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The crystallinity of bone mineral

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The degree of crystallinity of human femoral cortical bone from 16 to 83 years of age was determined by various X-ray and infra-red methods. It was established that an X-ray integral index method and an infra-red peak comparison method were the most reliable. Both methods indicated that the crystallinity of bone material varies between 51 and 59%, with no significant change with age. A new X-ray line was noted at $2\theta = 43°$ in bone of age greater than 50 years.

1. Introduction

It has been widely recognized that the mineral which is included in hard tissues in general and in bones in particular is not entirely crystalline. This can be observed from the diffuse X-ray wide-angle pattern, which is attributed to the very small size of the crystallites and to the presence of an amorphous phase. Many approaches have been tried to determine the nature of that amorphous phase [1, 2]. Chemical analysis and X-ray powder determinations have ruled out the presence of materials other than calcium phosphates of the apatite type [3]. The accepted explanation is that in addition to a crystalline phase similar to hydroxyapatite ($Ca_5(PO_4)_3(OH)$), there is an amorphous calcium phosphate phase in the mineral of bones [4].

The purpose of this study was to determine the degree of crystallinity of cortical bone and its change with age. The three methods used were (a) an infra-red method, (b) an X-ray method based on two lines, and (c) an integral index X-ray method, which has not hitherto been applied to bone.

2. Materials

The study was carried out on human, male, femoral cortical bones from 16 to 83 years in age and in a normal condition. These bones were stored (from months to years) in a deep freeze at $-10°$ C. Some studies [5] have shown that the ultrastructure of bones is not altered by the length of storage in a deep freeze. Samples were sawn from the bones along the long axis of the bone and in the middle of the shaft. For the infra-red method the bone samples were powered under alcohol with a dental drill (diamond discs), rotating at low speed to prevent heating. For the other two methods the samples were polished on ground glass (to provide a smooth surface). Care was taken during the polishing not to enhance any preferred orientation already present in the bone, by polishing the section circularly. The standards used in this study (see Fig. 2) were (a) a mineral apatite powder (fluoroapatite ($Ca_5(PO_4)_3F$) from Ontario, Canada, which was used as a pure crystalline material, and (b) an amorphous calcium phosphate [7], which was prepared by precipitation of the following mixture:

300 ml 0.016 M $CaCl_2$ in buffer solution

300 ml 0.01 M $(NH_4)_2HPO_4$ in buffer solution

400 ml (0.05 M NH_4OH) at pH 10.5.

The three components were added at room temperature and the precipitated calcium phosphate was then washed in the buffer solution (3 times) in acetone (3 times) and then stored in a desiccator. This variety of calcium phosphate amorphous was used rather than the synthetic type described by Eanes *et al.* [6], which has a similar X-ray pattern to bone (see Fig. 2).

3. Methods

3.1. The infra-red method

This method was devised by Termine and Posner [1] and is based on the fact that the orthophosphate ion (PO_4^{-3}) absorbs infra-red radiation

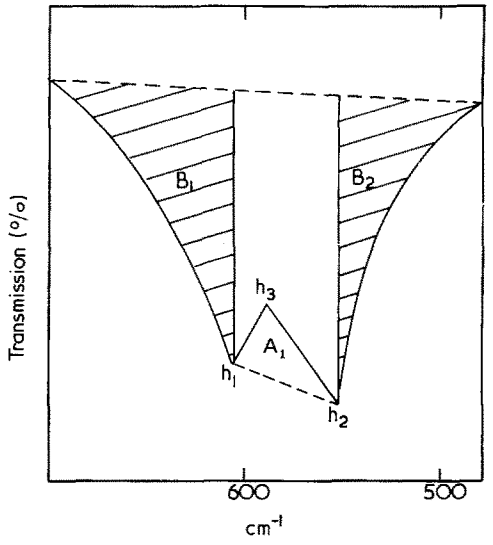

Figure 1 The infra-red method.

at wave numbers between 500 and 600 cm^{-1} for both amorphous calcium phosphate and for crystalline apatite. Amorphous calcium phosphate gives a broad and symmetrical single absorption band in this region, while crystalline apatite partially splits the degeneracy of this absorption band into a well-defined doublet. Mixtures of amorphous calcium phosphate and crystalline apatite exhibit various degrees of resolution of the doublet depending on their weight fraction [4]. The method used to estimate the crystallinity consisted of measuring area A_2 (the whole area under the double peak and the base line) and A_1 (see Fig. 1) and assigning their ratio as the splitting fraction. By assuming that a standard specimen of mineral crystalline fluorapatite is 100% crystalline, one can compare the splitting fraction of that standard with the various bone samples. Termine and Posner adjusted the total absorption, so that transmission was between 90 and 85% at the base line, and only the spectra that gave a minimum transmission of 45 to 50%, as measured at the midpoint of the absorption band (base line to constructed line), were used for splitting fraction analysis.

The instrument used for the present studies was an infra-red spectrophotometer Perkin Elmer 457. Bone samples or mineral fluorapatite were mixed with nujol and the paste was pressed between caesium iodide windows. Once the samples had been run on, the results from the infra-red spectrophotometer were digitalized and normalized on a computer to correspond to an 80% transmission base line and 45 to 50% transmission at mid-point between the two peaks (scaling). The splitting fraction as defined by Termine and Posner did not give any significant results because it was not possible to distinguish between the mineral fluorapatite standard and the bones specimens (certain bones gave a higher A_1/A_2 ratio than apatite).

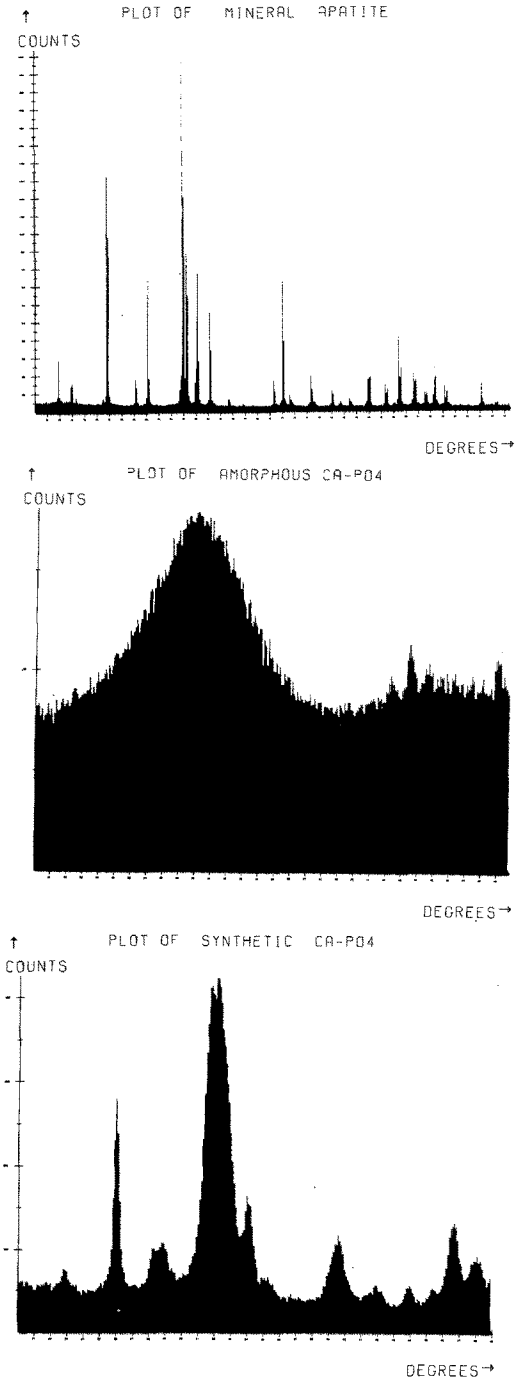

Figure 2 X-ray diffraction pattern (2θ : 20°–50°).

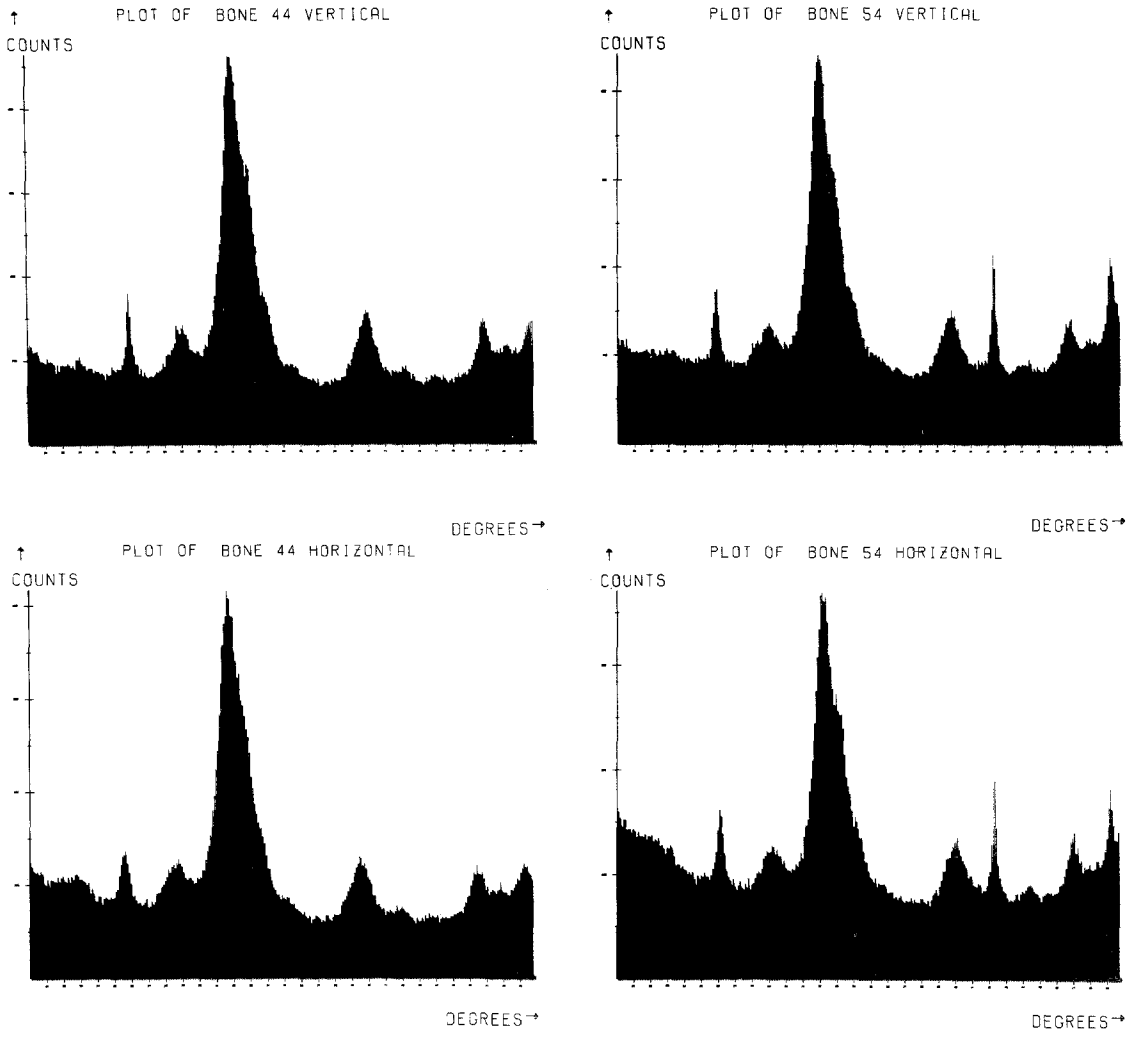

Figure 3 X-ray diffraction pattern (2θ : 20°–50°).

Alternatively we may define the crystallinity as:

$$\%\,\text{crystallinity} = \frac{h_1 - h_3}{h_2 - h_3} \times 100, \qquad (1)$$

where h_1 = height of the first peak, h_2 = height of the second peak, and h_3 = height of the depression (in this ratio apatite gave 100%) (see Fig. 1).

A second method of measuring crystallinity is to compare the ratio of B_1/B_2 in bone to the B_1/B_2 ratio in the fluorapatite standard (with B_1 and B_2 defined in Fig. 1), the latter being taken to represent 100% crystallinity. (see Fig. 1). The reason for choosing half the peaks area (B_1/B_2) is to prevent an uncertain mathematical separation of the peak doublet into two independent peaks [7].

3.2. An X-ray method based on two lines

This procedure, developed by Harper and Posner [2], depends on the direct proportionality existing between the X-ray peak intensity of a crystalline component in a mixture and its weight fraction. Hence, our estimate of the weight fraction of a crystalline phase is obtained from a comparison of the peak intensity of this phase with the corresponding peak intensity from a sample with 100% of the crystalline phase, under identical experimental conditions. Then, we have that

$$I_b/I_c = W_c = 1 - W_{Am} \qquad (2)$$

where I_b is the corrected integrated area of non-overlapping X-ray peaks (002 and 130) of bone and I_c is the integrated area (I.A.) of the same peaks of the standard fluoroapatite. W_c is the crys-

talline fraction and W_{Am} the amorphous one. If bone mineral is considered as a binary mixture of crystalline and amorphous phases, then the ratio I_b/I_c can be represented as follows:

$$I_b/I_c = (W_A\mu_A)/(W_A(\mu_A - \mu_{Am}) + \mu_{Am}) \quad (3)$$

where W_A = weight fraction of crystalline apatite, μ_A = absorption coefficient of apatite and μ_{Am} = mass absorption coefficient of the amorphous calcium phosphate. If we assume that $\mu_A = \mu_{Am}$ then Equation 3 reduces to Equation 2. Harper and Posner applied a correction (μ) to the peak area of the bone:

$$\mu = \frac{W_m(\mu_m - \mu_n) + \mu_n}{W_m\mu_n} \quad (4)$$

where W_m = ash fraction taken as 0.6 [7], μ_n = average mass absorption coefficient of the non-mineral constituents (water, collagen, etc), and μ_m = average mass absorption coefficient of the bone mineral. Then

$$\mu_m = \frac{((Ca/P)_{th}/(Ca/P)_f)10A_{Ca}\mu_{Ca} + 6A_P\mu_P + 26\mu_0A_0}{((Ca/P)_{th}/(Ca/P)_f)10A_{Ca} + 6A_P + 26A_0} \quad (5)$$

where $(Ca/P)_{th}$ = theoretical Ca/P of hydroxyapatite which is 2.1566 ($Ca_{10}(PO_4)_6(OH)_2$), $(Ca/P)_f$ = Ca/P ratio found (between 1.79 and 1.96 from [7], μ = mass absorption coefficient of the atom designated by the subscript, and A = atomic weight of the atom designated by the subscript. The value of μ_n was calcualted from a chemical analysis of the organic matrix of human bone reported by Woodward [8]. Thus $\mu_n = 10.26\,W_{H_2O} + 7.98\,W_c$, where W_{H_2O} was taken as 0.082 and W_c as 0.199 and $\mu_n = 2.429$.

The specimen were examined on an Hilger and Watts X-ray powder diffractometer, operated at 45 kV and 16 mA with Ni filtered Cu$K\alpha$ radiation, with 0.2 mm detector slit and 0.1° divergence slit and in the reflection mode. The integrated areas were taken on the whole peaks 0 0 2 and 1 3 0 in our calculation because there is no theoretical reason to use 0 0 2 and half 1 3 0 peaks, as proposed by Harper and Posner [2]. The 0 0 2 (at $2\sigma = 26°$) and 1 3 0 (at $2\sigma = 40°$) lines were recorded by a step by step counting at 0.04° intervals each count being of 40 sec.

3.3. The integral index

Wakelin *et al.* [9] devised a method based exlcusively on the measurement of X-ray intensity differences in order to determine the crystallinity of various cotton cellulose samples. Their procedure expresses numerically the degree of ordering in a given specimen relative to the minimum and maximum values that are observed in a sampling of various cotton celluloses. Hence this method constitutes a particularly appropriate device for characterizing the degree of ordering with reference to a given crystalline and a given amorphous standard.

As for the X-ray method, the specimens were examined on a Hilger and Watts powder diffractometer at 45 kV and 16 mA with Cu$K\alpha$ (Ni filtered) radiation with 0.2 mm detector slit and 0.1° divergence slit and in the reflection mode. For the integral index, the differential intensity measurements must encompass an angular range that includes most of the crystalline peaks. The range for bone in our study goes from $2\theta = 20°$ up to 50°. The angular increment was taken as 0.1° and 40 s per count which is small enough to sample even the sharp peaks of mineral apatite. This method compares, by ratio, the included area between the bone specimen and the amorphous standard curves with that between the crystalline and the amorphous standard.

The difference described above (B-A) and (C-A) are accordingly summarized without regard for sign to provide an estimate of crystallinity (C_i). The integral index may be represented as follows:

$$C_i = \frac{\sum_{2\theta_0}^{2\theta_m} |B - A|}{\sum_{2\theta_0}^{2\theta_m} |C - A|} \quad (6)$$

where B = intensity of the bone sample at that point, A = intensity of the amorphous sample at that point and C = intensity of the crystalline sample at that point. The sample B was taken at each point as the average between $B\uparrow$ and $B\rightarrow$ (which means that the same sample of bone was run in two mutually perpendicular directions, the first one ↑ with the X-ray parallel to the main axis of the bone and the second one → with the X-ray perpendicular to it, to prevent error due to preferred orientation of the specimen).

The counting rate of the counter was never high enough to necessitate any correction for non-linear response, while the incoherent scattering need not to be taken into account since it is the same for

TABLE I Infra-red data, first method

Sample	Integrated area	Peak 1(h_1) (height)	Peak 2(h_2) (height)	Depression (d)	Crystallinity ratio (%) ($h_1 - d/h_2 - d$)
Apatite	4095	45	45	38	100
Bone 16	2668	26	32	23	33
Bone 23	4216	40	49	35	55
Bone 29	2283	26	31	21	50
Bone 32	3051	33	39	28	45
Bone 44	4636	47	55	40	47
Bone 55	1817	20	24	17	43
Bone 59	2716	29	35	24	45
Bone 70	2175	24	29	20	45
Bone 83	2882	31	36	25	55
Bone 97	5030	55	62	47	53

TABLE II Infra-red data, second method

Sample	Peak 1(B_1) (area/2)	Peak 2(B_2) (area/2)	Ratio (B_1/B_2)	Crystallinity (%)
Apatite	1023	1558	0.6566	100
Bone 16	472	1278	0.3693	56
Bone 32	593	1670	0.3550	54
Bone 44	1064	2996	0.3551	54
Bone 48	800	2515	0.3181	48
Bone 59	528	1369	0.3869	59
Bone 70	369	950	0.3884	59

TABLE III The μ correction

Sample	μ_m	Correction
Bone 16	89.22	1.0231
Bone 23	86.29	1.0184
Bone 29	87.98	1.0180
Bone 32	86.46	1.0192
Bone 44	88.68	1.0187
Bone 54	86.96	1.0186
Bone 62	87.29	1.0192
Bone 74	87.29	1.0187
Bone 83	87.29	1.0206

crystalline and amorphous regions and is then eliminated by computing the differential intensities.

4. Results

In Tables I and II the results of the crystallinity measurements by the first and the second infra-red methods are given. One can see that the spread of data was greater in the first method. In the second method the crystallinity varied from 48 to 59% and there was no noticeable change with age.

Table III shows the results of the μ correction. It can be seen that this correction is, in fact, negligible (within 2%) because it falls well within the limits of experimental error. For this reason the correction has not been applied in the integral index method.

The crystallinity results shown in Table IV include areas under lines 0 0 2 and 1 3 0 of two measurements taken at right angles (↑ and →). The intensities (I.A.) were calculated using a programme (peak) which fits a high power polynomial curve to the peak and substracts the background. The results show a wide spread and are generally lower than those from the integral index method, in which, as shown in Table V, the crystallinity varies between 51 and 57%, with no noticeable change with age. However, bone over fifty years of age does exhibit a new X-ray line at $2\theta = 43°$ attributable to carbonate deposition [7], which can perpahs be explained by a rearrangement in the crystal lattice (see Fig. 3).

5. Discussion

Of the four methods used to determine crystallinity, the integral index is the most reliable because it takes into account the whole angular range of X-ray diffraction pattern. In the case of the X-ray method based on two lines the lower percentage of crystallinity is probably due to the fact that only two lines are taken into account. In

TABLE IV The X-ray method based on two lines

Sample	002	130	I.A.	W_c(%)	W_{Am}(%)	W_c(%) corrected
Apatite	186.	78.	264.			
Bone 16 ↑	28.12	62.73	85.775	32.49	67.51	33.24
Bone 16 →	29.15	57.55				
Bone 23 ↑	37.53	68.88	92.842	35.17	64.83	35.81
Bone 23 →	35.40	43.88				
Bone 29 ↑	24.65	78.71	107.965	40.90	59.10	41.63
Bone 29 →	35.82	76.75				
Bone 32 ↑	35.58	82.97	105.44	39.94	60.06	40.71
Bone 32 →	25.81	66.53				
Bone 44 ↑	33.48	76.92	105.15	39.83	60.17	40.57
Bone 44 →	27.00	72.00				
Bone 54 ↑	31.52	82.92	107.83	40.84	59.16	41.60
Bone 54 →	29.21	72.01				
Bone 62 ↑	54.35	83.24	141.285	53.12	46.48	54.54
Bone 62 →	55.43	89.55				
Bone 74 ↑	30.84	91.01	119.05	45.09	54.91	45.94
Bone 74 →	32.06	84.19				
Bone 83 ↑	32.78	93.59	112.35	42.56	57.44	43.43
Bone 83 →	25.37	72.96				

TABLE V Integral index

Sample	crystallinity	Sample	crystallinity
Bone 16 =	50.97%	Bone 54 =	54.36%
Bone 23 =	52.76%	Bone 62 =	56.56%
Bone 29 =	54.75%	Bone 74 =	55.54%
Bone 32 =	55.00%	Bone 83 =	52.96%
Bone 44 =	54.68%		

the infra-red method there are two ways of measuring crsytallinity, of which the one which takes into account the half area of the peaks is more significant than that which only deals with the ratio of the peak heights, as peak height is very dependent on concentration. The conclusion from these measurements is that crystallinity of the bone is best measured by the integral index method, does not change significantly with age and is estimated to be in the region between 50 and 60%.

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