the carbinol dehydrated easier, and the bath was maintained at a lower temperature (185°). From 212 g. of *o*-fluorophenylmethylcarbinol and 2 g. each of potassium bisulfate and hydroquinone, there was obtained 109 g. (76%) of *o*-fluorostyrene which boiled at 32–34° (3 mm.); d^{20}_4 1.030; $n^{20}D$ 1.5197. Forty-eight grams of the carbinol was recovered.

Anal. Calcd. for C_8H_7F: C, 78.68; H, 5.74. Found: C, 78.68; H, 6.02.

m-Fluorophenylmethylcarbinol.—From 99 g. of the *m*-fluorobenzaldehyde there was obtained 83 g. (74%) of *m*-fluorophenylmethylcarbinol boiling at 120–121° (45 mm.); d^{20}_4 1.1201; $n^{20}D$ 1.5012.

Anal. Calcd. for C_8H_7OF: C, 68.57; H, 6.43. Found: C, 68.96; H, 6.66.

m-Fluorostyrene.—The preparation of this compound was identical with the procedure followed for the *ortho*-derivative. From 220 g. of the carbinol there was obtained 144 g. (80%) of *m*-fluorostyrene which boiled at 30–31° (4 mm.); d^{20}_4 1.025; $n^{20}D$ 1.5173. Twelve grams of the carbinol was recovered.

Anal. Calcd. for C_8H_7F: C, 78.68; H, 5.74 Found: C, 78.73; H, 6.03.

p-Fluorophenylmethylcarbinol.—From 99 g. of *p*-fluorobenzaldehyde there was obtained 94 g. (84%) of *p*-fluorophenylmethylcarbinol which boiled at 122–123° (45 mm.); d^{20}_4 1.122; $n^{20}D$ 1.5001.

Anal. Calcd. for C_8H_9OF: C, 68.57; H, 6.43. Found: C, 68.20; H, 6.52.

p-Fluorostyrene.—In a manner similar to that used in preparing the other fluoro isomers, there was obtained from 260 g. of the carbinol 172 g. (81%) of *p*-fluorostyrene, b. p. 29–30° (4 mm.); d^{20}_4 1.024; $n^{20}D$ 1.5158. Seventeen grams of the carbinol was recovered.

Anal. Calcd. for C_8H_7F: C, 78.68; H, 5.74. Found: C, 78.21; H, 5.89.

3,4-Dichlorophenylmethylcarbinol.—3,4-Dichloroacetophenone, prepared according to Roberts and Turner,[9] was reduced by the method of Marvel and Schertz.[7] From 110 g. of 3,4-dichloroacetophenone there was obtained 98 g. (91%) of 3,4-dichlorophenylmethylcarbinol, b. p. 127–128° (2 mm.); d^{20}_4 1.318; $n^{20}D$ 1.5628.

(9) Roberts and Turner, *J. Chem. Soc.*, 1855 (1927).

Anal. Calcd. for $C_8H_8OCl_2$: C, 50.26; H, 4.19. Found: C, 50.19; H, 4.24.

3,4-Dichlorostyrene.—A mixture of 150 g. of 3,4-dichlorophenylmethylcarbinol, 1.5 g. of hydroquinone, and 1.5 g. of potassium bisulfate was placed in a 500-cc. modified Claisen flask and immersed in an oil-bath held at 210–215°. The pressure, initially at 110 mm., was gradually reduced to 15–20 mm. where the styrene–water mixture distilled at 130–150°. After working up the product in the usual manner there was obtained 90 g. (83%) of 3,4-dichlorostyrene boiling at 69–70° (4 mm.); d^{20}_4 1.256; $n^{20}D$ 1.5857. Thirty-one grams of carbinol was recovered.

Anal. Calcd. for $C_8H_6Cl_2$: C, 55.48; H, 3.47. Found: C, 55.72; H, 3.71.

2,5-Dichlorophenylmethylcarbinol.—2-Chloro-5-nitrobenzaldehyde was prepared according to the method of Erdmann.[10] The nitro group was reduced and replaced by chlorine exactly as described for the preparation of *m*-chlorobenzaldehyde in "Organic Syntheses."[11] From 110 g. of 2,5-dichlorobenzaldehyde there was obtained 110 g. (83%) of 2,5-dichlorophenylmethylcarbinol, b. p. 107–109° (2 mm.); m. p. 63–64°.

Anal. Calcd. for $C_8H_8OCl_2$: C, 50.26; H, 4.19. Found: C, 50.20; H, 4.09.

2,5-Dichlorostyrene.—This compound was prepared exactly according to the directions given for 3,4-dichlorostyrene. From 170 g. of 2,5-dichlorophenylmethylcarbinol and 1.7 g. each of hydroquinone and potassium bisulfate, there was obtained 44 g. (37%) of 2,5-dichlorostyrene. Thirty-nine grams of the carbinol was recovered. The product boiled at 72–73° (2 mm.); d^{20}_4 1.4045; $n^{20}D$ 1.5798.

Anal. Calcd. for $C_8H_6Cl_2$: C, 55.49; H, 3.47. Found: C, 55.55; H, 3.51.

Summary

The preparation of *o*-, *m*- and *p*-fluorostyrene, *o*-, *m*- and *p*-chlorostyrene, 3,4-dichlorostyrene and 2,5-dichlorostyrene has been described.

(10) H. Erdmann, *Ann.*, **272**, 153 (1893).

(11) "Organic Syntheses," John Wiley and Sons, Inc., New York, N. Y., 1943, Coll. Vol. II, p. 130.

NORTH ADAMS, MASS. RECEIVED APRIL 21, 1944

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY AND BIOLOGICAL ENGINEERING, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

X-Ray Diffraction Studies on Protein Fibers. I. The Large Fiber-Axis Period of Collagen

BY RICHARD S. BEAR

Most of the detailed deductions regarding protein fiber structure have been derived from wide-angle X-ray diffraction patterns, which yield information concerning the common inter-atomic distances of small magnitude. It has been known for some time that these fibers also exhibit small-angle patterns, resulting from very large structural periodicities. This paper reports in detail the evidence regarding the long spacings of collagenous tissues, briefly described in a previous Communication.[1]

Clark and Schaad,[2] on the basis of observation of diffractions corresponding to spacings of 202, 107, 72.6 and 57 Å., plus unspecified higher orders, concluded that the collagen large fiber-axis period was probably 216, 432 Å., or other multiple, favoring the second figure. Corey and Wyckoff[3] considered that their measurements of spacings at 103, 70.1, 54.6, 33.6, 26.9, 24.1 and 21.6 Å. indicated a period of 330 Å. The value 216 is one-third and 330 nearly half that now believed to be correct, but these early experiments lacked both the angular resolution and accuracy necessary to determine the true period.

Subsequent to the first report of the 640 Å. collagen period[1] it has been supported from two

(1) R. S. Bear, THIS JOURNAL, **64**, 727 (1942).

(2) G. L. Clark and J. A. Schaad, *Radiology*, **27**, 339 (1936).

(3) R. B. Corey and R. W. G. Wyckoff, *J. Biol. Chem.*, **114**, 407 (1936).

directions. Electron micrographs[4] have shown that a period of this average size can be seen in a variety of collagenous fibrils. Kratky and Sekora,[5] apparently unaware of the prior work of this Laboratory, have confirmed the correct value by means of X-ray diffraction.

Astbury has from time to time attempted to deduce the true collagen period by a combination of speculative arguments involving the prominent meridional short-spacing arc at 2.86 Å. and interpretation of amino acid analyses of gelatin after the manner of the Bergmann–Niemann periodicity theory. Originally he concluded that the probable value was about 838 Å.,[6] or nearly twice the figure favored by Clark and Schaad. Aside from the difficulties facing Astbury's point of view as discussed below, it is apparent from the facility with which he adjusted his calculations to accept the 640 Å. period[7] that at the present time his theories do not offer deductions which are prescribed with sufficient precision to be of value.

While correct evaluation of the fiber-axis period of collagen is important, one also desires knowledge of the variations in the period induced by chemical or physical manipulation, since information of this sort can be of aid in interpreting the large spacing. Hitherto unpublished observations regarding phenomena of this sort are described in the present paper.

Experimental Methods

From the diffraction standpoint the procedures are chiefly extensions of the usual methods for photographing fiber patterns of organic substances with Cu Kα radiation. Fine pinholes or slits, long collimating tubes, and large specimen-to-film distances are employed. These in turn entail long exposure times so that available radiation should be used efficiently. In the following paragraphs are discussed some of the more important experimental considerations, with a brief description of the equipment used to date.

Radiation Characteristics and Specimen Thickness.—The X-ray tubes used were of the General Electric Ca-6, Cu-target type, which has sealed-in, tungsten-filament construction and Be windows. For monochromatization Ni foil (0.015 mm. thickness) was employed in order to maintain a moderate level of Cu Kα intensity. In cases where it became necessary to ascertain whether the general radiation was the cause for an observed phenomenon, two films were placed in the cassettes and separated by an Al filter of 0.25 mm. thickness. This filter allowed the major portion of the effects of the general radiation alone to be registered on the second film.

It can be shown that when, as in the present experiments, the specimen is wide enough to cover the entire cross section of the incident beam, the optimum thickness is $1/\mu$ cm., where μ is the linear absorption coefficient of the material for the X-rays.[8] For proteins in general the value of μ for Cu Kα radiation indicates specimen thicknesses of about 1.5 mm. This approximate thickness was built up by combining material under a microscope. Excessively thick specimens strongly absorb reflections due to Cu Kα radiation, thus reducing their intensity relatively to the corresponding reflections of the more penetrating general radiation. The latter artifacts then obscure the central regions of the pattern.

Adequate Angular Resolution.—The system of apertures serving to define the X-ray beam must yield sufficient angular resolution to permit clear distinction of the position of one diffraction order from that of a consecutive one, even when these are not immediately adjacent to the center. For example, the 640 Å. collagen spacing not only has its first order removed by only eight minutes of arc from the central beam but consecutive orders are separated by the same angle. At small angles of reflection according to Bragg law the difference in the diffraction radii for two consecutive orders is $\lambda s/d$, where λ is the radiation wave length, s the specimen-to-film distance, and d the fundamental spacing observed. A convenient test to apply to a collimating system is that of determining whether it is such as to prevent the maximum width of the central beam at the photographic film from being larger than this value of $\lambda s/d$. Because higher orders are apt to be faint, yet require about the same angular resolution, their registration with adequate resolution is particularly difficult.

To reduce exposure times the specimen-to-film distance should be kept at a minimum. The smallest convenient yet adequate distance is determined by the magnitude of the spacing to be measured, the accuracy of measurement desired, and the ability of the investigator to prepare and align fine enough pinholes and lead beads (the latter being used to stop the undiffracted beam just before the photographic film). A rough rule for a suitable specimen-to-film distance is that it shall be equal (in mm.) to half the largest Bragg spacing (in Å.) required to be examined.

The Collimating System.—With suitable specimen-to-film separation and angular resolution selected, there remains some choice of pinhole or slit dimensions and of collimating tube length (distance between defining apertures). The proximal pinhole (next to the tube) should be larger than the distal, beam-defining one to allow use of as much tube-target area as is feasible. If the distal aperture is too fine, however, the intensity diminishes. Permissible collimating tube lengths depend on whether they can be evacuated to cut down air absorption.

In addition to the two beam-defining pinholes or slits, a guard aperture located behind the distal opening, just before the specimen, is particularly important in small-angle work in order to cut off secondary radiation emitted by the inside collimating-tube walls or the edges of the distal pinhole or slit. In the experiments here described the guard opening was fixed to each of the interchangeable rear apertures at about 19 mm. distance and made open just sufficiently to clear the beam for all contemplated uses of the combination.

With pinhole collimations able to resolve spacings as high as 600–700 Å. in every direction on the film, exposure times for protein fibers become of the order of days and weeks. Employment of slits permits more effective utilization of the available radiation without corresponding deterioration of the angular resolution in one direction of the pattern. In addition, the closely spaced, line-shaped diffractions thus registered become more easily distinguished and measured than are the corresponding spot images of the pinhole pattern.

Below are cited typical systems employed in the present studies. The proximal aperture, distal aperture, collimating tube length, and specimen-to-film distance are cited in that order, dimensions being in mm.:

(1) 0.6, 0.3, 60, 30–60. Resolution 60–75 Å. This system was used in wide-angle work and for preliminary examination of specimens.

(2) 0.6, 0.3, 180, 60–100. Resolution 150–190 Å.

(3) 0.3, 0.3, 180, 100–200. Resolution 240–310 Å.

(4) The above were usually employed as pinhole systems. For higher angular resolution slits were used: 0.14, 0.14, 180, 200–300. Resolution 670–740 Å. The narrow

(4) F. O. Schmitt, C. E. Hall and M. A. Jakus, *J. Cell. Comp. Physiol.*, **20**, 11 (1942); THIS JOURNAL, **64**, 1234 (1942).

(5) O. Kratky and A. Sekora, *J. makromol. Chem.*, **1**, 113 (1943).

(6) W. T. Astbury, *J. Int. Soc. Leather Trades' Chem.*, **24**, 69 (1940).

(7) W. T. Astbury, in "Advances in Enzymology," Interscience Publishers, New York, N. Y., 1943, vol. III, pp. 91–96.

(8) M. J. Buerger, "X-Ray Crystallography," John Wiley & Sons, Inc., New York, N. Y., 1942, p. 180.

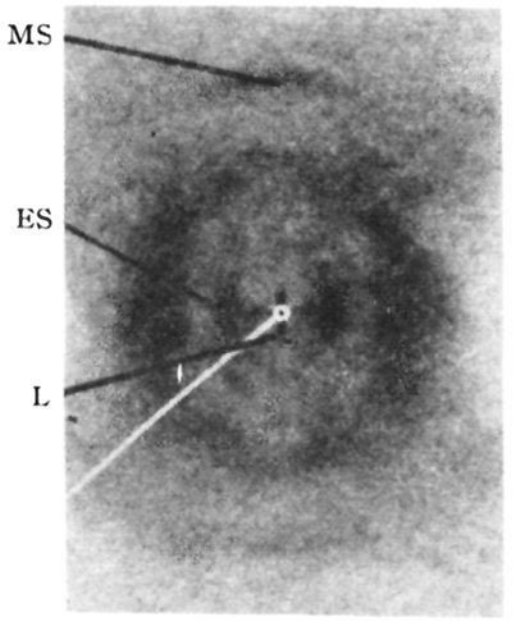

Fig. 1.

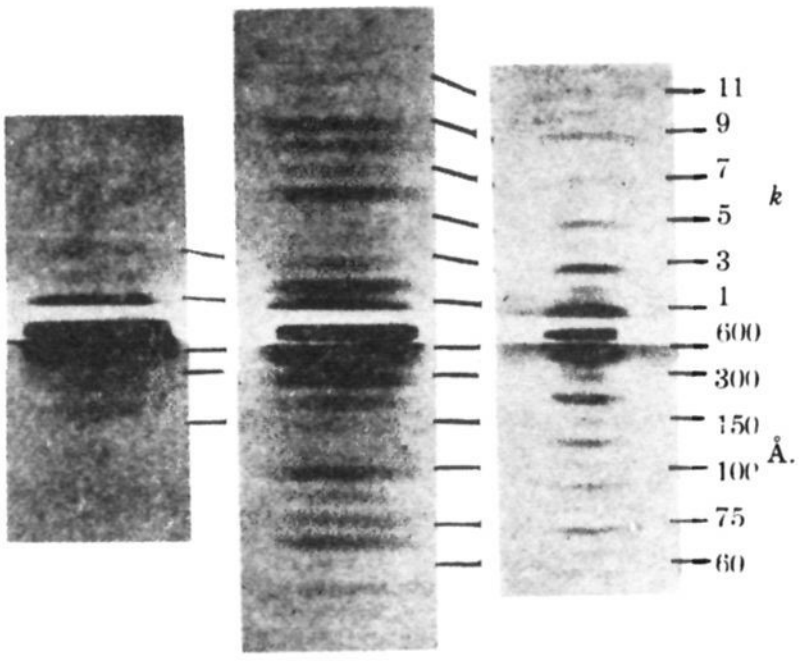

Fig. 2.

Fig. 1.—Wide-angle pattern of beef *tendo Achilles*, originally taken at a 5 cm. specimen-to-film distance. The fiber axis of the tendon is vertical. MS is the meridional arc of spacing 2.86 Å. and ES the equatorial arc at 11 Å., these being the short spacings frequently referred to in the text. L indicates the small-angle diffractions, featured at greater magnification in Fig. 2.

Fig. 2.—Small-angle patterns of a, reversibly shortened, formalin-treated rat tail tendon (sample 23 of Table II); b, dried beef *tendo Achilles;* and c, moistened kangaroo tail tendon. The photographs, while taken at 26, 26 and 30 cm. specimen-to-film distances, respectively, have been differently enlarged during reproduction so that they are now at the same angular magnification, one which is about 12.7 times that of Fig. 1. The scale on the upper half of the figure shows positions of odd orders, while that of the lower half indicates rough values of spacings. The fundamental periods are for a 550 Å., for b 640 Å., and for c 675 Å.

dimensions of the slits were as given, the long dimensions being 2 to 3 mm.

(5) All of the above systems were operated in air. An all enclosed outfit, normally using slits and constructed to permit evacuation, had the following critical dimensions: 0.14, 0.14, 330, 300. As thus employed it readily resolved orders of 600–700 Å. spacings, and should have been capable of examining up to about 1200 Å. A bead holder, adjustable from the outside, was placed in the film holder portion and aligned photographically.

Experimental Results

Description of the Collagen Long-Spacing Pattern.—The most complete description of the collagen long-spacing pattern can be given for dried beef tendon. Figure 1 shows the short-spacing pattern as photographed at a 5 cm. specimen-to-film distance. Near the center are to be seen the long-spacing diffractions which, when magnified using better angular resolution and slits, appear as shown in Fig. 2b. Measurements of the diffractions are given in Table I, from which it is clear that the fundamental fiber-axis period is about 640 Å. under these conditions. While on many films the first order can be seen distinctly, its diffraction diameter cannot be measured accurately, hence is not used to estimate the fundamental period.

Table I lists only the first twelve orders of this spacing because these are of sufficient intensity to be registered clearly on most photographs. Of these orders, the first, second, sixth, ninth and eleventh are most prominent. Occasionally orders with indices from 13 to 21, inclusive, can be seen faintly, of which the seventeenth and twentieth appear to be strongest. On more intense patterns, which lack sufficient angular resolution, however, to differentiate consecutive orders clearly, it can be shown that orders at least as high as about the thirtieth exist.

TABLE I

SMALL-ANGLE MERIDIONAL DIFFRACTIONS OF DRY BEEF *Tendo Achilles*

Measured spacing, Å.	Order index, k	k times spacing, Å.	Intensity description[a]
(640)	1	(640)	10
320	2	640	7
215	3	645	3
160	4	640	2
127	5	635	1
107	6	642	7
91.7	7	642	3
80.4	8	643	4
70.8	9	637	5
63.8	10	638	2
58.3	11	641	4
53.2	12	638	2

[a] The numbers describing order intensities have only qualitative significance, the higher values being assigned to the more intense orders.

TABLE II

SUMMARY OF DIFFRACTION DATA ON COLLAGENOUS TISSUES

No.	Collagen source	Pretreatment	Large period, Å.	Principal short-spacing arcs, Å. Equatorial	Meridional
1	RTT	Water	680-W	..	..
2	KTT	Water	675-W	15.5	2.87
3	KTT	Run up to 60% alcohol	675-W	13.8	2.82
4	KTT	Run up to 80% alcohol	675-W	13.3	2.87
5	KTT	HCHO, then *N* acetic acid	665-W	15.5	2.86
6	Guinea pig bone	Decalcified in 5% HCl	665-D	11.5	2.86
7	KTT	Swollen in 1 *N* acetic acid	660-D	11.0	2.85
8	Mammoth tusk	Demineralized in 5% HCl	655-D	11.6	2.86
9	Beef spleen	Digested in 1% trypsin	655-D	..	..
10	RTT	HCHO, limited T-contraction	650-DF	..	..
11	KTT	Water	645-D	11.5	2.90
12	Human skin (derma)	Water	645-D	..	..
13	Pickerel swim bladder	Water	640-D	..	..
14	Beef cornea	Water	640-D	..	..
15	BTA	Water	640-D	11.1	2.85
16	RTT	Water	640-D	11.2	2.88
17	Beef ligament	Water, stretched 100%	640-D	..	..
18	BTA	Cleaned after Bergmann[9]	630-D	..	..
19	RTT	5% tannic acid	625-D	11.5	2.85
20	RTT	5% chromic chloride	625-D	11.9	2.85
21	Sheep intestinal submucosa	"Plain surgical gut"	615-D	..	..
22	RTT	HCHO	615-DF	..	..
23	RTT	HCHO, reversible T-contraction	550-S	11.5	2.85
24	RTT	HCHO, maximal T-contraction	Lacking	11.5	2.87
25	RTT	Maximal T-contraction, re-extended	Lacking	11.6	2.88
26	Gelatin	Oriented by tension	Lacking	11.1	2.85

EXPLANATION OF TABLE II

RTT = freshly dissected rat tail tendon, initially rinsed in water; KTT = dry strips of kangaroo tail tendon (kindly supplied by the Johnson and Johnson Co.), initially reswollen in water; BTA = beef *tendo Achilles* strips, rinsed in water. Other tissue samples (12, 13, 14 and 17) were preliminarily freed mechanically of as much extraneous non-collagenous matter as possible and rinsed in water. The guinea pig bone and beef spleen (6 and 9), for the preparation of which the author is indebted to Dr. B. S. Gould, required preliminary decalcification with acid or removal of other proteins by the action of trypsin. The mammoth tusk sample (8) was from a petrified specimen, supplied by Dr. J. W. M. Bunker, the estimated age of the tusk being about 20,000 years. This preparation also required removal of mineral constituents with acid. All samples, 6 and below, for which the table lists chemical pretreatments were subjected to the reagents for many hours and finally thoroughly washed in water.

Pretreatments involving HCHO employed 10% formalin (overnight), followed by prolonged washing in water. "T-contraction" refers to the thermally induced contraction in length resulting from subjecting the wet tendon strips to water at 80 to 100°. Specimen 10 underwent "limited" contraction (15%), being forcibly prevented from contracting further and dried at 80° to maintain the contraction. Specimen 23 was contracted in boiling water and then allowed to re-extend spontaneously as it cooled to room temperature. Specimen 24 underwent maximal contraction (64%) and the contraction was maintained by drying at elevated temperatures. Specimen 25 was not treated with formalin, but simply heated in water to secure maximum contraction, then forcibly re-extended and dried at its original length.

The "plain" surgical gut ("boilable") was used as it came from the usual surgical package, after blotting and drying to remove the tubing fluid (sample 21). Gelatin strips were moistened with water and hung up to dry, with tension increased as the moisture evaporated in order to induce orientation (sample 26).

All of the preparations numbered 6 and higher were photographed dry, having been hung up under moderate tension, to prevent curling, and dried thus in air. Samples photographed wet with fluids (1 through 5) were prepared by tying them to fine glass fibers (to maintain extension) and inserting the whole into glass (Pyrex) capillaries of thin wall (0.02–0.035 mm. thickness). An excess of the fluid last noted in the pretreatment column was added and the ends of the capillaries then sealed to prevent drying.

The large collagen periods (column 4) were sought in all preparations by using diffraction systems (4) or (5). Large periods are given to the nearest 5 Å., error being estimated to be of the order of 1%. Diffraction order intensifications of the patterns are classified in column 4 according to whether they resemble the typical wet (W), usual dry (D), or the modified dry (DF) cases described in the text. With example 23, marked S, only the first three diffraction orders were observed.

Diffraction system (1) was used to examine the short spacings of many of the specimens, the results for the two principal wide-angle arcs (see Fig. 1) being given in columns 5 and 6.

Variations in the Collagen Long Spacing.—Table II summarizes the results obtained to date regarding the small-angle diffractions of collagen, listing examples in the order of decreasing fundamental periods. The table is fairly self-explanatory, so that only the principal correlations need be considered.

(1) The dry, minimally treated tissues (examples numbered 11 to 17, inclusive) all possess

(9) M. Bergmann, *J. Biol. Chem.*, **128**, 217 (1939). Instead of freezing and grinding, thin intact tendon strips were used.

collagen fibrils whose fiber-axis periods are in a narrow range around 640–645 Å. This is observed in spite of the impossibility of ensuring that in every case the fibrils were subjected to the same tension during drying. Similarities of these patterns extend to the relative intensities of the diffraction orders, so that one can speak of a typical order intensification for dry collagen. This is not to say that small variations in order intensities do not occur, and in the clearer tendon patterns minor fluctuations are observable, particularly with the fainter orders. In all examples classified D in Table II the first, sixth, and ninth orders are particularly prominent, and others do not depart too widely from the relations given for beef tendon in Table I.

(2) The samples photographed in moistened condition (numbered 1 through 5) have the longest observed periods (665 to 680 Å.). Furthermore, in these cases there is a typical order intensification, consisting of a most striking alternation of intensities over the first ten orders, odd ones being strong and even ones weak. An example, taken with diffraction system (5), is shown in Fig. 2c, measured spacings and relative intensities being listed in Table III. Variations from the relative intensities roughly indicated in Table III are not readily apparent for the cases designated W in Table II. Beyond the tenth order the diffractions are weak but the regular alternation of intensities appears to be lost.

TABLE III

SMALL-ANGLE MERIDIONAL DIFFRACTIONSS OF MOISTENED KANGAROO TAIL TENDON

Measured spacing, Å.	Order index, k	k Times spacing, Å.	Intensity description[a]
(675)	1	(675)	10
342	2	684	4
225	3	675	8
168	4	672	2
136	5	680	6
113	6	678	2
97.2	7	680	4
83.1	8	665	1
74.5	9	671	6
67.4	10	674	2
61.2	11	673	2
56.0	12	672	2

[a] The numbers representing intensity possess only qualitative significance. They are not to be compared with the corresponding figures of Table I.

(3) Examples 6 through 8, whose pretreatment involved subjecting them to acid, are intermediate between the two classes cited above. Their periods (655 to 665 Å.) approach those of moistened collagen, but the order intensifications correspond to the condition under which they were photographed.

(4) Examples 19 through 22 constitute a group of preparations which have undergone treatments similar to tanning. They are characterized by low fundamental periods (615 to 625 Å.) and, for the most part, the D type of order intensification. "Plain" surgical gut is not out of place in this group because in the process of manufacture processes resulting in light tanning and extensive dehydration are used. Clark and Schaad[2] noted a similar low period with chrome-tanned collagen, but expressed their observation in terms of the supposed fundamental period of 432 Å., which became 408 after tanning.

(5) Examples 5, 10, 22 and 23 all experienced treatment with 10% formalin. This has the effect of reducing collagen swelling in water and in aqueous acid solutions. Specimen 22 shows this effect by the fact that air drying leads to the low period of 615 Å. Results with sample 5 indicate that after formalin treatment the tendon is unable to swell, even in acetic acid, to the point where its period (665 Å.) is as large as that normally attained by untreated collagen in water alone (675 to 680 Å.). Formalin-treated specimens, such as 10 and 22 which were photographed dry, yield order intensifications resembling the normal D type in having prominent first, sixth and ninth orders, but differ most strikingly in that the second orders are unusually faint. This type of modified dry pattern is indicated by DF in Table II.

(6) Formalin treatment also has the effect of preparing collagen fibers which will undergo reversible thermal shortening.[10] The following is a detailed description of the behavior of example 23 which underwent this phenomenon: The wet rat tail tendon fiber was left in 10% formalin for 24 hours, washed in water several hours, and then suspended briefly in boiling water. The original length of 40 mm. decreased to 12.5, or a shortening of about 70% took place. After slowly cooling to room temperature the final spontaneously reacquired length was 35 mm. or 87% of the initial extension. After drying under a minimum of tension (to keep the fiber straight) diffraction patterns were obtained. The first three orders (S, Table II) of a fiber-axis period of 550 Å. were observed, the first being very intense and the next two rather faint (Fig. 2a).

The behavior of example 23 may be contrasted with that of sample 10. The latter preparation was forcibly prevented from contracting more than about 15% and then dried at high temperature (80°C.) in order to avoid relaxation. It was visibly apparent that some portions of the fiber underwent full contraction to take up slack, while others remained taut. The 650 Å. period observed is undoubtedly that of the unshortened portions, which apparently have undergone a small amount of stretching.

Conditions under which the Long Spacings Are Observable.—Clark and Schaad[2] have indicated that swelling of collagen in acid or alkali,

(10) For a good discussion of swelling and thermal shortening of collagen fibers, and of the effect of formalin treatment thereon, see A. Küntzel and F. Prakke, *Biochem. Z.*, **267**, 243 (1933).

which results in multifold expansion to a gel-like state, does not destroy the small-angle pattern as it is observed after subsequent deplumping and drying. They also reported that thermal and chemical shortening of collagen completely abolishes the large spacings, which cannot be restored by forcibly re-extending the shortened fiber. Oriented gelatin strips also do not exhibit the long spacings, although the short-spacing pattern of this material appears to be identical with that of collagen. These observations have been confirmed in this Laboratory, as is shown in examples 7, 24, 25 and 26 of Table II, with the reservation described in connection with the reversibly shortened, formalin-treated tendon (example 23 of Table II). Even in this case the regularity of the structural features was reduced by the shortening to the point where only three diffraction orders were obtained.

Schmitt, Hall and Jakus[4] have shown with the electron microscope that under the conditions of their examinations the collagen long-spacing was quite variable (400–1000 Å.), though the average value of their measurements (rat tail tendon) was 644 Å., agreeing well enough with the X-ray value for the dry tendon. Undoubtedly intact and undamaged collagen fibrils normally do not possess great variation in period, as is shown by the large number of diffraction orders observed.

In several instances it was found that preliminary mechanical distortions destroyed the discreteness and orientation of the diffractions, making their registration more difficult and sometimes impossible. In a more positive way also the X-ray method points to the variability of the collagen large period, as is apparent from the results cited above, which indicate values ranging from 550 to 680 Å.

The statement of Wyckoff and Corey,[11] that long-spacing diffractions can be obtained from rat tail tendon collagen reprecipitated from dilute acetic acid solutions, could not be confirmed in the present experiments, using filtered solutions. The point is probably immaterial, however, since Schmitt, Hall and Jakus[4] succeeded in showing that from these filtered solutions typical fibrils showing the large structural period can be obtained by neutralization or precipitation with salt.

That rat tail tendon collagen yields small-angle diffractions with difficulty after precipitation is in line with findings of Nageotte and Guyon[12] regarding the relative susceptibilities to dissolution in acid exhibited by tendons from various animals. It has been observed during the present studies that the relatively resistant acid-swollen beef tendons can be macerated into dilute suspensions (though the collagen will not pass through filter paper) and subsequently reprecipitated to reform fibers exhibiting the small-angle diffractions.

(11) R. W. G. Wyckoff and R. B. Corey, *Proc. Soc. Exp. Biol. Med.*, **34**, 285 (1936).

(12) J. Nageotte and L. Guyon, *Compt. Rend. Assoc. Anat.*, 1934 Réunion, p. 408.

Results of the above type are examples of the facts that the collagen small-angle diffraction is typical of intact collagen fibrils, and that different collagenous materials exhibit varying resistance to damage by mechanical or chemical means. The possibility of X-ray demonstration of the large collagen fiber-axis period depends upon avoiding excessive injury to the fibrils, hence this method of study is limited to examining such phenomena as are undergone rather uniformly by an appreciable portion of the fibrils of a sample.

Relation of the Principal Collagen Short Spacings to the Macroperiod.—In order to examine the possibility that relationships between the short and long spacings of collagen exist, many of the preparations whose small-angle patterns have been described were also examined at wide diffraction angles. The two principal diffractions of each of the wide-angle patterns thus obtained are listed in Table II, columns 5 and 6.

There is no readily apparent connection between the changes in the long period and those of either the 11–15 Å. equatorial spot or the 2.86 Å. meridional arc (see Fig. 1). The equatorial spacing, which is well known to vary from 10 or 11 Å. in the dry state to 15 Å. or more in the wet condition,[13] does resemble the macroperiod roughly in that both increase with hydration. The series of examples 2, 3 and 4 indicate, however, that noticeable changes in the equatorial arc can occur without appreciable alterations of the large period. Furthermore, over all of the dry samples, with the large period changing from 550 to 665 Å., the equatorial spacing remains at 11–12 Å., which may be regarded as practically constant, considering the difficulties of measuring this spacing accurately and the possible variations due to small differences in hydration.

It is more reasonable to expect the 2.86 Å. meridional arc to change with alterations of the similarly oriented macroperiod. Nevertheless, in preparations which do not show long periods at all and in those whose periods range from 550 to 680 Å., the short meridional spacing remains essentially constant. The accuracy of measurement of the 2.86 Å. spacing in the present photographs is about 1 to 2%, while the radial width of this diffraction never corresponds to more than about 4 to 5% of the spacing. It is, therefore, quite impossible to doubt that for changes in the macroperiod amounting to at least 20% no corresponding alteration in the short meridional spacing takes place.

Similar remarks could be made regarding other collagen short-spacing diffractions, but a more complete consideration of this problem is reserved pending the results of further study.

Discussion

The number of tissues and animals whose collagen yields the typical small-angle pattern suggests

(13) K. Hermann, O. Gerngross and W. Abitz, *Z. physik. Chem.*, **B10**, 371 (1930).

that this phenomenon is characteristic of collagenous structures wherever found in the vertebrates. Schmitt, Hall and Jakus[4] demonstrated this fact for an even wider range of animals, including one example of an invertebrate (squid). The present result with spleen, whose reticular fibers are frequently considered as differing from the collagenous fibers of skin, bone and tendon,[14] suggests that the basic structure of the reticular fibers must resemble that of other collagenous fibers rather closely.

Ligaments possess largely the protein elastin, which Astbury[6] has shown gives only an amorphous and unoriented pattern, even after considerable stretching. Such oriented components as exist in ligaments have been considered to be collagen, and the present finding of the collagen small-angle diffractions, faintly registered on the beef ligament pattern, supports this conclusion.

The result with the mammoth tusk, though possibly not surprising, is of some interest as indicating that the collagen structure is stable over long periods of time.

From the standpoint of the meaning of the collagen macroperiod in terms of precise structural details, the evidence is as yet inconclusive. It seems clear, however, that a study of the large fiber-axis period will yield information more directly linked to certain of the colloidal properties of collagen than have similar wide-angle investigations in the past. Often the latter have been disappointing in that the short spacings remain little affected during various physical and chemical alterations of the collagen, the exception being the 11 to 15 Å. arc which is altered during ("intramicellar") swelling.

The results of the present survey concerning the large collagen period show that the small-angle diffractions are influenced by the presence or absence of water, acid and "tanning" agents, and by temperatures which result in shortening, thus offering the opportunity of studying these phenomena in greater detail than has been possible. In particular, it seems probable that the elongations and contractions in the lengths of whole collagen fibers during swelling and thermal shortening are more directly related to the corresponding variations in the macroperiod of the constituent fibrils, than they are to alterations of the meridional short spacings, which are extremely reluctant to show any change whatsoever.

To date it has been impossible to fit the short-spacing diffractions into any unequivocal structural explanation. The short-spacing pattern will be considered in greater detail in another paper. At this time it is desired only to point out that one practice recently in vogue, namely, that of describing the several short-spacing meridional arcs as extremely high orders of the large fiber-axis period, is not very illuminating.

(14) A. A. Maximow and W. Bloom, "A Textbook of Histology," pp. 71–73, W. B. Saunders Co., Philadelphia, 1942. See also J. Nageotte and L. Guyon, *Am. J. Path.*, **6**, 631 (1930).

Astbury[7] and Kratky and Sekora[5] have wished to describe the 2.86 Å. arc as the 220th order of the large fiber-axis period. Aside from the difficulty of proving precisely what indices are to be assigned, this view also runs into the following trouble. With the diffraction method the large fundamental period has been found to vary between 550 and 680 Å. in specimens whose prominent short-spacing arc at 2.86 Å. remains apparently unchanged in position. Thus it would become necessary to assign indices to the 2.86 Å. arc varying between about 192 and 238, depending on the particular sample, though the significance of this diffraction undoubtedly remains the same.

Facts of this sort suggest that the short-spacing portion of the pattern represents structural features of but a part or parts of the whole material involved in producing the long-spacing diffractions. Though the prominent small interatomic spacings of these fibril portions will undoubtedly "couple" with the large period, so that a short-spacing arc may be produced by intensification of appropriate high orders of the large period, the values of the particular large indices observed become of little significance. It would seem wiser to consider the short spacings as related to small pseudo-cells which may not be found *throughout* the entire macroperiod of the fibril.

The electron microscope studies of Schmitt, Hall and Jakus[4] contain observations on a stretched collagen fibril which suggest that the constituent unit longitudinal arrays ("protofibrils") are normally far from being maximally extended, nor do all portions along the fibril appear to possess the same degree of extension. The chemical data of Bergmann and Stein[15] indicated that a dual frequency might be required for the distribution of proline in collagen and gelatin. These electron microscope and chemical results support the view that collagen may possess more than one chemically and configurationally defined fraction of the structurally repeated large period. With this in mind the inadvisability of trying to relate large diffraction indices to the frequencies of amino acids derived from chemical analysis of *whole* collagen or gelatin becomes particularly obvious.

The number and sharpness of the small-angle collagen diffractions offer hope that eventually one-dimensional Fourier analyses of the density distribution along the typical fibril may be possible. Variations in order intensities would be useful in determining phase relations of the various F factors, while electron micrographs will be helpful in deciding between possible alternative structures. The data are insufficient as yet to permit a systematic attempt in this direction, though qualitative conclusions can be drawn readily from casual inspection of the relative order intensities.

(15) M. Bergmann and W. H. Stein, *J. Biol. Chem.*, **128**, 217 (1939).

In both wet- and dry-tendon patterns the first order is by far the most intense, hence the electron density in either case is likely to approximate a cosinusoidal function of position along the fibril. With the wet tendon, odd orders are much more intense than even ones, so that in this condition the lengths (measured along the fibril) of denser and less dense regions should be nearly equal, the division being made where density becomes greater or smaller than the average. In the dry material the alternation of intensities is lost, which means that the alternating bands of unequal density should be of unequal length. These deductions agree well with electron micrographs of collagen fibrils,[4] on which can be seen the alternation of dense and light bands of average total period (length of one light and one dark band) about 644 Å. Rough estimates from the electron micrographs of the dry fibrils indicated that the denser and lighter bands are indeed normally of unequal length, the former being the longer.

Normally on the pattern of dry tendon there is a progressive diminution of intensity observed for the first five orders (Table I). Similarly, except for the alternation of intensities between odd and even orders, the first eight orders progressively fade on the diagram of wet-tendon (Table III). These effects undoubtedly represent the ways in which the various terms of a Fourier series, each term related to the corresponding order intensity, modify the cosinusoidal first approximation for electron density mentioned above, the result being a smooth distribution of electrons with period of 640–680 Å. Superimposed on this relatively slowly changing density distribution there are probably important fluctuations possessing subperiods of the order of 70 to 110 Å., as is evidenced by the abnormally high intensities of the ninth order on the wet-collagen pattern and of the persistent sixth and ninth orders of the dry-collagen diagram.

The fact that diffraction orders as high as the thirtieth are in evidence indicates that the structure of intact fibrils is reproduced in each period to details of the order of 3% of the fundamental repeating pattern, hence that the analysis of the order intensities could yield particulars of the structure down to 20 Å.

In a direction transverse to the fiber axis the diffraction evidence for structure is poor and it must be concluded that order in this direction is not great. In fact the largest lateral spacing that can be seen is that of the intense equatorial arc which varies between angles corresponding to 11 and 15 Å., depending on water content. Clark and Schaad[2] described an equatorial small-angle streak lying within the position of this arc on the patterns of alkali-treated tendon. A similar streak is also found on moist-collagen patterns, but attempts to resolve any structure from it with the present apparatus have been negative. In addition, it appears to possess a large component of general-radiation artifact. Similar doubts also apply to the faint equatorial arcs (19.9, 30.0 and 47.6 Å.) reported for kangaroo tendon by Corey and Wyckoff.[3] These spacings are not constantly represented on all tendon photographs, and Clark and Schaad have shown that a similar diffraction (48 Å.) found associated with the collagen of intestinal gut is caused by the presence of a wax-like impurity.

Schmitt, Hall and Jakus[4] noted the tendency of collagen fibrils to fray longitudinally into very thin subfibrillar strands still possessing the banded appearance. The ultimate collagen unit imagined to be gotten by this fraying process, carried to a limit, was termed the "protofibril." From the X-ray diffraction standpoint the absence of well-defined, large lateral spacings suggests that the collagen protofibril is indeed very thin, possessing the width of very few polypeptide chains, or that the protofibrillar widths exhibit considerable variability. In this respect collagen differs notably from other fibrous proteins (the keratins and clam muscle, to be described in a subsequent paper), which possess quite definite and prominent large spacings transverse to the fiber axis.

Summary

The results of a survey of the small-angle diffractions obtained from a variety of collagenous tissues of a number of vertebrate animals are presented. It is shown that a characteristic large fiber-axis period is possessed by all of these materials, similarities extending from the magnitude of the macroperiod to the relative intensities of the diffraction orders exhibited.

The collagen large period is 640 Å. long, as measured from photographs of normal dry material. Variations from 680 to 615 Å. are introduced by appropriate treatment, involving wetting at the upper limit and treatments resembling tanning at the lower.

Relative diffraction order intensities differ remarkably between moistened and dried samples, the wet preparations showing a striking alternation of intensities, with odd orders predominant. The order intensities suggest that normally the electron density distribution along a collagen fibril consists of a primary, slowly changing, nearly cosinusoidal variation in density, having a period of 640–680 Å. Superimposed thereon are secondary fluctuations, prominent among these being sub-periods which are 70 to 110 Å. in extension along the fibril. For dry samples the orders extend out to angles corresponding to about the thirtieth order, indicating a considerable regularity in the structure.

Physical and chemical manipulations which destroy this regularity abolish the possibility of observing the small-angle diffractions by means of X-rays. The maximum spacing alteration which has been observed with X-ray techniques (reduction of the period to 550 Å.) was secured with a formalin-

treated preparation which had been nearly reversibly shortened and re-extended by a heating and cooling cycle.

Throughout all manipulations that have been tried there is no apparent relation between variations in the large period and the diffractions observed at wider angles. This is interpreted to mean that the wide-angle (short) spacings are related to but a portion or portions of the entire matter constituting the macroperiod.

The long spacings of collagen promise to be more directly related to certain of the colloidal properties of this protein than are the short spacings used to study such phenomena in the past. Correlation is excellent between the X-ray information and electron microscope results regarding the large period of collagen.

CAMBRIDGE, MASS. RECEIVED MAY 8, 1944

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

Anomalous Effects in the Titration of Phosphoric Acid with Calcium Hydroxide

BY ISIDOR GREENWALD

In 1923, Wendt and Clarke[1] reported that the titration of a solution of phosphoric acid with one of calcium hydroxide yielded anomalous results, in that, after a little more than one equivalent of calcium hydroxide had been added, further addition *lowered* the *p*H. After shaking for two hours, this effect was lessened, and after ten days it had disappeared. They advanced the explanation that the increasing acidity upon the addition of calcium hydroxide was due to the fact that the precipitate of calcium monohydrogen phosphate first formed was changed into tri-calcium phosphate and that the amount of acid thus set free was greater than was required to neutralize the hydroxide added. Similar results were also obtained by Holt, La Mer and Chown[2] and by Farnell.[3] Holt, La Mer and Chown analyzed the precipitates obtained in one series of experiments, in which this anomalous effect had practically disappeared after ten days of continuous shaking at 38°. The ratio of calcium to phosphorus, by weight, was close to 1.94, that calculated for tri-calcium phosphate. They believed these analyses confirmed the hypothesis of Wendt and Clarke. While they mentioned another series of experiments in which "a slight irregularity remained" after shaking intermittently for six months and then continuously for two months, they made no attempt to explain why this irregularity should persist for so long a time.

It should be quite obvious that, while the hypothesis of Wendt and Clarke might explain the increase in acidity during the titration, it cannot explain the subsequent *diminished* acidity in the same mixtures after six hours or ten days.

The clue to a satisfactory explanation is given by those results of Holt, La Mer and Chown, in which the anomaly persisted for eight months. In the first five columns of Table I, there are presented the pertinent data taken from the paper by Holt, La Mer and Chown. The values in the following columns are calculated from these.

TABLE I

TITRATION OF PHOSPHORIC ACID WITH CALCIUM HYDROXIDE

Data of Holt, La Mer and Chown[2] (pp. 537 and 550)

	Initial	Final			Precipitated			
Number	$[Ca^{++}]\,10^3$	$[Ca^{++}]\,10^3$	$[P]\,10^4$	*p*H	$[Ca]\,10^3$	$[P]\,10^3$	[Ca]/[P]	[Ca]/[P]
M_4	5.89	5.30	10.65	5.06	0.59	0.15	4	0.44
M_5	6.64	5.00	10.3	5.03	1.64	0.5	3.3	1.14
M_6	7.34	4.55	10.3	4.94	2.79	0.5	5.6	2.29
M_7	8.10	4.15	8.39	4.86	3.95	2.41	1.64	1.54
M_8	8.80	4.00	7.74	4.84	4.80	3.06	1.57	1.74
M_9	9.56	3.50	6.97	4.87	6.06	3.83	1.58	2.23

It will be noted that, with the exception of M_6, the value for [Ca]/[P] in the precipitate fell regularly with the addition of $Ca(OH)_2$. This exceptional value is not determined very accurately, being derived from small differences between rather large numbers. However, it can scarcely be less than 4, unless the value for either $[Ca^{++}]$ or [P] in the liquid be in error. Examination of the fourth column indicates that this is probably the case. This is the one instance in which the addition of calcium hydroxide did not lower [P].

We believe that the failure of these particular mixtures to attain equilibrium even after six months was due to the fact that the precipitate contained no crystals having the apatite structure, but consisted entirely of $CaHPO_4$ and $Ca(OH)_2$; or of $CaHPO_4$ and some compound having Ca:P > 1.5. In the last column of the table, we have calculated the amount of precipitated $Ca(OH)_2$, upon the assumption that all the precipitated P was present as $CaHPO_4$. With the exception of M_6, the *amount* of such precipitated $Ca(OH)_2$ increases regularly, although the ratio, Ca:P, diminishes.

That precipitates of tri-calcium phosphate are apt to contain an excess of calcium hydroxide is well known, but it has been observed previously only at a *p*H above 6 or 7. Even with a large excess of calcium hydroxide, the highest Ca:P

(1) G. A. Wendt and A. H. Clarke, THIS JOURNAL, 45, 881 (1923).

(2) L. E. Holt, V. K. La Mer and H. B. Chown, *J. Biol. Chem.*, 64, 509 (1925).

(3) R. G. W. Farnell, *J. Soc. Chem. Ind., Trans.*, 45, 343 (1926).