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# TWO NEW STAINING PROCEDURES FOR QUANTITATIVE ESTIMATION OF PROTEINS ON ELECTROPHORETIC STRIPS

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## SUMMARY

Two new procedures are described for the estimation of protein by direct photometry on electrophoretic strips. The protein complexes of procion brilliant blue RS and coomassie brilliant blue R250 are shown to follow Beer's law up to 50 and 20 $\mu$g/cm, respectively. The lower limits of detection are 2 and 0.5 $\mu$g/cm. Within these ranges the absolute amount of protein can be estimated within a single test with an accuracy of about $\pm 10\%$. The major contribution to the error arises from uneven application of the samples. Relative concentrations within a mixture of proteins can be evaluated to an accuracy better than $\pm 3\%$.

Technical details of the procedures and of the equipment required are given in full, and their areas of usefulness discussed.

## INTRODUCTION

The advantages of zone electrophoresis are often largely lost by the need to elute separated fractions for reliable assay, or by using methods of detection that are essentially qualitative or at best approximate. In the course of work on the antibody fraction of immune sera this need for a quantitative micromethod became only too evident. We decided therefore to develop a staining procedure which (a) was sensitive enough to allow estimation of proteins in microgramme quantity, (b) would follow Beer's law over a reasonable range of concentrations, (c) could be performed directly on paper and cellulose acetate strips, or on agar and starch gel, (d) could be evaluated by simple, commercially available equipment. To this end a good number of standard dyeing techniques used in the textile industry were tested, and two families of dyes found which met all of our starting criteria. We report here on the methods deemed optimal, and give the evidence required to establish the scope and reliability of the new techniques.

## MATERIALS AND METHODS

### *Electrophoresis equipment*

The apparatus used was similar to that described by KOHN[1], which is a modification of GRASSMANN's apparatus[2]. The tank, made of Perspex, had internal dimensions of 32 × 18 × 5 cm, and was capable of taking four bridges side by side,

each carrying two strips 2.5 cm wide. Two partitions were inserted between the electrode compartment and the compartment in contact with the bridges, as we have found that without this arrangement the pH of the buffer in the outer compartments tended to change on prolonged electrophoresis. To avoid sagging of the strips which invariably led to collection of condense water on their undersurface, we stretched three fine nylon threads 3 cm apart across the bridges; these gave the needed horizontal support during electrophoresis. As a consequence, it was possible to reduce the air space around the strips to a minimum, and thus ensure quicker saturation of the vapour phase and obviate excessive evaporation and the resultant uneven movement in different regions of the zone*.

*Buffer system*

The Tris (8.0 g/l)–boracic acid (8.0 g/l)–EDTA (6.0 g/l)–sodium azide (0.8 g/l) buffer was essentially that described by ARONSSON AND GRÖNWALL[3], and used throughout the main experiments. The choice was made after comparing several commonly used buffer systems and finding that separation of serum components was both the cleanest and most rapid in this buffer, thus confirming ARONSSON AND GRÖNWALL's claims. The buffer has a pH of 8.95, and its conductivity is 3.4 $m\Omega^{-1}$.

*Support*

Cellulose acetate strips (Oxoid Div., London, E.C. 4) of 36 × 5 cm size were quartered, giving four strips of 18 × 2.5 cm. (Cutting up is best done on wet strips, to avoid cracking of the brittle material.) The strips were floated on buffer, with their "O" sides up: by soaking from one side no air was trapped within the capillary spaces. This process is complete within a few minutes, after which the strips were submerged and stored in the buffer at room temperature.

*Electrophoresis*

Before being laid on the bridges, the wet strips were lightly passed between two sheets of absorbent tissue. At least 1 h was allowed for equilibration between mounting the strips and switching on the current. The samples, usually 4 µl, were applied with a capillary pipette, along a line 2 cm long We have tried several commercially available applicators without satisfaction. These, designed for paper strips, rely on the sample being soaked up instantly on application. On cellulose acetate even volumes as small as 1 µl/cm take about 1 min to soak in, and therefore gradual delivery and spreading by hand was found preferable. However, even with the greatest care it is difficult to perform this step reproducibly, and the unevenness of application will be shown to be the major contributor to the error component of the technique.

Electrophoretic runs were done at a potential gradient of 10 V/cm. Due to the low conductivity of the buffer system, the current per strip amounted to 0.75 mA only, and thus the heating effect arising from 3 $mW/cm^2$ was negligible. Consequently, tests could be conducted at room temperature (18–20°).

*Scanning*

The stained, dried cellulose acetate strips were floated on a paraffin fraction of appropriate refractive index (Flozene 85) according to KOHN[1], and thus rendered

* Working drawings of the equipment are available on request.

transparent. The central 6 cm of the strip, carrying the protein pattern, was cut out and slipped into a scanning cell: a $7.5 \times 2.5$ cm microscopic slide mounted on a rim 2 mm high, and sealed along one of the long and the two short edges to a $6 \times 9$ cm photographic plate. The resulting chamber, of $71 \times 23 \times 2$ mm$^3$ volume, was filled with Flozene before receiving an electrophoretic strip. Thus, within the cell, there was no refractive interface in the path of the light beam. All patterns were evaluated by a recording strip-scanner (Beckman/Spinco "Analytrol"), using the film densitometer attachment. The scanning cell was clamped directly to the carrying plate of the micro-analyser. We employed the smallest slit supplied with the equipment (0.1 mm), and scanned at a speed of 1.86 cm/min that is, about 3 min per strip. This amounts of a magnification factor of 4.65-fold. All measurements refer directly to the areas under the curves, as integrated by the machine. Checks were made only for areas less than 0.5 cm$^2$, in which case the millimetre squares on the scanning sheet were counted.

*Test samples*

Six protein preparations were used in standardizing the staining techniques: bovine serum albumin, Armour Fraction V, twice recrystallized; human serum albumin, Nutritional Biochemicals Co., crystalline; ovalbumin, Nutritional Biochemicals Co., twice recrystallized, salt-free; rabbit $\gamma$-globulin, Armour Fraction III containing 80 % $\gamma$-globulin, the rest of the plasma proteins being reduced to different extent; lysozyme, L. Light & Co. Ltd; insulin (bovine), Commonwealth Serum Laboratories, "pool C", twice recrystallized.

All test samples were made up to about 20 mg/ml concentration in buffer; the true concentrations were derived from the Kjeldahl nitrogen value multiplied by the factor appropriate for the respective substance. In the case of rabbit $\gamma$-globulin, an inhomogeneous preparation, the conventional average factor 6.25 was used.

## RESULTS

*Principles of screening and basic tests*

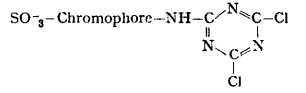
When choosing the dyes, our first criterion was set by the equipment we wished to use for evaluating electrophoretic patterns. The scanner is supplied with filters between 450 and 650 m$\mu$, at 50-m$\mu$ steps. The "matched" filters of our instrument were found to differ from each other by as much as 25 m$\mu$ in their transmittance maxima, and by as much as 15 m$\mu$ from their rated peak values. We had to look therefore for dyes whose protein complex would show broad absorption maxima around the mean values set by the filters, and preferably in the range from 550 to 650 m$\mu$ where the highest sensitivity of the photocells falls. The second criterion was set by the need for quantitative evaluation, and hence only such dyes and staining procedures were considered which resulted in absorbancy proportionate to protein concentration. The third criterion demanded that the dye should show no affinity for fully acetylated cellulose, and thus leave a clear background on which the stained protein would stand out. Simplicity was regarded as a further requirement, automatically ruling out a large class of dyes bound to the substrate after complex mordanting and fixing reactions.

The basic experiments were conducted on replicate strips: 1 $\mu$l of rabbit serum was electrophoresed for 150 min, giving a separation of about 4.5 cm between the

albumin and γ-globulin peaks. 50–100 such strips were prepared at a time and submitted to factorial experiments in which the concentration of the dye, the time of staining, the diluent, and the medium and time of differentiation were the variables. After eliminating several large classes of dyes, among them the commonly used amido black, lissamine green, azocarmine and bromphenol blue, we retained two groups of compounds as most promising. The reactions of these will be described in what follows.

*Procion blue*

*Dyeing characteristics*: The procion dyes, released in 1956, have the general structure



The Cl-substituted triazinyl group is highly reactive and will combine with hydroxyl, amino and amide groups through elimination of HCl. There is further evidence, obtained by independent methods in several laboratories[4–6], that such substitution occurs also at the peptide bonds of nylon; it is most likely therefore that similar combinations are formed with proteins, too. At alkaline pH these dyes react with water: it is essential therefore to use freshly prepared, acidic baths for dyeing. Since covalent bonds are formed, with the equilibrium well towards the dye–protein complex, the process may be regarded as quantitative and, for practical purposes, irreversible under ordinary conditions.

Of the set of triazinyl dyes procion brilliant blue RS (ICI) was chosen as it had the highest colour intensity of those tested, with a broad peak of absorption centred on 602 mμ. The absorption was found to be 88 and 93 % of the maximum at 575 and 625 mμ respectively. It is also worth noting that the absorption spectra of the free dye and of its protein complexes do not differ appreciably, a fact that follows from the insulation of the chromophoric moiety of the dye molecule from its triazinyl ring, through which coupling takes place.

*Standard staining procedure*: After experimenting with dye-baths set at particular hydrogen-ion concentrations and containing various salts in order to shift the partition of the dye in favour of the protein, we found that rather better results were obtained by avoiding aqueous solutions and making up procion blue in methanol. As a result, all protein fractions stained as intensively, yet the background did not retain the dye and fixation of proteins as a preliminary to staining was rendered superfluous. The procedure found optimal is as follows: (a) After electrophoresis immerse the cellulose acetate strips directly into the dye-bath (5 g/l procion brilliant blue RS and 20 ml/l concentrated HCl in methanol) for 5 min at room temperature. The time is not critical: twice as long steeping will give the same results. (b) Without drying transfer the strips to a bath of pure methanol for 5 min. Most of the background will be removed, the stained bands of protein become clearly visible. (c) Repeat step (b), to remove last traces of dye from the cellulose acetate. (d) Dry strips, either stretched on wooden frame or, preferably, in a photographic glazer, at 60° for about

5 min. By the second procedure the curling of the strip at the edges can be avoided, and thus subsequent insertion into the scanning cell is facilitated.

*Relationship of absorbancy and concentration*: Four proteins (ovalbumin, bovine serum albumin, human serum albumin and lysozyme) were made up to concentrations ranging from 0.2 to 2%, and 4-$\mu$l volumes of each dilution applied over 2 cm width of cellulose acetate strips. After 30 min electrophoresis at 10 V/cm potential gradient, the strips were stained with procion blue, dried, made transparent, and scanned at the wavelength of 600 m$\mu$. Fig. 1 gives the results of one such experiment, obtained by using cam B2 of the photometer. By this arrangement absorbancy is recorded, and the concentration–response relationship will be linear over the range where Beer's law is obeyed.

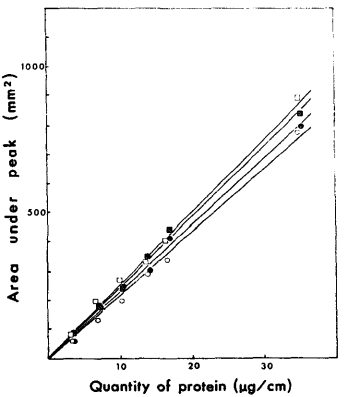



Fig. 1. Absorbancy scans of proteins stained with procion blue. Bovine serum albumin (■—■), human serum albumin (●—●), lysozyme (□—□), and ovalbumin (○—○) were electrophoresed on cellulose acetate strips, stained with procion blue and scanned at a wavelength of 600 m$\mu$ in a Spinco "Analytrol" integrating photometer. The curves are fitted by the method of least squares. Each point represents the mean of four determinations.

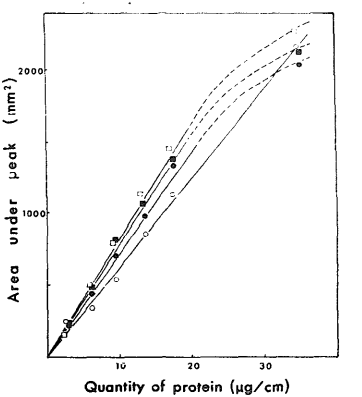



Fig. 2. Transmittance scans of proteins stained with procion blue. Bovine serum albumin (■—■), human serum albumin (●—●), lysozyme (□—□) and ovalbumin (○—○) were electrophoresed on cellulose acetate strips, stained with procion blue and scanned at a wavelength of 600 m$\mu$ in a Spinco "Analytrol" integrating photometer. The curves are fitted by the method of least squares. Deviations from Beer's law are indicated by broken lines. Each point represents the mean of four determinations.

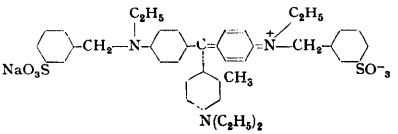
Evidently, the curves are well fitted by straight lines over their whole length, and all extrapolate to zero concentration. This combination of findings indicates that Beer's law may be taken as a valid descriptive model of the optical behaviour of procion blue–protein complexes, at least over the range tested. This extends from absorbancy readings of 0.07–1.50, and corresponds to 3–40 $\mu$g protein applied across 1 cm width.

The same strips were also scanned with cam B1, where the recorded response

is proportionate to transmission. At low levels of absorption the relationship should be indistinguishable from linear since $\ln(1-c) = -c$, provided $c \ll 1$. However, as the initial rate of change here is much steeper than when absorbancy is being plotted against concentration, the sensitivity of the method should be appreciably increased over the appropriate range. The curves in Fig. 2 are seen to be linear up to concentrations corresponding to an absorbancy of 0.6, *i.e.*, to at least 20 $\mu$g of protein/cm of strip. The level of detection is lowered to about 1 $\mu$g protein/cm. The scatter about, the curves is, on the whole, greater than in the concentration—absorbancy plots largely due to the uncertainty of the readings at the lower end of the scale.

*Coomassie blue*

*Dyeing characteristics*: A number of triphenylmethane dyes were found to give strongly coloured protein bands without irreversibly combining with cellulose acetate. Of these we found coomassie brilliant blue R 250 (ICI) most suitable, on account of its exceptionally high colour intensity and of the position of its absorption peak. This dye, belonging to the magenta family, is classified in the Colour Index under the name of acid blue 83 (CI 42660) and has the structure




In slightly acid media, the dye-anion is electrostatically attracted to the $NH_3^+$-groups of the protein, and within this primary combination van der Waals forces hold the reactants together. The dye–protein complex is firm, although fully reversible by dilution under appropriate conditions of pH. It is known that on drying the dye or its protein complex migrate towards the hotter surface, a point to be remembered when designing or evaluating practical procedures.

Coomassie blue dissolved in 0.01 M citrate buffer at pH 3 has its peak of absorption of 555 m$\mu$, with 74% and 83% of the maximum absorbed at 525 and 575 m$\mu$ respectively. The protein–dye complex is characterized by a peak slightly broader than that of the free dye, and a maximum at 549 m$\mu$. Apart from its high selective absorption, it was this almost negligible spectral shift between the free and bound dye-anion which led us to choose it for our purposes.

*Standard staining procedure*: Unlike procion blue, coomassie blue dissolved in methanol does not differentiate between proteins and cellulose acetate, and thus has to be used in aqueous solutions. Consequently, the proteins have to be fixed to the supporting strip before staining. The procedure found optimal is as follows: (a) Fix protein by immersing electrophoretic strip in 200 g/l sulphosalicylic acid in water, for 1 min. (b) Without rinsing transfer strips to dye-bath (2.5 g/l coomassie brilliant blue R 250 in glass-distilled water) for 5 min. (The use of water free from traces of heavy metals is essential, as these fundamentally alter the staining properties of the dye and prevent its subsequent removal from the background.) (c) Remove back-

ground stain by four rinses of 5 min each in distilled water. (d) Dry strips either on wooden frame or, preferably, in a photographic glazer at 90° for 15 min.

*Relationship of absorbancy and concentration*: The same four proteins as used in the testing of procion blue were made up at concentrations ranging from 0.01 to 1.0 %, and 4 $\mu$l of each was applied over 2 cm width of cellulose acetate strips. After electrophoresis at 10 V/cm for 30 min the strips were fixed in sulphosalicylic acid and stained according to the standard procedure described above. The dried strips were made transparent and scanned at 550 m$\mu$, first by using cam B2 (absorbancy recorded) and then by using cam B1 (transmission recorded). The results are shown in Figs. 3 and 4.

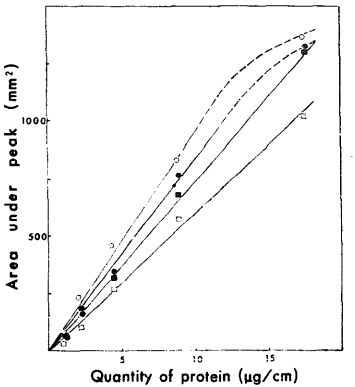



Fig. 3. Absorbancy scans of proteins stained with coomassie blue. Bovine serum albumin (■—■), human serum albumin (●—●), lysozyme (□—□) and ovalbumin (○—○) were electrophoresed on cellulose acetate strips, stained with coomassie blue and scanned at a wavelength of 550 m$\mu$ in a Spinco "Analytrol" integrating photometer. The curves are fitted by the method of least squares. Deviations from Beer's law are indicated by broken lines. Each point represents the mean of four determinations.

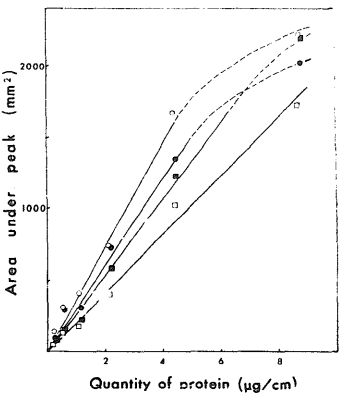



Fig. 4. Transmittance scans of proteins stained with coomassie blue. Bovine serum albumin (■—■), human serum albumin (●—●), lysozyme (□—□), and ovalbumin (○—○) were electrophoresed on cellulose acetate strips, stained with coomassie blue and scanned at a wavelength of 550 m$\mu$ in a Spinco "Analytrol" integrating photometer. The curves are fitted by the method of least squares. Deviations from Beer's law are indicated by broken lines. Each point represents the mean of four determinations.

Although, due to its high colour intensity, coomassie blue was evaluated on a set of test proteins 2–10 times more dilute than the series used for procion blue, deviations from Beer's law become obvious from the absorbancy reading of 0.8 upwards, corresponding to concentrations of 0.5 % or higher (Fig. 3). However, all the curves pass through the origin and hence may be used for quantitative estimation of protein-bound dye over their linear limb. Significant readings can be made in the concentration range of 0.05–0.5 % of the original solutions, or between 1 and 10 $\mu$g protein applied across 1 cm width.

Recording transmission (Fig. 4) leads to similar conclusions. Indeed, the scope of quantitative evaluation here is greater, extending over a 20-fold range, from 0.2 $\mu$g to 4 $\mu$g of protein/cm width. Even though the intercepts are estimated with rather lower accuracy than on the absorbancy *versus* concentration plots, the curves are once again seen to extrapolate to zero, thus validating direct estimation of protein-bound dye over the lower, linear parts of the plots.

*Quantitative estimation of proteins*

*Differences between substrates*: As a basis of comparison two commonly used chemical methods were chosen: the determination of nitrogen by the micro-Kjeldahl procedure, and the photometric method of LOWRY *et al.*[7], based on the titration of tyrosyl residues. A 1% solution of crystalline bovine serum albumin served as standard, and all concentrations will be expressed in these terms. The molecular weights of the test substances ranged from $6 \cdot 10^3$ (bovine insulin) to $1.7 \cdot 10^5$ (rabbit $\gamma$-globulin). Each of these proteins was highly purified and, with the exception of rabbit $\gamma$-globulin, crystalline or at least electrophoretically homogeneous (Table I).

TABLE I

COMPARISON OF METHODS FOR THE ESTIMATION OF PROTEINS

| *Protein* | *Micro-Kjeldahl* | *Lowry* | *Procion blue* | | *Coomassie blue* | |
|---|---|---|---|---|---|---|
| | | | *Absorbancy* | *Transmittance* | *Absorbancy* | *Transmittance* |
| Bovine serum albumin* | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Human serum albumin | 0.99 | 0.96 | 0.97 | 0.94 | 1.15 | 1.16 |
| Ovalbumin | 0.98 | 0.99 | 0.90 | 0.89 | 1.33 | 1.39 |
| Rabbit $\gamma$-globulin | 1.00 | 0.81 | 1.03 | 0.97 | 1.06 | 1.05 |
| Lysozyme | 1.16 | 1.09 | 1.05 | 1.05 | 0.82 | 0.75 |
| Insulin** | 0.98 | 1.16 | >0.41 | >0.42 | >0.52 | >0.61 |

* The figures represent means of 10–12 determinations, and show the estimated protein content in terms of the bovine serum albumin standard.

** The procion and coomassie blue values are underestimates, due to loss of insulin during the dyeing procedure.

As expected, the estimates of concentration obtained by the four techniques differ from each other according to the kind of protein tested. The Kjeldahl-nitrogen values differ appreciably only in the case of lysozyme, a protein known to be of considerably higher than average nitrogen content. The average difference from the bovine serum albumin standard is $\pm 7\%$, while LOWRY's method is seen to deviate by $\pm 12\%$. Averaged over the first five proteins, the procion blue values have a scatter of $\pm 6\%$ in terms of the standard, and the coomassie blue estimates deviate by $\pm 21\%$. The readings for insulin are inordinately low after either of the staining procedures. However, control tests showed that both insulin and its procion blue-complex are soluble in methanol, and sulphosalicylic acid does not fix this protein

to cellulose acetate. Thus the low readings are due to leaching out of the substrate into the dye-bath, and hence the inclusion of insulin into the average would be unfair. The same holds for some small peptides on which we performed similar experiments—an alternative procedure covering these will be published separately.

If the proteins tested may be regarded as a representative set, this would mean that a quantitative estimate of an unknown protein would fall between 0.86 and 1.16 with 95% probability, using the Kjeldahl procedure, between 0.76 and 1.24 when assayed by the LOWRY test, between 0.87 and 1.11 when recording absorbancy with procion blue, and between 0.85 and 1.09 when assayed by transmittance. Coomassie blue would cover the range of 0.70–1.45 and 0.60–1.54 for absorbancy and transmittance respectively. With known substrates, of course, definition of a factor would allow transformation of results obtained with any one technique into those of any other, or into terms of dry weight. The figures obtained with lysozyme show up the particular limitations of coomassie blue: the chances of complementariness between the relatively rigid, plate-shaped dye molecule and the small proteins of partly random secondary structure are low, and the intensity of staining falls away disproportionately when compared to the dye–albumin or dye–globulin complexes. However, even after disregarding reactions with the smallest substrates, coomassie blue would still show a variation of 0.85–1.42 and 0.80–1.50 in terms of the bovine serum albumin standard. Procion blue, on the other hand, compares well with the two chemical methods. Indeed, its range of variation is less than that of the LOWRY technique, due no doubt to the more even distribution of sites within proteins at which the dye can form bonds than the distribution of their tyrosyl residues.

*Accuracy*: Tests under this heading were performed on a "globulin fraction" of rabbit serum, originally prepared for the estimation of its antibody content. It was chosen because it contained comparable quantities of albumin, $\alpha$-, $\beta$- and $\gamma$-globulin, and thus the size of the four protein bands could be estimated without the risk that any of them would fall on the non-linear limbs of the response curves. The preparation contained 1.28 mg N/ml, as determined by the micro-Kjeldahl procedure, and thus 8.0 mg of protein/ml. Four dilutions (0.25:1, 0.50:1, 0.75:1, 1:1) were made up and each applied to 2 $\mu$l volumes/cm across 6 cellulose acetate strips. The total quantity of protein applied was thus 4, 8, 12 and 16 $\mu$g/cm width. After a run of 2 h at a potential gradient of 10 V/cm, a third of the strips was stained with procion blue, a third with coomassie blue and a third for sake of comparison with one of the popular staining techniques, with amido black. Each of the strips was scanned—both for absorbancy and transmission—at two levels of the bands, to allow independent evaluation of the evenness of applying the samples. This provided 32 estimates at each of the 4 levels of concentration, with each of the three dyes. The experimental data are given in full (Fig. 5), and the percentual distribution of the main classes of serum proteins in this preparation has also been worked out from each of the scans (Fig. 6). The error of the percentual estimates was obtained by submitting the data to an analysis of variance, while the slopes of the regression lines, fitted by maximum likelihood methods, were compared by covariance analysis. The latter also served to test the significance of the regressions and to detect deviations from linearity.

The procion blue readings show good linearity throughout, even when transmittance was recorded: the highest concentration of protein in any one peak (5 $\mu$g/cm

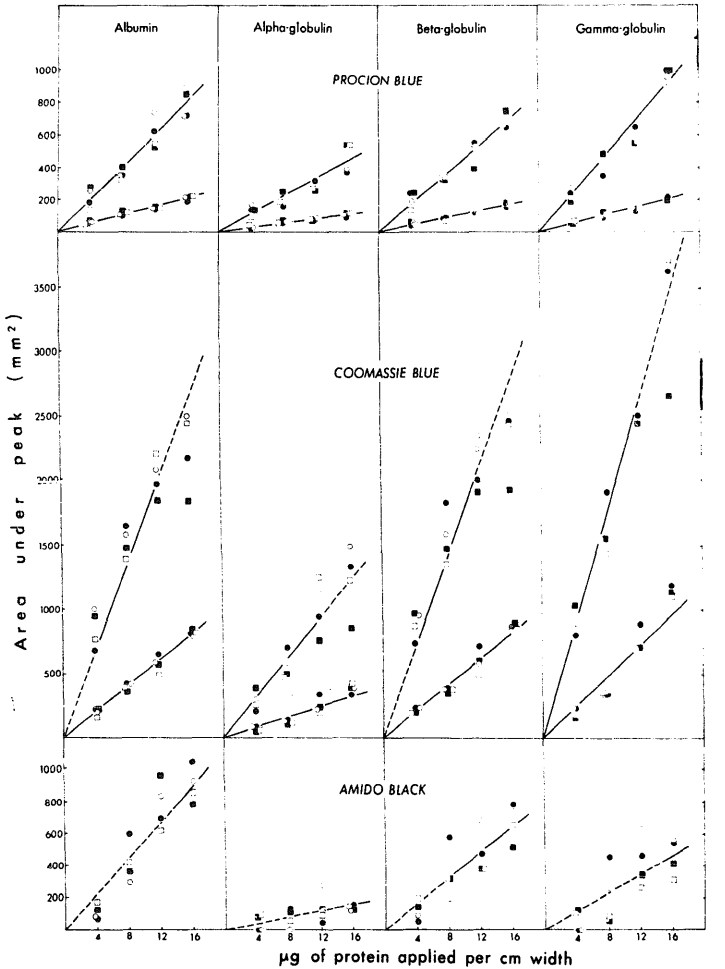



Fig. 5. Quantitative estimation of serum proteins. Dilutions of a "globulin fraction" of rabbit serum were electrophoresed for 2 h at a potential gradient of 10 V/cm, in the ARONSSON–GRÖNWALL buffer, on cellulose acetate strips. The protein bands were stained with procion blue, coomassie blue or amido black and scanned at 600 mµ, 550 mµ or 550 mµ wavelength respectively in a Spinco "Analytrol" integrating photometer. The steeper curves represent scans for transmittance,

width, for the $\gamma$-globulin band of the 1:1 sample) still falls on the straight limb of the response curve. Moreover, all lines intercept the ordinate at points insignificantly different from zero. Thus, the slopes may serve as direct estimators of protein concentration in each of the bands. These slopes carry errors of $\pm 9.3$, $\pm 15.3$, $\pm 8.3$ and 7.6% for the albumin, $\alpha$-, $\beta$- and $\gamma$-globulins respectively, when transmission is recorded (overall average $\pm 10.1$ %). The corresponding errors for absorbancy readings are $\pm 8.2$, $\pm 21.9$, $\pm 13.9$, $\pm 10.7$, averaging $\pm 13.6$ %. The somewhat increased variance of the theoretically superior absorbancy scores stems largely from the uncertainty of evaluating the $\alpha$-globulin peaks of the most dilute samples, where about 0.3 $\mu$g of protein/cm width had to be estimated.

Coomassie blue gives consistently linear plots when absorbancy is recorded, and the errors of the slopes for albumin, $\alpha$-, $\beta$- and $\gamma$-globulin come to $\pm 4.9$, $\pm 11.1$, $\pm 8.4$ and $\pm 8.1$%, respectively (average $\pm 8.1$ %). Readings of transmission on the same strips significantly deviate from linearity for the higher concentrations of albumin and $\beta$-globulin, the two fractions moving in very narrow bands and thus characterized by high colour intensity on staining. Also, the best fitting straight lines computed from these two sets of data intercept the ordinate at points significantly different from zero. If the curves are constrained to pass through the origin, the estimated slopes carry errors of $\pm 11.0$, $\pm 13.6$, $\pm 5.5$ and $\pm 8.7$%, averaging $\pm 9.8$ %.

Amido black does not emerge as a satisfactory dye for the evaluation of protein concentration on electrophoretic strips: all plots are non-linear, and three of the four curves do not pass through the origin. If errors were computed, they would average over $\pm 25$ %, even without constraining the curves to pass through the zero point. Furthermore, evaluation by absorbancy measurements is not feasible in this concentration range: the two lower quantities of protein tested (2 and 4 $\mu$g/cm) gave no significant readings for $\alpha$- and $\gamma$-globulin, and a greatly distored overall pattern was observed even at the higher concentrations. These effects are evident also in the transmittance readings, the only ones plotted in Figs. 5 and 6.

On analysis of the components of error, the overwhelming contribution of differences between scans on the same strip stands out. On the average, about 60% of the variance is accounted for by this source. Errors arising from deviations from linearity, from differences between replicate tests and from the interaction of these factors would add up to an overall error not exceeding $\pm 4$ % for either procion or coomassie blue, scanned either for transmittance or absorbancy. And none of the individual sources would cause more than $\pm 2$ % error on their own. Clearly, the most promising avenue to future improvement of the technique leads through a method that would ensure uniform application of samples over the width of the cellulose acetate strips.

The main source of error is minimized if relative concentrations only have to be estimated. An evaluation of the data in this form (Fig. 6) shows that the average standard deviation of the coomassie blue results is $\pm 1.3$ % for transmission readings

*(Continued legend Fig. 5.)*

the flatter ones for absorbancy. The lower two dilutions stained with amido black did not permit evaluation by absorbancy readings. The curves are fitted by the method of least squares. Deviations from Beer's law are indicated by broken lines. The symbols ■ and □ refer to readings at two levels of the first strip, and the symbols ● and ○ to readings on a replicate strip.

(range: 0.8 % for $\beta$-globulin; 1.6 % for albumin), and $\pm$ 1.9 % for absorbancy (range: 1.0 % for $\beta$-globulin; 3.7 % for $\gamma$-globulin). The percentage is in terms of the total quantity of protein applied. The corresponding data for procion blue are $\pm$ 2.9 % (range: 2.5–3.4 %) for transmittance and $\pm$ 2.0 (range: 1.5–2.3 %) for absorbancy. The amido black estimates carry partly overlapping, large errors and differences of less than 20 % in terms of overall concentration cannot be asserted with any degree of confidence.

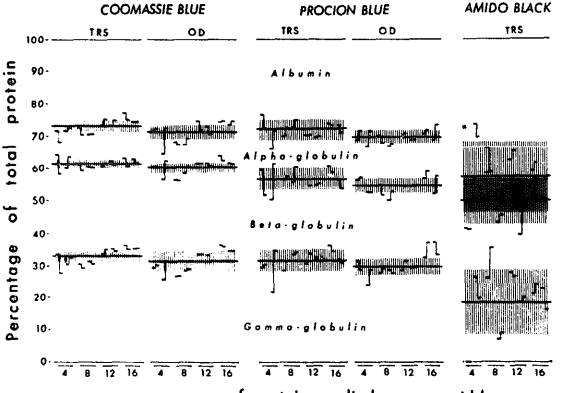



Fig. 6. Relative estimation of serum proteins. The average percentage of the protein fractions is shown as horizontal lines surrounded by shaded areas of 2 standard deviations' width. The short lines connected vertically stand for two readings on the same strip, and correspond to the symbols ■, □ and ●, ○ of Fig. 5, respectively. TRS = transmittance, OD = absorbancy.

*Reproducibility*

The relevant experiments were of the type presented in Table I: six different proteins were made up at six concentrations each, ranging from 1.0 to 0.1 %. 2 $\mu$l of these solutions per cm width were applied to two cellulose acetate strips, electrophoresed, stained with procion or coomassie blue, and scanned both with the cam linear for absorbancy and the one linear for transmittance. The test was repeated a few days later, using the same protein preparations, but freshly made-up reagents for the dyeing procedure. Straight lines constrained to pass through the origin were fitted to the experimental readings by the method of least squares. An analysis of variance provided estimates of variation from sample to sample and from day to day, providing thus a measure of the reproducibility of the two staining procedures as well as of the two methods of evaluation. A summary of the findings is given in Table II, in the form of percentual errors arising from single experiments or attached to tests done on different days. (The errors refer to the slopes fitted to the experimental points, each being based on 6–8 degrees of freedom. The standard error of a single determination would thus be $\sqrt{6}$ to $\sqrt{8}$, that is, 2.5–2.8 times greater than the values shown in the Table.)

TABLE II

REPRODUCIBILITY OF THE QUANTITATIVE STAINING PROCEDURES

| | *Procion blue* | | | | *Coomassie blue* | | | |
|---|---|---|---|---|---|---|---|---|
| | *Error I** | | *Error II** | | *Error I** | | *Error II** | |
| | *Absorbancy* | *Transmittance* | *Absorbancy* | *Transmittance* | *Absorbancy* | *Transmittance* | *Absorbancy* | *Transmittance* |
| Bovine serum albumin | 2.1 | 3.0 | 10.8 | 3.1 | 1.3 | 10.3 | 8.9 | 11.0 |
| Human serum albumin | 1.9 | 2.2 | 1.5 | 2.0 | 1.7 | 8.1 | 5.5 | 12.8 |
| Ovalbumin | 2.3 | 4.3 | 3.9 | 2.4 | 3.1 | 9.5 | 7.9 | 11.5 |
| Rabbit $\gamma$-globulin | 2.7 | 3.0 | 4.9 | 0.8 | 2.7 | 6.6 | 2.9 | 4.0 |
| Lysozyme | 1.6 | 1.8 | 4.8 | 1.6 | 2.7 | 4.4 | 2.4 | 6.5 |
| Insulin | 5.5 | 5.9 | 5.7 | 5.4 | 3.4 | 3.5 | 4.4 | 7.6 |
| Average percentual error | $\pm 3.03\%$ | | $\pm 3.91\%$ | | $\pm 4.77\%$ | | $\pm 7.13\%$ | |

* The figures represent the standard percentual errors derived from a set of measurements on the listed protein preparations within a single experiment (Error I), and the common error of two such sets obtained on different days (Error II).

The results demonstrate the superior reproducibility of the procion blue technique as compared with coomassie blue—or for that matter, with any dye that forms relatively weak adsorptive complexes with its substrate. The least controllable steps of any dyeing procedure are those where excess dye has to be removed in order that a high contrast be achieved between the stained zones and the support. The procion blue–protein bonds stand up well to washing, while apparently large enough fractions of coomassie blue dissociate at this stage to increase appreciably the error of the procedure, even under carefully controlled conditions.

## DISCUSSION

The two new techniques for estimating the quantity of protein directly on electrophoretic strips were developed and tested on cellulose acetate as support. Once the basic information was available, however, a number of parallel tests were run also on filter paper, agar and starch gel. Both procion and coomassie blue proved as satisfactory under these conditions as in the experiments presented above, and we may conclude therefore that the two staining procedures are equally well suited to work on any of the more popular supports used for zone electrophoresis at present.

Compared with other available techniques, staining with coomassie blue stands out by virtue of its great sensitivity. When recording transmittance, as little as 0.5 $\mu$g of protein/cm width (*i.e.*, less than 1% of the protein in 1 $\mu$l of serum) can be estimated to better than $\pm 10\%$ accuracy. Procion blue is about three times less sensitive, while azocarmine, amido black and bromphenol blue are 5–10 times less so. Transmittance readings in this range show satisfactory linear relation to concentration, and are hence to be preferred when minority components of mixtures or dilute solutions of proteins are to be evaluated. Determination of absorbancy was

found, by and large, four times less sensitive than transmission measurements; its value lies in the extended range of concentrations over which the area under the peaks is directly proportional to the quantity of proteins stained by either of the two techniques developed above. Whereas coomassie blue would be the dye of choice when the demand was for sensitivity, the procion blue technique is superior by the criterion of reproducibility and accuracy. The dye–protein bond of the latter being covalent, there is little risk of day-to-day variation between tests or of variation due to operators. Neither overstaining nor overdifferentiating alters the amount of procion blue bound, in marked contrast to most other procedures where the delicate balance between bleaching the background and losing some of the protein-bound dye can be maintained only by the most rigorous standardization of techniques. Another feature of procion blue, and possibly of triazinyl dyes in general, is their uniform behaviour towards peptides and proteins of widely differing physical and chemical characteristics. This property is well documented by the evidence of Table I: the factor relating intensity of staining to dry weight varies less than half as much as it does with coomassie blue, and less than a tenth of what can be expected of Amido black (*cf.* Figs. 5 and 6), provided its erratic performance on plasma proteins may be taken as an indication of general behaviour.

The main shortcoming of the techniques is the unsolved problem of evenly applying microlitre volumes of fluid to poorly absorbent surfaces. The overall error of measurement is more than doubled by this factor: its elimination would allow an accuracy of rather better than $\pm 5\%$ in estimating protein in bands of 1–20 $\mu$g dry weight content. The magnitude of the error arising from uneven application of the samples can be best seen from the difference in accuracy when the same data are evaluated in absolute and relative terms. While the standard deviation of a single reading in Fig. 5 is about $\pm 10\%$, the relative protein content of the four serum fractions can be determined with a standard deviation of about 2.5% (Fig. 6), using the same experimental observations. As a consequence, the best practical approach available at present is multiple scanning of the same strips, covering preferably their entire width. Until a satisfactory method of applying the test samples is found, this is the only way of salvaging all the quantitative information these techniques can yield.

Both procedures are simple in their final form, and suitable for routine and large-scale work. The areas in which the two dyes can be employed to best advantage partly overlap. We have used coomassie blue and cellulose acetate as support where the greatest sensitivity was required and where quantitative considerations are only secondary. Thus in electrosyneresis[8] and electrodieresis[9] of immune complexes, and in testing the purity of antibody fractions. For similar reasons, LAVER[10,11] has followed by this method the separation of influenza virus into its subunits, and the purification of the viral enzyme. For quantitative assay of serum proteins procion blue is preferable, partly because it shows more uniform affinity towards the various fractions than do other staining procedures, and partly because it is free from day-to-day variation. Also, in this case sensitivity is no major concern since a microlitre of serum spread over one centimetre width happens to fall in the optimum range of this technique, with no significant deviations from Beer's law by any of the fractions. Procion blue has been used for comparisons of serum and milk proteins by LASCELLES[12] and GARLICK[13] and by us in immunochemical experiments[14–16].

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