Chamot, E. M. & Pratt, D. S. (1909). *J. Amer. chem. Soc.* **31**, 922.
Chamot, E. M. & Pratt, D. S. (1910). *J. Amer. chem. Soc.* **32**, 630.
Clayton, B. E. (1949). Personal communication.
Cohen, S. L. & Marrian, G. F. (1934). *Biochem. J.* **28**, 1603.
Engel, L. L., Slaunwhite, W. R. jun., Carter P. Nathanson, I. T. (1950). *J. biol. Chem.* **185**, 255.
Haenni, E. O. (1950). *J. Amer. pharm. Ass.* **39**, 544.
Kenny, A. P. (1952). *Biochem. J.* **52**, 611.
Kober, S. (1931). *Biochem. Z.* **239**, 209.
Marlow, H. W. (1950). *J. biol. Chem.* **183**, 167.
Marrian, G. F. (1938). *Bull. N.Y. Acad. Med.* **15**, 27.
Pinnow, J. (1915). *Z. Elektrochem.* **21**, 380.
Pinnow, J. (1917). *Z. Elektrochem.* **23**, 243.
Szego, C. M. & Samuels, L. T. (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 263.
Szego, C. M. & Samuels, L. T. (1943). *J. biol. Chem.* **151**, 587.
Venning, E. H. (1952). *Ciba Foundation Colloquia on Endocrinology*, **2**, 144. London: J. A. Churchill Ltd.
Venning, E. H., Evelyn, K. A., Harkness, E. V. & Browne, J. S. L. (1937). *J. biol. Chem.* **120**, 225.
Werner, E. A. (1933). *Analyst*, **58**, 335.

Continuous Direct Photometry of Dyed Materials in Filter Paper with Special Reference to the Estimation of Proteins Separated by Electrophoresis

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The estimation of protein fractions after their separation by electrophoresis in filter paper and subsequent staining may be carried out by excision, elution, and colorimetric estimation (Cremer & Tiselius, 1950) or by direct photometry, either intermittent (Grassmann, Hannig & Knedel, 1951) or continuous. Direct photometric methods have the advantage of being less laborious and also of requiring less material.

A brief account of an apparatus and technique for direct continuous photometry has already been given (Crook, Harris & Warren, 1952). In the present paper the underlying problems involved in such a technique are discussed, and a detailed account is given of an apparatus which has given satisfactory results in practice.

CONSIDERATIONS IN DESIGN

In designing an apparatus and a technique of measuring continuously the optical-density distribution in a strip of filter paper, many decisions have to be made between possible alternative solutions of the problems that arise. Some of these are straightforward, such as the choice of moving part, since it is obvious that movement of the paper past a fixed optical system and photocell is the simplest arrangement. Similarly, although a cylindrical paper carriage is more compact, a flat carriage is much simpler to construct and to retain the paper upon.

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At first sight, measurement of the amount of colour by reflexion from the dry paper might appear preferable to the measurement of transmission, since it avoids all the complications of the scattering of light by the fibres of the paper and the consequent necessity for an immersion fluid. However, although it is easy to perceive qualitative differences in reflexion by the unaided eye, preliminary experiments showed that there were quantitative irregularities in the amount of light reflected, due presumably to variations in the surface texture of the paper and to differences in the degree of penetration of protein and dye into the paper. Thus a transmission system which allows the light to pass through all the dyed protein is the only admissible type.

The paper must, however, be rendered as translucent as possible to reduce light scattering, i.e. it must be made to approach an optically homogeneous medium by the use of an immersion fluid. Theoretically, except for a small residual effect due to differences in the dispersion of immersion fluid and paper which would cause the combination to act as a Christiansen filter, an immersion fluid with a refractive index equal to that of the paper would reduce the scatter to zero provided the paper was homogeneous. However, since the paper is not homogeneous and scatter can only be reduced to a minimum, the refractive index of the immersion medium is not critical provided it is in the neighbourhood of 1·55, which is the approximate refractive index of cellulose. On trial, media with refractive indices in this region were found to be

sufficiently effective in minimizing scatter in filter paper (Whatman no. 1). It is, of course, important to remove all air trapped in the interstices of the paper.

The presence of residual scattering makes it necessary to consider carefully the layout of the optical system. The fact that the photocell must 'see' only a restricted length of paper requires the introduction of a slit parallel to the short axis of the paper strip. The best restriction is obtained by a narrow slit very close to the paper on the photocell side, since this prevents scattering of light into the photocell from adjacent areas of the paper. However, variations in scattering power of the paper then appear as variations in optical density and are recorded by the photocell as though they were variations in the amount of protein. Such variations may be sufficiently serious to make this positioning of the slit undesirable. It therefore appeared preferable to use the slit to restrict the illuminated area. By the use of a broad photocell placed close to the paper most of the scattered light is picked up. Variations of the scattering power of the paper become of minor importance and the system measures the light absorbed irrespective of the scattering. A small residual effect remains owing to differences in absorption within the paper of obliquely scattered light, according to whether scattering takes place through a clear or through a dyed region. This tends to depress the transmission of a clear region surrounded by heavily dyed areas and vice versa. This is a disadvantage of having the slit between the light source and the paper, and is obviated by having the slit immediately in front of the photocell. However, this effect was less important than variations in the scattering power of the paper.

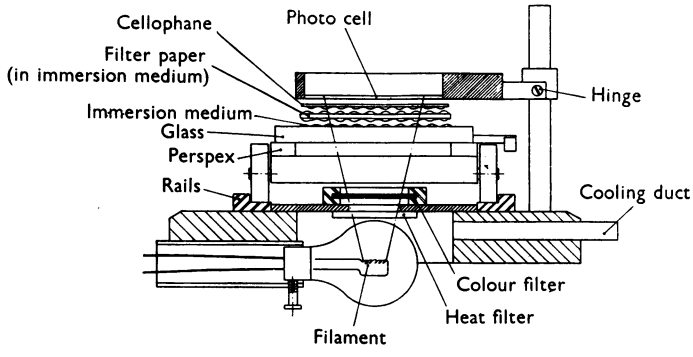
Correct restriction of the area of the paper 'seen' by the photocell demands a sharply outlined image of the slit. Such a requirement, combined with high light intensity, demands an elaborate optical system of condenser and focusing lenses for its complete realization. The next best solution is to remove lenses altogether from the system, relying on minimizing the distance between source and paper to obtain maximum intensity and placing the slit as close as possible to the paper to reduce the penumbra to a minimum. Such a system has the advantage of simplicity and compactness.

The restriction of the illumination by the slit makes inevitable the amplification of the photocell output before it is available to work a recording milliameter, even if a photomultiplier is used. The choice of photocell type therefore depends upon the available amplifiers, the necessary sensitivity, and the geometry of the optical system. The last two requirements are best satisfied by a selenium barrier layer cell, because of its high sensitivity compared with the simple vacuum and gas-filled emission cells and because its large sensitive area and absence of glass envelope make it possible to bring the sensitive surface close to the paper. It is generally regarded as not possible to amplify the output from one of these cells because of their low internal resistance. However, it can be done very satisfactorily with a Tinsley d.c. amplifier since this is of the galvanometer input type, particularly if the output of the cell only varies slowly as in the present case. A magnetic amplifier would also be suitable. A possible disadvantage of the Tinsley amplifier is its high input resistance—of the order of 2000 Ω. It is generally stated that selenium barrier layer cells do not show a linear current/light relationship if the resistance in the external circuit exceeds 50–100 Ω. However, the large area cell used here responds linearly within the limits of our detection when combined with a Tinsley d.c. amplifier having an input resistance of 2300 Ω.

DESCRIPTION OF APPARATUS

The apparatus embodying these features is shown in Fig. 1. The paper, soaked in the immersion fluid, is carried upon a thin glass plate which is in turn supported by the framework of the trolley. The trolley runs on rails fixed on the top of the wooden stand below which is supported the light source. The trolley carries along one edge a strip of toothed rack which engages with a pinion of a synchronous clock motor which is fixed to the wooden base. The rack disengages on lifting the trolley, which can therefore be placed at any convenient position along the length of the track. The trolley is driven at the rate of 1 in./min. Since the chart of the recording milliameter is also driven by a synchronous motor, paper and chart always remain 'in step'. The slit, at the level of the top of the wooden base, has adjustable jaws and its effective length may be varied by laying on top of it brass masks with openings of various sizes between 5 and 25 mm. The light source is an 18 w, 6 v car-headlamp bulb with straight filament. This is mounted below the baseboard with the filament parallel to the slit and with the glass bulb almost touching the heat filter, which is carried in a frame attached below the slit carrier. Since it is important that the light should remain of constant intensity, it was found necessary to use a large-capacity battery as a current source. Constant-voltage transformers are useful if the mains have small fluctuations. The photocell, measuring $1\frac{1}{2} \times 2$ in., is mounted in a shallow frame carried on a short pillar. Its height can be adjusted so that whatever the thickness of the glass plate, etc., the sensitive surface is only 2–3 mm. above the filter-paper strip. A hinge is provided so that the cell can be swung out of the way while the trolley and its burden are being manipulated. The light filters are laid loosely on the slit. The whole of the space above the baseboard is enclosed by a light-tight box with an easily removable lid.

The d.c. amplifier and recording milliameter used have been those which are normally part of the Tinsley polarographic equipment. The polarograph control box has also been used to provide sensitivity control and zero-setting facilities.



Section through optical system

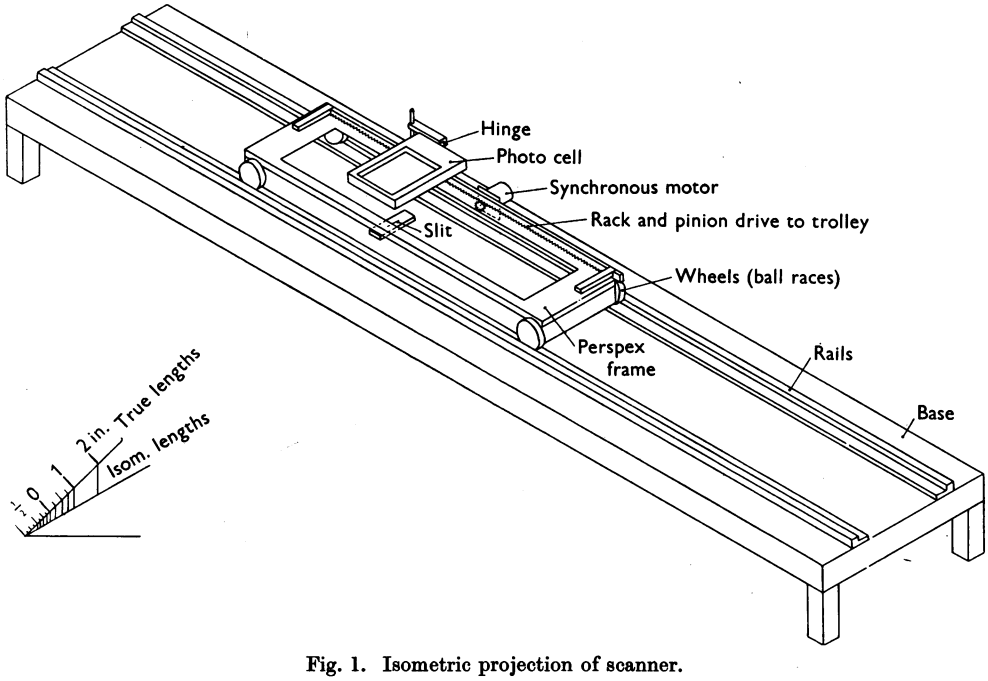


Fig. 1. Isometric projection of scanner.

To enable the recordings to be analysed, each is enlarged approximately 2½ diameters by means of an episcopic projector. The recording, covered by a thin sheet of glass, is supported on a table in the light box where it is illuminated by four 60 w bulbs placed at the corners of the box. The lens, which is a Dallmeyer F 2·9, 8 in. focal length, focuses an enlarged image at the top surface of a sheet of plate glass covering a hole cut out of a table beneath which the light box is placed. A sheet of tracing paper is placed on this glass and the image followed round in pencil. Black curtains assist the procedure.

Preparation of strips

In experiments using serum or plasma, strips of no. 1 Whatman paper of area 38 × 5 cm. were used. For electrophoresis, Perspex tanks as described by Flynn & de Mayo (1951) were used. In the majority of experiments, approximately 5 μl. of serum and plasma were used on each strip. A veronal buffer of pH 8·6 and ionic strength 0·05 was usually employed, and a current strength of about 0·1 mA/cm. width of paper. A current-regulator was incorporated in the circuit in order to maintain a constant current through-

out the duration of the electrophoresis, which was usually carried out overnight for a period of about 16 hr. The strips were then dried in an oven at 100–110° and then stained.

It was found that the most convenient dye was azocarmine B, used according to the technique of Plückthun & Götting (1951). After being washed, the protein components appeared as red bands on a completely white background. The use of 'naphthalene black 12B.200' (Flynn & de Mayo, 1951) was also investigated. The chief disadvantage of this dye is that even after very protracted washing the background is still distinctly blue. When strips dyed with naphthalene black are used for direct photometry, the background colour introduces complications which are absent with azocarmine (cf. Fig. 9). Bromophenol blue, which is a satisfactory dye with the elution technique, is unsuitable for direct photometry because of its liability to change colour on exposure to the atmosphere.

After the final wash, the dyed strips were again dried in an oven at 100–110° and were then ready for impregnation with the immersion fluid. The immersion fluid used throughout these experiments was a mixture of equal volumes of α-bromonaphthalene and liquid paraffin (sp.gr. 0·880–0·895). A mixture of these two liquids was first suggested by Grassmann *et al.* (1951) and was found to be effective in reducing scatter, and has the added advantage of having no deleterious effect on Perspex. The dried paper strips are immersed in the mixture in a dish or beaker which is then transferred to an empty vacuum desiccator and evacuated with a water pump for about 20 min. A number of strips may be treated in this way simultaneously and if not required immediately for photometry should be left soaking in the liquid.

For photometry the strip is removed from the immersion fluid, drained momentarily, and laid flat on a thin glass plate (7·5 × 27 cm.). A strip of Cellophane is then placed over the whole paper strip and firmly pressed into close contact so as to exclude air trapped between the filter paper and the glass and between the filter paper and the Cellophane. The strip can now be transferred to the trolley and a recording made.

CALIBRATION

A first essential in calibrating the instrument is to ensure that the chart reads linearly in terms of light falling upon the photocell. In recording milliameters working on the Murday system, such as that supplied with the Tinsley Polarograph, the chart is not evenly divided. However, it is substantially linear between 10 and 90 divisions (Fig. 2), and in these experiments has usually been used between 20 and 80 divisions. To check the linearity of the photocell-amplifier-recorder combination, an Ilford neutral wedge was placed on the trolley and a recording of the light transmitted was made as it moved across the slit. The routine which was followed in this and all other recordings was the following. With the driving motor stopped, the wedge and trolley are positioned so that the slit image is in a clear portion of the wedge and the absorbing part of the wedge or paper will be carried over the slit when the driving motor is started. It is necessary to have the light on for these adjustments and in any event it is preferable to have the light on continuously, since this ensures a constant voltage from the battery and that the photocell has reached a steady state. Normally, the photocell is disconnected from the amplifier except during a recording. When the adjustments are complete, the light-proof lid is replaced, the recorder chart started and the recorder pen adjusted to division 20 by means of the zero-setting control. The photocell is now switched into the amplifier and the light intensity adjusted by means of a series resistance until the pen is at 80. Initially this may require adjustment of the sensitivity of the amplifier. Usually it has been found to be satisfactory to use this at the 1 μA range, that is, so that a 1 μA input from the photocell gives a full-scale deflexion. The trolley motor is now switched on and as the wedge or paper is traversed across the slit the milliameter records the decrease of photocell

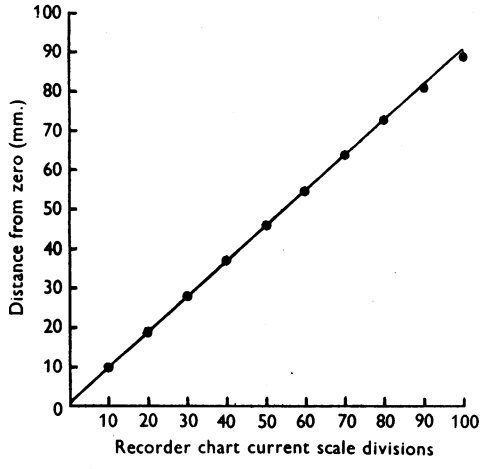


Fig. 2. Graph showing essential linearity of recorder chart current scale divisions over the normal working range of 20–80 scale divisions.

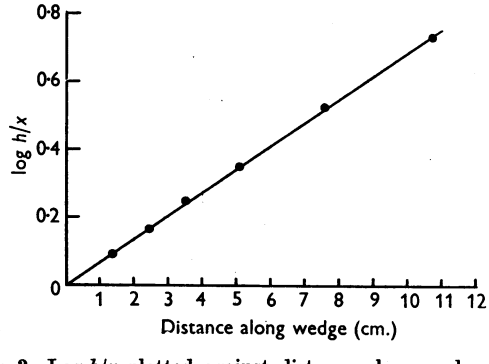


Fig. 3. Log h/x plotted against distance along wedge for a recording of light transmitted during transit of an optical wedge. h = excursion of milliameter with full light intensity; x = excursion with absorbing medium.

current. Since the optical density of the neutral wedge varies linearly with length, if 'h' is the excursion of the milliameter with full light intensity and 'x' is the excursion with an absorbing medium in the light path, a plot of log h/x should be linear for a recording from the wedge provided the photocell-amplifier-recorder combination is linear. Fig. 3 shows that the response of the combination is substantially linear.

It appears to have been generally assumed by other workers (e.g. Grassmann *et al.* 1951; Latner, Braithwaite & Nunn, 1952) that the light absorption by dyed protein in an immersed filter paper obeyed Beer's law. However, the system is optically very complex, both because of the residual scattering of light and also because the protein-dye complex is not uniformly distributed, being in the form of irregular aggregates of dyed protein of different sizes distributed non-uniformly within the depth of the paper. It seemed essential, therefore, to test experimentally the validity of Beer's law.

Serial dilutions in saline of a solution of dried, citrated human plasma were prepared. Filter paper disks (12·5 cm.) were saturated with these dilutions by placing a disk in the bottom of a Petri dish and quickly pouring on the diluted plasma. It is essential that the dish and the paper should be dry initially and that the paper be completely wetted at once, otherwise non-uniform impregnation with protein results. After soaking for a few minutes, the papers were dried and dyed in the usual way. From the central, uniform portion of each disk an area 6 × 5 cm. was cut out and divided into three strips, 2 × 5 cm., numbered 1, 2 and 3. Nos. 1 and 3 were placed in test tubes and the dye was eluted from them by treatment with 0·1 N-NaOH and measured in a photoelectric colorimeter, care being taken to ensure that the dilution of the dye was sufficient to bring the readings into the range where Beer's law was obeyed. The concentration was expressed in arbitrary units based on the colorimeter reading and the dilution. Strip no. 2 was soaked in immersion fluid and, in close juxtaposition with similar strips from other plasma dilutions, was laid upon a glass plate to form a graded series of increasing depth of colour—an 'optical wedge' of dyed protein. At the beginning and end of this 'wedge', strips of immersed but otherwise untreated paper were placed to provide a base-line. The whole was then placed on the trolley and a recording made. A typical result is shown in Fig. 4. The sloping tops of the steps are not due to non-uniform dyeing of the strips but to the effect discussed earlier of the surrounding dyestuff upon the light absorption, and possibly also to pen-friction in the recorder. The value of the current at the centre of the strip can be taken as the correct value of 'x' for each dye concentration. When log h/x is plotted against concentration of dye in arbitrary units, a straight line should result if Beer's law is being obeyed. As can be seen from Fig. 5 this is very far from being true. There are appreciable deviations at quite low dye concentrations.

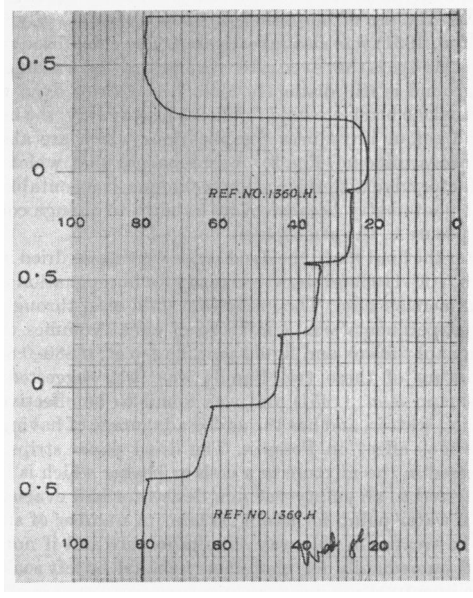


Fig. 4. Photograph of actual recording of azocarmine 'step-wedge'. Milliameter adjusted to 80 scale divisions for transmission through undyed paper. 20 scale divisions correspond to complete extinction. The wedge contained five steps made by serial dilution of 1, 1/2, 1/4, 1/8, 1/16.

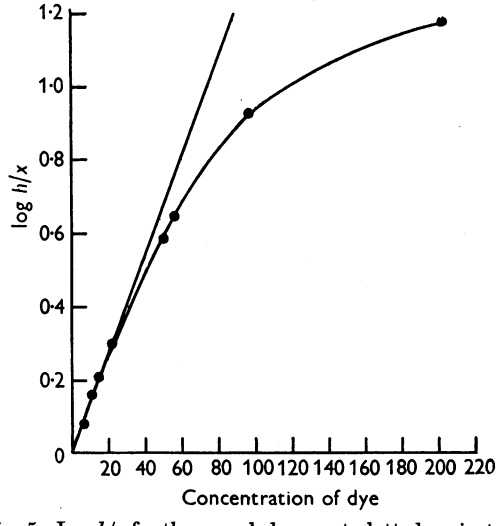


Fig. 5. Log h/x for the recorded current plotted against the concentration of dye (in arbitrary units) actually on the paper, as determined by elution. The straight line is calculated from Beer's law.

In attempts to find a suitable law to express the relation between current and dye concentration, it was found empirically that the relation between the decrease in photocell current 'y' and the dye concentration in arbitrary units is well represented over the working range by a rectangular hyperbola of the form:

$$y=\frac{ac}{b+c},$$

where 'c' = concentration and 'a' and 'b' are constants. This can be more readily seen by taking reciprocals, when

$$1/y=\frac{b+c}{ac}=\frac{b}{a}\frac{1}{c}+\frac{1}{a}.$$

Thus, when $1/y$ is plotted against $1/c$ (Fig. 6), a straight line results, of which the intercept on the ordinate is $1/a$ and the slope b/a. From this plot, the constants for the best fit of a set of experimental points are readily deduced. It must be emphasized that the hyperbolic relation no longer holds under conditions of extremely dense staining.

Later it was found to be unnecessary to impregnate the paper with protein before applying the dye. Simple dipping of filter paper disks in serial dilutions of dye solution followed by drying was found to give identical calibration curves. Nor in the case of azocarmine nor naphthalene black, two dyes with quite different absorption spectra, was the curve altered by changing the dye, provided the recording was made with a light filter passing only the major absorption peak of the dye used. Unsuitable filters or the absence of a filter gave curves falling below that in Fig. 6. Fig. 6 includes points obtained from wedges of protein dyed with azocarmine B, with azocarmine B without protein, and with naphthalene black without protein.

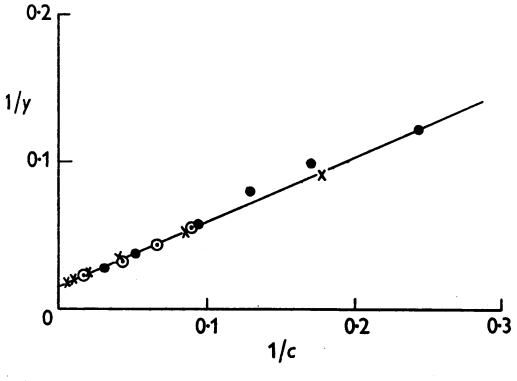


Fig. 6. Reciprocal of the decrease of photocell current ($1/y$) plotted against the reciprocal of the dye concentration ($1/c$). ● = naphthalene black; ○ = azocarmine B, × = azocarmine B + protein. Ilford filters 608 (napthalene black) and 624 (azocarmine B).

It is thus obvious that assuming the applicability of Beer's law will lead to grossly erroneous results, the errors being greatest when comparing a high with a low concentration.

The pronounced curvature of the calibration curve has further important implications for the slit dimensions and the amount of protein placed upon the paper. Since the decrease in photocell current for large concentrations of dye is small in proportion, the presence of small amounts of extraneous radiation will cause a large error when calculated in terms of protein. Use of a wide slit for scanning bands whose edges rise sharply is therefore liable to introduce serious 'optical dilution', resulting in a lowering in the height of the recorded peak. It has been found necessary to have the slit less than 1 mm. wide to avoid this effect with the strips used in these experiments.

Similarly, it at first appeared best to limit the protein band to less than the total width of the paper and to scan with a slit whose length was sufficient to project beyond the bands and ensure complete integration of all the colour. However, the diluting effect of the undyed paper at the ends of the slit reduced the albumin peak seriously, and it was found to be necessary to use a slit short enough to take in only the central uniform portion of the bands.

Increasing the amount of protein too much results, not only in poor strips due to 'overloading', but also brings the albumin peak into the flat portion of the calibration curve and makes the record insensitive to variations in the amount of albumin. This last effect becomes important at lower concentrations than does 'overloading' and for best records, strips should be run which would be judged rather too pale for comfortable examination by eye.

ANALYSIS OF CURVES

When a paper strip carrying bands of dyed protein resulting from the electrophoresis of, say, normal human serum is run through the recorder, there results an 'electrophoresis diagram' closely resembling that obtained with the classical Tiselius equipment. Some typical results are shown in Fig. 7. The chief differences are (1) that the albumin peak is considerably depressed owing to the flattening out of the calibration curve at high dye concentrations and (2) that here each peak represents the total protein making up that component, whereas in the classical diagram it represents the concentration change due to adding that component to a mixture of other components. The total area under a peak is a function of the total dye in that part of the paper, which in turn represents the total amount of protein in the particular serum protein component. The chief question that arises in attempting to assign a given area under the curve to

a particular protein is, what is the dividing line between it and its nearest neighbours. Three chief methods of deciding upon the separation of the components have been used in analysing the classical Tiselius diagrams. The oldest is simply to drop perpendiculars from the troughs of the curve to the base-line and to regard the area enclosed between each adjacent pair of perpendiculars as a measure of the amount of that component. Alternatively, if the peaks are symmetrical in outline, the inner edges of the albumin and γ-globulin peaks can be obtained by reflecting the outer edges about the perpendicular from the peak (Svedberg & Pedersen, 1940). Subtraction of these inner 'tails' from the total curve gives a residual curve for the remaining components and the process can be repeated with this. A third method depends upon the assumption that, in the absence of distorting influences, the shape of the peak is due to the diffusion of the boundary and therefore should resemble a normal Gaussian error curve. It is possible to fit such curves to the diagram by the methods of Labhart (1947) and of Wiedemann (1947). The results obtained by these more elaborate methods do not differ very much from those obtained by simple perpendicular dropping, and in any case they are not applicable here, since the peaks are not symmetrical and do not fit normal error curves. Perpendicular dropping was, therefore, used in this work and has the advantage that it is the simplest and most consistent of the methods for drawing arbitrary lines of demarcation and is, in fact, the method which results when elution methods of analysing the paper strips are used. Its chief disadvantage is that it tends to exaggerate a minor component which occurs as a shoulder on the side of a major component, e.g. the α_1-globulin in most sera.

As the calibration curve is not linear, the area under a peak is not necessarily directly proportional to the total amount of dye, i.e. to the total amount of protein in the particular components. However, the initial part of the curve (up to one-fourth of the total range) is effectively linear, and for peaks which fall below this level the area of the peak can be taken as an estimate of the amount of the component present within the experimental error. With most strips, certainly those dyed with azocarmine B, it is easy, and indeed desirable for other reasons, to arrange the total amount of protein so that only the albumin peak rises above the straight portion of the calibration curve. The remaining components can therefore be estimated by direct planimetry.

A simple graphical method was developed for correcting the albumin peak so that its area becomes

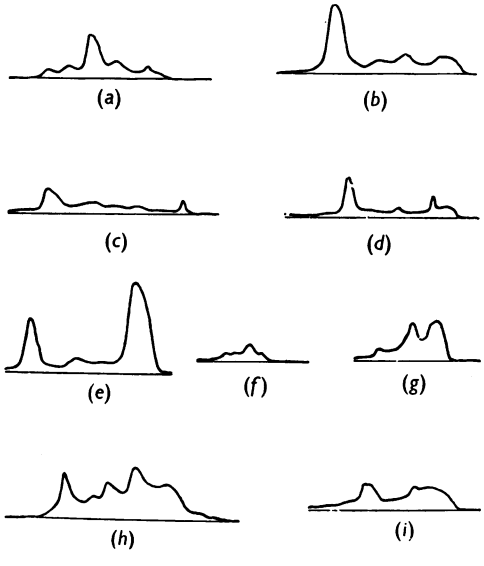


Fig. 7. Tracings of illustrative recordings: (*a*) human nephrotic serum; (*b*) normal human serum; (*c*) foetal goat plasma; (*d*) maternal goat plasma; (*e*) human lymphatic leukaemia serum; (*f*) human milk in phthalate buffer pH 6·0; (*g*) goat colostrum without caseinogen; (*h*) partially purified hexokinase preparation; (*i*) goat colostrum. With the exception of (*f*) all samples run in barbitone buffer pH 8·6. The fastest moving component appears on the left of the tracing.

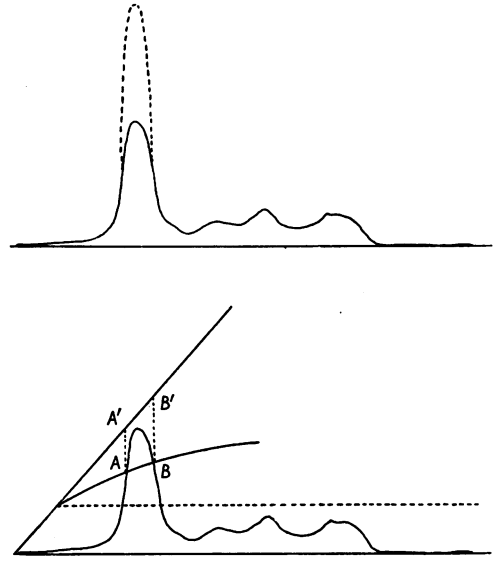


Fig. 8. Diagrams showing method of using calibration curve for correcting high peaks for deviation from Beer's law. Points of interception of a high peak with the calibration curve (*A* and *B*) are replotted vertically on the linear projection of the initial part of the calibration curve (lower diagram). By lateral movement of the peak this process may be repeated and the corrected peak constructed as shown by the dotted curve in the upper diagram.

directly proportional to the amount of albumin, which can therefore also be measured by planimetry. The calibration curve is redrawn so that its vertical scale is enlarged by the same factor as are the tracings obtained by projection from the original records. The concentration scale is quite arbitrary. The initial straight portion of this curve is projected as shown in Fig. 8. The tracing of a record is then placed over the calibration curve and projected straight portion, and adjusted so that its base-line coincides with the base of the calibration curve. In most instances the albumin curve will cut the calibration curve at two points, say A and B. If the decrease in photocell current due to these two concentrations of dye had been directly proportional to the dye concentration, the points would have occurred at the positions vertically above these and upon the projected straight line at A' and B', respectively. By shifting the tracing sideways, keeping its base-line co-incident with the base of the calibration curve, another pair of 'proportional points' can be found, and so on until a sufficient number has been plotted to allow a freehand curve to be drawn connecting them. This will merge into the original albumin curve at the height at which the calibration curve diverges from its initial linear portion. If the calibration curve and projected line are drawn on graph paper, finding the points vertically above the intersections is very simple and the whole process of 'correcting' the albumin peak by this method only occupies 3 or 4 min. The corrected albumin peak thus obtained, since the height of every point on it is directly proportional to the dye concentration at that point, has an area directly proportional to the total amount of the albumin, and is ready for planimetry.

This last operation is carried out by placing the tracing on a flat surface beneath a thin sheet ($\frac{1}{16}$ in. or less) of Perspex, which provides an excellent surface for the planimeter to work on and holds the tracing flat and prevents it from moving about.

It is because of the necessity of correcting the record that naphthalene black is an unsuitable dye. With azocarmine B, the background is completely undyed and only the albumin peak reaches into the region of substantial curvature of the calibration curve. With naphthalene black, on the other hand, even prolonged washing does not remove the dye from the paper, and the electrophoresis diagram is therefore superimposed upon a considerable continuous background which raises all of the peaks into the region of curvature. A tedious correction of the whole curve by the method described above is then necessary. Fig. 9*b* shows a record from a naphthalene-black dyed strip, with the corrections, for comparison with the azocarmine-B dyed strip in Fig. 9*a*. Strips of undyed but immersed papers at each end of the naphthalene-black strip are included in order to provide a base-line from which the corrections are made, and the final areas under the peaks are from this base-line.

REPRODUCIBILITY OF RESULTS

It was found that the reproducibility of the recordings obtained from repeated runs on a given strip was good. That is, the actual curves recorded are, for all practical purposes, superimposable. However, in considering the quantitative evaluation of components in a given serum, there are several stages in the procedures for recording and analysing the curves where errors might be introduced. In

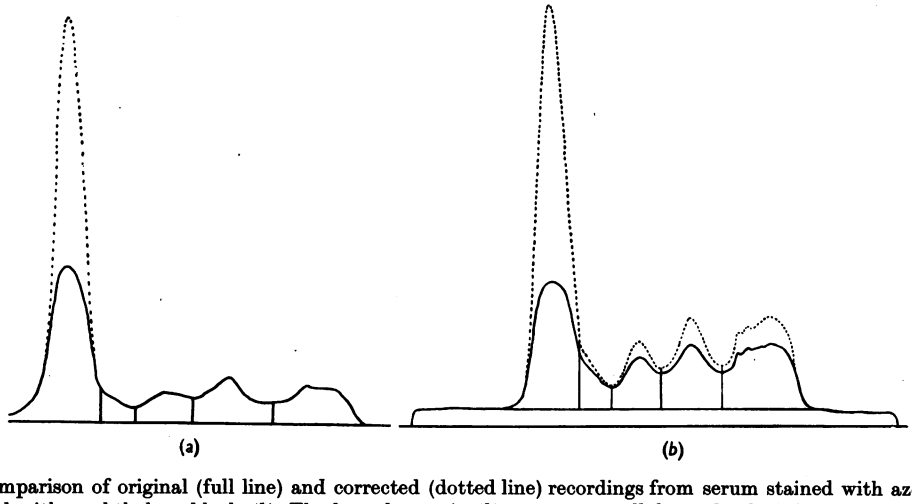


Fig. 9. Comparison of original (full line) and corrected (dotted line) recordings from serum stained with azocarmine B (*a*) and with naphthalene black (*b*). The low plateau in (*b*) represents cellulose-adsorbed dye not removed by the washing process.

Table 1. *Analysis of variance for thirty-two replicate recordings on the same sample of serum*

(Eight separate strips were prepared and each was run four times through the photometer.)

Fraction	Mean % of total protein	Components of variance: Between strips σ_B^2	Components of variance: Between runs σ_W^2	Total variance	S.D.
Albumin	60·5	3·02	2·50	5·52	2·35
α_1-Globulin	3·9	0·15	0·57	0·72	0·85
α_2-Globulin	8·7	0·89	2·18	3·07	1·75
β-Globulin	10·2	0·82	0·30	1·12	1·06
γ-Globulin	16·7	1·09	0·75	1·84	1·36
	100·0				

a detailed examination of the procedure from this point of view, it was found that the variation in results largely arose from the inherent difficulties of that part of the analysis involving the delimitation of the fractions on the diagram. Replication experiments indicated that the standard deviation of the percentage of any given component was of the order of 1–2 % of the total protein. A typical result is shown in Table 1. In this experiment eight replicate strips were run on a single sample of serum from one subject. No attempt was made to apply exactly the same quantity of serum to each strip. The strips were stained with azocarmine, washed and dried in the usual way. Four independent recordings were then made from each strip, the strip being re-immersed between each recording. The thirty-two recordings thus obtained were then analysed independently and the variance of the percentage of each fraction was divided into two components. One component, σ_B^2, represents the variation between individual strips and σ_W^2 that between replicate recordings on the same strip. These two components are of the same order of magnitude for each fraction.

DISCUSSION

This investigation has shown that, with attention to certain important points, it is possible to analyse human serum by paper electrophoresis with the same order of error as that obtained with the classical Tiselius apparatus, but using apparatus of about one-tenth the cost. Also, at least ten times as many analyses can be performed in the same time. The question therefore arises as to how well the results of the older established and more elaborate apparatus are reproduced by the newer and simpler techniques. In attempting to discuss this question it is important that certain points should be kept clearly in mind.

The first point to be remembered is the arbitrary nature of the fractions separated by the classical procedure. It is well known that although, in general, electrophoresis of human serum under standard conditions produces only five components (albumin, α_1-, α_2-, β- and γ-globulins), many more proteins than this are actually known to be present. Thus, Cohn and his collaborators (e.g. Cohn, Oncley, Strong, Hughes & Armstrong, 1944; Cohn *et al.* 1946) have separated more than a dozen fractions differing clearly in their physico-chemical properties, a large number of enzymes are known to be present in serum, and most sera contain a host of antibodies of one sort or another. Many of the peaks on the classical electrophoresis diagram must therefore represent mixtures of proteins and this has been demonstrated for the α- and β-globulins by Cohn's fractionations. Comparison of electrophoresis with ultracentrifugation and salt precipitation serves only to emphasize the arbitrary nature of any of the fractions produced by whatsoever method from so complex a mixture as serum.

Considerations such as these suggest that the regions below the troughs in the classical diagrams also represent mixtures and may contain components other than those represented by the adjacent peaks. A recent experiment by Kunkel & Tiselius (1951), in which serum was separated electrophoretically in filter paper in two directions at right angles, supports this.

In analysing schlieren diagrams quantitatively, it is generally assumed that the regions below the troughs represent mixtures of the components forming the adjacent peaks caused by spreading of the peaks owing to diffusion and errors in the separation procedure, and contain no other components. Indeed, in most instances, no other assumption would be possible in the absence of other data as to the true number of protein components present, and even with such data there would be no obvious method for assigning particular areas to particular components. It is obvious that a large number of different combinations of components could give rise to the same electrophoretic diagram.

These facts are generally admitted by those working on the electrophoresis of proteins. However, there is no doubt that, in practice, they are

largely ignored in interpreting electrophoresis diagrams or in arguments concerning the relationship of the results of electrophoresis to other evidence about the properties of proteins and protein mixtures. Thus, it is frequently reported that, in a partially purified protein preparation, 'the main component is accompanied by x% of another protein of lower mobility', the implication being that each is a single entity. Often this assumption is subsequently found to be justified, but not invariably so.

It is often assumed that the classical electrophoresis diagram provides an absolute standard to which other methods of separation should be referred. However, in view of the facts mentioned above, there could be no *a priori* expectation that electrophoresis in filter paper with subsequent dyeing should produce results identical with those obtained in the classical apparatus. The fact that the picture obtained for normal sera separated in filter paper and subsequently dyed resembles very closely that generally obtained by the older technique can be regarded as nothing more than an interesting and useful coincidence.

Whether the two techniques will always give qualitatively similar pictures remains to be determined. It is certain already that there are quantitative differences. In discussing the reasons for these differences and the criteria to be used in assessing the paper electrophoresis diagrams, a second point must be borne in mind, namely, the arbitrary nature of the quantitative interpretation of both the classical and paper electrophoresis diagrams.

Apart from the assumption that only the major components are present and that it is possible to fix lines of demarcation between these, two other major assumptions are made in converting the areas under a classical electrophoresis curve into their equivalents in protein. The first is that the change in refractive index at the boundary between components is caused solely by changes in the concentration of protein, and the second is that the specific refractive increments of all components are the same, or alternatively that the specific refractive increments of all globulin components are equal to that of γ-globulin, and that these remain the same from one serum to another, in health and in disease, and are independent of the admixture of varying quantities of other components.

Neither of these assumptions is true. The presence of the so-called δ and ϵ anomalies makes quite clear that changes in the buffer and salt concentrations do occur. It is generally assumed that no such anomalies are associated with the protein components, other than the β-peak on the descending limb where the occurrence of the so-called β-anomaly has usually caused investigators to confine their quantitative analyses to the ascending limb. However, particularly in the albumin and γ-globulin components where there is a junction between buffer and buffer + protein, the occurrence of Donnan equilibria and of changes in pH and electrolytic conductance would make it inevitable that there are substantial changes in the concentration of buffer and salts, i.e. that these peaks are due partly to protein and partly to 'anomalies'. This has recently been confirmed for the albumin peak by Svensson (1943), Perlman & Kaufmann (1945) and by Koling, Perring & Hogness (1946). Svensson's results suggest that the proportion of albumin which, for an average normal serum, is usually measured as approximately 60 %, should be approximately 56 % when allowance is made for the accompanying anomaly. Although investigations have not yet been made, there is no reason to assume that similar effects are absent at the other peaks.

Little information is available on the variation of the refractive index increment of the different components, or of how this is affected by the environment. However, it is unlikely that lipoproteins, some of which are known to contain up to 75 % lipid, would have the same value for this constant, as, say, albumin. Moreover, proteins other than those present in serum are known to vary considerably in this respect (e.g. Putzeys & Brosteaux, 1936).

Nothing that has been said above should be interpreted to mean that the assumptions are not useful or even that other assumptions are possible. They have only been enumerated to demonstrate that the usual quantitative interpretations of the classical electrophoresis diagram can have no claim to absoluteness and indeed represent an entirely empirical method of analysis giving strictly comparable results only when carried out under carefully standardized conditions. As this is almost never done, most of the data in the literature which have been produced by this apparatus can only be approximately compared.

Quantitative evaluation of dyed-paper electrophoretic diagrams involve assumptions similar to those necessary for the classical diagrams. Again it must be assumed that only the major components are present and that there is a clear-cut line of demarcation between them. In addition, it has to be assumed that only protein takes up the dye, and, if the results are to be reported in terms of per cent protein, that all components take up the dye to the same degree. Alternatives to this will be discussed later. In addition, a further assumption has until now been made by most workers in this field, namely, that the relation between the concentration of the dye and the photocell current is that described by Beer's law. This last assumption has been shown

in this paper to be invalid and to lead to serious under-estimation of the amount of dye in the albumin band. Using an elution method, and bromophenol blue as their dye, Cremer & Tiselius (1950) and Kunkel & Tiselius (1951) found it necessary to multiply the globulin components by factors of about 1·6 to bring their results into line with those of the classical method. The question arises as to whether it is advantageous to apply such correction factors so as to bring the paper results into line with the classical values, or alternatively, whether calibration factors based on nitrogen, dry weight, or on some other property of the fractions should be used.

It appears to the authors that correction factors are neither necessary nor desirable. Even though it may be possible to determine correction factors for each peak for converting paper values into classical values, little is gained, since the classical values are not strictly comparable among themselves; nor is there any guarantee that these factors will remain the same from one serum sample to another or from one disease to another. In any event, the lack of any fundamental basis for the classical values suggests that it would be preferable to build up a new set of data based entirely upon the paper technique.

The alternative proposal that the amount of dye absorbed by any component should be calibrated in terms of nitrogen or dry matter, etc., is open to exactly the same objections, namely, the difficulty of separating the components in order to carry out the calibrations and the uncertainty that the calibrations will be unvarying from one protein mixture to another or from one disease to another. Indeed, it is well known that the proportion of nitrogen in the various serum components varies considerably.

It is therefore concluded that expression of the analyses in terms of the total amount of dye adsorbed by components separated and dyed under standard conditions is at present the most satisfactory basis for measurement. There is nothing in this to preclude the investigation of changes in the composition of sera and other protein mixtures under various conditions, whether these changes are due to variation in the actual amounts of the components or in their relative ability to adsorb dye. Indeed, whatever the basis of measurement, there is little to distinguish these two factors unless more than one criterion is applied simultaneously.

SUMMARY

1. An apparatus is described for the continuous direct photometry of dyed materials in filter paper, which has been successfully employed for the estimation of proteins separated by electrophoresis.

2. The applicability of Beer's law to the absorption of light by dyed protein in an immersed filter paper was tested experimentally. It was found that, outside of a very restricted range, large deviations occur.

3. A method of calibration is described by which the deviation from Beer's law may be estimated and compensated for.

4. The relation between the results of electrophoretic analysis of proteins in paper and in free solution are discussed.

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REFERENCES

Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L. jun. & Armstrong, S. H. jun. (1944). *J. clin Invest.* **23**, 417.

Cohn, E. J., Strong, L. E., Hughes, W. L. jun., Mulford, D. J., Ashworth, J., Melin, M. & Taylor, H. L. (1946). *J. Amer. chem. Soc.* **68**, 459.

Cremer, H. D. & Tiselius, A. (1950). *Biochem. Z.* **320**, 273.

Crook, E. M., Harris, H. & Warren, F. L. (1952). *Biochem. J.* **51**, xxvi.

Flynn, F. V. & de Mayo, P. (1951). *Lancet*, **2**, 235.

Grassmann, W., Hannig, K. & Knedel, M. (1951). *Dtsch. med. Wschr.* **76**, 333.

Koling, J. L., Perring, J. D. & Hogness, K. R. (1946). *Arch. Biochem.* **11**, 345.

Kunkel, H. G. & Tiselius, A. (1951). *J. gen. Physiol.* **35**, 89.

Labhart, H. (1947). *Experientia*, **3**, 37.

Latner, A. L., Braithwaite, F. & Nunn, R. A. (1952). *Biochem. J.* **51**, x.

Perlman, G. E. & Kaufmann, D. (1945). *J. Amer. chem. Soc.* **67**, 638.

Plückthun, H. & Götting, H. (1951). *Klin. Wschr.* **29**, 415.

Putzeys, P. & Brosteaux, J. (1936). *Bull. Soc. Chim. biol., Paris*, **18**, 1681.

Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*, p. 296. Oxford: Clarendon Press.

Svensson, H. (1943). *Ark. Kemi Min. Geol.* **17A**, no. 14.

Wiedemann, G. (1947). *Helv. chim. acta*, **30**, 892.