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Paper Electrophoresis as a Quantitative Method

SERUM PROTEINS

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A number of workers have recently questioned the validity of paper electrophoresis as a method for the separation and quantitative estimation of serum proteins. The four main problems are: (1) albumin 'tailing' due to irreversible adsorption of albumin on paper during its migration (Kunkel & Tiselius, 1951; Merklen & Masseyeff, 1952; Sommerfelt, 1953); (2) variation in dye binding capacity with the amount and area of application of protein (Franglen & Martin, 1954; Hardwicke, 1954); (3) fading and incomplete elution of the commonly used dyes bromophenol blue and amidoschwarz 10B (Flynn & de Mayo, 1951; Pezold & Peiser, 1953; Franglen & Martin, 1954); and (4) the lack of a linear relationship of dye concentration to photocell response in the direct scanning of dye on paper (Van Os, 1953; Crook, Harris, Hassan & Warren, 1954). In addition, there has been widespread disagreement as to the magnitude and usefulness of 'correction factors' for the conversion of the results of paper electrophoresis expressed in terms of dye binding to those of moving boundary electrophoresis expressed in terms of refractive index increment.

A study of these and some related problems is presented here. On the basis of this work, a detailed procedure for the paper electrophoretic analysis of serum proteins has been developed.

MATERIALS AND METHODS

Paper electrophoresis of serum. This was carried out on Whatman 3MM paper strips 2·9 × 30·0 cm. in a hanging strip type cell (Williams, Pickels & Durrum, 1955). The paper was saturated with barbital buffer pH 8·6 ionic strength 0·075 and allowed to drain in position in the cell for 15 min. Serum (0·01 ml.) was then applied from a micropipette to the apex of the strip directly over the silicone-coated glass supporting rod. Separation was carried out with a constant current of 8 mA for 8 strips (90–100 v, about 3–3·5 v/cm.) for 16 hr. at room temperature, avoiding any marked inequalities of temperature from drafts, sunlight, etc. The strips were then removed from contact with the feed wick, spread flat, and dried in an oven at 110–120° for 30 min.

Staining. The strips were transferred to a staining rack which holds them apart, and placed in an aqueous dye bath containing 0·01% bromophenol blue, 5% acetic acid (v/v) and 5% $ZnSO_4$, $7H_2O$ (w/v) for 16 hr. at room temperature. They were rinsed 3 times for 5, 5 and 10 min. in 2% acetic acid (v/v) without agitation, and then for 2 min. in a bath of 2% sodium acetate (CH_3.COONa, $3H_2O$, w/v) and 10% acetic acid (v/v). After being blotted gently on clean filter paper (S & S 470), the strips were transferred to a drying rack which touches them only on the ends and dried in the oven for 8–10 min. at 110–120°. A typical pattern is shown in Fig. 1.

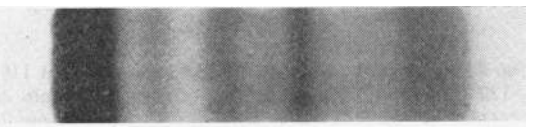

Fig. 1. A typical paper electrophoretic pattern of serum proteins prepared by the technique described.

Elution. The strips were cut into the desired segments and eluted in 0·01 N-NaOH for 30 min. with occasional agitation, thoroughly mixed, and read within 1 hr. in a Beckman model DU spectrophotometer at 590 mμ. in 1 cm. cuvettes. The monochromator was calibrated with a mercury lamp.

Scanning. Dyed strips were scanned with an automatic recording and integrating scanner (Durrum & Gilford, 1954) after the strips had been 'cleared' by dipping in liquid petrolatum (mineral oil), allowed to drain for 2–18 hr., and blotted. After scanning, the petrolatum was removed with ether.

Protein was determined by the biuret method (Gornall, Bardawill & David, 1949) or by micro-Kjeldahl determination of nitrogen.

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RESULTS

Drying of strips

Most of the published procedures for paper electrophoretic analysis of serum proteins have not specified the exact conditions under which the proteins were dried after separation and prior to staining. It might be expected that variations in the amount of heat applied to oven-dried strips would lead to different degrees of denaturation and unfolding of the peptide chain of the protein, thus allowing access of dye to a varying number of dye binding sites.

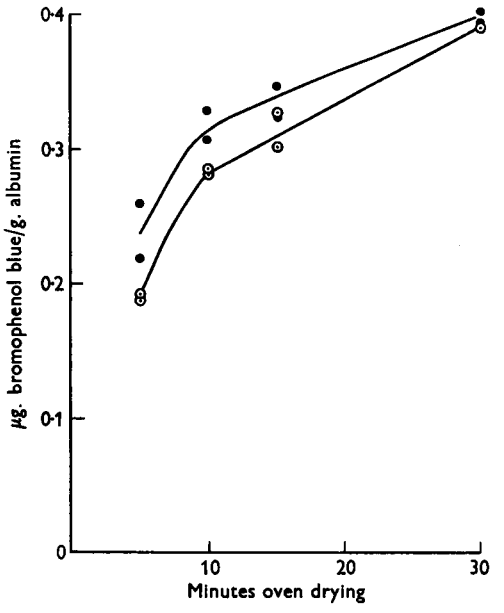

Fig. 2. Effect of the length of time of oven drying at 110–120° on the dye uptake of human serum albumin on paper. ●—●, 0·15 mg. in 0·01 ml.; ⊙—⊙, 0·30 mg. in 0·02 ml.

Fig. 2 shows the influence of the length of time of oven drying at 110–120° on the amount of dye bound by human albumin. In order to obtain bands of a relatively pure protein on paper at concentrations and with areas of application similar to those encountered with an electrophoretic separation, but without the problem of albumin 'tailing', the protein was streaked on paper which previously had been moistened with buffer and blotted gently. The strips were then dried for 8–30 min. at 110–120° and carried through the standard staining procedure. Although the strips were dry after 5–8 min. at this temperature, the dye uptake increased considerably with further heating, and furthermore the dye bound/g. of protein was not the same for the two different amounts of protein studied until after 30 min. heating.

This effect is less marked or absent for globulins. Consequently it is possible to produce widely varying dye binding ratios for albumin and globulin depending on the amount of heating. Thus, in another experiment the ratios of dye bound by γ-globulin to dye bound by albumin were 1·2 when the strips were dried at room temperature, and 0·75 when dried in the oven (see also Fig. 2, chap. 18, Block, Durrum & Zweig, 1955).

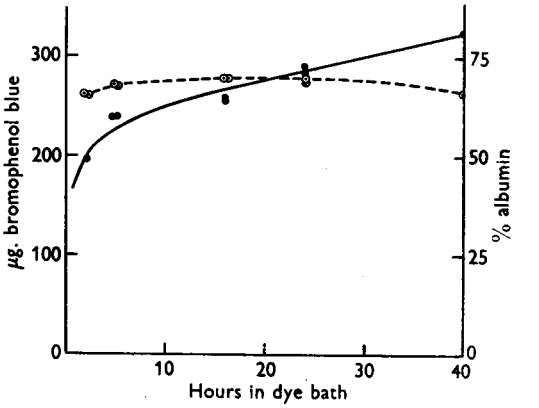

Fig. 3. Dye binding by electrophoretically separated serum on paper strips with increasing time in staining bath. ●—●, Total dye bound; ⊙—⊙, percentage of total dye bound by albumin fraction.

Staining time

In Fig. 3 is shown the influence of the length of time in the dye bath on dye uptake by serum separated on paper strips. The amount of dye bound increases rapidly for the first 5 hr., and then slowly up to 40 hr.; the relative amounts of dye taken up by the albumin and globulin fractions are constant from 5 to 24 hr. Experiments with varying amounts of pure albumin streaked on paper moistened with buffer have shown that at 2 hr. the larger amounts of protein bind less dye/g. than the smaller, but at 16 hr. a constant amount of dye is bound/g. of protein. Sixteen hr. was selected as the most satisfactory staining time since it avoids the initial period of rapidly changing dye uptake and allows sufficient time to approach equilibrium.

Staining periods as short as 10 min. have been used by a number of workers, usually with more concentrated dye solutions (e.g. Cremer & Tiselius, 1950; Grassmann & Hannig, 1952). It is unlikely that dye uptake can approach equilibrium in such a short period (Flynn & de Mayo, 1951). In addition, the use of a more dilute solution for a longer time

facilitates removal of background dye and consequently reduces the rinsing error (cf. next section). A lightly coloured area in the centre of the dense albumin band due to incomplete staining has not been encountered under the conditions described here.

Rinsing

An ideal rinsing procedure would remove all of the dye mechanically trapped in the fibres of the paper and none of the dye attached to protein through ionic or other bonds. In practice, this goal has not been achieved, but can be approached fairly closely by using a dilute dye solution for staining and by slightly modifying our previously described rinsing technique (Durrum, Paul & Smith, 1952).

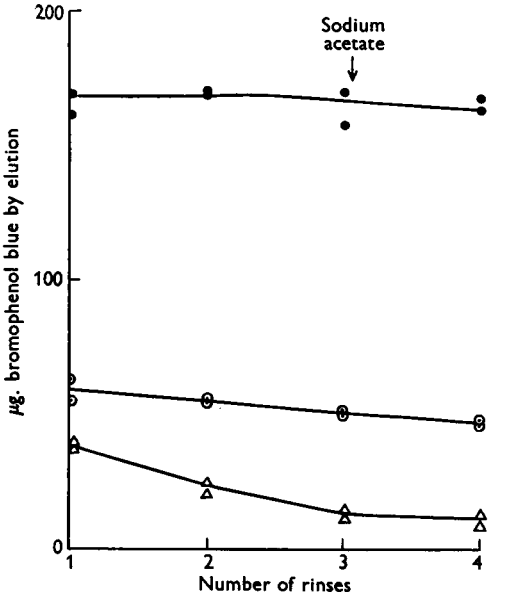

Fig. 4. Effect of rinsing on dye bound by the albumin and globulins of normal serum on paper after electrophoretic separation. Blank values for corresponding segments of protein-free paper were subtracted from each value. Rinses were 5, 5 and 10 min. in 2% acetic acid then 2 min. in 10% acetic acid–2% sodium acetate. ●—●, Albumin; ⊙—⊙, globulin; △—△, 10 cm. blank paper.

Fig. 4 shows the loss of dye from serum separated on paper strips during three rinses of 5, 5 and 10 min. in 2% acetic acid, and one rinse of 2 min. in 10% acetic acid and 2% sodium acetate. In this experiment, blank values for a corresponding length of protein-free filter paper have been subtracted from each value to correct for dye mechanically trapped in the paper. Dye loss is slightly greater from the globulin than from the albumin fraction of the serum. Although this may be due partly to the fact that the globulins are spread out over a much greater area than the albumin, it is also due to a less tight binding of dye by certain globulins. Table 1 shows the dye binding by purified albumin, γ-globulin, and crude β-lipoprotein ('top fraction' prepared by ultracentrifugal flotation of serum in a medium of density 1·063 (Lindgren, Elliott & Gofman, 1951)) after one rinse and after the complete rinsing procedure. Although only 4 and 7% of the dye was lost from albumin and from γ-globulin respectively, there was a 25% loss of dye from β-lipoprotein during the last three rinses. The final

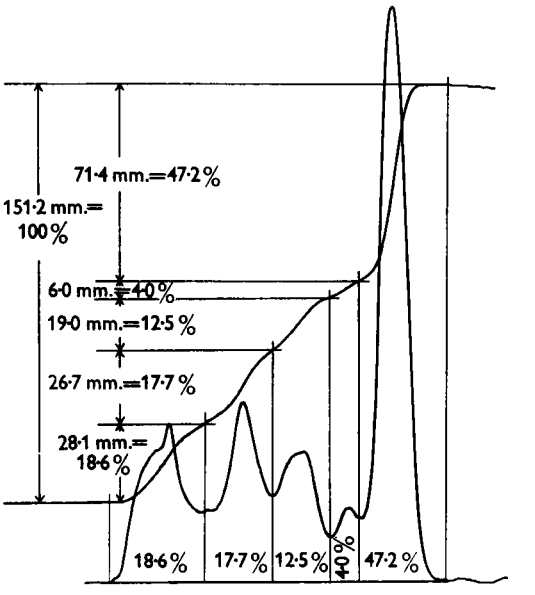

Fig. 5. Method of analysis of a scan pattern of serum proteins. Lower curve: optical density of the dye bound to the various protein fractions. Upper curve: integral of the optical density, showing the method of division to obtain the percentage of the total dye bound by each of the protein fractions.

Table 1. *Dye binding by protein fractions after one and four rinses*

Values expressed as g. bromophenol blue/g. protein as determined by biuret. The absolute figures for β-lipoprotein are only approximate, due to the interference of turbidity with the biuret determination. Blank values for dye in corresponding segments of protein free filter paper have been subtracted in each case. Each value is an average from two determinations on each of two different amounts of protein.

	Albumin	γ-Globulin	β-Lipoprotein
After one rinse	0·491	0·380	0·420
After complete rinse	0·469	0·353	0·315
Percentage of dye lost	4	7	25

rinse impregnates the paper with sodium acetate. When the acetic acid is evaporated, the dye is left as the blue sodium salt and needs no further treatment before scanning.

Although there is some fading of bromophenol blue on paper over a period of weeks, some 6-year-old serum strips still appear to be as deeply stained as ever, and there is no change in the relative amount of dye in stained albumin and globulin over a period of 2 months (Table 2). Nevertheless, for quantitative work it is our practice to elute or scan strips within a week of staining.

Table 2. *Constancy of the relative amounts of bromophenol blue in the albumin and globulin fractions of eight different serum strips over a two month period*

Figures represent percentage albumin as determined by scanning.

	Kept in room light				Kept in dark			
Strip number ...	1	2	3	4	5	6	7	8
After staining	52·6	52·5	54·6	61·4	52·2	54·5	52·3	60·7
After 1 week	52·9	52·5	56·4	62·4	52·7	54·5	52·4	60·2
After 2 months	52·2	51·6	54·5	61·1	51·2	52·2	51·0	56·9

Elution

Franglen & Martin (1954) have objected to the use of bromophenol blue because of fading of this dye in alkaline solution. We have encountered no difficulty in the routine use of 0·01 N sodium hydroxide for elution of this dye provided the samples are read within 1 hr. after elution. Bromophenol blue (Fisher Scientific Co.) measured at 1·7 and 17 mg./l. at a room temperature of about 25° and exposed to room light, showed only 5 % fading after 5 hr. with no difference in the percentage loss at the two concentrations.

Dye concentration was measured with the Beckman model DU spectrophotometer at 590 mμ., the absorption maximum for bromophenol blue in 0·01 N sodium hydroxide. Our value of 974 for $E^{1\%}_{1\,cm.}$ and $6{\cdot}52 \times 10^4$ for the molecular extinction coefficient of bromophenol blue, based on a molecular weight of 670 and assuming the commercial product to be completely pure, is in satisfactory agreement with the value of $6{\cdot}6 \times 10^4$ given by Franglen & Martin (1954).

Scanning

Using a scanner of their own design and Ilford filters for their measurements, Crook *et al.* (1954) have shown that azocarmine B and naphthalene black on paper do not follow Beer's law, and Van Os (1953) has reached similar conclusions under somewhat different conditions with a monochromatic light source. Franglen & Martin (1954) have shown that preparations of azocarmine B dye contain as many as six components. The applicability of Beer's law to bromophenol blue (99 % homogeneous by electrophoresis at pH's 8·6 and 2·3) on paper has been studied with the scanner described by Durrum & Gilford (1954). This instrument plots both the optical density of the material scanned and an integral of this curve. The percentage of each fraction, as measured by the area under the optical density curve, is estimated by measuring the rise in the corresponding segment of the integral curve (Fig. 5). Fig. 6 shows that the ordinates defined by the integral are proportional to the density areas as measured by the weight of the paper enclosed by the curve. The variations shown are no greater than the variations in weight of different pieces of graph paper.

Since the Coleman Junior spectrophotometer and the Bausch and Lomb type 33-29-25 colorimeter do not give a linear optical density response to increasing concentrations of bromophenol blue even in solution, these studies were carried out with a Beckman model DU monochromator as light source, with the light beam focused so that even illumination of the paper was obtained. Solutions of dye measured with this instrument obey Beer's law up to optical densities of over 1·0 with either a 1·0 or a 10 mm. light path. A tungsten light source filtered through a Bausch and Lomb second-order interference filter with an 8 mμ. half-width, and a Corning 3385 yellow filter (to cut out the third-order pass band of the interference filter) gave practically identical results. Measurements on paper were made at 600 mμ. with the monochromator or 604 mμ. with the filter, to allow for the 10–15 mμ. higher absorption maxima of dyes on paper as compared to dyes in solution as observed by Pezold & Kofes (1954) and confirmed in this laboratory by Dr E. R. B. Smith (unpublished).

In order to measure dye on paper under conditions similar to those encountered with scanning of dyed serum proteins, the dye was applied in bands rather than over a whole piece of paper. This procedure avoids any inequalities in dye distribution which might result from dipping a paper into a dye solution and then drying it. The paper was moistened with the sodium acetate–acetic acid rinse solution, blotted, streaked with various concentrations of

dye, oven dried, oiled, and scanned. The edges of the strip which were not in the light path of the scanner were cut off, and the dye bands were eluted from the 23 mm.-wide strip remaining. Thus only that quantity of dye which was actually scanned was eluted and measured.

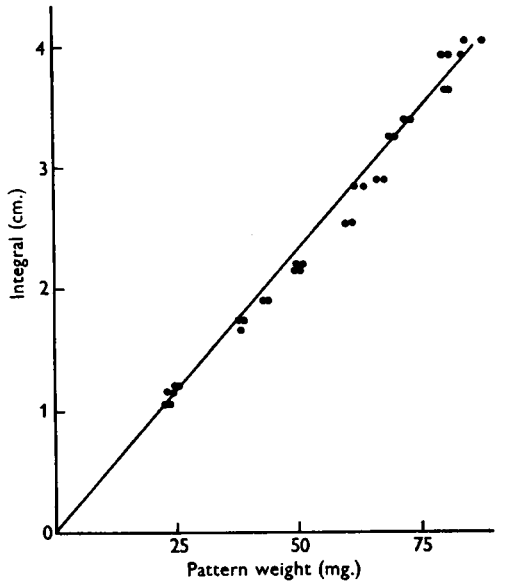

Fig. 6. The relationship of distance measured on the integral curve to area under the optical density curve, as measured by the weight of paper under the latter curve.

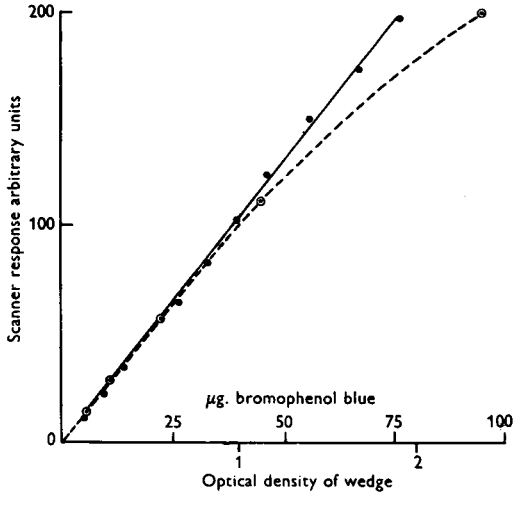

Fig. 7. Scanner response to a calibrated optical wedge, ●—●, and to increasing amounts of bromophenol blue streaked on paper, ⊙—⊙. The wedge was calibrated on a Macbeth-Ansco densitometer with the red filter. Dye concentration was measured by elution.

Fig. 7 shows that the scanner response to optical density is essentially linear as measured by a calibrated neutral density wedge, whereas there is a marked fall off of response to high concentrations of dye on paper. These dye concentrations were selected to fall in the same range of optical density as the wedge. The curve shown was determined with the filtered light source; experiments with the Beckman monochromator using a 0·1 mm. slit width gave almost identical results. Use of white light or light passed through wider band filters resulted in a slightly greater deviation from linearity, whereas monochromatic light of wavelengths other than that of the absorption maximum of the dye gave results close to those at 600 mμ. (see also Figs. 6 and 8, chap. 18, Block *et al.* 1955). Dry

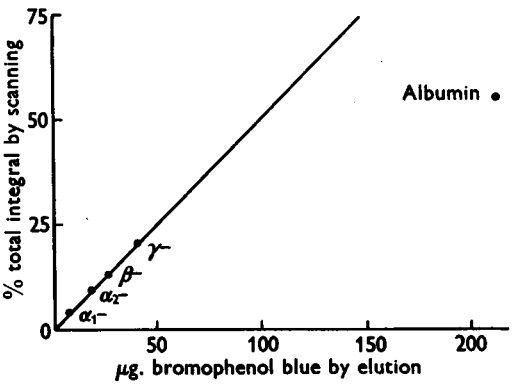

Fig. 8. The relationship of scanner response to amount of dye bound by various protein fractions as determined by elution. Data from an analysis in quadruplicate of pooled sera from forty normal blood donors.

paper produced a considerably greater deviation than paper which had been 'cleared' by dipping in mineral oil; addition of up to 60% α-bromonaphthalene to the mineral oil (Grassmann & Hannig, 1952) produced a negligible further change. The results of these experiments are not reported in detail since the variations in the curve under these different conditions are of much smaller magnitude than the deviation from linearity observed under the best conditions. This suggests that the deviation observed is due to the nature of the dye on paper rather than to the inadequacy of the instruments used to measure it. For routine purposes we have settled on the use of mineral oil for a 'clearing' agent and the filter combination described for a light source.

The effect of this scanning error on the analysis of serum under these conditions is shown in Fig. 8 in which are compared the elution and scan values for the protein fractions of a pooled serum from forty

normal blood donors analysed in quadruplicate. The scanner correctly measures the concentration of dye in all fractions except the densely stained albumin, in agreement with the results of Crook *et al.* (1954) for the dye azocarmine B (but not for naphthalene black, which they found required a correction for all fractions).

As these authors have pointed out, it is not surprising that dye on paper does not obey the same laws as dye in solution. Observation of a densely stained piece of paper with a light directly behind it will show that light is transmitted very unevenly through the stained paper fibres; i.e. in some areas this will appear as a deviation from Beer's law; the situation is analogous to that encountered with a light leak in ordinary transmission measurements.

Stoicheiometry of protein staining

Franglen & Martin (1954) have demonstrated that with their technique of staining there is not a linear relationship between protein concentration and dye uptake, and furthermore, that dye uptake varies depending on the area of paper to which the protein is applied. They conclude that under these conditions dye uptake is not a valid measure of protein concentration on paper.

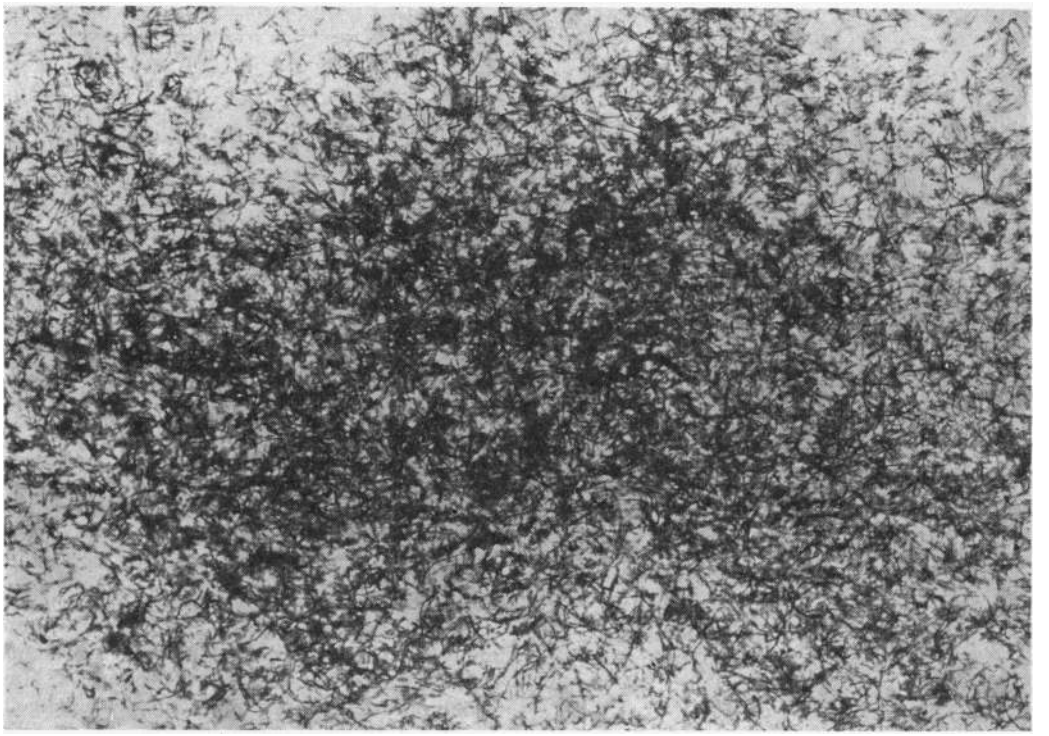

Fig. 9. A photograph of a portion of the stained albumin band of normal serum on paper, showing how dye and protein coat the individual paper fibres, and how this uneven distribution allows light to be transmitted through the interstitial spaces of the paper without encountering dye. The photograph was taken under conditions similar to those encountered with scanning, using transmitted light and a 617 mμ. interference filter. The optical density of this area is 1·7, measured with the same filter. The figure represents about 1 sq.cm. of paper.

of the paper there is 0 % transmission and in other areas perhaps 50 % (Fig. 9). With high dye concentrations these areas of relatively high transmission will account for a large fraction of the measured light and result in a greater amount of transmission than would be observed with uniformly distributed dye. On converting transmission to optical density,

Fig. 10*A* shows the dye binding of purified human albumin which was streaked on paper moistened with buffer, using the staining technique described above. The dye uptake for increasing amounts of protein approaches linearity closely, and there is a negligible difference in the dye binding of 100 μg. of protein spread over different sized areas

of paper by application in volumes of 0·01 or 0·03 ml. A similar experiment with globulin fractions separated by electrophoresis on a continuous flow 'curtain' (Durrum, 1951) showed that a linear relationship holds for these proteins also (Fig. 10*B*).

Several other workers have previously described staining procedures that appear to give a linear relationship between dye and protein concentration (Kunkel & Tiselius, 1951; Grassmann & Hannig, 1952; Röttger, 1952; Pezold & Peiser, 1953; Sommerfelt, 1953; Hardwicke, 1954; Mackay, Volwiler & Goldsworthy, 1954).

Dye binding by protein

Different proteins have been reported to have widely varying ratios of dye binding capacity to other properties used for protein measurement. These are usually expressed as 'correction factors' by which the dye binding value for total globulin is to be multiplied to permit comparison of results with refractive index or other units, and which vary from 1·0 to 1·6 or more with different staining and dye measuring techniques (Cremer & Tiselius, 1950; Kunkel & Tiselius, 1951; Plückthun & Götting, 1951; Esser, Heinzler, Kazmeier &

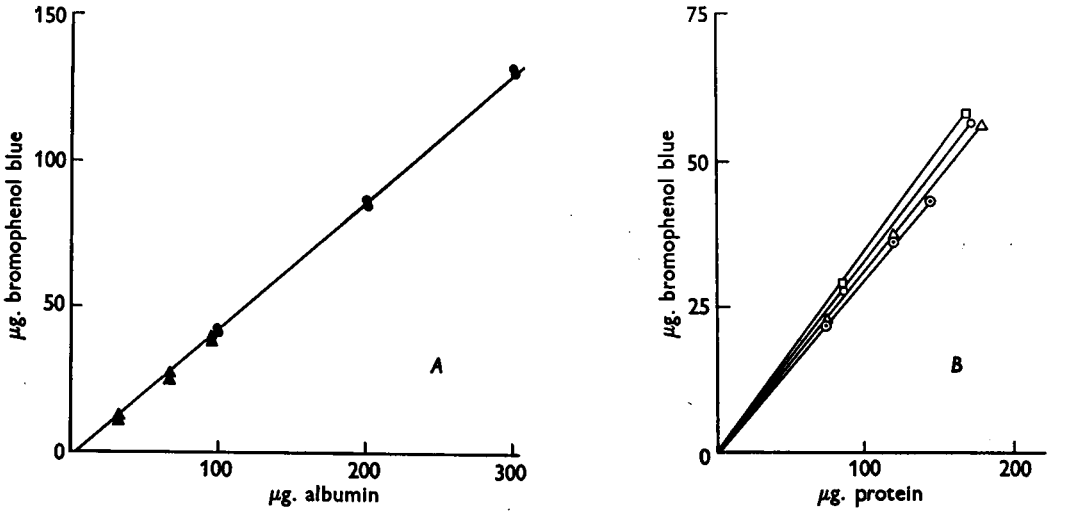

Fig. 10. (*A*) Dye binding by Squibb human albumin (lot 618R) on paper. ▲—▲, 0·33% solution; ●—●, 1·0% solution. (*B*) Dye binding by globulin fractions from a continuous flow 'curtain' electrophoretic separation of serum. Samples applied to paper in 0·04 and 0·08 ml. ○—○, α_2-globulin; □—□, β-globulin; ⊙—⊙, β- + γ-globulin; △—△, γ-globulin.

With bromophenol blue the 'blank value' for the amount of dye bound to protein free filter paper is negligible compared to the amount of dye bound by the concentrations of protein encountered in serum. However, if the dye binding of very dilute proteins on paper is measured, it becomes necessary to decide whether or not to subtract such a 'blank' value. Ordinarily we have not subtracted this value, on the somewhat arbitrary assumption that any charged groups on the paper which bind dye will also bind any protein with which they come in contact, and thus become covered up and inaccessible to dye. Careful inspection of stained, blank filter paper shows that the residual dye is bound to easily visible specks of impurities, rather than being simply trapped in the paper fibres.

Scholtan, 1952; Grassmann & Hannig, 1952; Pezold & Peiser, 1953; Sommerfelt, 1953; Conn & Klatskin, 1954; Hardwicke, 1954; Latner, Molyneux & Rose, 1954; Mackay *et al.* 1954). Köiw, Wallenius & Grönwall (1952) have reported such factors for individual globulin fractions ranging as high as 8·1.

A summary of several experiments to study these differences using the bromophenol blue staining technique described above is given in Table 3. The first column shows the amount of dye bound compared to biuret value of serum protein fractions separated by electrophoresis on a continuous flow 'curtain'. The fractions were lyophilized, dialysed against 0·1 M sodium chloride, streaked on paper strips previously moistened with buffer, and stained. Any insoluble material was removed by

Table 3. *The relationship of dye binding to biuret, nitrogen, and refractive index values for different proteins*

The source of the protein fractions is described in the test.

	'Curtain' fractions g. dye/g. protein	Purified proteins g. dye/g. protein	Serum on strips* g. dye/g. nitrogen	Normal serum % total dye	'Factor' for refractive index
Albumin	—	0·38	—	70·8	1·0
	0·53	—	3·1	—	—
α_1-Globulin	—	—	—	2·7	2·6
α_2-Globulin	0·32	—	1·3	5·7	1·9
β-Globulin	0·34	—	2·1	8·3	2·1
γ-Globulin	0·31	0·28	1·3	12·6	1·1
Bovine albumin	—	0·39	—	—	—

* Abnormal serum.

centrifuging and discarded before the biuret determination or streaking so that the fractions are not necessarily representative of whole serum. The second column shows dye values for purified human albumin (Squibb lot 618 R), γ-globulin (Squibb) and crystalline bovine albumin (Armour) compared to biuret (see also Table 1). The third column shows the dye binding values compared to nitrogen content of the various fractions of a serum which was separated on paper strips with borate buffer pH 8·55 and stained. Nitrogen determinations were carried out on corresponding segments of companion strips and corrected for the nitrogen content of the paper. This blank value was decreased by first soaking the paper for 30 min. in N hydrochloric acid, then in changes of distilled water until neutral to pH paper, and finally in the borate buffer. The fourth column shows the values, in percentage of total dye by elution, for an analysis in quadruplicate of pooled sera from normal blood donors. In the last column are shown the 'correction factors' that would be needed if it were desired to convert the dye binding values to the refractive index values for electrophoresis of normal sera as reported by Armstrong, Budka & Morrison (1947) (based on the absolute amount of dye bound, not percentage).

It is clear that the bromophenol blue binding capacity, as compared to biuret value, nitrogen content, or refractive index increment, is not the same for different proteins. These differences in dye uptake may be accounted for by the nature of the dye binding. The most important linkage between bromophenol blue and a protein would be expected to be between the single sulphonic acid group of the dye and the basic groups of the protein.

To demonstrate the importance of amino groups in dye binding, the proteins in a sample of serum were deaminated with nitrous acid or acetylated with acetic anhydride; the effectiveness of this treatment was indicated by an increase in electrophoretic mobility of all the serum fractions amounting to 30 % for the albumin. The dye uptake of these proteins was decreased by both treatments (58 % by deamination and 23 % by acetylation in one experiment).

The role of amino groups in dye binding is further suggested by the greater dye uptake of the basic protein clupein as compared to albumin (Grassmann & Hannig, 1952), by the very slight dye uptake of the acidic protein pepsin, and by a comparison of the dye uptakes of some purified proteins with their known amino acid compositions. Thus the relative dye values for human serum albumin, bovine serum albumin, and γ-globulin are 1·34, 1·38 and 1·00 (as compared to biuret, Table 3), while the number of free amino groups in these proteins is 1·42, 1·48 and 1·00 moles/1000 g. respectively (Brand, 1947; Stein & Moore, 1949).

However, amino groups are not the only dye binding sites for bromophenol blue. We have never succeeded in completely eliminating dye uptake by deamination or acetylation, and such non-ionic substances as Tween 40 (polyoxyethylenesorbitan mono-oleate) are stained by this dye. This type of staining may be tentatively ascribed to an attraction of the non-polar portion of the dye molecule to other non-polar substances which is analogous to the uptake of lipid dyes by lipoprotein. The weakness of this type of linkage may account for the considerable amount of dye lost from β-lopoprotein during rinsing (Table 1). Thus is should not be expected that proteins with different free amino group and lipid contents should have dye binding capacities directly proportional to other properties.

Albumin 'tailing'

It has been generally recognized that 'tailing' or irreversible adsorption of albumin or other proteins on paper is one of the major difficulties encountered with electrophoresis in this medium. The exact magnitude of this error in the analysis of serum proteins, although estimated by several workers (Kunkel & Tiselius, 1951; Merklen & Masseyeff, 1952; Sommerfelt, 1953; Hardwicke, 1954), is difficult to measure because of the small amounts of protein involved, and furthermore must be deter-

mined for any given set of conditions because of variations among papers, buffers, temperatures, and even albumins (Gabrieli, Goulian, Kinersly & Collet, 1954).

We have confirmed Sommerfelt's (1953) observation that the absolute amount of 'tailing' is approximately constant regardless of the amount of albumin which migrates over a given area of paper, so that it may be regarded as an 'unrolling of a carpet' over which the other fractions may travel with relatively little adsorption.

Two methods are available for the measurement of 'tailing': a direct measurement of the amount of albumin bound to the paper behind the albumin peak, and an indirect measurement of the difference between the amounts of protein expected and found in the albumin peak after movement of known amounts of albumin over a given area of paper.

Table 4 shows the results of an experiment with a highly purified crystalline bovine serum albumin making use of both of these methods. Both methods require that the albumin preparation used be free of any globulin contaminants that would be included in the measured 'tail', and that it be adsorbed by paper to the same degree as albumin in serum—i.e. is not partially denatured or otherwise changed in its adsorption characteristics during purification. The direct measurement of dye bound in the 'tail' is subject to two types of error—the loss of dye from this very dilute protein during rinsing, and the uncertainty regarding subtraction of a 'blank' value for dye bound by the paper. In view of these uncertainties the agreement between the two methods is satisfactory and albumin 'tailing', under the conditions given in the table, is of the order of 1 μg./sq.cm.; this amounts to about 5% of the albumin in normal serum under the conditions of separation given above.

Table 4. *Albumin 'tailing'*

0·01 ml. of 0·5%, 1·0% and 3·0% crystalline bovine serum albumin (Armour) moved 8·8, 8·8 and 9·0 cm. respectively on Whatman 3MM paper 3 cm. wide. After staining, 7·5 cm. of 'tail' and the main albumin band were eluted separately. Subtraction of dye binding values for corresponding segments of blank paper would decrease by about half the value for 'tail' as measured directly.

Protein applied (μg.)	Recovered in albumin band (μg.)	Difference (μg.)	Recovered in 'tail' (μg.)	'Tail' (μg./sq.cm.)
300	279	21	26	0·9–1·2
100	82	18	30	0·8–1·3
50	26	24	29	1·1–1·3

A simpler approach, but one which depends on the assumption of constancy of the amount of adsorption at very low protein concentrations, is to determine that dilution of normal serum at which the albumin peak is no longer visible (i.e. is completely adsorbed) after a run under standard conditions. This point has been found to be at about a 1:30 dilution, corresponding to 'tailing' of about 3% of the albumin.

Reproducibility

In order to test the reproducibility of the complete separation, staining, and dye measuring procedure under conditions of routine analysis of clinical specimens, three different sera were analysed along with routine work on eight separate runs over a period of 2 weeks; each run was stained, rinsed, and scanned separately. The results are summarized in Table 5. The standard deviations for the various fractions are similar for the elution and for the

Table 5. *Reproducibility of the complete procedure*

Three sera were analysed along with routine strips 8 times over a period of 2 weeks. Figures are given as percentage of total dye by elution and scan, uncorrected.

		Elution					Scan				
Serum		Albumin	α_1-G.*	α_2-G.	β-G.	γ-G.	Albumin	α_1-G.	α_2-G.	β-G.	γ-G.
S-1474	Average	67·3	2·9	11·2	10·2	8·3	50·2	4·3	16·6	15·4	13·2
	Range	64·4–70·1	2·3–3·4	9·5–12·3	8·7–11·7	6·9–9·6	46·8–52·8	3·1–5·1	14·6–17·8	13·4–16·9	12·2–15·1
	S.D.	2·6	0·4	0·8	0·9	1·0	2·3	0·6	1·1	1·1	1·1
X-343	Average	72·6	1·8	8·8	9·9	6·8	56·2	2·6	13·5	15·7	12·1
	Range	69·7–75·7	1·2–2·1	7·6–10·1	7·7–11·4	5·9–7·6	53·5–58·8	1·7–3·1	12·7–14·6	13·4–17·5	11·1–13·8
	S.D.	2·7	0·3	0·7	1·3	0·7	1·8	0·5	0·8	1·5	1·0
X-342†	Average	78·0	3·5	8·0	7·9	2·4	62·8	5·8	14·0	13·7	3·8
	Range	75·6–80·9	2·8–4·1	7·0–9·1	6·6–8·9	1·7–2·8	58·2–66·2	4·2–6·4	12·1–16·2	11·4–17·7	2·6–5·0
	S.D.	2·2	0·5	0·9	0·9	0·5	2·8	0·2	1·3	2·1	0·8

* G. = globulin.

† Serum from a patient with hypogammaglobulinemia.

scanning techniques, although slightly greater for the latter. These figures are similar to those reported by Crook *et al.* (1954) and Mackay *et al.* (1954), and slightly greater than those of Sommerfelt (1952); however, these authors apparently were comparing strips from a single run. Variation in the individual steps of the procedure is somewhat less. For instance, considering the albumin fraction only, the standard deviations given in the table for the complete procedure are 2·3, 1·8 and 2·8% by scanning; for eight strips of the same serum which were run, stained, and scanned together they were 1·8 and 1·5% in two different experiments, and for one strip scanned 12 times the standard deviation was 0·4%.

DISCUSSION

In order to obtain a stable and reproducible dye uptake by proteins on paper it has been found necessary to control the heat denaturation of the protein, the time of staining, and the rinsing of background dye from the paper; variations in these factors make the comparison of various staining techniques difficult. For example, varying the time of denaturation may alter the apparent albumin/globulin ratio through an almost twofold range due to the different effects of heating on albumin and globulins, and excessive rinsing of dye may lower the globulin value considerably since dye is rinsed more easily from the globulins than from albumin.

The procedure described satisfies the following necessary criteria for this type of analysis: (1) it provides a linear relationship between dye uptake and protein concentration which is not dependent on the area of application of protein; (2) it allows a satisfactory reproducibility in separate determinations of the percentage distribution of albumin and the globulins; and (3) it provides a sufficiently intense stain to minimize errors due to background variations. The two main sources of error under these conditions are the loss of dye during rinsing and the unavoidable subjective element involved in dividing the final pattern into its components. Albumin tailing produces a further small error, but this may be easily corrected if desired. The lack of linearity in the relationship between dye concentration on paper and scanner response for the high optical densities produced by stained albumin may also be corrected either by construction of a calibration curve for known amounts of albumin as determined by elution, or by incorporation of a correction factor into the scanner itself. Although it would probably be possible to avoid this correction by use of a smaller quantity of serum or a larger paper in order to remain within the linear range of the instrument, either of these changes would increase albumin tailing, the rinsing error, and the ratio of background to protein bound dye.

Comparisons of classical electrophoresis as measured by refractive index increment and paper electrophoresis as measured by dye binding have yielded widely varying results depending on the staining technique employed. With the procedure reported here the relationship between dye binding capacity and biuret value, nitrogen content, or refractive index is not the same for different proteins, and since acid dye binding appears to be related to the content of free amino groups and lipid of proteins, no constant relationship should be expected. Much of the work reported on this subject needs re-examination with regard to the reliability of the staining procedure employed.

It is an interesting coincidence that under the conditions described, the factor required to correct the scan value for albumin of normal serum to the elution value is close to the 'conversion factor' relating globulins to refractive index, so that uncorrected scan values alone will give a protein pattern not very different from that obtained by free electrophoresis. Grassmann & Hannig (1952) have observed a similar situation with the dye amidoschwarz 10B.

Although the ratio of dye uptake to other properties of proteins varies by more than twofold for different proteins and probably even more for pathological proteins, these variations are by no means a unique characteristic of dye binding values. The refractive index increment, although closely related to dry weight, varies up to fourfold when compared to the nitrogen content of different proteins (Armstrong, Budka, Morrison & Hasson, 1947).

Because of these difficulties involved in the interconversion of results from one system of units to another, it would appear preferable to establish normal values for paper electrophoresis of serum with a standardized staining technique (Table 3).

The direct determination of nitrogen in separated protein fractions is an alternative procedure that avoids most of the objections to staining (Levin & Oberholzer, 1953). However, for most purposes the staining technique is adequate and certainly is more practical.

SUMMARY

1. The quantity of bromophenol blue dye bound by denatured proteins on filter paper is dependent on the time of heat denaturation of the protein, the time of staining, and the amount of rinsing.

2. The amount of the variations is different for different proteins.

3. Bromophenol blue on paper does not follow Beer's law when directly scanned. For stained normal serum, under the conditions described, the deviation requires correction for only the densely stained albumin fraction.

4. Albumin 'tailing' under the conditions described amounts to 3–5 % of the total albumin of normal serum.

5. A staining procedure is described which gives a linear relationship between protein and dye concentration.

6. The relationship of dye binding capacity to other properties of proteins commonly used to measure concentration varies more than twofold among different proteins.

7. Normal values for serum protein fractions measured by the technique described, and the results of a study of the reproducibility of the method under conditions of routine use, are presented.

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Potato Pyrophosphatases

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The early studies with malt sprouts (Lüers, Zychlinski & Bengtsson, 1931), takadiastase and rice bran (Kurata, 1931; Uzawa, 1932; Munemura, 1933) have established the existence of acid pyrophosphatases in higher plants. Pfankuch (1936) prepared by ethanol precipitation a highly active extract from potato and sugar beet which showed phosphatase and pyrophosphatase (PP-ase) activities optimally at the pH of the press juice, viz. pH 5·8–5·9. Giri (1937) also prepared such active extracts from germinated soya beans and found that they hydrolysed pyrophosphate, glycerophosphate, and hexose diphosphate at an optimum pH of 5·1–5·5. Fleury & Courtois (1937) made a comparative study of phosphatase and pyrophosphatase activities of a number of vegetable tissues and found that pyrophosphate was hydrolysed at an optimum pH of 5·6–5·8 by all the tissues; takadiastase, however, required a more acid medium for optimum activity.

Until recently it was generally believed that vegetable tissues contain PP-ases that are active only in acid medium, but in 1951, one of us (Naganna, 1951*a*) reported the occurrence of a potent alkaline PP-ase in fresh extracts of potatoes. This enzyme requires the presence of Mg^{2+}, without which it is inactive. In this respect it resembles those specific PP-ases which are reported to be present in yeast

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