SHORT COMMUNICATIONS

On the suitability of Light Green as a stain for the quantitative evaluation of proteins in paper electrophoresis

The quantitative evaluation of proteins in paper electrophoresis is based on the equal dye-binding capacity of different protein fractions. Light green (LG), first proposed by CASPANI AND MAGISTRETTI[1], was claimed to satisfy such a criterion. Among the various protein stains used in paper electrophoresis that do not satisfy the above-mentioned fundamental criterion for suitable dyes LG seems to be a special one.

In this paper an attempt is made to ascertain whether LG is suitable for the quantitative estimation of proteins. This involves an investigation of linearity of dye uptake as a function of protein concentration as well as of the protein surface area on paper.

In addition, the general properties of the dye, and the best conditions of staining and elution are presented.

Materials and methods

Light green (LG) is a derivative of crystal violet in which a seventh methyl group has been introduced. It is a basic dye, mol. wt. 458. The LG used in our experiments was a Geigy (Switzerland) product. In order to develop the full colour, the spectrophotometric analysis of the dye was made at room temperature in 0.1 *N* NaOH, 20–25 min after addition of glacial acetic acid (3%, v/v); a Beckman spectrophotometer model DU was used.

Serum proteins. In our experiments pure human albumin and γ-globulin (kindly supplied by Istituto Sieroterapico Italiano) were used. Two 5% stock solutions were prepared in 0.15 *M* NaCl, from which dilutions of 0.5, 1.0, 2.0, 3.5% were made. The protein concentration was checked by the biuret method, according to GORNALL *et al.*[2].

Paper. The paper used throughout our experiments was Whatman No. 1 for chromatography.

Procedure. The measurement of dye uptake by proteins and the calculation of results were made according to the methods described in previous papers[3,4,5]. The complete procedure can be summarized as follows: preparation of proteins spots on filter paper strips by varying the amounts of each standard dilution; staining; rinsing; drying; measurement of surface area of each spot; elution and measurement of the dye eluted from each spot. Staining, rinsing, drying and elution are described in detail.

Results

Absorption spectrum. Fig. 1 shows the absorption spectrum of LG in 0.1 *N* NaOH after addition of glacial acetic acid (3%, v/v). The spectral analysis was made 30 min and 4 h after preparation of the dye solution. The absorption spectrum shows a maximum at 630–635 mμ and, at this wavelength, $E^{1\%}_{1cm}$ is 32.2.

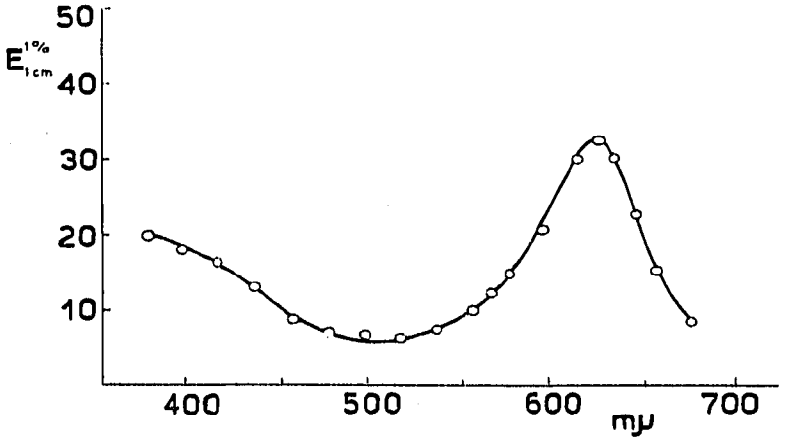

Fig. 1. Absorption spectrum of LG in 0.1 *N* NaOH after acidification with glacial acetic acid (for details see text).

Chromatography. Qualitative chromatographic analysis of the Geigy product used in our experiments, showed the presence of two green coloured components (R_F = 0.114 and 0.159). Chromatographic assay was made by the ascending technique using *n*-butanol–acetic acid–water (5:1:4, v/v).

Staining. The dye bath was an aqueous solution containing 0.5 g % LG and 10 g % trichloroacetic acid. The dye concentration was determined experimentally on the basis of a constant amount of dye bound by a standard quantity of protein in 40 min. The dye concentration suggested by previous authors[1] is in fact too low, and the staining procedure very time-consuming.

Rinsing. The rinsing liquid consisted of a 10% aqueous solution of trichloroacetic acid. Rinsing was repeated 3 times, for about 30 min, without agitation. The strips were then blotted gently on clean filter paper, and dried at room temperature.

Elution and colour development. The dye is eluted in 0.1 *N* NaOH for 30 min with occasional agitation. The elution liquid is colourless and becomes green on acidification. 3 ml of glacial acetic acid per 100 ml of eluate is the optimal amount that may be added to the elution liquid. The maximal intensity of the colour is reached 20–25 min after acidification. The dye concentration was estimated at 630 mμ, by comparison with a standard curve. The volume of the elution liquid was carefully chosen, in order to keep to the linear tract of the curve.

Dye uptake by proteins. Fig. 2 and Fig. 3 show the LG uptake by albumin and γ-globulin, respectively, in terms of μg LG/μg protein and μg LG/mm² spot as a function of protein concentration. Fig. 2 shows that a linear relationship exists for both proteins. It should be noted that

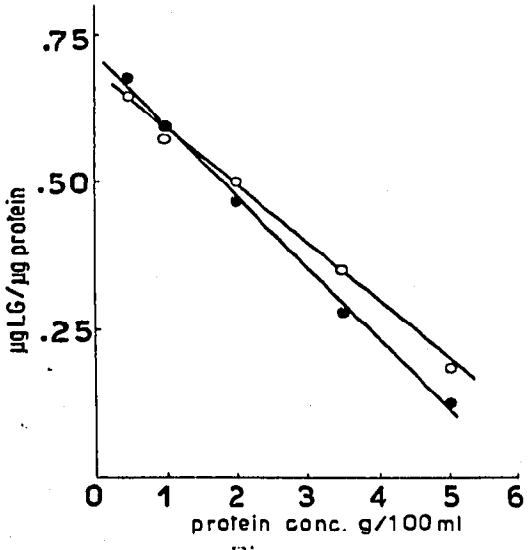

Fig. 2

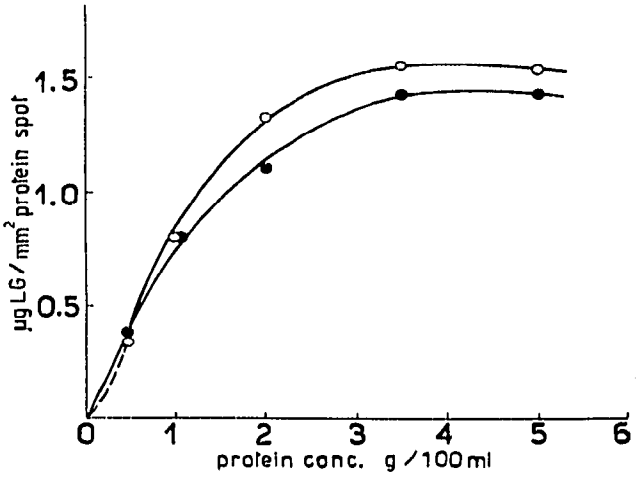

Fig. 3

Fig. 2. Uptake of LG by albumin (—○—○—) and by γ-globulin (—●—●—) per unit weight.
Fig. 3. Uptake of LG by albumin (—○—○—) and by γ-globulin (—●—●—) per unit area.

the behaviour of albumin and γ-globulin with regard to LG is very similar in the range 0.5–2.0 g protein %. Fig. 3 shows the course of the μg LG/mm² spot ratio with increasing protein concentration. According to the figure, the dye-binding capacity of γ-globulin seems to be smaller than that of albumin at higher concentrations, where a saturation value is attained at 2% for both proteins On the other hand, at a protein concentration of less than 1%, the behaviour is the reverse.

Discussion

Although the average LG uptake by proteins is relatively high as compared with that of bromophenol blue[3], and nearly similar to that of Azocarmine B[3,4] and Amidoschwarz 10 B[6], the extinction coefficient for LG is the lowest, so that the advantage offered by the first characteristic is cancelled out by the second.

The fundamental reason why LG is unsuitable for the quantitative estimation of proteins in paper electrophoresis, both by elution and, consequently, by scanning, is the non-proportionality between dye uptake and protein concentration, even when some other factors such as time and temperature of drying, time of staining etc., are kept constant.

Although the dye uptake is somewhat similar for both proteins over a rather wide range of concentrations, which was also observed in the case of bromophenol blue[3] and nigrosine[5], it is not possible to take advantage of this fact, because of the steepness of the curve in Fig. 2.

From this simple case, involving only two proteins, it can be inferred that in a more complex

system, such as blood serum, the quantitative results obtained by paper electrophoresis with LG will not be reliable.

The only advantage of LG is the absence of a coloured background.

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Nigrosine as a stain for quantitative estimation of serum proteins in paper electrophoresis

To the various dyes used for staining proteins in paper electrophoresis, another one has recently been added, namely nigrosine (NIG), which was proposed by ORTEGA in a short note[1].

The present paper deals with a study of the general properties of this dye, particularly its interaction with serum proteins on paper, and the results are compared with work carried out in recent years[2,3].

The main purpose was to check the dye-binding capacity of proteins at varying protein concentrations and surface areas on paper.

Materials and methods

Nigrosine. This dye is not a pure chemical compound, but a mixture of at least two dyes, induline violet and a yellow dye. The first is the sodium salt of a sulphonic acid derivative of the alcohol-soluble induline (indulines are phenylamine derivatives of safranines[4]). The NIG used in our experiments was a Merck product, which after qualitative chromatographic analysis appeared as a mixture of two deep blue components.

The absorption analysis of the dye in 0.01 *N* NaOH was made in a 1-cm cell, at room temperature, in the Beckman spectrophotometer model DU.

Serum proteins. Human albumin and γ-globulin fractions (by the courtesy of Istituto Sieroterapico Italiano) were used. The electrophoretic homogeneity was checked by free electrophoresis according to Tiselius. Two 5% stock solutions of the proteins were made in 0.15 *M* NaCl. The protein concentration was determined according to GORNALL *et al.*[5].

Paper. Whatman No. 1 was used in all experiments.

Estimation of dye uptake. Dilutions ranging from 0.5 to 5% were prepared from the albumin and γ-globulin stock solutions. By means of an Agla microsyringe each solution was applied, in duplicate, to paper strips 4 cm wide, as spots of 5, 10, 20, 30, 40 µl. The paper strips were divided by pencil into squares of 4 × 4 cm, and the protein solutions were applied in the center of each square. The protein spots were first dried in air, then at 105° in the oven for 15 min. Subsequently, they were placed for 10 min in a solution of NIG (1% in 1% acetic acid). The paper strips were washed in 1% acetic acid until the background paper was pale blue, after which they were allowed to dry at room temperature. The long and the short axis of the spots were accurately measured, and the surface area was calculated on the assumption that, when not perfectly circular, the spots were approximately elliptical. The paper strips were cut in square pieces (4 × 4 cm) and the dye eluted by soaking them in 10 ml of 0.01 *N* NaOH for 20–25 min.

Recovery experiments showed that the elution was almost complete. Whenever the extinction exceeded 0.800, the resulting blue solutions were diluted by adding known amounts of 0.01 *N* NaOH.

The amount of nigrosine eluted from each spot was determined spectrophotometrically at 560 mµ by comparison with a standard curve. This curve was prepared with solutions of NIG in 0.01 *N* NaOH with regularly increasing concentrations from 0 µg to 50 µg per ml; the slope of the curve was 0.0112.