DESCRIPTION OF A DENSITOMETER FOR DIRECT COLORIMETRY OF IRREGULARLY SHAPED CHROMATOGRAPHIC SPOTS

R. J. WIEME
Laboratory of the Medical Clinic, University of Ghent (Belgium)*

Correct photometry of coloured spots requires a uniform distribution of the absorbing material over the entire slit length. This severely limits the applicability of this otherwise convenient method of quantitative evaluation. In paper electrophoresis the difficulty is overcome by applying the mixture to be analysed along a line at right angles to the direction of the electrophoretic separation. But a regular distribution is not always maintained during the run, so that great colorimetric errors may ensue when direct colorimetry is attempted. This was clearly pointed out by GRASSMANN who also gives a mathematical expression for the error[1]; at higher optical densities this may easily reach 50%.

In paper chromatography it is even more difficult to obtain perfectly shaped spots; round ones are in any case not very suitable for direct colorimetry when a fixed slit length is used.

This error could be minimised by using a very small optical slit so that it can be assumed that the absorbing material is equally distributed over this area. The whole coloured spot could be scanned in this way and the optical densities integrated. Some attempts have been made in this direction but they do not seem to have introduced any improvement[2]. We shall describe here an apparatus, constructed with relatively simple means, which gives satisfactory results with spots of various shapes, and which lends itself to the direct colorimetry of a projected image of electropherograms made on an ultramicro-scale.

MODE OF OPERATION

The coloured material is linearly scanned by a spot of light of 2 × 2 mm, obtained by rotating a slit of 2 mm in front of a slightly curved stationary slit of 25 × 2 mm.

Between the stationary slit and a sensitive photocell, the translucent chromatogram is moved 2 mm at a time. The classical method of scanning in the direction of the run is maintained, but at each step of this "resolving scanning" the coloured material distributed along the stationary slit is scanned by the rotating slit. This "integrating cross-scanning" furnishes a value, read on a galvanometer, that is plotted in the usual manner on linear graph paper as the amount of substance at a

* Director, Prof. P. REGNIERS.

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given distance in the direction of the run. The area under the curve so obtained, is a direct measure of the total amount of substance present in the spot. To make the deflection of the galvanometer directly proportional to the amount of substance distributed along the stationary slit, the logarithm of the amount of light that has been absorbed, has to be integrated and not the amount itself. In histospectrophotometry similar problems arise[3], but the function transformer developed by LOMAKKA[4] cannot be applied here, because of the inertia of this mechanical device. Finally we adapted a photomultiplier circuit, described by SWEET[5] (see also CLINK[6]), which makes use of the exponential relationship that exists, within certain limits, between the sensitivity of a photomultiplier cell and the voltage applied at its dynodes. Conversely, to maintain the output of a photomultiplier cell constant under varying conditions of illumination, the applied voltage must be changed and its magnitude is a direct measure of the logarithm of the amount of light falling in, and thus of the amount of absorbing substance, if the law of Lambert-Beer holds.

In this model the width of the spots that can be read is restricted to 25 mm by the dimensions of the photocathode of the "931 A" cell. Since this was sufficient for our work, we did not attempt to improve upon it, although this must be possible by interposing some optical system between the chromatogram and the photocell. Alternatively, other types of cells with larger photocathodes, could be utilised.

The scanning method lends itself to the direct scanning of the projected image of small electropherograms and coloured tissue preparations. This may be of interest to histochemists. We have used the integrating densitometer for the reading of electropherograms of tissue proteins made on an ultramicro-scale[7].

TECHNICAL DETAILS

I. *Mechanical lay-out* (Figs. 1 and 2)

The black rotating disk is directly fixed on the axis of an induction type motor with shaded pole (electric gramophone motor, ± 2700 r.p.m.). At the circumference of

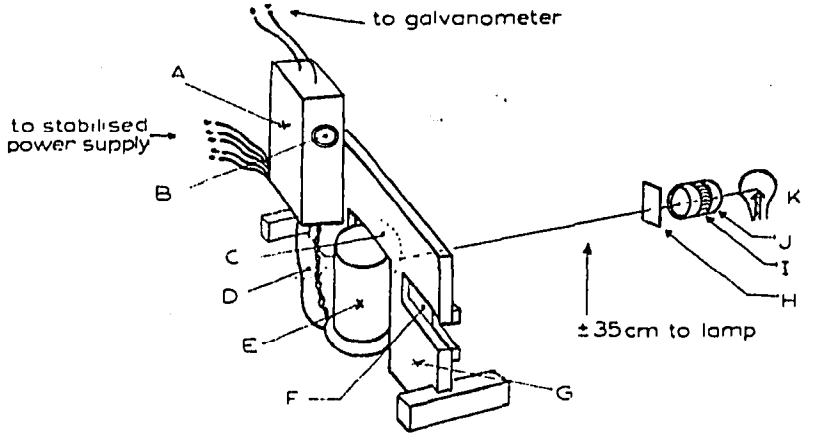

Fig. 1. Mechanical lay-out of the integrating densitometer. A = housing for the 6AG5 tube; B = set zero knob; C = projection of rotating disk; D = connections to photocell; E = photomultiplier housing; F = chromatogram clamped between glass plates; G = wooden frame; H = filter; I = diaphragm; J = objective; K = 6 V lamp.

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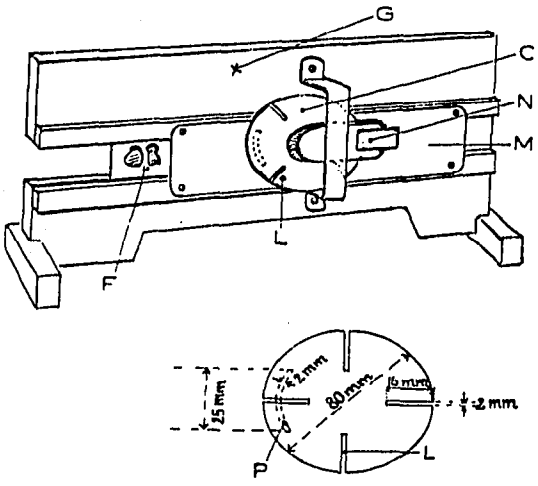

Fig. 2. Mechanical lay-out of the integrating densitometer. C = rotating disk; F = chromatogram; G = wooden frame; L = rotating slit; M = metal plate with stationary slit; N = motor; P = stationary slit.

the disk are situated four equidistant slots of 2 mm width and equal depth so as to maintain perfect wheel balance. At each turn of the disk the stationary slit is scanned four times. In order to obtain an even illumination of the slits we used a good photographic objective (photographic enlarger Meopta, objective Belar, $I = 4.5$, $f = 5.5$ cm) with built-in diaphragm, by means of which the main light level can easily be varied between relatively large limits. The light source, a low voltage tungsten filament lamp (General Electric, 13–23, 6 V, 2 A) fed from a heavy duty storage battery, is placed at the focus of the objective. An interference filter for the desired wavelength, interposed in the lightpath, serves as monochromator. The photomultiplier cell, in its metal housing, is placed as near as possible to the paper to be scanned so that no diffused light is lost. In the housing a rectangular slot of 2.5 × 0.5 cm is cut in front of the photocathode and carefully centered. The unit is countersunk in the wall in order to minimise the amount of direct light falling in. This is important as the direct light may alter the working points of the electronic circuit. With the same aim we interposed a screen (not shown in the figures) between the light source and the rotating slits.

II. *Electronic circuit* (Fig. 3)

The source of high tension is efficiently stabilised and can be set at any value between 400 and 1000 V. This output voltage has to be carefully chosen and fixed. An EF42 pentode was used, but any high slope pentode could be substituted provided the socket isolation is good. The anode of the photomultiplier cell is connected to a + 105 V point via a resistance of $10 \cdot 10^6$ ohms and is also directly attached to the grid of the 6AG5 tube whose cathode is at ground potential. The dark current polarises the grid slightly negative relative to the cathode (— 2 V). When light strikes the photocell this current tends to increase and makes the grid more negative, increasing the internal resistance of the pentode. As a result the voltage applied to the photocell

is drastically reduced. This voltage change is a logarithmic function of the amount of light falling in. In fact, large voltage pulses of constant frequency and width, but of varying shape, are obtained at the cathode of the photomultiplier tube and it is an easy matter to integrate them by a circuit composed of R and C in series. The integrated voltage is applied to a sensitive galvanometer. Its scale is linearly divided and marked

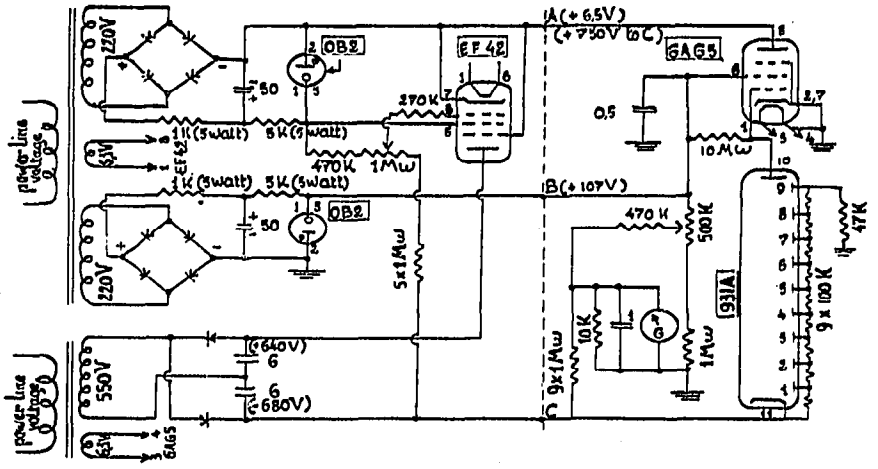

Fig. 3. Electronic circuit of the integrating densitometer. All C values in μF; all resistors of the ½ watt type, unless otherwise specified. All V values measured with vacuum tube voltmeter; input R = 25 Mω. G = galvanometer (Multiflex, Lange, Berlin; sensitivity $4 \cdot 10^{-9}$ A per mm deflection, R = 1300 ω, set to 1/10 of this sensitivity).

from 0–100. By introducing a voltage of opposite polarity, the spot is brought to zero on a blank. When a spot is scanned the integrated voltage changes and the galvanometer index deflects to a value that gives, in arbitrary units, the amount of material present. Stability is high; slight jerks of the galvanometer may be noticed, especially at high optical densities, but these are due to irregularities in the noise voltages and do not interfere seriously with the accuracy of the galvanometer readings. There should be no drift after a warming up period of 15 minutes. When this is not the case the cause should first be sought in an exhausted storage battery.

III. *Final adjustments*

These, although simple, have to be made carefully, as they are essential for a good quantitative response. First of all the stabilised voltage is set at about 720 V. Then the general light level is adjusted so that when a blank is scanned a really high impedance voltmeter reads approximately — 5.5 V at the grid of the pentode. Finally the spot of the galvanometer is brought to zero by the zero-set potentiometer. Then readings can be taken.

IV. *Accuracy*

The accuracy was checked in the following manner:

(1) Equal amounts of a coloured substance applied as spots of various shapes should give equal integrated values with this densitometer. So, 10 or 20 μl of a saturated solution of amido black in methanol containing 10% (v/v) acetic acid, were applied

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as uniformly as possible on dry Whatman No. 1 paper. As extremes spots elongated in the direction of the step by step scanning (3 cm × 1 cm; B spots) were compared against ones that were very short, but extended over the entire slit length (1.5 cm × 2.5 cm; A spots). After oiling the paper, each spot was scanned twenty times. Table I

TABLE I

DIRECT COLORIMETRY OF SPOTS OF VARIOUS SHAPES, CONTAINING EQUAL AMOUNTS OF ABSORBING SUBSTANCE

Amount of amido black, satd. soln. in methanol	*Densitometer*	*A spots**	*B spots**	*Difference*
		mean of 20 readings		
20 µl	Integrating type	1840 units $s = 38$	1837 units $s = 34$	3 $s_{diff} = 11.5$
	Bender and Hobein (slit width reduced to 2.5 cm)	6298 units $s = 95$	5083 units $s = 105$	1215 $s_{diff} = 32$
10 µl	Integrating type	860 units $s = 18$	817 units $s = 19$	11 $s_{diff} = 5.8$
	Bender and Hobein (slit width reduced to 2.5 cm)	3302 units $s = 85$	3199 units $s = 127$	103 $s_{diff} = 34$

* For A and B spots see text.

$$s = \sqrt{\frac{\Sigma x^2}{n-1}} \; ; \quad s_{diff} = \sqrt{\frac{s_1}{n_1} + \frac{s_2}{n_2}} \, .$$

shows the results. The standard deviation was of the same order (2%) as the one obtained with a Bender and Hobein densitometer on the same spots. With this latter apparatus, however, a systematic error is introduced especially at higher optical densities and the differences between the means exceed the 5% level of significance. With the densitometer described here, this level is not exceeded so that we may safely assume that spot shape as disturbing factor has been eliminated. It may be of interest to emphasize the fact that with other simple direct colorimeters the systematic error will probably be even greater, as the constructors of the Bender and Hobein photometer have purposely introduced some compensation by increasing the illumination at the center of the optical slit.

TABLE II

PAPER ELECTROPHORESIS OF A HUMAN SERUM (ROUND SPOTS)

Densitometer	*Albumin*	α_1	α_2	β	γ *globulin*
Integrating type	51.8% $s = 1.37$	4.5% $s = 0.45$	14.0% $s = 0.62$	13.1% $s = 0.63$	16.4% (mean of 10 scannings) $s = 0.75$
Bender and Hobein (slit width reduced to 2.5 cm)	40.9% $s = 0.33$	7.5% $s = 0.36$	13.7% $s = 0.39$	12.7% $s = 0.56$	25.0% (mean of 10 scannings) $s = 0.41$
Elution	51.1%	6.9%	11.3%	10.6%	19.9% (mean of 2 readings)

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(2) Electropherograms obtained by applying human serum as a small round spot, were read with the two types of densitometers and compared against the values obtained by elution of the cut-out fractions followed by ordinary colorimetry (staining with amido black, colorimetry at 578 mμ). Table II shows clearly that, with the Bender and Hobein apparatus, too low a value is obtained for the albumin fraction, which is in fact the fraction present in the highest concentration. On the other hand, the performance of the integrating densitometer compares favourably with that of the elution technique. The minor variations in the globulin fractions are probably due to the incertainty in the choice of the cutting lines.

SUMMARY

A uniform distribution of the absorbing material over the entire optical slit is essential for photometry of coloured spots. Irregularly distributed substances could, nevertheless, be measured correctly by using a very small slit, which is moved over the entire spot area, and integrating the total amount of absorbing material. A scanning densitometer fulfilling these requirements, is described. Data are presented demonstrating a good performance in the case of irregularly shaped spots and of actual electropherograms with irregular lateral distribution. With this apparatus it is possible to scan the magnified image of a coloured preparation, so that its application in histochemistry is suggested. The author uses the scanning densitometer in the quantitative estimation of electropherograms made on an ultramicro-scale in agar gel.

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