A NEW AUTOMATIC RECORDING DENSITOMETER FOR PAPER CHROMATOGRAPHY

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INTRODUCTION

A versatile instrument of high sensitivity for the quantitative estimation of substances on paper chromatograms, which would be capable of dealing quickly with large numbers of such chromatograms and give a permanent record of the results, is highly desirable.

HACKMAN AND LAZARUS[1] have recently discussed the types of densitometers reported in the literature, but none of these seemed to fulfil our requirements.

The advantages of the instrument to be described are that it uses a fluorescent lamp as a light source and it maintains a direct spatial relationship between the chromatogram and the chart record. The fluorescent lamp gives an even illumination and the lack of heat enables the light to be mounted in contact with the paper. The relatively high output of light in the blue region from this type of lamp ensures a good contrast for red spots; lack of blue sensitivity being a disadvantage of incandescent lamps, particularly if used in conjunction with a photocell having an S1 response. The high electrical sensitivity and stability of the instrument enabled us to dispense with the use of dimethyl phthalate for making the paper translucent.

INSTRUMENTATION

The instrument consists of three basic parts (Fig. 1):

1. Light source and photocell assembly;
2. Amplifier and driver unit;
3. Chart recorder.

1. *The light source and phototube unit* (A) consists of a 15 watt 9 inch fluorescent tube, with the phototube (B) arranged parallel to the lamp and just above it. The phototube (type 90 AV with S4 spectral response) is housed inside two concentric brass cylinders each having a 4 mm slit cut longitudinally; the slit being adjustable by rotating the inner cylinder using a reduction drive and a graduated knob (C). The balancing phototube is under the light and is arranged similarly to the top housing.

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Fig. 1. Automatic recording densitometer.

Coarse balancing is effected by adjusting the slit width. The paper is kept in place by guides 2 inches apart and a roller which prevents buckling. The photocell has a sensitive length of about 2.5 cm; our usual spot width being about 1.5 cm, accurate centralisation of the spot is not necessary.

2. *Amplifier and driver unit* (*D*). This consists basically of a bridge circuit comparing the impedance of the two photocells, a tuned amplifier, detector and driver stage for the chart recorder (Fig. 2).

The rectified 50 c/s signal from the photocells is developed across the 10 megohm resistor. The output at this point is heavily filtered with the 0.01 μF condenser to

Fig. 2. All capacitances are in microfarads, all resistances are in ohms, kiloohms or megohms.

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reduce interference from the light source which is modulated by about 10% and exhibits a very spiky waveform. The output is fed into an amplifier tuned to slightly below 50 c/s. It is then further amplified in the phase splitter, detected and the rectified signal filtered and passed to the grids of the driver stage. A shift voltage is added at this point to give a fine balance.

This circuit is only one of many possible and is the result of modification of an existing instrument. However, it has proved trouble-free and very stable.

3. *Chart recorder.* A standard dual speed, 5 mA Evershed and Vignoles bench chart recorder (E) was used with a slot (F) cut in its back level with the platen. A shelf was extended out from the back with the light source and phototube assembly mounted at its far end.

A length of chart slightly longer than the chromatograph paper is cut and the chromatogram stapled to one end. The chromatograph paper is fed back between the phototube and light source and the chart fed through the back of the recorder and over the drive wheels.

The recorder pulls the chart under the pen at a speed of 6 inches per minute, at the same time pulling the chromatogram over the light source. This system obviates any slip or mismatch between the chromatogram and the chart record.

The instrument can be adapted for radio chromatograms by replacing the light source and phototube unit with Geiger-Müller tubes and connecting the recorder to the output of a ratemeter. For use in this connection the slow drive on the recorder is used.

APPLICATION

Quantitative estimation of amino acids and related compounds

All standards were chromatographically pure, and reagents (B.D.H.) were of L.R. and A.R. standard. Whatman No. 1 Chromatography Paper was used for most determinations, though other grades of paper could also be used. This was ruled with a pencil so that the starting line was 1½ inches from the bottom of the paper and the width was divided into 2 inch wide "strips". These were separated after application of the detecting reagent. At least three such strips were run simultaneously in the one determination. They were spotted either with three standards at different concentrations or with one standard, a control and a test solution.

The spot was applied with a "Shandon" pumpette fitted with a suitable "Shandon" micropipette. Various solvent systems have been used. However, it was found that for the estimation of amino acids in toad sartorii, *n*-butanol–*n*-propanol–0.05 *M* HCl (1:2:1) gave a clear separation by the ascending technique in 16–20 h. The chromatograms were dried in a draught oven at 50° for 30 min. The paper was then dipped in a solution of ninhydrin in acetone, to which pyridine had been added[2]. It was found that this method was necessary to produce a constant colour especially in the case of the acid solvents. KAY *et al.*[3] have discussed the "quantification" of the ninhydrin reaction and have used a caustic spraying mixture to give the alkaline reaction. We have found that under these conditions the spray damages the paper

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rendering subsequent techniques difficult and unreliable. The paper was then heated again at 50° for 30 min. This produces even and reproducible colours. The paper was cut in two inch strips and scanned as described with the densitometer. Fig. 3 shows a typical tracing obtained during an investigation into the effects of abnormal constituents of Ringer on the excised muscle of the toad, *Bufo marinus*. Here we see that

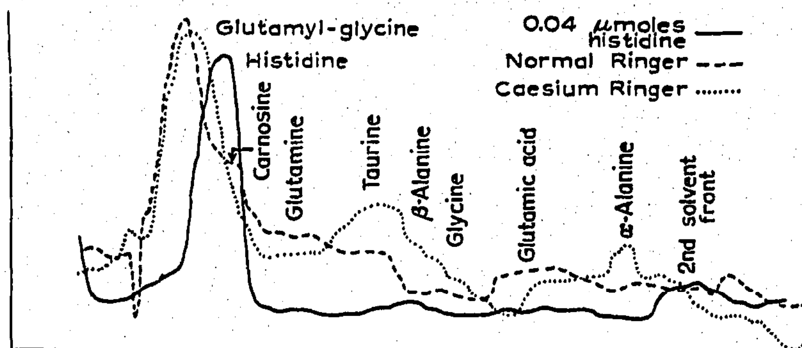

Fig. 3. Typical recording.

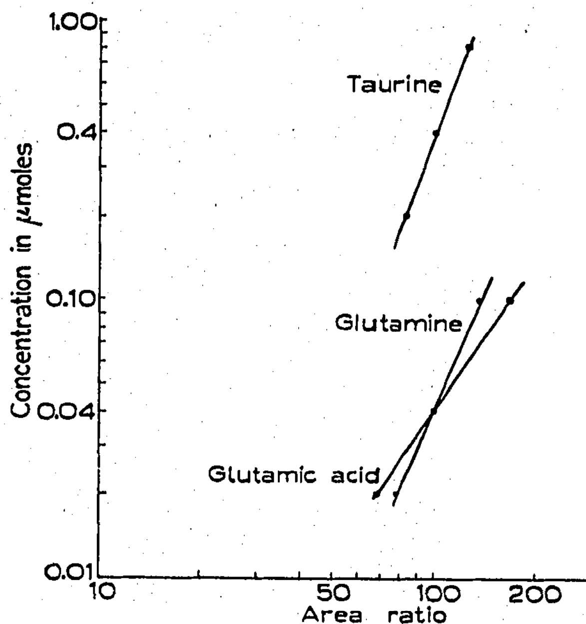

Fig. 4.

addition of large amounts of caesium has caused a breakdown in the carnosine and glutamine with a consequent increase in histidine, β-alanine and glutamic acid.

For well defined peaks such as glutamyl-glycine and histidine in Fig. 3, measurement of areas from the pen recording gave a quantitative estimate of material on the paper scanned. The standard, control and test were recorded on the same chart in different colours. For smaller quantities it was not always possible to investigate new solvents to give a sharper separation; therefore, providing a reasonable separation was obtained, a "carrier technique" was used. This consisted of adding the same

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quantity of standard to both the control and test samples; and allowing for that standard in reading the final result. By this method, between 0.005 and 0.040 μmoles of most amino acids could be estimated. When no clear separation occurred, the balance of the curve was drawn by hand[4].

The area under the curve was estimated with an "Allbrite" planimeter, which gave the area in arbitrary units. It was found that variations in the colour produced through deviations from the detection procedure could be compensated by running a standard, whose area was designated as 100, with each paper.

Typical calibration curves are shown in Fig. 4. It will be seen that a plot of the area against the concentration on log–log paper produces a linear calibration. The accuracy of the method is $\pm$ 3%.

Other uses

The densitometer has also been used for quantitative estimation of the red colour produced by the reaction of carnosine, histamine and histidine with diazotized sulphanilic acid reagent.

The method of AMES AND MITCHELL[5] was modified to eliminate the sodium

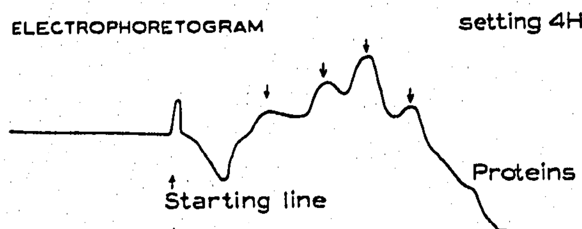

Fig. 5.

carbonate spray, by introducing liquid ammonia into the draught chamber and allowing for full colour development. Purines also gave linear calibrations when the photograph obtained with ultraviolet photography was submitted to the scanning technique. Full details of this method are described by us in another paper[6].

The scanning of photographs of starch electrophoretograms has been undertaken by Miss HELEN SILBERMAN of the Biochemistry Department. Clear records have thus become available for the estimation of proteins by this technique.

Semi-quantitative results were also obtained for molybdate blue in phosphate estimations and for proteins separated by electrophoresis and detected with bromphenol blue in saturated mercuric chloride solution[7]. A typical record is shown in Fig. 5.

CONCLUSION

The instrument has the following advantages over other models; it has a high sensitivity, a positive one-for-one spatial correspondence between chromatogram and chart, and does not heat the paper.

The densitometer described has proved highly satisfactory in use over the past

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year in the evaluation of a great variety of substances. It has proved useful qualitatively in precise determinations of the R_F values of amino acids, phosphates, purines, nucleotides, proteins and alkaloids and has been used in estimation of amino acids.

The instrument has a wide range of possible applications which covers colorimetric, photographic and radiometric techniques, and should find extensive use in industry and research.

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SUMMARY

An automatic recording densitometer is described which has high sensitivity and eliminates the need for making the paper translucent. It can be adapted to scan chromatograms using colorimetric, photographic or radiometric techniques. It provides a positive 1:1 correspondence between record and paper and has an overall accuracy of better than $\pm 3\%$.

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