The Interaction of Dyes with Proteins on Paper with Special Reference to Paper Electrophoresis

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In attempts to develop quantitative techniques for the analysis of serum proteins separated by electrophoresis on filter paper, many workers have estimated the amount of dye taken up by the different protein fractions after staining. When comparisons have been made with analysis by the classical technique, quantitative differences have been noted (Cremer & Tiselius, 1950; Kunkel & Tiselius, 1951; Schneider, 1951; Grassmann & Hannig, 1952). Cremer & Tiselius used a factor of 1·6 to correct the difference on the grounds that the globulins had a lower binding power for bromophenol blue than had the albumin. This empirical factor was also used by Flynn & de Mayo (1951). Kunkel & Tiselius (1951) published a comparison of albumin and globulin uptake of bromophenol blue using a three-point plot. Grassmann & Hannig (1952) and Grassmann, Hannig & Knedel (1951), describing a procedure for the quantitative estimation of serum proteins using 'Amidoschwarz 10B' (naphthalein black 12B 200 of Imperial Chemical Industries Ltd.), pointed out the difference between albumin and γ-globulin in binding this dye. Martin (1952) stressed the gross differences in dye-binding capacity that existed between the various serum proteins and criticized the use of empirical correction factors derived from normal sera when studying abnormal sera. Slater & Kunkel (1953), though they give no data, clearly share this doubt about existing quantitative procedures, for they make no attempts at quantitative measurement of individual proteins in a study of urinary and serum proteins, and in their discussion write: 'it appears likely that quantitative procedures applied to filter-paper electrophoresis may be subject to considerable error....'.

The increasing use of filter-paper electrophoresis for separation of serum proteins, together with the very clearly expressed doubts by workers about the validity of the quantitative results, suggested that an attempt should be made to check some of the assumptions on which quantitative analysis is based.

MATERIALS AND METHODS

Dyes. Naphthalein black 12B 200, I.C.I. Ltd.; azocarmine B, from G. T. Gurr; bromophenol blue, and bromocresol green from British Drug Houses Ltd.

Absorption curves of the dyes in 0·01 N-NaOH were made in 1 cm. cells at room temp. in the Hilger Uvispec spectrophotometer standardized by hydrogen lines at 656·3, 486·1 and 434·0 mμ. Two such curves were plotted for each dye, one within 20 min. of the dye solution being made up, and the other after it had stood in a closed vessel in the dark for a day at room temp.

Qualitative analysis of the dyes used was carried out by descending filter-paper chromatography using *n*-butanol–acetic acid–water (5:1:4, v/v).

Serum proteins. Human albumin and γ-globulin fractions were prepared by the ether fractionating technique of Kekwick & Mackay (1954) and the composition was checked by electrophoresis according to the classical method of Tiselius. The proteins were taken up in 0·15 M-NaCl to give eleven albumin solutions ranging from 0·7 to 9·8% (w/v) and five γ-globulin solutions ranging from 0·3 to 5·0% (w/v). The protein concentrations were checked by estimating N (micro-Kjeldahl) using the factor 6·25 to convert to protein.

Paper electrophoresis was carried out in 0·06 M sodium diethylbarbiturate buffer (pH 8·6) (Flynn & de Mayo, 1951) by a method similar to that described by Franglen (1953). The paper used throughout this work was Whatman no. 1 as sold for chromatography.

Measurement of dye uptake by protein on filter paper. Each protein solution was pipetted on to dry sheets of filter paper by an Agla micrometer syringe (Trevan, 1925) as spots of 5, 10, 15, 20, 25, 30, 35 and 40 μl.; four spots at each volume were applied. The protein spots were dried at 105° for 30 min. They were then dyed for 10 min. in a solution of bromocresol green (0·2%, w/v) in 10% (v/v) ethanolic glacial acetic acid (Franglen, 1953). The pH of this solution was 3·2 by glass electrode standardized against 0·05 M potassium phthalate buffer (pH 4·00) (British Standards Institution (1950) 1647). The sheets were washed in 10% (v/v) aqueous acetic acid (pH 2·3) for 20 min., four changes of washing solution being used. At the end of this procedure, the background paper was clear of dye, and the sheets were blotted and allowed to dry at room temp.

The long and short axes of the spots were measured to the nearest mm. and from this the area was calculated to an accuracy of within 10%.

Constant areas of 3 × 3 cm. enclosing each dyed spot were cut from the sheets. The squares were eluted of dye by soaking in 5·0 ml. of 0·01 N-NaOH for at least 60 min. Recovery experiments showed that no less than 97% of the dye was eluted from the paper. Where necessary, the resulting blue solutions were diluted by adding known amounts of 0·01 N-NaOH to give $E^{613\,m\mu}_{1\,cm.}$ not greater than 0·500. The $E^{613\,m\mu}_{1\,cm.}$ of each solution was determined by means of a Unicam spectrophotometer S.P. 350. Over the range 0·000–0·500 $E^{613\,m\mu}_{1\,cm.}$ these solutions of bromocresol green showed approximately 5% deviation from Beer's Law, but

by reference to a calibration curve from sixteen different solutions of known composition, this deviation was allowed for when the bromocresol green concentration was calculated from the $E^{613\,m\mu}_{1\,cm.}$.

Calculation of results. For each concentration of protein, two plots were made. These consisted of the weight of dye eluted from each spot plotted against: (1), the weight of protein applied to the paper; (2), the area of each protein spot. These plots approximated closely to straight lines passing near or through the origin, the slopes being respectively equal to: (1), the weight of dye bound to unit weight of protein; (2), the weight of dye eluted from unit area of protein spot. To get as accurate an estimation as possible of the slope, straight lines were fitted on to the plots by the method of least squares.

EXPERIMENTAL

The absorption curves in 0·01 N-NaOH of the four dyes commonly used for staining protein on filter paper are shown in Fig. 1 (*a–d*). The absorption maximum of bromocresol green is at 613 mμ., of bromophenol blue 595 mμ., of fresh naphthalein black 610 mμ., and of azocarmine B 520 mμ. It will be seen that at these wavelengths, the order of decrease of $E^{1\%}_{1\,cm.}$ is bromophenol blue, bromocresol green, naphthalein black and azocarmine B, the maximum absorption of a 1% solution of azocarmine B being less than one-fifth of that of a 1% solution of bromophenol blue. The molecular extinctions (ϵ) for bromophenol blue and bromocresol green, the two dyes for which this figure could be expressed, were $6{\cdot}6 \times 10^4$ and $4{\cdot}57 \times 10^4$ respectively. This figure also shows that the absorption of both naphthalein black and bromophenol blue diminish appreciably on standing; a comparison of the rate of fading of bromophenol blue with that of bromocresol green is shown in greater detail in Fig. 2. Bromophenol blue is more stable in 0·01 N-Na_2CO_3, as pointed out by J. Hardwicke (personal communication).

Chromatographic analysis of samples of azocarmine B used in this work demonstrated that the dye contained at least six components, as shown in Fig. 3. Similar analysis of bromocresol green demonstrated one sharply defined component. Analysis of bromophenol blue suggested trace impurities.

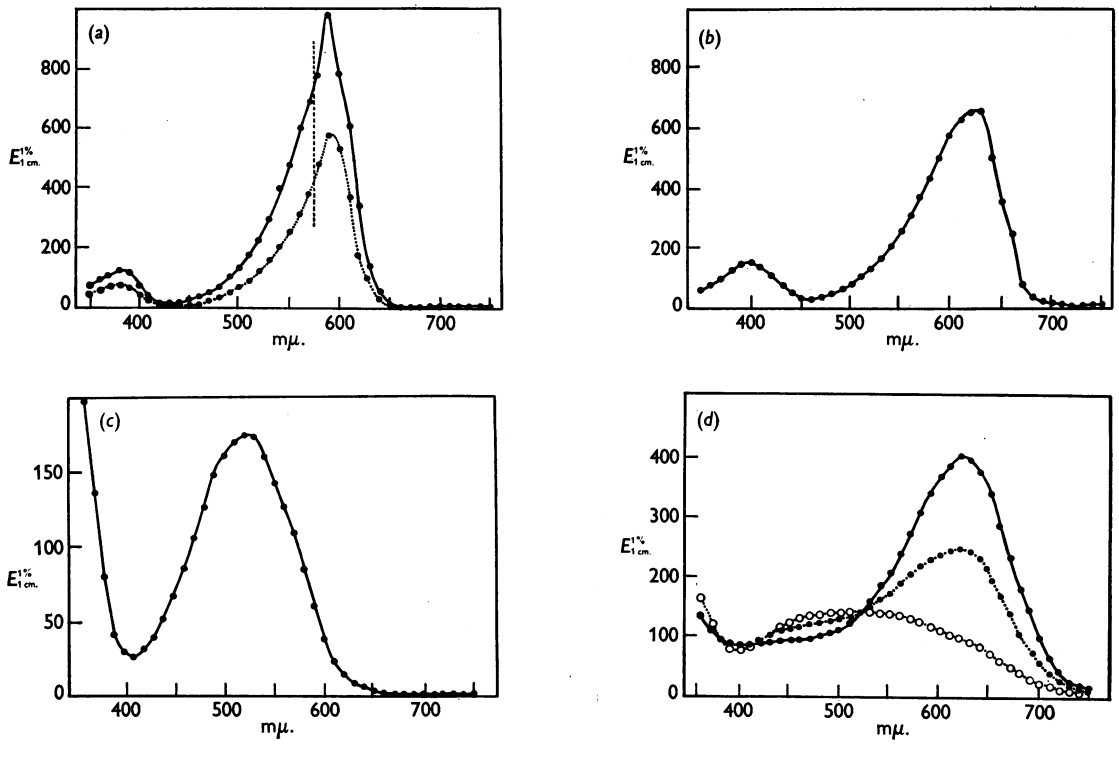

Fig. 1. (*a*) Absorption curves of bromophenol blue (1%, w/v, in 0·01 N-NaOH). ●—●, within 20 min. of solution; ●- - -●, 26 hr. after solution. Vertical dashes represent the wavelength used for measurements of bromophenol blue by Kunkel & Tiselius (1951). (*b*) Absorption curve of bromocresol green (1%, w/v, in 0·01 N-NaOH) within 20 min. and at 26 hr. after solution. (*c*) Absorption curve of azocarmine B (1%, w/v, in 0·01 N-NaOH) within 20 min. and at 26 hr. after solution. (*d*) Absorption curve of naphthalein black 12B 200 (1%, w/v, in 0·01 N-NaOH). ●—●, within 20 min. of solution; ●- - -●, 26 hr. after solution; ○- - -○, 54 hr. after solution.

Fig. 4 shows the result of a comparison of bromocresol green uptake of albumin with that of γ-globulin presented in terms of (*a*) g. dye uptake/g. protein against concentration of protein applied to the paper, and (*b*) g. dye uptake/mm.^{2} protein spot area against concentration of protein solution. It will be seen that the curves obtained from the two proteins are neither linear nor superimposed. Fig. 5 shows the data presented differently. Here the ratio of dye uptake of γ-globulin to dye uptake of albumin for a standard area (Fig. 5*a*) and a standard weight (Fig. 5*b*) are plotted against γ-globulin concentration, plots being shown for a series of concentrations of albumin. The results emphasize the impossibility of obtaining reasonably accurate analysis of a two-component system by dye-uptake techniques.

This experiment does not mirror precisely the conditions of analysis of mixtures in practice. Solutions were prepared therefore containing vary-

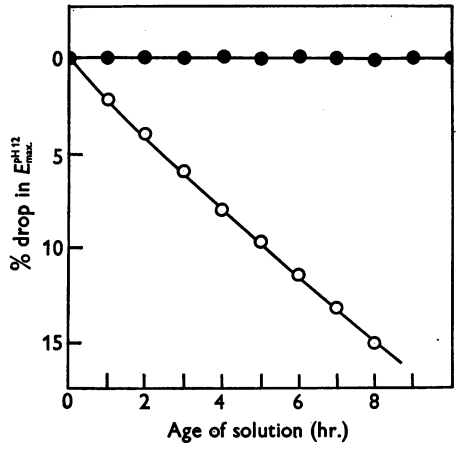

Fig. 2. Comparison of the alteration of extinction with time (1%, w/v, dye in 0·01 N-NaOH). O—O, bromophenol blue; ●—●, bromocresol green.

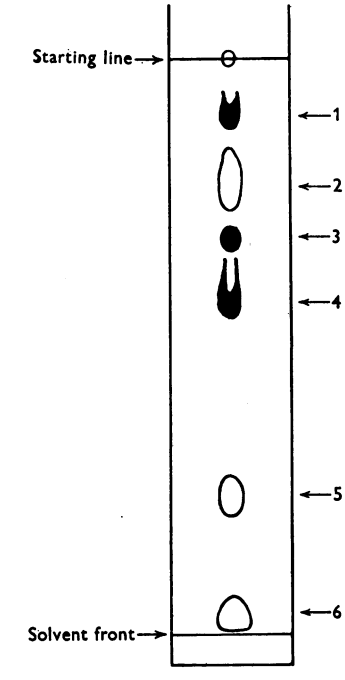

Fig. 3. Drawing of filter-paper chromatogram of azocarmine B run in aqueous butanol–acetic acid mixture. Black spots represent areas of red fluorescence under light of 253·7 mμ.; white spots represent areas of orange fluorescence.

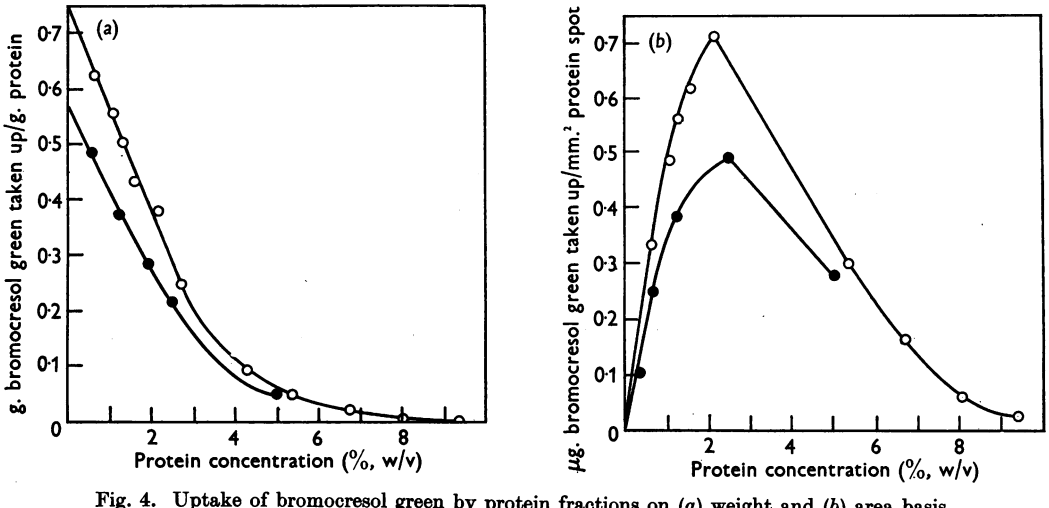

Fig. 4. Uptake of bromocresol green by protein fractions on (*a*) weight and (*b*) area basis. O—O, albumin; ●—●, γ-globulin.

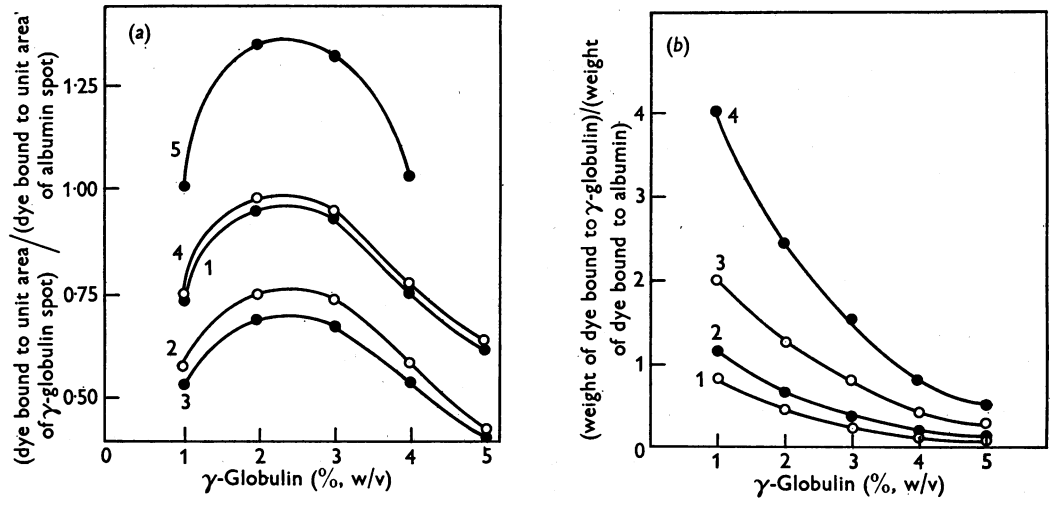

Fig. 5. (*a*). Ratio of bromocresol green bound by unit area of γ-globulin to bromocresol green bound by unit area of albumin spot plotted against concentration of γ-globulin at albumin concentrations (%, w/v) shown beside each curve. (*b*) Ratio of bromocresol green bound by unit weight of γ-globulin to bromocresol green bound by unit weight of albumin plotted against concentration of γ-globulin at albumin concentrations (%, w/v) shown beside each curve.

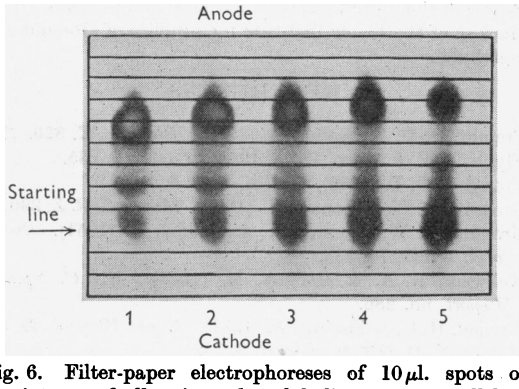

Fig. 6. Filter-paper electrophoreses of 10 μl. spots of mixtures of albumin and γ-globulin run in parallel in 0·06 M sodium diethylbarbiturate buffer (pH 8·6) for 12 hr. at a potential gradient of 3·8 v/cm. and dyed with bromocresol green. Grid lines 1 cm. apart.

	Albumin (g./100 ml.)	γ-Globulin (g./100 ml.)
1	9·6	1·0
2	7·7	2·0
3	4·8	3·0
4	3·8	4·0
5	2·0	5·0

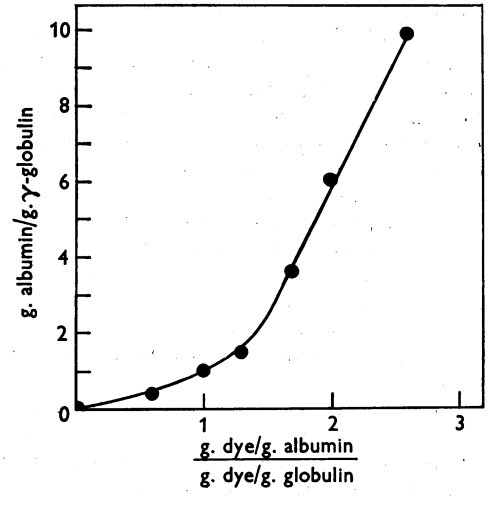

Fig. 7. Plot of true ratios of albumin to γ-globulin against ratios calculated directly from bromocresol green uptake (see Fig. 5 and text).

ing proportions of albumin and γ-globulin. 10 μl. of each of these solutions were then subjected to simultaneous electrophoresis on paper. Fig. 6 shows a photograph of one such run, and Fig. 7 shows graphically the plotting of the ratio of albumin to γ-globulin arrived at by dye elution plotted against the true ratio. It will be seen that a linear relation is achieved at ratios which only occur rarely in human serum-protein analyses.

DISCUSSION

Previous writers (see Tiselius & Flodin, 1953) have observed the discrepancy between quantitative analysis using staining techniques on paper and the classical methods in solution. The majority have sought to circumvent this discrepancy by the introduction of empirical factors based on the deviations demonstrated in normal sera with the tacit assumption that these factors are adequate when the ratios of the components are altered, as

they are in many disease states. This assumption demands, if nothing else, a direct linear relationship between individual protein concentration and dye uptake. Some attempt at a more fundamental approach has been made by Kunkel & Tiselius (1951). They have published plots of dye intensity against protein concentration, contrasting albumin and γ-globulin stained with bromophenol blue, though, unfortunately, they did not mention any correction for the fading of bromophenol blue in 0·01 N-NaOH, nor did they explain the use of 575 mμ. as the selected wavelength for estimation after extraction, when it appears that the maximum absorption for bromophenol blue is nearer 600 mμ. Bromocresol green, a close analogue of bromophenol blue, is free from the hazard of fading exhibited by both naphthalein black 12B and bromophenol blue and is homogeneous on chromatography in aqueous butanol–acetic acid mixture unlike azocarmine B. It would seem therefore to be the dye of choice if dye-elution or scanning techniques are to be used.

There is, however, another and more fundamental reason for doubting the validity of the techniques than the unfortunate choice of relatively unsuitable dyes. The plot of the dye bound to a protein against the concentration of that protein is not linear. Moreover, the characteristics of the relation of dye binding to protein concentration differ from one protein to another. Further, if two protein solutions of known concentrations are mixed in predetermined proportions and are then separated by paper electrophoresis, the proportions of these two components calculated by dye-binding techniques fail to tally with the predetermined ratios. Moreover, when a series of predetermined mixtures is examined, there is no consistent relationship which enables a correction factor to be applied.

If this be so when a two-component system is studied, it does not seem reasonable to suppose that a system of four components or more will be capable of more exact analysis. It would seem, therefore, that quantitative data based on the use of dyes with proteins separated by electrophoresis on paper needs drastic reassessment. Though the method in its present form has considerable use as a qualitative tool, the present data throw very grave doubts on the value of quantitative analyses produced by existing staining techniques.

SUMMARY

1. The spectral absorption characteristics of bromophenol blue, bromocresol green, azocarmine B, and naphthalein black 12B have been examined. Azocarmine B is heterogeneous on chromatography on filter paper.

2. The dye-binding characteristics of human albumin and human γ-globulin with bromocresol green have been studied individually and in mixtures of known composition. The results suggest that existing quantitative methods of analysis by paper-strip electrophoresis require reassessment.

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