THE DETERMINATION OF SMALL, IRREGULAR AREAS BY A PHOTOELECTRIC METHOD

by

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Introduction:

Mainland (9) (8) has drawn attention to the fact that there has been a recent tendency for histology and cytology to develop as quantitative studies. It is unnecessary to search exhaustively through the literature of the past two decades in order to find abundant evidence that quantitative data and even statistical methods have been used rather widely in the various fields of microbiology. This might be regarded as a natural trend, for as Lord Kelvin has said, "When you can measure what you are speaking about and express it in numbers, you know something about it, but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind." *

In biology, quantitative data are commonly concerned with size. The methods that have been used to determine it, however, have been somewhat primitive. They have consisted mainly of linear measurements. Recent examples are included

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* Tables for Statisticians and Biometricians: Part 1, 1925, Karl Pearson.

in the publications of Price-Jones (13), F. P. Parker et al (12) on human red blood cells, of Savage, Williams and Fowler (17 (18) on bovine and equine spermatozoa, and of Bailey (2), Levine (6), Newton and Johnston (1) on the spores of wheat rust fungi.

With the aid of a camera-lucida, Price-Jones (13) drew outlines of red blood cells at 1000 diameters magnification. The diameters of these outlines were measured with a millimeter scale and the results expressed as microns. In dealing with cells of reasonably uniform shape this method is satisfactory but, when irregular forms are concerned, such as the blood cells found in some types of anaemia, simple linear measurements do not apply. The irregularly shaped cells would have to be avoided - a procedure which would be unsound in proportion to the relative number of such cells.

The method of F. P. Parker et al (12) was a slight improvement. Images of red blood cells were projected at 500 diameters, they were measured in two directions, in millimeters, and the sums of these measurements were used to express the diameters in microns. It is evident, however, that considerable error was unavoidable in thus attempting to express the area of irregularly shaped cells.

An improvement on this method was employed by Mainland (9) in the measurement of ferret pronuclei. This worker

assumed the shape of the nucleus to be ellipsoid. The three axes were measured and the sectional area and the volume arrived at by the aid of a formula. This method was extremely laborious and time consuming. The author made the following statement: "It should be noted that this formula is only applicable to bodies of this approximate ellipsoidal shape, in which the axes do not differ very widely from each other. For a long, thin body the formula would probably give results with an error greater than that inherent in the observations." Knowledge of this would naturally lead to selection which is contrary to the statistical principle that the observations must be made on a random and representative sample.

Mainland (7) also compared a few other methods for arriving at areas. He established mathematically that for areas up to 5 square millimeters the "squared paper method" was the best. However, as the areas increased beyond that size the value of this method rapidly diminished.

In this general field the use of a planimeter for the measurement of areas has been dealt with by Scammon and Scott (19). By measuring discs from one to four centimeters in diameter, they found that the coefficient of variability amounted to about 8.3% for the smallest and to 0.84% for the largest. These errors are too large to be disregarded in quantitative work. Scammon and Scott also investigated the

area-weight method of Hammar. (4).

This worker projected images on heavy, well made paper, and traced their outlines. He subsequently cut these out and weighed them. He then computed the areas from the weight of the cut-outs by the application of the weight-area ratio derived from samples of the paper.

At least three sources of error present themselves when this method is used. First the error of cutting, second the error of moisture content and therefore in the weight of the paper, and thirdly, variations in the thickness of the sheets of stock used for tracing. The error of cutting is small when the work is done by a careful worker, but it hardly can be obviated entirely by any method. Moreover, it is reasonably constant when one person does all the cutting. The error of moisture content, however, is very significant. Scammon and Scott note the work done by Houston, Carson and Kirkwood (5) on bond and ledger paper. They found that there was a rapid, though not constant, absorption of moisture by this material. There was also a change in the area of the paper due to its expansion with an increase of moisture in the air. Atwell and Woodworth (1) found that part of the variation caused by moisture was overcome by storing the cut-outs in a desiccator for a week before weighing, and removing a few at a time, since certain makes of paper absorb a large percent-

age of their moisture content within twenty minutes after exposure to air.

Scammon and Scott also studied the variability in thickness of commercial bond paper. Two sets of fifty discs each, 18 and 4 millimeters in diameter, were cut at random with a steel tool from a sheet of stock, known as Resolute Ledger No.32. These were weighed on an analytical balance. The results showed that the coefficient of variability was between 2.0 and 2.5% in the case of the large discs and, about 3.0% for the smaller ones. A similar set of discs cut from "Eastman's Kodaloid No.3" (material used in the making of photographic film) showed coefficients of variability from 0.31% to 0.33%.

Obviously the weight-area method is laborious and time consuming. Moreover the use of Kodaloid in tracing say 500 individual outlines from a large population would be extremely expensive. Something more rapid and less expensive is required before measurements of this kind are likely to be made by other than a very limited number of workers.

Savage and Jamieson (16) evolved a more rapid method for obtaining comparative areas of isolated cells. The apparatus consisted of a vacuum type photoelectric cell, insensitive to red light, connected to a battery and a suitable galvanometer. The images of certain rust spores, stained red,

were projected seriatim with a microscope at 500 diameters on to the cathode of the cell and the resulting decrease in light read from the galvanometer scale.

This method fulfilled its purpose only in part. The objects dealt with differed in staining intensity as well as in area, while the photoelectric cell responded to a complex of both of these attributes. The workers named made no attempt to separate these.

Savage and Isa (15) increased the sensitivity of the photometric method by amplifying the photoelectric current. This was done with an "FP-54" valve and an appropriate circuit. The apparatus was used in the same manner as above (16). With the equipment at their disposal it was imperative to use the house supply current (A.C.) for operating the projection lamp. During the daytime the voltage fluctuated between 100 and 115, consequently, it was necessary to work between the hours of one and five A.M. when the variation was at a minimum. The results obtained with this apparatus were again expressed as an area-staining intensity complex, though the staining intensity could be indicated mathematically to some extent.

Savage, Goulden and Isa (14) modified the technique and apparatus of the above (15) and were able to separate the area-staining intensity complex. The apparatus consisted of a modification of the balanced bridge circuit of DuBridge in a specially built photometer, having an indicated sensitivity of about 7000 centimeters per lumen to the light of a "Mazda"

lamp. This apparatus, although satisfactory in many respects, had a few undesirable features. Due to the complicated nature of the circuit, its control consumed much time during the course of a set of readings. Because the images were projected at 3000 diameters magnification, the intensity of the light striking the "measuring cell" was very low and consequently the total deflection of the galvanometer was comparatively small. Moreover, the position in which the photometer had to be operated rendered the measurements of area awkward. No precautions were taken regarding the moisture content of the cut-outs.

Their method of separating area from the area-staining intensity complex was as follows. The latter was obtained first. To quote from the text:-

"a. The area-staining intensity complex.

An image of a red-blood cell, at 3000 diameters magnification, was focused on the projection screen immediately above the measuring eye. Here its outline was carefully drawn in pencil on a piece of thin, white Bristol board, 2" x 3". The photometer, having been balanced and checked, the image was then moved into the eye and the galvanometer deflection written (in red) on the card beneath the cell outline.

This was repeated until 400 images (and cards) had been dealt with. The procedure was very tiresome and required from 7 to 9 hours of careful team work on the part of the two observers.

b. Area.

To render them opaque, the cards were subsequently painted a dull black on the sides which contained no data. Then the outlines were carefully cut out with scissors and, for safe-keeping, each was placed between the pages of a book together with the stub of the card from which it came.

The comparative areas of the "silhouettes" were obtained by moistening each one slightly and placing it against the glass plate which covered the measuring eye. There it adhered and, because the light conditions were the same as those which maintained during the observation of stained cells, the resulting deflection indicated the area of the corpuscle at the magnification employed. As in the previous series of observations, the galvanometer deflections were written on the card stubs, but this time black ink was used to avoid the possibility of confusion."

Scope:

The present account deals with the construction and use of a photometer for measuring small, irregularly-shaped areas. It was designed to be simple, reasonably accurate, and to operate with ease and rapidity.

Preliminary Experiments:

The most simple apparatus was considered to be a flat photoelectric surface, on which opaque silhouettes would be placed and which could be connected to a suitable galvan-

ometer. The Weston Photronic Cell at once suggested itself. The surface was adequate in size, the use of batteries would be eliminated, and the voltage generated before a bright light would be ample to give a suitable deflection if used with a moderately sensitive galvanometer. A preliminary trial showed, however, a serious defect in the cell. The photoelectric surface was not equally sensitive in all its parts before a homogenous source of light. Accordingly another method was tried.

The Weston Cell was mounted in a box so that it faced a two inch circular aperture, which was covered with ground glass. Fair diffusion was produced by the glass, but due to this diffusion, the galvanometer response was reduced to such an extent as to become inadequate. It was therefore necessary to resort to photoelectric cells and an amplification Circuit.

The second trial with the Weston Cell presented an immediate problem. In the first place, the total flux in the photometer box had to be distributed evenly. Second, it had to undergo a decrease in direct proportion to the areas of the silhouettes placed between its aperture and the source of light.

There were at least two possible ways of reaching a solution. One was by diffusing the outlines of the silhouettes through a ground or flashed opal glass. How-

ever, it was found that, when a photoelectric cell was mounted in a box so that it faced an aperture covered with flashed opal glass, a slight but appreciable shadow was cast by an opaque object placed on the glass. This was hardly noticeable until the object was moved.

The second method consisted of allowing the light to diffuse by reflection from a white matte surface into the photo cell. This is the usual procedure in the construction of photometer spheres. Actually, it gave the more satisfactory result.

Description of Photometer.

The assembly finally constructed was a rectangular wooden box about 10 x 10 x 18 inches, completely covered with sheet aluminium and divided internally into two approximately equal compartments by a vertical partition. One part constituted the photometer, the other contained the amplifier.

The photometer was lined with white matte paper. Two Burt cells (16), connected in parallel, were mounted horizontally on the vertical partition so that their cathodes were directed toward a central area on the bottom of the box. This area was located directly below an aperture in the lid, above and midway between the photo cells. The aperture was rectangular and measured 25 by 45 millimeters. It was placed so that its smallest dimension lay in a straight line joining the two cathodes. This tended to nullify the slight difference in the responsiveness of the photo cells.

The light used to operate the photometer was a small, spiral tungsten filament in a spherical envelope. It was mounted in a standard screw socket at a fixed distance of 18 inches from the aperture of the photometer. At 6 volts, it consumed 4.3 amperes. This current was provided by a battery of four, heavy duty accumulators, connected in parallel and provided with a suitable rheostat.

A cylindrical shield of thin, black iron surrounded the light laterally. It was adjusted permanently, so that while the aperture of the photometer was amply illuminated, the operator's head and neck were in deep shadow.

Description of Amplifier:

After having experimented with a number of amplifiers, the circuit of DuBridge and Brown (3) with a slight modification was finally adopted. Figure 1 indicates the circuit. The thermionic valve was an "FP-54" designed by Metcalf and Thompson (10). The galvanometer was of the old d'Arsonval type. It had a sensitivity of 0.003 micro-amperes and a period of 3 seconds. An Ayrton shunt was connected with it. The grid leak used was a tested resistance calibrated at 250 megohms. It was obtained from the S.S. White Co., and was of unknown composition.

Construction:

In constructing the amplifier the usual precautions were observed. All connections were carefully soldered with resin-core solder, and the leads made as short as possible. The standard radio resistances used were insulated from the

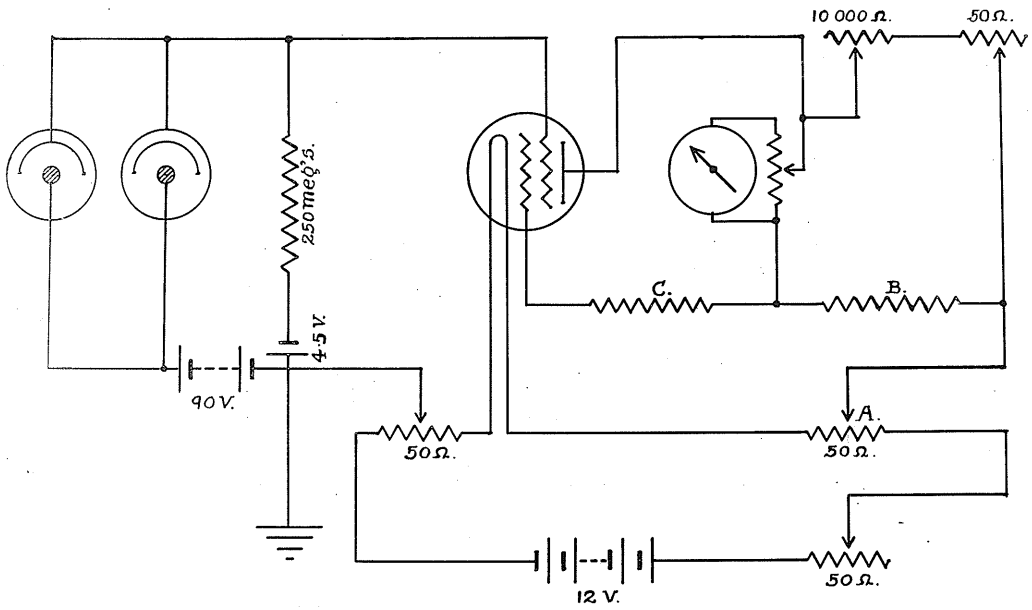

FIGURE 1.

Diagram of the circuit.

metal panel by the use of rubber grommets. The circuit and metal box were grounded by a wire leading to a copper-tipped iron rod driven 4 feet into the earth.

Adjustment:

The nature of the circuit and the construction of the valve called for certain preliminary adjustments. The rheostat was set to cause a drop of 2.5 volts across the filament of the valve. The variable plate resistances were advanced about half-way across their arcs of travel (to allow for considerable adjustment) and the potentiometer at "A" (see Figure) turned until a potential of 6 volts was applied to the plate, as measured on a sensitive voltmeter attached to the negative side of the filament.

"B" and "C" were fixed resistances which caused the potential to drop to 6 and 4 volts, respectively. When the galvanometer was placed in the circuit, it required very little adjustment of the plate resistance to bring it to zero on the scale.

Since comparative increments of light (or shadow) were to be measured, it was fundamentally important that the grid potential-plate current relationship be linear. A sensitive Weston D.C. voltmeter was connected between the negative side of the filament and the grid resistance. Negative bias was applied in steps of 0.5 volts and the resulting galvanometer deflections recorded. The grid potential-plate

current curve is given in Figure 2. The graph shows a linear relationship between -5.0 and -8.5 volts. Consequently, the negative grid bias was set at the latter figure and the resulting galvanometer deflection balanced out by varying the plate resistance. It is of interest to note that the linearity of the curve was arrived at with the Ayrton set at 0.01. When the photometer was used for routine measurement, the Ayrton was set at 1.0. Consequently, the change in the grid potential-plate current relationship, due to a photo-electric current, was small even when the galvanometer deflection swept across the entire scale of 50 centimeters. This assured that the linearity held throughout the range of measurement.

Behaviour:

By connecting the photoelectric cells first, directly to the galvanometer and secondly, to the galvanometer through the amplifier, it was determined that the latter gave an amplification of approximately 500 times.

Calibrated with a standard tungsten filament lamp, the photometer had a sensitivity of approximately 1500 millimeters per lumen when the Ayrton was set at 1.0.

To test the response to increasing increments of shadow, a set of opaque discs was employed. These were cut from stiff, black paper and ranged from 5 to 20 millimeters in diameter. When placed one at a time on the glass covering

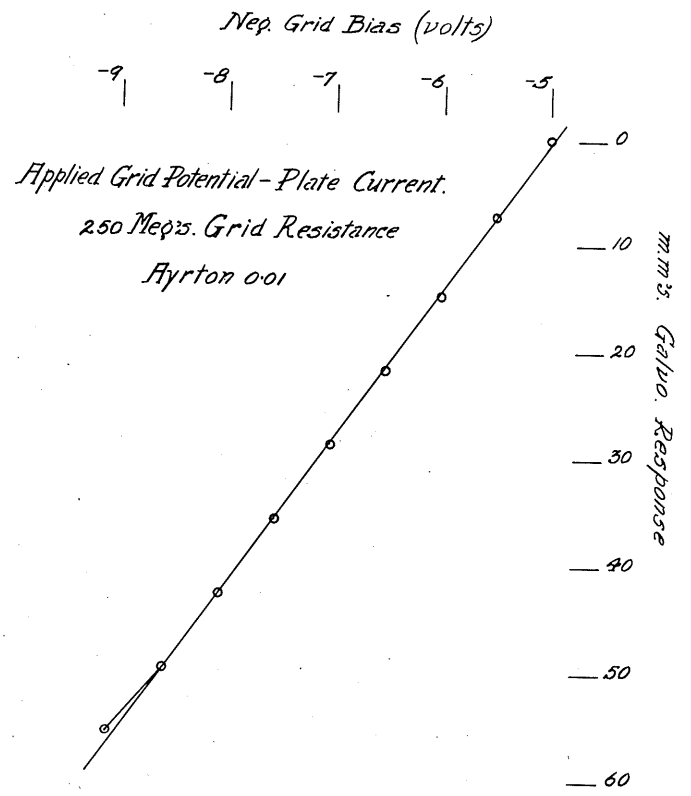

FIGURE 2.

Graph showing the nature of the grid potential - plate current response.

the aperture, they showed that the light cut off was directly proportional to the squares of their dimensions.

The same discs were used to demonstrate the consistency of the response. Each disc was placed in position a number of times and, in consequence, it was found that the amount of variation in the response was less than 1%. This is considered to be well within the limits required for the work contemplated.

Operation:

When the apparatus was to be used, both the amplifier and the photometer were turned on a half hour before any readings were attempted. This procedure was necessary to allow the apparatus to reach a state of stability. With the photoelectric cells in darkness, the Ayrton was set at the plate resistance. (The day-to-day variation in this respect amounted to very little). The photometer was then exposed to the light and the galvanometer deflection adjusted to zero at the other end of the scale by varying the resistance which controlled the light's intensity. The galvanometer scale was marked to read from either end or from the centre. After making these adjustments, the apparatus was ready for use.

"Dark zero" remained remarkably steady during the course of any set of measurements; "light zero" was checked at each reading.

Figures showing the frequency distributions of the areas of silhouettes representing bovine sperm heads at

3000 diameters are contained in the accompanying thesis by the writer. The readings were made at the rate of about 100 per hour.

Summary.

A photo-electric method has been described whereby the comparative areas of small, irregularly shaped silhouettes may be obtained conveniently, rapidly and with sufficient accuracy for biometrical purposes.

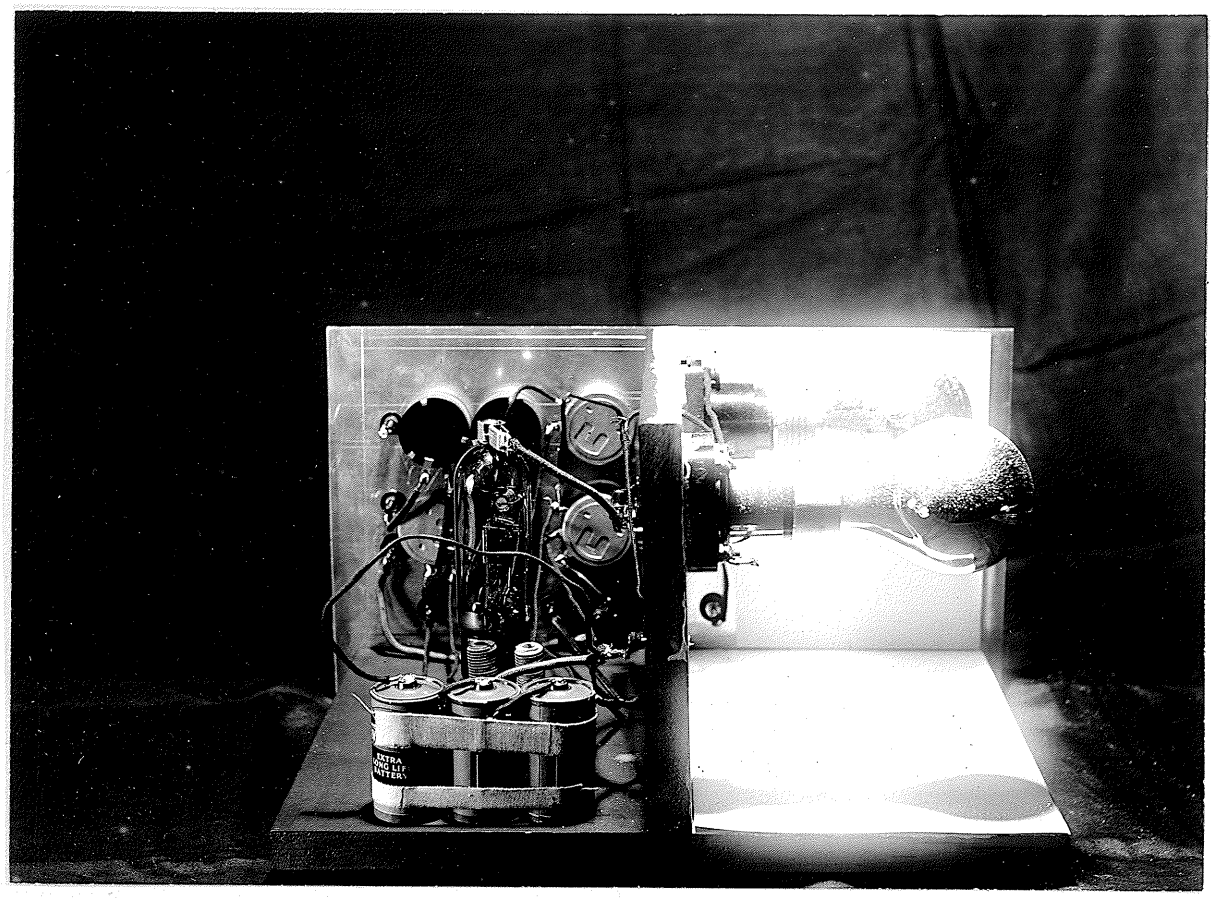

PLATE 1.

Photograph of the apparatus with outer cover removed. (The Burt cells are powdered with chalk to prevent excessive reflections).

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