THE MEASUREMENT OF THE DIAMETER OF ERYTHROCYTES BY THE DIFFRACTION METHOD.

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This paper describes a modification of the diffraction method of measuring the diameter of erythrocytes. The diffraction method was introduced by Pijper(1) in 1919, although it had previously been used by Young. Pijper employs a white light source, and either projects the spectra produced by the film of cells upon a screen, or photographs the spectra and makes measurements from the plate. The formula which he uses for obtaining the cell diameter from these measurements is only an approximate one, but he has developed the method in a most elegant manner, and has extended its uses to the measurement of the dimensions of bacteria, etc.(2, 3). This original method has been much improved by Millar(4), who has designed an instrument by means of which the measurement of the position of the various diffraction bands can be made with great accuracy, and who has supplied in addition the correct formula for the calculation of the cell diameters. Millar finds that the values given by the formula are from 10 to 20 p.c. too high, and at the same time is unable to detect the change in diameter of the cells which occurs when they dry(5). He concludes that the method is not a reliable means of determining the absolute size of red cells, and that although small changes in size can be detected, the extent of these changes in absolute units must remain a matter of doubt. Millar's paper also contains a most valuable discussion of the limitations of the method and of some of the difficulties encountered.

Recently a simple modification of the diffraction method has been described by Emmons(6). The spectra are produced by a film of dried cells which is inserted into a small instrument (an "eriometer"), supplied with a white light source and with a simple device for determining the size of the various diffraction bands. Emmons claims that this method gives results correct to $0{\cdot}1\,\mu$, and that no change in diameter of the cells can be observed when they dry(7).

1. Method.

The principle of the method to be described is that the diffraction spectra are produced by passing monochromatic light through the film of red cells, the monochromatic light being derived from the various lines of the mercury arc.

The diffractometer used is one constructed exactly as described by Millar, but with two slight modifications in the telescope. (i) Instead of the opaque half diaphragm in the telescope eyepiece, this eyepiece is fitted with two cross threads at 90°, oriented in such a way that the one always transects the image of the pinhole while the other lies tangentially to the various diffraction bands of the spectrum. When the telescope is in the zero position the intersection of the two threads appears in the middle of the image of the pinhole. These cross threads can be adjusted more accurately to any desired position than can the opaque diaphragm, and in this method there is little glare from the central image. (ii) The clamping screw of the telescope is replaced by a rack and pinion by means of which the position of the telescope can be adjusted to 0·1° on the scale.

The instrument is enclosed on three sides so as to cut off all scattered light, and is adjusted exactly as described by Millar. It may be mentioned that the position of the spectra is not very sensitive to slight alterations in the distance between the pinhole and the collimating lens of the instrument.

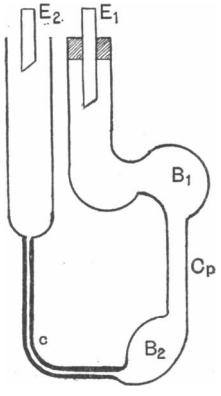

Fig. 1.

The source of illumination employed is a mercury vapour arc of special design, the appearance of which is shown in Fig. 1. The lamp is constructed from quartz tubing of about 1 cm. bore. This tubing is drawn out into a capillary *Cp* about 2·5 cm. long and 3 mm. in internal diameter, within which the arc is formed; at either end the capillary opens out into bulbs B_1 and B_2, one of which communicates with an exceedingly narrow capillary *c*. The purpose of this latter constriction is to damp the oscillations of the arc. Electrodes E_1 and E_2 are inserted into the open ends of the tube, one of the electrodes being held in position by a cork. The whole tube is filled with mercury. The electrodes are attached to a 110 volt D.C. circuit with a 60 watt lamp in series, and the arc formed by heating the capillary *Cp* with a Bunsen flame. It is desirable to have a small

fan in the neighbourhood of the arc to keep it cool, and under these circumstances it will burn steadily for hours. Should it go out, it usually re-forms spontaneously as it cools.

The intensely luminous filament which appears in the capillary *Cp* is now focussed on the slit of a spectrometer. In this way the spectrum of the mercury arc is obtained, and by means of a lens inserted between the prism of the spectrometer and the pinhole of the diffractometer, any one of the lines of the spectrum can be focussed on the pinhole of the latter instrument. Considerable care is necessary in the adjustment and alignment of the various parts of the diffractometer and spectrometer, but once these preliminary adjustments have been made it is only necessary to start the arc before use.

Before passing to the detailed consideration of the results obtained by the method, it may be as well to describe briefly the appearances seen in the telescope of the diffractometer under different conditions of illumination, when the apparatus is properly adjusted and when a thin dried film of human red cells is placed on the diffractometer stage.

(i) The principal lines of the Hg spectrum are the two yellow lines with wave lengths of 5770 and 5791 Å.U. respectively, the green line with a wave length of 5461 Å.U., the blue line at 4358 Å.U., and the violet line at 4078 Å.U. It is both difficult and unnecessary to separate the two yellow lines, which may be treated as a single line with a wave length of 5780 Å.U.; the violet line yields spectra to which the retina is comparatively insensitive, and thus we are left with three effective lines, the yellow, the green, and the blue.

When the film of dried red cells diffracts the approximately monochromatic light supplied by the two yellow lines, the pinhole appears brilliantly lit with yellow light and surrounded by a circular area of considerable intensity. There is, however, none of the glare associated with the light from the carbon arc, and it is easy to look directly at the image of the pinhole on which the cross threads intersect when the telescope is in the zero position. This yellow circle is surrounded by the first diffraction minimum, which appears as a circular dark band with fairly well-defined edges. This band is quite black at the points where the illumination is least; the illumination within it increases from its centre outwards, and undergoes a rather sharp increase at the edges. Surrounding this diffraction minimum is the first diffraction maximum, a yellow band of considerable luminosity. This is again surrounded by the second minimum, the position of whose edges is difficult to determine, and this again by the second diffraction maximum, a yellow band which

can only just be seen. It is quite easy to set the cross threads at the points in the maxima and minima where the illumination is greatest or least respectively, but the edges of the bands are a little deceptive, for the illumination changes rapidly at a point a little within the true edge of the band.

When green light is diffracted, the picture is much more brilliant. The first minimum is very intense, and the point within it at which the illumination is least can be readily determined. The first maximum is a brilliant green circle; the second minimum can be easily recognised, and so can the fainter maximum beyond.

The diffraction pattern with monochromatic blue light is equally clear, and at first sight even sharper than that with green light. The first and second maxima are completely black, while the two visible maxima are of an intense dark blue colour. Experience shows, however, that it is not so easy to set the cross threads in definite positions within the bands as it is in the case of green light, and that reliable measurements can be obtained only from the positions of the first minimum and of the first maximum.

The violet line of the Hg spectrum also gives a diffraction pattern which shows a faint first minimum and a fainter first maximum, but the sensitivity of the retina to this colour is so low that the necessary measurements are unobtainable. The same remark applies to the pattern seen with the red line of the mercury arc.

(ii) When the diffraction pattern is examined with the light of the mercury arc itself instead of with the monochromatic light derived from its various lines, a series of alternating yellow and blue bands appear. No distinct minima can be seen, for the yellow and blue bands overlap to some extent, nor is there any sign of the green diffraction maximum which should correspond to the green line of the arc spectrum. The limits of the yellow and of the blue bands can be measured with a considerable degree of accuracy, and such measurements bring out two very interesting points.

The first point is that there is only an approximate correspondence in position between the yellow band of the arc spectrum and the first yellow maximum with monochromatic light, and a scarcely closer correspondence between the blue band of the arc spectrum and the first blue maximum with monochromatic light. This will be seen by an inspection of Fig. 2, in which the position of the respective bands is shown; it will be observed that (*a*) the yellow and the blue bands of the arc spectrum do not correspond in position, except roughly, to the yellow and blue

first maxima with monochromatic light, and (*b*) that the green first maximum is missing from the diffraction pattern obtained with the light of the mercury arc as a whole. Measurements of the position of the limits or of the points of greatest luminosity within the bands of this latter diffraction pattern would accordingly give incorrect values for the diameter of the cells for no other reason than that the position of the bands is replaced from the true one owing to colour-mixing and overlapping.

The second point of importance is that there appears in the diffraction pattern obtained with the light of the mercury arc as a whole a very bright yellow band which has no apparent counterpart in the patterns obtained with monochromatic light. The position of this band, marked *B*, is indicated in Fig. 2, and it will be seen that it lies nearer

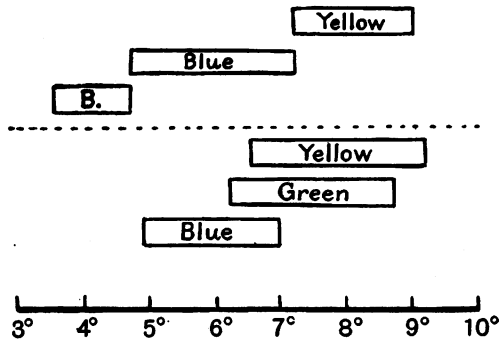

Fig. 2. Diffraction bands produced by human red cells. The positions of the 1st maxima for the monochromatic light of the blue, green, and yellow lines of the mercury arc are shown below the dotted line. Above the dotted line are shown the bands which appear when the same preparation of cells diffracts the light of the whole mercury arc. The band *B* is yellow in colour and corresponds to no maximum for monochromatic light. The green band which appears with monochromatic light is missing from the diffraction pattern.

to the axis of the optical system than does either the first blue band of the mercury arc diffraction pattern or the first blue maximum obtained with monochromatic light. In appearance it is absolutely indistinguishable from a diffraction band, but its position alone indicates that it cannot be one, for it lies nearer the optical axis than does the first diffraction band of a shorter wave length. Calculation of the position of the maxima and minima for the various wave lengths which make up the light of the arc shows that this yellow band really corresponds to the first minimum for the blue and violet lines of the arc, and that its appearance is due to the blue and violet components of the composite

light falling to a minimum intensity in this position. That this explanation is correct can be shown by inserting a potassium bichromate filter in the path of the light; this procedure causes the yellow band *B* to disappear altogether, for potassium bichromate cuts out the blue and violet lines of the mercury arc and therefore removes their first minimum. At the same time it allows the yellow lines to pass, and the fact that the band *B* disappears under these circumstances is proof that it is not a true diffraction band at all.

In the diffraction pattern obtained with the light of the mercury arc as a whole we have therefore the remarkable occurrence that a yellow band, superficially indistinguishable from a yellow maximum, represents a first blue-violet minimum. The conclusion to be drawn from the fact that such a misleading appearance occurs, even when light of a comparatively simple composition is used, must be that it may be difficult or impossible to identify correctly the various bands seen in the diffraction pattern when white light is employed. The difficulty will, of course, be increased by the fact that overlapping of the bands must occur under these circumstances to an extent impossible to determine.

(iii) When the light of the carbon arc is used, the coloured bands which make up the diffraction spectrum have exactly the same appearance as that described by Millar. As this investigator observes, the violet ends of the higher order spectra overlap the red ends of the lower ones, and it is very difficult to form a satisfactory judgment of the correct position of the telescope. In addition to this, the colour mixing and the comparative faintness of the spectra, due principally to the flooding of the field with white light, make it difficult to determine the exact wave length of the light which is being subjected to measurement. As Millar points out, an improvement can be effected by the use of filters. We have used for this purpose (*a*) an eosin filter which passes the yellow portion of the spectrum, (*b*) a double filter of neodynium chloride and potassium bichromate, which passes the green part of the spectrum only, and (*c*) the Zettnov filter used by Millar. The use of these filters purifies the diffraction spectra considerably, but even under these circumstances it is difficult to determine the position of the various bands. The difficulty is increased by the fact that the whole field becomes flooded with light of the same colour as that of the filter.

Emmons uses in his eriometer a source of white light, and his apparatus is therefore working under the greatest possible disadvantage[1].

[1] Emmons figures one diffraction pattern obtained with monochromatic light, but uses white light for his measurements.

The spectra obtained are certainly brilliant, but they are very impure, the impurity arising from the fact that the colours overlap as a result of the cells not being all of the same size. Under these circumstances it must be a very difficult matter to determine which particular wave length is being used in making the measurements. In addition to this, the diffraction pattern contains a yellow and an orange band lying within the first violet maximum, bands which are spurious in the sense that the band *B* is spurious. How many other such bands contribute to the impurity of the spectra is impossible to say. The eriometer would be greatly improved if an eosin filter were included in its construction, for this at least gives a series of comparatively pure yellow maxima and minima.

Just as the position of the maxima and minima supplies us with a measure of the mean size of the red cells of the population which produces the diffraction pattern, so the variation in the illumination intensity within the various maxima and minima supplies us with data regarding the scatter of the population about the mean. The study of this aspect of the problem, however, involves the use of special mathematical and experimental technique, and will not be considered in this paper.

2. The applicability of the theoretical formula.

Millar gives as the relation between the diameter of the cells and the position of the bands of the diffraction pattern

$$2a = z/\pi . \lambda . \operatorname{cosec} \theta .$$

Here a is the radius of the cell of average size, θ the diffraction angle, and z/π a constant for any one maximum or minimum. When applied to measurements made in conjunction with white light from the carbon arc, Millar finds that the expression gives results from 10 to 20 p.c. too high.

In order to show that this formula is more strictly applicable when monochromatic light is used, we give below a table of results obtained from the diffraction pattern produced by dried human cells. The film of cells was made in the usual way and allowed to dry in the air of the room; it was then placed on the diffractometer stage and the measurements made without it being moved. After completion of the measurements, the size of the cells was determined photographically in the usual way. The mean diameter was found to be $7{\cdot}6\mu$.

		θ	M
Yellow	1st minimum	5·3	7·7
	1st maximum	7·2	7·5
	2nd minimum	9·4	7·9
	2nd maximum	11·6	7·7
Green	1st minimum	5·2	7·4
	1st maximum	6·8	7·5
	2nd minimum	9·2	7·6
	2nd maximum	11·1	7·6
Blue	1st minimum	4·0	7·6
	1st maximum	5·2	7·8
	2nd minimum	7·4	7·6
	2nd maximum	8·9	7·6

Each one of these measurements was made for the point of greatest or least intensity within the maximum or minimum respectively, and all values for the mean diameter of the cells (M) are in μ. So far as these figures are concerned, it will be seen that single readings give results correct to within $\pm 0{\cdot}2\mu$, the average of a number of readings having, of course, a much higher degree of precision.

The following table gives a few values for rabbit cells, the mean diameter of which was ascertained by photographic measurement to be $6{\cdot}4\mu$.

		θ	M
Yellow	1st maximum	8·5	6·4
Green	1st minimum	6·1	6·3
	1st maximum	8·0	6·4
Blue	1st maximum	6·3	6·5

The next table gives a few values for the dried cells of the sheep. As soon as we come to cells so small as this, considerable difficulty is met with in the making of the films, for if the films are thick they dry to give exceedingly poor spectra, whereas if they are very thin the spectra have a very low intensity.

		θ	M
Yellow	1st minimum	8·6	4·7
Green	1st minimum	8·0	4·8
	1st maximum	10·9	4·7
Blue	1st minimum	6·6	4·6

The dried cells of this animal were found by direct measurement to have a mean diameter of $4{\cdot}7\mu$.

Finally, we give four values for the dried cells of *Hemitragus jemlaicus*, the diameter of which was found by direct measurement to be $3{\cdot}2\mu$. The cells of this animal are among the smallest of those of any of the deer family after acclimatisation at sea level.

		θ	M
1st minimum	Yellow	12·2	3·3
	Green	11·0	3·5
	Blue	9·0	3·4
1st maximum	Blue	12·3	3·5

It is, however, exceedingly difficult to obtain films which give a good diffraction pattern when the cells are so small as this, and even in the best films it is difficult to make out the limits of the various bands.

These results are sufficient to show that the diffraction method gives remarkably good results when monochromatic light is used, and that the formula given by Millar is applicable. When the diffraction pattern is obtained with the light of the whole mercury arc, however, the results are not nearly so good, for the coloured bands do not exactly correspond to the maxima given with monochromatic light, as Fig. 2 shows. When white light and the Zettnov filter is employed, we agree with Millar that the formula gives results which are too high by about 10 to 15 p.c., the discrepancy being no doubt due to the great difficulty in determining the predominant wave length and the position of the diffraction bands. White light and an eosin filter give somewhat better results, but even this combination falls far short of monochromatic light.

When white light is employed without a filter, as in Emmons' eriometer, it is a difficult matter to determine the wave length corresponding to the points of maximum intensity within the various diffraction bands. Emmons claims an accuracy of $0{\cdot}1\,\mu$, and gives a table in which the values for the diameter of the cells of four animals, as measured by diffraction, are compared with the diameter as measured by a projection method. The results agree very closely, the greatest error being only 1·8 p.c., but as we shall show below that the eriometer does not detect differences amounting to 10 p.c., the correspondence is to be regarded more as evidence of good fortune than as evidence of the accuracy of the method.

3. The effect of drying.

In discussing the applicability of the theoretical formula, we have confined ourselves to measurements of dried cells, for such measurements are not the subject of dispute. We have now to consider the statement of Emmons that the drying of erythrocytes is not accompanied by a diminution in diameter. This statement has been made by several observers, while a shrinkage has been claimed to occur by an almost equal number(8, 9, 10). So far as the recent observations go, the position may

be summarised by saying that Millar and one of us find by direct measurement that there is a 10 to 20 p.c. shrinkage on drying, and that Millar and Emmons, working independently, are unable to find evidence of shrinkage by the diffraction method. Millar concludes from this failure that the diffraction method suffers from imperfections, but Emmons, although he is aware of Millar's results and of his interpretation, concludes that only a negligible shrinkage occurs and that the direct measurements are unreliable.

We agree with Millar and with Emmons that it is not possible to detect a diminution in diameter on drying from the study of the diffraction pattern produced in conjunction with white light. When monochromatic light is used, on the other hand, the results are very different, for the drying of the cells is accompanied by a marked change in the position of the diffraction maxima and minima. This change can be followed as the cells dry, in the case of the cells of man and of the rabbit at least, and may be demonstrated in the following simple manner. A film of cells is made on a slide, and the film while still wet is placed on the stage of the diffractometer. A reading is made at once, the most convenient reading being that for the middle of the first maximum or of the first minimum. It is often possible to measure the position of the edges of the 1st maximum and minimum in addition. The film is then allowed to dry without being in any way disturbed, and readings again made; in this way the diffraction pattern for the wet cells is formed by the very same cells as contribute to the formation of the pattern for the dry cells. Results of this procedure are shown in the following table, the figures being for the middle of the first green minimum.

	θ wet	θ dry	M wet	M dry
Man 1	4·6	5·2	8·3	7·4
„ 2	4·5	5·0	8·5	7·6
„ 3	4·5	5·1	8·5	7·5
„ 4	4·5	5·0	8·5	7·6
„ 5	4·4	5·0	8·7	7·8
„ 6	4·3	4·9	8·9	7·8
„ 7	4·3	4·8	8·9	8·0
Rabbit 1	5·3	6·0	7·3	6·4
„ 2	5·5	6·1	7·0	6·3

These figures are sufficiently numerous to show that shrinkage takes place on drying, and it is, indeed, a simple matter to observe the first diffraction minimum moving outwards as the drying occurs. Since neither the diffractometer in conjunction with white light[1] nor the

[1] Millar, it should be noted, does not claim that his method can determine differences in absolute units.

eriometer of Emmons are able to detect this change, it is to be concluded that neither of these methods is sufficiently sensitive to demonstrate with certainty differences which amount to 10 or 15 p.c.

In connection with these results two points require to be emphasised. (i) The extent to which the cells shrink on drying varies somewhat, even under what appear to be the same conditions. This variation may be related to the fact that many films give a very poor diffraction pattern on drying, presumably owing either to irregular shrinkage of the cells or to numbers of the cells overlapping. This difficulty is particularly liable to be encountered if the films are made thick. (ii) Even when monochromatic light is employed, the diffraction method does not, in our experience, give satisfactory results for cells in plasma. Usually the diffraction bands are not quite sharp, and this gives rise to difficulties in making the readings. When the cells are suspended in undiluted plasma, rouleaux formation is very troublesome. When they are suspended in plasma diluted with NaCl, it is difficult to avoid crenation occurring. The best suspension medium is probably a mixture of plasma and citrate, as used by Millar, but even in this fluid some cupping of the cells is often apparent, and one cannot be certain that changes of shape and size have not occurred. Diffractometer measurements on such preparations generally show a slightly smaller diameter than that found by photography; in the case of human cells, for example, photography will show a mean diameter of $8{\cdot}7\mu$, while the diffraction method will show a diameter of about $8{\cdot}3\mu$. The latter method is accordingly not so reliable in the case of fresh preparations as it is in the case of dried films, the errors arising, however, from the difficulty in obtaining satisfactory preparations.

We have not been able to demonstrate shrinkage on drying in the case of the cells of the sheep, goat, or *Hemitragus* when diffraction methods are used. The difficulty here consists in making really satisfactory films which give good spectra both in the wet and in the dried state, for films which give good spectra while the cells are wet usually give very poor diffraction patterns when dry. In the case of the goat and of *Hemitragus* it is difficult to demonstrate shrinkage even by photographic methods, for the limit of resolution amounts to about 10 p.c. of the major axis of the cell. Emmons has selected the cells of the former animal for the one photographic measurement which he records, although by this selection the difficulties are placed at a maximum. He states that the result of this measurement is to show that the goat cell is smaller in plasma than it is in the dried state, but does not support the

finding either by details or by figures. We presume, however, since our technique is stated to have been followed, that the difference observed was greater than $0{\cdot}2\mu$, and, as the difference was also observed by the eriometer, that it was of the order of $0{\cdot}4\mu$ at least. Even were we not disposed to dispute this result, we are unable to see that it is compatible with Emmons' own statement that the diameter of the dried cell is a dimension which is a very close approximation to the corresponding dimension of the cell as it exists in the circulating blood, for the finding appears to us to lead to exactly the opposite conclusion.

Using the eriometer as a means of measuring the diameter of dried cells, Emmons arrives at certain far-reaching conclusions regarding the relation of hæmoglobin content to surface area of the circulating cell and of the red cell count to red cell diameter. It is not our purpose to discuss these relations at this time, but it will be understood that in so far as they rest upon eriometer measurements, the conclusions stand disputed.

SUMMARY.

1. A modification of the diffraction method for measuring the diameter of erythrocytes is described. This modification makes use of monochromatic light derived from the individual lines of the mercury arc.

2. It is shown that the formula deduced for the position of the diffraction bands produced by a haphazard distribution of cells is applicable when monochromatic light is used.

3. This method detects the change in diameter of erythrocytes on drying.

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