A New Method for Scanning Electrophoretograms

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A new instrument is described for evaluating electrophoretograms on various media. The same densitometer can be used to evaluate spots on chromatographic strips and for colorimetric analysis of solutions. The versatility of this instrument makes it a useful adjunct to any analytical laboratory.

While scanning techniques for paper electrophoretograms are available, the introduction of new and varied electrophoretic systems (agar gel and starch gel) begets the necessity for more versatile scanning and quantitating devices. One instrument that seems to fulfill this need is a commercially available automatic recording and integrating densitometer*; a prototype of the present model was described by Latner (4).

The instrument, a reflectance densitometer, is designed for quantitative evaluation of electrophoretic patterns obtained on paper, on starch, cyano, and agar gels, and on cellulose acetate. Paper chromatographic systems, spot test analyses, autoradiographs, and colored solutions can all be evaluated. Indeed, any system can be quantitated in which variation in color intensity is a measure of the chemical composition or concentration of the sample. A detailed account of the use and limitation of this new instrument for evaluating electrophoretograms is presented.

Methods

Electrophoresis Systems Studied

1. Agar gel on cellulose acetate. Essentially, the method of Zak (9) was employed. Tris-(hydroxymethyl)-aminomethane-borate-EDTA

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buffer (60.5 gm., 4.6 gm., and 4.0 gm./L., pH 8.9) was substituted for the original buffer and used both for dissolving the Noble agar (0.15%, w/v) and for filling the cell compartments. Protein samples were exposed to 250 v/10 cm. for 2 hr.

2. **Agar gel on microscope slide.** A combination of the methods of Zak (9) and Wieme (8) was used. The current was applied at 90 v for 25 min., followed by 250 v for 27 min. This double-voltage technic seemed to align the protein fractions first before migrating them on the slide at the increased voltage. The agar gel solution was 0.2% (w/v) in the Tris-borate-EDTA buffer.

3. **Starch gel electrophoresis.** The original method described by Smithies (7) was modified by the use of a discontinuous buffer system as suggested by Poulik (6). We further modified Poulik’s technic by changing the molarity of the buffers. After trying several concentrations of Tris-citrate buffer, a 0.023M solution (pH 8.65) gave optimum separation in our hands. This buffer concentration for the gel was prepared by dissolving 2.786 gm. (0.023M) of Tris (Sigma 121) and 0.420 gm. (0.002M) of citric acid in 1 L. of glass-distilled water. The compartment buffer (pH 8.42) was approximately 10× stronger in molarity (0.209M) and compounded by adding 12.90 gm. (0.209M) of reagent-grade boric acid crystals and 4.5 ml. of 10N NaOH (0.045M) to 1 L. of distilled water. Current was applied for 3-4 hr. at 6 v/cm.

4. **Paper electrophoresis.** The commercially available (Beckman, Spinco Division) Durrum cell for paper electrophoresis was used in these studies. Two buffer systems, Veronal (0.075 ionic strength) and the Tris-borate-EDTA, as described for agar gel above and as described by Aronsson and Gronwall (1), were used. Directions described in Beckman brochure RIM-5, as procedure B, was followed. Schleicher and Schuell 2043-A mgl paper strips were used as paper supports.

**Power**

A Heathkit power supply (Heath Company, Benton Harbor, Mich.) Model PS-3, was used as the voltage source for the agar gel and starch gel media. The Beckman Duostat power supply was used for paper electrophoresis.

**Staining**

Following electrophoretic separation of the protein in the various gel media, fixation was accomplished by immersion of the gel in metha-
not for 10-15 min. The respective strips were then stained with amido black 10B according to Smithies (7). After paper electrophoresis, plasma fractions are coagulated by heat fixation at 110-120° for 30 min. The standard Spinco method as outlined in the Beckman manual, RIM-5, was followed in the staining of the paper strips.

**Scanning Techniques**

All the scanning was done with the Chromoscan (Fig. 1). The instrument is an automatic recording and integrating reflectance densitometer. It is particularly appropriate for the agar gel and starch gels since reflection rather than transmission of light is a necessary means of analysis. The instrument is fitted with an automatic integrator which records the relative and/or absolute area values. The pattern obtained by scanning the separated proteins is controlled by four variables; (a) the kind of light filter used, (b) the slit width of the aperture, (c) the slope of the grey wedge or cam, and (d) the gear ratio of sample movement to recording pen.

A variety of fixed slits and filters are available and readily interchangeable. For scanning fine structures in such media as starch gel, it was necessary to use a slit width of 1/20 mm. This aperture could be made even smaller by partially covering the opening with black tape. The filters to be used are selected by a trial-and-error process, though the operator probably would have an idea of which wavelength

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**Fig. 1.** Physical layout of the instrument (Chromoscan).
would be most absorbed by the specimen. The adjustment of the specimen position and the baseline is individually controllable. These adjustments can be accomplished with a minimum of effort and training. Less than two hours of instruction is necessary before being able routinely to assay samples.

The gears which control the ratio of specimen movement to the drum recorders are easily adjusted to any one of the three gear combinations available. The 1:1 ratio would give a short scan with the peaks close together, as shown in Fig. 2. As the gear ratio is increased to 3:1 or 9:1, the scan is spread wider. For most purposes, we have used the 1:1 ratio. While the resulting pattern is more pictorial of the electrophoretogram, the integration on small, highly spiked peaks is more tedious.

In order to achieve linear response for specific dyes and supporting media, it may be necessary to calibrate the instrument. If calibration shows that the cam corrections are necessary, a systematic and simple procedure is outlined in the manual accompanying the instrument. Because of the high sensitivity of the instrument, low concentration of dye binding can be used, which in turn leads to small correction factors. The height of the pen response is a function of the cam angle. Generally, a cam which gives a deflection no greater than two-thirds of the maximum pen deflection (140 mm.) for the densest peak is used.

![Fig. 2. Illustration of a micro starch gel electrophoretogram of human plasma scanned at the three gear ratios available on the instrument. A red filter and the 25/59 wedge were used.](image)
Figure 3 is an illustration of the effect of cam angle on the resulting pattern. If the cam angle is too large (25/59), the densest peaks cannot be measured accurately since the limit of the pen's excursion is reached. If the angle is too shallow (25/28), small bands are not differentiated.

**Fig. 3.** Illustration of a starch gel electrophoretogram of mouse liver extract stained for esterase fractions. The gel was scanned using a 3:1 gear ratio, red filter, narrow aperture, and variable wedges as indicated.

A special carrier for mounting gel and paper specimens is provided. For gel media, we found it most convenient to place the specimen on an opaque glass, cover the gel with a piece of clear glass, and invert this into the carrier. Care must be taken to keep the gel moist and to remove all air bubbles before beginning the scan. Also, care must be exercised with thick gel so as not to compress and thus destroy the separation of two closely allied fractions. The clear glass plate was also placed in front of paper sample when wrinkled, not flat, or not sufficiently wide. The carrier then is placed in the holder of the scanner. Through an aperture at the top of the instrument, the specimen and recording drum can be aligned by control knobs before beginning the scan. As the pen records the graphic picture, the integrator records a relative number. The electrophoretogram to be quantitated is scanned once and then the total integrations are noted. The drum is reversed and the pen placed at the starting point again. The integrator is then cleared and a second scan made, stopping the scanning process at each peak or ledge, and noting the number of integrations recorded at each interval. The integrator is cleared at each stopping
point. The baseline reading is obtained as described in the instruction manual and subtracted from the counter readings. These net recordings may then be expressed as a per cent of the whole by dividing the reading of the fraction by the reading for the total and multiplying by 100.

The values obtained by this method, in common with all other similar methods, do not give a measure of protein concentration; they represent only the amount of dye picked up by the protein fraction. The amount of dye may reflect protein-binding capacity rather than protein concentration. A representative specimen may be seen in Fig. 4.

A summary of 24 protein patterns of human plasma obtained from published works has been compiled by Ehrmantraut (2). The mean values for the five major protein fractions are:

<table>
<thead>
<tr>
<th>Protein</th>
<th>Value</th>
<th>Approximate Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>49.2%</td>
<td>(43.3%)</td>
</tr>
<tr>
<td>Alpha 1</td>
<td>4.1%</td>
<td>(6.4%)</td>
</tr>
<tr>
<td>Alpha 2</td>
<td>8.4%</td>
<td>(14.4%)</td>
</tr>
<tr>
<td>Beta</td>
<td>11.4%</td>
<td>(15.9%)</td>
</tr>
<tr>
<td>Gamma</td>
<td>16.6%</td>
<td>(19.0%)</td>
</tr>
</tbody>
</table>

The percentages we obtained from the Durrum cell, using Veronal buffer and amido black, are recorded in parentheses and approximate his mean values. As pointed out by Ehrmantraut, each laboratory must establish its own normal range for the particular buffer and dye system used.

Fig. 4. Scan of a paper electrophoretogram illustrating the integration and calculation of the protein fractions.
Discussion

We have used this instrument in our laboratory for over two years and have found it a most useful addition to our analytical armamentarium.

Early experiences with this instrument, however, were marred by several electronic problems. These annoyances have largely been overcome with the cooperation of the manufacturer and resultant changes have since been incorporated into standard production models.

One problem which still persists is overheating of the lamp during routine use. By limiting scanning time to less than two hours, this trouble can be averted. This disadvantage is not serious since approximately 12-15 specimens can be scanned during this interval. After allowing the light to cool down to room temperature, a second group can then be evaluated.

Our experience has shown that the adjustment for establishing baseline readings is important. If the baseline is too high (>10 counts per inch), the sensitivity is too low and an incomplete scan results, and if too low, it is overly sensitive and surface defects and unevenness in the sample are represented as protein fractions. Once the baseline is established for a given sample, repeated scans of the electrophoretogram are identical.

We found it advantageous to use India ink in the pen holder. The India ink is heavier and flows better than the recording ink accompanying the instrument. The substitution has the added advantage of making the scans more prominent for photographic purposes. Regular bond typewriter paper was also substituted for the graph paper provided. The pen "rode" better on the absorbing bond paper surface than on the glossy graph paper.

The sensitivity of the instrument is very high. Scans of the agar gel media as shown in Fig. 5 and 6 indicate that even protein bands which are extremely close are measurable. In instances in which the bands are extremely narrow and very close together, the instrument at these settings does not indicate an actual peak but rather a ledge, as in the gamma area on the celluloid. These particular scans were obtained with a gear ratio of 1:1, a blue filter, and the narrowest aperture (1/20 mm.) available to us. The scan of these bands could be separated more by using a gear ratio of 3:1. This procedure of changing gear ratios facilitates the scanning and integration of closely allied areas. In the starch gel media, where up to 15 bands for human plasma are separated, the instrument is capable of indicating each fraction.
Conventional paper electrophoretic strips can easily and quickly be evaluated. Both in Veronal and especially in the Tris-borate-EDTA buffer systems, subfractions of major bands were resolved. These subfractions were difficult to distinguish with the naked eye.

Ingle and Minshall (3), after reviewing the literature with respect to photometric evaluation of chromatograms, have concluded that estimations based on transmission measurements involve greater er-

Fig. 5. Scan of an agar gel electrophoretogram of human plasma illustrating the resolution of protein on a small plastic strip. A blue filter, 1:1 gear ratio, 25/59 cam, and the narrowest slit width available (1/20 mm.) were used for scanning the protein fractions.

Fig. 6. Human plasma fractionated on agar gel on a microscope slide and scanned with a 1:1 gear ratio, a 25/59 cam, and the narrowest aperture (1/20 mm.).
rors than did measurements by reflection. A previous report on the use of a transmission densitometer (5) pointed out that the discrimination by the densitometer was not as great as the resolution obtained in the gel. Our experience has shown that by reflective densitometry the discrimination of the various major bands into subfractions by this instrument is possible.

Because of the above observations, the Chromoscan, a reflectance densitometer, seems to be an ideal instrument for measuring gel and paper electrophoretograms as well as performing evaluations on chromatograms.

References