

Presence of whitlockite in the mineral substance of annealed fish-bones: character and possible significance

Fish
Annealed bones
Phosphates
Calcium
Magnesium
Whitlockite
Poissons
Os Calcinés
Phosphates
Calcium
Magnesium
Whitlockite

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ABSTRACT

Bones from 18 species of fish annealed at a temperature of 800°C, were analyzed and their whitlockite content determined. Both diffractometric and chemical features of this phase are given and discussed. For most of the species examined, a statistical interdependence appears to exist between total Mg and whitlockite content. Certain differences have also been observed between the behaviour of the cranial portion of the skeleton on the one hand, and the remaining bones on the other. The relative abundance of whitlockite in annealed fish bones reflects a corresponding abundance of amorphous Ca-phosphate in the natural bone-tissues. Some biogeochemical implications are briefly outlined.

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RÉSUMÉ

Présence de whitlockite dans les os de poisson calcinés : caractéristiques et signification possible

Les produits obtenus par calcination à 800°C du tissu osseux de 18 espèces de Téléostéens ont été analysés. Les teneurs de whitlockite ont été déterminées; les caractéristiques diffractométriques et chimiques de cette phase sont rapportées et discutées. Pour une bonne part des échantillons examinés, on a trouvé une relation d'interdépendance statistique entre les teneurs totales de Mg et celles de whitlockite. Il y a été possible de mettre en évidence quelques différences de comportement entre les os du crâne et, respectivement, les autres parties du squelette. La relative richesse en whitlockite des os de poissons, calcinés, est à mettre en rapport avec la richesse correspondante en phosphate de Ca amorphe du tissu osseux naturel de ces animaux. Quelques implications biogéochimiques de cette observation ont été brièvement signalées.

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INTRODUCTION

Trace-element distribution in sediments depends to a great extent on the mineralogical and chemical constitution of marine-organism inorganic remains. During an extensive investigation of such materials, we had the occasion to examine the constitution of the skeletal tissues of some twenty species of fish (Classis *Osteichthyes*).

Due to the primarily biogeochemical nature of this research, our study was limited to a chemical and diffractometric examination of bone tissues, after complete removal of any organic matter.

MATERIALS AND METHODS

All the analyzed fishes came from Mediterranean Sea.

Only adult specimens were selected, with dimensions strictly close to the typical size of each species. No malformations, visible traces of any disease or parasitic presence were observed, and gonad inspection indicated that no examined individual was in its spawning-time.

The entire skeleton of examined fishes was treated with 120 volumes analytical grade H_2O_2 to remove organic matter, then quantitatively washed, air dried and finally ground to 250 mesh.

After this treatment, all the specimens gave the well-known, faint and broadened X-ray powder pattern of a very poorly crystalline apatite. After annealing at $800^\circ C$ for 3 hours, sharp diffractograms were observed, with many well-defined hydroxylapatite and $\beta-Ca_3(PO_4)_2$ (whitlockite) peaks. Unit-cell dimensions were thus measured by means of a very high resolution step-scanning technique and least-squares refinements.

Starting from measured hydroxylapatite/whitlockite intensity ratios, the percentages of the two phases were evaluated, together with the Ca/P atomic ratios, by the technique suggested and used by Legros *et al.* (1977) for the analysis of bone mineral components. A calibration curve relating to these determinations is shown in Figure 1.

Final values for each species comprise an average of 5 or 6 individual measurements. The accuracy and precision limits attained are quite satisfactory; for Ca/P ratios, the standard deviations σ range from 0.0037 to 0.0007; while for the whitlockite estimated percentages, the standard deviations of the means σ/\sqrt{n} lie between 1.0 and 0.2. We are thus in full agreement with the assertion by Legros *et al.* (1977), that the reliability of this X-ray technique is much higher in comparison with traditional wet chemical methods, even when macro-samples are available.

Measured unit-cell edges and volumes of the whitlockite obtained by fish-bone annealing were always significantly smaller with respect to the stoichiometric

compound. From these observed unit-cell shrinkages, Mg-content within the whitlockites was evaluated. Comparison was made with pure $\beta-Ca_3(PO_4)_2$ and synthetic solid solutions with regard to the composition $(Ca_{0.905}, Mg_{0.095})_3(PO_4)_2$ which appear to correspond to the solubility limit of Mg in the whitlockite structure (calibration curve in Figure 2). This limit, together with the observed shrinkage rate of the unit-cell due to the substitution of Mg^{2+} for Ca^{2+} , agree with the results obtained by Jensen and Rowles (1957), with those of Fuchs (1969) and with the figures recently quoted by Calvo and Gopal (1975); we may note, however, that Ito (1968) has been able to synthesize whitlockites up to the composition $Ca_7Mg_2(PO_4)_6$.

From the results of this work, the substitution of Mg^{2+} for Ca^{2+} in whitlockite seems to affect not only unit-cell dimensions, but also the parametric ratio c_0/a_0 . In fact the data we obtained point to a clear and positive correlation between MgO estimated content in whitlockite and the c_0/a_0 ratios of its cell. The r value results 0.770, largely significant at < 0.001 probability level. This regularity confirms the reliability of diffractometric MgO determinations.

The total amounts of MgO in calcined fish bones was determined by wet chemical techniques (atomic absorption), together with the total P_2O_5 (spectrophotometric method). No iron or other elements were detected with the exception of Sr, which ranged between 1,180 and 2,330 ppm (X-ray fluorescence method).

Generic and specific nomenclature adopted in this work follows the catalogue of Mediterranean fishes compiled by Tortonese (1963).

RESULTS

Diffractometric determinations results are set out in Table 1. Only *Boops boops* and *Mugil cephalus* gave no whitlockite reflections; we at present have no expla-

Figure 1

Calibration curve for determining Ca/P atomic ratio as function of the measured hydroxylapatite/whitlockite intensity ratio. This curve is referred to 2.81 and 2.86 Å peaks of hydroxylapatite and, respectively, whitlockite.

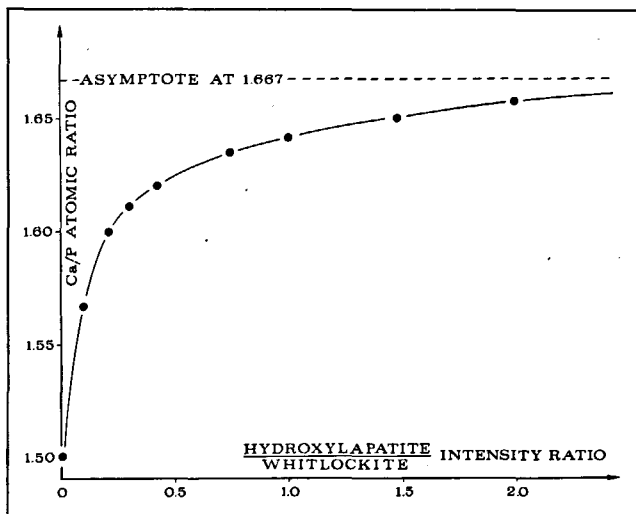


Figure 2

Calibration curve for determining MgO per cent as function of whitlockite unit-cell volume. Equation of the working least-squares straight:

$$(\%)MgO = 133.415715 - 0.037696 V;$$

$$r = -0.9993.$$

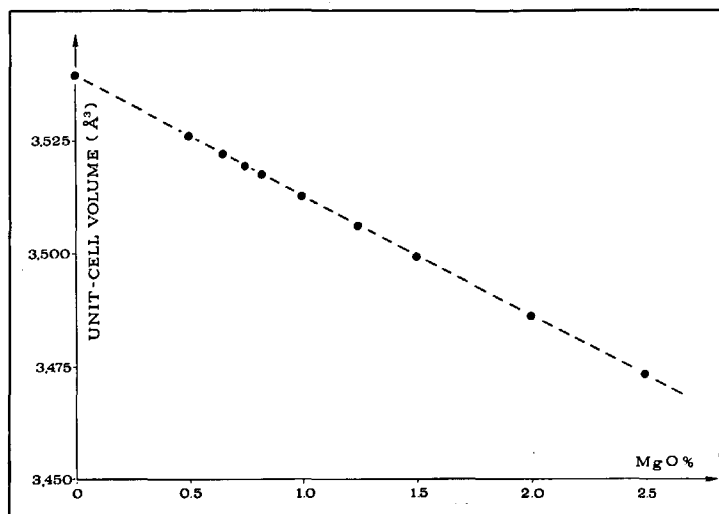


Table 1
X-ray measurements on 800°C annealed fish-bones.

Species	Ca/P	Phases (%)			Whitlockite hexagonal unit-cell dimensions			Estimated Mg-contents in whitlockite		
		H	W		a_0 (Å)	c_0 (Å)	V (Å ³)	(a)	(b)	(c)
1, <i>Lophius budegassa</i> Spin.	1.5852	25.8	74.2		10.451	37.202	3518.9	1.97	0.77	0.57
2, <i>Mullus surmuletus</i> L.	1.6001	33.4	66.6		10.450	37.198	3517.9	2.04	0.80	0.53
3, <i>Trigla lyra</i> L.	1.5894	27.9	72.1		10.449	37.196	3517.0	2.15	0.84	0.61
4, <i>Mullus barbatus</i> L.	1.5983	32.4	67.6		10.448	37.193	3516.1	2.22	0.87	0.59
5, <i>Phycis phycis</i> (L.)	1.5736	20.9	79.1		10.447	37.190	3515.1	2.33	0.91	0.72
6, <i>Uranoscopus scaber</i> L.	1.6123	40.8	59.2		10.444	37.183	3512.4	2.58	1.01	0.60
7, <i>Stromateus fiatola</i> L.	1.6400	63.6	36.4		10.440	37.166	3508.1	3.01	1.18	0.43
8, <i>Pagellus erythrinus</i> (L.)	1.6356	59.3	40.7		10.439	37.162	3507.1	3.09	1.21	0.49
9, <i>Crenilabrus tinca</i> (L.)	1.6253	50.3	49.7		10.437	37.155	3505.1	3.29	1.29	0.64
10, <i>Scorpaena scrofa</i> L.	1.6368	60.4	39.6		10.436	37.154	3504.3	3.37	1.32	0.52
11, <i>Merluccius merluccius</i> L.	1.6066	37.2	62.8		10.434	37.148	3502.4	3.55	1.39	0.87
12, <i>Onos tricirratu</i> s (Brünn)	1.5896	27.9	72.1		10.432	37.140	3500.3	3.75	1.47	1.06
13, <i>Lithognathus mormyrus</i> (L.)	1.6398	63.4	36.6		10.431	37.137	3499.4	3.82	1.50	0.55
14, <i>Serranus cabrilla</i> (L.)	1.5985	32.5	67.5		10.430	37.133	3498.3	3.93	1.54	1.04
15, <i>Trachinus radiatus</i> Cuv.	1.6284	52.8	47.2		10.426	37.116	3494.0	4.36	1.71	0.81
16, <i>Gobius niger</i> jozo L.	1.6282	52.6	47.4		10.424	37.112	3592.3	4.51	1.77	0.84
17, <i>Boops boops</i> (L.)	1.5000	—	—		—	—	—	—	—	—
18, <i>Mugil cephalus</i> L.	1.5000	—	—		—	—	—	—	—	—
U. <i>scaber</i> (cranial bones)	1.6109	39.9	60.1		10.446	37.186	3514.1	2.43	0.95	0.57
U. <i>scaber</i> (non-cranial bones)	1.6237	49.0	51.0		10.423	37.109	3491.4	4.58	1.80	0.92

X-ray estimated MgO-contents in whitlockite:

(a), as atom per cent of cation.

(b), as weight per cent of MgO in whitlockite.

(c), as whitlockitic MgO weight per cent referred to the total sample.

nation for this anomaly. For the remaining species, whitlockite content in calcined bones ranges between 36.4% (*Stromateus fiatola*) and 79.1% (*Phycis phycis*). The unit-cell dimensions (hexagonal coordinates) obtained for all the examined whitlockites are also presented, together with the diffractometrically-determined Mg-contents, given as atom per cent of cation as well as MgO per cent in whitlockite. No lattice constants of hydroxylapatite are set out, since

Table 2
Chemical determinations on 800°C calcined fish-bones

Species	Total Mg-contents		P ₂ O ₅ (%)	Sr (ppm)
	1	2		
1, <i>Lophius budegassa</i> Spin.	1.36	41.9	45.00	1605
2, <i>Mullus surmuletus</i> L.	0.76	69.7	44.52	1820
3, <i>Trigla lyra</i> L.	0.90	67.8	45.57	2085
4, <i>Mullus barbatus</i> L.	0.80	73.8	44.48	1810
5, <i>Phycis phycis</i> (L.)	1.41	51.1	44.88	1770
6, <i>Uranoscopus scaber</i> L.	1.01	59.4	44.53	1185
7, <i>Stromateus fiatola</i> L.	0.75	57.3	43.64	n. d.
8, <i>Pagellus erythrinus</i> (L.)	0.71	69.0	43.52	1560
9, <i>Crenilabrus tinca</i> (L.)	1.05	61.0	44.16	1785
10, <i>Scorpaena scrofa</i> L.	0.93	55.9	43.75	1540
11, <i>Merluccius merluccius</i> L.	1.24	70.2	44.62	1515
12, <i>Onos tricirratu</i> s (Brünn.)	1.77	59.9	45.61	2330
13, <i>Lithognathus mormyrus</i> (L.)	0.91	60.4	43.75	1890
14, <i>Serranus cabrilla</i> (L.)	1.36	76.5	44.41	1535
15, <i>Trachinus radiatus</i> Cuv.	1.08	75.0	44.02	1330
16, <i>Gobius niger</i> jozo L.	1.01	83.2	43.71	2330
17, <i>Boops boops</i> (L.)	1.51	—	42.43	1350
18, <i>Mugil cephalus</i> L.	1.55	—	41.84	1955
U. <i>scaber</i> (cranial bones)	1.02	55.9	44.67	1185
U. <i>scaber</i> (non-cranial bones)	1.24	74.2	43.36	1195

1, Total MgO per cent in calcined fish-bones.

2, Whitlockitic MgO as per cent of total MgO.

n. d., not detected.

none of the specimens gave a significant difference with respect to stoichiometric Ca₁₀(PO₄)₆(OH)₂.

No relationships are observed between the phylogenetic location of different species and the amounts of whitlockite in their calcined bones, or Mg-contents in whitlockites. These contents (Table 1, seventh and eighth columns) show, however, a clearly bimodal distribution. The first group (species 1 to 6 in the Tables) includes species with a well-developed cranial ossification, whose cranial bones exceed by far the remaining parts of the skeleton in weight. In this group, the average MgO-content in whitlockite is 0.87 ± 0.09%.

For the species belonging to the second group, the cranial bones constitute but a comparatively small fraction of the whole skeleton. Here, we observe slightly more scattered MgO-values; their average results 1.44 ± 0.20%.

In order to test the possible interdependence between lower Mg-contents in whitlockite and preponderance of cranial bones in the whole skeletal mass, cranial and non-cranial bones of *Uranoscopus scaber* have been separately analyzed: 0.95 and 1.80% MgO were found respectively.

MgO-contents chemically detected in total calcined bones are listed in the first column of Table 2. These figures show that only a fraction of the Mg present in fish-bone calcination products is contained in the whitlockite structure. This fraction ranges from 41.9 to 83.2% of total MgO amount (second column), and from 0.43 to 1.06 as weight per cent on total calcined bones (last column of Table 1).

Chemically detected P₂O₅ contents are also given in Table 2 (third column). A strong positive correlation obviously exists between chemical P₂O₅ and diffracto-

metric whitlockite percentages; nevertheless we preferred, for the reasons outlined above, not to use these figures for the evaluation of the mineralogical composition. The P_2O_5 -values determined for *B. boops* and *M. cephalus* give results of 42.43 and 41.84% respectively. These figures may be compared with the calculated value for pure hydroxylapatite: 42.39%.

The Sr-contents in total calcined bones (Table 2, last column) appear to be quite independent of any other compositional parameter or the phylogenetic location of the species. It may be interesting to observe that the values determined for different anatomic parts of an identical species (*U. scaber*) or for different species of a same genus (*Mullus*), agree with each other within experimental errors (± 25 ppm).

DISCUSSION

The actual nature of the mineral species whitlockite and the true significance of Mg^{2+} and H^+ cations within its structure have been the subject of recent speculations (Mason, 1971; Gopal, Calvo, 1972; Gopal *et al.*, 1974). More recently, Calvo and Gopal (1975) stated: "With hindsight, it appears that the literature regarding synthetic and biological calcium phosphate species of this kind would have been less confused if the name whitlockite had been restricted to those species containing essential hydrogen".

Whatever may from a strictly taxonomical point of view be the most correct usage of the name "whitlockite", there is no doubt that any experimental ascertainment of the structural role played by hydrogen is extremely difficult in biological whitlockite-type materials. We thus prefer an extensive usage of this name, as is customary in osteological literature.

In comparison with hydroxylapatite, whitlockite appears to be a quite uncommon constituent of animal calcified tissues. So far, it has been encountered in pathological calcifications, especially those found in human teeth and in the adjoining salivary ducts and glands (Jensen, Danø, 1952; Jensen, Rowles, 1957; Vahl *et al.*, 1964; Gatter, McCarty, 1967), but also in some urinary calculi (Lonsdale, Sutor, 1972).

On the other hand, the presence of whitlockite in annealed bone mineral substance is well established. Very abundant literature exists on this subject, from the classic review by Carlström (1955) the recent work of Legros *et al.*, (1977). It is now generally admitted that the initial material, which on annealing recrystallizes to whitlockite, is an amorphous $Ca_3(PO_4)_2$, coexistent in the untreated bone tissues with a very scarcely crystalline hydroxylapatite.

The role of Mg in stabilizing whitlockite with respect to hydroxylapatite has, however, been emphasized by a number of authors including for example Trautz *et al.* (1954) and Gatter and McCarty (1967), as regards biogenic whitlockites. Moreover, Jensen and Rowles (1957) report a Mg-content ranging from 6 to 8 atom per cent of cation for whitlockites from pathological concrements.

It may be of some interest to verify whether any relationship exists between the whitlockite we observed in annealed fish bones and the Mg-contents in these materials.

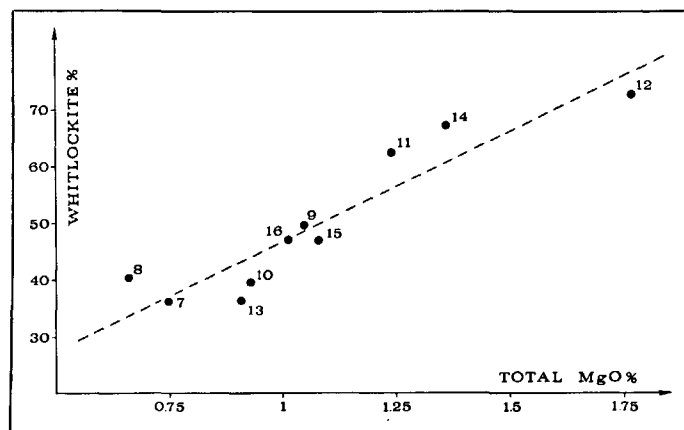
As we have pointed out above, Mg-contents are significantly lower in fishes which possess strongly developed cranial bones. The difference between the mean MgO-values of each group, together with the corresponding standard deviations, lead to a very high student's t value ($t = 6.29$). No doubt, from this statistical point of view, we may treat these two groups as two clearly distinct populations. They also differ, at a fairly good probability level, on the basis of whitlockite-contents (Student's $t = 3.41$; P , between 0.01 and 0.001).

The first population (species from *Lophius budegassa* to *U. scaber* in the Tables; MgO-contents between 0.75 and 1.01%; mean whitlockite-content 69.8%) shows no interdependence between whitlockite percentages and Mg-contents, both in whitlockite and in total calcined bone.

In the second population (species from *S. fiatola* to *Gobius niger jazo*; MgO-contents between 1.18 and 1.78%; mean whitlockite-content 50.0%) there remains an absence of interdependence between whitlockite per cent and MgO per cent within the whitlockitic phase. On the other hand, very strong linear positive correlation appears between whitlockite per cent and total MgO per cent within total calcined fish bone (Fig. 3). The correlation coefficient $r = 0.928$ is significant at $\ll 0.001$ probability level. A clear correlation of the same type obviously exists also between whitlockite per cent and whitlockitic MgO percentages within total calcined bones (figures in the last column of Table 1).

As the distinction between these populations is based on the cranial bones/total skeleton weight ratio, we may assume that the outlined differences between two groups of species reflect the differences between two somewhat dissimilar kinds of skeletal tissues. It appears indeed likely that certain bio- and physicochemical phe-

Figure 3
Linear correlation between whitlockite per cent and total MgO per cent in the annealed bones of examined fishes with weak cranial ossification. Equation of interpolated least-squares straight: $(\%)W = 8.1906 + 38.6766 (\%MgO)$; $r = 0.9284$.
Numbers of the species as in Tables 1 and 2.



nomena may occur in a somewhat different manner in the cranial portion and in the other parts of the fish skeleton. With particular regard to the whitlockite contents after annealing and to the Ca/P ratios, a different behaviour has been recently pointed out for different kinds of bone tissue and accordingly to the bone formation process (Legros *et al.*, 1977).

For the cranial bones, we merely observe an overall trend to lower Mg-contents and, at once, to give by annealing higher whitlockite amounts. For non-cranial skeleton, on the contrary, a positive statistical interdependence clearly appears between total Mg within bone mineral and whitlockite amounts built up by annealing. Such interdependence is obviously to be extended to the amorphous $\text{Ca}_3(\text{PO}_4)_2$ if we assume that a whitlockitic crystalline phase can grow essentially at expense of this latter, as seems well established at the present stage of our knowledge.

Whitlockite amounts within annealed fish bones appear rather irregular, but are often much higher in comparison with the mammals (Termine, Posner, 1967). In the above outlined perspective, amorphous $\text{Ca}_3(\text{PO}_4)_2$ must be more abundant, as a rule, in fishes than in mammalian bones. This observation agrees with the results obtained by E. Lopez *et al.* (1970) for immature *Anguilla anguilla* in normal physiological conditions: amorphous Ca-phosphate = 72.2%, measured in conformity with the method of Harper and Posner (1966).

Like all non-crystalline mineral substances, this amorphous $\text{Ca}_3(\text{PO}_4)_2$ appears rather unstable and easily mobilizable even within living animals. In the case of *A. anguilla*, this has been demonstrated by means of hormonally-induced demineralization experiments (Lopez, 1970; Lopez *et al.*, 1970). While hydroxylapatite contents remained unaffected, a strong decrement of amorphous Ca-phosphate was then observed, probably due to a phenomenon of halastasic decalcification.

CONCLUSIONS

The presence in fish bones of relevant amounts of easily mobilizable Ca-phosphate may be of interest from the biogeochemical point of view. In fact, during the early fossilization stages, a significant fraction of the fish bones embedded within a sediment will be rapidly dissolved. By no means insignificant amounts of P, and eventually of a number of minor elements, will be dispersed in this manner into the whole sediment. Similar phenomena due to a natural early-diagenetic dissolution of metastable mineral phases of biogenic nature are of recognized importance with regard, for example, to aragonitic shells enclosed in calcarenitic (though not in silty or clayey) sediments.

A different problem concerns the phenomena which may determine the eventual interdependence between Mg-contents and whitlockite production by annealing.

Setting aside the puzzling question posed by the anomalous behaviour of *B. boops* and *M. cephalus*, no simple solutions arise from our results. Nevertheless, certain observed statistical regularities would appear to suggest that this argument merits further consideration.

Acknowledgments

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