

# THE FOSSILIZATION OF HUMAN BONE: CALCIUM, PHOSPHATE, AND CARBONATE

BY

S. F. COOK

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## CONTENTS

	PAGE
Introduction .....	263
Material used .....	264
Results of direct analysis .....	264
Ion exchange in fossil bone .....	272
Conclusions .....	278

## TABLES

1. Mean values for bone constituents .....	266
2. Calculated values for calcium and inorganic matter .....	270
3. Carbonate and phosphate in fossil bone .....	273
4. Uptake of $P^{32}$ by a series of large pieces of bone .....	274
5. Release of radiophosphate from rat bones .....	275
6. Uptake of radioactive carbonate by fossil bone .....	276
7. Release of phosphate and carbonate from fossil bone .....	277
8. Uptake of radioactive phosphate and carbonate by suspensions .....	278

# THE FOSSILIZATION OF HUMAN BONE: CALCIUM, PHOSPHATE, AND CARBONATE

BY

S. F. COOK\*

## INTRODUCTION

FOR MANY years scientists with such diverse interests as physical anthropology, archaeology, and paleontology have been concerned with the process whereby fresh animal or human bones, subsequent to the death of the organism, become so altered as to resemble stone. It has generally been assumed that the original bone substance has undergone a replacement by inorganic matter from the soil in which it was buried, frequently referred to as "mineralization" or "fossilization." Few details concerning the mechanism of this change have been available. Recently, however, investigations carried out along a number of parallel lines in the laboratories of the Division of Physiology of the University of California School of Medicine have shed new light on the problem. Simultaneously, methods have been developed which aid in securing information concerning not only dead or fossilized, but also living, bone. This report covers merely a part of the investigation.

A recent contribution of fundamental significance is the electron micrographic study by Mrs. Elizabeth P. Barbour.<sup>1</sup> She was able to obtain photographs of very thin bone fragments by means of the electron microscope, photographs with magnification of the order of 60,000 diameters. Five samples were studied: a femur of a laboratory rat, a normal human femur from a cadaver, a human femur of relatively recent prehistoric time, perhaps five hundred years old, a femur from an archaeological site of the so-called Early Central California period, possibly six thousand years old, and a femur from an ancient site on the south coast of California.<sup>2</sup> All five showed a very characteristic structural pattern resembling a honeycomb, with light spaces on the negatives surrounded by dark walls. The diameter of the light spaces ranged from 0.0062 to 0.0225 $\mu$ , whereas the bone cavities hitherto regarded as smallest, the canaliculae of the Haversian system, average 0.3 $\mu$  in diameter.

For reasons which are discussed in her paper Mrs. Barbour concludes that the discontinuous light spaces probably consist of organic material together, perhaps, with water, whereas the continuous dark wall-like network is composed of inorganic compounds. These compounds may be presumed to include primarily calcium phosphate and carbonate organized in some orderly structure.

\* The writer wishes to express his gratitude to the Viking Fund, Inc., of New York for a grant which made this investigation possible and to the Committee on Research, University of California, for further financial assistance. H. S. Gladwin, Gila Pueblo, and T. Dale Stewart, U. S. National Museum, kindly supplied prehistoric bones from the Southwest.

<sup>1</sup> A Study of the Structure of Fresh and Fossil Human Bone by means of the Electron Microscope, *Amer. Journ. Phys. Anthro.*, n.s., 8:315-330, 1950.

<sup>2</sup> The recent prehistoric bone came from the so-called Hotchkiss site (CCo-138) in the San Joaquin delta region. The early Central Valley bone is from the Windmill site (Sac-107) on the upper Cosumnes River. The fifth bone, excavated in 1948 by Mr. A. E. Treganza, is from the Topanga site (LAn-1) near Santa Monica.

In a sense the findings of Mrs. Barbour merely confirm the conclusion long since reached by investigators of bone who used purely chemical methods: bone consists of two clearly distinct types of compounds—inorganic or mineral, and organic. The electron micrographic data, however, clearly delimit the spatial relationship of the two components and make it very clear that in studying post mortem changes the fate of the organic and the inorganic materials must be followed separately. In this paper attention is directed principally toward the inorganic fraction.

### MATERIAL USED

The experimental material reported on in this paper consisted of a series of bones from each of several archaeological sites. In tables 1 to 3 analytical results are given as mean values for these sites; hence the samples are not individually identified. However, lists of all the California skeletal specimens used have been made, the specimens identified by their numbers, and the burial numbers given in the catalogue of the University of California Anthropology Museum. The lists are on file, and may be consulted in the records of the University of California Archaeological Survey, Berkeley.

The sites investigated and the cultural periods represented are as follows:

*CCo-138*: Delta region, Late period, phases I and II.

*Sac-21*: Central Valley, Late period.

*Sac-6*: Central Valley, Late period.

*CCo-141*: Delta region, Late period.

*Sac-43*: Central Valley, Middle period.

*Sac-151*: Central Valley, Middle period.

*Sac-104*: Delta region, Middle period.

*CCo-137*: Coast ranges, near Concord, Middle period.

*Tul-18*: San Joaquin Valley, Middle period.

*SJo-68*: Central Valley, Early period.

*SJo-142*: Central Valley, Early period.

*Sac-107*: Central Valley, Early period.

*SJo-56*: Central Valley, Early period.

*Fre-28*: San Joaquin Valley, Early period.

*Hum-118*: Patrick's Point, Humboldt Bay, Late period.

*Marin Co.*: Two lots of bones were used, one of Late period, the other from the Middle period, the bones all being derived from a number of sites in Marin County.

*LAn-1*: The Topanga site, near Santa Monica, of very early origin.

*SBa-7*: A site at Carpinteria on the Santa Barbara Channel, of Oak Grove culture.

*Elden Pueblo, Arizona*, a series obtained through the courtesy of Dr. T. Dale Stewart from the United States National Museum collection. The bones have been dated as from 708 to 855 A.D.

*Hawikuh, N. Mex.*, a series of similar origin, dated from 1381 to 1480 A.D. The National Museum catalogue numbers of the individual samples are on file with the University of California Archaeological Survey.

*White Mound, N. Mex.*, a series obtained through the courtesy of H. S. Gladwin, Gila Pueblo, Globe, Ariz., dated 750 to 800 A.D.

*Soda Canyon, N. Mex.*, also obtained from H. S. Gladwin, dated 950 to 1116 A.D. The catalogue numbers for these series are also on file.

### RESULTS OF DIRECT ANALYSIS

Apart from small quantities of other elements and compounds the inorganic portion of bone consists of calcium phosphate and calcium carbonate.

For more than a century, to be sure, the presence of small quantities of fluoride

has attracted the interest of chemists, mineralogists, and biologists, an interest which has by no means subsided. It was felt, however, that the fluoride problem merited special and separate consideration. Hence the present study is limited to those compounds constituting the major portion of bone in the quantitative sense. Discussion of the fluoride content of fossil bone is therefore postponed.

During the past two years we have carried out in the laboratory of the Division of Physiology a series of analyses upon a group of ten to thirty samples each of human bone from 17 Central California, 2 Southern California, and 4 Southwestern archaeological sites. In all instances the solid bone from the central part of the femur was employed.<sup>3</sup> The analyses included determinations of elementary calcium and phosphorus together with carbonate as carbon dioxide. A preliminary partial series and a later complete series based upon 8 of the 17 Central California sites, all of them actually from the lower Sacramento River and delta region, indicated a progressive alteration of the bone with age. Thus the older material appeared to contain more calcium and more carbonate than the younger specimens, whereas the phosphate remained substantially constant. These relationships, together with information concerning the organic portion of the bones, were utilized to establish a numerical index, which, with few exceptions, could be employed to locate the site of origin upon a relative time scale.<sup>4</sup>

When the geographical range was extended, however, to include archaeological sites in areas with different climatic and soil characteristics, certain deviations appeared which made it necessary to examine more carefully the mechanism of alteration in the inorganic bone constituents.

To estimate calcium and phosphorus whole bone was ashed, and these elements were thus converted into their oxides. Although the method of ignition at high temperatures is not favored by investigators concerned with the problem of crystal structure, particularly of fresh bone, more elaborate procedures were not deemed essential to the elementary analysis of fossil bone. Carbonates were estimated by acid treatment of unashed samples and the water content was found by drying similar samples at 90° C for twenty-four hours.

During ashing the water is driven off, calcium carbonate is converted to calcium oxide with release of carbon dioxide gas, and all organic matter is oxidized. Since the loss in weight on ashing—or the ignition loss—can be taken as the sum of the organic matter, carbon dioxide and water, and since the weights of carbon dioxide and water are known, the total weight of the organic matter can readily be computed.<sup>5</sup> It may be noted that water is here considered as part of the inorganic frac-

<sup>3</sup> The technical procedures included grinding and ashing the bone sample followed by the ceric sulfate titration method for calcium and the colorimetric method for phosphorus. Nitrogen was determined by the micro-kjeldahl method and carbon dioxide by liberation from the sample with weak acid and absorption by the commercial preparation "caroxite." These methods are described in some detail in a recent paper: R. F. Heizer and S. F. Cook, *The Archaeology of Central California: A Comparative Analysis of Human Bone from Nine Sites*, Univ. Calif. Publ. Anthro. Rec., 12: 85-112, 1949.

<sup>4</sup> *Ibid.* Also Cook and Heizer, *The Quantitative Investigation of Aboriginal Sites: Analysis of Human Bone*, Amer. Journ. Phys. Anthro., n.s., 5: 201-220, 1947.

<sup>5</sup> Work now in progress renders it probable that the values for organic matter will have to undergo some revision. This in turn is due to the fact that the relation between removable water and the bone protein and apatite framework is not yet fully clear. Nevertheless, the error introduced by the method of water determination used hitherto, i.e., drying at moderate heat, is relatively small and will not invalidate the conclusions drawn from the data as here presented.

TABLE 1  
MEAN VALUES FOR BONE CONSTITUENTS

Site	Per cent of whole bone						Per cent of inorganic fraction							
	Organic matter	Ca	P	CO <sub>2</sub>	H <sub>2</sub> O	H <sub>2</sub> O	Ca	P	CO <sub>2</sub>	H <sub>2</sub> O	Ca	P	CO <sub>2</sub>	H <sub>2</sub> O
<b>Central Valley and Delta</b>														
<b>Late period</b>														
CCo-138.....	14.72	30.87	12.07	4.82	6.05		36.20	14.15	5.65					7.09
Sac-21.....	15.01	31.12	12.03	3.80	5.61		36.62	14.15	4.47					6.60
Sac-6.....	9.52	31.79	14.26	4.75	4.74		35.11	15.76	5.25					5.24
CCo-141.....	21.68	23.17	10.40	4.94	7.14		29.58	13.28	6.31					9.12
<b>Middle period</b>														
Sac-43.....	13.14	30.22	12.79	5.33	5.59		34.93	14.78	6.16					6.46
Sac-151.....	9.27	33.47	13.24	6.18	4.09		36.85	14.59	6.81					4.51
Sac-104.....	4.85	30.90	13.40	4.94	3.59		32.45	14.08	5.19					3.77
CCo-137.....	10.27	28.85	11.40	7.67	4.92		32.15	12.70	8.55					5.48
Tul-18.....	18.65	31.60	10.86	5.27	6.74		38.82	13.35	6.48					8.29
<b>Early period</b>														
SJo-68.....	9.97	32.89	13.43	6.14	5.12		36.50	14.92	6.62					5.69
SJo-142.....	4.41	35.80	12.88	9.56	3.49		37.42	13.47	10.00					3.65
Sac-107.....	3.92	35.50	14.01	9.67	3.40		36.95	14.58	10.18					3.54
SJo-56.....	11.20	32.00	12.60	5.62	4.88		36.02	14.18	6.22					5.49
Fre-28.....	2.42	31.80	12.70	6.35	2.56		32.33	13.03	6.32					2.63



Central and Northwest Coast

Late period

Marin Co.*	10.53	29.64	12.60	3.49	4.57	33.11	14.08	3.90	5.11
Hum-118	14.54	30.80	12.70	3.75	5.39	36.04	14.86	4.39	6.31

Middle period

Marin Co.*	9.77	31.14	12.85	4.20	4.52	34.52	14.24	4.66	5.01
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South Coast

Early period

LAn-1	3.42	35.78	14.42	2.50	3.13	37.04	14.93	2.59	3.24
SBa-7	6.24	35.25	12.40	6.51	3.75	37.19	13.22	6.94	4.00

Southwestern United States

Late period

Elden Pueblo	18.36	29.02	11.99	3.62	6.00	35.52	14.67	4.43	7.34
Hawikuh	27.50	23.41	10.25	3.10	7.45	32.30	14.14	4.28	10.28
White Mound	21.71	29.81	10.78	4.08	6.35	38.03	13.75	5.21	8.10
Soda Canyon	23.82	29.59	10.31	3.80	6.98	38.82	13.53	4.99	9.16

\* Each of these series consisted of a mixed lot of bones from several sites.

tion, an assumption which is probably erroneous. However, this procedure has been adopted by other investigators and may be followed for convenience in calculation.

The results for organic matter, calcium, phosphorus, carbon dioxide, and water, expressed as per cent of whole bone, are shown in table 1. The values for each site represent the means for several samples, usually 10 to 30, and are placed in the first five columns of the table. The sites are arranged according to relative age<sup>6</sup> (as determined by archaeological evidence) and geographical location.

It will be observed that the per cent of calcium appears to vary inversely with the organic matter and, since the organic matter diminishes with age, one might initially infer that the calcium increases with the age of the bone. Indeed, the correlation coefficient between these two factors, for the means of 23 sites, equals  $-0.74$ . However, this relationship actually demonstrates merely that calcium, as one component of the entire inorganic fraction, varies inversely as the organic matter. A real change in the calcium content alone could be established much more effectively by an increase in relation to other inorganic, rather than organic, constituents. To test this possibility calcium, phosphorus, carbon dioxide, and water have been computed as per cent of the inorganic fraction and the results placed in the last four columns of table 1.

Inspection of the values for calcium now reveals no marked trend toward increase or decrease in passing from the recent to the early sites. The mean percentages for 10 sites of the Late period, 6 of the Middle period, and 7 of the Early period are respectively 35.13, 34.95, and 36.20. The small differences between the three figures have no statistical or other significance. So far as time, per se, is concerned, therefore, there is no alteration in the calcium content.

For phosphorus the corresponding values pertaining to the same three groups of sites are 14.24, 13.95, and 14.04 per cent. The inference is thus valid that the bone content of this element also bears no consistent relation to age. With respect to carbon dioxide there is a slight increase in the older sites, the mean percentages for Late, Middle, and Early sites being respectively 4.89, 6.30, and 7.02. If we select only those sites lying in the lower basin of the Sacramento and San Joaquin rivers, the values become 5.42, 6.08, and 8.28. It was this increase of  $\text{CO}_2$  with age of burial which led Professor Heizer and myself, in previous work, to consider carbonate content an entity which could be used as an index for the estimation of relative age. However, the extension of analyses to include other geographical areas has made it clear that there is not necessarily such an increase in  $\text{CO}_2$ . It is likely that in the Central Valley local soil conditions favored carbonate accumulation, whereas the opposite was true elsewhere. At the Topanga site, known by cultural evidence to be very ancient, the bones contained even less carbon dioxide than may be found in the normal fresh human skeleton. Thus the relative carbonate content loses all universality as a criterion of age.

<sup>6</sup> The terms Late, Middle, and Early are applied generally throughout Central and Northern California to the three principal periods of aboriginal civilization, based on cultural sequences. The expressions are as yet not capable of translation into terms of absolute time units, for example, years. Moreover, the cultural periods are not necessarily contemporaneous in different localities. The subdivision of sites given in table 1 and elsewhere cannot therefore be regarded as definitive but must be considered simply the best estimate or guess possible at present. If some idea of the probable time span involved is desired, a purely tentative estimate would be, in years before the present: Late, 200-2,000; Middle, 2,000-4,000; Early, 4,000-8,000.

Although the inorganic constituents of these long buried bones cannot be systematically connected with age, it is apparent from table 1 that they have undergone some kind of alteration. Otherwise the variation in calcium, phosphorus, and carbon dioxide content from site to site has no reasonable explanation. In order to gain further insight into the nature of such alteration the analytical data have been recalculated and estimated in table 2. In the first column of the table, designated A, there is shown for purposes of comparison the average content of calcium found by analysis in the inorganic fraction of the bones. In columns B and C are placed theoretical values for calcium calculated by molecular proportion from the measured phosphate and carbonate content.

In order to make such calculations it is necessary to consider the form in which the salts may be present. The modern literature on the crystal structure of bone is voluminous.<sup>7</sup> It would be tedious and profitless to recapitulate here in detail the theories which have been advanced with respect to the exact mode of combination of calcium, phosphate, and carbonate, particularly since this investigation has produced no new evidence bearing on the problem. However, if we attempt to compute the calcium content of fossil bone, using phosphate and carbonate as a point of departure, we must tentatively at least adopt some hypothesis concerning possible structure. Accordingly, it was considered logical to examine alternative assumptions, the second being based upon the two most widely discussed theories. These assumptions are: (1) that in fossil bone the calcium and phosphorus are combined as simple tricalcium phosphate  $[\text{Ca}_3(\text{PO}_4)_2]$  plus calcium carbonate and (2) that the bone retains the apatite structure according to the empirical formula  $[\text{Ca}_{10}\text{OH}(\text{PO}_4)_6]$  for hydroxyapatite and that calcium carbonate is not incorporated in the molecule. The sets of values obtained for calcium under these two assumptions are shown in columns B and C in table 2. In columns D and E are given the corresponding differences, that is, calcium excess or deficit obtained by subtracting the calculated values in columns B and C from the observed quantities shown in column A.

The means for the columns D and E are + 2.73 and - 0.32. The standard error for both means is  $\pm 0.54$ . It thus appears that for the twenty-three sites as a whole the second assumption is in closer conformity with the results, than is the first. The calcium found by analysis can best be accounted for as in combination with phosphorus as an apatite and with carbon dioxide as the carbonate. Even if these data cannot be regarded as conclusive, they nevertheless create a strong presumption that the apatite structure of the original living bone persists for long periods in the soil without significant alteration. Such an hypothesis would be in conformity with the results obtained by electron micrography. Mrs. Barbour, in the investigation to which previous reference has been made, was able to show that the geometrical pattern of three fossil bones (from CCo-138, Sac-107, and LAn-1) was identical

<sup>7</sup> Reference may be made to the following articles or reviews which have appeared in recent years and which serve as a guide to the work carried on in the field: Charles Huggins, The Composition of Bone and the Function of the Bone Cell, *Physiological Reviews*, 17: 119-143, 1937; Milan A. Logan, Recent Advances in the Chemistry of Calcification, *ibid.*, 20: 560, 1940; W. D. Armstrong, Biochemical and Nutritional Studies in Relation to the Teeth, *Annual Review of Biochemistry*, 11:441-464, 1942; Franklin C. McLean, Physiology of Bone, *Annual Review of Physiology*, 5:79-104, 1943; M. J. Dallemagne and J. Melon, La Localisation de l'apatite et du phosphate tricalcique dans l'émail dentaire, *Archives de Biologie*, 57:79-98, 1946.

TABLE 2  
CALCULATED VALUES FOR CALCIUM AND INORGANIC MATTER

Site	A Observed calcium as per cent of inorganic matter	B Calculated per cent calcium assuming tricalcium phosphate	C Calculated per cent calcium assuming hydroxyapatite	D Calcium deficiency or excess (A-B)	E Calcium deficiency or excess (A-C)	F* Total calculated inorganic matter assuming tricalcium phosphate	G* Total calculated inorganic matter assuming hydroxyapatite	H Inorganic matter excess (100-F)	I Inorganic matter deficit or excess (100-G)
CCo-138.....	36.20	32.54	35.64	+3.66	+0.56	90.64	95.14	9.36	+ 4.86
Sac-21.....	36.62	31.47	34.57	+5.15	+2.05	87.48	91.98	12.52	+ 8.02
Sac-6.....	35.11	35.28	38.68	-0.17	-3.57	95.66	100.86	4.34	- 0.86
CCo-141.....	29.58	31.43	34.23	-1.85	-4.38	89.74	93.94	10.26	+ 6.06
Sac-43.....	34.93	34.20	37.40	+0.73	-2.47	94.26	98.86	5.74	+ 1.14
Sac-151.....	36.85	34.38	37.58	+2.47	-0.73	92.89	97.39	7.11	+ 2.61
Sac-104.....	32.45	31.97	35.02	+0.48	-2.57	87.55	91.85	12.45	+ 8.15
CCo-137.....	32.15	32.38	35.08	-0.23	-2.93	88.42	92.32	11.58	+ 7.68
Tul-18.....	38.82	31.69	34.64	+7.13	+4.18	89.73	93.83	10.27	+ 6.17
SJo-68.....	36.50	34.87	38.52	+1.63	-2.02	95.36	100.26	4.64	- 0.26
SJo-142.....	37.42	35.15	38.05	+2.27	-0.63	93.67	97.87	6.33	+ 2.13
Sac-107.....	36.95	37.45	40.65	-0.50	-3.70	99.86	104.36	0.14	- 4.36
SJo-56.....	36.02	33.33	36.43	+2.69	-0.41	90.77	95.17	9.23	+ 4.83
Fre-28.....	32.33	31.13	34.08	+1.20	-1.75	82.65	86.65	17.35	+13.35
Marin Co.....	33.11	30.89	33.70	+2.22	-0.59	84.27	88.67	15.73	+11.83
Hum-118.....	36.04	32.72	35.95	+3.32	+0.09	90.49	95.09	9.51	+ 4.91
Marin Co.....	34.52	31.79	34.89	+2.73	-0.37	86.79	91.09	13.21	+ 8.91
LAn-1.....	37.04	31.25	34.45	+5.79	+2.59	83.73	88.33	16.27	+11.67
SBa-7.....	37.19	31.71	34.75	+5.48	+2.44	85.82	90.82	14.18	+ 9.18
Elden Pueblo.....	35.52	32.37	35.57	+3.15	-0.05	90.70	95.10	9.30	+ 4.90
Hawikuh.....	32.30	31.25	34.34	+1.05	-2.04	90.71	95.01	9.29	+ 4.99
White Mound.....	38.03	31.79	34.34	+6.24	+3.69	88.64	92.84	11.36	+ 7.16
Soda Canyon.....	38.82	30.73	33.63	+8.09	+5.19	88.21	92.31	11.79	+ 7.69
Mean.....	.....	.....	.....	+2.73	-0.32	.....	.....	.....	+ 5.66
S.E. of Mean.....	.....	.....	.....	±0.54	±0.54	.....	.....	.....	± 0.88

\* For explanation of values in this column, see discussion in text, especially p. 271.

with that of fresh bone in that the spacing of the light and dark components did not differ. Thus the mean distance from the center of one light area to that of another varies by no more than  $\pm 5$  per cent in all four bones.

Although the fundamental submicroscopic and molecular structure of the bone is retained during fossilization, it is clear from tables 1 and 2 that minor fluctuations from site to site do occur. These may be accounted for in part by analytical, experimental error or random variation among the particular samples used to determine the mean values for each site. But it is also probable that some of the larger deviations represent real differences between localities. Thus we may contrast the quantities (column E, table 2)  $-3.57$  for Sac-6,  $-4.38$  for CCo-141, and  $-3.70$  for Sac-107 on the one hand with  $+4.18$  for Tul-18,  $+2.59$  for LAn-1, and  $+5.19$  for Soda Canyon on the other. There is no known factor associated with age in years or with material culture to which these variations can be ascribed. Hence it remains only to attribute them to different physical and chemical environmental conditions at the various localities which seem to have induced secondary changes in the composition of the buried bones.

At this point it is worth while to examine the total inorganic content of the bones. When buried they contained, apart from organic material and water, primarily calcium phosphate and carbonate. The small quantities of magnesium, chloride, fluoride, etc., which may have been present would not affect the general situation. If at the present time, as has been already suggested, the fossil bones still retain the calcium salts in no very profoundly modified condition, and if no other changes have occurred, then the sum of the calcium phosphate and calcium carbonate should still represent substantially the total *inorganic* fraction of the bone, quite irrespective of any alteration in the *organic* fraction. In other words, the sum of the calculated calcium phosphate and calcium carbonate should equal nearly or exactly 100 per cent of the inorganic matter. To test this proposition the indicated sums have been performed for each of the twenty-three sites (assuming that the phosphorus is combined both as tricalcium phosphate and as hydroxyapatite) and the results placed in columns F and G of table 2. As a final step the values shown in these two columns have been subtracted from the value for total inorganic matter, expressed as 100 per cent, and the differences entered in columns H and I.

If the algebraic difference between the calculated calcium salts and 100 is positive, then it must be assumed that some other type of inorganic—or at least, ignition stable—material is present. If the difference is negative, we must invoke experimental error or seek some other explanation. The mean difference for all the sites, based upon a probable hydroxyapatite structure is  $+5.66$  per cent  $\pm 0.88$ . Hence it appears that throughout the series approximately 5 per cent of the inorganic substances recovered after ignition consists of material accumulated from the soil during the interment of the bones. The chemical nature and exact quantity of such material must depend upon the composition of the mound matrix.

It is to be regretted that complete quantitative analysis of the bone samples has not been performed and that the physical and chemical characteristics of the sites themselves are still largely unknown. Nevertheless some comment is possible. Sites Sac-6, Sac-43, Sac-151, SJo-68, SJo-142, and Sac-107 all show negative or very low positive values in column I, table 2. This is an indication of little or no infiltration

by foreign noncalcareous substances, although it does not exclude the accumulation of lime salts from the soil solution. Now all these six sites are of a type which occurs frequently in the Central California Valley, consisting almost entirely of midden refuse with very little admixture of extraneous soil. They uniformly have a high content of lime. Thus it has been found<sup>8</sup> that the mean carbonate content for sites SJo-142, SJo-68, and Sac-43, derived from 30 or more soil samples from each mound, was respectively 7.92, 23.72, and 4.81 per cent. Long periods of exposure to such high levels of calcium carbonate could conceivably mean an infiltration of this material into the bone. Any such foreign or extrinsic carbonate would then be included in the calculations above together with the carbonate which originally constituted part of the bone structure, and this would increase the computed value for total calcium salts.

On the other hand, the Orwood no. 2 site (CCo-141) lies in and is surrounded by an acid peat bog. The matrix of Tyler Island no. 2 site (Sac-104) and of the Tranquillity site (Fre-28) consists predominantly of a sandy river deposit rather than undisturbed habitation midden. The Topanga site (LAn-1) appears to contain original mound deposit, now altered to almost an adobe clay in which there are only faint traces of carbonate. Bones from these four sites all show a high percentage of accumulated noncalcareous material (values in column I, table 2 are respectively +6.06, +8.15, +13.35, and +11.67). This noncalcareous material may perhaps consist of such soil derivatives as silicates and other salts of iron or aluminum. It is noteworthy, as a supplementary observation, that the first group described (SJo-142, etc.) produces bones which to visual inspection are compact, dense, white, and chalky, whereas the second group is characterized by light, porous, brown-to-black bones of almost woody consistency. Further evidence that we are dealing here with variation in the inorganic fraction is supplied by the electron micrographs of bones from the Sac-107 site and the Topanga site (LAn-1). Although the basic geometrical pattern of each conforms to that found in normal specimens, there were quantitative differences. Thus the thickness of the walls in the sample from Sac-107 is very much greater than in fresh bones, whereas in the Topanga (LAn-1) sample the walls are much thinner. At the same time the carbonate content of the bone from Sac-107 (expressed as CO<sub>2</sub>) was 10.18 per cent and that of the Topanga sample 2.59 per cent. In summary, it appears highly probable that, whether or not the substances concerned are exactly those here suggested, alteration in the inorganic bone constituents has proceeded in more than one direction. Moreover, the nature of this alteration seems contingent upon the chemical characteristics of the soil in which the bones have been embedded.

### ION EXCHANGE IN FOSSIL BONE

It is now appropriate to introduce certain experimental data which furnish a clue to one possible mechanism whereby changes in the inorganic components of fossil bone may have been accomplished. We may consider primarily the two important acid radicals, phosphate and carbonate.

A glance at table 1 demonstrates that there is considerable variation from one

<sup>8</sup> From the unpublished results of José Setzer, "Chemical Analysis of Indian Mounds," Thesis, Univ. Calif., 1947.

locality to another in both phosphate and carbonate content. On the other hand, the relative constancy of the primary positive element, calcium, implies that the total of these two anions fluctuates very little. This is also evident from a computation of the sums of phosphorous and carbon dioxide as found by analysis and compared with the corresponding ratios (see table 3). Thus the mean of the sums is 20.01 per cent with a coefficient of variation of  $\pm 8.53$  per cent of the mean. The

TABLE 3  
CARBONATE AND PHOSPHATE IN FOSSIL BONE

Site	Per cent phosphorus in inorganic fraction	Per cent CO <sub>2</sub> in inorganic fraction	Sum of P and CO <sub>2</sub>	Ratio P:CO <sub>2</sub>
CCo-138.....	14.15	5.65	19.80	2.50
Sac-21.....	14.15	4.47	18.62	3.16
Sac-6.....	15.76	5.25	21.01	3.00
CCo-141.....	13.28	6.31	19.59	2.10
Sac-43.....	14.78	6.16	20.94	2.40
Sac-151.....	14.59	6.81	21.40	2.14
Sac-104.....	14.08	5.19	19.27	2.71
CCo-137.....	12.70	8.55	21.25	1.49
Tul-18.....	13.35	6.48	19.83	2.06
SJo-68.....	14.92	6.62	21.54	2.25
SJo-142.....	13.47	10.00	23.47	1.35
Sac-107.....	14.58	10.18	24.76	1.43
SJo-56.....	14.18	6.32	20.50	2.24
Fre-28.....	13.03	6.52	19.55	2.00
Marin Co.....	14.08	3.90	17.98	3.61
Hum-118.....	14.86	4.39	19.25	3.39
Marin Co.....	14.24	4.66	18.90	3.05
LAn-1.....	14.93	2.59	17.52	5.76
SBa-7.....	13.22	6.94	20.16	1.91
Elden Pueblo.....	14.67	4.43	19.10	3.31
Hawikuh.....	14.14	4.28	18.42	3.31
White Mound.....	13.75	5.21	18.96	2.64
Soda Canyon.....	13.53	4.99	18.52	2.71
Mean.....	.....	.....	20.01	2.63
Coeff. var.....	.....	.....	$\pm 8.53$	$\pm 30.44$

mean of the ratios is 2.63 with a coefficient of variation of  $\pm 30.44$  per cent. Thus the variability of the ratios is nearly four times as great as that of the sums.

It has been postulated that an accumulation of calcium carbonate or of non-calcareous material is conditioned by the soil content. It is similarly possible that fluctuation in the relative quantities of carbonate and phosphate may also be attributed to the substances forming the mound matrix, and furthermore that the bone may have lost as well as gained. This hypothesis has been tested by examination of the exchange of ions between bone and an external solution.

It has long been appreciated that there is a continuous overturn of diffusible ions, particularly phosphate and carbonate, between the living bone and the blood plasma, and considerable progress has been made in the study of the dynamics of

the process. The physical and chemical conditions, however, are very dissimilar when the completely dead bone lies surrounded by soil. Hence our knowledge of the reactions proceeding during life cannot be carried over directly to the non-living state. For present purposes, indeed, it should be sufficient to show that exchange of ions, specifically anions, can occur and does occur, without attempting to elucidate the kinetics of the reactions or to measure the equilibria involved. In an attempt to answer this question directly a few simple experiments were performed by the use of radioactive tracers and the standard counting technique.<sup>9</sup>

1. A series of 14 bones was selected, at random except for a more or less equal

TABLE 4  
UPTAKE OF P<sup>32</sup> BY A SERIES OF LARGE PIECES OF BONE

Bone no. and site	Dry weight of bone (in gm.)	Per cent of the activity lost by the solution after:				
		15 min.	20 hrs.	68 hrs.	168 hrs.	336 hrs.
1 Col-1.....	3.274	+3.1	-35.9	-87.9	-97.4	-99.4
33 Col-1.....	2.937	0.0	-44.3	-84.9	-94.4	-98.1
5 Col-1.....	6.250	-3.1	-52.8	-87.5	-96.0	-98.8
32 Col-1.....	5.423	-1.8	-35.0	-91.3	-96.3	-98.8
9 Sac-66.....	4.876	-1.1	-51.7	-79.0	-89.8	-94.6
10 Sac-66.....	5.556	-7.0	-63.2	-81.6	-95.5	-98.0
14 Sac-43.....	8.652	-3.5	-61.7	-85.3	-93.9	-97.1
15 Sac-43.....	4.512	-6.0	-57.7	-86.3	-90.0	-95.8
27 SJo-142.....	6.312	-3.6	-65.2	-86.9	-93.2	-96.9
28 SJo-142.....	4.915	-5.1	-54.7	-79.9	-90.0	-95.9
21 SJo-56.....	8.797	-4.6	-69.0	-77.7	-93.0	-98.0
22 SJo-56.....	10.092	-3.7	-67.4	-87.6	-94.0	-97.2
23 SJo-68-3.....	6.544	-2.4	-73.3	-90.8	-96.7	-98.3
23 SJo-68-12.....	5.897	-2.4	-58.9	-87.9	-96.1	-98.8
Mean.....	.....	-2.6	-56.5	-85.3	-94.0	-97.5

representation according to probable age. From each bone a single piece was removed and weighed. No attempt was made to secure exact uniformity in either shape or size. Each piece was then placed in a container with 50 cc. of radioactive phosphate solution, which consisted of a tracer quantity of P<sup>32</sup> in the form of the phosphate and incorporated with Na<sub>2</sub>HPO<sub>4</sub> so that the total quantity reached  $3 \times 10^{-7}$  moles. At the end of specific time intervals one cubic centimeter of the solution was removed, placed on a metal plate and dried in an even film. The radioactivity of the dry material was then determined with a Geiger-Muller counter. The results are found in table 4, expressed in terms of the per cent of activity which had been lost by the solution after each time interval as compared with a standard control solution which contained no bone. In other words, the values represent the per cent of the total radioactive phosphate which had been taken up by the bone.

Certain details are worth mentioning. The larger pieces tend to remove the radio-

<sup>9</sup> The radioactive elements were kindly supplied by Professor Joseph G. Hamilton of Crocker Laboratory, Berkeley. The rat-feeding experiments were performed by Dr. K. G. Scott of the Division of Radiology, University of California School of Medicine, who generously donated his time for the purpose.



phosphate somewhat more rapidly than the smaller ones. Thus at 20 hours the correlation coefficient between weight of bone sample and per cent of activity lost is equal to + 0.646, a value of moderately high significance. However, apparently by 68 hours, and certainly by 168 hours, these differences have disappeared and the relation between size and accumulation of radiophosphate is purely random. Because of the decay of the  $P^{32}$  the measurements had to be discontinued at the end of 336 hours, but the trend of the data up to this point indicates that an equilibrium would eventually have been reached in the vicinity of a 98 to 99 per cent loss of activity by the solution. However, the probable arrival at a state of equi-

TABLE 5  
RELEASE OF RADIOPHOSPHATE FROM RAT BONES

Sample no.	Dry weight (in gm.)	Suspension medium	Activity as counts per sec. per gm. of bone in 50 cc. of suspension medium after:		
			1 day	8 days	16 days
1.....	2.826	Dist. aq.	89.2	240.5	225.0
2.....	3.795	Dist. aq.	46.9	115.6	137.0
3.....	3.598	NaHCO <sub>3</sub>	150.5	396.0	487.0
4.....	4.242	NaHCO <sub>3</sub>	110.0	262.0	278.0
5.....	3.276	Na <sub>2</sub> CO <sub>3</sub>	143.5	278.0	345.0
6.....	4.219	Na <sub>2</sub> CO <sub>3</sub>	102.3	189.6	235.0
7.....	2.907	KH <sub>2</sub> PO <sub>4</sub>	384.0	987.0	1272.0
8.....	4.159	KH <sub>2</sub> PO <sub>4</sub>	365.5	686.0	847.0
9.....	3.646	Na <sub>2</sub> HPO <sub>4</sub>	363.5	677.0	745.0
10.....	4.056	Na <sub>2</sub> HPO <sub>4</sub>	366.5	698.0	719.0
Mean for distilled water.....			68.1	178.1	181.0
Mean for NaHCO <sub>3</sub> and Na <sub>2</sub> CO <sub>3</sub> .....			126.7	281.2	336.2
Mean for KH <sub>2</sub> PO <sub>4</sub> and Na <sub>2</sub> HPO <sub>4</sub> .....			283.4	762.0	895.8

librium argues, not for a unidirectional accumulation of material, but for a balance wherein there is an interchange between nonactive phosphate from the bone and active phosphate derived from the exterior. Such a hypothesis requires proof that phosphate ions which are originally laid down as an integral component of bone can be released and can diffuse into an external solution. Such proof is furnished by the second experiment.

2. Several rats were maintained for one week on a diet which contained innocuous quantities of  $P^{32}$  in the form of Na<sub>2</sub>HPO<sub>4</sub>. At the end of this time they were killed and the leg bones cleaned and dried. These bones were then suspended in a series of flasks, each of which contained 50 cc. of a nonradioactive solution. Specifically, two flasks had distilled water, two  $10^{-2}N$  NaHCO<sub>3</sub>, two  $10^{-2}N$  Na<sub>2</sub>CO<sub>3</sub>, two  $10^{-2}N$  KH<sub>2</sub>PO<sub>4</sub>, and two  $10^{-2}N$  Na<sub>2</sub>HPO<sub>4</sub>. After one, eight, and sixteen days, one cubic centimeter of the medium was withdrawn from each flask and its activity determined. The results will be found in table 5. Although no attempt was made to control the exact amount of  $P^{32}$  ingested or to use precisely the same weight of bone in each experiment, two facts seem sufficiently well established. (1) The radioactive phosphate which was incorporated in the inorganic bone substance during

life was released with apparent ease and rapidity after death. There can be no question, therefore, that the ion can be liberated from the apatite crystal. (2) Although the data are not as satisfactory as one would desire, nevertheless there is a clear tendency for more phosphate to be liberated to the carbonate and bicarbonate solutions than to distilled water and more to the phosphate solutions than to those containing carbonate. The conclusion thus seems justified that the presence of inactive external ions facilitated the release of active ions from the bone. This may well have been owing to replacement or interchange of the anions.

3. In the third series of determinations the objective was to ascertain whether the carbonate ion would be assimilated by fossil bone in a manner resembling that

TABLE 6  
UPTAKE OF RADIOACTIVE CARBONATE BY FOSSIL BONE

Sample*	Dry weight (in gm.)	Per cent of activity lost by the solution after:			
		10 min.	4 hrs.	24 hrs.	120 hrs.
1.....	2.771	- 5.6	-10.2	-38.6	-57.5
2.....	3.390	- 8.7	- 8.6	-35.7	-46.6
3.....	2.865	-10.4	.....	-40.6	-35.2
4.....	2.126	-11.0	-16.2	-36.7	-59.1
5.....	2.833	- 4.2	-14.5	-38.6	-56.0
6.....	3.056	-11.0	-10.4	-35.0	-48.7
Mean.....	.....	- 8.5	-12.0	-37.5	-50.5

\* All samples are from site Col-1.

in which phosphate ion is taken up. For this purpose radioactive carbon ( $C^{14}$ ) was used as  $Na_2CO_3$  at a concentration of  $3 \times 10^{-7}$  moles in 50 cc. of solution. The procedure was the same as in experiment 1. The bone samples were all derived from a single recent site (Col-1). The results are to be found in table 6. Unfortunately, owing to loss of radioactive carbon through escape of  $CO_2$  from the containers, the test could be continued only five days. Nevertheless during that interval it became evident that the bone can remove carbonate from solution nearly as rapidly as it can remove phosphate. Hence there is no reason to doubt that, if ion exchange occurs, carbonate can be substituted for phosphate and vice versa.

At this juncture the attempt was made to repeat experiment 2, using radioactive carbonate in lieu of phosphate. This, however, was found impossible, since the carbonate assimilated through the digestive tract of the rats was so rapidly released through the respiratory system in the form of  $CO_2$  that the bones on autopsy had accumulated very little  $C^{14}$ ; so little, indeed, that it could scarcely be detected by the Geiger counter. As a result no further endeavor was made to establish radioactive carbon in living rat bone.

4. Since radioactive carbonate cannot easily be incorporated in living bone, it was decided to observe the release of both phosphate and carbonate from fossil bone after these ions had been absorbed from an external solution. To this end a large sample from site Col-1 was ground into a fine powder and separated into eight lots weighing 1 gm. each.

In setting up the experiment two series of five bottles were used. The first was designated H1-H4 plus one control, the second K1-K4 plus a control. In each bottle of series H was placed  $3 \times 10^{-7}$  moles of radioactive carbonate and in series K an equal quantity of radioactive phosphate. To all except the controls was then added 1 gm. of bone. After standing two weeks the original solutions were removed, except from the two controls. The bone was thoroughly but rapidly washed with distilled water. Then to bottles H1, H2, K1, and K2 were added 50 cc. of  $10^{-2}$ N NaCO<sub>3</sub> and to bottles H3, H4, K3, and K4 were added 50 cc. of  $10^{-2}$  Na<sub>2</sub>HPO<sub>4</sub>. The radioactivity appearing in the eight bottles containing replacement solutions was then measured at intervals and expressed as per cent of the activity in the corresponding control (for data see table 7).

TABLE 7  
RELEASE OF PHOSPHATE AND CARBONATE FROM FOSSIL BONE

Solution designation*	Activity in replacement solutions, expressed as per cent of control after:			
	3 hours	1 day	4 days	19 days
H1 + H2, mean.....	7.6	10.1	13.6	13.6
H3 + H4, mean.....	9.6	11.9	18.5	19.0
K1 + K2, mean.....	7.6	15.2	17.2	13.4
K3 + K4, mean.....	13.1	28.3	34.5	38.0

\* For solution designations, see text.

From the results it is clear that radioactive phosphate and carbonate ions leave the bone and enter the external medium, thus directly reversing the process by which they originally entered. The final value in terms of per cent of control is evidently determined by the relative concentrations of active and inactive ions inside and outside the bone substance. However, since the total concentration of inactive PO<sub>4</sub> and CO<sub>3</sub> external to the bone was much greater than that of the active ions, it is very unlikely that the active ions would have been released against a concentration gradient without replacement by the inactive. Again, we must conclude that an exchange of ions is in progress. Moreover, despite minor quantitative differences, the ion species plays a relatively insignificant part.

5. Since it is established that anions can enter and become intimately associated with fossil bone, there arises the question whether this process is dependent upon the molecular arrangement itself, that is, whether it is contingent upon the existence of the apatite structure. To gain information concerning this matter an experiment was performed with a purely artificial system. Pure suspended tricalcium phosphate and pure calcium carbonate were exposed to solutions of radioactive phosphate and carbonate. Initially, 5 gms. each of powdered Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> and CaCO<sub>3</sub> were shaken in a flask with distilled water and then allowed to settle for several hours. The fluid was next decanted and the process repeated. After approximately ten treatments a suspension of the solid was obtained which was substantially clear of particles of colloidal dimensions because the remaining material settled within five minutes in such a way as to produce a perfectly clear supernatant liquid. The component particles of the suspension possessed no characteristic crystal structure ;

they were merely loose molecular aggregates of highly insoluble tricalcium phosphate and calcium carbonate (solubility of both approximately 0.02 gm. per lit.).

After the final decantation each suspension was divided into two equal parts. To one part was added 50 cc. of water containing  $3 \times 10^{-7}$  moles of radioactive phosphate and to the other the same quantity of radioactive carbonate. All the suspensions were shaken and, as soon as settling was complete, samples of the fluid portion were withdrawn for counting. The data in table 8 show that the active ions were absorbed by the suspensions with extreme rapidity at first, and later more slowly. A final equilibrium would probably have been reached, had the experiment continued for a longer period. Since the actual concentrations of the substances were

TABLE 8

UPTAKE OF RADIOACTIVE PHOSPHATE AND CARBONATE BY SUSPENSIONS  
(Per cent of activity lost by solution to the suspended solid when the solution contained active phosphate and active carbonate)

Time after mixing	Active phosphate		Active carbonate	
	Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	CaCO <sub>3</sub>	Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	CaCO <sub>3</sub>
5 minutes.....	81.9	53.4	35.5	15.1
2 hours.....	95.2	72.7	....	....
4 hours.....	96.0	82.4	51.1	26.9
1 day.....	98.4	89.5	64.7	40.8
3 days.....	98.8	90.1	75.8	44.4
6 days.....	98.9	92.3	83.7	47.2
20 days.....	99.4	92.8	....	....

not known with certainty, the kinetics of the process was not studied in detail. With respect to the influence of structure the conclusion is justified that the assumed apatite organization is not a determining factor in the uptake or removal of anions.<sup>10</sup>

### CONCLUSIONS

The experiments performed with radioactive tracers warrant the conclusion that the diffusible anions, phosphate and carbonate, can enter and leave dead or fossil bone with very great facility. It is further probable that such movement is based upon an interchange of ions between the bone substance and the external solution rather than upon a unidirectional accumulation or depletion of a single ion species. Fossil bone, therefore, behaves in a manner fundamentally similar to living bone.

Analyses of more than four hundred samples of fossil bone of different ages and from widely separated localities show a remarkable constancy in content of calcium as referred to the total inorganic matter. At the same time, the relative quantities of phosphate and carbonate fluctuate over a much wider range, although the sum of these two components is fairly constant.

<sup>10</sup> Several other experiments were performed which indicated that the velocity of initial uptake of phosphate and carbonate is modified by the state of subdivision of the bone as well as by its total quantity. These factors appear to be without influence on the final equilibrium. On the other hand, the total quantity absorbed is dependent upon the external ionic concentration. Since, however, these experiments are still incomplete, any comprehensive report concerning them is best deferred until they can be repeated and confirmed.

For these reasons, therefore, it may be maintained that the primary participation of the major mineral components in the process of fossilization involves an exchange or replacement of substance rather than an accumulation or depletion, although the existence of depletion processes on a secondary scale cannot be denied.

It must be recognized that, so far as the determination of the age of fossil bone is concerned, the inorganic constituents (save, perhaps, fluorine) are of little immediate value. The composition of any particular specimen as it comes into the laboratory will have been determined, not by the lapse of time since the death of the person or animal, but by the mutual interaction between the bone and the substances in the soil surrounding it, together with the physical conditions characteristic of the environment subsequent to the bone's burial. If a temporal sequence based on changes in inorganic components is to be established, a combined study of the bones and the corresponding soil chemistry will be necessary.