X-RAY DIFFRACTION STUDIES ON THE ULTRASTRUCTURE OF BONE

by

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Previous studies on the ultrastructure of bone using polarised light[1], X-ray diffraction[2,3,4,5] and electron microscopy[6,7,8,9] have demonstrated a high degree of order in the arrangement of structural components in this system, and indicated a close relationship between the organisation of the apatite particles and the collagen fibres.

Most of the X-ray diffraction work has been devoted to a study of the crystal structure of the apatite component, but complete agreement has not yet been reached[10,11,12]. In the wide-angle diffraction pattern of intact bone, many of the reflections are poorly defined, because of the small crystallite size, and a reliable indexing of the reflections and determination of the unit cell dimensions has been possible only with heated bone samples in which the crystallite size has been greatly increased. Even here the treatment has been indirect, the diffraction rings from powdered bone specimens being indexed in relation to the pattern of fluorapatite[2].

In bones in which the collagen fibres are well aligned, the wide-angle X-ray reflections show good orientation, the *c* axis of the hexagonal unit cell being directed along the long axis of the bone and parallel to the collagen fibre axis.

From an initial electron microscope study[6] of fragmented bone, and more recently of thin sections of bone, ROBINSON AND WATSON[7] have suggested that the apatite is present in the form of tabular crystals, the most probable "mature" crystal length being 350 to 400 A, the width of the same order as the length, and the thickness between 25 A and 50 A. The spacing of these crystals appeared to be associated with the periodic banding along the collagen fibres.

From an earlier consideration of the low-angle diffuse scatter[4] of X-rays from compact bone it was possible to suggest precise axial dimensions for the crystalline particles, and these were found to be elongated in the direction of the *c* crystallographic axis, the long axis being orientated parallel to the collagen fibre axis. In deducing the dimensions of the particles, a number of assumptions had to be made concerning their shape and distribution, and the inter-particle interference. These assumptions seemed to be justified by the observed form of the low-angle scatter, and by the fact that precise dimensions could be readily deduced by applying the theory of independent particle scatter in a well-ordered system[15]. Further consideration and discussion with other workers using the low-angle diffuse scatter methods have strengthened the justification for applying the simple theoretical treatment to this system, and it has also been possible

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make a more detailed estimation of the probable limits of error*. The particle dimensions given in previous publications[3,4] were calculated using the relationship $R = 0.83 S\sqrt{a}$, where R was the radius along a principal axis of the particle, S the sample to film distance, and a the slope of the log I/r^2 relationship along the corresponding axis of X-ray scatter. The factor 0.83 was calculated assuming a particle shape approximating to an ellipsoid of revolution as was suggested by the shape of the particle scatter from longitudinal bone sections. The shape could in the extreme case correspond to a uniform cylinder, and this, from considerations of the conditions of crystallite growth, might appear to be the more likely shape. For such a uniform cylinder, the constant in the relationship given above would be reduced to 0.74 for the diameter, but the length would not differ appreciably from the case of the ellipsoid of revolution. Thus, the diameter might be as high as 75 A as given in previous publications, or as low as 50 A should the particle shape be that of a uniform cylinder. The length of the particle should be very close to the value of 210 A given previously.

The suggestion of a fundamental tabular-shaped crystallite[6,7] is not in keeping with the form of the low-angle scatter of X-rays. A random or radial orientation of such particles in the plane perpendicular to the long axis would be expected to destroy the linearity of the log $I - r^2$ curve, whilst an ordered arrangement should be reflected in the shape of the particle scatter from cross-sections of bone. The X-ray diffraction data can be most readily interpreted in terms of a particle of symmetrical cross-section.

The fact that the low-angle scatter was diffuse and that no definite diffraction maxima were observed was assumed to be due to a slight irregularity in the spacing of particles so that well-defined diffracting planes for X-rays were not produced. Recently, a study has been made of the diffraction patterns of fish bones, and here a much more precise arrangement of structural components is indicated (Figs. 1 and 2). Definite diffraction peaks have been observed in the low-angle pattern which permit a more direct estimation of particle size. The wide-angle pattern, too, particularly in the case of heated specimens, showed improved definition so that a direct indexing of the wide-angle reflections could be made, and accurate unit cell dimensions deduced. These improved patterns, together with a study of the effects of heating, are reported in this paper.

MATERIALS AND METHODS

Longitudinal and cross-sections (0.2 mm thick) of ribs from perch and from pike, and of membrane bones from pike were obtained by grinding. Intact specimens were examined, and also specimens from which the collagen had been removed by refluxing with ethylene diamine in a Soxhlet distillation apparatus for 24 hours. The complete removal of the collagen was confirmed by nitrogen determination, and by the fact that no carbonisation occurred on heating.

In addition, a study was made of the effects of heating on the form of the low-angle scatter and diffraction. In some experiments, the specimens were heated for 1 hour in a furnace at a controlled temperature between 200° C and 600° C, and the diffraction then examined at room temperature, whilst in others, the bone specimens were mounted in a platinum heating foil on the diffraction camera, and serial exposures made during heating. Both intact bone and ethylene diamine extracted bone were used in these experiments.

The low-angle scatter was recorded photographically at about 100 mm sample to film distance. Details of experimental equipment and procedure are as described in a previous publication[4]. Wide-angle patterns were recorded by microcamera, by cylindrical powder camera (diameter 57.4 mm), and by high precision, 190 mm diameter powder camera (Hilger).

* We acknowledge in particular the detailed discussion of the problem with Dr. G. POROD, Institute of Theoretical and Physical Chemistry, University Graz, Austria.

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RESULTS

The phase contrast photograph (Fig. 1) shows the parallel alignment of the collagen fibres in decalcified fish bone. Fig. 2 shows the similar alignment of structural components in a section of intact bone. This precise alignment seen in the optical microscope is also reflected at the molecular level as indicated in the wide-angle diffraction pattern of intact fish bone (Fig. 7) which shows a well orientated apatite pattern and also an equatorially orientated 11.5 A collagen reflection.

The low-angle scatter of X-rays from the normal bone sections showed marked asymmetry, indicating an elongation of the particles in the direction of the long axis of the bone. The asymmetry was most marked in the case of the membrane bone of the pike (Fig. 3), and with this specimen well-defined diffraction bands were seen in the direction perpendicular to the elongation of the particle scatter. The most intense band gave a spacing of about 220 A, and a much fainter band was observed at about 130 A. A photometer trace along the long axis of the particle scatter (corresponding to short particle dimension) showed a pronounced shoulder (Fig. 13). If this shoulder should reflect a definite Bragg diffraction at this point then the spacing would be approximately 68 A.

In the low-angle pattern of the specimen from which the collagen had been removed by extraction with ethylene diamine (Fig. 9) this shoulder in the scattering curve was no longer observed. The intensity of scatter along the major axis showed a regular decrease with increase in scattering angle which gave a relationship between log I and r^2 which was approximately linear. From the slope (α) of this line a particle diameter of about 65 A was calculated assuming the particle to approximate to an ellipsoid of revolution[4]. The well-defined diffractions in the direction perpendicular to the long axis of scatter persisted even after the complete extraction of the collagen. Exposures at longer sample to film distances showed intense and well-defined reflections at 650 A and 218 A, but the 130 A and longer spacings were weak and difficult to record.

Heating the sections of intact membrane bone of pike to 200° C for 1 h produced a blackening of the specimen and an appreciable intensification of the low-angle scatter (Fig. 4). This appeared to be shortened along the long axis and extended along the short axis but not so much as to obscure the diffraction band at 220 A. The decrease in intensity of scatter along the long axis was regular, and a particle diameter of approximately 60 A was deduced. With increase in temperature, the intensity and spread of scatter in the short dimension was increased (Fig. 5) and the relationship between log I and r^2 along the axis showed increased deviations from linearity. Calculations of approximate particle dimensions showed a tendency for the apparent diameter to increase. When the spread of scatter in the other direction made possible a direct evaluation along the short axis, the initial values for 2 R were of the order of 100 A. Above about 350° C, when the colour of the specimen showed an obvious removal of carbon, the fall off in intensity with scattering angle became more and more steep, and the spread of the scatter restricted (Fig. 6) until it was no longer resolved at a sample to film distance of 100 mm. The same sequence of changes was observed both in the experiments in which the specimen was heated in a furnace and then cooled before recording the diffraction pattern, and those in which the specimen was maintained at high temperature during exposure.

When the ethylene diamine extracted pike bone was heated above 400° C, striking

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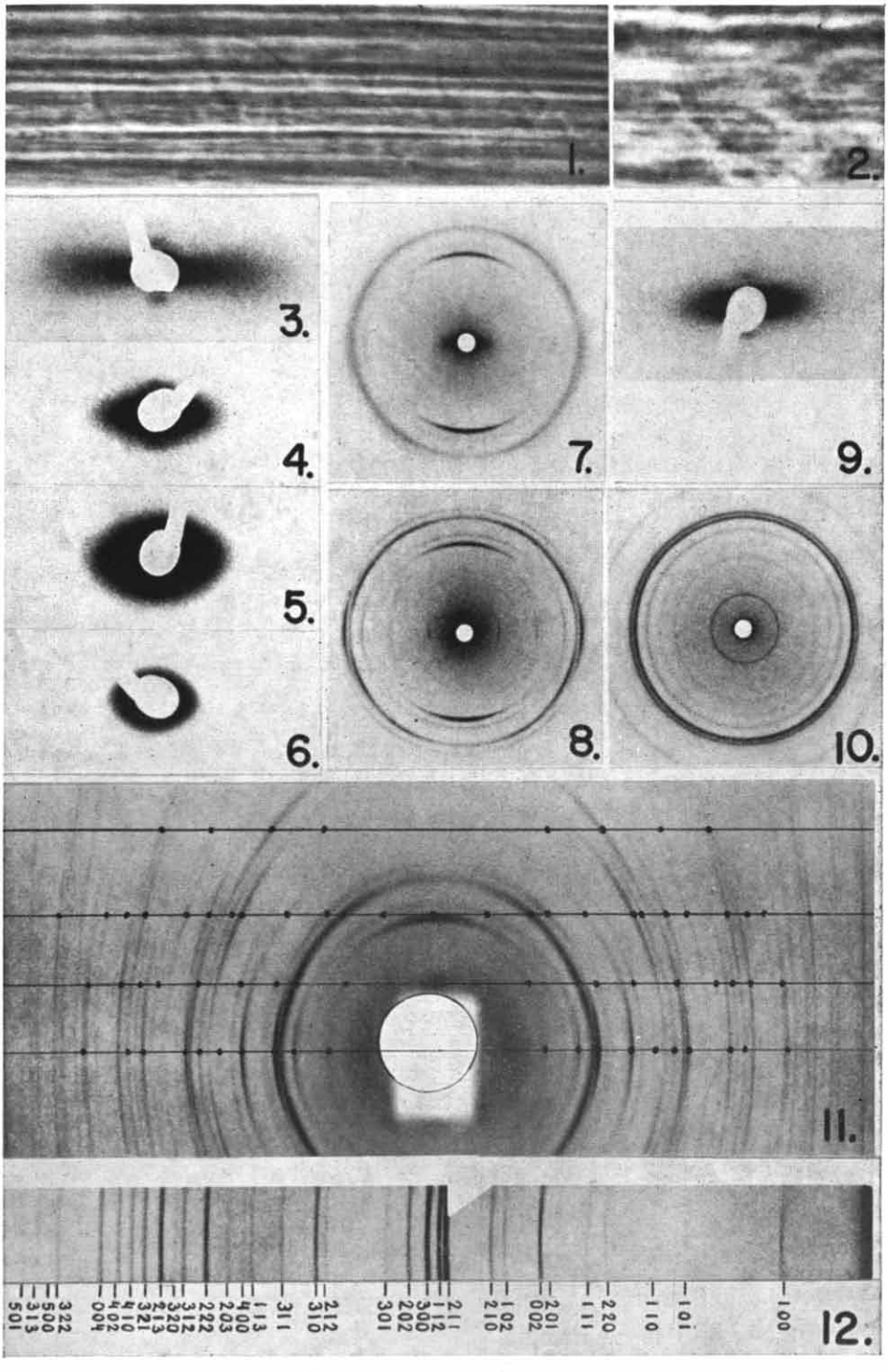
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changes were observed in the relative intensities of the meridional low-angle reflections. The intensity of the second order diffraction increased until, after heating to 500° C for one hour, it became more intense than the third order. Eventually, both second and third orders faded, and above 600° C only the first order diffraction at 650 A remained. At higher temperatures, this spacing apparently increased, but the changes could not be followed closely because of the difficulty of resolving this spacing.

The wide-angle patterns did not show any appreciable changes until the carbon was removed from the specimens at about 400° C, but above this temperature, when the specimen became white and brittle, the definition of the diffraction rings was markedly improved (Figs. 8 and 10) and an accurate indexing of the reflections became possible. To facilitate indexing, rotation photographs (Fig. 11) of bone which had been heated to 600° C for 1 h were obtained in a cylindrical camera (57.4 mm diameter). These showed the layer lines, and in the illustration (Fig. 11) the centres of the reflections are marked with dots. The indexing of some of the lines in the powder diagram obtained with the Hilger camera is shown in Fig. 12. The hexagonal unit cell was found to have axes measuring $a = 9.41$ A and $c = 6.87$ A.

DISCUSSION

The wide-angle diffraction pattern of fish bone shows the same set of reflections as has been seen in other types of bone and related to the apatite component, but with better orientation than that seen in previous patterns, thus indicating a more precise alignment of crystallites. The improvement may be associated with the fact that the fish bone resembles in many ways a single Haversian system, and in fact an improved orientation in the pattern of human bone has been obtained from an isolated single Haversian system[14]. In addition, the phase contrast photographs show a better alignment of collagen fibres in fish bone as compared with the mammalian bones studied previously. This too contributes to the better alignment of the apatite particles.

As in previous studies, the asymmetry of the particle scatter is such as to suggest an elongation of the apatite crystallites in the *c* direction, and parallel to the fibre axis of the collagen. In the case of the perch ribs and those of pike bone refluxed with ethylene diamine or heated to 200° C for short periods, the scatter in the direction

Fig. 1. Phase contrast photograph of decalcified perch rib. × 400.
Fig. 2. Phase contrast photograph of intact pike rib. × 400.
Fig. 3. Low-angle diffraction pattern of longitudinal section of pike membrane bone, showing meridional reflection at 218 A, and diffuse equatorial reflection superimposed on particle scatter at 68 A.
Fig. 4. As in 3, but specimen heated to 200° C for 1 hour.
Fig. 5. As in 3, but specimen heated to 300° C for 1 hour.
Fig. 6. As in 3, but specimen heated to 400° C for 1 hour.
Fig. 7. Wide-angle diffraction pattern of longitudinal section of intact perch rib, (fibre axis vertical) showing meridional orientation of 002 reflection of apatite and equatorial collagen (11.5 A) reflection.
Fig. 8. As in 7, but specimen heated to 700° C for 1 hour.
Fig. 9. Low-angle diffraction pattern of longitudinal section of pike membrane bone after removal of collagen with ethylene diamine. Shows meridional reflection at 210 A, but diffuse equatorial reflection is no longer observed.
Fig. 10. Wide-angle diffraction pattern of cross-section of perch rib heated to 700° C for 1 hour. Note absence of orientation and marked changes in intensities of reflections as compared with Fig. 8.
Fig. 11. Wide-angle rotation photograph of longitudinal section of perch rib after heating (700° C for 1 hour). Layer lines are marked, and centres of reflections indicated by dots.
Fig. 12. Rotation powder diagram of heated perch bone showing indexing of the reflections.

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corresponding to the particle diameters can be treated according to the procedure outlined previously[4] for systems of ellipsoidal shaped particles, and values between 60 and 66 A are obtained for these diameters ($2R$). These values are only slightly lower than those obtained previously in the study of human bone, and are significantly close to the dimension obtained by treating the shoulder in the scatter along the long axis from pike membrane bone as indicating the presence of a Bragg reflection superimposed on the diffuse particle scatter. Fig. 13 shows how two such scattering curves could be added to produce the observed effect. The appearance of this reflection suggests that the particles are packed in a uniform array laterally, and gives strong support to the method employed for deducing the particle dimensions from the continuous scatter. The fact that the shoulder in the scattering curve for the long axis of scatter of normal bone disappears when the collagen is either extracted or disorganised by heating, might at first suggest that it is associated with the collagen rather than with the apatite. However, collagen itself does not give such a reflection in this region, and as the arrangement of apatite crystallites is probably closely associated with that of the collagen fibres, it would not be surprising if their organisation were disturbed by the removal of collagen. On the other hand, the diffraction bands at 650 A and 220 A in the direction

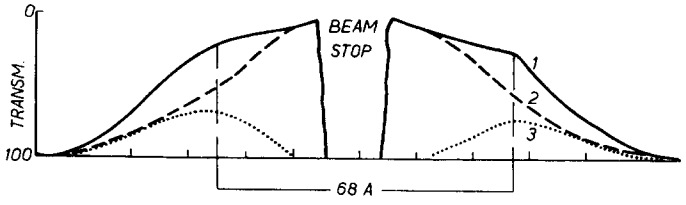

Fig. 13. Diagram showing photometer trace along long axis of scatter from longitudinal section of pike membrane bone before (1) and after (2) extraction of collagen with ethylene diamine. Curve (3) is obtained by subtracting curve 2 from curve 1.

of the long axis of the apatite particle remained well-defined and were in fact more intense after the removal or disorganisation of the collagen, thus suggesting a precise and stable spacing of the apatite particles in the direction parallel to the collagen fibre axis. The fundamental 650 A repeating unit in this direction is indistinguishable from that of the collagen period, but the intensity of the diffraction and the pattern of relative intensities is markedly different, leaving no doubt as to the dependance of the low-angle reflections on the organisation of the apatite component. The outstanding intensity of the 218 A reflection in the apatite may indicate a special significance for this spacing, and it can in fact be readily related to the dimension of 210 A deduced for the basic particle length from an earlier study of the continuous low-angle scatter. Evidence for a specific reinforcement of this third order diffraction is also obtained from the heating of decollagenised bone, where, under conditions producing growth or fusion of the crystallites, the emphasis can be moved from the third order diffraction to the second order, and eventually concentrated on the fundamental spacing alone. A ready explanation of all diffraction effects can be obtained by assuming particles of 210 A to 220 A in length, aligned in approximately parallel array but forming well-defined crystallographic planes only at intervals (about 650 A) corresponding to the lengths of three such basic units. There can be little doubt of the close relationship between the form of crystallization of the apatite component and the structure of collagen, and that the

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coincidence of the fundamental repeating unit in the apatite structure with the collagen period is a direct result of this relationship. The fact that the length of the basic apatite particle corresponds to only one third of the collagen period would seem to emphasise the importance of a division into three very similar sub-units of the fundamental repeating unit of collagen itself. The third order reflection is of outstanding intensity in the collagen diffraction pattern, and electron micrographs often show a banding within the collagen period, the number of inter-period bands varying from three to six with different preparations. In certain cases, notably with reprecipitated collagen[16] and in tissue cultures[17], the fundamental period observed in the electron micrographs has been 220 A instead of the usual 640 A. Thus, there would appear to be definite grounds for suggesting a close relationship between the centres of crystallization for the apatite and the chemical structure of the collagen, and these findings concerning the length of the apatite particles may reflect the spacing of important chemical groups along the collagen chains.

If one considers the development of bone, one can readily picture the crystallization of the mineral component as being initiated at certain regularly repeating points along the surface of the bundles of collagen, and the crystallites growing to a length corresponding approximately to the distance between centres of crystallization. In such a case, the repetition of units in the direction of the collagen fibre would be expected to be more precise than in the direction at right angles to it, and this in fact appears to be the case in the bone specimens studied, the long axes of the particles being capable of forming regular diffraction planes so as to produce well-defined X-ray reflections, whilst the diameters normally show sufficient disorganisation to give rise to a continuous scatter.

The changes with heating, of the intensities of the low-angle reflections of the ethylene diamine extracted bone, besides emphasising the initial specific reinforcement of the third order diffraction, also indicated that the growth or fusion of the crystallites in the direction of the long axis is somewhat slower than in the short direction, and that the changes are at first confined within the fundamental unit. In other words, an initial growth or fusion of the sub-units can take place without affecting the fundamental repeating unit.

The arrangement of apatite particles along the collagen fibre is almost certainly continuous, and a basic particle length of about 210 to 220 A would appear to be much more satisfactory than the value of 350 to 400 A suggested by ROBINSON AND WATSON[7] from E.M. studies. It is noted that in the latter work, the particle length most frequently found was about 180 A, but these were assumed to be fragments of the larger particles. Our results suggest that the larger plaques which are seen so frequently in electron micrographs may be formed by the association of a number of the more basic particles and reflect a higher stage of the ultrastructural organisation.

The changes in low-angle scatter observed when the bone sections are heated seem to follow a fairly regular sequence for which a qualitative explanation can readily be found. The initial spread of the scatter is probably associated with the carbonisation of the collagen, the formation of carbon particles within the specimen producing a second scattering phase, possibly of non-uniform particle size, which increases the spread and intensity of scatter. At this stage, the scatter is composite and would not be expected to respond to any simple theoretical treatment. When all the carbon has been removed, the system is presumably again of apatite alone, and the contraction and increase in

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steepness of the scattering curve seem to indicate a fusion to give larger and larger crystallites. That the phenomenon is one of fusion and not recrystallization is indicated by the fact that the same sequence of changes is observed during continuous heating as with discontinuous heating, and that the orientated wide-angle pattern persists throughout.

The improvement in definition of the wide-angle pattern brings up the layer lines quite clearly, and an unequivocal indexing of the reflections for θ up to about 45° is possible. This, together with the careful measurement of the positions of the powder rings in the high precision camera leads to accurate dimensions for the principal axis of the unit cell. The indexing is identical with that employed by STÜHLER[2] for values of θ between 10° and 30°. POSNER AND STEPHENSON[13] have employed a slightly different indexing for their synthetic hydroxyapatites.

From the crystal structure it is clear that the substance examined is an apatite, and in fact, the strong reflections seen in the normal bone pattern can be identified precisely from the patterns of the heated bone. However, this does not exclude the possibility that the fusion of the crystallites on heating may be associated with a slight chemical change in the structure of the apatite.

ACKNOWLEDGEMENTS

We are grateful to Professor ARNE ENGSTRÖM for his constant interest in this work and for much helpful discussion, and to the Rockefeller Foundation for financial assistance.

SUMMARY

In the low angle diffraction pattern of certain types of fish bone, definite BRAGG diffractions have been observed which tend to confirm the particle dimensions deduced from the continuous low angle scatter given by less well-ordered specimens. The particles are probably rod-shaped, the principal axial dimensions being about 65 A and 220 A. The long axis is aligned parallel to the fibre axis of the collagen, and the coincidence of the long axial dimension with the spacing corresponding to the intense third order diffraction of collagen is thought to emphasise the close relationship between the form of crystallization of the apatite and the chemical structure of collagen.

Heating bone sections to a temperature of 500 to 600° C seems to cause a fusion of the crystallites, and this produces a marked improvement in the definition of the wide angle pattern. In this pattern the layer lines were brought out very clearly, and a direct indexing of the reflections was carried out and precise unit cell dimensions deduced (a = 9.41 A, c = 6.87 A).

RÉSUMÉ

Dans les images de diffraction sous angle faible de certains types d'os de poissons, les auteurs ont observé des diffractions de BRAGG définies qui confirment les dimensions de particules calculées à partir de la dispersion sous angle faible donnée par des échantillons moins bien orientés. Les particules sont probablement en forme de bâtonnets, les dimensions axiales principales étant d'environ 65 A et 220 A. Le grand axe est orienté parallèlement à l'axe de la fibre de collagène, et la coïncidence entre la dimension du grand axe et l'espacement qui correspond à la diffraction intense de troisième ordre du collagène semble confirmer qu'une relation étroite existe entre la forme de cristallisation de l'apatite et la structure chimique du collagène.

Le chauffage de sections d'os à des températures de 500° à 600° C provoque la fusion des cristallites, et entraîne une amélioration nette de la définition des images sous grand angle. Dans ces images, les couches apparaissent très distinctement et un repérage direct des réflexions a été effectué ainsi qu'un calcul précis des dimensions des unités cellulaires (a = 9.41 A, c = 6.87 A).

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ZUSAMMENFASSUNG

Bei den Kleinwinkelstreuungsdiagrammen gewisser Typen Fischknochen wurden bestimmte BRAGG-Streuungen beobachtet, die die von kontinuierlicher Kleinwinkelstreuung, wie sie von weniger gut geordneten Arten gegeben wird, abgeleiteten Teilchengrössen zu bestätigen scheinen. Die Teilchen sind wahrscheinlich stäbchenförmig, die Grössenordnung der Hauptachsen liegt bei ungefähr 65 A und 220 A. Die lange Achse ist parallel zur Faserachse des Kollagens angeordnet und die nahe Verwandtschaft zwischen der Kristallisationsform des Apatits und der chemischen Struktur des Kollagens wird durch das Zusammenfallen der Dimension der Längsachse mit dem der intensiven Streuung 3.Ordnung des Kollagens entsprechenden Netzebenen abständen betont.

Ein Erhitzen der Knochenschnitte auf Temperaturen von 500–600° C scheint eine Fusion der Kristallite zu verursachen und dies ergibt eine beträchtliche Verbesserung bei der Definition der Weitwinkeldiagramme. Bei diesen Diagrammen kommen die Schichtlinien sehr klar heraus. Es wurde eine direkte Registrierung der Reflektion durchgeführt und die genauen Dimensionen der Einheitszelle abgeleitet (a = 9.41 A, c = 6.87 A).

REFERENCES

[1] W. J. SCHMIDT, *Naturwissenschaften*, 24 (1936) 361.
[2] R. STÜHLER, *Fortschr. Gebiete Röntgenstrahlen*, 57 (1938) 231.
[3] A. ENGSTRÖM AND J. B. FINEAN, *Nature*, 171 (1953) 564.
[4] J. B. FINEAN AND A. ENGSTRÖM, *Biochim. Biophys. Acta*, 11 (1953) 178.
[5] J. B. FINEAN AND A. ENGSTRÖM, *Experientia*, (In press).
[6] R. A. ROBINSON, *J. Bone and Joint Surg.*, 34-A (1952) 389.
[7] R. A. ROBINSON AND M. L. WATSON, *Anat. Record*, 114 (1952) 383.
[8] CH. ROUILLIER, L. HUBER, E. KELLENBERGER AND E. RUTISHAUSER, *Acta Anat.*, 14 (1952) 9.
[9] W. SCHWARZ AND G. PAHLKE, *Z. Zellforsch, u. mikroskop. Anat.*, 38 (1953) 475.
[10] M. J. DALLEMAGNE, *Metabolic Interrelations*, Trans of the Fourth Conference of the Macy Foundation. 1952. p. 154.
[11] D. MCCONNELL, *ibid.*, p. 169.
[12] S. B. HENDRICKS, *ibid.*, p. 185.
[13] A. S POSNER AND S. R. STEPHENSON, *Nat. Bureau of Standards Report* No. 1455.
[14] Unpublished data.
[15] A. GUINIER, *X-ray Crystallographic Technology*, English translation by T. L. TIPPELL. Edited by K. LONSDALE, F.R.S. Hilger and Watts Ltd. London, 1952.
[16] J. GROSS, *Metabolic Interrelations*, Trans of the Fourth Conference of the Macy Foundation. 1952. p. 32.
[17] K. R. PORTER AND P. VANAMEE, *Proc. Soc. Exptl. Biol. Med.*, 71 (1949) 513.

Received August 29th, 1953