ESSAY

on

"The Diffraction Method of Measuring Erythrocytes"

by

W. G. Millar

for

The Gunning Victoria Jubilee Prize

in

Physiology   1925

Department of Physiology

Edinburgh University

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The Dean of the Faculty of Medicine

Dear Sir,

I submit the enclosed paper on
"The Diffraction Method of Measuring Erythrocytes" for com-
petition for the Gunning Victoria Jubilee Prize in Physiology.

I remain, Sir,

Yours faithfully,

W. Gilbert Millar

The Diffraction Method of Measuring Erythrocytes.

Pijper has described (1,2) a method of measuring the diameters of erythrocytes based on the formation under suitable conditions, of diffraction spectra by the cells.

In Pijper's apparatus the light from a carbon arc is rendered parallel by a suitable lens and then allowed to fall normally on a suspension of the cells. The parallel light is received on a second lens which forms an image of the arc on an opaque screen placed in the principal focal plane of the lens. Some of the light is diffracted by the cells and is brought to a focus on the screen as a series of circular spectra arranged concentrically about the image of the arc.

By measuring the radius of one or other of these spectra the mean size of the cells producing it can be calculated.

From elementary principles Pijper derives the following expression for the mean diameter of the cells :-

$$2a = \frac{n\,\lambda\,\sqrt{f^2 + r^2}}{r}$$

where $2a$ is the mean diameter, n the number of the spectrum measured, counting from the centre, λ the wave-length of the light corresponding to the particular part of the spectrum chosen, f the equivalent focal length of the second lens and r the radius of the spectrum. a and λ are expressed in micra, f and r in millemetres. The symbols have been slightly

modified from the original, the better to correspond with the notation
to be employed later.

This formula may be further modified to a
form similar to that used in optics by substituting for r and f,
the angle θ at which the diffracted light is inclined to the axis.
If instead of writing $\dfrac{r}{\sqrt{f^2 + r^2}}$, we put $\sin \theta$, the formula becomes:-

$$2a.\sin \theta = n \lambda. \qquad . \qquad . \qquad . \qquad . \qquad . \qquad .(1)$$

Pijper derives this simple expression by considering the field of
cells as equivalent to a linear grating and neglecting the distances
between the cells, which, as he rightly points out, are negligible
since the effects due to them cancel out on the average.

On the other
hand it is not admissible to treat of a collection of discrete
circular bodies as though they were lines of indeterminate length and
the derivation of a formula connecting the mean diameter of disc with
the diffraction angle (θ) is by no means simple. It is, however,
amenable to mathematical treatment and I am indebted to Prof. C.G.
Darwin for the following analysis.

Neglecting for the moment the
effect of the distances between the discs, the total effect will be
the sum of all the individual effects.

Consider the effect produced
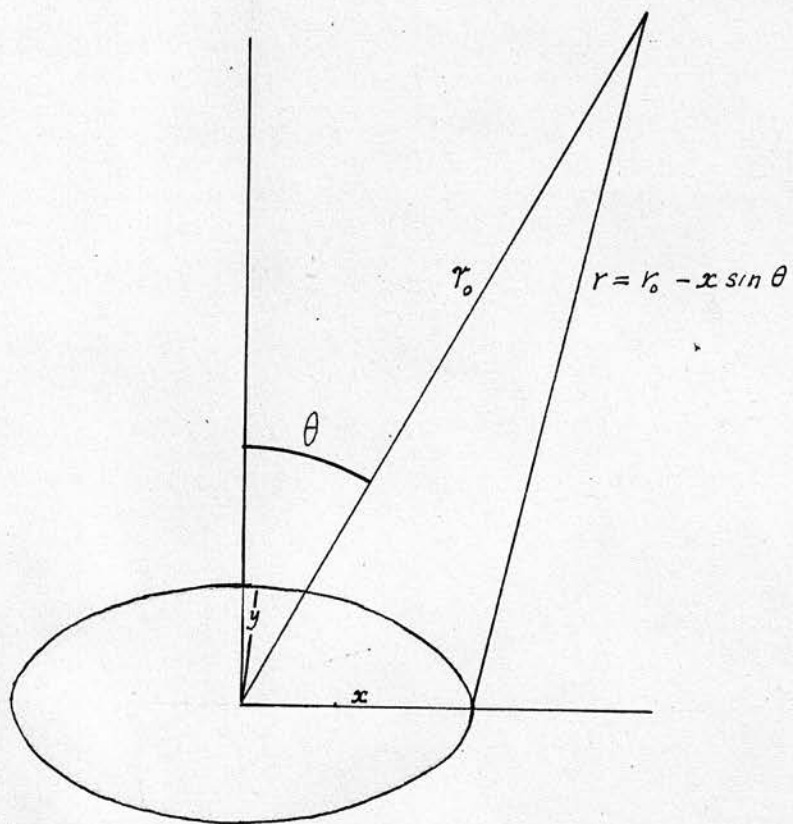by a single disc (Fig.1) of radius a observed in a direction at an

Figure 1.

angle θ to the normal or axis.

From an element of surface ds, determined by co-ordinates x, y, comes a wave proportional to

$$\iint exp - \imath k \sqrt{} \; [(r\sin\theta - x)^2 + (y^2 + r^2\cos^2\theta)] dx dy$$

where $k = 2\pi/\lambda$ and $r = r_o - x\sin\theta$, r_0 being the distance from the centre of the disc to the point at which the spectrum is formed. Putting $x = \rho\cos\phi$ this is nearly

$$\int_0^a \int_0^{2\pi} d\phi. \, e^{-\imath k(r - x\sin\theta)} \, \rho d\rho$$

$$= 2\pi e^{-\imath k r} \int_0^a J_0 \, (k\rho\sin\theta)\rho d\rho$$

$$= 2\pi e^{-\imath k r} \frac{1}{k \sin\theta} \int_0^{ka\sin\theta} z(1 - \frac{z^2}{2^2} + \frac{z^4}{2^2 \cdot 4^2} = \cdot \quad \cdot \quad \cdot). \, dz$$

where $z \doteq ka\sin\theta$

$$= 2\pi e^{-\imath k r} \frac{ka\sin\theta}{k^2\sin^2\theta} J_1 \, (ka\sin\theta)$$

$$= 2\pi a^2 e^{-\imath k r} \frac{J_1 \, (ka\sin\theta)}{ka\sin\theta}$$ which vanishes for $J_1 \, (ka\sin\theta) = 0$,

and which is therefore the condition for minimum illumination.

The illumination will be at a maximum when $\dfrac{J_1\,(ka\sin\theta)}{ka\sin\,\theta}$ is at a

maximum, that is when $J_2\,(ka\,\sin\theta)=0.$

To prove that, with a haphazard arrangement of discs, the influence of the intervening spaces cancels, let there be N centres on 1 sq.cm. say the first at $x_1\,y_1$, etc. Then at p_0 distance from the centre we have for this one

$exp\,-ik(p_0-x_r\sin\theta)$ as its factor, or

$e^{-ikx_r\sin\theta}$

The whole lot will give amplitude

$$\sum_{p=1}^{N}\,(e^{-ikx_r\sin\theta})$$

and intensity

$$\left(\sum_{p=1}^{N}\,e^{iq}\right)\left(\sum_{s=1}^{N}\,e^{-ikx_s\sin\theta}\right)$$

where $q=ak\sin\,\theta$

$$=N+\sum_{p}.\sum_{s}\,2\,\cos\,q(x_r-x_s)/a$$

$$=N+N(N-1)\,\cos\,q\,(x_r-x_s)/a$$

If we average the last expression
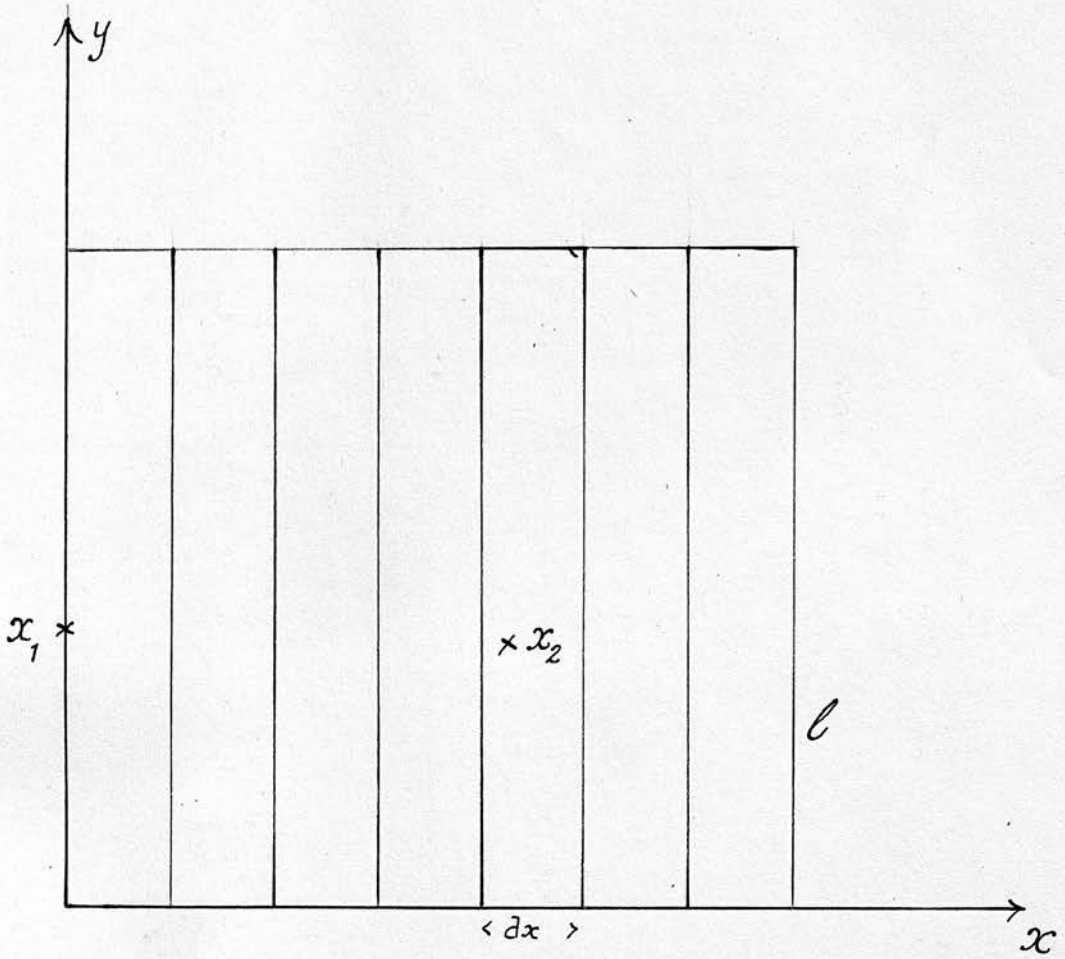
Figure 2.

it is easily seen to vanish, so that the total effect is simply the
sum of the effects due to the discs.

To average, take x_1 fixed and
let x_2 be anywhere (Figure 2).

Its chance of being in a range dx at
distance x is

$$\frac{dx}{l}$$ where l is the breadth

therefore average is

$$\int_{-\frac{l}{2}}^{+\frac{l}{2}} \cos q.\frac{x}{a}.\frac{dx}{l}$$

$$= \quad 2\frac{a}{ql}.\ \sin\frac{ql}{a}$$

This vanishes on the average
since the sign is as much positive as negative and to take in the
edge values would amount to counting up the diffraction pattern of
the edge of the field - obviously not wanted.

Hence to obtain an ex-
pression connecting diameter of cell, wave-length of light and angle.
of diffraction, the expressions $J_2(ka.\sin\theta) = 0$, and $J_1(ka.\sin\theta) = 0$
have to be solved. Reintroducing z for the values of $ka.\sin$ which
satisfy the equation, the formula becomes

$$z = \frac{2\pi a}{\lambda}.\ \sin\theta$$ or

$$2a.\sin\theta = \frac{z}{\pi}\lambda \quad . \quad . \quad . \quad . \quad . \quad (2)$$

a form very similar to Pijper's with the substitution of the series of values of z/π for the simple numbers of the spectra.

The values of z/π derived from the second order Bessel function (J_2) correspond to the bright spectra, those from the first order (J_1) to the intervening dark spaces. These values may be derived from tables of Bessel functions. Those for the first five spectra and spaces are given in the Table below.

TABLE I

Order of Spectrum	Value of z/π	
	Spectrum	Space
1	1.634	1.220
2	2.679	2.233
3	3.698	3.238
4	4.709	4.241
5	5.716	5.243

In the higher order spectra the dark spaces lie more and more accurately midway between the spectra.

Bergansius (3) gives similar figures based on standard optical forms, but does not appear to have adapted the treatment to a haphazard distribution of cells, preferring to measure cells from parts of the film where they may be regarded as in contact. Under these con-

conditions he applies Pijper's formula. This he finds to give
readings that are too high. This procedure is open to the ob-
jection that it is based on assumptions that cannot easily be
fulfilled in practice and restricts the parts of the film that are
available for measurement.

Adaption of the Principle to Practice.

It is evident from the above reasoning, that the theory under-
lying Pijper's diffraction method is fundamentally sound although
it appears that he has allowed an error to enter his formula.
There are also a good many points on which Pijper's apparatus may
be criticised, some of which, it is only fair to add, the origin-
ator recognized at the outset. Attention will therefore be direct-
ed more to those points wherein Pijper's own suggested improve-
ments seem open to criticism.

In the first place the spectra are
not very intense and hence are difficult to see on the opaque
screen. To overcome this Pijper has steadily increased the size
of his arc carbons to get more light. This certainly increases
the intensity of the spectra but it also entails other disadvant-
ages which more than counterbalance the gain. The central image
of the arc is much bigger so that there is more glare and the
spectra themselves are broader, increasing the difficulty of
measurement which is already sufficiently great. His suggestion
to use the sun as an illuminant is theoretically sound since the
light from it may be taken as parallel and it is of course, far

more intense than any arc. Unfortunately this source is far too
uncertain in this country to be of much practical use; owing to
the rotation of the earth some form of heliostat would be necessa-
ary to keep the image stationary. The impurity of the spectra
obtained is far too great to permit of the seeing of the Fraun-
hofer lines which Pijper suggests as a possibility.

In the ab-
sence of any suitable method of increasing the actual intensity
of the spectra, it remains to find a more sensitive method of
detecting them than an opaque screen.

Bergansius (*loc cit*) has
substituted ground glass, through which he views and measures
the spectra. He has also increased the focal length of the
collimating lens and uses a small 4 ampere arc which he finds
bright enough. In this way the size of the central image of the
arc is decreased and its brightness increased, while the spectra
are correspondingly clearer.

Apart from the intensity of the
spectra, the use of any screen for the linear measurement of
the spectra involves a knowledge of the focal length and other
optical constants of the second or projection lens to a degree of
accuracy not easily obtainable , in order to reduce the linear
measurements to the trigonometrical functions of the diffraction
angle.

In view of these considerations it appears preferable
to measure the diffraction angle directly by means of a telescope
provided with suitable rotation and angular scale.

This has been done in the apparatus about to be described, in which certain other inconveniences of the original apparatus are also overcome, making the arrangement a good deal more handy to use.

Description of the Diffractometer.

The general principle of the instrument is as follows. The image of the illuminant A (Figure 3) is focussed on a pinhole I which is situated at the principal focus of a parallelising lens C. The parallel beam of light emerging from C is thrown on a totally reflecting prism P and reflected at right-angles, vertically upwards through the suspension of cells E. Here the light is diffracted, the greater part passing on parallel to the optic axis but some going to form the diffraction spectra in directions corresponding to the diffraction angles. The parallel light is received by a telescope T which is rotatable about a horizontal axis which lies in the plane of the cell suspension. By swinging the telescope until the desired part of the spectrum is in the centre of the field the diffraction angle can be read off on the scale S.

The collimating system of pinhole and lens consists of a brass tube C (Figure 4) into one end of which is screwed an anastigmat photographic lens of 15 cms focal length and aperture about $f/6$. This gives a parallel beam of about 25 mm diameter when used with its iris fully open, or less when the iris is closed down. The pinhole is made at the intersection of two perpendicular straight lines in a piece of ferrotype sheet or
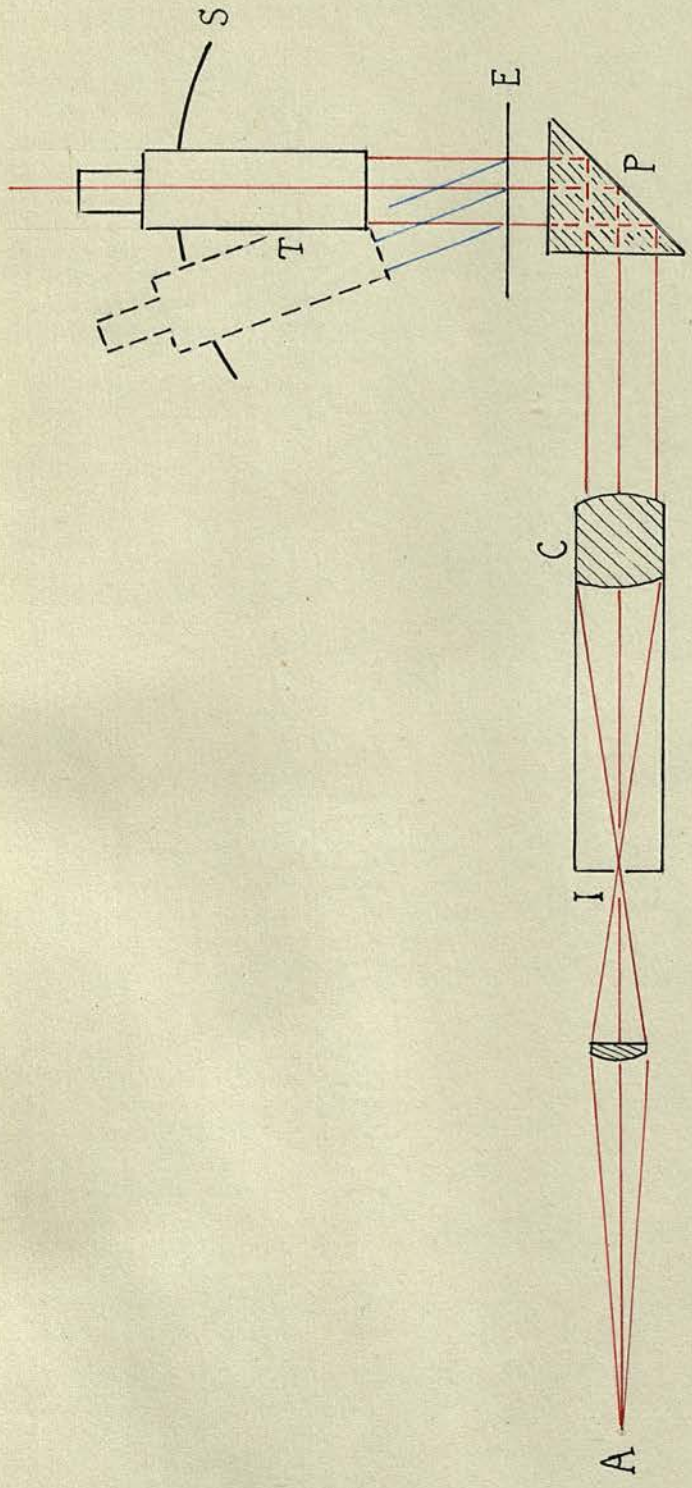
Figure 3.

N.B. For convenience of illustration, the plane of rotation of the tele-
scope is represented in the plane of the paper, instead of perpen-
dicular to this. The diffracted light is represented in blue, one
half only of the spectrum being shown.

stencil brass, and is held in the end of a tube which slides on the first tube, by a cap. The sliding adjustment permits of the placing of the pinhole accurately at the principal focus of the lens in a manner which will be described under *Adjustment of the Apparatus* below.

The collimator is held in a V-grooved holder H. which is pivotted about a vertical axis with clamp, and is provided with small levelling screws.

The holder for the prism and slide of cell suspension consists of a framework on three levelling screws S, which carries a plate-glass stage to the under surface of which the prism is cemented with Canada balsam. The prism is optically worked and the shorter sides are each 25 mm long.

The telescope T is supported vertically so that its optic axis is vertically over the central point of the reflecting surface of the prism, and so that the image of the pinhole is formed in the centre of its field of view when the scale Sc reads zero.

The horizontal axis of rotation of the telescope, R is screwed into a right-angled extension of the base which carries the collimator and stage. It is very slightly tapered to ensure an accurate fitting and has an axial hole drilled through it, by means of which the stage can be adjusted.

The scale Sc is divided in degrees and the telescope carries a pointer or vernier. The
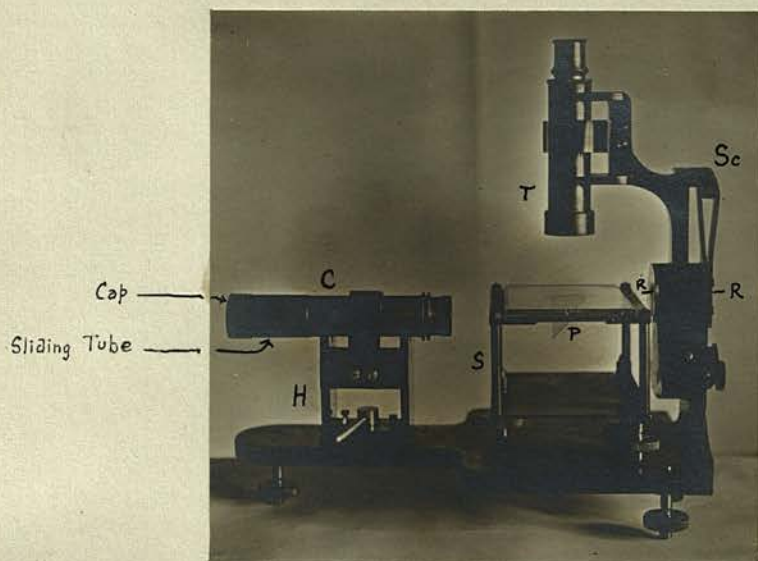
Figure 4.

zero of the scale is in the centre and the divisions extend to
30 degrees in either direction.

Adjustment of the Apparatus.

1. The stage is first set perpendicular to the optic axis of the
telescope when this is in its central position, and lying in the
plane of the axis of rotation of the telescope..To carry this out
with a sufficient degree of accuracy, a slide is laid on the top
of the stage and viewed through the hole in the telescope axis.
the margins of this hole are provided with cross lines which inter-
sect in the centre of the hole. The eye is held accurately cent-
ral with the hole, and the two screws on the near side of the
stage adjusted until the near edge is collinear with the horizon-
tal line. The front screw of the stage is then adjusted until
the far edge of the stage is on the same level as the near edge
and the line of sight grazes along the top surface of the slide,
which is thus in the plane of the telescope's rotational axis.
Further slight adjustments may have to be made to the rear screws
in order to get the setting quite right.

2. The top surface of the stage is now levelled to the horizon
by laying a spirit level on it and centring the bubble by means
of the levelling screws of the base.

3. The telescope is adjusted in the usual manner by focussing
on a distant object, after which the focus is kept constant.

4. The pinhole is placed in the principal focus of the collim-
ating lens in the following manner. A disc of finely ground
glass is inserted into the cell for the pinhole with the ground

surface facing the lens and is held in this position by the cap.
The lens, at full aperture, is directed towards a distant object
and the image of this focussed on the ground glass by moving the
sliding tube. A low power hand lens aids in obtaining an accurate
adjustment. The ground glass is now removed, care being taken
not to alter the position of the sliding tube, and the pinhole
diaphragm inserted in its place. The cap is put on lightly and t
the pinhole adjusted so that the cross lines on the diaphragm are
in continuity with those marked on the cap. When this is done
the pinhole is accurately central, and in the principal focus of
the collimating lens. The collimator is now put into its place
in the collimator holder, with the lens end facing the stage.

5. The collimator holder is now adjusted. An image of a not
too intense light source is focussed on the pinhole with a bulls-
eye or other lens and the telescope set to zero. The collimator
holder is then swung on its pivot until the image of the pinhole
lies in the centre of the field of the telescope, the levelling
screws of the holder being used to adjust the "up and down" pos-
ition. The holder is then clamped, care being taken to ensure
that the adjustment is not altered by so doing.

6. The centre of the field of the telescope may be indicated in
the usual manner with cross lines in the focus of the eyepiece,
but owing to the glare from the central image of the pinhole when
a carbon arc is used, dazzling the eye, it is found preferable
to cut off one half of the field by an opaque semi-circular dia-
phragm, the diameter of which lies accurately in the centre of
the field, and the centre of the diameter indicated by a cross

line. To ensure that the edge of the half shade is central with the field, swing the telescope until the image of the pinhole is bisected by the opaque edge. Then rotate the eyepiece through 180 degrees. The pinhole should remain bisected. If it does not move the telescope until it is, then the mean between the two readings gives the true zero. Alternatively the half shade diaphragm may be adjusted in the eyepiece by the method of trial and error and secured in its proper position by means of soft wax.

Preparation of Cell Suspension and Films.

Pijper in his original description recommends the use of a shallow wedge-shaped cell to contain the cellssuspension, owing to the ease with which the right thickness may be obtained. It has, however, been found preferable with the apparatus described above to use a simple slide and cover glass preparation. The most suitable thickness of film is easily ascertained by a few trials.

The slides on which the preparation is made are of thin glass about $3\frac{1}{4}"\times2\frac{1}{4}"$ obtained by cutting in two of discarded photographic quarter-plates. The covers are stout and measure about $2\frac{3}{4}"\times1\frac{3}{4}"$. To obtain a given distribution of cells over the extent of the sl slide, it is obvious that the film may either be a thin one of a relatively concentrated suspension or a thick one of a dilute suspension. It has been found that the more satisfactory is to have a fair thickness of fluid, for in the greater bulk of fluid the cells tend to distribute themselves more evenly over the film. The latter should not however, be so thick that the cover

slips about.

The formula derived for the size of cell is based on a haphazard distribution but it must be remembered that the distribution over any appreciable area of film is assumed similar to that over any other area of sufficient size. Consequently the aim should be to obtain a film which gives substantially the same reading throughout its extent. In spite of all care slight differences will be found. Thus, however rapidly the cover is applied after the drop of suspension is put on the slide, there is a tendency for this area to give a slightly higher value for the cell diameter, while the edges of the film tend to show slightly lower values. This is presumably due to the tendency of the small cells to be carried further than the large ones by the fluid spreading over the slide.

There is another tendency of the cells which seriously militates against accuracy, unless steps are taken to control it. This is the formation of rouleaux. The complete rouleaux formation is not so serious as its early stages of overlapping for the values obtained from a film with many rouleaux are so high as at once to arouse suspicion. A certain amount of overlapping may, however, put the average diameter up an appreciable but not very conspicuous amount and render results quite worthless. This drawback is so serious that up to the present it has not proved possible to get reliable readings from cells in plasma. But even in saline there is liable to be sufficient overlapping to vitiate results and in consequence no film should be used for

measurement until it has been examined under the microscope and
seen to be free from overlapping of any noticeable degree.

Measurement.

The process of measurement is extremely simple. The apparatus
having been put into adjustment an image of the positive crater
of a small carbon arc lamp, is projected on the pinhole by means
of a bullseye. If a plain uncorrected bullseye is used the convex.
surface should be directed towards the lamp, which may be at a
distance of 18 inches or so. By this means the spherical aber-
ration of the uncorrected lens is materially reduced and more
light obtained.

Before applying the eye to the telescope, the
latter should be swung a little to one side, so that the image of
the pinhole is cut out by the half shade, for this light is ex-
tremely bright and dazzles the eye badly. The telescope is now
cautiously brought nearer the centre until the first spectrum is
seen, that is the one nearest the pinhole image. It is always
necessary to pick up this spectrum to avoid counting the spectra
wrongly. The red and yellow will be well seen if the film is of
suitable thickness, but the blue end is usually obscured by the
glare from the central image. The slide is shifted about to
bring all parts of it into the illuminated area, the extent of
which is easily seen on the film by the naked eye, and can be
adjusted to a suitable size (an even fraction of the whole film)
by means of the iris diaphragm in the collimating lens. As the
slide is shifted, the first spectrum is watched. It should not

show any marked alteration in diameter in different parts of the
film, if it does the film should be discarded and another made.
If the film is satisfactory, the telescope should be swung further
out until the spectrum whose diameter is required appears in the
field. The higher the order of spectrum used, the more accurate
the method, but in practice there is so much blurring of the
spectra by the overlapping of wave -lengths and the intensity of
the light falls off so rapidly, that usually the second or third
give the most satisfactory results. The desired point of the
spectrum is brought on the edge of the half shade which acts as
a cross line and the reading taken. The half shade is then re-
versed by turning the eyepiece through 180 degrees and the teles
scope swung over to the opposite side of zero. The corresponding
part of the other half of the same spectrum is brought on the half
shade and the scale again read. The mean of the two readings
is the diffraction angle for light of the wave-length correspond-
ing to the part of the spectrum taken. The value of this wave-
length and of the sine of the diffraction angle are substituted in
the equation, together with the appropriate value of z/π and the
diameter of the cells found. Readings should be taken over the
whole extent of the film to avoid errors due to uneven distrib-
ution and the mean of these taken as the diffraction angle. About
eight readings can be taken on each film on the size of slide and
cover used.

      The chief difficulty in the actual measurement lies
in the choice of the particular part of the spectrum. The most
obvious way is to

obvious way is to use the part corresponding to the D lines but this is by no means easy to judge. The spectra being caused by a number of objects of differing size are not pure and the colours in consequence are rather diffuse. In practice it has been found advantageous to measure from that part of the second spectrum where the yellow vanishes as it merges on the red. If for this part the wave-length is taken as $.6\mu$ the diameters obtained agree very well with those found by direct measurement by photomicrography on the same suspension. This is somewhat arbitrary but two considerations support its adoption. In the first place the violet end of the third spectrum overlaps the red of the second and causes a cut-off of the yellow at rather too small a diffraction angle, so that the taking of an apparently too high reading tends to compensate for this. Secondly, owing to the possibility of a slight degree of overlapping of the cells, the method tends to give values that are persistently too high. This is also to some extent counterbalanced.

None the less it is very desirable that this disadvantage be removed as much as possible. The most satisfactory method would be the employment of monochomatic light. This would result in much sharper spectra owing to the absence of overlapping of wave-lengths (except in very high order spectra) and some certainty as to the true wave-length corresponding to the spectrum measured. Moreover the absence of spectra of other colours would enable fainter higher order spectra to be used for measurement, with a resulting increase in accuracy.

It is, however, a matter of very great difficulty to obtain monochromatic light of sufficient intensity to be suitable for use with the method. The ordinary sources are far too dull. The carbon arc with sodium cores to the elements provides an intense sodium flame, but the cored carbons are not obtainable in the small size required for the 4 ampere lamp. Through the courtesy of the General Electric Company, the writer has been enabled to procure hollow carbons for packing with sodium compounds, in order to test the value of the sodium arc, but unfortunately they were not available in time to embody the results in this paper.

The other method of obtaining approximately monochromatic light is to use a source of white light, such as the ordinary carbon arc, and to filter this light by means of suitable colour screens. In this case also, it is not easy to find a very satisfactory means, for the ordinary colour screens which give a sufficiently sharp "cut" in the spectrum, are so dense and reduce the intensity even of the wave-lengths that are passed, so materially, that they prevent the spectra from being seen at all. The most promising line of investigation here is the use of cells containing coloured liquids, the intensity of colour of which is easily controllable. The best of those examined up to the present is the colour filter of Zettnow, used half strength. The solution used consists of 32% copper nitrate and 3% chromic acid in water, and is used in a thickness of 5 mm. This filter gives a fairly sharp cut. The red below λ = 6400 A.U. and all wave-lengths shorter than λ = 5200

are held back, while the intermediate wave-lengths are passed
with sufficient intensity to be visible in the second or third
order spectra. The easiest part to read when using this filter
is the last of the red, there being less scattered light at the
outer end of the spectrum than towards the centre, where the
pinhole image causes a certain amount of glare.

It will be seen
that there is abundant scope for improvement in the method along
these lines and the outlook is sufficiently promising.

Results.

The following results are added to illustrate the use of
the "diffractometer". In all cases the end of the red of the
second order spectrum was taken, using the Zettnow filter. For
this part, $\lambda = .64\mu$ and the value of z/π is 2.68. For convenience
of tabulation and calculation the formula (2) page 8, is re-
arranged and becomes,

$$d = \frac{z}{\pi} \lambda . cosec\ \theta \qquad z/\pi.\lambda \text{ having the value 1.7}$$

1. *The influence of hypotonicity of saline on the diameter of
    human erythrocytes.*

The dilutions of saline are made up
from a stock solution of 1% sodium chloride. A series of tubes
is put up each containing 0.25 cc of two-days-old guinea pig
serum. The serum prevents the assumption of the Goughian form

(see below)

by the cells, and being old does not tend to the production of
rouleaux. The proportion is that found to give the best results
and care was taken, by observations over periods far in excess of
those taken in the experiments, to make certain that the foreign
serum had no apparent action on the cells, other than those de-
sired. The necessary quantities of stock saline and water were
added to give a total volume of 2 cc after 0.5 cc of blood was
run in. The resulting figures are tabulated in Table II.

TABLE II

Saline %	Diffraction Angle θ	$cosec\,\theta$	d (μ)
0.85	11° 30'	5.02	8.53
0.60	12° 30'	4.62	7.85
0.50	14° 0'	4.13	7.02
0.40	14° 0'	4.13	7.02

It is evident from the above figures that the diameter of
the human red cell diminishes as the tonicity of the medium falls.
This confirms the findings of Ponder and Millar (4) using direct
photomicrography for the measurement of human erythrocytes in
hypotonic plasma, and is added evidence of the correctness of
a theoretical discussion on the subject by Ponder (5). The
latter shows mathematically that, assuming the human erythrocyte
to be of the nature of a balloon, that is a thin membrane en-
closing a fluid or semi-fluid substance, a slight increase in

volume of the contents will be followed by a decrease in the
equatorial diameter, which is that always referred to under the
term "diameter" when dealing with red cells.

Such a reaction is
inconceivable on the hypothesis that the red cell is a sponge-
like mass of stroma, in the interstices of which the haemoglobin
is contained, unless the holders of this hypothesis are prepared
to assume a very special and improbable structure of the stroma
in order to explain it. This they are evidently not willing to
do, since the more prominent work by them on this point, seeks to
show that the diameter of the red cell increases with an increase
of volume, Price-Jones (6), Fischer (7).

It has been shown else-
where by Ponder and Millar (4) that Price-Jones's results can
be explained by the deficiencies of his method, and it is not
necessary to go more fully into it here.

It is claimed, therefore,
that the evidence available points irresistibly to the fact that
the mammalian erythrocyte must be regarded as a bag of fluid, as
maintained by Schwann and Schafer, and not as a sponge-like body
as Rollett and his supporters contend.

2. *The influence of isotonic saline on the shape and diameter*
    of the human erythrocyte.

Gough (5) has shown that when

mammalian erythrocytes are suspended in isotonic saline solutions they first assume the shape of spheres. On standing or on the addition of 2% of serum, the cells resume their normal discoid shape.

Cells were suspended in 0.85% sodium chloride and measured by the diffractometer in the state of spheres, ellipsoids and also in the normal disc form, the shapes being checked by direct microscopical examination of the actual films measured. The results are contained in Table III below.

TABLE III

Shape	Diffraction Angle θ	cosec θ	d (μ)
Spheres	$16^\circ 0'$	3.62	6.15
Ellipsoids	$13^\circ 30'$	4.28	7.28
Discs	$11^\circ 0'$	5.24	8.90

These results show that when a red cell assumes the Goughian form of a sphere, it does so without change of volume. The volume of a sphere of diameter 6.15μ is approximately 108 cubicμ which is the volume of a red cell of diameter 8.9μ if the cell is taken to be an ellipsoid of rotation (Ponder 9).

The Goughian change is very difficult or impossible to reconcile with the stroma conception of the erythrocyte, for the change, since it involves no alteration of volume, cannot be considered due to swelling of the cell and being reversible, implies an extraordinary

elasticity of the hypothetical stroma. Gough considers it due
to alterations in surface tension, to which agency he also at-
tributes the bi-concave shape of the normal erythrocyte. He
does not suggest in what manner this can take place, nor does
Norris (10) whose viws he supports, enter into any details as
to the mechanism of the action of surface tension. It is by
no means easy to see how the action of surface tension can lead
to any other but the spherical form, and until more accurate and
fuller information is available concerning the nature of the
membrane of the erythrocyte, it seems unlikely that any satis-
factory explanation of this very important phenomenon will be
forthcoming.

In view of the fact that the erythrocyte must be
considered as a balloon-like body, the bi-concave shape is ex-
tremely difficult to explain. There seems no evidence that there
is any internal structure that could hold the bi-concavities after
the manner of guy-ropes, for dark-ground illumination has uniform-
ly failed to reveal anything of the sort, even when the full
aperture of highly corrected immersion objectives is used, as
has been shown by the writer in other paper (11). It seems,
therefore, that, for the present, one must postulate a definite
structure in the membrane itself which is capable of resisting
the strains imposed on it. These strains are produced by stresses
due to the fact that the surface is not at a minimum for the
enclosed volume and are of the order of 2.5×10^{-5} ergs (Ponder 12)
What this structure may be cannot be suggested in the light of

present knowledge of the erythrocyte and its membrane.

3. *The mean diameter of the red cells of the sheep.*

For the purposes of this experiment the cells were suspended in 0.85% sodium chloride, the films being examined under the microscope to exclude the presence of the Gough phenomenon. The results of measuring two different suspensions from different animals are given in Table IV.

TABLE IV

No.	Diffraction Angle θ	cosec θ	d (μ)
1	18° 20'	3.18	5.42
2	18° 40'	3.12	5.30

These values agree very well with those usually accepted, when it is borne in mind that the ordinary tables of diameter of red cells refer to the dried cell and not to the fresh. The figures are a little on the high side, but this is the inherent tendency of the method owing to the liability to overlapping of the cells, which may be overlooked when present to a slight extent, unless particular care is taken.

Conclusion.

The strength of the suspension and the size of the fields

are

measured are such that about one million cells averaged at each
reading of the instrument and eight readings are taken on each
preparation. It is this power of averaging very large numbers
in a short space of time, that forms the chief advantage of the
method. Changes in size, provided any alteration in the extent
of overlapping of the cells can be avoided, which is usually the
case, can be followed with great rapidity and with an accuracy
that compares favourably with any method other than the labori-
ous one of photomicrography. There is, however, always a lit-
tle uncertainty as to the accuracy of absolute measurements,
owing to the tendency to overlapping which has been emphasised
in the earlier parts of this paper. When every care has been
taken, by microscopical examination, to exclude this source
of error as far as is possible, the limit of accuracy should
not, in the writer's opinion, be placed any finer than ± 0.4 µ;
at least, under present conditions.

On the other hand, the
method shows promise of improvement in this respect, and it
may be claimed that, even in its present imperfect state, the
method has sufficient advantages to make it of value to the
haematologist.

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