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REFERENCES

1 F. G. E. PAUTARD, *Biochim. Biophys. Acta*, 28 (1958) 514.
2 E. FAURÉ-FREMIET, *J. Protozool.*, 4(2) (1957) 96.
3 A. BISHOP, *Quart. J. Microscop. Sci.*, 71 (1927) 147.
4 J. T. RANDALL, *Nature*, 178 (1956) 9.
5 F. C. MCLEAN, *Science*, 127 (1958) 451.
6 J. VON KÓSSA, *Beitr. pathol. Anat. u. allgem. Pathol.*, 29 (1901) 163.
7 F. FEIGL, *Z. anal. Chem.*, 61 (1922) 454; 74 (1928) 386; 77 (1929) 299.
8 A. MILLARD AND F. G. E. PAUTARD, *4. Internationale Kongress für Elektronmikroskopie*, Berlin (1958), in the press.
9 A. MUGGLETON AND J. F. DANIELLI, *Nature*, 181 (1958) 1738.
10 D. CARLSTRÖM, A. ENGSTRÖM AND J. B. FINEAN, *Symposia Soc. Exptl. Biol.*, 9 (1955) 85.
11 H. FÉRNANDEZ-MORÁN AND A. ENGSTRÖM, *Biochim. Biophys. Acta*, 23 (1957) 260.
12 R. S. LULL, *Organic Evolution*, Macmillan & Co., New York (1929), p. 427.

THE SIZE AND SHAPE OF THE APATITE CRYSTALLITES IN BONE AS DETERMINED FROM LINE-BROADENING MEASUREMENTS ON ORIENTED SPECIMENS

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SUMMARY

Line-broadening measurements on X-ray diffraction patterns from oriented specimens of bone with a well-ordered internal structure have shown that the line-broadening effects observed are caused both by a limitation in size of the apatite crystallites and by lattice distortions. The results indicate that the apatite crystallites in bone form long rods which are probably deformed by mechanical forces. The rods have an average diameter of 40–45 Å and their average length is in the order 600–700 Å. These findings are regarded as being much more reliable than those previously deduced from X-ray data.

INTRODUCTION

Several trials have been made earlier in order to deduce the crystallite size of the bone salt from the broadening of the Debye–Scherrer lines in X-ray diffraction patterns of powdered specimens. A first rough estimation by DE JONG[1] in 1926 showed that the crystallites were very small, probably containing not more than a few hundred apatite molecules. BALE, HODGE AND WARREN[2] found in dentin an average size of 240 Å,

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and for bone STÜHLER[3] gave the size limits from 31 Å to 290 Å. More refined measurements on the only resolvable diffraction line, the (00·2) reflection, in powder patterns of bone or dentin have given values pointing towards a crystallite size slightly over 200 Å (Table I).

TABLE I

Crystallite size from the (00·2) reflection (Å)	*Specimen*	*Reference*
about 200	Human dentin	TOVBORG-JENSEN AND MÖLLER[4]
290	Human dentin	TRAUTZ *et al.*[5]
230	Human adult bone	CARLSTRÖM[6]
158–217	Human fetal bone 15–40 weeks	WALLGREN[7]
215	Human adult bone	HOLMSTRAND[8]
215	Rabbit bone	HOLMSTRAND[8]

From powder X-ray diffraction patterns of bone it has been found that the apatite crystallites must have an elongated shape. The (00·*l*) reflections are namely much sharper than the (*hk*·0) reflections. The latter have in fact such a low peak intensity and are so broadened that they cannot be resolved from adjacent lines. For these reasons measurements on their broadening have never been carried out, and earlier values of the crystallite size refer only to the long dimension of the apatite particles. For the calculations from line-broadening data one presumption, however, had always to be made, namely that the observed effects were entirely due to the limitation in size of perfect crystallites and that no imperfections or strain contributed to the observed line breadth. In order to investigate this point and at the same time obtain a value for the short dimension of the apatite crystallites, line-broadening measurements were carried out on bone specimens having a high degree of orientation of the apatite crystallites. Diffraction patterns recorded from such specimens sectioned in two mutually perpendicular directions should give diagrams consisting of almost pure (*hk*·0) or (00·*l*) reflections with a considerable gain in peak intensity and resolution. Greater accuracy in the measurements of the line-broadening could thus be achieved and it should also be possible to record reflections at greater angles.

MATERIAL AND METHODS

In an earlier investigation[9] it was found that some long fish bones showed a remarkably high degree of orientation of the apatite crystallites, so that their *c* axes were essentially directed parallel to the long dimension of the bones. In the present investigation fresh ribs of bream (*Abramis brama* L.) were used. After the bones had been cleaned and dehydrated, close-packed bundles of the parallel-stacked straight parts of these long bones were embedded in methyl methacrylate. Cross sections and longitudinal sections (about 1 mm thick) were sawed from the embedded bundles and the sections were subsequently ground perfectly flat, and trimmed so that they fitted into the specimen holder of the X-ray diffractometer. Dried bream ribs were also ground to a fine powder for the recording of an unoriented diffraction pattern. Pure silicon powder and a synthetic fluorapatite having a crystallite size averaging 1 μ served as

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reference substances in order to furnish the geometrical breadth. The diffraction patterns and line profiles were recorded with a Philips X-ray diffractometer (PW 1010 – PW 1050 – PW 1051) and Ni-filtered Cu-radiation was used. For a faithful recording of the diffraction peaks a fixed number of counts were recorded at close angular intervals, and the counting rate was kept low enough to obtain a good correction for coincidence loss in the Geiger counter, the dead time of which was found to be 150 μsec. Corresponding diffraction lines of the bone specimens and the fluorapatite were recorded under identical conditions, and in some cases suitable Cu Kβ reflections of silicon were also used.

RESULTS

Parts of the diffraction patterns of the bone specimens are seen in Figs. 1–3. The characteristic pattern of unoriented bone (Fig. 1) clearly shows that the only line suitable for line-broadening measurements, the (00·2) reflection, is of rather low intensity and that apart from the high peak at 32° 2θ, largely composed of the (12·1), (11·2), (30·0) and (20·2) reflections, all other reflections are smeared out. The longitudinal bone sections, *i.e.* the specimens where the apatite crystallites were mainly oriented with their c axes parallel to the specimen holder, gave diffraction patterns having quite a different appearance (Fig. 2). Here the greatly broadened (hk·0) reflections show up quite clearly, but the strongest (hk·1) reflections, namely (12·1) and (23·1), show up to a lesser degree. All other reflections, including (00·2), have disappeared completely. The broadening of the recorded reflections is, however, so great that the only one which is not severely influenced by the superposition of adjacent lines is the (13·0) reflection at 40° 2θ. There is in fact one more "free" (hk·0) reflection in the pattern, the (10·0), (not shown in the figure) but its low intensity and angular position (10.8° 2θ) prevent its use for accurate line-broadening measurements. In Fig. 3 is shown a diffraction pattern of a bone specimen oriented in such a way that the apatite crystallites are oriented mainly with their c axes perpendicular to the surface of the specimen holder. Here, the (00·l) reflections, (00·2) and (00·4), are of outstanding intensity and sharpness. All other reflections are depressed and the only ones which are visible have low hk indices such as (10·2), (11·2), (11·3) and to a lesser degree (20·2) and (12·3). Because of the excellent orientation even the (00·8) reflection at 128° 2θ could be recorded.

The line profiles of the (13·0), (00·2), (00·4) and (00·8) reflections corrected for the slope of the background and for the non-linearity of response of the GM-tube are shown in Figs. 4–7. Some reflections of low intensity interfering with (00·2), (00·4) and (13·0) had to be subtracted, but in the case of (00·8) no such correction was carried out since the contribution of the only line which could possibly interfere, the (34·6)-(23·7)-doublet, was estimated to be of minor importance. The curves were subsequently corrected for the CuK$\alpha_1\alpha_2$-separation according to the method described by DU MOND AND KIRKPATRICK[10]. After having been corrected for the α_1-α_2-separation the widths of the reference peaks of fluorapatite agreed within the limits of experimental error ($\pm$ 0.005° 2θ) with the widths of the silicon CuKβ-reflections at comparable angles. Using the half-maximum breadths, which could be more accurately measured than the integral breadths, the true line breadths ($\beta_{1/2}$) were determined using the correction curve for instrumental broadening given by KLUG AND ALEXANDER[11]. The result of

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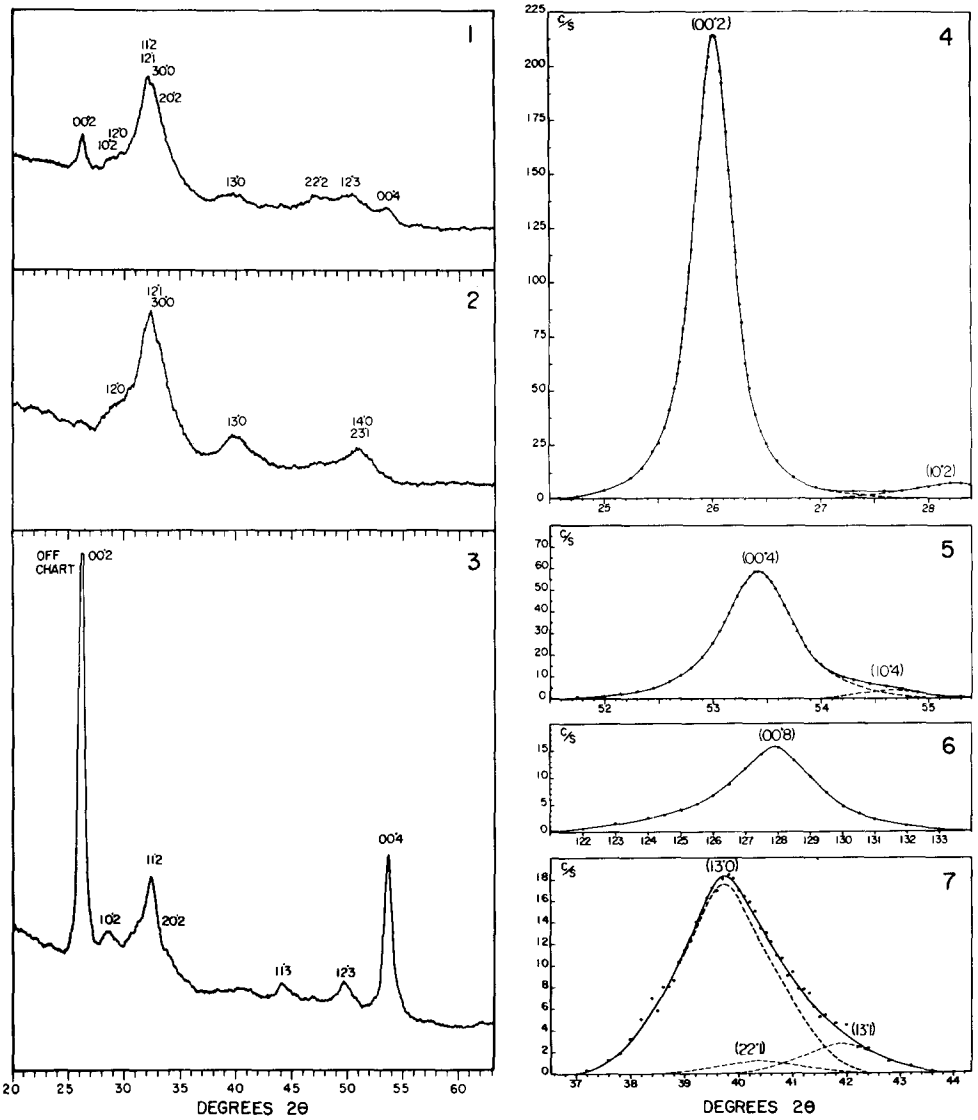

Figs. 1–3. Parts of X-ray diffraction patterns of fish bone. Fig. 1. Unoriented, powdered specimen. Fig. 2. Longitudinally-sectioned specimen. Fig. 3. Cross-sectioned specimen. Only the strongest reflections in each diagram are indexed. Note the enhancement of the (*hk*·0) and (00·*l*) reflections in Fig. 2 and Fig. 3, respectively, as compared with the pattern of the unoriented specimen (Fig. 1). Figs. 4–7. The line profiles of the (00·2), (00·4), (00·8) and (13·0) reflections corrected for the coincidence loss in the Geiger tube and for the slope of background. The subtracted background amounted to 41, 22, 107 and 51 counts/sec for the respective curves. The intensities of the reflections are not directly comparable since the line profiles were not recorded under the same instrumental conditions. Each dot represents 12,800, 25,600, 102,400 and 25,600 counts for the curves 4–7, respectively.

these calculations are given in Table II. Using the half-maximum line breadth the constant (K) in the well-known Scherrer formula $D_{(hkl)} = (K \cdot \lambda \cdot 57.3)/(\beta_{1/2} \cdot \cos\theta)$ was assigned the value 0.9. $D_{(hkl)}$ is here defined as the effective thickness of the crystallite

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TABLE II

(hkl)	Observed breadth		Breadth corrected for α_1-α_2-separation		Pure half-maximum breadth, $\beta_{1/2}$	$D_{(hkl)}$ (Å)
	Bone	Fluorapatite	Bone	Fluorapatite or silicon		
(13·0)	2.03	0.168	2.02	0.097	1.99 ± 0.10	42.5 ± 2
(00·2)	0.43	0.122	0.42	0.077	0.38 ± 0.02	215 ± 10
(00·4)	0.76	0.201	0.73	0.095	0.69 ± 0.02	129 ± 5
(00·8)	3.0	—	2.8	0.21	2.6 ± 0.15	66 ± 5

in a direction perpendicular to the reflecting planes, λ is the wavelength of the radiation and θ the Bragg reflection angle. The factor 57.3 converts $\beta_{1/2}$ to radian measure. The crystallite size $D_{(hkl)}$ for the measured reflections is given in Table II. The values given represent average dimensions and are mutually comparable, but their absolute magnitude might be wrong by about 10 %, depending upon the uncertainty of K in the Scherrer equation.

DISCUSSION AND CONCLUSIONS

The average length of the apatite crystallites in fish bone, as deduced from the broadening of the (00·2) reflection, is in perfect agreement with earlier values found from bone specimens of other origin (Table I). This finding, and the fact that powder patterns of all kinds of bone or dentin are visually indistinguishable, makes it quite certain that the results deduced from fish bone have a general validity. Investigations of the line-broadening from mammal bone and dentin are already in progress, and the results will be described elsewhere.

The most striking finding from the results in Table II is the obvious discrepancy between the length of the bone crystallites found from the (00·2) reflection and the length deduced from the broadening of the (00·4) and (00·8) reflections. Therefore it is clear that the small crystallite size alone cannot cause the observed broadening. If the line-broadening were due only to a limitation in the number of reflecting planes, the experimental values of $\beta_{1/2}$ would coincide with the function $k_1/\cos\theta$. On the other hand, if the line-broadening depended entirely upon strain or crystal imperfection, the observed values would satisfy the function $k_2 \cdot \tan\theta$. When choosing the values 0.37 and 1.65 for the constants k_1 and k_2, respectively, the curves intersected at the value of $\beta_{1/2}$ for the (00·2) reflection. It is seen in Fig. 8 that the experimental values of $\beta_{1/2}$ for (00·4) and (00·8) do not fall on either of these curves. A curve with the equation $k/\cos\theta + k' \cdot \tan\theta$ could, however, be fitted to these experimental values when k and k' were given the values 0.1235 and 1.0972, respectively. This means that the observed broadening is due partly to a reduction of the crystallite size and partly to slight variations in the length of the c axis of the unit cell, depending upon lattice distortions. From the value of k, the true crystal length in the c axis direction of the apatite crystallites was found to be 640 Å, and from k' the variation in length in the c axis was estimated to be within the limits of 6.81 Å to 6.95 Å.

With regard to the short dimension of the bone crystallites (42.5 Å) deduced from the broadening of the (13·0) reflection, no precise comparison could be made with other (hk·0) reflections. A rough estimation, however, of the broadening of the faint

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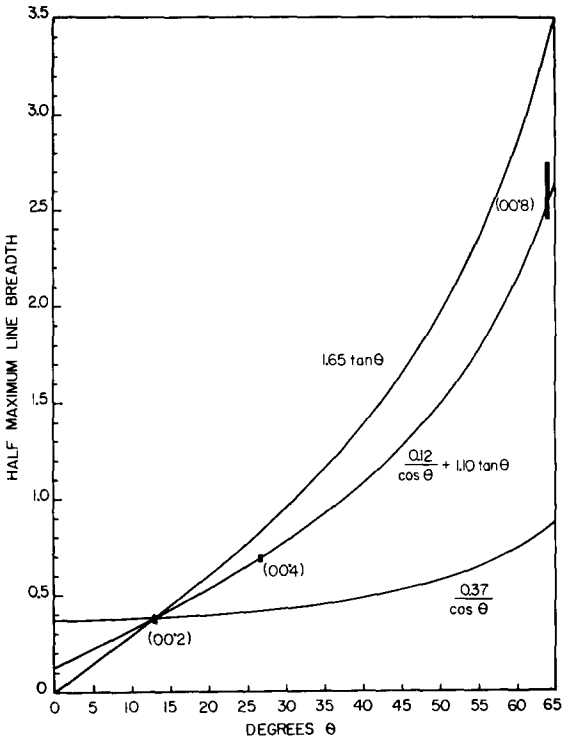

Fig. 8. Diagram showing the pure line breadth ($\beta_{1/2}$) of the (00·l) reflections plotted against the Bragg reflection angle (θ). The experimental values (black rectangles) fit the equation $k/\cos\theta + k' \cdot \tan\theta$ when $k = 1.1235$ and $k' = 1.0972$. This shows that the line-broadening is due partly to a limitation of the crystallite size and partly to lattice distortions.

(10·0) line and the unresolved (23·1), (41·0), (41·1) line indicated that the broadening of (13·0) depends almost entirely upon a limitation of the crystallite size in the direction of the a axes. Furthermore it can be stated that the cross-section of the crystallites must be roughly circular, because of the shape of the (13·0) reflection, and because all (hk·0) reflections showed the same magnitude of broadening. It is evident that the exact configuration of the cross section of the crystallites cannot be determined, but it seems likely that it should have the shape, more or less, of a regular hexagon.

Summarizing the results, it can be concluded that the apatite crystallites in fish bone form thin rods having an average length of 600–700 Å and an average diameter of 40–45 Å. The small variations observed in the length of the c axis can be due to crystal imperfections, and/or mechanical strain, but from the shape of the crystallites it seems most probable that it is the strain which is the more important. Such long and thin rods can easily be deformed, and even a slight bending should give the observed effects. It might be mentioned that TRAUTZ *et al.*[5] found a considerable discrepancy between the length of the apatite crystallites in tooth enamel as calculated from the (00·2) and (00·4) reflections. The values obtained were 870 Å and 480 Å, respectively. From the findings in the present investigation it is likewise very probable that the crystallites in tooth enamel are considerably longer than was previously assumed from line-broadening data. From an electron-microscopic investigation of tooth enamel it

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has in fact been found[12] that the apatite crystallites probably have lengths in the order of 50,000 Å to 100,000 Å.

The present picture of the apatite crystallites in bone differs considerably from those of earlier models. Line-broadening measurements have indeed indicated the elongated shape of the crystallites, but their lengths have invariably been found to be around 220 Å and their widths have never been measured. From investigations of the intensity distribution in the low-angle particle scatter of bone specimens with a high degree of crystallite orientation, the bone crystallites were also found to be rod-shaped, with a diameter of 60–75 Å[6,13,14]. This result is in fair agreement with the present finding, but the less accurate determination of the length of the crystallites gave a value of 210–220 Å. Studies with the electron microscope on bone tissue have given rather divergent pictures of the bone crystallites. Thus ROBINSON[15,16] and ROBINSON AND WATSON[17,18] found plate-like crystallites with a length and width of 350–400 Å and a thickness of 25–50 Å. Oval-shaped crystallites with dimensions ranging from 150 Å to 1300 Å have also been described[19]. From electron-microscopic studies of ultra-thin sections of compact bone, it has recently been postulated[20,21] that the apatite crystallites are rod-shaped and have a diameter of about 40 Å and a length of approximately 200 Å. A quite different concept of the ultrastructural organization of the inorganic fraction of bone has been put forward by CAGLIOTI, ASCENZI AND SANTORO[22]. Using replica techniques they concluded that the bone salt formed a homogeneous body with holes having a maximum diameter of 200 Å. This view is quite unacceptable, as is shown from the results obtained from electron microscopy of thin sections of compact bone as well as from X-ray data.

The nature of the possibly close relationship between the bone crystallites and the organic matrix is still unsolved. From X-ray diffraction investigations it has long been known that the apatite crystallites are always oriented so that their long axes are parallel to the direction of the collagen fibres. JACKSON AND RANDALL[23] found in an electron-microscopic investigation of embryonic bone that the first-formed apatite crystallites are attached to the collagen, and that they are localized at specific bands of the collagen fibres. *In vitro* experiments indicate the same relationship[24]. Hence it is very probable that certain groups on the polypeptide chain of collagen act as crystallization centers, in such a manner that the crystallites are spaced 640 Å apart along the fibres. It is tempting to associate the found average length of the apatite crystallites (640 Å!) with the repeating period of collagen, but as this figure is certain only to about $\pm$ 100 Å, it might be a pure coincidence. It must be kept in mind that only a fraction of the bone crystallites can be in direct contact with the collagen fibres and the orientation of the long apatite crystallites might equally well be explained by mechanical as well as by stereo-chemical concepts.

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References p. 53.

REFERENCES

1 W. F. DE JONG, *Rec. trav. chim.*, 45 (1926) 445.
2 W. F. BALE, H. C. HODGE AND S. L. WARREN, *Am. J. Roentgenol. Radium Therapy*, 32 (1934) 369.
3 R. STÜHLER, *Fortschr. Gebiete Röntgenstrahlen*, 57 (1938) 231.
4 A. TOVBORG-JENSEN AND A. MÖLLER, *J. Dental Research*, 27 (1948) 524.
5 O. R. TRAUTZ, E. KLEIN, E. FESSENDEN AND H. K. ADDELSTON, *J. Dental Research*, 32 (1953) 420.
6 D. CARLSTRÖM, *Acta Radiol. Suppl.*, 121 (1955).
7 G. WALLGREN, *Acta Paediat., Suppl.*, 113 (1957).
8 K. HOLMSTRAND, *Acta Orthopaed. Scand., Suppl.*, 26 (1957).
9 D. CARLSTRÖM AND J. B. FINEAN, *Biochim. Biophys. Acta*, 13 (1954) 183.
10 J. W. M. DU MOND AND H. A. KIRKPATRICK, *Phys. Rev.*, 37 (1931) 136.
11 H. P. KLUG AND L. E. ALEXANDER, *X-ray Diffraction Procedures*, John Wiley and Sons, Inc., New York, 1954, p. 509.
12 M. L. WATSON AND J. K. AVERY, *Am. J. Anat.*, 95 (1954) 109.
13 A. ENGSTRÖM AND J. B. FINEAN, *Nature*, 171 (1953) 564.
14 J. B. FINEAN AND A. ENGSTRÖM, *Biochim. Biophys. Acta*, 11 (1953) 178.
15 R. A. ROBINSON, *Trans. Macy Conf. on Metabol. Interrelations*, 3 (1951) 271.
16 R. A. ROBINSON, *J. Bone and Joint Surgery*, 34 A (1952) 389.
17 R. A. ROBINSON AND M. L. WATSON, *Anat. Record*, 114 (1952) 383.
18 R. A. ROBINSON AND M. L. WATSON, *Trans. Macy Conf. on Metabol. Interrelations*, 5 (1953) 72.
19 W. SCHWARZ AND G. PAHLKE, *Z. Zellforsch.*, 38 (1953) 475.
20 H. FERNÁNDEZ-MORÁN AND A. ENGSTRÖM, *Nature*, 178 (1956) 494.
21 H. FERNÁNDEZ-MORÁN AND A. ENGSTRÖM, *Biochim. Biophys. Acta*, 23 (1957) 260.
22 V. CAGLIOTI, A. ASCENZI AND A. SANTORO, *Biochim. Biophys. Acta*, 21 (1956) 425.
23 S. F. JACKSON AND J. T. RANDALL, in *Bone Structure and Metabolism*, Ciba Foundation Symposium, London, 1956, edited by G. E. W. WOLSTENHOLME AND C. M. O'CONNOR.
24 M. J. GLIMCHER, A. J. HODGE AND F. O. SCHMITT, *Proc. Natl. Acad. Sci. U.S.*, 43 (1957) 860.

STUDIES ON PHOTOSYNTHETIC PHOSPHORYLATION

III. RELATION BETWEEN PHOTOSYNTHETIC PHOSPHORYLATION AND REDUCTION OF TRIPHOSPHOPYRIDINE NUCLEOTIDE BY CHLOROPLASTS

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SUMMARY

The photochemical reduction of TPN by isolated chloroplasts was investigated. A comparison of the rate of TPN reduction with that of photosynthetic phosphorylation provided evidence that the generation of ATP in the presence of vitamin K_3 or FMN is not coupled with the reoxidation of TPNH by the oxidized product of the photolysis of water. Photosynthetic phosphorylation could proceed unimpaired under conditions in which the chloroplasts had lost their ability to reduce TPN. On the other hand, TPN reduction could be considerably stimulated by a chloroplast extract which did not affect photosynthetic phosphorylation. These results are discussed in relation to the recent finding that the reduction of TPN by chloroplasts is accompanied by ATP formation.

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