FURTHER EVIDENCE CONCERNING THE PERIODIC STRUCTURE IN COLLAGEN*§

by

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The existence of a large period in collagen and other fibrous proteins has been demonstrated many times by electron microscopy[1] and by X-ray diffraction[2,3,4]. Electron micrographs have shown a characteristic intraperiod fine structure [5,6,7] which could be observed best with proper staining conditions. X-ray diffraction results, particularly those of BEAR[8] and his collaborators, have shown that the intensities of the diffraction orders, and hence the periodic fine structure, is related to the collagen source and to the preparative treatment it has received. BEAR[9] has also shown that valuable information concerning collagen structure is obtainable by considering the shapes of the X-ray diffraction spots.

In principle it would be expected that an analysis of the intensities of the X-ray spots should lead to more detailed structural information than that obtained from electron micrographs, because preparative artefacts are not as great and also because the number of diffraction orders that is usually available results in better resolution than is given by electron microscopes. However, structural information is not readily available from order intensities because knowledge concerning scattering phases is lacking. This is not serious when dealing with molecules made up of only a small number of atoms but it is almost an insurmountable obstacle for complicated structures like proteins. PATTERSON distributions[10] are usually constructed and these can sometimes be interpreted in terms of molecular structure***. Even when a direct interpretation

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*** In the PATTERSON synthesis the X-ray intensities are made the coefficients of a Fourier series. PATTERSON has shown that the function $A(x)$ which the series represents in the 1-dimensional case is also equal to

$$\frac{1}{L}\int_{\text{period length}} \varrho(\eta)\cdot\varrho(x+\eta)\,d\eta$$

where ϱ is the charge density and L is the length of the period in the structure under discussion. With the help of this equation, $A(x)$ is readily interpretable. The principal contribution to $A(x)$ comes when both $\varrho(\eta)$ and $\varrho(x+\eta)$ are large. Thus a maximum in $A(x)$ at the point x_1 means that there are two maxima in the density distribution whose distance apart is x_1.

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of the X-ray PATTERSON distributions is not apparent, they may be used to check whether molecular models deduced from other than X-ray information are consistant with the X-ray data. In particular, PATTERSON distributions can be constructed from electron micrographs and checked against Pattersons deduced from X-ray data.

We have determined small angle X-ray diffraction intensities from several specimens of beef tendon collagen and where possible have taken corresponding electron micrographs. PATTERSON distributions were determined in each case.

EXPERIMENTAL METHODS

The X-ray data were taken with a continuously evacuated X-ray tube operating at 30 kilovolts and 20 milliamperes. Cu K_α radiation, monochromatized by means of balanced filters, was used in conjunction with a slit system and a Geiger counter detector as has been described previously[11]. Slit widths were adjusted so that the angular width of the individual diffraction orders was about one-third the separation between orders, hence the overlap of peaks was small.

The method of obtaining the intensity of the diffraction orders was as follows: At each angle we first measured the diffracted intensity through a nickel filter and then through a cobalt filter whose absorption at long and short wave-lengths was identical with that of the nickel filter. The difference in intensity through the two filters was plotted as the diffracted intensity due to Copper K_α radiation. The resulting curves showed the diffraction peaks superimposed on a background due to the presence of a small amount of continuously scattered X-radiation. The intensities we quote are the peak heights measured above this background.

Statistical effects introduce an uncertainty of about 5% in the case of the more intense orders and about 15% for the weakest orders. Photographic evidence shows[8] that particularly for dry, stained specimens some error may be introduced into the intensity measurements due to the shape of the diffraction spots. While it is not readily feasible to assess this error quantitatively, it appears from published X-ray patterns that the error introduced is smaller than our statistical uncertainty. The number of observed orders, 28 in the case of the wet collagen specimens, was limited by the geometry of the apparatus. In the case of the dry collagen specimens the high orders were easily visible but were not sufficiently strong to warrant their use in the calculations.

Our micrographs were taken with an RCA EMU 2b microscope using compensated polepieces and no objective aperture.

Samples for X-ray diffraction were 10 × 10 × 1 mm sections of beef Achilles tendon. The specimens to be stained were immersed in a 0.2% solution of phosphotungstic acid at pH 3 for periods up to 105 minutes and were then rinsed in distilled water. Higher concentrations of stain in the specimens would have decreased the length of the fundamental period and, more important from our point of view, would have also increased the errors due to the shapes of the diffraction spots. Corresponding samples for electron microscopy were out from these sections, fragmented, sedimented by centrifugation, and re-suspended in distilled water. The X-ray specimens that we call dry were air-dried under spring tension in a specimen holder that could be enclosed during the X-ray run to insure that the humidity near the specimen remained constant. The relative humidity for dry specimens was about 40% and corresponded to a fundamental period of 645 A for both stained and unstained specimens. Wet specimens were studied in the same specimen holder. They were wet by allowing them to come to equilibrium with saturated air. The fundamental period was 675 A for stained and unstained wet samples. Repeated trials showed that the fundamental period and order intensities change in a continuous fashion when specimens are allowed to contract from 675 A to 645 A by drying. These runs were made using a two-crystal spectrometer[12] to collimate the beam and to detect the diffracted radiation. Consequently, the fundamental period could be determined with considerable precision.

RESULTS

Table I gives order intensities obtained for a typical collagen specimen under five different conditions: wet, unstained; wet, 15 minute stained; wet, 105 minute stained; dry, unstained; dry, 15 minute stained. The specimen had been cut from the tendon and alternately wet with distilled water and dried for a period of several weeks with no noticeable changes in the order intensities of the wet specimen. Fig. 1 shows a portion

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of the diffraction data for this specimen when wet and stained for 105 minutes. Other wet specimens gave very similar spacings and relative intensities. The peaks in the specimen when dried were as well resolved but they were far weaker in absolute intensity. Analogous intensity relationships held for stained, wet and dry specimens.

TABLE 1

DIFFRACTED INTENSITIES FROM BEEF TENDON COLLAGEN

Diffraction order	*Wet, unstained*	*Wet, stained 15 min*	*Wet, stained 105 min*	*Dry, unstained*	*Dry, stained 15 min*
1	100.0	100.0	100.0	100.0	100.0
2	0.77	1.90	1.52	0.2	21.2
3	7.72	23.0	2.72	9.0	10.9
4	0.38	0.71	1.06	2.3	9.0
5	3.50	31.4	62.2	4.0	25.0
6	0.76	2.11	2.10	2.6	5.8
7	1.04	8.08	17.1	2.0	5.8
8	0.66	7.58	14.5	2.9	9.6
9	1.56	9.00	24.6	2.9	10.8
10	0.48	2.90	4.33	0.6	1.0
11	0.17	1.30	4.08	1.7	0.3
12	0.64	1.80	7.19	1.2	0.3
13	0.02	0.90	8.52	0.4	5.8
14	0.05	0.10	0	0.1	1.2
15	0.22	6.20	13.2	0.5	2.3
16	0.12	0.71	2.89		1.7
17	0.21	1.69	3.80		2.6
18	0.14	1.00	4.70		1.0
19	0.06	1.96	3.03		1.1
20	0.56	3.69	10.8		0.8
21	0.36	0.59	0.76		
22	0.20	1.00	1.96		
23	0.03	0.31	0.76		
24	0	0.31	0.59		
25	0.13	0.40	2.60		
26	0	0.31	1.37		
27	0.05	0.10	0.30		
28	0.02	0.01	0.30		

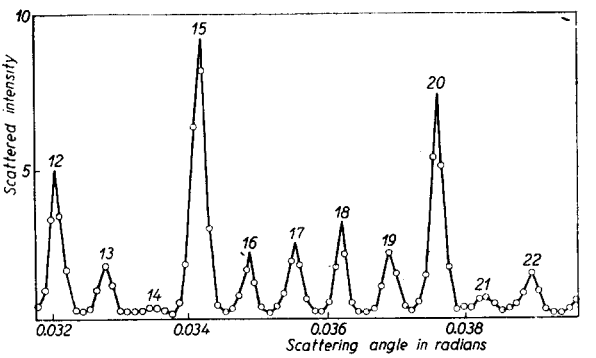


Fig. 1. A portion of the diffraction curve for wet, 105-minute-stained beef tendon collagen showing the 12th through the 22nd orders.

Portions of the specimen before and after staining were cut from the section for electron microscope examination. Many fibrils showed the characteristic 650 A funda-

mental period but in most fibrils we were unable to detect a part or all of the fine structure shown so beautifully in some of the specimens of SCHMITT AND GROSS[6]. In the minority of fibrils we observed a fine structure which consisted of as many as 8 dark bands within the period. In the notation of SCHMITT AND GROSS[6] we observed bands a, b_1, b_2, c_1, c_2, d, e_1, and e_2. In Table II we list the approximate positions of these bands. The less dense regions about d and e were more apparent than the rest. SCHMITT AND GROSS also found similar fine structure in unstained specimens, but the contrast in our micrographs was inadequate to confirm this result. In Fig. 2 we plot an idealized density distribution along the length of the collagen fibril as suggested by Table II. For the purpose of later comparison we also plot a density distribution which consists of only one dense band within the fundamental period.

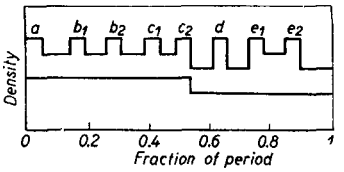


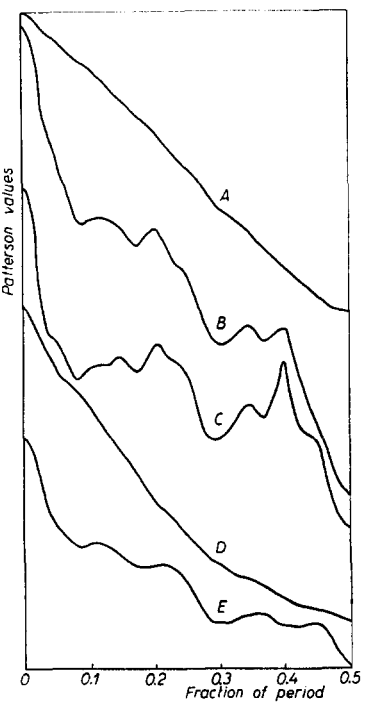
Fig. 2. Idealized density distribution along the length of the collagen fibril (upper curve). Idealized density distribution consisting of one dense band whose widtl is 53% of the total period (lower curve).

TABLE II

AVERAGE BAND POSITIONS OBSERVED IN PTA-STAINED BEEF TENDON COLLAGEN

Band	*a*	b_1	b_2	c_1	c_2	*d*	s_1	e_2
Band Position	0.140	0.280	0.400	0.525	0.630	0.775	0.865	1.000

Fig. 3 shows the PATTERSON distributions for the 5 X-ray diffraction patterns of Table I. Fig. 4 shows the PATTERSON distributions for the density distributions of Fig. 2*. In analyzing the PATTERSON distributions it should be noted first that the Patterson for the wet, unstained specimen (Fig. 3, Curve A) is monotonic. It consists of a linearly decreasing portion from 0 to 0.47 L where L is the length of the collagen period, and a horizontal portion from 0.47 L to 0.50 L. This shape suggests that there is no fine structure evident within the fundamental period, and that furthermore the molecular structure responsible for this pattern consists of a nearly rectangular distribution of high electron density of width approximately 0.50 L within the period. Thus, there is one band per period. The Patterson shown for comparison in Fig. 4 (Curve B) is for a band width of 0.53 L.



* It should be stated that a structure in which the bands were regions of low, rather than high, density would have the same Patterson distribution.

Fig. 3. Patterson for wet, unstained collagen (Curve A)
Patterson for wet, 15-minute stained collagen (Curve B)
Patterson for wet, 105-minute stained collagen (Curve C)
Patterson for dry, unstained collagen (Curve D)
Patterson for dry, 15-minute stained collagen (Curve E)

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The detailed peak structure found for wet, stained specimens (Fig. 3, Curves B and C) shows that the electron density here must vary greatly over small distances along the fiber axis. The 105 minute stained collagen Patterson suggests that there may be bands separated by 0.05 L, 0.11 L, 0.15 L, 0.20 L, 0.25 L, 0.34 L, 0.40 L, and 0.45 L while the 15 minute stained preparation shows less well resolved PATTERSON peaks. At present we cannot specify the disposition of bands within the fundamental period in either case.

While not revealing a rectangular density distribution, the curve for dry, unstained collagen (Fig. 3, Curve D) is also monotonic and so again reveals no intraperiod fine structure. SCHMITT AND GROSS' result, in which they find fine structure in unstained collagen, thus suggests that smaller variations in density may be apparent by microscopy than are revealed by X-ray data when they are converted to PATTERSON distributions.

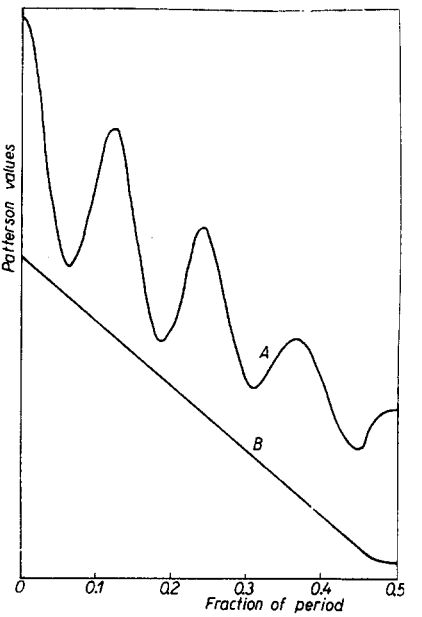

Fig. 4. Patterson for the upper electron density curve of Fig. 2 (Curve A); Patterson for the lower electron density curve of Fig. 2 (Curve B)

The X-ray Patterson for dry, stained collagen (Fig. 3, Curve E) shows considerable evidence for intraperiod fine structure. We find PATTERSON peaks at 0.12 L, 0.22 L, 0.36 L, and 0.46 L suggesting there are bands within the period separated by those distances. The electron microscope Patterson for our 8-band fine structure (Fig. 4, Curve A) similarly shows 4 peaks in nearly the same positions, these being 0.12 L, 0.24 L, 0.36 L and 0.50 L; however, the shapes of the peaks are considerably different. The latter result is not unexpected in view of the assumed idealized electron microscope structure. If one wanted to compare peak shapes, then detailed information concerning the electron density distribution within each band would be required. In the idealized structure, if we were to make the bands narrower, the position of the fourth PATTERSON peak would shift to a lower abscissa while the positions of the other peaks would remain substantially unchanged. If we changed the positions of our bands to a small extent we would get a corresponding change in the positions of the PATTERSON peaks but the number of peaks would remain at 4. The results suggest that the intra-period detail in the micrographs and the diffraction order intensities arise from the same structural features of the collagen and that these features are not appreciably different if the specimen is dispersed as in microscopy or intact as is the case in diffraction.

We are indebted to Professor W. W. BEEMAN, Department of Physics, for allowing us to use the facilities of his X-ray laboratory.

SUMMARY

PATTERSON distributions were constructed from the X-ray diffraction patterns produced by the large periodicity in beef tendon collagen. The Pattersons were interpreted in terms of the intra-period fine structure observable in electron micrographs of collagen. It is suggested that there may

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be a single high density band in wet, unstained collagen and a very detailed band structure in wet, stained collagen. The Patterson from dry, stained collagen is in satisfactory agreement with one calculated from an idealized structure suggested by electron micrographs.

RÉSUMÉ

Des distributions de PATTERSON ont été construites à partir de diagrammes de diffraction de rayons-X produits par la longue périodicité dans le collagène de tendon de bœuf. Les distributions de PATTERSON ont été interprétées en function de la structure fine de la période observable dans les micrographies électroniques de collagène. On suggère qu'il peut se trouver, dans le collagène mouillé et non teint, une bande unique à grande densité et, dans le collagène mouillé et teint, une bande à structure très détaillée. La distribution de PATTERSON du collagène sec et teint est en accord satisfaisant avec celle calculée à partir d'une structure idéalisée suggérée par des micrographies électroniques.

ZUSAMMENFASSUNG

Die Röntgendiagramme der grossen Periode von Rindersehnenkollagen wurden nach der Methode von PATTERSON analysiert. Die PATTERSON-Analysen wurden im Sinne der intra-periodischen, elektronenmikroskopischen Feinstruktur des Kollagens erklärt. Es wird vorgeschlagen, dass im nassen ungefärbten Kollagen ein einzelnes dichtes Band, im nassen gefärbten Kollagen dagegen eine Bandstruktur, die viele Einzelheiten zeigt, vorhanden ist. Die PATTERSON-Analyse des trockenen gefärbten Kollagens ist in guter Übereinstimmung mit einer PATTERSON-Analyse, die aus einer von Elektronenmikrographien hergeleiteten idealisierten Struktur berechnet wurde.

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