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An X-ray Study of α-Keratin. II. Diffractometer Measurements of the Complete Diffraction Pattern of Canadian Porcupine Quill

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The variation of scattering power has been measured along a set of directions in reciprocal space inclined at angles to the fibre axis increasing in steps of 10° from 0° to 90° inclusive. A focusing transmission-specimen technique and filtered Co $K\alpha$ radiation were used. The radial intensity distribution derived from the measurements is quite similar to that of serum albumin described by Arndt & Riley.

Introduction

Up to the present time the X-ray diffractometer has been little used in the measurement of the diffraction patterns of fibrous proteins. It appears, however, to provide a promising technique for such studies, for which it offers many advantages over conventional photographic methods. It is not intended to imply that the diffractometer should replace film recording of fibre patterns in all applications, but rather that it is an extremely valuable adjunct to the older methods. The most informative results are obtained by working the two techniques together on the same substance under investigation. Some useful characteristic features of the diffractometer fibre technique are made evident through a comparison with the established photographic technique.

Most materials showing a fibre diffraction pattern are also fibrous in physical form. In the camera it is both natural and convenient to mount such specimens parallel to the axis of the specimen-support and normal to the X-ray beam, the patterns so recorded being analogous to single-crystal rotation diagrams. Photographs obtained with this simple technique, which has been the standard practice for thirty-five years, serve adequately for the identification of fibres and for the study of transformations and variations in crystallinity. Difficulties arise, however, when an attempt is made to analyse a structure in detail. The best hope of accomplishing this lies with the best-ordered fibres, those whose diffraction patterns approach most closely the single-crystal rotation diagram. Use of the simple normal-beam photographic technique under such conditions is incompatible with an adequate collection of diffraction data, since the better developed the pattern the more reflections there are that do not enter the tore swept by the Ewald sphere. Yet it has been customary to describe only the normal-beam pattern of many comparatively well-ordered materials; this has led, as a consequence, to the long neglect of the significant high-angle 1·5 Å meridional reflection. In order to observe this and other reflections outside the tore, the fibre axis must be inclined to the X-ray beam at various angles other than 90°, and a number of photographs should be taken to ensure that the whole region within the limiting sphere has been covered. The experimental time requirements are thereby multiplied, and the patterns so obtained lack the simple geometrical interpretation of the normal-beam photographs. It is in these circumstances that the diffractometer method may be used to advantage.

In the diffractometer the X-ray source, the specimen and the detector together define the directions of incident and diffracted rays. It follows that the normals to planes contributing to the diffracted intensity being recorded must lie in the scanning plane and bisect the angle between the lines joining source to specimen and specimen to detector. Thus, for every angular setting of the detector, the direction of these plane normals is precisely defined in space. If the specimen is rotated about the diffractometer axis at half the angular speed of the detector, the reflecting-plane normals then remain fixed in direction relative to the physical axes of the specimen. By varying the initial orientation of the specimen systematically, the diffracting power may be explored along any chosen reciprocal-lattice direction. For example, with the fibre axis parallel to the diffractometer axis the equatorial reflections, from paratropic planes, are measured, and with the fibre axis aligned with the above-mentioned bisector it is the reflections from diatropic planes which are recorded.

This paper gives an account of diffractometer measurements made on quill tips of Canadian porcupine, employing a special technique suitable for fibre studies. A number of directions in reciprocal space has been examined sufficient for the description of the whole diffraction pattern. The average radial

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intensity distribution has been derived from these measurements and compared with the distribution for serum albumin given by Arndt & Riley (1955).

Diffraction technique

All measurements were made using a focusing transmission-specimen technique (Lang, 1952). Since this has been fully described elsewhere (Lang, 1955) it is necessary here only to indicate its essential principles. The method may be regarded as complementary to the well-known Brentano focusing method for reflection specimens. In the present case a thin, flat specimen is used in symmetrical transmission with a diverging incident X-ray beam. Rays diffracted at a given glancing angle appear to diverge from a virtual focus which remains at a constant distance from the diffractometer axis, a distance equal to that between the source of diverging X-rays and the diffractometer axis. This virtual source of diffracted rays is viewed by a multiple non-parallel receiving-slit assembly consisting of a large number of separate collimating channels each directed towards the virtual focus. The resolution with which the diffraction pattern is recorded depends on the collimation ratio of an individual slit element, whereas the intensity received, for a given degree of collimation, depends upon the number of channels in the slit assembly. It follows that the intensity is increased many-fold in comparison with a single-receiving-slit arrangement. A resolution comparable with that of the standard reflection arrangement may be achieved; in the present instance the distribution versus scattering angle 2θ of the intensity transmitted by the slits had a width at half maximum of less than 0·2°. A beam aperture of several degrees may be employed at the lower diffraction angles without impairing the resolution. In the work here described a standard 'Norelco' diffractometer (Parrish & Hamacher, 1952) was used without modification other than the replacement of the reflection-specimen support by a transmission-specimen support and the attachment of the multiple-slit assembly in front of the Soller slits on the counter arm. The aperture of beam utilised was limited to 1° by the mounting of these Soller slits.

Co $K\alpha$ radiation filtered by 0·0006 in. iron foil was used at 40 kV.P. and 10 mA. The X-ray tube current was electronically stabilised and the line voltage supply to the X-ray generator was well regulated. The specimen could be rotated in its own plane to bring the quill axes into any desired orientation with respect to the diffractometer scanning plane. The angle, ϱ, made by the fibre axes with this plane is also the polar angle of the direction in reciprocal space being examined. In each record the angular range 4° to 90° 2θ was covered, corresponding to a range of interplanar spacings from 26 Å to 1·26 Å. An exception was the equatorial record ($\varrho = 90°$), where the low-angle limit was 2·5°, corresponding to 41 Å. The counting rates varied over a wide range. Peak values registered were about 250 per second, whereas at the highest scattering angles studied the rate fell to about 12 per second. The measurement procedure was greatly facilitated by the use of the 'Norelco' Counting-rate Computer (Parrish & Hamacher, 1952). This device functions on the 'constant count' principle, so that each measurement is made with the same standard deviation due to statistical fluctuations. It plots on the strip-chart the counting rate derived from the time required to accumulate the fixed count. At the conclusion of the counting and recording operations the diffractometer is advanced by a chosen small step and the cycle is repeated. By this means it was possible to make the collection of diffraction data entirely automatic, the diffractometer working unattended throughout 24 hr. periods.

The preparation of the specimen

Over a hundred tips of Canadian porcupine quill were used in the specimen. The average length of each tip was about 2 mm. and they tapered to a sharp point from a maximum diameter of $\frac{1}{2}$ mm. The tips were mounted on a sheet of Du Pont 'Mylar' polyester film 0·00025 in. thick, covering altogether an area of about 10 mm. square, and were fixed in position by a few drops of a dilute solution of collodion in amyl acetate. They were laid out in rows, head to tail like sardines. Care was taken to make the specimen as uniform as possible, and particularly to maintain a common axial direction for all the tips. To further this end, tips chosen to be laid together were matched in pairs. The resulting specimen could by no means be regarded as homogeneous; it is, however, a particular advantage of the focusing transmission technique that a large area of specimen is irradiated, with consequent reduction of effects due to irregularities. In the present case not fewer than about fifty quill tips were always in the beam.

The choice of absorption correction to be applied presents some difficulty. Consider a single quill. This may be regarded as a plate specimen of variable thickness when set for $\varrho = 0°$ and as a cylindrical specimen of variable radius when set for $\varrho = 90°$. A parallel array of quills set for $\varrho = 90°$ behaves as a specimen intermediate between a cylinder and a slab. The transmission coefficients of the specimen, set respectively for low and for high scattering angles, were obtained by measuring the intensity of filtered Co $K\alpha$ radiation diffracted by an aluminum sheet, with and without the quills placed in the incident beam. In both orientations of the specimen about 61% of the radiation was transmitted. The variations in transmission when the specimen was rotated in its own plane were not significant. This measurement corresponds to a value of $\mu R = 0{\cdot}3$ for a cylindrical specimen, but, owing to some passage of radiation through interstices between quills, the effective value of μR was probably a little higher. A rough calculation confirmed that the ab-

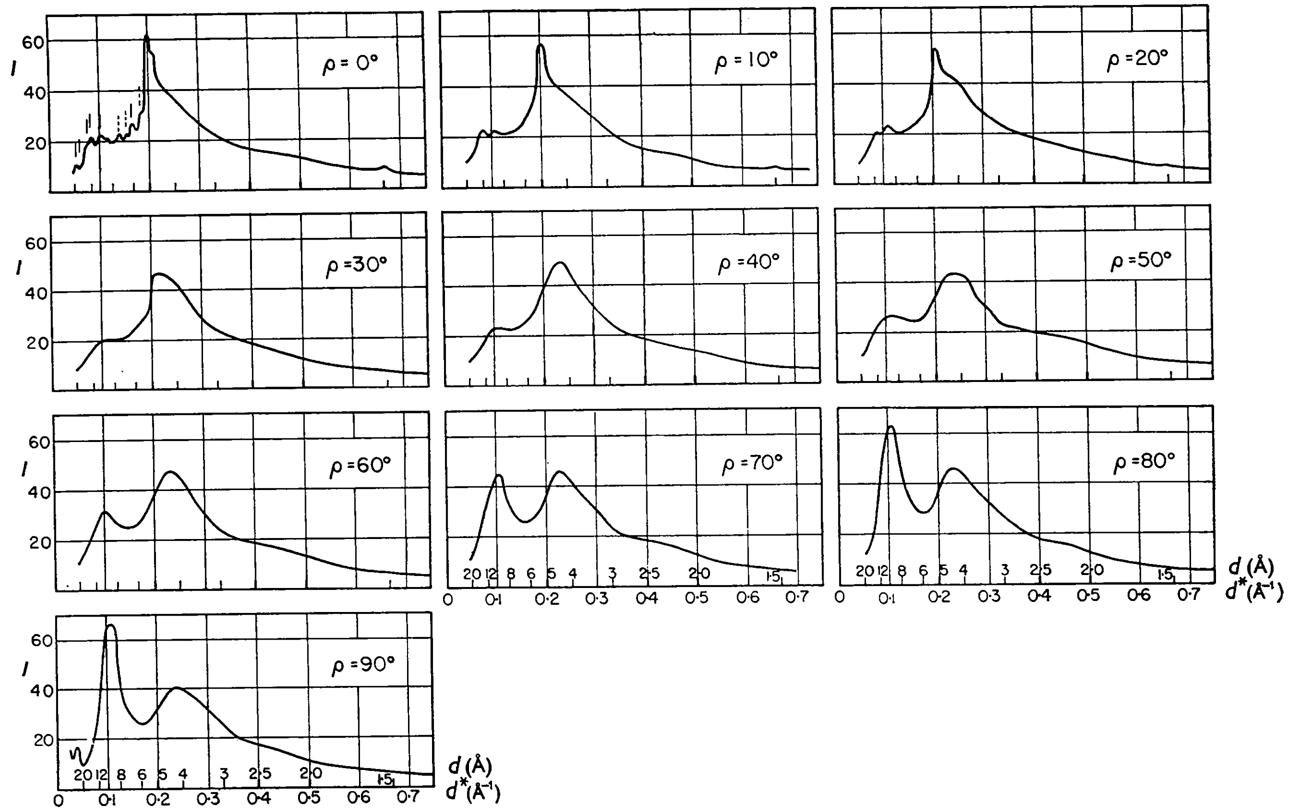

Fig. 1. Diffractometer records. Intensity in arbitrary units.

sorption factor should be approximately constant within the angular range studied, and accordingly no correction was applied. It is believed that the ratio between the intensity of the low-angle peaks and that recorded at $2\theta = 90°$ is not likely to be in error by more than a few per cent on this account.

The quills were stored in a vacuum desiccator over phosphorous pentoxide for some time before use, but during X-ray examination no attempt was made to control the surrounding atmosphere. The temperature varied between 21° C. and 32° C. and the relative humidity between 35% and 70%.

The measurements

Fig. 1 shows the form of the diffractometer records taken with ϱ increasing by steps of 10° from $\varrho = 0°$ (the meridional pattern) to $\varrho = 90°$ (the equatorial pattern). The intensity, in arbitrary units, is plotted versus $d^* = 2 \sin \theta/\lambda$, in units of reciprocal Ångströms. Corresponding values of d, the interplanar spacing in Ångströms, are also shown along the abscissa axis. The background, due to 'Mylar' film, air scatter and cosmic rays, has been subtracted in these curves. The spectra were scanned in steps of 0·25° 2θ and 3,200 counts were accumulated at each position, with the exception of the ranges 2·5° to 25° 2θ on the equator and 4° to 31° 2θ on the meridian, where the steps were 0·125° 2θ and 12,800 counts were accumulated in each measurement. The probable error due to statistical fluctuation with a count of 3,200 is 1·1% and with a count of 12,800, 0·6%. From the ten records shown in Fig. 1 a contour map has been built up indicating the distribution of scattering power in reciprocal space. Owing to the cylindrical symmetry about the fibre axis the complete diffraction pattern can be represented on a single quadrantal section of reciprocal space. On the contour map, Fig. 2, the cylindrical coordinators ζ and R are in units of reciprocal Ångströms.

It will be noticed that Figs. 1 and 2 do not show some of the fine detail that can be observed on good photographs of porcupine quill. How does this lack of resolution of the diffractometer records come about? It is not due to any inadequacy of the X-ray optical arrangements, for the resolution of the slit system and the fineness of steps of scanning are both more than sufficient for the study of this pattern. Rather it is statistical fluctuations which have caused small real differences of scattering power to be obscured. In this connection it is instructive to contrast the capabilities of the photographic and diffractometer techniques in regard to the detection of weak features. The eye can detect a change of photographic density of 0·01 when

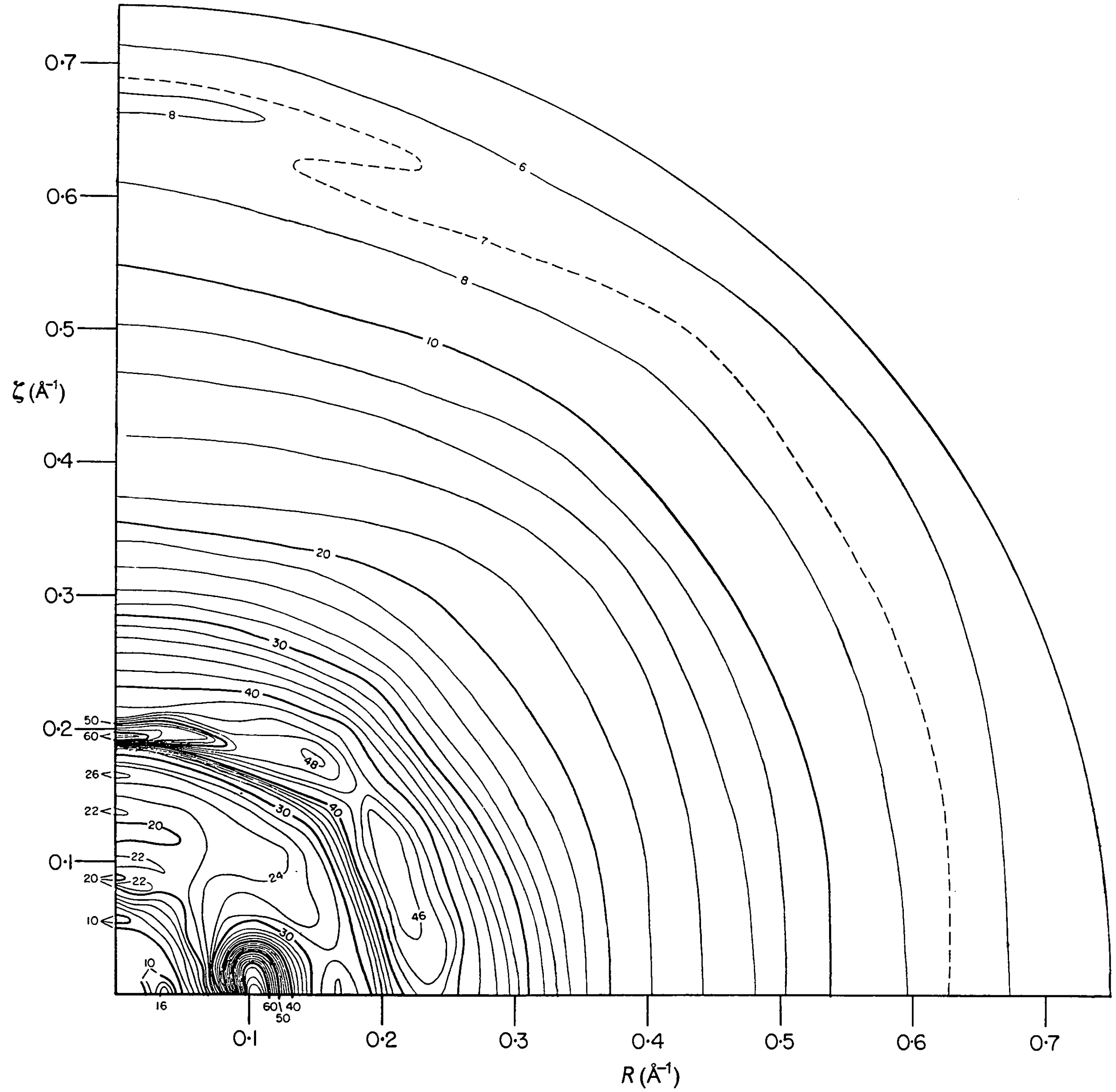

Fig. 2. Distribution of diffracting power in reciprocal space. Contour levels in arbitrary units.

this is fairly abrupt, such as is the case with a diffraction line or spot. Within the best working range for contrast the value of γ for Non-Screen X-ray film is about 4, hence the above change of density corresponds to a relative change of X-ray intensity of 1/200. Suppose that in a step-by-step diffractometer scan the background intensity is sufficiently slowly varying to be known with some certainty from the trend of a number of adjacent measurements. Then a reading higher than this background by twice the standard deviation, σ, of the count, N, can be regarded as significant, since its probability of occurrence by chance is less than 1 in 40. Now σ equals $\sqrt{N}$, so it follows that N must be made equal to $400^2 = 160{,}000$ in order to reach the sensitivity of detection possessed by the photographic film. If no assumptions can be made about background level and it is required to judge at the same significance level whether the excess of one count over another is real, then their difference must be greater than $2\sqrt{2}.\sigma$, and if this difference is 1 part in 200 each count must be not less than 320,000. The latter is, however, an unfavourable case unlikely to be encountered in practice, for in assessing the significance of one high count it can usually be compared with at least several others in the vicinity. If the experiments here described were to have been performed with a sensitivity similar to a film record then the number of counts accumulated would have to have been about ten times greater than that used (12,800) in the critical regions. Such an increase is entirely feasible, since removal of the Soller slits would allow increases in vertical and horizontal beam divergence sufficient to raise the intensity by at least an order of magnitude, and no increase in experiment time would then be required. It should be remembered that the above-quoted film sensitivity is only realized under favourable exposure conditions, and for quantitative intensity measurement the diffractometer can be expected to give the better results, provided suitable X-ray geometry and counting procedures are employed. The value of the records here presented is that they provide quantitative information on the relative intensities of the principal features of the quill diffraction pattern. They show, for instance, the relative magnitude and localisation of the 1·5 Å meridional reflection, and the intensity relations between meridional and equatorial reflections.

Indexing the meridional reflections

Film and diffractometer measurements of spacings of some meridional reflections have been made. The photographic experiments were done on a single quill tip using filtered Cu $K\alpha$ radiation and a specimen-to-film distance of 6 cm. On the trace for $\varrho = 0$ shown in Fig. 1, continuous lines indicate the positions of peaks with spacings equal to or less than 5·18 Å listed as meridional reflections of African porcupine quill by MacArthur (1943). Some other reflections, meridional or near-meridional, observed on photographs, are indicated by dashed lines. A list of meridional reflections of African porcupine quill has also been given by Bear & Rugo (1951).

The 1·5 Å meridional reflection is rather diffuse, the width at half maximum being about 1° 2θ. The diffractometer measurements indicate that the spacing is 1·48±0·005 Å. This is 1/3·5 the spacing of the 5·18 Å meridional arc. It appears that all meridional reflections can be indexed satisfactorily with an axial repeat, c, of between 197 and 198 Å; the lower figure has been chosen here. On the coiled-coil model for α-keratin this long spacing may be identified with the major-helix axial repeat. The model does not require that the spacing of the 5·18 Å meridional reflection be an exact submultiple of this, though it is convenient for calculation of the diffraction pattern if such is the case. Table 1 lists the meridional spacings reported by MacArthur, and by Bear & Rugo, together with the writer's film and diffractometer measurements. The spacings and intensities within the range covered by the latter were obtained from the diffractometer charts, except for the weaker reflections only observed photographically. The values for the higher d values are those of Bear & Rugo.

Table 1. *Meridional spacings of porcupine quill*

(Indexed with c = 197 Å.)

Intensity	d (Å)	d^* (Å^{-1})	lc^* (Å^{-1})	l	Observations* M	BR	L_p	L_c
6	66	0·015	0·015	3	×	×		
1	49	0·020	0·020	4		×		
2	39	0·026	0·025	5		×		
4	27·4	0·036	0·035	7	×	×		
2	24·5	0·041	0·041	8		×		
2	22·0	0·045	0·046	9		×		
4	19·8	0·050	0·051	10	×	×		×
3	18·1	0·055	0·056	11	×	×		×
1	15·2	0·066	0·066	13		×		
2	13·1	0·076	0·076	15	×	×	×	×
4	12·4	0·081	0·081	16	×	×	×	×
2	10·4	0·096	0·096	19	×	×	×	×
1	7·92	0·126	0·127	25			×	
1	7·33	0·136	0·137	27			×	
1	7·07	0·141	0·142	28			×	×
2	6·58	0·152	0·152	30			×	×
2	6·16	0·162	0·162	32	×		×	×
2	5·67	0·177	0·178	35	×		×	×
15	5·18	0·193	0·193	38	×		×	×

* M: MacArthur; BR: Bear & Rugo; L_p: Lang (photo); L_c: Lang (chart).

Radial intensity distributions

An investigation of the nature of the polypeptide-chain configuration through the study of X-ray scattering radial intensity distributions of a number of proteins has recently been reported by Arndt & Riley (1955). The data here presented can be transformed to give the X-ray scattering radial intensity distribution of α-keratin. It is necessary to compute a weighted sum of the scattering measurements taken along all directions in reciprocal space, the weighting to be the same as that which would apply if the fibres were in completely random orientation, as in an ideal powder specimen. The weighting factor is simply sin ϱ. The distribution thus obtained is shown as a histogram in Fig. 3 after all intensities have been divided by the polarisation factor $1+\cos^2 2\theta$. It may be compared

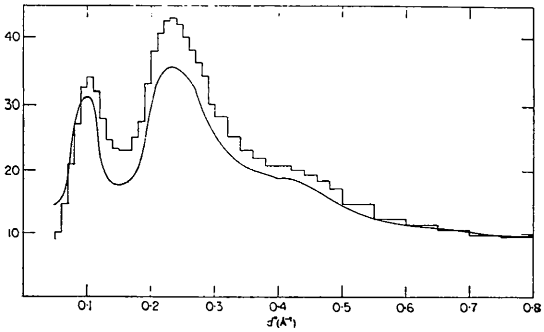

Fig. 3. Radial intensity distributions. Histogram: porcupine quill; continuous curve: serum albumin (Arndt & Riley).

with the continuous curve which is the distribution for serum albumin taken from Arndt & Riley's paper. This later distribution Arndt & Riley regard as typical of the α-proteins. In comparing the distributions it should be remembered that there is some uncertainty regarding the effect of absorption in the porcupine quill specimen, hence the scale of the histogram may change by a few per cent between high and low angles. However, the general run of the two distributions is very similar; the differences are roughly of the same magnitude as those found by Arndt & Riley between individual proteins of the α-class. It may be enquired what effect the use of non-monochromatic radiation has had on the α-keratin measurements. It is believed that the effects are very small. Certainly for the most reliable results a proportional counter with pulse-amplitude discrimination should be used, as was done by Arndt & Riley. However, in the special case of a thin transmission specimen used with filtered radiation, geometrical conditions are particularly favourable for the rejection of unwanted wavelengths. Such white radiation that is short enough to be appreciably transmitted by the β filter passes practically unabsorbed, and hence unscattered, through the specimen. It is necessary to consider only a small band of wavelengths from $\lambda = 1{\cdot}74$ Å, the K absorption edge of iron, to an upper limit of about $\lambda = 2{\cdot}1$ Å at which the absorption of specimen, air path, filter, and counter window together effectively cut off the intensity. Since a clean X-ray tube was used the energy of white radiation in the band must have been a small fraction of that of the characteristic.

It is of interest to consider why the 1·48 Å reflection has not been observed in radial intensity distributions of α-proteins. With fibres of the α-class it is a marked feature of the diffraction pattern, occurring as a localised meridional reflection. Its peak height in the case of the Canadian porcupine quill was 20% above neighboring scattered intensity. An estimate of the geometrical factors involved suggests that on randomising the fibre orientation to give a radial intensity curve, this peak intensity would be reduced a hundred fold. Now in order to detect confidently an increase of intensity only 0·2% above the local value, adopting the significance level considered in the earlier discussion, an accumulation of 10^6 counts is required at the point under observation. By suitable choice of beam divergences a counting rate at the scattering angle corresponding to $d = 1{\cdot}48$ Å of 500 per sec. may be obtained: this was the value in the early diffractometer measurements reported by Perutz (1951). Even with this high rate, a counting period of 35 min. would be needed to reach 10^6 counts, and during the course of the measurements strict control of specimen temperature and humidity, and of X-ray output, would be necessary. It is thus clear that very refined experiments would be required to find the 1·48 Å peak on radial intensity distribution curves.

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