascularity, hydration, density, and ash content occurring in even a small section of a single bone. Contrary to the sharp lines of demarcation of the diagram, there is an infinite series of rates of fluid exchange, ionic exchange, and recrystallization from very fast in areas of new calcification to very slow in established Haversian systems in adult compact bone.

B. The equilibrium between blood and bone

In the subsequent discussion, high or low levels of calcium and phosphate in the blood refer to high or low concentrations of Ca^{++} and HPO_4^{--} ions. Analyses for total calcium and inorganic phosphate provide only a rough indication of the actual ionic concentrations because of the many chemical states which these ions may assume in the blood:[6]

$$\text{Calcium proteinates} \rightleftharpoons Ca^{++} \rightleftharpoons CaHPO^0_{4\ \text{aqueous}} \rightleftharpoons \text{colloidal phosphate}$$

$$\Updownarrow$$

Calcium citrate complex,
Calcium malate complex, etc.

$$H_2PO_4^- \rightleftharpoons HPO_4^{--} \rightleftharpoons CaHPO^0_{4\ \text{aqueous}} \rightleftharpoons \text{colloidal calcium salt}$$

[6] The calcium–protein interaction has been well established for some time (222). The soluble, relatively undissociated citrate complex of calcium is also well established (167, 222, 350). While an undissociated 1:1 complex of calcium and phosphate exists (28, 145, 146, 259), there is no evidence that the complex is monomeric.

The old question of whether the blood is saturated with bone mineral (222) can now be answered with some assurance. Because the blood and the bone are in dynamic equilibrium, the blood may be considered to be "saturated" with bone mineral. However, an isolated sample of blood or serum can "hold" considerably greater quantities of calcium and phosphate than occur normally. The concentrations of calcium and phosphate in the blood are well below the point of spontaneous precipitation, i.e., well below the K_{sp} for $CaHPO_4$ (330). Very recently (185), it was again shown that the total phosphate concentration can be increased greatly, at least 700 per cent, without the formation of colloidal precipitates (141, 185, 219, 249). An older report (249) indicated that the ion product normally is only one-twentieth of the thermodynamic K_{sp}, but there are some uncertainties in assigning activity coefficients.

At first thought, it seems paradoxical that blood is saturated with respect to the solid phase but far below the point of spontaneous precipitation. However, this does not represent a violation of thermodynamic principles. The situation exists because the solid phase (hydroxyapatite) is not identical with the initial precipitate ($CaHPO_4$) and because the solid phase itself cannot be formed directly by ionic collisions. Since it is unlikely that the solid phase of bone mineral is formed by a precipitation process, the seeming paradox may be considered irrelevant from the physiological point of view.

Because the hydroxyapatite system shows a great variability in solubility and because no single K_{sp} governs its behavior, the bone mineral does not "fix" or control the levels of calcium and phosphate in the blood. The available crystals adjust to varying blood levels by varying slightly in composition, particularly in surface composition.

Within physiological limits, the bones cannot provide for inorganic homeostasis in the absence of osteoblastic and renal activity, but they do provide a buffer-like action (117) in preventing sudden changes in blood composition, specifically in the concentration of Ca^{++}, HPO_4^{--}, H_3O^+, and CO_2. Thus, the rapid injection or removal of amounts of calcium, exceeding by several times the quantities normally found in the circulation, causes only small changes in concentration (160). This passive assistance results from two phenomena: (*a*) the surfaces of the bone crystals available to the circulation are in constant dynamic equilibrium with the blood and (*b*), in quantity, this surface mineral far outweighs the mineral content of the body fluid. This is clearer if a few *approximate* calculations are given for calcium as an example, assuming the body weight to be 70 kg.

Skeleton (10 per cent of the body weight)	7 kg.	Blood (7 per cent of the body weight)	4.9 kg.
Available skeleton (30 per cent of the skeleton)	2.1 kg.	Serum (50 per cent of the blood)	2.45 kg.
Calcium (20 per cent of the available skeleton)	420 g.	Calcium (0.01 per cent of the serum)	0.25 g.
Surface calcium (10 per cent of the calcium)	42 g.		

On this basis, the physicochemical reserve of surface calcium is about 170 times the amount in the circulating blood in adults and considerably more than this in children.

Not all of this reserve should be considered to be readily available. As the blood levels of calcium or phosphate fall, the available crystalline surfaces will equilibrate with the new environment and, in doing so, will tend to return these blood constituents to their former levels. If the levels continue to fall, however, the crystalline surfaces will continue to equilibrate and can stabilize at *any* blood levels set by the absorptive, hormonal, renal balance of the organism. It is doubtful that even the available crystals will *dissolve* completely at levels compatible with life. Synthetic apatite preparations are stable at calcium and phosphate levels of a few parts per million, and a review of the older literature convinced Logan (222) that the bone salt will not dissolve at physiological levels of Ca^{++} and HPO_4^{--}. For these reasons, cellular activity must be responsible for massive demineralization, for remodeling and reworking the skeletal architecture, and perhaps for the *complete* dissolution of any single crystal.

C. Acid–base balance

The total skeletal mineral represents a tremendous reserve of alkali and, accordingly, can be considered to be a second line of defense supporting and supplementing the action of the buffers of the blood in preventing a rise or fall in the plasma pH (117).

Acidosis is usually characterized by an increased $[H_3O^+]$ and a decreased $[HCO_3^-]$ in plasma. The bones respond to acidotic states, produced in a number of different ways, by exhibiting a reduced carbon dioxide content, frequently with little change in the residual calcium:phosphorus ratio $\left(\frac{Ca - CO_3}{P}\right)$ (45, 124, 135, 149, 188, 195, 237, 343). The net contribution of the bone crystals to the blood is therefore either $CaHCO_3^+$ or $CaCO_3^0$, depending on the state of the carbon dioxide in the solid phase. In terms of the surface-exchange mechanism this may represent the substitution of surface calcium by hydronium ion:

$$\text{Bone:CaHCO}_3 + \text{H}_3\text{O}^+ = \text{Bone:H}_3\text{O} + \text{CaHCO}_3^+$$

If the bone gives up carbonate ion, it is necessary to leave a surface position vacant (which seems permissible) or to alter the residual calcium:phosphorus ratio:

$$\text{Bone:}\frac{\text{Ca}}{\text{CO}_3} + \text{H}_2\text{O} = \text{Bone:H}_2\text{O} + \text{CaCO}_3$$

Clarification is needed here, but the net effect is easily understood: the bones absorb hydronium ions and give up fixed base.

The reverse situation, in which the carbon dioxide content of bone increases in alkalosis, has also been observed (237, 371).

Of interest in this connection are the classical observations of Shear and

Kramer (328) on the carbon dioxide content of rat bones at various ages and their discovery that new calcification is low in carbon dioxide. Since all crystals, especially new crystals, reflect the composition of the medium, it follows that the fluids in the calcifying areas must also be low in carbon dioxide. This is not inconceivable. Calcification takes place in relatively avascular acellular areas. Furthermore, as the crystals are forming, the overall reaction is the hydrolysis of neutral $CaHPO_4$ to basic calcium phosphate with the liberation of acid. In fact, contrary to general opinion, it has recently been demonstrated (285)

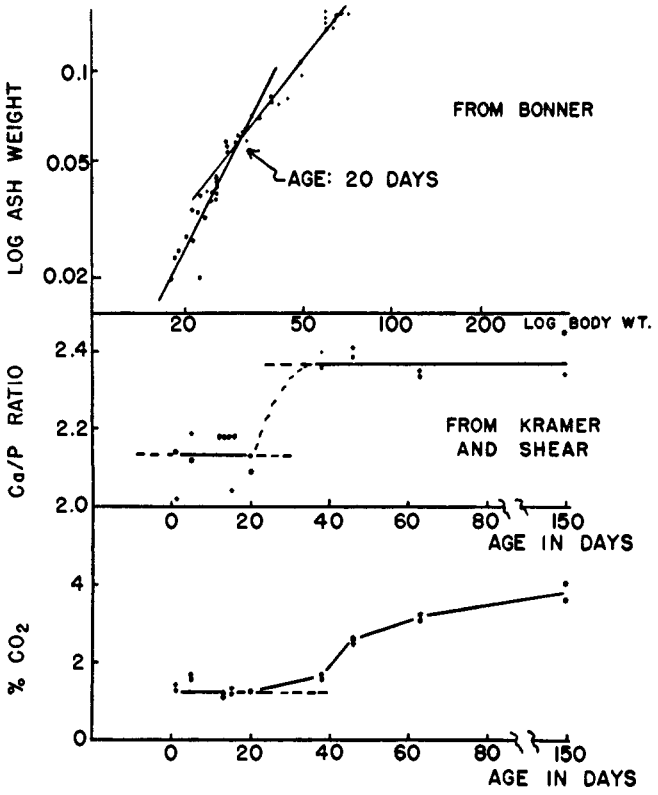

FIG. 8. Data showing the correlation of growth rate of rats (32) and the calcium:phosphorus ratio and carbon dioxide content of new calcification (328). Note the break occurring at 20 days in all three graphs.

that calcification occurs more readily in tissue cultures of bone maintained at a pH of 7.0–7.3 than in those at pH 7.8–8.0. A fall in pH, locally, would raise the pCO_2 and give a lowered $[HCO_3^-]$. This situation is analogous to the acidotic state. The crystals should have a low carbon dioxide content and exhibit maximal substitution of hydronium ions for surface calcium ions (low calcium:phosphorus ratio). An interesting correlation which supports this speculative development is presented in figure 8. In rats at 20 days (30 g.), there is a sudden fall in the rate of new crystal formation; at exactly the same period there is observed

a marked increase in the calcium:phosphorus ratio (indicating a pH rise) and the carbon dioxide content gradually rises to "normal" values for the more adult rat. It is a general rule, though not invariable (329), that the calcium:phosphorus ratio is low in sites of new bone formation, as stated in a review by Dallemagne (79).

D. Bone composition in relation to diet

In spite of the dynamic equilibrium between the bones and the circulation, it is difficult to vary markedly the composition of bone by dietary means (292, 305, 331, 345, 369, 371). The most effective approach is to alter the animals, acid–base balance (55, 67, 135, 149, 188, 343). As noted above, the carbon dioxide content of bone falls dramatically in acidosis. The residual calcium:phosphorus ratio $(Ca - CO_3)/(PO_4 - Mg)$, however, remains relatively constant at approximately 1.5. There are two reasons for this resistance to change: (*a*) in any but very young animals, the mineral composition would change only in a small portion of the skeleton and would not be revealed by analyses of the total bone, and (*b*) the composition of blood varies within rather narrow limits because of the efficiency of the active regulatory mechanisms.

Apart from changing the acid–base balance, the most dramatic dietary effects are seen in very young rats (337, 339) and with low salt rations (45, 375). Under these circumstances, the active regulatory mechanisms do not have an adequate supply of the minerals required for maintaining homeostasis, and the blood composition may therefore vary markedly from normal.

There is one excellent demonstration of the bone–blood equilibrium as influenced by diet (337). In one dietary group, the bones exhibited a residual calcium:phosphorus ratio of 1.33, the lower limit of variability of the apatite structure! The results were correctly interpreted to demonstrate that the PO_4:CO_3 ratio of bone is determined by the PO_4:CO_3 ratio of the blood. This equilibrium has also been demonstrated *in vitro* (*cf.* figure 5).

The whole problem of the effects of diet on bone composition requires reinvestigation, but there is sufficient evidence at hand (72, 237, 239, 268) to conclude that, within structural limits, the composition of the bone may reflect changes in the composition of the blood as induced by diet.

E. The skeletal deposition of radioisotopes of calcium and phosphate

Radioactive phosphorus was the first of the artificially produced radioisotopes to become available for biological investigation. From the early animal experiments with this isotope, Hevesy and associates concluded that ionic exchange was an important process by which the skeleton rapidly incorporated the bulk of an injected dose. The concept of recrystallization was also used in their interpretation of long-term isotope uptake (171). This represented the first demonstration of the importance of surface reactions in the metabolism of the skeleton. Since Hevesy's classical experiments, increasing numbers of investigators have relied on concepts of surface chemistry to explain the results of isotopic studies of bone metabolism.

The overall metabolism of calcium is much less complex than that of phos-

phate, which is found in a large variety of metabolically active organic substances in soft tissue. Nonetheless, the patterns of skeletal deposition of radiocalcium and radiophosphate are so similar that these isotopes may be considered interchangeable in the subsequent discussion.

(1) Deposition

While isotopes are useful in studying the absorption of mineral substances and the effects of diet (54, 56, 95, 129, 140, 159), this review is concerned only with the processes by which isotope enters the mineral phase of bone following its appearance in the blood. One of the primary mechanisms of isotope fixation is ionic exchange (4, 7, 14, 27, 57, 66, 150, 158, 159, 168, 169, 170, 171, 220, 275, 288, 298, 333, 334, 376). However, in animal experiments it appears that the exchange of bone mineral is not uniformly rapid, and various investigators have postulated different fractions or different rates of exchange to explain their results (158, 171, 235, 275, 333). The suggestion that bone crystals may recrystallize (171), later confirmed and extended (114, 271, 272, 273, 334), provides an explanation for the different rates of skeletal incorporation of isotope, and these two processes, exchange and recrystallization, may account for most of the isotope found in the bones of adult animals. In young growing animals, however, the formation of new crystals is quantitatively very important (32, 56, 57, 154, 220).

The distribution of isotope is not uniform throughout the skeleton but varies in different bones and in different parts of the same bone (11, 103, 106, 137, 138, 158, 171, 218, 232, 333, 334). The greatest concentrations of radioactivity are invariably found in areas of active growth, in the most recently deposited mineral (4, 5, 14, 27, 57, 58, 66, 107, 114, 154, 220, 288, 376). This preferential isotopic uptake has been shown in non-vital sections *in vitro* (4, 5, 27, 217) and is, therefore, the result of passive physicochemical reactions such as exchange and recrystallization. In established bone, the deposition of isotope, though considerable, is much less than in the areas of growth (103, 106, 137, 138, 158, 171, 218, 333, 334), and the age of the animal determines the pattern of distribution. This pattern, as shown by radioautography, changes from a diffuse general transient distribution in the young animal to a spotty distribution of discrete loci of intense radioactivity in the adult. The location of these loci has been shown to correspond exactly to individual Haversian systems which are of relatively recent origin and which exhibit densities lower than the surrounding bone, as measured by opacity to soft x-rays (3, 4, 114, 376). These findings have emphasized the importance of the resorptive processes and reconstruction of Haversian systems in the isotope deposition in both young and old bones (2, 6, 114, 137, 138, 288). A beautiful example of this anatomic specificity and correlation between density and isotope incorporation is given in figure 9. To learn the reasons for this remarkable variability in isotope fixation, several groups have studied the effects of various pretreatments on the exchange of isotope by tissue sections *in vitro* (4, 57, 376). Low-temperature ashing increases the fixation of isotope generally but diminishes the differences between different areas (114,

376). This has been observed with powdered bone also (271). Incineration at high temperatures abolishes the exchange (280, 376), probably by fusion of the crystals to large aggregates (300). Interesting results have been obtained by partial extraction of isotope-containing sections (376), but the interpretation of these data is difficult. While much remains to be learned, the following generalization may provisionally be made: the rates of exchange and recrystallization are greater, the lower the density, the greater the hydration, the newer the crystals,

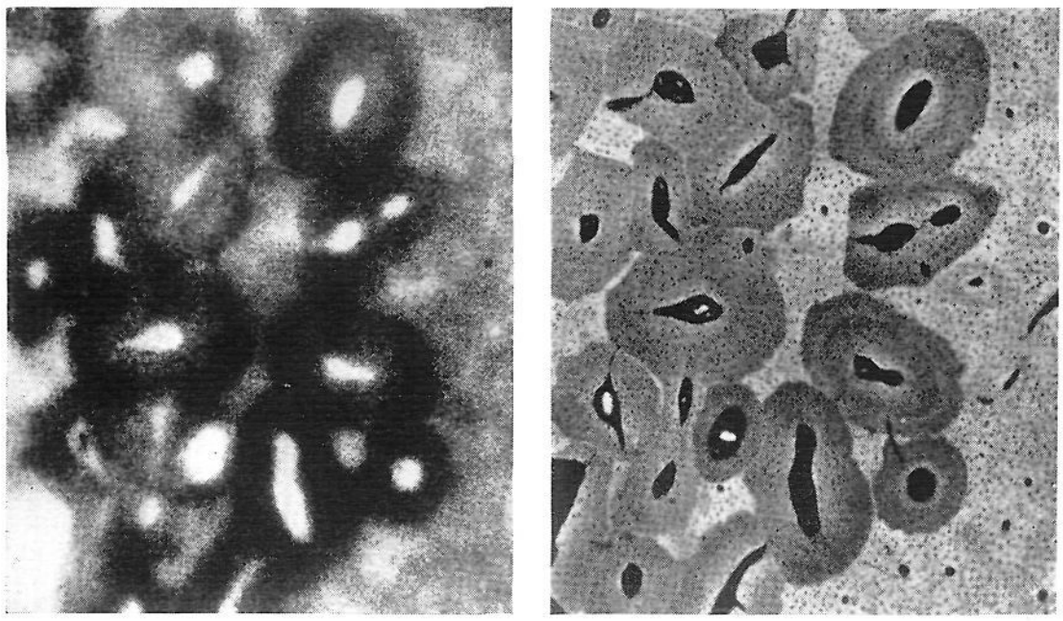

FIG. 9. Comparative photographs of radioisotope deposition and of opacity to soft x-rays. The radioautogram is on the left; the x-ray photograph on the right. Note the correlation between isotope concentration and density. These photographs (reference 6, figure 5) are reproduced through the courtesy of Springer Verlag.

TABLE 4

The effect of age on skeletal fixation of isotope

GROUP	APPROXIMATE AGE	PERCENTAGE OF TRACER IN SKELETON		
		Ca^{45} (69)	$Sr^{89,90}$ (69)	P^{32} (32)
	days			
Adult	170	31.7	29.1	16.7
Young	55	73.4	71.9	50.1
Very young	15			89.8

and the better the fluid exchange. There is a great need for the development of quantitative techniques of radioautography (110) for clarification of this problem.

The total skeletal deposition falls with age in direct relation to the declining reactivity of the skeleton (32, 69, 95, 154). Representative data are assembled in table 4 to illustrate this change. Low-phosphorus rickets, too, has a profound effect on the skeletal affinity for injected isotope (56, 58, 69, 106, 158, 234, 276). It seems that, on the deficient diet, skeletal maturation cannot occur. The skeletons of rachitic rats, therefore, exhibit the high reactivity characteristic

of growing bone, fixing a large percentage of isotope. This has been demonstrated with isotopic calcium (56, 58, 69, 158), strontium (69), phosphate (58, 106, 234), and uranium (276).

(2) Mobilization

Mobilization of skeletally deposited isotope represents something of a paradox. While the incorporation of injected material takes place quite rapidly, mobilization occurs very slowly suggesting, superficially, that the system is irreversible (56, 58, 66, 95, 275). This paradox can be resolved only by a consideration of the kinetics of the overall system.

The rate of isotopic exchange is always a function of the difference between the specific activities (SA)[7] of the two fractions interacting. As a rough approximation of the situation, three fractions of a given ion pool may be designated according to ultramicroscopic location: (a) the ion in the extracellular fluid, (b) the ion in the surfaces of the available bone crystals, and (c) the ion in the interior of the available crystals. The rate of net transfer of isotope from one fraction to another may be represented as follows:

$$R_{a-b} = K(SA_a - SA_b)$$

and

$$R_{b-c} = K(SA_b - SA_c)$$

Shortly after the administration of isotope, the rate of incorporation will be rapid, because the SA differences are necessarily large. After a few days or weeks, however, the system approaches the equilibrium state where $SA_a = SA_b = SA_c$ and no net transfer of isotope can occur. The only reason isotope leaves the bone at all is that the animal consumes, daily, variable amounts of non-isotopic ion in the food. This lowers the SA of the circulating fluids, causing the direction of isotope transfer to reverse. However, in terms of the total ion in the skeleton, the amounts taken in and excreted daily may be quite small. Thus the rate of removal of skeletally fixed isotope must be slow because the SA differences are small.

From these considerations, it follows that the retention of isotope in the young growing animal is nearly complete. The proportion of ion in available bone is relatively enormous compared to that of the circulation, most of the dietary intake is retained for growth, and very little ion, labelled or not, is excreted. Though the isotope is retained, the specific activity of the bones falls as more and more non-isotopic material derived from the diet is added to the growing skeleton. In the adult, only a fraction of the skeleton is in equilibrium with the blood, a larger amount of the ion administered is presented to the kidney, and a greater percentage is excreted. After SA equilibrium is reached, the blood SA

[7] Specific Activity (SA) is an expression of the concentration of isotopic atoms, thus:

$$\frac{Ca^{45}}{Ca^{40} + Ca^{45}}$$

Most commonly the expression "counts per minute per mgm" is used with radioisotopes.

keeps on falling as it is diluted by the non-labelled ion absorbed from the food. Since the adult is not in positive balance, there is a large excretion of the ion by the kidney. In its passage through the body the non-labelled dietary material draws isotope from the bones as it reverses the *SA* relationships.

This general relation between age and deposition and mobilization is well illustrated by the work of Copp (69) and of Bonner (32).

The overall picture of isotope retention is also affected by physiological processes not yet mentioned. In growth, the new crystals that are formed shortly after injection will "bury" isotope of very high activity and this activity can be released only very slowly by total recrystallization. As the endochondral calcification is invaded and destroyed this "buried" isotope is released, but it is redistributed and redeposited as it mixes with the circulating fluids. The importance of growth and reconstruction (2, 6, 137, 138, 288) on isotope distribution has been emphasized recently, and proof that these processes are dominant in the very young animal has been furnished by Leblond and associates (220), who were able to confirm and extend the classical theories regarding the mechanism of longitudinal growth of bone by means of radioautography.

F. Deposition of foreign ions

The term "foreign" refers to all those ions found to deposit in bone except Ca^{++}, PO_4^{---}, and H_3O^+. Thus, the list of foreign ions includes a number of physiologically important substances: sodium ion, magnesium ion, carbon dioxide, and citrate ion, as well as a large number of unphysiological elements: fluoride, strontium, lead, radium, barium, yttrium, uranyl, plutonium, americium, cerium, zirconium ions, etc. The entire list may be divided into three general categories:

Group I: Cations which deposit in the mineral crystals of bone by heteroionic exchange for surface calcium. These cations show a pattern of skeletal deposition, mobilization, and redistribution qualitatively very similar to that of radiocalcium, though they may differ with respect to distribution in soft tissue. Probably the principal factor in determining non-skeletal distribution is the degree to which, under physiological conditions of pH, ionic strength, etc., the cation hydrolyzes to form a colloid (313, 314, 315).

The bone deposition of all these cations is a very fast process, but further generalization is not possible. The percentage of the dose fixed by the skeleton and its mobilization thereafter depend on a number of factors poorly understood and rarely studied. The overall system may be represented diagrammatically as in figure 10.

With the exception of lead and uranyl ions, information is meager and assignments to this group must be considered provisional.

Uranyl ion: Uranyl ion has probably been the most exhaustively studied (274, 276, 278, 279, 283) of the bone-seeking ions. One uranyl ion displaces two surface calcium ions, and the mobilization of skeletal uranium is relatively rapid (276). This suggests that uranyl ion cannot fit into the apatite crystal structure but remains on the surfaces.

Strontium ion: Equimolar exchange of strontium ion for calcium ion both

in vivo and *in vitro* is well established (155, 177, 191, 198, 361). Strontium ion is so similar to calcium ion in its metabolism it has been used clinically to combat osteoporosis (332). It is nearly non-toxic and is incorporated *into* the crystals of bone (229, 231).

Lead ion: Though details are lacking, lead ion goes to bone because of exchange for calcium ion (17) and not, as commonly misstated, by deposition of colloidal lead phosphate. Such a colloid would deposit in the reticuloendothelial system but not in the skeleton (313, 314, 315). It may be incorporated *into* crystals (229) by recrystallization or crystallization.

Sodium ion: Further information is desirable but there is fair evidence (92, 157, 165, 166, 180, 199, 213, 270) that sodium ion exchanges for calcium ion on an equimolar basis. Whether sodium ion can occupy positions within the crystal

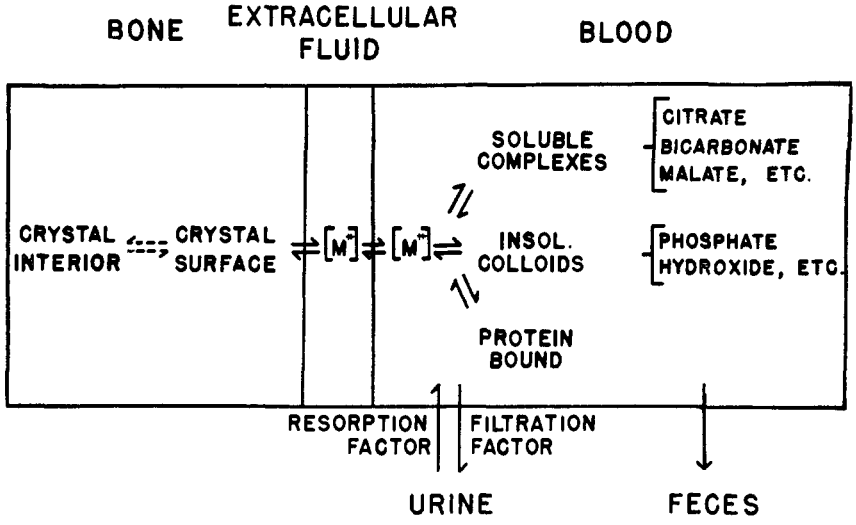

FIG. 10. A diagram illustrating the principal factors governing the distribution and excretion of foreign cations.

lattice is not established, but the *in vivo* exchange (112, 344) corresponds well with data on carbon dioxide exchange (48, 212, 266, 316). A conservative guess would be that sodium ion is mostly surface-limited.

Magnesium ion: Only fragmentary evidence is available for this element. The fact that magnesium ion is found in the crystal surfaces (92) and is "adsorbed" from solution by precipitates of basic calcium phosphate (215, 216) suggests that it exchanges with surface calcium. There is surprising agreement in the literature that the magnesium content of bone decreases markedly with age (75, 92, 153, 240, 261, 347). This may be a suggestion that crystal size increases with age.

Radium, thorium, and beryllium ions: The assignment of radium ion (13, 122, 181, 182, 284), thorium ion (342), and beryllium ion (323) to Group I is tentative, based entirely on the pattern of their distribution in the skeleton. No direct

experimentation has been found in the literature which supports or denies the possibility that these cations undergo heteroionic exchange for calcium.

Group II: A number of cations have been shown to deposit in the organic portion of bone,—the osteoid matrix. The specific affinity of the osteoid for these ions has not been explained. It is not known, for example, whether the affinity for these ions is due to the presence of a particular component in osteoid, such as chondroitinsulfuric acid, or whether the ions deposit because of specialized local conditions of pH, etc. Since one member of the series may displace another (312, 317), it is preferable to consider the deposition as a reaction with groups in the matrix, groups that are specific chemically or spatially. This series of "bone seekers" includes americium (151, 324, 365), plutonium (68, 318, 363, 365, 373), yttrium (68, 152, 196, 197, 230, 318, 373), barium (152), zirconium (151, 152), cerium (68, 151, 152), and gallium (108, 109).

Group III: A small number of anions are found to concentrate in bone. The most important of these are carbon dioxide, fluoride ion, and citrate ion. All three appear to be bound by the bone mineral by a process of surface exchange.

The heteroionic exchange of carbon dioxide for surface phosphate has been discussed earlier (see Section V,C). Of interest here is the pattern of distribution of administered $C^{14}O_2$ (12, 48, 266, 335), showing that the growing areas of bone exhibit the greatest accumulation of isotope (12, 325). This is confirmatory evidence that the fixation process involves ionic exchange.

Fluoride ion can substitute isomorphically for hydroxyl groups of the apatite structure, as discussed in previous sections. There is some evidence (282) that the mechanism of fixation involves an initial exchange of fluoride with hydroxyl and/or bicarbonate ions in the crystalline surfaces, but further clarification is needed. While the information on the mechanism of fluoride-fixation is meager, the literature on the fluoride content of bones and teeth, the effect of diet, etc., is too extensive to be reviewed here.

Unlike fluoride ion, citrate ion does not possess a structure that can satisfy the spatial requirements of the apatite lattice. It seems likely, therefore, that citrate ion is limited to heteroionic exchange at the crystal surface (166). The evidence to support this view, however, is very weak (215, 216). Most of the interest in citrate ion has been concerned with complex ion formation (167, 350), the content of bones and teeth (25, 59, 61, 348, 356, 378), the correlation between blood levels of calcium and citrate ions (1, 134, 311, 359), the relation between citrate ion and the action of vitamin D (156, 208, 289, 336), and the enzymatic synthesis and degradation of citrate ion in viable bone (104, 105).

VIII. CONCLUSIONS

Subject to modification and, with the risk of oversimplification, the present knowledge concerning the nature of the mineral phase of bone may be summarized as follows:

The crystals of bone are minute tablets, 25–50 Å. thick, approximately 400 Å. long and nearly as wide. In the intact bone, these crystals are found to be closely associated with the collagen, lying between the characteristic banding of

the fibers, with the long crystal axis (and the *c*-axis) parallel to the longitudinal direction of the fiber.

These crystals are comprised of calcium, phosphate, and hydroxyl ions arranged in a hexagonal lattice structure which diffracts x-rays to give a pattern characteristic of the apatite minerals. This lattice structure is not of fixed composition but may undergo some isomorphic substitution, particularly at the surface.

The specific surface area of bone mineral is enormous, because of the minute size of the crystals. To obtain measurements it is necessary to remove the organic material by heat treatment; therefore, the observed values of about 100 $m.^2/g.$ are minimal.

Because of this enormous area, surface phenomena dominate the chemical behavior of the bone mineral. One of the most important processes yet demonstrated is ionic exchange. The surface ions have been shown to be in equilibrium with the solution bathing the crystals. By heteroionic exchange, many nonlattice ions are bound by the crystals: hydronium, sodium, fluoride, carbon dioxide, and citrate. The crystals become highly hydrated in aqueous medium because of a boundary charge and the presence of exchangeable ions. The extreme thinness of the crystals permits an interchange of ions within the crystal with ions in solution, a process termed recrystallization.

The variability of the lattice structure, and the crystal surfaces especially, does not permit the application of the usual solubility principles. No single K_{sp} governs the solubility of either bone crystals or the basic calcium phosphates. However, the K_{sp} of $CaHPO_4$ sets a solubility maximum, above which precipitation occurs. Present data indicate that calcification *in vivo* involves a catalyzed crystallization rather than a precipitation, as frequently postulated.

All evidence is consistent with the belief that the skeleton and the body fluids are in equilibrium. The bones do not regulate the blood levels but they may provide considerable buffering action with respect to $[Ca^{++}]$, $[HPO_4^{--}]$, and $[H_3O^+]$. Thus, changes in blood composition induced by diet are reflected by the skeleton, especially in acidosis. The deposition of radioiosotopes confirms the dynamic equilibrium between blood and bone. Furthermore, recent studies with isotopes have shown dramatic variations in reactivity from bone to bone and from one microscopic area to another within a given bone. These data point up the fact that the crystal surfaces become less and less reactive with increasing age of the crystals. Foreign elements that concentrate in the skeleton do so by one of two processes: (*1*) a surface exchange with ions in the mineral crystals or (*2*) a specific but uncharacterized deposition in the organic or osteoid portion.

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