IMPROVED PROFILES OF ELECTRON DENSITY DISTRIBUTION ALONG COLLAGEN FIBRILS

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ABSTRACT In order to evaluate fully the meaning of small-angle X-ray diffraction data from collagen fibers in terms of the distribution of molecular substance along fibrillar axes, it is necessary to have some means of determining the phase angles of the several components of the axial diffraction series for combination with measured amplitudes in the formulation of a Fourier series expressing the fibrillar electron density profile. This investigation has developed strip models for fibrillar axial structure based on reported electron micrographic descriptions of how stainable bands and molecular overlap zones ("backgrounds") are located along the fibrils. These models permit the calculation of phases for use with the experimental amplitudes. Once band descriptions (identical widths and density heights plus relative locations) were fixed, three parameters dealing with background width, height, and location were varied to refine the models until they were reasonably capable of accounting theoretically for the observed diffraction amplitudes. Further minor adjustments, indicated by the initial results, finally produced models and profiles for dry and moist kangaroo tail tendon (KTT). The results show that the X-ray and electron optical conclusions regarding collagen fibrillar axial structure are in essential agreement down to a resolution of about 45 Å.

INTRODUCTION

Several attempts have been made to derive the distribution of matter along collagen fibrils by analysis of the axial series of small-angle X-ray diffraction orders of the large (600–700 Å) structural period (Kaesburg and Shurman, 1953; Tomlin and Worthington, 1956; Bear and Morgan, 1957; Ericson and Tomlin, 1959; Ellis and McGavin, 1970). Of course, the pattern of "bands" along the fibril can be directly photographed by means of electron microscopy, and several quantitative descriptions of the locations of the bands have appeared (see particularly Schmitt and Gross, 1948; Burge and Randall, 1955; Nemetschek et al., 1955).

One hopes that eventually such studies, along with chemical investigation of the sequence of amino acids in the collagen molecules, with allowance for molecular "staggering" (see further below) will identify the significance of the bands in chemical terms. The electron optical views provide most directly a visualization of the structure, with various electron stains helping to distinguish some aspects of side-chain distribution. The stains have not thus far been specific enough, nor has the

resolution been sufficiently fine, to provide the desired detail. In addition, the results may be related to normal conditions of the living state only grossly because of the necessarily harsh conditions of observation.

The small-angle X-ray diffraction procedures intrinsically offer the possibility of determining the structure under physiological conditions, with potentially sufficient resolution. Execution of such a program is handicapped however, by the well-known difficulty that the phases of the diffraction orders can not be directly determined, so that totally independent plots of fibrillar electron density profiles are not possible. In addition, the method requires, for best resolution, that the fibrillar structure be sufficiently perfectly reproduced in every repeated unit along the fibril so that the diffraction orders of sufficiently high index are observable. This has, so far, not been the case.

Nevertheless, there is some merit to efforts to apply the small-angle diffraction information to the fullest extent possible, if only to indicate to what degree the electron micrographic conclusions are reliable indications of the native structure. This paper presents a reconsideration of this objective, providing essentially a sequel to the earlier report of Bear and Morgan (1957), who derived electron density profiles of dry and moist mammalian collagen fibrils from a combination of electron micrographic and small-angle X-ray diffraction information, using strip models and optical diffractometry.

This new study employs accurately defined models and computer-assisted derivation and refinement of the models and related electron density profiles. It considers criteria for acceptable solutions and determines these for two collagen structures, namely ones for dry and moist KTT. Results are essentially those reported previously for the dry KTT but an important revision of the profile for moist KTT has been achieved.

Both of these structures have been derived without assumption of a center or cross-sectional plane of symmetry, which is manifestly not present in electron micrographs of collagens and is unlikely from current views of collagen molecular structure and the manner of aggregation to form fibrils. Several other similar investigations have unfortunately and improperly assumed symmetry in order to simplify the phase determination problem (Kaesburg and Shurman, 1953; Tomlin and Worthington, 1956; Ellis and McGavin, 1970). Because of the centrosymmetry of a major aspect of fibrillar structure (the backgrounds described below), this simplification permits consideration of this feature, but it does not reliably deal with the important finer bands revealed by electron stains and contributing significantly to diffraction orders beyond about the third.

PROCEDURES

Transforms (which are proportional to diffraction order amplitudes or to square roots of intensities) are functions of real (model) structure and the reciprocal (dif-

fraction) structure. In strip models, the single jth strip contributes an amplitude to the hth diffraction order proportional to

$$F_j(h) = \rho_j d \int_{u_{1j}}^{u_{2j}} \exp(i2\pi hu)\, du,$$

where d is the repeating period of the total structure, ρ_j is the constant electron density of the strip, and u is a variable of axial position through the strip expressed as a fraction of d. The integration extends from one side of the strip, u_{1j}, to the other, u_{2j}. In these considerations the strips can not be referred to an origin which is a center of symmetry, so that

$$F_j(h) = A_j(h) + iB_j(h),$$

where

$$A_j(h) = \rho_j w_j d \operatorname{sinc}(\pi h w_j) \cos(2\pi h u_{0j}),$$

and

$$B_j(h) = \rho_j w_j d \operatorname{sinc}(\pi h w_j) \sin(2\pi h u_{0j}).$$

The width of the strip is $w_j = u_{2j} - u_{1j}$, and its center is located at $u_{0j} = (u_{1j} + u_{2j})/2$. The symbol sinc x stands for $\sin x/x$ (cf. Worthington [1969] who was, however, considering centrosymmetric models for nerve myelin, where this is appropriate).

The transform of the total model is the sum of the transforms of the several strips constituting one repeating unit:

$$F(h) = A(h) + iB(h),$$

where

$$A(h) = \sum A_j(h),$$

and

$$B(h) = \sum B_j(h).$$

In what follows the special strip representing background is given the subscript b instead of j, but the summations include it also.

One can write for computational processing:

$$F(h) = [A^2(h) + B^2(h)]^{1/2} \exp i\Phi(h),$$

where $\Phi(h)$ is a phase for the hth order, given by $\tan \Phi(h) = B(h)/A(h)$ with the signs of $B(h)$ and $A(h)$ used to place Φ in the correct trigonometric quadrant.

A common test for the success of a model is the minimization of the residual

$$R = \frac{\sum_h | F_0(h) - F_c(h) |}{\sum_h F_0(h)}.$$

Refinement of a model consists of choosing parameters so that the differences between square roots of observed and calculated intensities, the amplitudes $F_0(h)$ and $F_c(h)$, respectively, for all orders, are reduced to as near zero as possible. In order to do this the observed and calculated intensities must be similarly scaled. It is well known that corrections of the observed intensities corresponding to the Lorentz (geometrical conditions of the pattern registration) and X-ray polarization factors are not necessary under the usual procedures employed in small-angle observations with collagen (cf. Blaurock and Worthington, 1966). It is, however, necessary to scale the two sets of intensities so that the sums within each set are identical.

Finally, with parameters of a model adjusted to a minimal residual we combine the observed amplitude data with phase angles calculated from the model to determine an electron density profile, which yields $\rho(u)$, the density of electrons (in arbitrary units) at each location u through the typical macroperiod ($u = 0$–1).

$$\rho(u) = \sum F | (h) | \cos [2\pi hu + \Phi(h)].$$

Although most interest attaches to the result developed when the observed amplitudes $F_0(h)$ and calculated phases $\Phi(h)$ are used, there is some value to obtaining also a profile in which calculated amplitudes $F_c(h)$ are employed. The difference between the two profiles then indicates where the experimental intensities are suggesting some departure from the postulated strip model. This process is equivalent to the use of "difference Fourier plots," commonly employed by crystallographers for similar purposes. The electron density profiles, since they lack the zero-order ($h = 0$) term, then only indicate departures from average density in arbitrary units.

Insight into how strip parameters influence diffraction amplitudes can be gained by noticing that the term sinc $(\pi h w_j)$ of the single rectangular strip transform is unity for $h = 0$ but becomes zero whenever hw_j is a positive or negative integer. Consequently, whenever a strip is of width which is some integral multiple m of $1/| h |$, the strip will contribute no amplitude to the m/w_jth orders. For example, if w_j is 1/15 (or 0.067), as in the bands strips used here, then the amplitudes provided to diffraction orders whose indices are multiples of 15 will be zero for all band strips, independent of their location in the model macroperiod. This characteristic of strips also causes the background strip developed below for wet collagen, whose width approximates one-half, to contribute little amplitude to the lower even orders,

corresponding to observation. In the dry collagen model the background width is nearly four-fifths, hence orders whose indices are multiples of five will receive little amplitude from the background, as also is observed. The adjustments of background parameters to achieve minimal residuals involve departures from these simple considerations to develop relationships between bands and backgrounds which provide appropriate amplitudes at all orders under consideration.

As did Bear and Morgan, in this study we limited the range of diffraction orders under consideration to those with indices 1–15, which includes the strongest observed diffractions yet reported. Higher orders are relatively very weak, and indeed, published values for intensities are so low that results drawn herein would be little influenced by them. Nevertheless, it should be recognized that consideration of experimental difficulties with regard to appropriate tilting of the fibrous specimens so that proper relations with the sphere of reflection cause optimal diffraction at higher angles, and the possible correction for the pseudotemperature decline of observed intensities at the higher orders, might in the future bring this information into greater significance and usefulness. At present the limitation to 15 orders has the result of setting the resolution of the electron density profiles to about $d/15$ or 43–45 Å, which is actually the band strip widths used here and approaches the resolution obtained in many electron micrographs of collagen fibrils.

If an initial strip model is close to biological reality, there is an expectation that refinement guided by reduction in the residual will eventually lead to the best structure. However, because of the continuous nature of the electron density profiles in such cases, lacking recognizable molecular structures, and the paucity of intense diffractions, there may be several possible starting models that will lead to reasonably low R factors. Hence, unlike the single crystal case, R factor considerations alone do not necessarily imply a correct solution to the diffraction problem. A "ground rule" that must be followed is that of keeping the number of variables to a minimum, i.e., keeping the model as simple as possible consistent with the available data. It is very tempting to vary all peak heights and widths independently, but this rapidly leads to a badly underdetermined situation.

Bear and Morgan (1957) readily achieved a reasonably satisfactory explanation of observed intensities for KTT from a rather simply derived starting model based on reported band positions gained from electron microscopy, with superposition of a more extensive background covering a number of the bands. Models of this kind would maximally require the following parameters for specification: n bandwidths w_j, where n is the number of bands; n locations of band centers u_{0j}; n band density magnitudes ρ_j; two locations, u_{1b} and u_{2b}, for the edges of the background strips; and ρ_b the density magnitude along the background; a total of $3n + 3$ parameters.

We have reduced the number of adjustable parameters, as did also Bear and Morgan, by fixing certain of these for the initial trial models as follows: n was limited to the seven major bands reported from electron microscopy and con-

ventionally designated as a_1, a_2, b_1, b_2, c, d, and e (Schmitt and Gross, 1948; Nemetschek et al., 1955); bandwidths were kept as the same single parameter fixed at $w_j = 1/15$, since this restricts band-scattered amplitudes to appreciable values within the first 15 orders whose observed intensities constitute the major diffraction being considered; band center locations were also fixed according to electron microscopic indications at $u_{0j} = 0.30, 0.39, 0.54, 0.64, 0.84, 0.00$, and 0.16, respectively, (band d is at the macroperiod origin); and all band density magnitudes were assumed identical, with a reference density of unity assigned thereto ($\rho_j = 1.00$). This leaves adjustment necessary for only three parameters during refinement: those defining background edges and density magnitude, namely, u_{1b}, u_{2b}, and ρ_b. Background widths, $w_b = u_{2b} - u_{1b}$, were further expected to approximate one-half and four-fifths for wet and dry states, respectively, for reasons given above.

In this investigation the calculations were performed on a Hewlett-Packard model 1900B programmable calculator (Hewlett-Packard Co., Palo Alto, Calif.), which is interfaced with a teletypewriter to provide output. This method has the advantage of ability to specify model parameters as accurately as desired, to permit survey of a large number of structures quickly, and to yield reliable quantitative results, in contrast to the dependence on accurate drawings, intricate darkroom operations, and qualitative judgments based on visual inspection, characteristic of the optical diffractometric procedures. The studies of Bear and Morgan used unpublished observed intensities for KTT determined by O. E. A. Bolduan and T. C. Furnas, Jr., which are in essential agreement with the published data of Tomlin and Worthington (1956) used in this study.

RESULTS

Preliminary considerations with dry KTT started with the parameters proposed by Bear and Morgan (model 1 of Table I; $R = 20\%$). Some satisfaction with the band locations and widths of this model was derived from various attempts to vary these parameters, none of which improved the residual. Modest refinement of the background coordinates, with one "forward" edge at $u_1 = 0.187$ and the other "rear" edge at $u_2 = 0.962$ (width accordingly being 0.775 and center at 0.575), with background height/band height ratio at 1.5, dropped the residual to 13% (model 2). No simple change was found to lower the R factor further, so these coordinates were taken as final at this stage. Fig. 1 *a* presents the corresponding electron density profiles based on calculated and observed intensities, both with calculated phases.

It was found during the course of this refinement, and during an attempted least square minimization of the R factor, that the parameters could not be considered independent of each other. That is, for a given ratio of background height/band height, R min would not in general be at the u_{1b} and u_{2b} corresponding to R min for another model. This difficulty may arise from the fact that the representation of bands and background by simple rectangular strip functions is somewhat arbitrary.

TABLE I

PARAMETERS AND RESIDUALS OF STRIP MODELS

Bands included	Background parameters					c_1 height	Residual R
	u_{1b}	u_{2b}	Width w_b	Centered at u_{0b}	Height		
							%
For dry KTT							
1. Basic set	0.230	1.010	0.780	0.620	1.50	—	20
2. Basic set	0.187	0.962	0.775	0.575	1.50	—	13
3. Basic set plus c_1	0.187	0.962	0.775	0.575	1.66	0.3	12
For hydrated KTT							
4. Basic set	−0.090	0.450	0.540	0.180	2.5	—	23
5. Basic set	0.397	0.847	0.450	0.622	2.5	—	26
6. Basic set minus b_1	−0.095	0.445	0.540	0.175	2.5	—	16
7. Same as 6 plus c_1	−0.095	0.445	0.540	0.175	2.8	0.5	13
8. Basic set minus d, a_1	0.397	0.847	0.450	0.622	2.5	—	15
9. Same as 8 plus c_1	0.392	0.847	0.455	0.620	2.5	0.5	13

Explanation: The basic set of bands centers them as follows: d, 0.00; e, 0.16; a_1, 0.30; a_2, 0.39; b_1, 0.54; b_2, 0.64; c, 0.84. The extra band c_1 is centered at 0.727 for dry and at 0.747 for wet KTT. Bandwidths are uniformly 0.067. u_{1b} and u_{2b} locate the forward and rear edges of the background, respectively. Locations and widths are expressed as fractions of the macroperiod. Background heights are given in relation to the heights for the basic band set, held at unity for all but c_1. The residual is defined in the text; minimal values identify favored models. Models 1 and 4 are those of Bear and Morgan (1957); others are introduced in this study.

Since the background occurs over a region where bands are most concentrated, its addition to the model in effect corrects for inadequacies of detail in the assumed bands. Variation of band parameters can then be compensated to some extent by background alterations.

The wet KTT provided a more interesting case. The original solution of Bear and Morgan kept the same band structure as in the dry case but changed the magnitude, width, and position of the background. The solution was obtained by selecting the best match between the model's optical diffraction pattern and the X-ray diffraction pattern. The background coordinates were $u_{1b} = -0.09$ and $u_{2b} = 0.45$ (i.e., width 0.54 and center at 0.18), with background height elevated to 2.5 times band height. The intensity match between optical and X-ray diffractograms was reasonably satisfactory, though the resulting model placed background peaks and troughs over almost entirely different bands from those indicated in the dry model. In addition, other investigations (Ellis and McGavin, 1970; Tomlin and Worthington, 1956; Ericson and Tomlin, 1959) have favored the complement (to unity) of the above 0.54 background width, or 0.46, which would be in better agreement with the original figure of 0.4 for the width of the denser molecular overlap regions proposed by electron microscopists (Hodge et al., 1965).

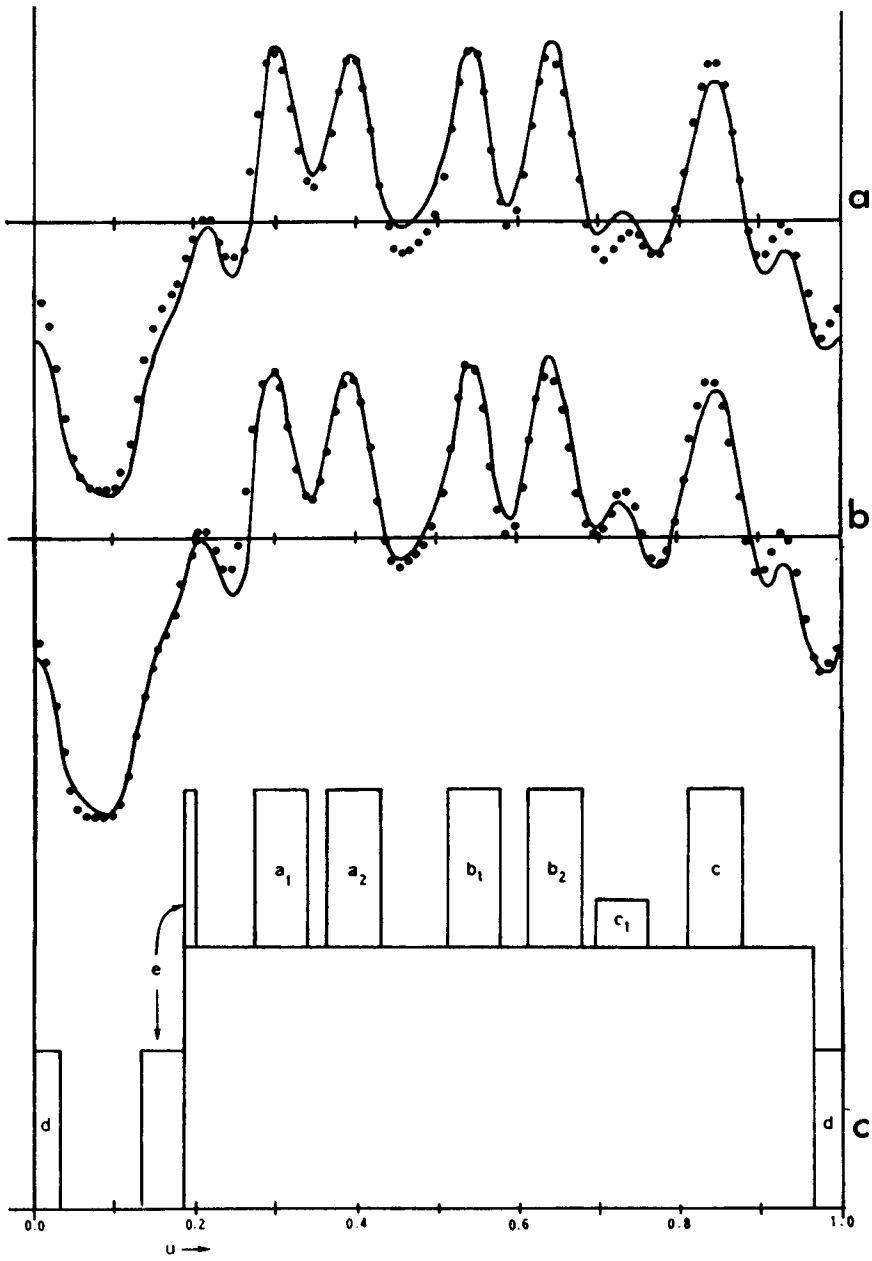

FIGURE 1 Electron density profiles for dry KTT. Solid lines are derived from observed amplitudes and calculated phases, dots from calculated amplitudes and phases. Abscissas are fractional positions within the macroperiod; ordinates are electron densities in arbitrary units above and below average values (horizontal lines) for each pair of curves: (*a*) model 1, (*b*) model 3, and (*c*) shows the strips of the model representing bands and background for model 3.

It is well known that two structures that are the negative of each other, in the photographic sense of possessing density in one where it is absent in the other, and vice versa, will yield identical diffraction patterns. Since in the moist collagen model the background strip dominates, it is possible that the earlier model mistakenly employed the negative of the correct background representation. In either case the background width remains near 0.5 so that, wherever placed relative to the bands, the background diffraction contributes heavily to odd orders. This is a notable characteristic of all known small-angle X-ray patterns of moist collagens (see Marks et al., 1949). Decision between positive or negative backgrounds rests upon, as Bear and Morgan were aware, locating one or the other relative to the bands so that the background amplitude reduces band contributions to make the even orders of modest intensity. This was particularly necessary at the sixth order, and the final choice made previously was based largely on this criterion.

In the present study a starting computation was made using the original Bear-Morgan parameters, model 4, which resulted in an R factor of 23%. Then background approximating the negative of the initial one was used. After variations in width and location seeking the minimum R, model 5 with $u_{1b} = 0.397$, $u_{2b} = 0.847$ (i.e., width 0.450, center at 0.622) was found with R of 26%. Neither of these is as good as might be hoped, but a calculation of residuals based on the sensitive even orders alone resulted in values of 19 and 46%, respectively, indicating why Bear and Morgan found their original solution more satisfactory.

It was clear that models 4 and 5 described above provide the best ones available as long as the simple rules for model formation initially adopted are maintained. To inspect these two models for possible minimal changes yielding more satisfactory residuals, the electron density profiles using observed and calculated amplitudes with calculated phases were compared. As Figs. 2 *a* and 3 *a* show, it then appeared that model 4 (Bear-Morgan model) might be improved if the b_1 band were greatly diminished in height, while in model 5 bands d and a_1 should be considerably weakened and possibly a new band added approximately midway between b_2 and c. Since the models for moist KTT collagen have assumed band structure to be carried over unchanged from the dry to the wet situations, which is without experimental confirmation, it seemed legitimate to accept the indications of the computations, so the diminished bands were removed from the models (models 6 and 8). It is quite possible that flooding of the natural fibrils with water may cause structural alterations which affect some band regions or reduce their density differences against surroundings. Computations with the models modified in these ways yielded R values of 16 and 15% for the modified models 6 and 8, respectively.

Further modification of models 6 (to 7) and 8 (to 9) introduced a new band between b_2 and c at half the normal band height. Experimental justification for this is found in the data of Nemetschek et al. (1955) who frequently observed a band designated c_1 in this region. These modified models 7 and 9 with some bands removed and c_1 introduced at low heights, yielded residuals of 13%.

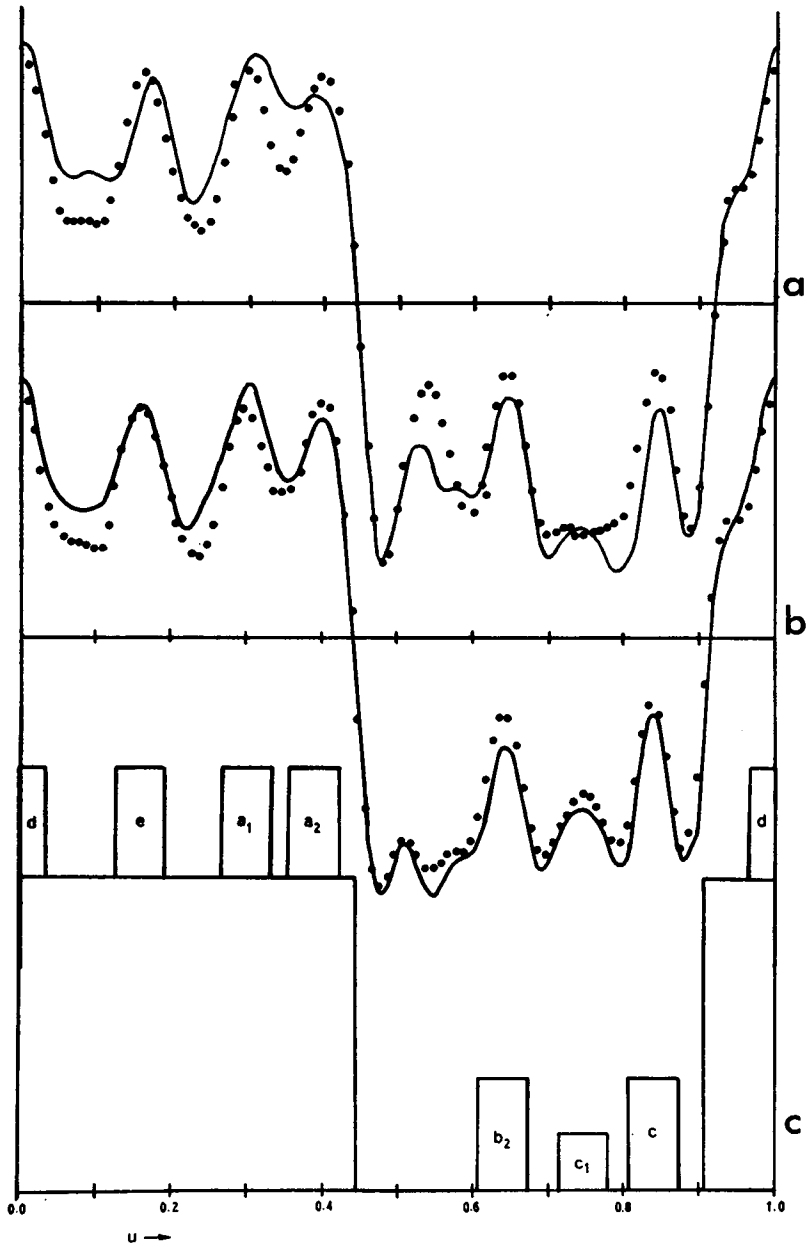

FIGURE 2 Using the same conventions as in Fig. 1, this figure shows results for (*a*) the Bear-Morgan model for moist KTT, model 4; (*b*) the same model modified, model 7; and (*c*) the strip model corresponding to model 7.

Since the residuals of the modified models 7 and 9 were still not convincingly selective, though somewhat improved, further testing by means of residuals based

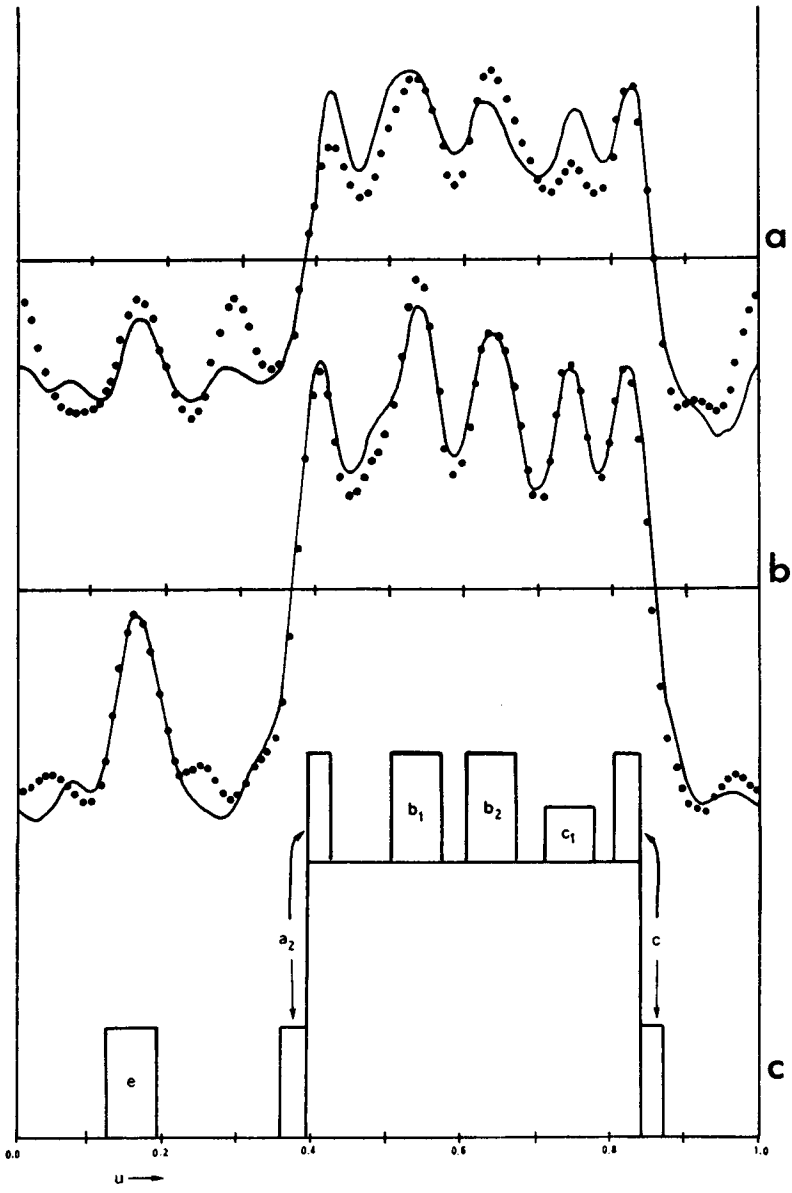

FIGURE 3 Using the same conventions as in Fig. 1, this figure shows results for (*a*) the initial model for moist KTT with background approximately inverted, model 5; (*b*) the fully refined model 9; and (*c*) the strip model corresponding to model 9.

only on the more sensitive even orders was made. These partial residuals were 17 and 26% for the two cases, respectively.

TABLE II

OBSERVED AND CALCULATED INTENSITIES AND CALCULATED PHASES FOR DRY AND WET KTT SMALL ANGLE DIFFRACTIONS

Order index	Dry KTT			Wet KTT		
	Observed intensities*	Model 3		Observed intensities*	Model 8	
		Intensities	Phases		Intensities	Phases
			rad			*rad*
1	1,000.	885.	2.7631	1,000.	987.	3.7377
2	349.	436.	3.4046	13.	26.	2.3329
3	194.	217.	−0.7964	141.	119.	2.9943
4	35.	17.	2.4454	8.	0.4	3.1236
5	13.	15.	−0.3547	38.	40.	−0.4734
6	70.	54.	0.1082	7.	7.	.6344
7	23.	34.	−0.3815	12.	21.	−1.1291
8	36.	41.	1.2395	9.	15.	1.1118
9	72.	73.	4.1887	22.	22.	3.9697
10	11.	24.	2.0767	7.	17.	2.9384
11	41.	52.	0.6075	3.	3.	0.6481
12	15.	6.	−1.5175	9.	8.	−1.3206
13	7.	9.	0.2424	0.5	0.3	0.3475
14	5.	3.	0.9618	2.	4.	3.3532
15	3.	7.	0.9439	1.5	1.	4.1660

* Data of Tomlin and Worthington (1956).

Though the modified Bear-Morgan model (model 7) still seemed to warrant some preference, this was not deemed sufficient to overcome its complete variance in background position relative to the dry situation. Nevertheless, it is interesting that two almost equally successful models can be formulated. It is unlikely that others which bear some resemblance to electron microscopical reality can be found. Accordingly, we consider that the one (model 9) which places background more like the situation in the dry condition (model 2) is preferable for the wet case. The preferred model for wet KTT is shown in Fig. 3 *b*.

Since the c_1 band's presence was indicated for the moist KTT structure, a return to the dry structure was suggested, with the minor modification of the band system. Some justification for it was apparent from comparison of profiles for observed and calculated amplitudes in Fig. 1 *a*. The strip model for dry structure, thus modified, yielded a slightly improved R of 12%. Fig. 1 *b* shows the profiles for this refined structure, model 3.

Table I summarizes the parameters of the strip models which have been cited above, along with the residuals appropriate to each. Table II compares the observed and calculated intensities for the dry and moist collagen models selected as best in

this investigation. Figs. 1–3 present the electron density profiles and the most advanced strip models for each set.

DISCUSSION

During the course of this work we have examined a number of other models departing materially from the ones described above, with attempted refinement. It was interesting to observe the behavior of the *R* factor as the refinement proceeded. If the starting model was grossly incorrect, the *R* factor would hover around the 50% mark, and no minor structural changes would improve it. Once a more or less correct structure was found, *R* would fall to about the 30% level. Correction of errors, such as extra bands, and improvement of the background would drop *R* to less than 20%, and then routine and relatively small changes would lower *R* to about 15%. We feel at this time that if *R* can not be brought below about 20%, the model is probably wrong in some fairly essential feature.

Again, because of the paucity of available data and because of the crudity of the models, great care must be used in the introduction of any parameters, and they must have a reasonable physical basis. Furthermore, as Ellis and McGavin (1970) have pointed out, an electron density profile based on observed amplitudes at whatever phases, can always be used as a model and parameters derived to describe it accurately, whereupon *R* would be reduced to zero. The result then is, of course, quite meaningless.

The present models have been based on electron micrographic evidence and rather readily could be refined, chiefly by means of variations in the breadth, height, and location of the background (three-parameter variation). Band descriptions generally remained unaltered, though the initial choice of bandwidth at 0.067 was designed to taper off order intensity so that band contributions became zero at the 15th order, where the series termination occurred. The only band variations allowed were the removal of one or two and the insertion and height variation of the relatively weak c_1 band toward the end of the refinements, when the computations as well as electron micrographic evidence suggested these might be permissable.

The essential results of this study can be seen in Figs. 3 *b* and 1 *b*, for KTT collagen in moist (physiological) and dry conditions, respectively. The electron density profile for dry KTT collagen is very nearly the same as that derived by Bear and Morgan (1957), differing slightly in the background coordinates and in the addition of the weak c_1 band. Change of major significance, however, has been made in the profile for moist KTT reported. This is in better agreement with the location of the background in the dry condition, as seems more reasonable than the earlier indication that the backgrounds of the two might be considerably out-of-phase.

In the physiological state, the macroperiod of the typical collagen fibril is about 670 Å, unless stretched (see Rougvie and Bear, 1953). The predominance of odd orders in the lower-order small-angle X-ray diffractions is the direct evidence that a

broad background, occupying 0.46 of the macroperiod or 308 Å, is present. Since quite widely different collagens which have yielded small-angle diffractions show this pronounced emphasis of the odd orders (Marks et al., 1949; Bear, 1952), this kind of background component of the fibrillar structure must be a relatively constant feature of those collagen fibrils which show axial periodicity of molecular arrangement (some "secreted collagens" do not; Bear, 1952).

Tomlin (1955) first described the evidence for a background strip to overlapping of molecular ends ("interstitial" segments) alternating with regions where molecular ends do not make contact ("defects") along the fibril. Subsequent investigations by electron microscopy (Hodge et al., 1965) indicated that molecular overlaps and end gaps are essential aspects of fibrillar structure resulting from the fact that lengths of collagen molecules are not integral multiples of the native fibrillar macroperiod. These studies indicated a molecular length of 4.40*d*, so that in addition to the molecular staggering required to develop the 670 Å native macroperiod from the longer (approximately 3,000 Å) molecular lengths (Schmitt et al., 1955), the ends of consecutive molecules must pull apart by 0.60*d* to fit the macroperiod, developing "hole zones" of this size, in each period, with corresponding fully overlapped zones of 0.40*d*. Since these figures are obtained from electron micrographs, they are probably somewhat in error, and one expects the X-ray values to be more appropriate for the physiological condition. Consequently, we conclude that collagen molecules are 4.46*d* or 2988 Å long in the native hydrated condition within fibrils.

The present model for moist KTT collagen assigns the complement (to unity) of the denser overlap zones (0.46*d*) to the less dense hole zones (0.54*d*). The decreased density is to be expected at the latter zones because some collagen molecular segments (electron density averaging 0.45 electrons per $Å^3$) are replaced by dilute aqueous solutions (0.34 electrons per $Å^3$). If one out of five molecular segments in a cross section at a hole zone is replaced by water, then the decrease in density overlap and hole zones is only about 5 %. This gives some idea of the ordinate scale in Fig. 3, which is otherwise arbitrarily scaled.

When the KTT collagen fibrils dry, their macroperiod normally shrinks to about 640 Å, though under extreme conditions it may become as low as 603 Å (Rougvie and Bear, 1953). Then the simple alteration of odd and even order intensities disappears and as Bear and Morgan (1957) showed, intensities of orders beyond about the third are in large part determined by the band structure. Nevertheless, it is still necessary to postulate a background, which must be taken in such a way as to develop the strong first three orders without significant influence near orders of indices 5 and 10. This requires either a background trough or crest of width about $d/5$. As shown in the original study and confirmed here, refinement leads to a background strip covering 0.78 of the macroperiod or normally about 500 Å. In other words, during drying the apparent overlap region has increased from 0.46 to 0.78*d*, or in absolute terms from 308 to 500 Å. Conversely, the hole zone gaps between molecular ends decrease from 362 to 140 Å. During drying 222 Å of original hole zone disap-

pears in favor of appearance of 192 Å overlap zone. It is well known that drying also corrugates the fibril (see Gross and Schmitt, 1948; Bolduan and Bear, 1951), since the overlap zones retain greater diameter than do the hole zones after drying. The changes of axial length at the borders between these two zones would conserve substance if the hole zone/overlap zone ratio of diameters were 0.93 after drying, on the assumption of equal compaction at both zones.

Since electron micrographs of collagen fibrils are necessarily achieved for dry material, it is somewhat surprising that the estimates of hole vs. overlap zone lengths obtained in this way have been fairly close to the X-ray values for moist fibrils (0.6/0.4 as compared with 0.54/0.46). They might have been expected to be nearer the 0.22/0.78 relationship indicated by the X-ray studies for dry fibrils. Indeed, Spadaro (1970) has examined rat Achilles tendon fibrils electron microscopically, unstained and negatively stained, concluding that earlier conclusions, based principally on negative staining, may have been in error. He would reverse the designation of the zones, arriving at a 0.42/0.58 ratio in a specimen of macroperiod 655 Å. Spadaro's claim, however, that in negative staining the stain emphasizes the overlap zones instead of the holes remains puzzling. In any event, it appears that modern methods of preparation for electron micrographic study may preserve hole/overlap relationships better than in simple drying of massive specimens (as done for X-ray studies).

A comparison of the models and profiles for wet and dry collagen gives rise to concern regarding the significance of the relatively much longer background in dry fibrils. It will be noticed from Figs. 1 and 3 that the process of drying causes a compaction of the hole zone such that bands *d* and *e* now become "plastered" against the ends of the background with the gap between *d* and *e* remaining as hole zone. Thus, the dry fibril's background probably has different significance from that of wet fibrils; the latter is a true molecular overlap zone, while the former consists of overlap plus compaction of hole zone. One wonders also whether the bands *d* and a_1, which are not apparent in the refined wet model, may not appear in the dry case from alterations occurring in the compaction process. Band *e* is the only one in that general region which remains under both conditions.

In any event, as to whether or not presently derived details can be interpreted in terms of molecular alterations with confidence, it should be cautioned that the crudeness with which strips can represent what is in reality a more smoothly varying molecular distribution presents some difficulties. It has been pointed out elsewhere above that, since the backgrounds occur where bands are most concentrated, their strip representation may correct to some extent for inadequacies of detail in the assumed bands. It seems more legitimate in molecular terms to represent the wet fibrillar background in terms of a strip, than in the dry case, since the predominance of lower odd orders in the diffraction patterns of wet collagen and the electron micrographs of negatively stained fibrils, showing relatively distinct borders to overlap

and hole zones, give consistent and compelling indications that the background is well defined.

In the course of this investigation we also investigated models in which the rectangular band strips were replaced by gaussian distributions of density about band centers and the background ends were given gaussian "rounding." The results offered no significant changes in the electron density profiles or improvement in R factors, so are not reported here. Possibly when profile resolution can be improved by inclusion of higher order diffractions the use of gaussian band and background edges will be justified.

Since the "positive" and "negative" relationships between the background and the bands gave rise to two nearly equally successful models for moist fibrils, it is necessary to consider whether the same situation exists for dry fibrils. Is the background of dry collagen correctly placed relative to the bands, or might a narrow negative of the background, as formerly proposed by Ericson and Tomlin (1959), provide a nearly equally appropriate solution? The most compelling reason for believing that the choice has now been made correctly for dry collagen, hence also for wet collagen, can be derived from examination of published electron micrographs and densitometer traces of stained fibrils. For example, Abb. 1, 2, and 15 of Nemetschek et al. (1955) show clearly that minimum density along phosphotungstate-stained fibrils occurs on either side of band *d*. Our profiles (Fig. 1 *b*) agree in this respect, though they tend to emphasize the trough between *d* and *e*.

Ericson and Tomlin (1959) have objected to the procedure of deriving band locations from stained fibril electron micrographs for use in interpreting diffraction patterns of unstained fibrils, as has been done here. Their study has the merit of attempting to employ diffraction data alone, on unstained and stained wet material, using Patterson profiles and difference profiles to locate the relative positions of stainable bands. The stained bands were then moved relative to background until residuals for diffraction intensities of the wet stained material were minimized. Finally, attempts were made to correlate the result with electron micrographs of the same (but dry) specimens, without notable success.

The conclusions of Ericson and Tomlin (their Fig. 10 *b*) resemble ours (Fig. 3 *b*) in several respects: in the adoption of background of width 0.46 of the macroperiod, in the location of one band (*e*) in the middle of the hole zone, and in the positioning of two strong bands (a_2 and *c*) near the edges of the background. Two other weaker bands on the top of the background bear no resemblance to our three (b_1, b_2, and c_1) and hence differ from the results of other electron micrographic studies.

Patterson profiles, although they develop directly from diffraction intensities and thus escape the phase difficulty, are notoriously lacking in ability to resolve interband vectors in the complex continuous density distribution encountered with collagen fibrils. For example, in the 7-banded initial model assumed here, there should be 22 interband vectors, with 19 different values showing in the Patterson profile, whereas

Ericson and Tomlin (1959) resolved only 6 for phosphotungstate-stained collagen, indicating considerable overlap and a consequent possibility of erroneously concluding that band structure is simpler than in reality.

In any event, studies based on diffraction data for stained material will still lack meaning for the native state if correspondence between electron micrographic evidence for band positions in stained specimens can not be carried over to aid the X-ray interpretations on unstained material. Indeed Spadaro's (1970) comparisons of unstained and stained material in the electron microscope indicate that the staining does indeed reveal the same locations of bands as in unstained preparations.

It is worth emphasizing that all investigations attempting to consider the significance of the small-angle axial X-ray diffraction of collagen have clearly indicated that the fibrillar structure must contain the two kinds of component, bands and background, for adequate explanation. Since the background is the grosser aspect, it is understandable that from the earliest beginnings of the electron microscopy of collagen relatively wide dense zones were resolved, designated *D* by Wolpers (1943) and *A* by Schmitt et al. (1942) and alternating with lighter zones, labeled *H* and *B*, by the same investigators, respectively. Grant et al. (1965) call the *A* zones bonding regions and the *B* zones nonbonding ones. The nomenclature of Tomlin and of Hodge et al. has already been mentioned.

Though often called bands, these relatively wide regions might better be called zones to distinguish them from the narrower bands revealed by positive electron stains such as phosphotungstate. Bands are believed to be locations of the relatively large acidic and basic residues (Bear, 1952) while the regions between bands (interbands) contain predominately the smaller and less polar residues, along with considerable amounts of imino acid residues (Hannig and Nordwig, 1967). Glycine is, of course, in both, as required by the triple chain coiled-coil molecular structure. Further relationships between chemical composition and the electron density profiles are difficult to establish at this time, though this may be achieved when profiles of better resolution are derived and studied in relation to amino acid sequences of the collagen molecule's three α-chains. The sequence information is now being accumulated in several laboratories and is expected to be available in the near future. Since much is known about how the three α-chains form the molecule, and how the molecules aggregate along the fibril, the correspondence between band locations of the profiles and the placement of clusters of electron-dense residues should be capable of examination.

The electron density profiles presented here for a specific collagen (KTT) should have some degree of general significance for all mammalian collagens, and may be regarded as probably similar to those of other vertebrate cases, such as the fibrils of teleostean fish ichthyocol. This follows from the fact that the most direct diffraction evidence related to band distributions is found in the relative intensities of the third and higher diffraction orders obtained from dry specimens; in the mammalian cases, and to a lesser degree in ichthyocol, the small-angle patterns are similar

in this respect (Bear, 1952). The more primitive elasmobranch fish elastoidin (from shark fins) and other collagens from echinoderm and coelenterate sources show marked variations from the typical mammalian collagen intensity distribution (Marks et al., 1949).

On the other hand, the part of the profile in the *wet* case which is contributed by the background or overlap zone configuration is probably of considerably more generality over all collagens which show macroperiods ("secreted collagens" do not; see Bear, 1952). The diffraction evidence for this feature is the alternation of order intensities, with odd orders strong, even orders weak, observed with wet specimens as far afield from vertebrate examples as in the collagen of the echinoderm *Thyone* (sea cucumber) studied by Marks et al. (1949).

All of these collagens are identifiable as such from their completely general wide-angle diffraction, which relates to their common molecular coiled-coil structure consisting of three polypeptide chains (see Rich and Crick, 1961). The implication of the widespread occurrence of similar native macroperiods and overlap zones is that molecular dimensions and modes of aggregation are also very nearly the same in all, despite differences due to chemical composition and side-chain distribution evident from the variations in band patterns.

Electron micrographs achieve band resolution normally through application of electron stains, while the small-angle X-ray studies are carried out on unstained fibers. This present investigation has, nevertheless, with relatively simple carry-over of information from the electron optical field, been reasonably successful in accounting for small-angle X-ray diffraction order intensities in both wet and dry unstained situations. These results may, conversely, be taken as evidence that the X-ray diffraction data support the observations in electron microscopy, at least to a resolution of about 45 Å.

Finally, it should be reiterated that the ordinates of the profiles in Figs. 1, 2, and 3 are completely arbitrary, and can not be compared between figures. The profiles represent variations above and below average fibrillar electron density, based on unit magnitude for most band heights above background. It is quite probably, as Ericson and Tomlin (1959) and others have suggested, that bands in wet fibrils depart in density from the absolute density of the overlap and hole zones by rather small amounts. Probably also, the difference is materially enhanced upon drying.

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