THE QUANTITATIVE ESTIMATION OF SUBSTANCES ON PAPER CHROMATOGRAMS

II. AN APPARATUS FOR THE RAPID QUANTITATIVE PHOTOMETRY OF PAPER CHROMATOGRAMS

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INTRODUCTION

The general rationale of direct quantitative estimation of substances on paper chromatograms has been discussed in the first paper of this series[1] and in earlier articles[2,3]. It is well-known that this method, commonly known as the "scanning" of paper chromatograms, fell into disrepute around 1954 because of the apparently insuperable difficulties encountered in attempts to reduce its errors to acceptable limits (see *e.g.*, refs. 2 and 4). In 1954, the author discovered numerous elementary mechanical and optical errors in a commercially available manual scanner for paper electropherograms and decided to reinvestigate the method[5]. This investigation led to a useful method for determining reducing steroids on paper chromatograms[6] and to the design and construction of a mechanically driven scanner which has been described previously but incompletely[2,3,7]. Although this apparatus is now over seven years old, it seems to possess numerous advantages over any commercially produced instrument available at present, and, in some aspects at least, to surpass other published instruments of this type (see Discussion). Although designed to be operated in conjunction with the chromatogram-processing machine described in the first paper of this series[1], it can be used quite independently whenever necessary or convenient. This paper will be devoted to a description of the apparatus, some variations that have been tried out, and to the general principles governing its design and method of operation. Special details of chemical techniques and solid-state versions of the electronics will be reserved for later papers in this series.

CONSTRUCTION OF THE APPARATUS

The apparatus consists of three main sections, the light-source and monochromator; the paper tracker and photomultiplier (the scanner proper); and the electronic recording apparatus.

(1) *Light source and monochromator*

The monochromator is based on the optical components and light path of the

Unicam (Cambridge, England) S.P. 900 Flame Photometer. A variety of light sources have been used, including tungsten lamps (6 or 12 V d.c.) for absorptiometry in the visual range of wavelengths, mercury arc for ultraviolet-excited fluorimetry, and a.c. and d.c. xenon arcs (Osram-Neco, XBO-150) for both absorptiometry and fluorimetry. The most reliable arrangement and the one used for the greater part of the working life of this scanner is described here.

The components of the S.P. 900 Flame Photometer's monochromator were secured by their usual mountings to an aluminum alloy base plate (0.635 × 47 × 36 cm). The sides and a top were fitted to the base to form a closed light-proof box (Fig. 1). For reasons of economy simple wedges and cams were used to control the width of home-made slits (Fig. 2), and the angle of the Littrow mirror required for wavelength selection (Fig. 3). A double quartz lens condenser (diameter 5.1 cm, focal length 5.1 cm) and plane diagonal mirror (7.5 × 5.1 cm) were mounted so as to form an image of the exit slit (S_2, Fig. 1) on the paper strip, in the horizontal plane 3.3 cm above the top surface of the lid which was pierced with a 5.1 cm diameter hole (Fig. 1c, H_2) on the axis of the diagonal mirror.

The light source used most frequently was a xenon arc (a.c., 375 W, Westinghouse type now obsolete, and later a d.c. arc, type XBO-150, Osram-Neco). This was mounted vertically using a large Tufnol (bonded plastic) base (0.635 × 15.2 cm diameter) to insulate the anode terminal from a subsidiary aluminum alloy base plate. The lamp was surrounded by a stout cylindrical metal housing perforated with ventilation louvres and supporting a crude focussing mirror made by aluminizing a 10.2 cm diameter watchglass. The outlet hole was directed toward the inlet slit (S_1, Fig. 1) and the light path between the two was covered by a removable metal cover. The igniter-circuit and power-supply to the xenon arc were mounted on the side of of the lamp away from the monochromator.

(2) *Paper-tracker and photomultiplier housing*

This was constructed as a separate unit which could be lifted on and off the lid of the monochromator (Fig. 3). Its optical axis was aligned with that of the diagonal mirror of the monochromator by the adjustment screw AS (Fig. 1b).

The photomultiplier (PMT) was housed in a bushing holding the base-connector, which slid into the side of the brass cylinder C (Fig. 3). The dynode resistances were soldered directly to the pins of the base-connector and covered with a light aluminum cover made out of a commercially available can. The main brass cylinder (C) was fitted to the top of the paper-tracking device by pressing it into a snugly-fitting hole in the well (W) of the latter.

The main frame of the paper-tracker consists of two 0.635 cm thick aluminum side plates (P, Fig. 3) fixed to aluminum spacing rods (8.1 cm × 2.5 cm diameter,

Fig. 1. Monochromator. (a) Plan view showing layout of main optical components and the position of the paper-tracking unit (1) and its photomultiplier housing (2). (b) Elevation of monochromator and paper-tracking unit. (c) Vertical section through axes of slits S_1 and S_2 to show arrangement of cross-beam (B) holding them, the condenser L_1 and the diagonal plane mirror, M_1. S_1 = entry slit; S_2 = exit slit; D_1 = first diagonal; D_2 = second diagonal; CM_1 = first collimating spherical mirror; CM_2 = second collimating spherical mirror; P = dispersing prism; LM = Littrow mirror (plane); L_1 = condenser; WS = wavelength adjustment knob; AS = longitudinal adjustment screw; H_1 = hole and cover for collimation of source S_1, S_2 and L_1; H_2 = hole in lid of monochromator below paper-tracking unit.

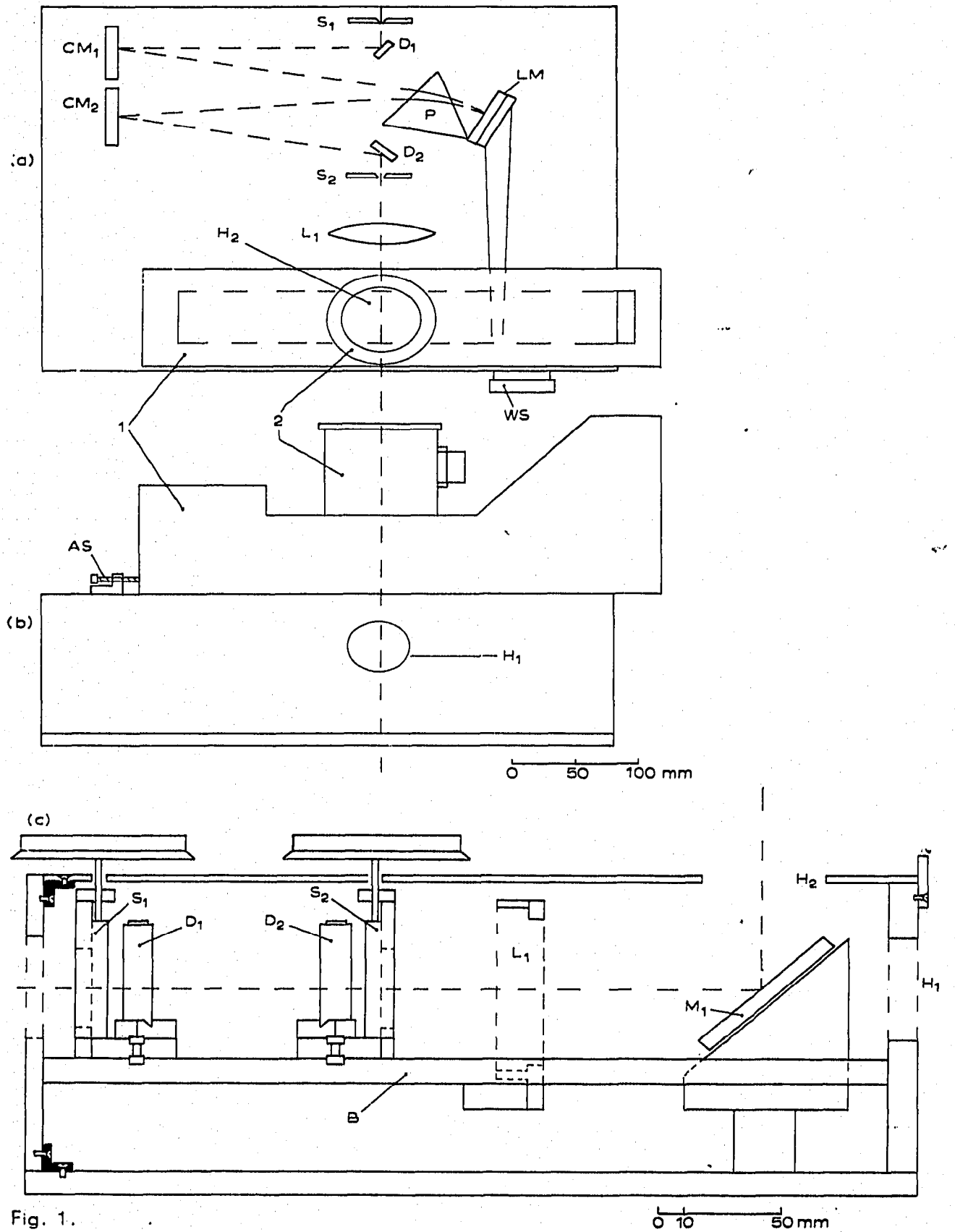
(a)
S1
D1
CM1
CM2
LM
P
D2
S2
H2
L1
1
2
WS
AS
(b)
H1
0 50 100 mm
(c)
S1
D1
S2
D2
L1
H2
H1
M1
B
0 10 50 mm

Fig. 1.

SP, Fig. 3; and 8.1 × 0.95 cm diameter, sp, Fig. 3). Knurled rollers (R_1–R_3, Fig. 3) drive paper strips 5.0–5.2 cm wide by means of spring-loaded idling rollers (r_1–r_3, Fig. 3) the paper sliding in the guide-rails (G) milled from aluminum alloy. The paper enters at R_1—r_1 *via* a metal guide chute (Fig. 4) and is ejected by rollers R_3—r_3. The correct direction and identical speeds of rotation of the drive-rollers are obtained by a train of fiber spur gears. These are fixed to the outside face of the tracker by spongy-bronze bearings on stub shafts screwed to the (back) main side-plate P.

The track of the paper is straight and horizontal in the region around the optical axis of the scanner and the guides G are supplemented by cross-plates and diaphragms (– – –, Fig. 3) to minimize the entrance of extraneous light. The end-plate of the tracker (EP, Fig. 3) is extended upwards to form the main body of the entrance paper-guide (Fig. 5). A flap of black paper closes the exit guide of the device and is lifted by each strip of paper as it emerges.

The front main side-plate (P_1) is pierced by two rectangular holes (3.8 × 0.6 cm) centered on the optical axis of the scanner which hold the slits and auxiliary filter holders S_3 and S_4 (Fig. 3). The well W whose upper ridged circumference receives the PMT housing C has a ridge machined at its lower end on which can be mounted the collimating lens L_2 and auxiliary optical components, including secondary filters for fluorimetry, and the field-stop FS.

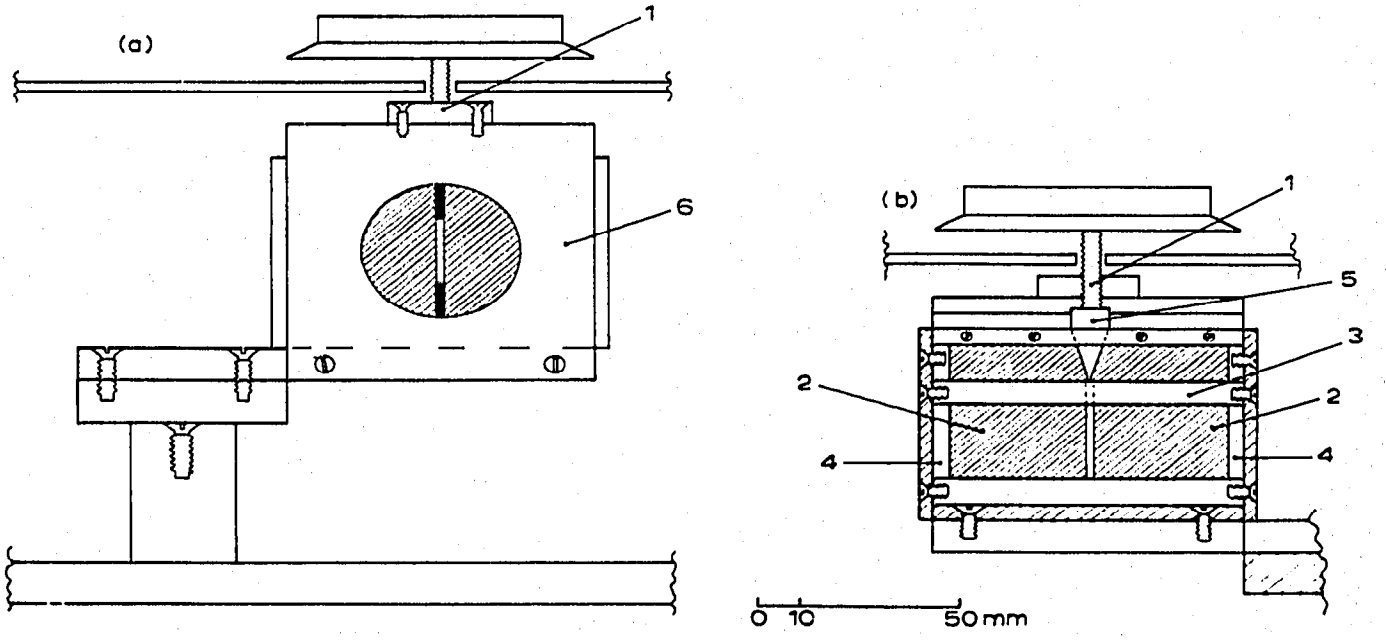

Fig. 2. Slit mechanism. (a) Incident and (b) emergent view of home-made slits for the monochromator. See Fig. 1c for sectional view and mounting beam. 1 = Screw adjustment with knob; 2 = jaws of slit; 3 = retaining cross-bar; 4 = flat springs; 5 = wedge driving slits apart; 6 = main plate supporting mechanism. In order to obtain a linear final image on the paper, the exit slit (S_2, Fig. 1a) was made linear and the entry slit (S_1, Fig. 1a) machined to a curve obtaining full correction of spherical aberration.

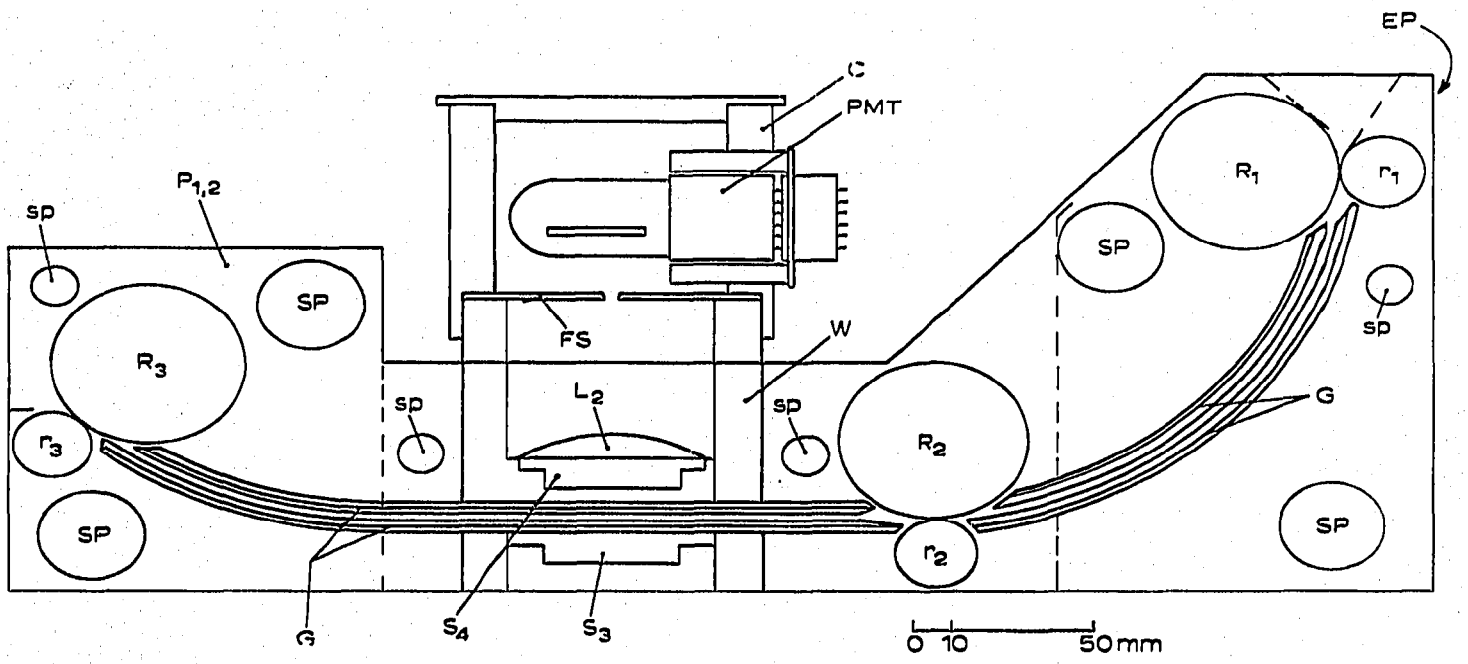

Fig. 3. Paper tracking-unit and photomultiplier housing. Diagrammatic elevation and section. See text for details. The entry guide for the paper strips is omitted from this view but is seen in Fig. 5.

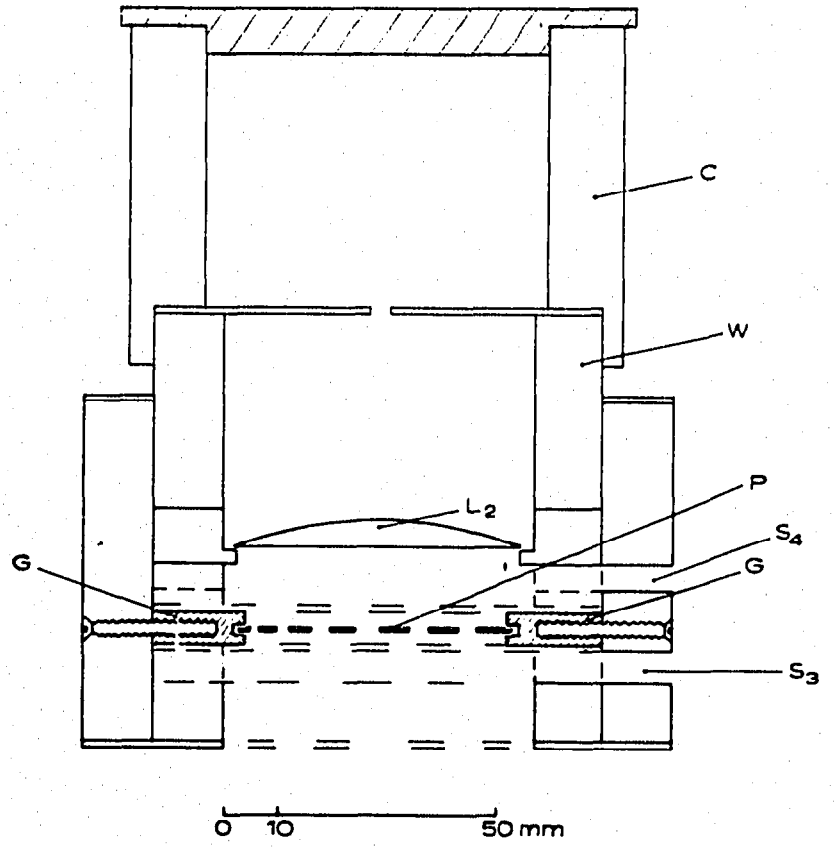

Fig. 4. Cross-section of photomultiplier section. C = Photomultiplier housing; W = well for lens, etc.; L_2 = collimating lens; G,G = guide rails for paper strip; P = paper chromatogram strip; S_3,S_4 = slits for second slits, filters, etc.

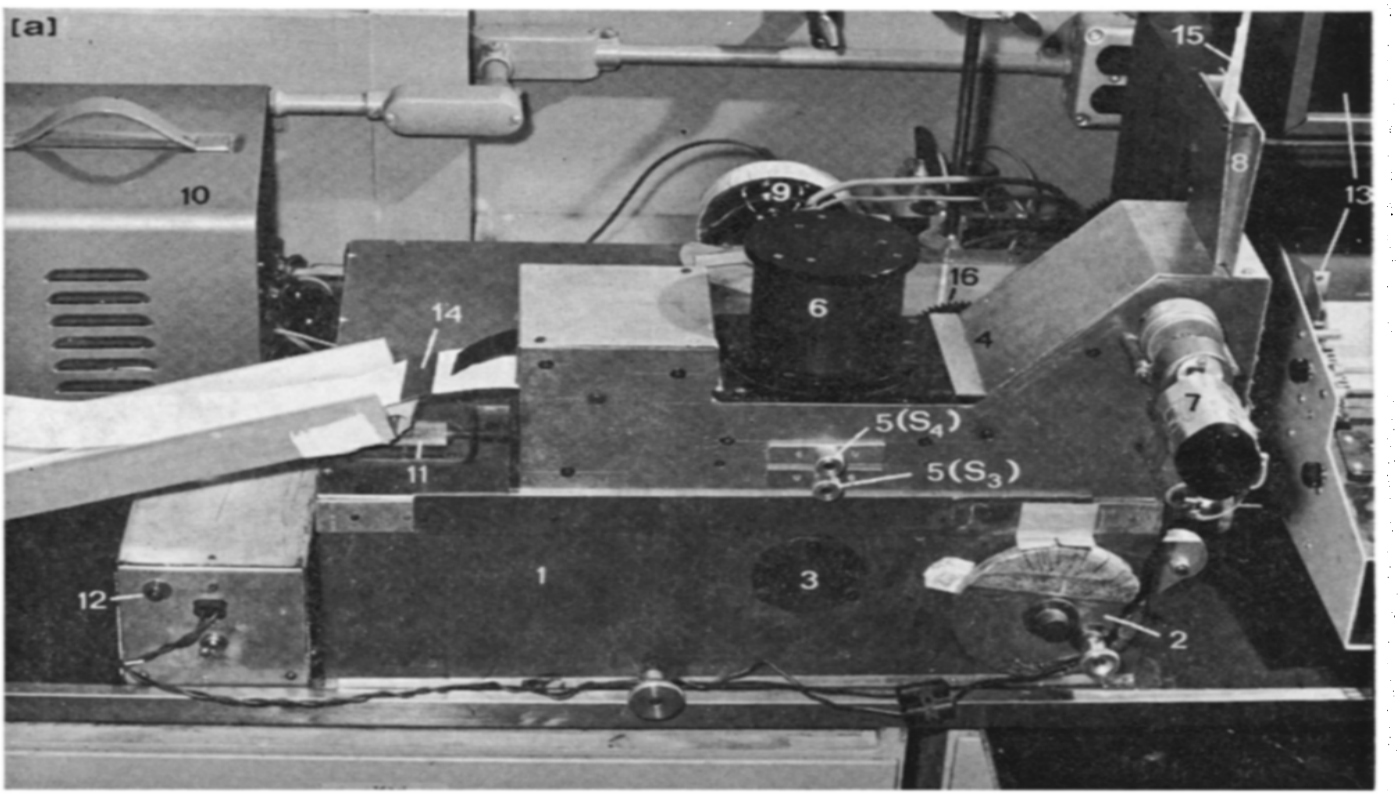

Fig. 5a. General view of the scanner. 1 = Monochromator; 2 = wavelength control; 3 = collimating aperture (covered) (H_1); 4 = paper-tracking unit; 5 = secondary slits (and filter) holder (S_3,S_1); 6 = photomultiplier housing (C); 7 = synchronous motor driving R_1; 8 = inlet guide attached to EP; 9 = tungsten lamp; 10 = stabilized rectifier and power supply for lamp; 11 = adjustment screw; 12 = magnetic clutch power supply for motor drive; 13 = recorder and electronic apparatus (see Fig. 5b); 14 = train of chromatograms emerging from scanner; 15 = last of the train entering scanner; 16 = one of fiber gears in train linking drive from R_1 to R_2 and R_3. Letters in brackets refer to codes of Fig. 3.

The top and ends of the tracker are closed with thin (approx. 0.2 cm thick) aluminum alloy plates screwed to the side-plates P_1 and P_2. These and other metal–metal joints were sealed internally before assembly with matt-black paper or felt, glued or taped to the metal. A cross-section of the PMT housing, paper guides and related parts is shown in Fig. 4.

(3) *Electronic and recording apparatus*

Various arrangements have been used successfully but all of those used for absorptiometry have been based on the circuit of SWEET[8], the output of which is approximately a linear function of the logarithm of changes of light intensity. Changes of output are thus directly proportional to changes in the optical density of media placed in the light path.

Earlier versions employed a tube circuit very similar to that of SWEET with an RCA-931A photomultiplier. This gave signals from chromatographic zones of average intensity in the range 0–100 mV which were recorded by a Sunvic (A.E.I., London, England) RSP2 potentiometric chart-recorder with a two-channel digital integrator. More recently we have used GORDY's solid-state version of SWEET's photometer[9] and used the output to drive a Bausch and Lomb VOM-6 potentiometric chart recorder with the range setting 0–2.5 mV. In this case the photomultiplier was the RCA-IP-21.

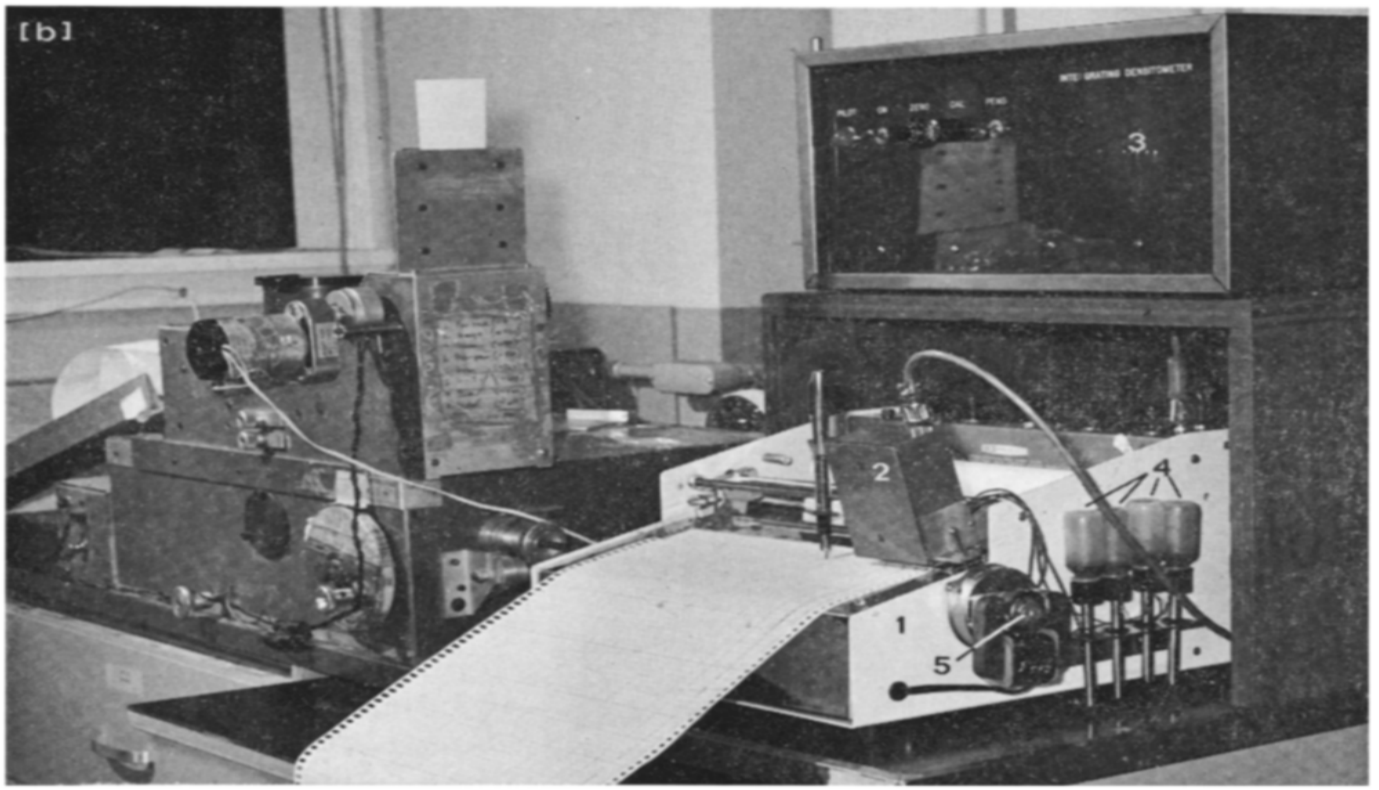

Fig. 5b. View of scanner with recorder and associated electronics. 1 = Heathkit EUW-20A chart recorder; 2 = 4-pen integral output unit; 3 = cabinet containing Sweet logarithmic output circuit, voltage-to-frequency converter, decade dividers, Schmidt triggers and pen drivers; 4 = ink wells; 5 = motor exteriorised to provide high-speed gearing to chart-drive.

Full details of the circuitry are given by GORDY *et al.*[9] In order to bring the output of GORDY's instrument on to the working range of the chart-recorder, however, an external backing-off voltage must be applied. This is conveniently provided by a standard mercury cell with a potential divider such as is commonly used for standardizing potentiometric recorders (*e.g.*, Eveready, E42N, 1.35 V). This is connected in the appropriate sense in either of the output leads to the recorder.

The most convenient arrangement is to place a good variable potentiometer of high precision (0–1000 Ω) in the output lead of a Sweet-type circuit as a potential divider. A smooth continuous variation in sensitivity is then obtainable so that an optical density change of 0.3 above the paper background which is nearly the maximum that can be measured reliably in practice, can be used to generate an output signal of up to 100 mV. A wide variety of commercially-available potentiometric chart-recorders can then be used with the apparatus.

Two aspects of the electronic apparatus are especially important. The paper is driven at 1 cm/sec through the tracker so that fast recorders are needed if accurate results are to be achieved. We have found that the A.E.I. Sunvic RSP2 specially modified to give full-scale deflection (f.s.d.) in 0.5 sec, the Bausch and Lomb VOM-6 rated at an f.s.d. of 0.5 sec, and to a lesser extent the Heathkit EU20A rated at an f.s.d. of 1.5 sec give reasonably good reproducibility with scanning speeds of 1 cm/sec and paper chromatograms 40–50 cm long. All these recorders however are extremely sensitive to stray a.c. noise so that it has usually been necessary to connect a capa-

citor of 200 μF across the input terminals of the recorder. Recently, trials with an Esterline Angus Lab Graph (Series S) recorder with an f.s.d. time of 0.2 sec have given the best results we have obtained so far.

A second important feature of the recording apparatus has been the provision of a cumulative integral record in digital form in parallel with the direct record of optical density. The A.E.I. Sunvic RSP2 provides an integral output in the form of two pens acting as "event markers" along one side of the chart. The solenoid-driven pens are fired by pulses from an inertia-less "integrating motor" which is driven by a voltage supplied from a retransmitting slidewire mounted on the same drum as the servo-driven potentiometer of the recorder itself. The rate of firing is directly proportional to the voltage provided by the retransmitting slidewire and hence to the voltage being recorded. The two pens are arranged to fire units and tens up to a certain level, above which the unit pen changes over to fire in hundreds.

The most successful arrangement has been a four-channel integral marker (units, tens, hundreds and thousands) which was driven, *via* a chain of decades, either by an external photoelectrically based integrating motor of very low inertia (Series 5300 Integrator, Electromethods Ltd., Stevenage, Herts., England), or by a solid-state voltage-to-frequency converter of the type described by HOWARD[10] driving the 4-pen unit *via* suitable dividers, decades and pen-drivers, the effective voltage being drawn from a retransmitting 1000 Ω potentiometer mounted on the servo-potentiometer of the Heathkit EU20A recorder, and a stabilized d.c. source.

All the electronic circuits that have been used with the scanner over the last eight years have been simple adaptations of standard or published apparatus. In the last year, solid-state modifications have been developed which offer considerable advantages in stability and reliability. This final version of the electronic system will be described in detail in the next paper of this series.

METHOD OF OPERATION

Collimation (See Fig. 1)

The light source is placed on the optical axis of the monochromator by sighting the (unlighted!) arc through the centers of the slits S_1 and S_2 *via* the hole H_1. The diagonals D_1 and D_2 are now inserted and the arc lighted. The focussing mirror of the lamp-house is then adjusted to give the best available diffuse image of the arc covering or filling the slit S_1. Vertical alignment is now checked and corrected if necessary by caliper measurement of the heights of the centers of the arc and of S_1. The components of the monochromator are now aligned in the usual way using card diaphragms marked with a convenient graticule to center images and beams on each component in the optical pathway. The lens L_1 is next aligned *via* the hole H_1. The diagonal mirror M_1 is now inserted and adjusted until a sharp image of the exit slit S_2 is obtained normal to the axis of the paper strip at the calculated position above the hole H_2. The lid of the monochromator casing is then placed in position and the diagonal mirror M_1 is now re-aligned if necessary, by centering the diffuse image of S_2 on a ruled paper diaphragm placed in the exit hole of the lid. The cover plate for H_1 is then fixed in place.

The collimation of the monochromator and light source is now checked by selecting a wavelength close to 580 mμ (yellow) and observing the image of S_2 with the eye close to the image plane. The eye is then moved to and fro along the image. At

this wavelength any serious misalignment is readily detected as vignetting at either end of the image, or as a change of apparent color over the length of the image.

The visual range of the monochromator can now be calibrated approximately on the wavelength dial by matching the color of the image with standard filters. If the standard wavelength drum control of the Unicam SP 900 is purchased, one adjustment with yellow light of 585 mμ is sufficient at this stage.

The scanner is now placed on the lid of the monochromator casing and 4.8 × 0.5 mm slits placed in the slit holders S_3 and S_4 (Fig. 3). Approximate alignment is now obtained by removing the PMT housing and looking down through S_3 and S_4. The screw (Fig. 1b) is now turned until the position of the scanner secures maximum illumination *via* S_3 and S_4, and the PMT housing is placed in position.

The final adjustment of the scanner and the PMT itself is made after checking the electronic equipment and will be described later.

Adjustment of the electronic equipment

If the SWEET circuit is based on electronic tubes it should include a delaying relay which switches on the E.H.T. supply only after the heater circuit of the power tetrode has brought the cathode to full emission[8], and preferably a fast safety cut-out relay which switches off or reduces the E.H.T. whenever the anode current from the PMT exceeds a safe threshold. This protects the photocathode from damage due to failure of the feedback controlling the dynode voltages, or to its possible inadequacy during accidental exposure to strong light. In any event it is best to minimize the light reaching the photocathode whenever the scanner is being adjusted or left to warm up by inserting a blank strip of filter paper in the scanner or by reducing the widths of S_1 and S_2 to less than 0.1 mm.

A blank strip of paper is therefore fed into the scanner by hand until the leading edge has just emerged from the exit rollers R_3—r_3 (Fig. 3). The slits S_1 and S_2 are now reduced to 0.3 mm width and a convenient visual wavelength selected on the monochromator dial. The power supplies to the SWEET circuit and to the potentiometric recorder are then switched on, and the usual period of warming-up is allowed to elapse if tube circuits are used.

The backing-off voltage is now adjusted to bring the potentiometric recorder to a reading of approximately 10 % of fullscale. (It is assumed that the recorder itself has been adjusted previously.) If the potentiometer controlling the backing-off voltage has to be turned too far towards either extreme of its range the slits S_1 and S_2 must be adjusted until the recorder can be brought to near zero without employing an extreme position of the backing-off potentiometer.

Using Whatman No. 2 or 3MM paper in the scanner, it will usually be found that a zero adjustment of the chart recorder will be obtained with slit widths (S_1 and S_2) of approximately 0.6 mm in the wavelength range 450–600 mμ.

The sensitivity is now reduced to a minimum by means of the potentiometer forming the potential divider in the output of the SWEET circuit. A small adjustment of the backing-off voltage may be required to zero the recorder. It will then be found that complete closure of the slits S_1 and S_2 produces a small or negligible increase in the reading of the chart recorder. The sensitivity control is then turned up until, after zeroing with the backing-off potentiometer, complete closure of the slits S_1 and S_2 just produces a full-scale deflection of the chart recorder. This setting of the sen-

sitivity control is then recorded or marked as the *lowest* practicable sensitivity of the instrument as a whole.

Because of the large background optical density of filter paper (see later) a check should be made before every use of the apparatus that the instrument is adjusted to be on the working range of the feedback controlling the dynode voltage of the PMT. Thus for wavelengths distant from the optimum of the photocathode's response the slits S_1 and S_2 must be widened so that the recorder is brought to zero with approximately the same backing-off voltage as obtained for the optimum wavelength. If the adjustment required to zero the recorder on changing wavelength is carried out entirely by changing the backing-off voltage, it is often found that the maximum response on closing slits S_1 and S_2 is less than 25 % of full-scale even at high sensitivity. The simplest way of ensuring that one is indeed on the working range of the instrument is to ensure that with the estimated starting slit-width and backing-off voltage needed to zero the recorder, a full-scale deflection is achieved by reducing S_1 and S_2 to less than 0.1 mm width.

Final optical adjustment

The recorder is brought to 50 % full-scale by reducing the width of S_1 or S_2. The position of the scanner is now adjusted with the screw (Fig. 1b) until a *minimum* reading is obtained on the recorder. If the original alignment was very faulty, or if the sensitivity setting is very high, readjustment of the backing-off voltage or further reduction of slit width (S_1 and S_2) may be needed to keep the recorder pen above zero.

On completion of this adjustment, the photomultiplier housing C is rotated in the well W to achieve a minimum reading, and finally the same manoeuvre is carried out rotating the PMT bushing (Fig. 3). These adjustments bring the axes of slits S_3 and S_4 (Fig. 3) on to the optical axis of the emerging beam of the monochromator, and the photomultiplier into the orientation securing optimal illumination of the photocathode. This minimizes the slit widths of S_1 and S_2 required to secure the full working-range at any wavelength, and maximizes the range of wavelengths that can be used with any given photomultiplier.

Checking the performance of the instrument

The machine is designed to give a signal proportional to the optical density of an absorbing zone on a paper strip and to produce a record on which the areas of peaks are directly proportional to the quantity of substance producing the peak. The sources of error are thus as follows:

(1) Limitations on the validity of the Beer–Lambert law as applied to absorbing materials deposited in a relatively thin medium of variable thickness and heterogeneous refractive index.

(2) Variations with time of the intensity, orientation, and wavelength of the light irradiating the chromatogram.

(3) Deviation of the output of the SWEET circuit from a strictly linear function of the logarithm of the intensity of light reaching the photocathode.

(4) Errors in the linearity and stability of the potentiometric chart recorder.

(5) Non-linearity of the integrating system (*i.e.*, either of the retransmitting slidewire or of the voltage-to-frequency converter itself).

(6) Time-lag in response of any part of the circuitry due to the large velocity at which the paper is scanned (1.0 cm/sec or 10-20 "effective zones"/sec).

(7) Variations with time of the velocity of the chromatogram through the scanner.

(8) Variations of chart-speed with time in the absence of a direct, time-controlled integrating system.

The first source of error is not an instrumental one and the features of the instrument designed to minimize the optical factors in this source of error will be discussed later.

As long as satisfactory mechanical rigidity has been assured by the usual means the second source of error can be checked by running the chart-recorder at a slow speed with the instrument switched on at high sensitivity and a stationary strip of paper in the scanner. Assuming that the stability of the SWEET circuit and the recorder have been checked and confirmed, any drift or more rapid shifts in the chart record will indicate instability of the illuminating system. The most likely cause of this is arc-jumping or variations in power supplies with xenon arcs, and filament vibration, overrated lamps, or variations in supply voltage with tungsten sources. The effects of arc-jumping and filament vibration are minimized by using the crude system described above to focus the arc on the slit S_1 which produces a diffuse image which is approximately 1.5 times as long, and perhaps as much as 10 times the width of S_1.

It is difficult to stabilize xenon arcs and much experience suggests that the more elaborate types of current-stabilizers (N.B. voltage stabilization is not adequate with these arcs) are not usually worth the moderate advantages they possess over the conventional stabilized power-supplies usually recommended by the manufacturers. Stability over periods of a few minutes is adequate since each chromatogram only takes 40–60 seconds to scan and average peaks are completely scanned in 2–5 seconds. It has usually not been difficult to secure stability to within $\pm$ 2 % full-scale deflection for periods of 30–60 minutes (equivalent to 40–80 chromatograms scanned) with conventionally stabilized xenon arcs, and to within $\pm$ 0.05 % with stabilized d.c. tungsten lamps.

The linearity of the response of the SWEET circuit has been discussed fully before[8,9]. Almost any desired degree of linearity can be achieved, although different types of photomultiplier design can affect the relative ease of doing so. The instrument described here was checked by using neutral grey filters of optical densities 1.00, 2.02, 3.01, and 4.01 which had been standardized by the National Physical Laboratory, Teddington, England. The three circuits that have been used during the seven and a half years' operation of the instrument were linear to within $\pm$ 2 % or better over the range optical density 1.0–4.0. Since the working range *with chromatographic zones* is usually only 0.5 optical density units or less (see later) and the effect of this error is reduced by a reasonable standard of uniformity of the methods of preparing and running paper chromatograms, this error is of small influence on the overall precision of the instrument.

The linearity and stability of the chart-recorders has been checked by standard electronic means. The linearity of the integrating system and its effective calibration curve was checked by feeding stable voltages to the recorder and running the chart for periods of 10–15 sec at each voltage. The integrating motor (Electro Methods Ltd.,

Series 5300) gave excellent results using a 1000 Ω retransmitting potentiometer and although rated at ± 1 % usually gave results within 0.5 % of linearity (Fig. 6).

Time-lag in the recorder is checked easily by running through the scanner a test chromatogram containing a moderately dense zone of light-absorbing substance, using a sensitivity-setting that gives 80–90 % full-scale deflection. The chromatogram is then passed through the scanner manually and positioned to give the maximum optical density of the peak. With the strip stationary in this position the chart is run for a few seconds to record this density as a short straight line. If the time-response of the system as a whole is adequate this reading will equal that obtained at the peak when the chromatogram was scanned at the normal operating speed. If the moving peak gives a result more than 1–2 % below the stationary recording the time-response of the recorder is probably at fault and should be checked electronically. If the recorder performs at its rated response-speed on a noise-free source pulse it is probable that the output of the SWEET circuit is conducting a.c. noise. If a simple filter fails to eliminate this (*e.g.*, a 20–100 μF capacitor across the output of the circuit) the nature and source of the a.c. interference should be sought with an oscilloscope in the usual fashion.

The source of a.c. interference is often eliminated by screening or improved earthing, but may sometimes be due to mains ripple or higher frequency noise in the power-supply to the xenon arc. Ordinary 50–60 cycles per sec mains ripple should not seriously affect a recorder protected by the simple filter described above. A "noisy" mains supply, however (*e.g.*, one supplying a large number of unsophisticated motors in a building), can produce serious effects, either directly *via* power supplies or stray capacitances to the circuitry, or indirectly *via* the xenon arc which is capable of "following" very high frequency oscillations of current.

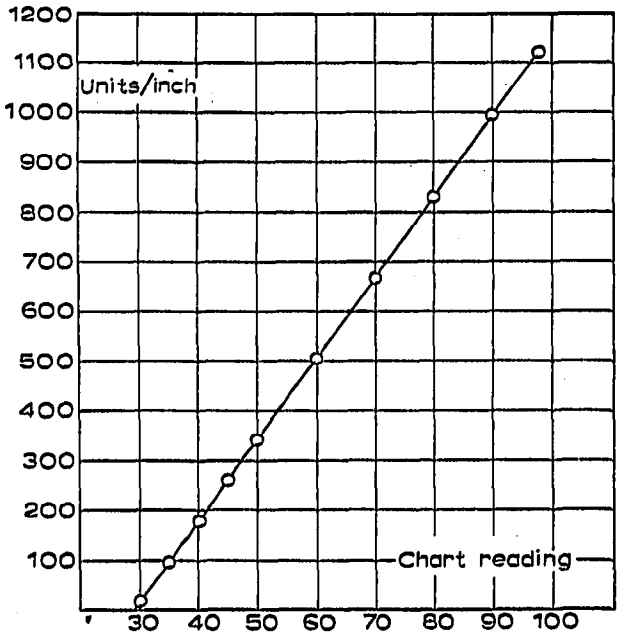

Fig. 6. Calibration curve for integrator. The input to the recorder was short-circuited and the pen manually placed at a desired chart-setting (zero at 28). The chart was then run at the usual working speed until approximately 10 cm of record had been obtained. This was repeated at various chart settings. The integrator's counting rate per length of chart was then obtained by counting over a minimum of 7.6 cm. (The results are expressed in inches (2.54 cm) which are the units used for the readings on the printed chart.)

The speed of the paper through the scanner is easily checked by ruling pencil lines at known intervals across a strip of Whatman No. 2 paper and repeatedly passing it through the scanner. If the speed of the chart of the recorder has been checked independently (*e.g.*, with a stroboscope or with an electronic pulse generator triggering pen) one can then measure the intervals between the pen deflections obtained from the pencil marks on the strip of chromatography paper. This suffices to provide estimates of the velocity of scanning and its variance. Using two different synchronous motors over the past seven and a half years, the variance has never exceeded 0.5 % for 10 cm intervals and has in fact been too small to measure precisely by this method. Any larger errors over intervals of this size would probably be the result of eccentricity or other machining faults in the main driving rollers of the scanner.

When these several characteristics and sources of error have been checked independently, the overall performance of the apparatus has been assessed by repeatedly scanning single test chromatograms containing zones of small, moderate and large optical density. It is best to test both moderately broad zones near the middle of such strips, and also the sharper zones found nearer the origin. Tests of this kind have invariably shown an unexpectedly high degree of reproducibility, suggesting that despite the numerous potential sources of errors in the whole system, the sources of systematic errors are reasonably stable and that the rest are truly random and mutually cancelling in their overall effect. Thus, even when the Heathkit EUW20A Recorder which is the slowest that has been used (f.s.d. rated at 1.5 sec), the standard deviation of the integrated value of peak area in five repeated scans of a moderately sharp peak (cortisone, estimated with alkaline blue tetrazolium) giving 75 % f.s.d. at the peak was only 0.87 % of the mean.

Absorptiometric characteristics of paper chromatograms

When off-axis scattered light is reduced to a minimum by the use of the slits S_3 and S_4, the collimating lens L_2, and the field stop (Fig. 3), the optical density of untreated filter paper (Whatman No. 2) was found to be approximately 2.5 in yellow light (585 mμ). This was measured as follows. The filter paper strip was fed into the scanner by hand and the recorder brought to 25 % f.s.d. with the backing-off potentiometer using a low sensitivity setting. The paper was then removed, and standardized neutral grey filters of optical density 2.02, 3.01 and 4.01 inserted between S_3 and S_4 (Fig. 3). The signals recorded on the chart for each filter were measured and plotted as an absolute calibration curve of optical density. The optical density of the paper strip was then read from this calibration curve. To overcome small errors due to variable positioning of the neutral filters these measurements were repeated three or four times for each filter and paper strip. The background-optical density of a strip of Whatman No. 2 paper treated with alkaline blue tetrazolium[6] was found by this method to be approximately 2.64 (range $\pm$ 0.01, four measurements). Untreated Whatman 3MM paper gave an optical density of 2.79 (range $\pm$ 0.005, three measurements).

Using the same procedure the optical densities of some typical colored zones on test chromatograms were measured. A 45 μg zone of tetrahydrocortisone approximately 18 cm from the origin of a 45 cm long chromatogram, filling the width of a 5.08 cm wide strip of Whatman No. 2 paper, and approximately 2.8 cm long (head to tail) was revealed with alkaline blue tetrazolium by the method of BUSH AND WILLOUGHBY[6]. The optical density at the peak's maximum was 0.19 optical density units above back-

ground. Subsequent use of GORDY's circuit which provides a direct meter readout in optical density units has confirmed these results. Zones containing approximately 80 μg of cortisone represent the upper limit of linearity of the effective calibration curve for this method. Zones of 2 μg represent the minimum quantity that is reliably detected on scanning records made at a sensitivity giving 90% full-scale deflection with 50 μg zones.

The scanning records at various sensitivity settings of an untreated strip of Whatman No. 2 paper are shown in Fig. 7. The record is characterized by a continuous series of short period fluctuations (approx. 6 cycles per cm of paper), less regular fluctuations in which cycles of 1–3 per cm predominate, and very irregular fluctuations 2–8 cm in length. If scanning records of the same strip of paper are made at different wavelengths the records are almost exactly superimposable, except for a small and smooth increase in the amplitude of the short period fluctuations in the ultraviolet. Very similar results have been obtained with Whatman 3MM paper in several experiments of this type.

Such records provide a good objective assessment of the "texture" of different grades of filter paper although the short period of variations in optical density will not be recorded faithfully at very high sensitivity-settings due to the relatively slow responses of pen-recorders. Thus the records for Schleicher and Schuell papers Nos. 287 and 2495 were extremely uneven, and those for Whatman papers No. 1, 3 and 4, moderately uneven. Whatman Nos. 2, 3MM, 20 and 520 possessed the smallest fluctuations in optical density and are probably thus the most suitable for this type of work.

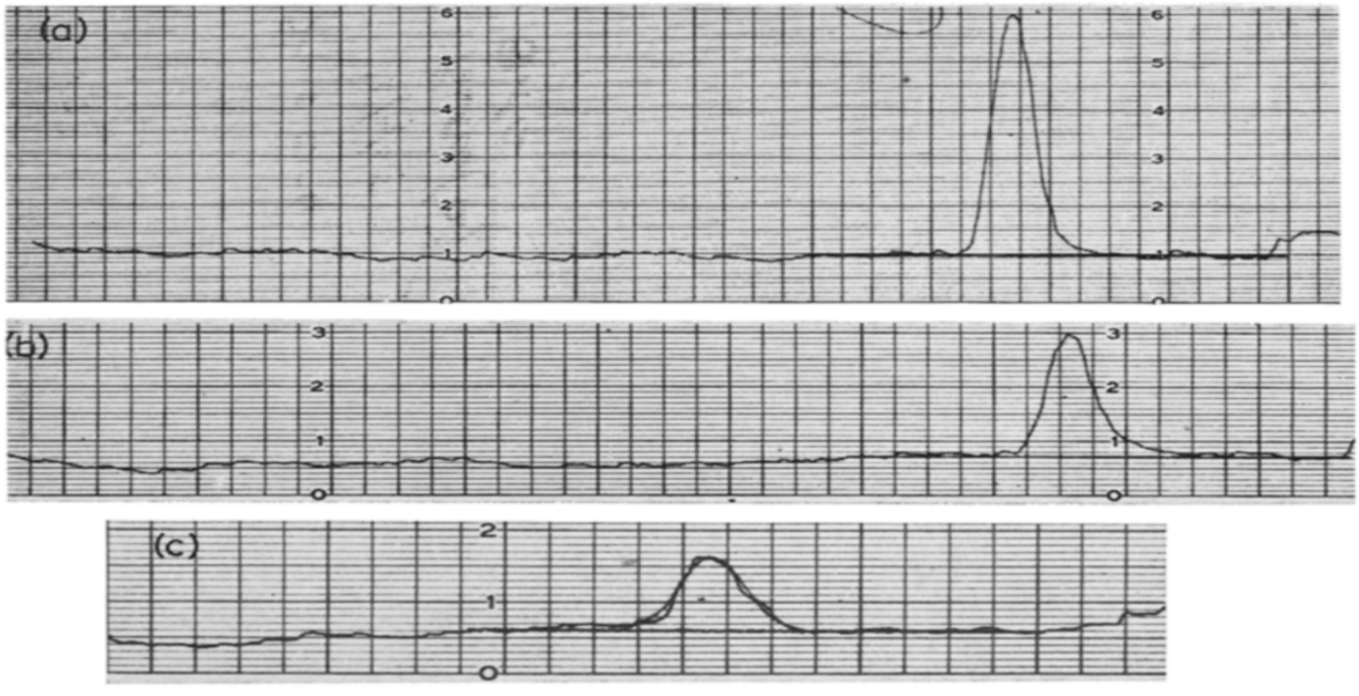

Fig. 7. Scanning records. (a, b, c) 25, 10 and 5 μg of 11-deoxycorticosterone with the alkaline BT method[6]. Low sensitivity, moderate damping, Bausch and Lomb VOM-6 recorder. Whatman No. 2 paper washed 36 h with 90% aq. methanol.

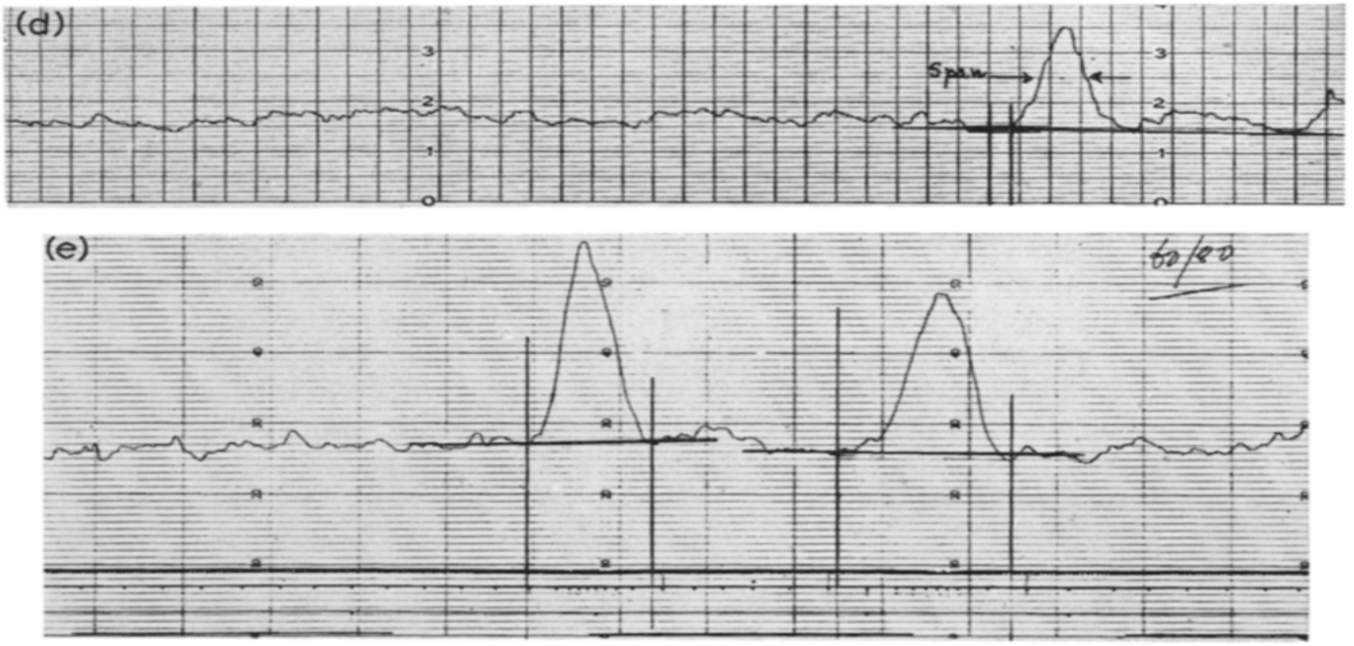

Fig. 7. Scanning records. (d) 5 μg deoxycorticosterone as in (c) but using minimal damping, moderate sensitivity, and unwashed Whatman No. 2 paper. (e) 10 μg each of androsterone (A) and dehydroepiandrosterone (DHA) with the Zimmermann reagent on washed Whatman No. 2 paper. Moderate sensitivity, minimal damping, A.E.I. Sunvic RSP-2 recorder. Chart record thickened by hand for photography. An earlier integrator was used in which the "thousands" pen is at the bottom and the "ones" at the top.

Adaptation of the instrument for fluorimetry

The instrument is not optically designed for fluorescence measurements but has been adapted successfully to the fluorimetry of N-DANSYL-amino acids [N-(1-dimethylaminonaphthalene-5-sulphonyl)-amino acids][11,12]. A wooden box with the same dimensions as the monochromator casing was constructed and fitted with a diagonal mirror in the position of M_1 of the monochromator (Fig. 1c). A mercury arc, filters, and a focussing quartz lens (as in GRAY AND HARTLEY[11]) were fitted and the scanner placed over the exit hole H_2 (Fig. 1c) and aligned in the same fashion as when used with the S.P. 900 monochromator. Secondary filters were mounted in S_4 or in the well below the PMT housing in place of the collimating lens, L_2, and field-stop, FS (Fig. 3). The PMT was now connected directly to the E.H.T. supply and amplifier unit of a Bowman-Aminco spectrophotofluorimeter and the oscilloscope output of the latter fed to the chart-recorder and integrator via a suitable potential divider.

While far from achieving optimal conditions, the apparatus was capable of detecting as little as 0.5×10^{-3} μmole of the majority of common amino acids, and the useful working range for their estimation was from $1.0\text{–}20.0 \times 10^{-3}$ μmole.

Routine operation for quantitative absorptiometry

Ideally, a batch of chromatograms is assembled which includes three or four on which suitable quantities of a standardizing substance have been run, and on which all the "unknowns" are present in quantities smaller than that of the largest standard. The chromatogram of the latter is fed into the scanner by hand after switching on the power supplies and selecting the appropriate wavelength on the monochromator dial.

The chromatogram is brought to a position at which a section of background is under the scanner's slits (S_3 and S_4, Fig. 3) and the pen of the recorder brought to a position between 2 % and 5 % of full-scale deflection by a combination of adjustment of slit width (S_1 and S_2, Fig. 1) and the backing-off potentiometer. The slits S_1 and S_2 are now closed to ensure that a full-scale deflection is obtained. If not, a wider initial slit width must be used and the backing-off voltage reduced. The chromatogram is then passed slowly through the scanner by manual rotation of one of the driving gears and the variation of the background is noted. If the pen of the recorder falls to zero at any point it is brought to just above zero (approx. 1 % of full-scale deflection) by reducing the backing-off voltage. When the zone of absorbing material passes under the scanner's slits a positive deflection of the pen is obtained. The sensitivity-setting and backing-off voltage are now adjusted if necessary until the maximum of the peak gives 90–95 % full-scale deflection, when the background has been restored to its original position. The rest of the chromatogram is now passed through the scanner by

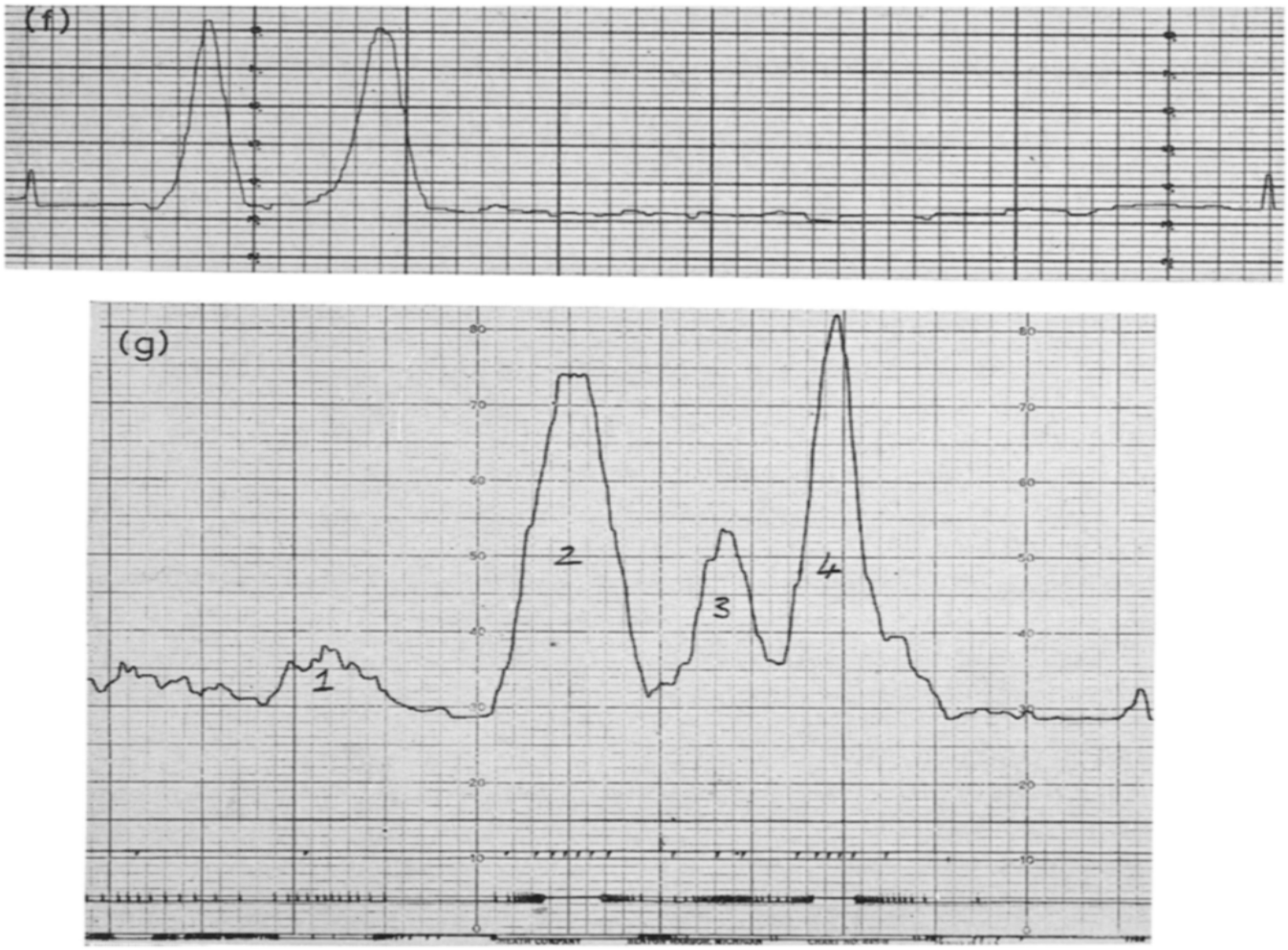

Fig. 7. Scanning records. (f) 25 μg of cortisol (f) and cortisone (E) by the alkaline BT method. Z_1, o, pencil lines at front and origin respectively. Esterline Angus Labgraph recorder, low sensitivity, minimal damping. Whatman SG81 paper, unwashed. (g) Typical record of the main reducing steroids of human urine using alkaline BT[6]. 1 = Cortisol; 2 = tetrahydrocortisone; 3 = *allo*tetrahydrocortisol; 4 = tetrahydrocortisol. Heathkit EUW-20A recorder with four-pen integrator (see text). Moderate sensitivity, minimal damping.

hand observing the background fluctuations, making further adjustments of the backing-off voltage if necessary to keep the background always above zero on the chart-recorder.

The chromatogram of the largest standard is now run through mechanically by engaging the magnetic clutch on the synchronous motor-drive of the scanner, and the chart recorder is switched on to obtain a record. A reasonably satisfactory standard chromatogram should give a record in which all "background" zones are within ± 3 % (f.s.d. units) of a mean which lies between 3 % and 5 % of full-scale deflection, and in which the largest peak has a maximum between 90 % and 98 % of full-scale deflection.

The chart recorder is now re-started and the chromatograms are fed by hand into the scanner in sequence. The chart is marked with a pencil to indicate the reference number of each chromatogram. With a long series of "unknowns" it is useful to provide a check on stability of the apparatus by re-scanning one or more of the standards once or twice during the middle or at the end of the series. The smoothest operation is secured by linking the "unknowns" strips head-to-tail with short lengths of adhesive tape and piling them in zig-zag fashion in a shallow tray at the level of the inlet to the scanner. The first strip is introduced by hand, following which the whole series is pulled through without further manual assistance. The beginning and end of each scanning record is conveniently indicated on the chart record by brief excursions of the pen to (or below) the zero line, caused by the sudden increases of light transmission in the gaps between successive strips; or by excursions to full-scale deflection if the strips are linked by opaque adhesive tape.

The problems that may arise during this procedure are of two sorts. The first are those due to instability or failure of any part of the electronic apparatus or light-source. It is useful to have spares of any particularly vulnerable components at hand for such emergencies. The second class of problems comprise those which arise from defects or intrinsically unavoidable features of the chromatograms. The commonest problem is the need to measure both large and small peaks on the same chromatograms. Sometimes one or two unknowns may contain unexpectedly large peaks which exceed the largest standard. Unless two recorders can be run in parallel at conveniently different sensitivities the only way to overcome the first problem is to carry out two series of scans at different sensitivities, including the standards so that two calibration curves can be constructed. Peaks overshooting full-scale deflection in the high-sensitivity run are neglected, and the smaller peaks are measured only from the record taken at high sensitivity.

An "unknown" peak which exceeds in height the peak given by the largest standard can be measured by extrapolation in the following manner. The strip containing the largest unknown is fed into the scanner by hand and the background level and sensitivity setting adjusted until the peak gives 90–95 % full-scale deflection. The scanner and chart-recorder are now run mechanically and all "excessive" unknowns are scanned at the new setting in company with the two or three largest standards. If the areas and extrapolated estimates of quantities obtained for the "unknowns" are within the range known from previous studies to give linear calibration curves with the absorptiometric method in question, these extrapolated values can be used with reasonable confidence.

In the case of methods involving a color reaction carried out on the paper, the limiting factors on the linearity of calibration curves will almost certainly be chemical

in origin and not optical or instrumental[2,7]. If, however, light-absorbing derivatives have been prepared by some standardized method before chromatography the limitations are confined to those of optical or instrumental origin. In such cases good or reasonable estimates of unexpected and excessive amounts of "unknowns" can be obtained by scanning at wavelengths away from the maxima usually used. Thus, in the absence of non-specific absorption, excessive amounts of keto-acid dinitrophenylhydrazones which are normally scanned at 390 mμ (BUSH AND HOCKADAY[13]) can be scanned in the visual range at approximately 480 mμ (at which the molecular extinction coefficient is much smaller) and estimated by an extrapolation of the calibration curve of the standards carried out at the same wavelength. It is more convenient when possible to use samples which contain quantities small enough to be in the normal working range of the instrument with the color reaction in question.

Processing the chart records

The basic details of this procedure have been discussed previously[7] so that the account here is confined to an example showing how the 4-channel digital read-out is used. Fig. 8 shows an untouched section of a chart-record containing a typical well-defined peak of large size (cortisone revealed by alkaline BT, approx. 50 μg) and below

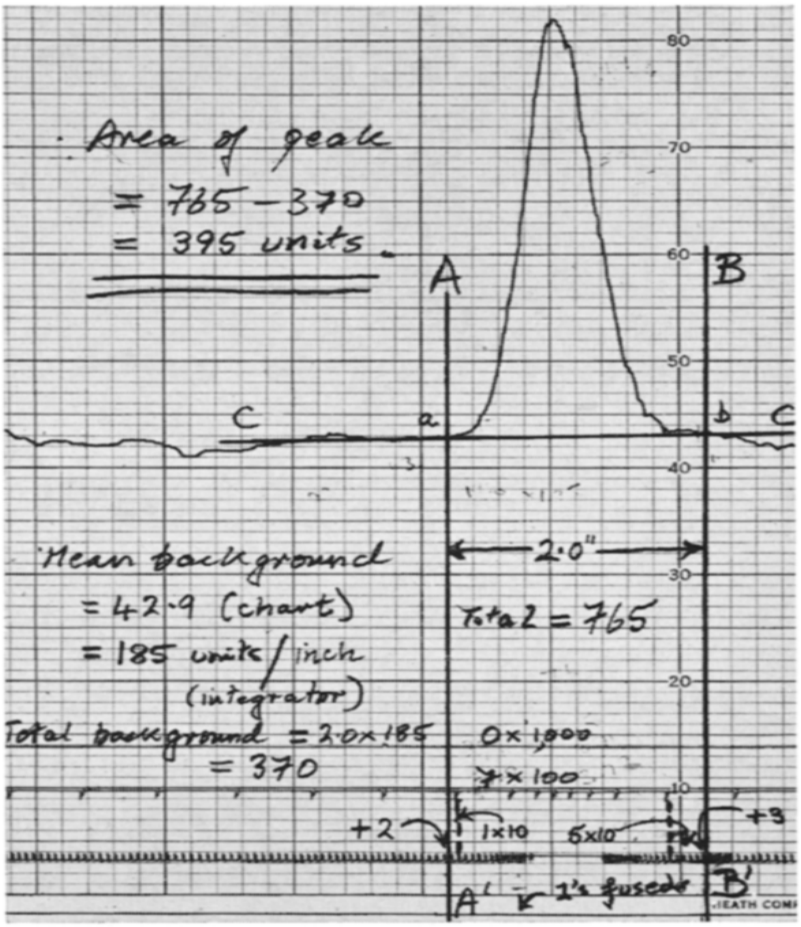

Fig. 8. Section of a scanning record of a paper chromatogram illustrating the method of calculating areas under a single peak from the 4-channel digital record. See text for full description.

it the cumulative integral record. The lines AA′ and BB′ were drawn at right-angles to the long axis of the chart so as to be close to the limits of the peak and yet to coincide with the nearest integral or simple decimal-fractional distance between them. The background level is taken as the line CC′. The first and last deflections of each pen between AA′ and BB′ were then lightly marked and the number of deflections counted and marked as shown by dotted lines so as to obtain rapidly the total number of "counts" between AA′ and BB′. The background was set higher than absolutely necessary in this run (the recorder is zeroed at 30 on the chart to make room for the 4-pen integrator unit), so that the "ones" pen was fused (rate > 25/sec). The units were estimated by eye from the "tens" pen using a magnifying glass ("+ 2" and "+ 3"). Artifacts, or difficulties of alignment between pens, are eliminated by checking sums across two adjacent pens or by counting back to areas where the record is absolutely clear.

The points *a* and *b* are now read from the chart as ordinates and their mean (42.9) taken as the level of the background under the peak. The firing rate of the integrator at this level (185 units/inch) was then read from the calibration curve of the integrator and multiplied by the length of chart between AA′ and BB′ to give the background "count". This was subtracted from the total count to give the digital value of the peak area.

Note that with isolated well-defined peaks and a good background the exact position of AA′ and BB′ is not crucial since any excess over the "true" width of the peak is compensated automatically by an increase in the total background count. Thus, to speed up computation it is best whenever possible to place AA′ and BB′ at a distance apart which gives a convenient value of the *length* by which the background firing rate must be multiplied to give the total background count. Using a slide-rule or a desk calculator such calculations can then be carried out very rapidly. Overlapping peaks are dealt with by an obvious extension of the method described earlier in detail[7].

If the background near the peak is excessively "noisy", the baseline for the peak is drawn according to an arbitrary rule as follows (see Fig. 7d). A length of background equivalent to approximately half the "span" of the peak (width at half the height of the maximum) is marked off ahead and behind the limits of the peak. A point midway along each of these segments of background is drawn at the mean of the highest and lowest values of background within each segment. The line joining these points is drawn and its intersections *a* and *b* with AA′ and BB′ are taken as the ordinates whose mean gives the best estimate of the baseline for the peak itself.

The digital values for the areas of the standards are used to construct a three or four-point calibration curve from which the quantities of unknowns are read once their areas have been calculated.

DISCUSSION

Principal features of design and construction

The present apparatus suffers from a number of limitations, but conforms better than most or all other available instruments of this type to the general principles required for an efficient absorptiometric scanner[2,5,7,14]. The most important features are as follows.

First it is essential to ensure that the incident light beam be monochromatic and

as nearly as possible in the form of parallel or only slightly convergent rays (see *e.g.*, BROWN AND MARSH[14] and EICHORN[15]). This is partly achieved in the present instrument by the combination of the lens L_1 and the slit S_3 (Fig. 3). Even more essential, the transmitted light must be well collimated and any non-perpendicular (off-axis), scattered light rejected. This is achieved adequately in planes parallel to the axis of the paper strip by the slit S_4, but the lens L_2 and field-stop FS (Fig. 3) are needed to eliminate non-perpendicular scattered light in the plane of the slit S_4. The positions of the stop and photomultiplier are designed to provide a diffuse patch of unfocussed light on the photocathode. This minimizes desensitization of the photocathode with age and avoids sensitivity to positional or directional variations in the photocathode's response to illumination.

Since the complete elimination of non-perpendicular scattered light is difficult or impossible to achieve, the position of the paper relative to the slits S_3 and S_4 (Fig. 3) must be maintained very precisely. This is achieved by the guiderails, G,G, of the scanner (Figs. 3, 4) which provide a groove 0.8 mm wide and 2.4 mm deep in which the edges of the paper strip slide. These grooves and the slits S_3 and S_4 also ensure that no light passes outside the edges of the strip.

While this arrangement is questionably not quite as satisfactory as to mount the strips in an accurately machined frame or drum (*e.g.*, LAURENCE[10]; Photovolt Corporation, Densicord 542), it appears to provide an adequately precise location of the paper strip during the scanning process, and has the very considerable advantage that unmounted strips can be passed directly and rapidly into the scanner either singly or as trains of strips linked together with adhesive tape.

Another important feature is the relatively high velocity of the strips in the scanner. The velocity of 1 cm/sec was adopted in order to match the first part of the combined apparatus described previously[1]. While the response-time of the SWEET circuit gives a large safety factor when using such velocities[8] with chromatograms of conventional size (30–60 cm long) the same is not true of some potentiometric recorders and integrators of the type described in this paper. There are however a number of suitable recorders now available which have f.s.d. times of 1 sec or less and several of these have proved entirely adequate to the task, with the exception of very sharp zones such as are found near the origins of typical paper chromatograms. It is probably best to use recorders rated at 0.5 sec f.s.d. or less, and preliminary experience with an Esterline Angus Series S Lab Graph recorder (f.s.d. $\leq$ 0.2 sec) has confirmed this view.

It would be entirely feasible at the present time to scan at even greater velocities. However, this is unlikely to be worthwhile, since the mechanical difficulties of moving the fairly fragile strips of paper at these speeds are likely to be considerable, and the speed of the present apparatus is already sufficient to produce results at a rate which can only be dealt with satisfactorily by a digital computer (see below).

The main defects of the present apparatus are as follows:

(1) The glass prism monochromator is inadequate for work in the ultraviolet below about 360 mμ as is the photomultiplier used at present (RCA-931A or IP-21).

(2) The optical pathway is poorly adapted to fluorimetry despite the very satisfactory results obtained with DANSYL-amino acids[12].

(3) The optical pathway requires a wastefully powerful light source for several reasons. First, the avoidance of trouble from the instability of xenon arcs is secured

by using a large, poorly focussed image on the entrance slit of the monochromator. Second, the elimination of incident and scattered light in directions non-perpendicular to the paper is achieved mainly by collimating slits, and the dimensions of the monochromator do not lend themselves to an inexpensive condensing system.

(4) The grooved paper guides produce an undesirable amount of friction to the entry of paper strips along the first curved part of the guides. The finely grooved section of the guides should be confined to the central straight portion of the paper's track immediately below the photomultiplier, and should preferably be highly polished.

(5) The lack of a cross-scanning device (WIEME[17]) means that good quantitative estimations can only be obtained with chromatograms prepared and run so that the resulting absorbing zones are regular bands at right-angles to the axis of the strip, and fill the whole width of the strip[2,7]. Techniques to secure such zones are available but require slightly more skill and time than those which will suffice for good zones in the form of "spots"[2].

A large number of scanners for paper and thin-layer chromatograms, and for electrophoretograms have been described (*e.g.*, refs. 19–23). The earlier generation of such instruments (up to around 1954) have been discussed previously[2,4,18]. One of the most satisfactory of these, and the only one in which a careful appraisal of the optical pathway appears to have been made, was that of BROWN AND MARSH[14]. This was designed to fit the Beckman Model DU spectrophotometer, the output of which was fed to a suitable potentiometric chart recorder. Both absorptiometric and fluorometric measurements could be made by using appropriate filters. These authors were the first to point out that the irregular background absorption of filter paper did not seriously affect the accuracy with which quantitative estimation could be carried out by this means. In another pioneering study REES, FILDES AND LAURENCE[16] defined carefully the requirements for accurate quantitative scanning of paper electrophoretograms of proteins stained with dyes such as bromphenol blue. WIEME[17] made a major contribution by devising a scanner which also scanned rapidly across the paper strip. The majority of other scanning instruments have been designed as attachments to commercially available spectrophotometers or fluorometers (*e.g.*, refs. 14, 22–24).

The latter and all the available commercially supplied scanners suffer from more or less serious defects. The three principal defects to be found in these instruments are:

(1) The optical pathway does not meet the requirements for efficient operation either as absorptiometers or fluorometers[2,7].

(2) The paper or thin-layer chromatogram must be mounted on a drum or linear frame for transport across the scanning beam of light.

(3) The movement is unnecessarily slow.

The last two defects make the procedure unnecessarily lengthy and tedious. The first makes the method almost totally dependent upon absolutely reproducible chromatography (*i.e.*, shapes of zones) for any accuracy of quantitative measurement, and it is not usually possible to obtain linear calibration curves.

The present instrument has been designed so as to avoid these defects. Unmounted strips are fed into the input orifice of the apparatus and scanned at 1 cm/sec. A continuous train of chromatograms linked by adhesive tape can be fed into the machine so that its operation is fully automatic for an hour or more, during which time up to 72 strips of 50 cm length can be processed per hour. The most important

point to be observed, however, is that the desired optical pathway is not difficult to achieve, but is *absolutely essential* if reliable performance is to be achieved.

The only commercially available paper chromatogram scanner which embodies many of the principles of the instrument described in this paper is the recently announced fluorimetric scanner of the Shandon Scientific Company attributed to BOULTON, CHARD AND GRANT[25,26]. As far as can be judged from the brief publications which are available, the Shandon instrument is a more or less direct copy of the fluorimetric adaptation described above (p. 171), apart from the addition of a digital data-output, a heating unit on the paper inlet to intensify the fluorescence of certain substances, and an altered packaging of the components[25,27]. It is not clear whether the Shandon scanner can be used for absorptiometric measurements. Until full details of this instrument are published, it is impossible to compare it with the instrument described here. However, all the specifications and details of performance that have been released so far[25–27] are, apart from the use of filters in place of a monochromator, *identical* with those of the instrument described in this paper. One must conclude that it probably resembles the latter in being of low efficiency for fluorimetric scanning because the main optical pathway was designed for *absorptiometric* purposes, a fact apparently not sufficiently understood by BOULTON *et al.*[25].

The optical characteristics of filter paper

The general subject of scattering in heterogeneous materials has been well reviewed by SHIBATA[28], and the consequences for the scanning method have been discussed previously[2]. One point of interest has emerged since the earlier article was written. Much previous work had shown that the apparent optical density of absorbing zones in heterogeneous media was much greater than in a homogeneous medium[28]. It is similarly a general rule that the apparent optical densities of colored zones on dry paper chromatograms and electropherograms are over twice those obtained when the paper is wetted with water or "cleared" with an oil of suitable refractive index[2,19]. This suggested that the high optical density of dry filter paper itself was largely due to internal scattering and absorption. The measurements with Whatman papers Nos. 2 and 3MM however (see above) suggest that reflection, or scattering in a relatively thin surface layer towards the incident light may be the main source of the high apparent optical density of filter paper. Thus, the optical density (or more strictly "attenuance"[28]) of Whatman No. 2 (0.160 mm thick) at 584 mμ was found to be 2.5: that of Whatman No. 3MM which is 0.318 mm thick was 2.79, a surprisingly small increase. This and the actual absolute values which have been obtained may well lead to a reconsideration of the relative advantages of using dried or "cleared" strips for scanning[2,16]. Thus, the two main advantages of using dried strip rather than "cleared" strips are an approximately doubled optical density of absorbing zones, and the omission of a time-consuming process which is both somewhat troublesome and a potential source of error. Since, however, the limitations on the sensitivity of the scanning method appear to be almost entirely a question of signal-to-noise ratio one could well afford a loss of optical density if a more than comparable reduction in "noise" were gained thereby.

Some operational characteristics of the method

The acceptability of the analytical accuracy of the method of direct scanning

of paper chromatograms has been argued previously in detail[2,7]. There seems little doubt that the present instrument performs with an overall instrumental and computational standard error of approximately ± 1 % as expected from the ratings of the electronic equipment that is employed. On the basis of past experience one can expect to be able to adapt any well-established absorptiometric method for quantitative scanning of paper chromatograms, as long as it does not employ reagents and conditions which destroy or caramelize filter paper. With the equipment described in this and a previous paper[1], 95 % confidence limits of from ± 2 % to ± 4 % should be achieved without too much difficulty. A considerable number of methods which employ aqueous reagents or other easily controlled conditions should be adaptable for the *manual* treatment of the chromatograms with reagent followed by the use of the scanner. This is the case for instance with the alkaline blue tetrazolium method for reducing steroids[6] and of BAROLLIER's ninhydrin–cadmium reagent for amino acids[2].

Although acceptable analytical performance compared with other chromatographic methods is an essential feature of direct scanning of paper chromatograms, and could not have been expected on the basis of published results up to 1954, the main advantage of the method is its speed and potential productivity. Although conventionally sized paper chromatograms are slow to run compared with gas–liquid chromatograms, large numbers can be run at the same time in cheap and relatively compact apparatus, and the running time involves no personnel working time[2,3]. The overall productivity of the scanning method is thus very much greater than any other existing method of quantitative estimation by chromatography for comparable capital and running costs. The present machine produces a chart record of a 50 cm chromatogram (equivalent to approx. 2000 theoretical plates for a typically overrun strip[29]) in 50 seconds, *i.e.*, at rates of 504 chromatograms in seven hours' running.

In fact, the present machine has never been used to process more than about 200 chromatograms in any one day even though it has serviced simultaneously up to four separate research projects most of its working life. The preparation of extracts suitable for chromatography, and the processing of the chart records are the rate-limiting steps in analytical procedures of this type. The present machine has processed approximately 25,000 chromatograms in its working life despite a considerable amount of "down time" spent on modification, development work, and transport between laboratories. In the last 12 months one research group carrying out a large scale survey of ten individual urinary steroids by a modification of the general fractionation scheme of BUSH AND WILLOUGHBY[6] has been able to process over 1500 urines (*i.e.*, approx. 5000 chromatograms) using this apparatus, at the same time as it was being used for two other major research projects.

Chromatograms of the usual size may contain ten or more separated substances that are detected by any one colorimetric method, but it is unusual to obtain well-separated zones with more than eight components on each strip. With many research problems one adopts a system giving five or fewer zones to be measured per strip. Direct measurements in our laboratory showed that a skilled worker takes approximately 1.8 min per zone for the computation of peak areas by triangulation (MOORE, STEIN AND SPACKMAN[30]) and 0.8 min for computation from the 4-channel integral record described here. For a batch of chromatograms 50 cm long containing an average of five zones per strip, therefore, 50 sec work scanning the strip produces 4.0 min computational work, plus approximately 10 min per batch of chromatograms for

construction of a calibration curve, checking dubious results, etc. Reading quantities from the calibration curve and tabulation of the results take an average 0.4 min per peak. Thus, a batch of 50 chromatograms would require approximately 42 min scanning time plus approximately 10 min for warming up and initial adjustments, or a total of 52 min work with the instrument. This would then need approximately 5 h of computation and tabulation. These figures agree well with the usual routine experience of our laboratory with this method.

It will be obvious that the problems of processing chart records of this type are entirely similar in principle to those of processing the record of gas–liquid or other types of column-effluent-monitored chromatography. Specialized digital computers such as the Infotronics peak integrator (Infotronics Corp., Houston, Texas, U.S.A.) have been used successfully for peak area computation in ion-exchange chromatography of amino acids and in gas–liquid chromatography[31]. The background "noise", however, is much greater from paper scanning than from liquid or gaseous effluent monitoring methods. This, and the rapid speed of the scanner described here, made it impossible to achieve reliably reproducible results with the Infotronics instrument during a recent trial. It seems likely that the best method of overcoming the bottleneck imposed by the computational part of the procedure is to convert the photometric signal to a convenient digital form on paper or magnetic tape and process this off-line with a general purpose digital computer. A variety of computer programs have been reported recently which greatly facilitate this part of the work[32–36]. Most of them have been used for the calculation of amino acid analyses using the automatic ion-exchange method of SPACKMAN, MOORE AND STEIN[30]. The calculation of quantities from peak areas on chromatogram records of whatever sort is complex unless attention is confined to "ideal" chromatograms with zero background drift and noise, and perfectly separated peaks[7,31]. There is little doubt, however, that the use of fast digital computers will enable the full productivity of chromatogram-scanners to be achieved[3]. A detailed consideration of this topic will be given in a later paper of this series.

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SUMMARY

Electronic apparatus for photometric scanners of paper chromatograms is described, together with its testing and use. The apparatus includes a logarithmic densitometer based on SWEET's circuit, a stabilized supply for a tungsten light source, a voltage-to-frequency converter for providing integral records, a four-pen event marker with decade-counters and pen-drivers to give a convenient 4-digit integral record alongside the direct chart record of the scan, and a stabilized voltage source for a retransmitting slidewire capable of providing a 0 to —10 V output from the scanner (*via* the recorder) suitable for driving the voltage-to-frequency converter or other types of analogue-to-digital conversion apparatus.

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