

THE QUANTITATIVE ESTIMATION OF SUBSTANCES ON PAPER CHROMATOGRAMS

I. A MACHINE FOR THE TREATMENT OF PAPER STRIPS WITH CHEMICAL REAGENTS

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INTRODUCTION

Almost as soon as partition chromatography on paper had been introduced by CONSDEN, GORDON AND MARTIN¹ attempts were made to determine substances on chromatograms by the direct measurement of the color density of the zones that could be produced with suitable color reagents. These early attempts have been reviewed in detail by BLOCK, DURRUM AND ZWEIG². In summary, it would be fair to say that reasonable estimations (approx. errors $\pm 10\%$) could be achieved by rigorous attention to details of technique but that by 1958 this method of approach had not been adopted to any great extent for practical problems in biochemical estimation (see also CHIBNALL³). Instead, quantitative estimation in conjunction with chromatography was carried out by the application of more or less conventional colorimetric methods to the effluents from columns, or to the eluates from segments of paper chromatograms cut out by hand. Continuous mixing of the effluent of a column with reagent, and monitoring the optical density of the mixture with a photometer and potentiometric chart recorder was developed for amino acids by SPACKMAN, MOORE AND STEIN⁴ and has become widely used as a result of the commercial manufacture of their apparatus.

The author re-examined the question of the direct estimation ("scanning") of colored zones on paper chromatograms in 1953 and devised an apparatus for mechanizing the procedure in 1955. It was found that certain general rules seemed to hold for the successful quantitative scanning of paper chromatograms whatever class of substances were being measured. Knowing these rules, it was relatively easy to establish quantitative methods of low error, and to devise reproducible techniques without a prolonged empirical search for the right conditions with each new colorimetric method. The course of this work has been described in two review articles (BUSH^{5,6}) and a book (BUSH⁷, Chap. 4) but many details remain unpublished or are scattered through papers published since 1957 (*e.g.* BUSH AND WILLOUGHBY⁸). In view of numerous private enquiries about the apparatus that has been devised it is hoped to collect this unpublished material in the series of papers of which this is the first. Schematic descriptions of the original apparatus and a discussion of the rationale

of the technique in general have been given previously and will only be discussed briefly in this series of papers (BUSH^{6,7}).

The first paper deals with the design and construction of a machine capable of treating paper chromatograms reproducibly with chemical reagents.

DESIGN AND CONSTRUCTION OF THE MACHINE

Early attempts and difficulties

The first machine was made in late 1955 and was similar in layout to the fourth model described in this paper. Crude as it was, this prototype sufficed to demonstrate the considerable reproducibility with which the reagent roller unit (BUSH⁷, Chap. 4) was capable of applying a film of liquid reagent to successive paper strips, and the efficiency with which color reactions could be carried out by using a zone in which hot air was forced at high speed over the paper.

The second model (Fig. 1) was designed to provide a much longer path in the heat-treatment zone and employed a zig-zag path of the belts to secure reasonable compactness. Many successful runs were made in 1959-1960 but the tension required

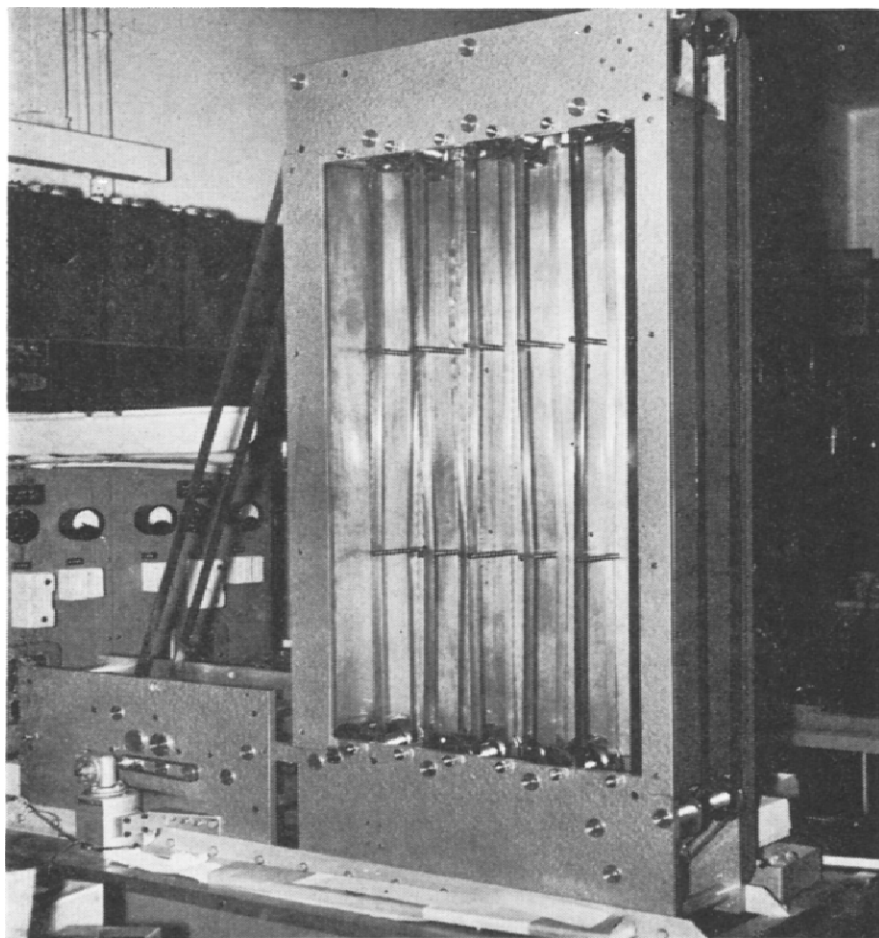


Fig. 1: The second model of the machine. The reagent applicator is at the left and the reaction-drying chamber at the right. The front panels of the latter are removed to show the zig-zag path of the belts. Heating units and blowers were attached to the front panels which also carried partitions dividing the chamber into six partly separated compartments.

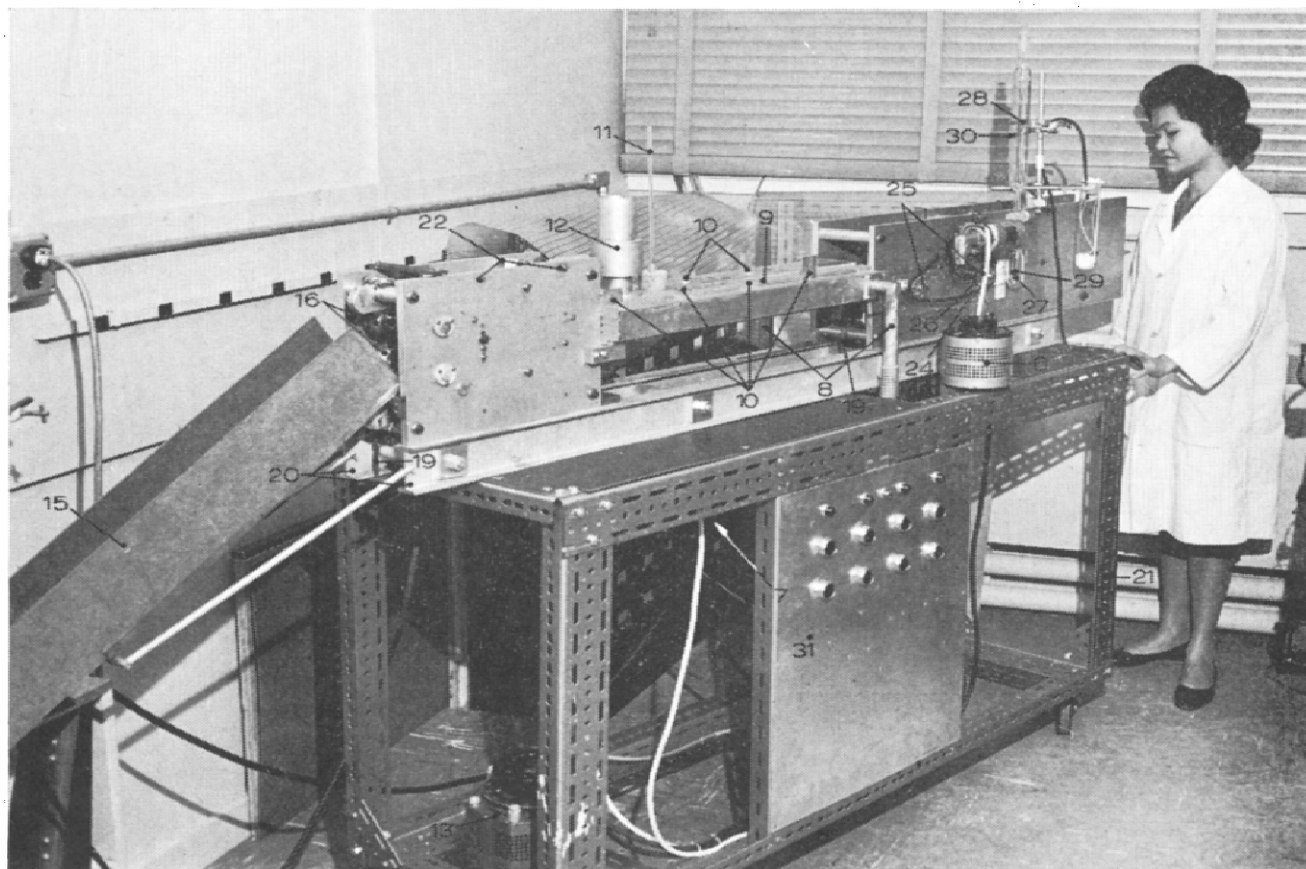


Fig. 2a. The fourth model described in the paper. The reference numbers are used throughout the paper. 6 = Variac controlling reagent-roller motor; 7 = heater-blower, beneath and behind top of carriage; 8 = hot-air conduits to reaction chamber; 9 = reaction chamber; 10 = slide valves to entry ports of reaction-chamber; 11 = thermometer; 12 = exhaust tube; 13 = variable transformer for heater; 15 = collecting tray for ejected strips; 16 = main driving pulleys; 19 = cross-bars of main supporting rails; 20 = main supporting rails; 21 = dexion frame on casters; 22 = tensioning auxiliary pulleys for belts; 24 = driving chain for reagent-rollers; 25 = sprockets on shafts to the two reagent-rollers; 26 = PTFE reagent feed tube to reagent-roller; 27 = constant-level chamber of reagent-feed; 28 = upper chamber of reagent-feed; 29 = lower chamber of reagent-feed; 30 = clamps supporting reagent feed; 31 = control panel.

to keep the belts running true on the one-flanged pulleys (approx. 15 kg) led to fairly rapid distortion of the stainless-steel belts by cold-flow, and their frequent replacement was uneconomical and time-consuming.

The third model reverted to a simple straight-through reaction chamber. An inadequately rigid base failed to maintain proper lateral stability of the belts. The fourth model employed the same overall design, corrected the remaining errors of the third, and has given excellent and reliable service since it was completed nearly two years ago. The belts have been replaced twice, and the machine has treated several thousand chromatograms.

Description of the fourth model

The machine is shown in Figs. 2 a, b and c and a schematic elevation in Fig. 3. It consists of three main sections. The first is the reagent-applicator unit (extreme right of Fig. 2). An aluminum guide-rail (1, Figs. 3 and 7 a) receives the strips of paper (5.1 cm

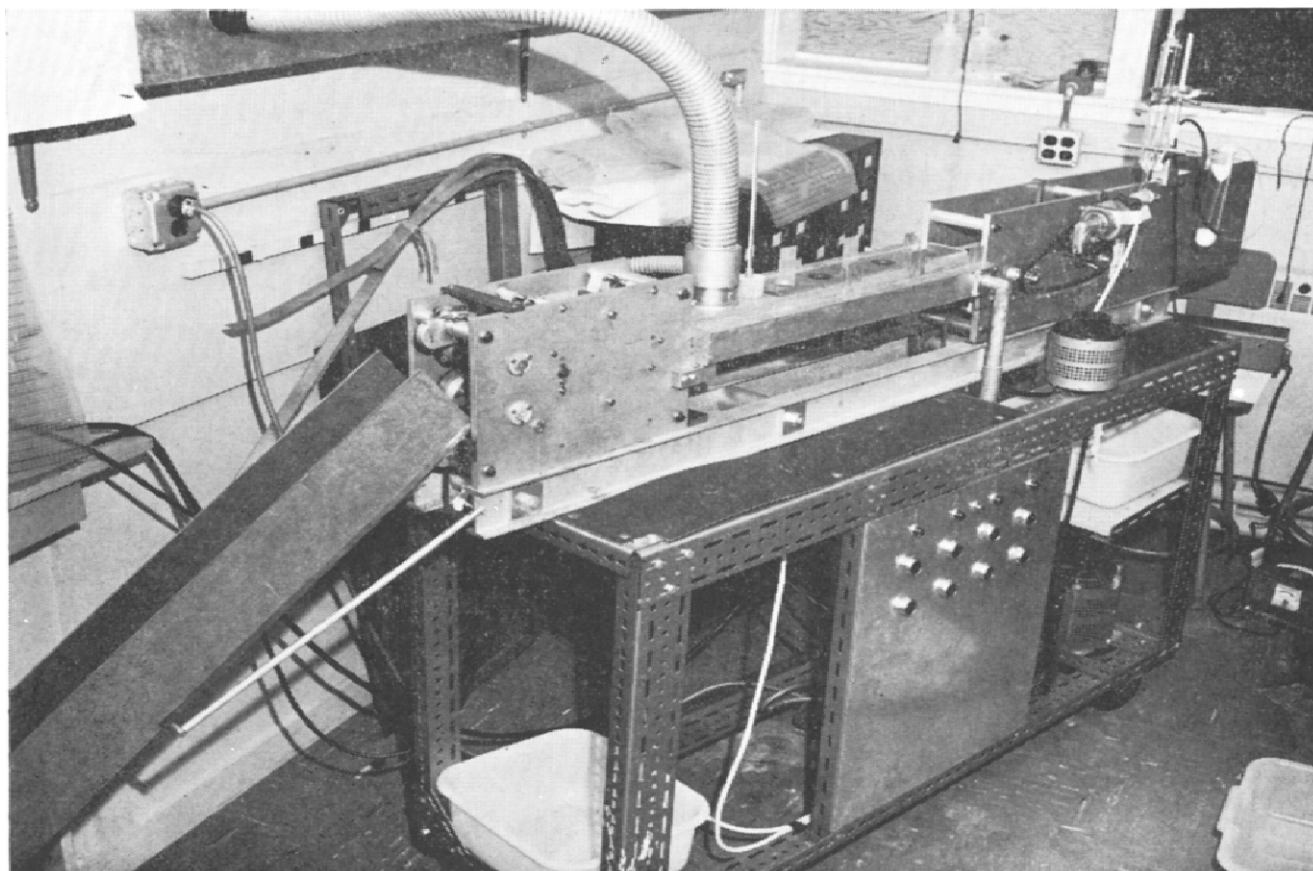


Fig. 2b. The fourth model equipped with an auxiliary exhaust system. The flexible metal hose is connected to an exhaust duct discharging through a window-board at the right. To the left, an opening to the room (not shown) is controlled as a bypass with a butterfly valve.

wide) which are pushed along until the edges of the leading part of the strip are gripped by the upper and lower belts on each side at their meeting point (2, Fig. 3). The paper is then gently flattened, if convex upwards, by the idling roller (3, Fig. 2c) which is made of polytetrafluoroethylene (PTFE). The paper then passes over the stainless-steel reagent-roller (4, Figs. 3 and 7a) in light contact with it and takes up the reagent from the film on the roller by capillary action. There is provision for a second reagent-roller (5, Fig. 3) but it has not been necessary to use it up to the present.

The speed of movement of the paper is fixed at 1 cm/sec which is determined by the motor speed, gearing, and diameter of the driving pulleys in the take-off unit described later. This speed is between 1.5 and 2.0 times the minimum speed required to ensure that the longitudinal capillary movement of typical liquid reagents in the paper is slower than the paper movement itself. This is necessary to ensure that the reagent does not move or distort zones of material on the chromatograms.

The speed of the reagent-roller is adjusted with a variable transformer (Variac, Duratrak, Type V5HM, Zenith Electric Co., Birmingham, England) and is usually in the range 40-120 r.p.m. with reagents of ordinary viscosity (6, Fig. 2a).

The paper, soaked with reagent, is now carried through the reaction-chamber or middle zone of the apparatus. This is a steel and aluminum box of rectangular cross-section and 65.5 cm long. The side-walls are double, the two outer compartments

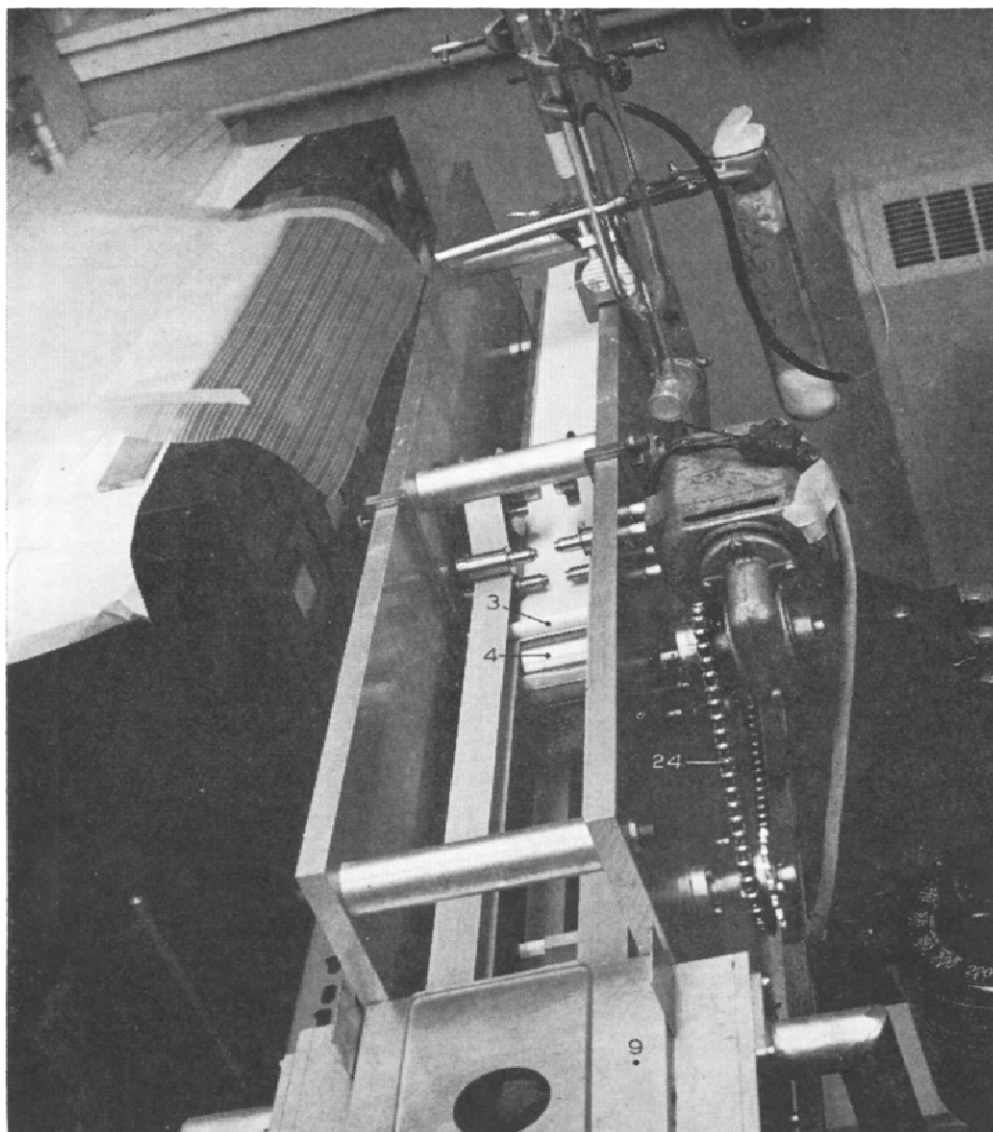


Fig. 2c. A view showing the reagent applicator unit with a chromatogram just about to make contact with the reagent roller; 3 = idling roller. Other reference numbers as in Figs. 2a and 3.

(cross-section, inside dimensions 2.54×3.81 cm) acting as conduits for hot-air supplied by the heater-blower (7, Figs. 2a and 3) via the Y-tube and connectors (8, Figs. 2a and 3). These conduits (9, Figs. 2a and 3) communicate with the reaction-chamber itself (inside cross-section, 11.5×5.0 cm) by entry ports 3.2 cm in diameter which can be opened or closed by simple slide valves (10, Figs. 2a and 3). The lid of the reaction chamber is perforated with inspection holes which are covered with a sheet of plate glass. Similar holes are used to hold a mercury-bulb thermometer (11, Fig. 2a) and the exhaust-tube (12, Figs. 2a and 3). The latter is a simple venturi tube driven by a centrifugal fan mounted behind the machine. In practice it has been found necessary to supplement this fan by connecting the exhaust-tube with flexible metal hose to a conventional exhaust fan discharging to the outside air (not shown in Fig. 2). The reaction chamber contains a strip heater (1.5 kW) in its floor for ballast heat but this has not been used frequently. A variety of patterns of heating and drying can be achieved by varying the setting of the variable transformer controlling the heater-

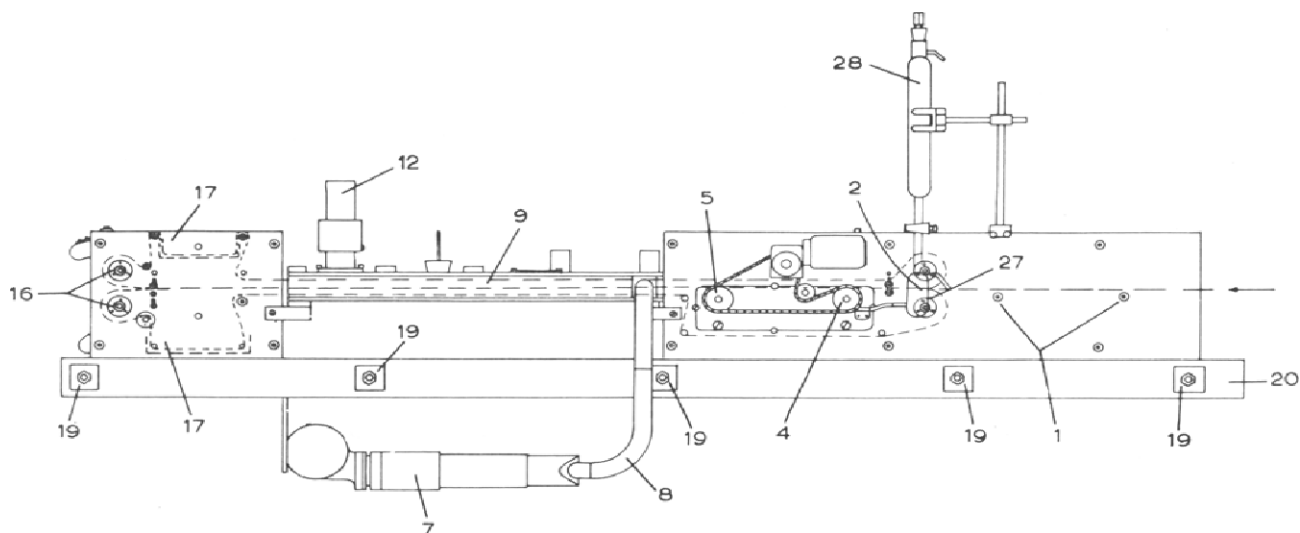


Fig. 3. Side-elevation of the fourth model excluding the supporting carriage. 1 = Supports for entry guide-rails; 2 = meeting point of upper and lower belts at which paper strips are gripped; 4 = sprocket and axle of first reagent-roller; 5 = sprocket and axle of second reagent-roller; 17 = washing baths for upper and lower pairs of belts. Other reference numbers as in Fig. 2a.

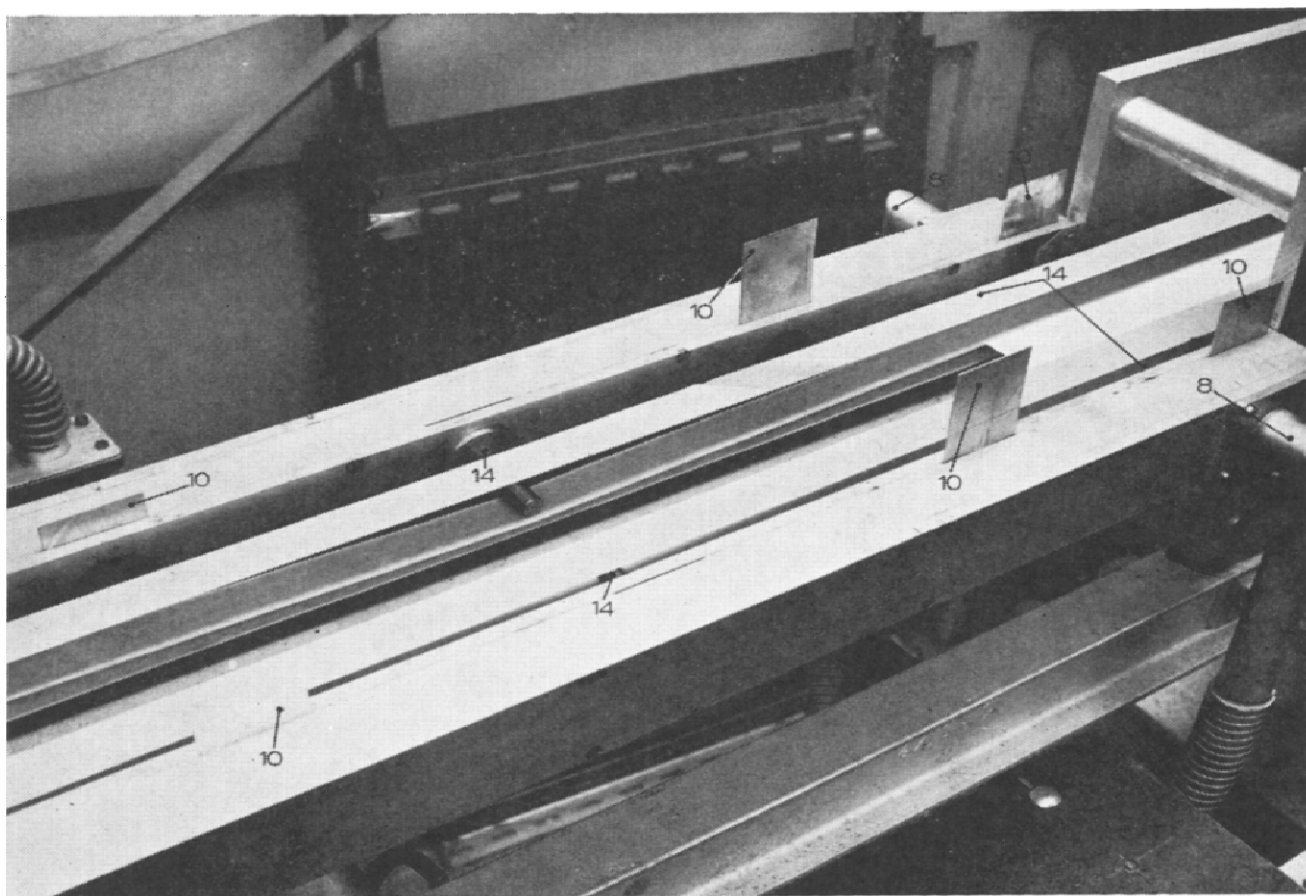


Fig. 4. The reaction-chamber with lid removed to show arrangement of the belting system. A strip of paper is entering from the right. The central guideposts (14) are visible; the first pair are not visible but their position is indicated by the reference numbers. (Other reference numbers as in Fig. 2a.) The upper and lower belts are seen on their return passage through the reaction chamber.

blower (13, Fig. 2a) and by using different settings of the 4 entry-ports (10, Figs. 2a and 3).

To ensure that the belts are pressed together and grip the edges of the paper strips in at least two positions on each strip (approx. 45 cm long) at all times, three guide posts (14, Fig. 4) are fixed to the sides of the reaction chamber. These are ridged to assist the maintenance of correct lateral alignment of the belts through the chamber, and are placed so as to produce a slight up and down deviation of the belts from a straight horizontal path. The third, middle, pair of guide-posts was only added to the machine recently. Although good performance was achieved with a straight, horizontal, path of the belts through the machine, strips were occasionally dislodged and lost in the reaction chamber due to an inadequate gripping action by the PTFE-coated fiber-glass belts which have an extremely low coefficient of friction. To minimize this problem the belts had to be run at rather high tension, thus increasing wear and reducing their useful life. Foolproof performance, with lower tension on the belts, has been achieved by the addition of the middle pair of guide-posts which deflect the belts from a straight path. The distances between the three pairs of posts are 27 cm and 28 cm.

The paper is passed out of the reaction chamber and ejected into the collecting tray (15, Fig. 2a) by the third section of the machine called the take-off unit. The two belts on each side of the paper disengage on passing through the main driving pulleys (16, Figs. 3 and 5) and are taken by means of small doubly-flanged auxiliary pulleys through two washing-baths (17, Figs. 3 and 6b) one for the upper, the second for the lower, belts. A stream of hot tap water is fed to these baths by tubes and sucked off

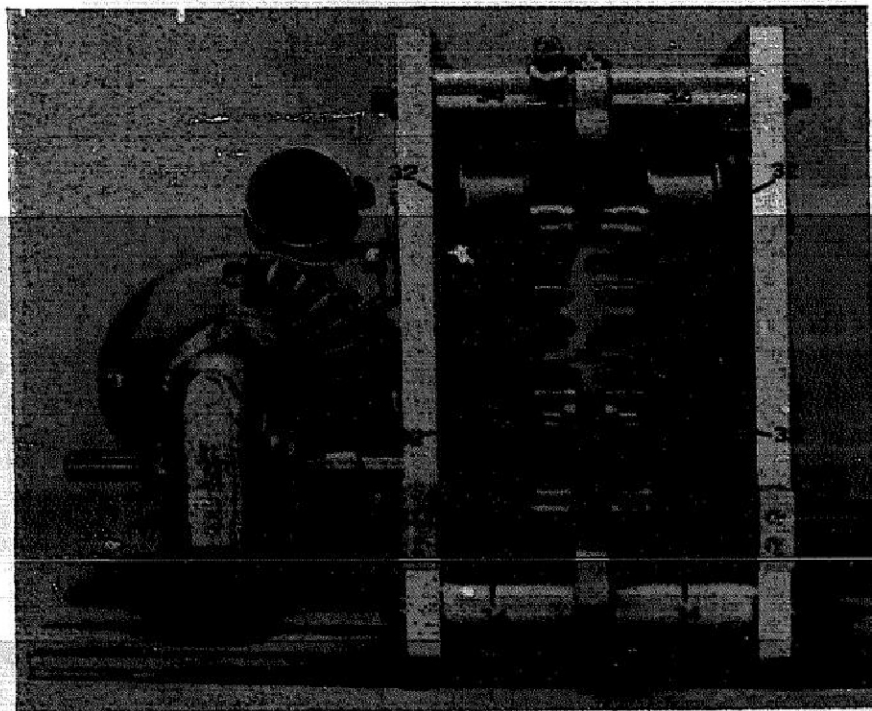


Fig. 5. End-view of main drive-pulleys of the take-off unit. Key as in Fig. 2a. The belts are removed for clarity. 32 = Spur gears on main drive-pulleys; 33 = common spur driving 32; 34 = rubber pressure rollers.

by a water-pump via drain tubes which maintain a constant level in the baths. Any drops of water are wiped from the belts as they emerge from the baths by small plastic-sponge wipers mounted on metal brackets (18, Fig. 6b). The belts then return to the reagent-applicator unit *through* the reaction-chamber so that they are completely dried before re-engaging and taking in fresh strips of paper from the loading guide-rail.

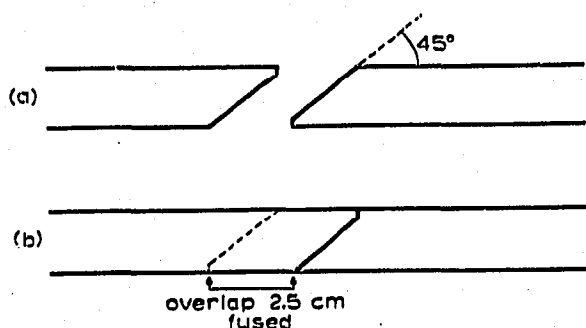


Fig. 6a. Diagram of method of joining the ends of the belts. The fusion was carried out by the manufacturer.

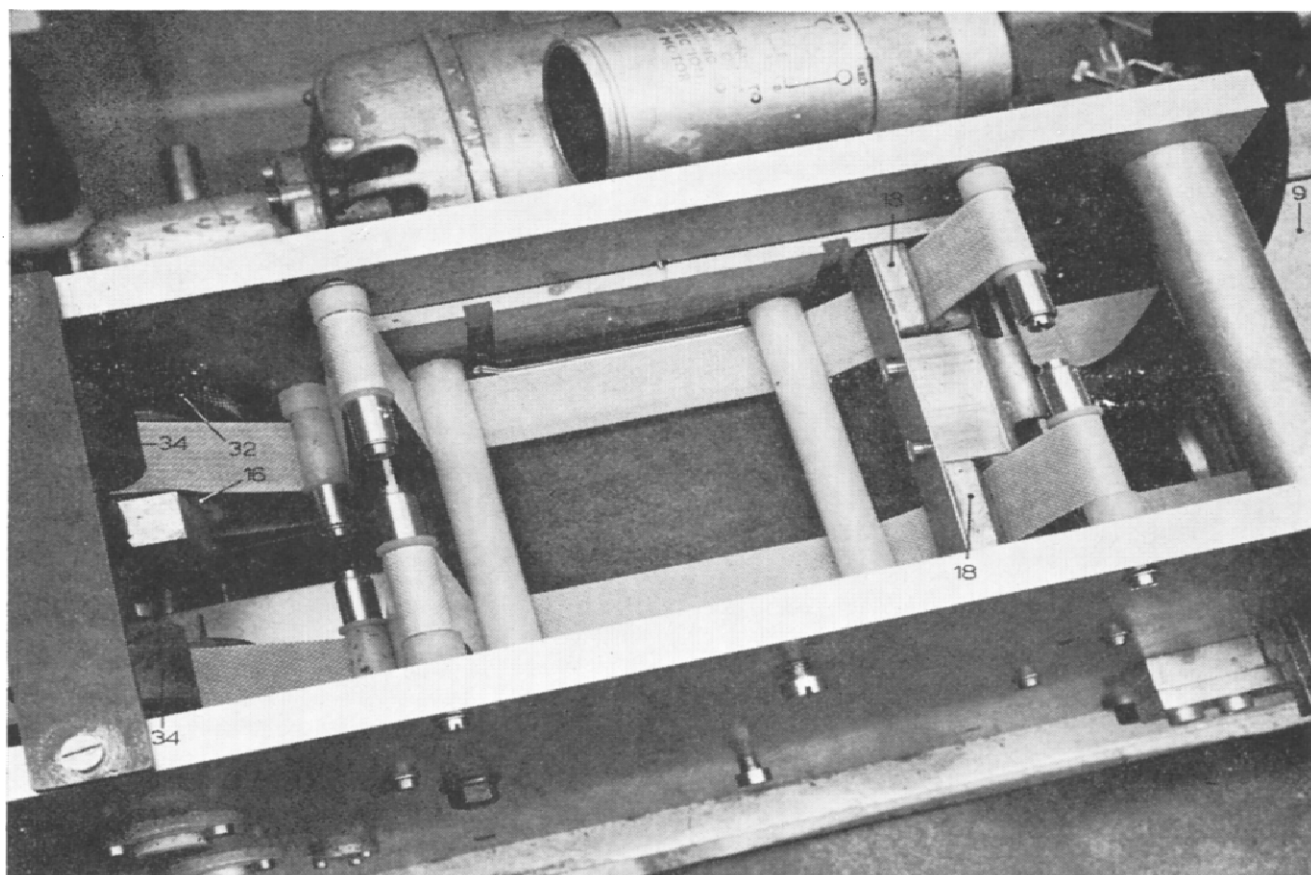


Fig. 6b. The washing-trough for the upper pair of belts. The lower washing trough is similar. The PTFE rollers in the trough are inserted by the vertical sections of the two grooves seen on the back wall of the trough, and similar grooves (not seen) on the front wall. The belts return to the right via the reaction-chamber (see Fig. 4). 18 = Plastic sponge wipers. Other numbers as in Figs. 2a and 5.

The heater-blower is a standard commercial unit rated at 2.0 kW on the heater, and moving approximately 325 l/min. The centrifugal fan is run at constant speed. The supply voltage to the heater is controlled by a variable transformer (Variac Type 100 RM, Claude Lyons & Co., Liverpool, England).

The reagent-applicator and take-off units are made on the watch-plate principle, the main side plates being of hard aluminum alloy 1.25 cm thick, joined together by 2.54 cm diameter mild-steel spacing rods to which they are fixed by counterbored screws. The pulley shafts are of steel (diam. 0.9 cm), and all bearings are of presoaked spongy phosphor-bronze mounted in steel bushes. The main driving pulleys are contained in the take-off units and their arrangement is shown in Fig. 5. Equal velocities of the two sides and of the upper and lower belts are obtained by the steel spur gears (32, Fig. 5) the lower pair of which is driven by a common long spur (33, Fig. 5). The motor is a synchronous motor of 46 kg·cm torque with built-in gear box and an axle speed of 36 r.p.m. (Parvalux Motors, Poole, Dorset, England).

The reaction-chamber is slung between the reagent-applicator unit and the take-off unit by means of brackets which allow the reaction-chamber to expand at high temperatures by a horizontal sliding movement.

Because of the low coefficient of friction of the PTFE-coated belts, rubber pressure rollers (34, Fig. 5) have been inserted to ensure that the belts do not slip on the steel driving pulleys.

The first and third units of the machine are rigidly fixed with cross-bars and steel-wire ties (0.63 cm diam., 19, Fig. 2a) around the bottom spacing rods, to a heavy rail (20, Figs. 2a and 3) made of two I-section steel girders (7.5 × 3.8 cm) bolted together against steel spacing rods (5.1 cm diam.). The whole apparatus can then be mounted on a fairly light frame (21, Fig. 2a) which moves on rubber casters.

The electrical controls are mounted on the front panel (31, Fig. 2). The order of switching is controlled by relays so that switches 1, 2, 3, and 4 (from left to right) can only be switched on in that order, and switching off any one switch automatically opens all switches to its right. The switches 1-4 control in order—the fan of the heater-blower, the heater of the heater-blower, the motor driving the reagent-roller, and the motor driving the belts.

The belts and their mountings are very important parts of the machine and need a detailed description. They are endless and arranged in two pairs. The upper belts are 324.1 cm long and the lower belts are 321.4 cm long. They are 2.23 cm wide and 0.023 cm thick. They are made of woven glass fiber coated and impregnated with PTFE. They are joined by overlapping and fusing, the area of overlap being an oblique parallelogram (Fig. 6a) to present minimum resistance to their passing through or over pressure-points on their paths through the machine. Two firms have been able to supply us with adequate belts after consultation with them. The first set were 0.030 cm thick (0.012 in.) and made by Tygadure Co. Ltd., Nottingham, England. The second pair were supplied by A.A.A. Plastics Ltd., Cambridge, Mass., U.S.A. and are 0.023 cm thick (0.009 in.). The operating tension of these belts has usually been in the range 1-3 kg as measured indirectly by displacement with a weight of 100 g.

All pulleys driving, supporting, or deflecting the belts are mounted on stub shafts which leave a clear central gap of 1 cm right down the length of the machine. This is a very considerable convenience in the servicing and replacement of belts since, after loosening the tensioning pulleys (22, Figs. 2a and 3), the belts can be lifted

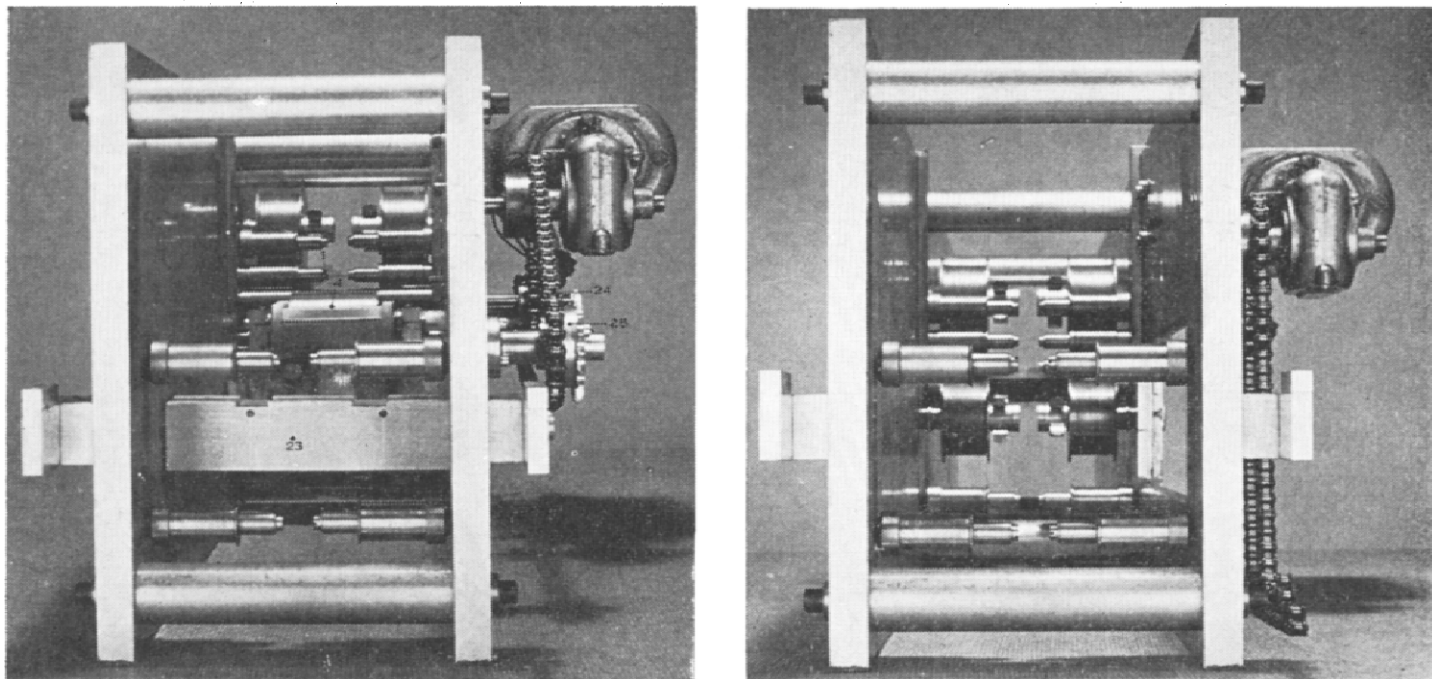


Fig. 7a. The reagent-applicator unit seen from the end normally attached to the entry of the reaction-chamber. The belts are removed for clarity. 23 = Cross-bar supporting reagent-trough and roller No. 2. The support for reagent-trough No. 1 is seen immediately behind 23. Other numbers as in Figs. 2a and 3.

Fig. 7b. Reagent-applicator unit with reagent-trough-roller unit removed.

off the pulleys and out of the machine via the gaps between the stub-shafts. This arrangement is clearly seen in Figs. 2c, 5 and 7.

In Fig. 7 the arrangement of the reagent-roller is seen. The loading guide-rail is seen at the end of the unit distant from the reader in Fig. 7a. The belts have been removed for clarity. The reagent-trough is made of PTFE end-plates with a near-circumferential groove into which is pressed an approx. $2/3$ -cylinder of PTFE radius 2.54 cm, length 5.1 cm, and thickness 0.18 cm. The trough is held together by 0.161 cm diameter steel-tie bars secured with nuts. The trough is mounted on a rectangular aluminum cross-bar (23, Fig. 7a) mounted in turn on a face-plate which is let into a rectangular hole in the front main plate of the reagent-applicator unit. The whole trough-roller section is demountable from the front main plate after disengaging the driving chain (24, Figs. 2a and 7a) from the sprockets (25, Fig. 7a) (see Fig. 7b). A PTFE tube (I.D. 0.30 cm, O.D. 0.50 cm; 26, Figs. 2a and 8) leads from the lower part of the front edge of the trough out through the subsidiary face-plate to the constant-level chamber of the reagent feed (27, Figs. 2a and 8).

The reagent-feed is a simple constant-level device with a capacity of approximately 180 ml. With the tap closed, the upper chamber (28, Fig. 2a) is filled with reagent and the stopper is inserted, well-lubricated with excess reagent or an inert solvent. The tap is then opened and reagent flows into the constant-level chamber and thence to the trough. Further fluid will only pass from the upper to the lower chamber when the level drops below the orifice of the control tube (29, Fig. 8), thus allowing a bubble of air to pass up into the upper chamber and displace the fluid downwards. The level is set by sliding the unit vertically in the laboratory clamps

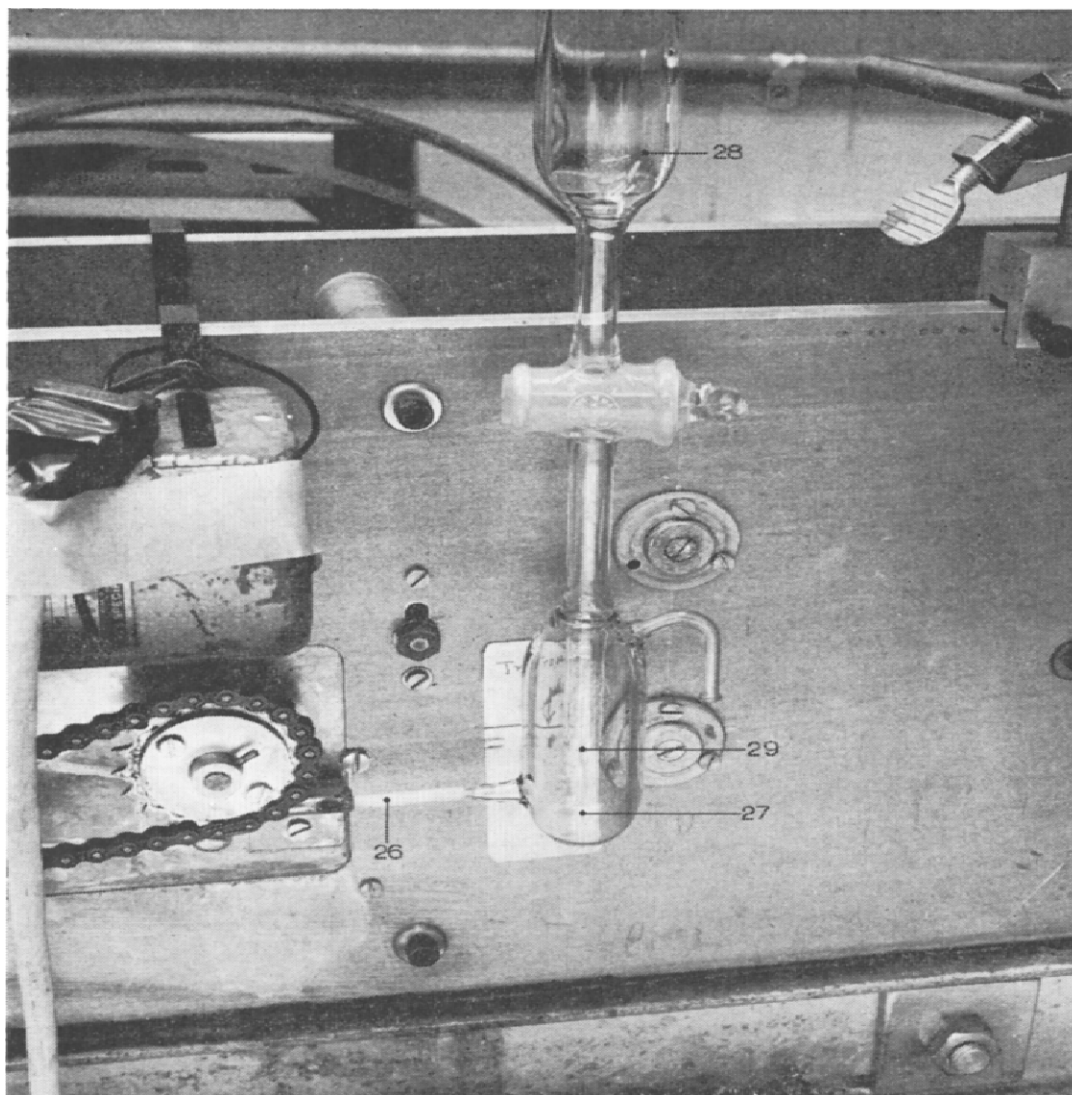


Fig. 8. Constant-level chamber and inlet tube to reagent-trough and roller. 26 = PTFE connecting tube to reagent-trough and roller; 27 = reagent feed constant-level chamber; 28 = reagent reservoir-feed, upper chamber; 29 = orifice of control inlet tube from upper chamber.

(30, Fig. 2a) until the orifice of the control tube is level with the mark indicating the desirable fluid level in the reagent trough.

OPERATION AND USE OF THE MACHINE

Method of operation

The machine was first subjected to calibration of the variable transformer (Variac) controlling the heater of the heater-blower unit. The room temperature (26°) was noted and the Variac turned to give maximum voltage. Ports 1 and 2 were opened and the exhaust fan switched on. After 15–20 min at an ambient temperature of 26° the thermometer in the reaction-chamber of the present machine reached a steady reading of 150° (uncorr.) which was checked four times at intervals of 5 min and showed a range of variation of approx. $\pm 0.2^\circ$.

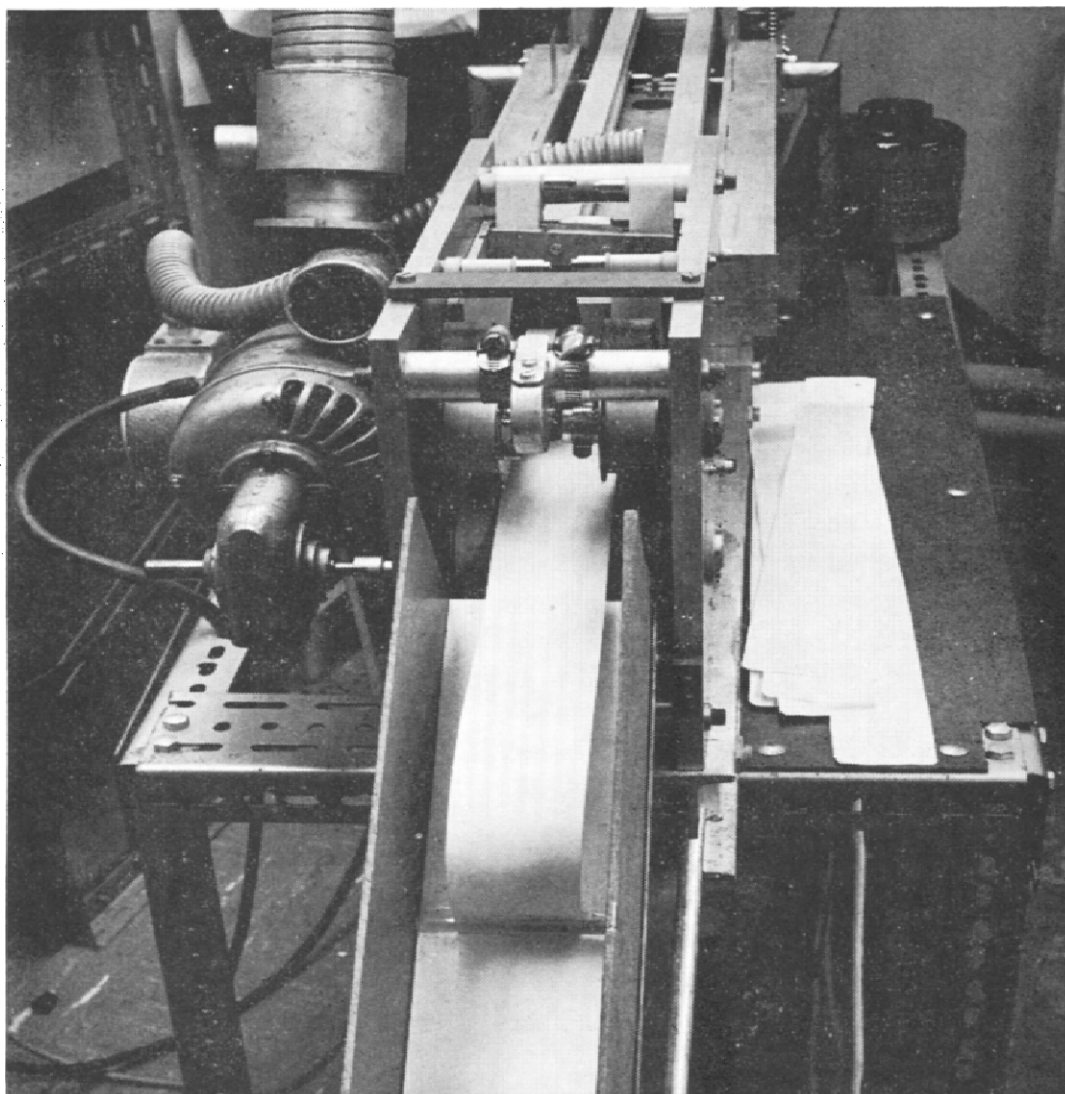


Fig. 9. View of ejection of a strip from the take-off unit showing the action of the ridge in the collecting trough. The strip is about to roll over and fall "tail-first" into the collecting tray.

The belt drive was then switched on and the temperature of the reaction-chamber fell in 10 min to a steady value of 148° . The belt-drive was kept running throughout the subsequent calibration. The Variac was turned back to 80 % of full-scale and a new steady temperature was reached in approx. 10 min. The process was repeated in approx. 10% steps on the Variac dial until a reading of 42° was obtained. A typical calibration is shown in Fig. 10.

This calibration curve is used in day-to-day operation of the machine to set the Variac to achieve the desired temperature. If necessary, calibration curves for a range of ambient temperatures can be made. After a 15-20 minute period allowed for warming up, a final adjustment of the Variac is made until the thermometer indicates the exact temperature that is desired for the reaction to be carried out. Usually the belt drive is switched on 5 min before using the machine to avoid unnecessary wear and tear. Final adjustment of the starting temperature *must* be made with the belts running.

Without servo-control of the temperature, however, this *starting* temperature will *not* be maintained when strips soaked with reagent start passing through the reaction-chamber. The proper starting temperature depends both on the thickness of the paper strips to be treated and the nature of the reagent, and is determined by trial and error when investigating the optimal conditions for each color or fluorescence

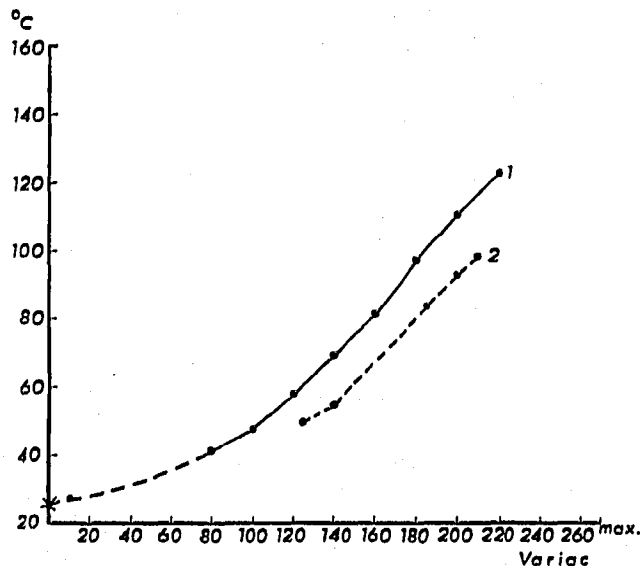


Fig. 10. Calibration curve for temperature of reaction chamber as a function of the setting of the Variac controlling the heater of the heater-blower unit. 1 = Room temperature 26°; 2 = room temperature 22°.

reaction. With the present machine a fall of 2–10° is seen with Whatman No. 2 paper when using reagents dissolved in common alcohols or solvents. Since control to within $\pm 0.5^\circ$ is essential for adequately reproducible results a simple technique has been devised to achieve a steady-state before feeding strips into the machine.

Having achieved the appropriate starting temperature, the reagent reservoir is filled with reagent, the stopper inserted, and the tap opened. The constant-level chamber and reservoir are usually kept at the appropriate level that fills but does not cause overflowing of the reagent-roller trough. One or two clean strips of filter paper, 56 cm long, of the type used for the chromatograms are now fed into the machine via the entry guide rails, and the temperature is observed by an assistant. If the temperature fails below the desired operating temperature for the reaction, the Variac is readjusted and further clean strips passed into the machine until the correct temperature has been achieved. Usually, one or at most two "leader" strips of this sort are needed, and the operating temperature is achieved without readjustment of the Variac.

The "leader" strip is also used to adjust the speed of the reagent-roller. The speed of rotation of the roller is adjusted, while watching the strip at its point of contact with the roller, until the trailing edge of the wetted zone holds a steady position several mm *behind* the actual point of contact with the roller itself, and is convex backwards in the area between the inner edges of the belts and the outer edges of the roller (see Fig. 11). This ensures maximal loading of the paper with reagent, without feeding excess reagent on to the edges of the paper and between the belts.

The chromatograms to be treated are now fed into the machine by hand, making sure that the gaps between the ends of successive strips do not exceed 1–2 cm. It is also possible and eminently satisfactory to link the strips head-to-tail in a zig-zag pack with clean cotton thread, or by slots cut with scissors. The first of the train is fed in manually after which the entire operation is automatic.

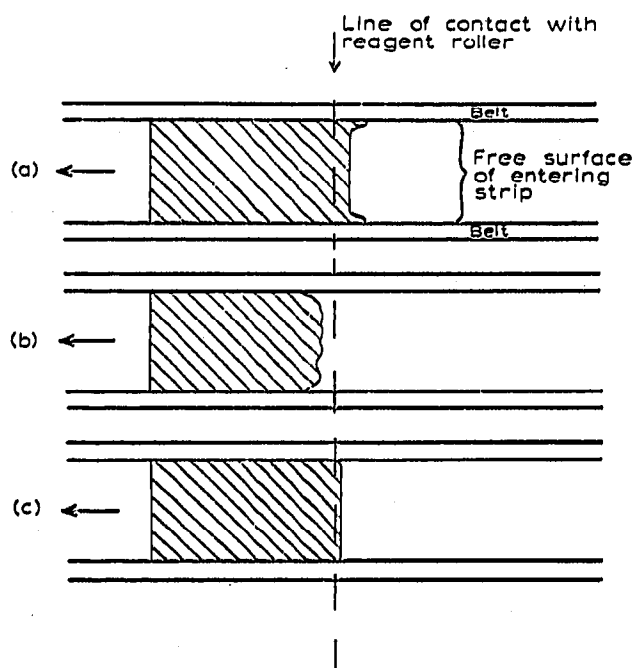


Fig. 11. Diagram of the pattern of wetting of strips with reagent when the speed of rotation of the reagent roller is (a) too large, (b) too small, (c) optimal.

As soon as the last chromatogram has entered the machine, a clean, 56 cm long strip of filter paper is fed in as a "trailer" strip to maintain a steady-state in the reaction-chamber while the last chromatogram is passing through. Without this precaution the temperature will rise and the last chromatogram will be overheated over at least its trailing half. With most reagents this will result in a rising background optical density along its length when the chromatogram is scanned.

If the exit of the machine is not directly coupled with a scanner (see later papers in this series) the treated chromatograms are now piled in order in the collecting tray and are ready for scanning or other procedures.

The speed of operation is determined by the length of the machine and the fixed velocity of the belts of 1 cm/sec. In the present model the entry and ejection points are 127 cm apart so that a 50 cm long chromatogram takes 177 sec to pass right through the machine. This, however, is the "dead time" for the first strip: after this time successive 50 cm strips emerge at one every 50 sec, *i.e.* at 72 strips/h. The "productivity" of such a machine is therefore very large. Allowing 30 min for warming up and the initial adjustment, and 30 min for cleaning up at the end of the day, it would be possible to treat 500 chromatograms 50 cm long in an 8-hour working day. A team of one skilled worker and a trained assistant could conveniently maintain such a full-time schedule, and in addition pass the treated strips through a suitable scanner if the device of linking the strips with scissor-cuts or cotton was employed.

If a series of different color reactions are to be used, approximately 20 min must be allowed with the present machine for the readjustment of temperature and cleaning of the reagent-roller, trough, and constant-level reservoir, for each change of color-reaction.

DISCUSSION

The need for rigorous control of conditions in the treatment of paper chromatograms with chemical reagents

The filter paper used for chromatography is an incompletely described form of hydrated or solvated cellulose. Any chemical reactions carried out *in situ* may be complicated in unknown ways by this circumstance, as well as by the fact of their being performed in a heterogeneous rather than in a homogeneous system. Extensive work on this problem has been carried out with amino acids^{3, 10, 18-20}. Many examples in the steroid field have been described by the author (BUSH^{5, 6, 7}) and will be described in more detail in later papers of this series. Briefly, it is well-known that different types of filter paper vary in properties that affect the running of chromatograms. In addition, its previous exposure to solvents and the atmosphere is also a source of considerable variation in properties (HANES *et al.*¹⁰, and earlier papers; TOMISEK AND ALLAN¹¹). This variation can be eliminated by appropriate pre-treatment of the chromatograms.

As with other aspects of the scanning method for quantitative estimation of substances separated on paper chromatograms, there are a number of relatively unexpected features which are of critical importance to the success or failure of the method as a whole. It must be re-emphasized (BUSH^{7, 8, 9}) that these are relatively easy to *control* once they have been discovered, and that most of them are common in greater or lesser degree to *all* colorimetric reactions which we have examined by this method. There is no doubt that mechanization of the treatment of paper strips with chemical reagents improves very greatly the degree of control achieved over those critical factors.

Assuming that removable impurities in the original filter paper and chromatographic solvents have been brought to a satisfactory and minimal level, there are two main factors affecting the design of a machine for treating chromatograms with chemical reagents. The first is that the most common deficiency of the conventional color reagents used in paper chromatography is that they are *too dilute* (BUSH^{5, 6}). A calculation has been given previously demonstrating just how serious this can be (BUSH⁷, Chap. 4). In all cases the use of a more concentrated reagent gives rise to a more or less serious increase in background color. In some cases this is so severe that the concentration of the reagent can only be increased to an extent which provides a marginal excess per unit area of the paper.

These two reasons alone make it necessary to ensure that any reagent is applied as evenly as possible over the *whole* length of *all* the strips of paper to be scanned.

The second main factor to be controlled is the reproducible heating and drying of the strips once the reagent has been applied. The even and reproducible heating of sheets or strips impregnated with color reagents presents many problems (BLOCK¹², OUGH *et al.*¹³). The complications are easily envisaged and will not be discussed in detail.

In order to solve these problems the machine to be described is designed to move strips of filter paper at a steady speed through a zone in which the reagent is applied to it by a roller and trough, followed by a zone in which they are exposed to heat and a moving air stream. This method has two sets of advantages. In the first place, it is easier to maintain temperature and other relevant conditions constant with time in small volumes of space, rather than attempt to control large volumes of space. In the second, a continuous, sequential movement of strips through the machine is more easily made automatic than the step-wise or batchwise treatment of strips. This approach is justified by the accumulated experience of industrial processes for the production of uniform materials in the form of sheets and strips, and for dyeing and processing paper, cloth, and plastics.

Mechanical requirements

The mechanical specifications of a satisfactory machine of the type under consideration are determined by a combination of mechanical and chemical problems which cannot always be treated separately. Thus, smooth and precise mechanical operation is required to ensure that the spatial relationships of the paper to the treatment zones of the machine are held constant. The working parts of the machine must also be chemically inert and resistant to corrosion. The paper must be supported in its passage through the machine in such a way that all parts are treated with reagent, heated, and dried in identical fashion. Preferably, heating and drying should take place simultaneously on both sides of the paper.

These requirements were met by a pulley and belt system which gripped the paper strips by their edges (BUSH¹⁴). This meant that the main bearing pulleys in the region where the belts gripped the paper could not have internal flanges. Auxiliary double-flanged pulleys and precise machining are essential to secure trouble-free running of the belts. A more expensive method, but suitable for a commercially produced model, would be the use of chain-belts made of die-cast or moulded units, or a perforated belt, running on grooved pulleys or on sprockets or pin wheels to provide lateral fixation of the belts.

To avoid twisting or off-axis movement of the paper strip, the pairs of belts gripping each edge of the paper must have exactly equal velocities. To avoid displacement of the paper strip by uneven vibration in the air-stream used for drying, the belts must be pressed together at several points in their passage so that the paper is held tightly in position and cannot move laterally by slipping between the pairs of belts.

General features of the design

The most important and possibly questionable features of the present machine are:

(a) The linear, sequential arrangement of the machine and thus of the process it carries out;

(b) The belting-system supporting and transporting the paper strips by their extreme edges; and

(c) The use of a solid roller and trough for applying the liquid reagent.

The main reasons for the linear arrangement of the machine, and of the continuous sequential treatment of strips have been described above. Furthermore,

gradients of important variables along the axis of the controlled space are not only tolerable but can be produced and used if this is desirable. A further advantage is that the machine can be directly linked with the scanner, thus achieving the desirable property of the system as a whole that each zone along the length of each strip is scanned at exactly the same time interval after completion of the reaction in that zone. This enables the use of relatively evanescent color or fluorescence reactions for quantitative work.

The belting system was designed with three main aims in mind. The first was to minimize or eliminate as far as possible all preliminary maneuvers with the paper strips such as mounting them on frames or cutting or folding them into complicated forms. This both saves a lot of time when many strips have to be processed and reduces the risk of getting the strips contaminated with dirt and foreign chemicals. Secondly, it was not considered feasible to provide reproducible treatment of the strips with reagent by sprays or feeds from above, without considerable expense on the development of such equipment. The paper must be fed with a film of reagent from below, and its central region must therefore be freely accessible. Finally, both optical and chemical requirements of the method as a whole make it desirable, if possible, to heat and dry the reagent-soaked strips from both sides at equal rates at all stages of the process. This also requires that the working area of the strips be fully exposed throughout their passage through the machine.

The use of a solid roller and trough rather than a porous roller with a sponge-action (*e.g.* HRUBANT¹⁵) was preferred for a variety of reasons. In the first place, the release of fluid by a porous roller depends on the capillary properties of *both* the paper and the roller and is very sensitive to variations in the pressure between the two. Second, the dead-space of such a system includes both the porous volume of the roller as well as the volume of fluid between the roller and its trough. Such a dead-space, and particularly its porous nature, increases the risk of slowly exchanged reagent deteriorating during the course of a run and of decomposition products being applied in greater quantity to the later than the earlier strips in a train. Finally, the production of a really reliably inert and durable porous material in roller form capable of resisting attack by a wide range of reagents is expensive and not too easy.

A criticism has been made fairly frequently that the author's apparatus cannot handle two-dimensional paper chromatograms. This criticism seems to have relatively little weight for the following reasons. My experience with the problems afforded by one-dimensional chromatograms suggested very early on that a machine capable of really good performance with two-dimensional chromatograms would be excessively expensive to develop. Moreover, the zones on two-dimensional chromatograms are more diffuse and often much more irregular than those on one-dimensional strips, which makes the already more complex problem of scanning a two-dimensional array of zones subject to additional sources of error which would have to be overcome. Finally, the use of sequences of one-dimensional chromatograms (column or paper) to obtain sub-fractions of an extract prior to final one-dimensional chromatography has numerous advantages over two-dimensional methods when quantitative work is in question.

Important details of design and construction

Many features of the present machine are not critical, and a detailed description

has not been given of those dimensions and features which can readily be ascertained approximately from the figures, or are obvious to a good machinist. Indeed, certain features are not optimal, having been forced upon me by financial and other circumstances, and will be discussed below. Here I shall simply emphasize those features which are of crucial importance to the achievement of a successful machine.

There is a great advantage in using simple, unpatterned, belts for transporting the paper strips. The manufacture of patterned belts (*i.e.* belts with some form of ridges or holes to give lateral fixation) from corrosion-resistant material of low specific heat is extremely expensive unless large-scale production is contemplated. In order to use simple unpatterned belts successfully, however, one must obtain considerable geometrical accuracy and rigidity over the whole length of the machine, and ensure that thermal expansion of the reaction-chamber does not disturb this. This is obtained by the following features:

(a) very accurate machining of the main plates of the first and last sections, their spacing rods, and the stub shafts mounting the pulleys;

(b) the provision of a very rigid, heavy base or rail to which the first and last units are secured;

(c) the suspension of the reaction chamber on brackets which allow sliding to take up thermal expansion;

(d) the provision of lateral guide-posts or rails for the belts in the reaction chamber, and a small deflection of the belts from a straight horizontal path to provide pressure-points gripping the edges of the paper strips.

If the defects mentioned below can be overcome, PTFE-coated woven fiber glass belts seem ideal from a number of points of view. They are relatively inelastic and show little stretching or distortion when used under reasonable tensions. They are chemically inert and resistant to chemical destruction. Even more important is their virtue of being unwettable and of low thermal conductivity and specific heat. The former property allows a very simple washing tray to maintain adequate cleanliness with endless belts, and prevents overloading of the edges of the strips with reagent. The latter property reduces the difficulty of maintaining a constant temperature of the reaction-chamber, and minimizes underheating or overheating of the edges of the strips of paper, which can otherwise be a nuisance under certain conditions.

A disadvantage of PTFE-coated belts is their low coefficient of friction. Contact over a large fraction of the circumferences of the main driving pulleys must be obtained by using auxiliary pulleys or rollers. Other alternatives are considered below.

The entry and exit of air to and from the reaction-chamber must be regulated by using fans or blowers at both points. Although the diaphragms and deflecting baffles are of use in confining hot air to the chamber itself, and in securing a uniform heating and drying action, they are not adequate to the task on their own. In the absence of an exhaust fan capable of matching the inlet blower, hot air blows out of the ends of the chamber. This can heat up the take-off unit to an undesirable extent and lead to distortion and excessive wear due to uneven thermal expansion. Even worse, the reagent trough in the applicator unit can be gradually heated up. This causes a slow change in the conditions to which successive paper strips are subjected, and in many cases causes evaporation or deterioration of the reagent in the trough itself.

A small detail of the collecting tray is important (see Fig. 9). The tray is con-

structed with a floor of Formica (melamine-plywood laminate with a low coefficient of friction on the hard upper surface) and hardboard sides. At any angle which ensures that the strips stay in the tray after being ejected from the machine, successive strips tend to stick on, and between, one another and pile up as an irregular many-layered sandwich, the "tail" of which stays close to the ejection point and blocks or hinders the ejection of succeeding strips unless the strips are continually pushed down the tray by hand. This is overcome by the ridge (Fig. 9) placed (for 50 cm strips) approximately 28 cm horizontally beyond and 14 cm vertically below the ejection point (*i.e.* the point at which the upper and lower belts disengage from the paper). The leading edge of the paper strip catches on the ridge, thus forcing the emerging strip to roll over as it is pushed out from the rear (see Fig. 8). If the ridge is positioned properly by trial and error, the center of gravity of the strip is beyond the ridge by the time the end of the strip is released at the ejection point and the strip rolls over on itself. This ensures that it drops smoothly and sharply down to the end of the tray, ending up with the leading edge at the *upper* end of the tray. The operator can thus devote his whole attention to loading the strips into the machine and checking the controls.

Plenty of scope must be available in the tensioning pulleys to obtain the proper tension in all four belts. The manufacture of continuous belts of the correct length is subject to error, and even the exact measurement of the long and tortuous path taken by the belts is not easy. Their specification must always allow a safe margin of slack, and the tensioning pulleys must have sufficient movement to take up the maximum amount of slack likely to be encountered. With adequate accuracy of the machining and alignment of the main components, and with the use of guideposts in the reaction-chamber, the adjustment of belt tension is not difficult and not too critical a factor in the reliable running of the belts (see also below).

Undesirable features of the present machine

While the fourth machine described above is a serviceable and apparently effective piece of apparatus, as will appear in later papers of this series, it contains a number of defects which should be corrected in future models.

First, it is not possible to use the present machine for the treatment of strip chromatograms with predominantly aqueous reagents. The method of supporting and transporting the strips fails to allow for the swelling of paper that takes place when reagents containing a large fraction of water are used, and also gives inadequate support to the central area of the paper. It is also difficult to secure complete drying of the strips in the short path-length of the reaction-chamber if the reagent contains more than 50% of water by volume.

The short, straight path, in the reaction-chamber is traversed in 65 sec. This, again, means that certain reactions which need prolonged exposure to the color reagent before finally drying the strips, cannot be completed in the machine. In this respect, the long zig-zag path used in the second model (Fig. 1) was greatly superior as it allowed a ten-minute exposure to the reagent.

These two defects are, however, not too serious for the following reasons. The majority of useful color and fluorescence reactions are carried out with reagents dissolved in organic solvents containing little or no water. Such reagents do not swell or weaken the paper strips sufficiently to prevent their efficient carriage by the belt system. Second, while many color reactions for paper chromatograms are normally

carried out by procedures which involve exposure to the reagent at room temperature, or with moderate heating, for several minutes or hours, the excellent control of conditions which is obtained by mechanizing the process allows one to increase the velocity of such reactions by using elevated temperatures. In addition, the application of a blast of hot air may in itself increase the speed and efficiency of color reactions on paper chromatograms by mechanisms which are not fully understood (see *e.g.* KOCHAKIAN AND STIDWORTHY¹⁶; SWALE¹⁷). Thus, for instance, better control of the ninhydrin reaction is usually achieved in conventional methods for paper chromatograms of amino acids by prolonged exposure (2–24 h) to the reagent at low or moderate temperatures, rather than by brief exposure to higher temperatures (*e.g.*, BARROLIER^{18,19} and PATTON AND CHISM²⁰). However, completely satisfactory results have been achieved with ninhydrin in the third and fourth machines described here by using a working temperature of 115°.

Another undesirable feature of the present machine is the absence, for reasons of economy, of any servo-control on air-flow and temperature in the reaction-chamber. The difficulties arising from this can be overcome to a large extent by the general technique described above, but reproducible operation of the machine tends to be difficult, or to require much preliminary adjustment, if the room temperature or main electricity supply varies appreciably.

It will be seen in subsequent papers that these defects, while making it essential to have reasonably skilled workers operating the present machine, by no means prevent it from being a serviceable instrument of considerable and practical usefulness.

Problems encountered in using the fourth machine

Most of the problems that have been encountered have been due either to the defects mentioned earlier or to difficulties with materials or the environmental conditions.

The belting system is designed to grip 5.08 cm wide strips by an overlap of their edges of 0.318 cm. Care must be taken to see that a batch of strips from the supplier is not used for chromatograms if they are more than 1.5–2.0 mm undersize in width. Oversized strips can be managed quite adequately as long as the guide-rails at the loading point are adjustable in width, which is easily arranged (Fig. 12).

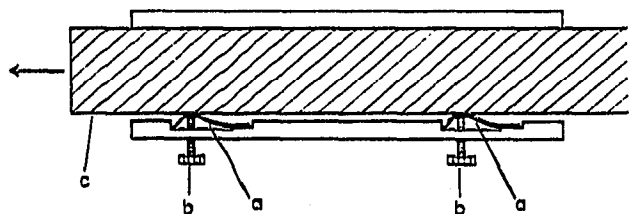


Fig. 12. Arrangement for providing an adjustable width to the entry guide-rails. a = Leaf spring inset on side face of guide rail; b = screw control; c = paper strip in guide rails.

Another problem is common to all methods of carrying out reactions on paper chromatograms, namely atmospheric contamination with agents affecting the reaction being carried out. This is particularly dangerous with this machine because of the relatively large air-flow through the reaction-chamber, and the necessary use of an exhaust fan leading to the outside air. Care must be taken to see that the outflow

through the exhaust system is balanced by an inflow of air to the room from an *uncontaminated* region of the atmosphere, and that other exhaust systems in the laboratory (e.g. hoods and air-conditioning systems) are either similarly balanced or are switched off during operation of the machine. A dust-filter on the intake to the heater-blower is desirable unless a dust-free atmosphere is obtained in the room by air-conditioning apparatus.

In the presence of rapid or extreme variations in ambient temperature, humidity, or mains voltage supply, it is difficult to achieve reproducible results with the present machine because of its lack of servo-controls. Under reasonable laboratory conditions in England and the U.S.A. such difficulties have not often been sufficiently severe to cause serious trouble. At worst a slightly lengthened period for adjustment of working temperature has been required.

Care is also needed in arriving at the optimum arrangement of the entry ports to the reaction chamber. The present machine with its single rather short reaction-chamber is not as flexible as is desirable. It is important that the exhaust system be adjustable so that inflow from the blower-heater is as nearly as possible exactly balanced by the exhaust outflow. If, in order to lengthen the time before each chromatogram is completely dried, one attempts to reduce the air-flow by reducing the total area of open entry ports *without* reducing the flow to the exhaust fan, colder room air will be sucked into the open ends of the reaction-chamber. This both lowers the temperature, and, more serious, tends to produce a laminar air-flow in the reaction-chamber with a central cold air stream, flanked by peripheral hot air streams from the entry ports (see Fig. 13). In the present machine, balance is achieved manually with a butterfly-valve which controls a bypass to the exhaust system. The pressure difference at the opening of the reaction-chamber is observed by means of a thin strip of tissue paper hanging in the opening which is brought to a vertical undisturbed position when the pressure difference is zero.

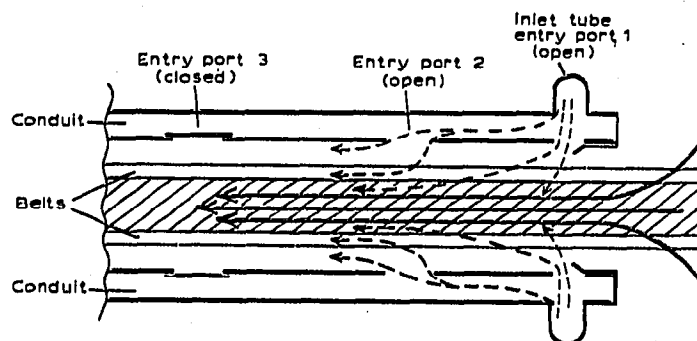


Fig. 13. Streamlining of cool air in the reaction chamber when inlet air-flow is less than exhaust outlet air-flow. Hot air as dashed lines, room air as solid lines.

The belt-washing baths have worked well, but the PTFE coating of the fiber glass belts is often not as continuous or as impervious as could be desired. The best way that I have found of testing such belts for selection is to soak them in 1.0 *N* KOH in 50% ethanol for 24 h at 23–28° and then rinse them well in cold tap-water for 5–10 min. The washed belts are now immersed in phenolphthalein (1% w/v in ethanol) which rapidly reveals as pink spots all serious defects through which KOH has penetrated. Although quite feasible technically, it is not usually economical to

obtain narrow belts of this sort which are covered with PTFE at their edges. Instead, they are cut from much wider sheets of PTFE-fiber glass and the cut edges can only be "sealed" with PTFE at great expense if success is to be guaranteed. Again the joints of the belts are a potential source of weakness. Mechanical strain is increased by the extra thickness and irregularity, and the fusion process sometimes removes some of the original PTFE coating and exposes the fiber glass to corrosive action.

The only economical solution to this problem, and to the similar one of preserving the stainless-steel reagent roller in good condition, is rapid and complete cleaning immediately after each use of the machine. The belts are most seriously endangered by flooding with strongly alkaline reagents, while the only reagent that has threatened to corrode the reagent roller in our experience has been 20 % w/v phosphomolybdic acid in alcohols. The stainless-steel roller has, however, lasted well and has not had to be replaced in eighteen months of frequent use.

Performance of the machine

The performance of the machine must be assessed in terms of two separate functions:

(a) The evenness of the application of reagents to different parts of each strip of paper and to different strips in a train of them.

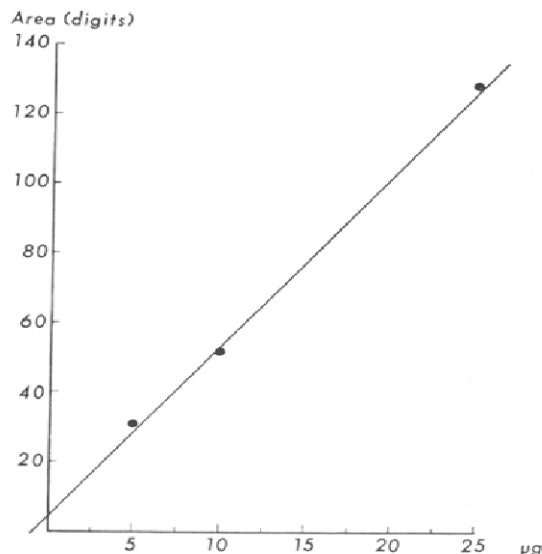
(b) The evenness of heating and drying the strips.

The first function has been assessed largely independently of the other by substituting a colored solution for the reagent, and setting the temperature of the reaction chamber at the minimum required to achieve almost, but not quite, complete drying of the strips. After treatment in the machine, they were hung horizontally (BUSH AND WILLOUGHBY⁸) in a hood to complete their drying. The strips were then scanned at the wavelength of maximum absorption of the dye that had been used. The first model was tested in this way using Sudan red in ethanol; later models were tested using the dye 4-amino-1-methylaminoanthraquinone (NEHER²¹). Such strips were compared with similar ones which were dipped by hand over the dye solution in a watchglass (BUSH AND WILLOUGHBY⁸) and dried horizontally in a hood. The scanner was used at a higher sensitivity than usual in order to accentuate variations in the artificial background color that had been produced.

The maximum variation in optical density over the length of any one strip treated by the machine (*i.e.* the "worst" strip) was invariably much less (5-10 fold) than the variation on the "best" strip of the series treated by hand. In view of the clearcut superiority of the mechanized process no statistical analysis of these records was carried out.

The evenness of heating and drying of the strips is best determined by using a color-reagent which requires a moderate temperature for completion in the 65 sec period in the reaction chamber, and which gives a very high background on over-heating. An even more critical test is provided by running the machine at a temperature *below* the optimum for such a reaction so that the reaction is incomplete. In these circumstances the calibration curve for a set of standards in the usual working range of concentrations is nonlinear and highly susceptible to any variation in the heating and drying conditions that were used. The best test of this sort is provided by the phosphomolybdic acid reagent, used for a wide range of hydroxylic steroids (KRITCHEVSKY AND KIRK²²) and other alcohols of high-molecular weight. Under the usual

conditions of heating the paper in an oven at 90° the reaction usually gives little color in 1.0 min, an optimum color contrast (*i.e.* zone/background = blue/yellow-green) in approx. 1.5 min, and a uselessly high background, in which the whole paper becomes deep blue-black in color, in approx. 2.2 min. It has been found almost impossible to control the conditions sufficiently by manual methods to obtain reliable quantitative results with this reagent by scanning, unless the much slower, low-temperature method of MARTIN²³ is used. As will be shown in paper 3 of this series the machine enables one



(a)

(b)

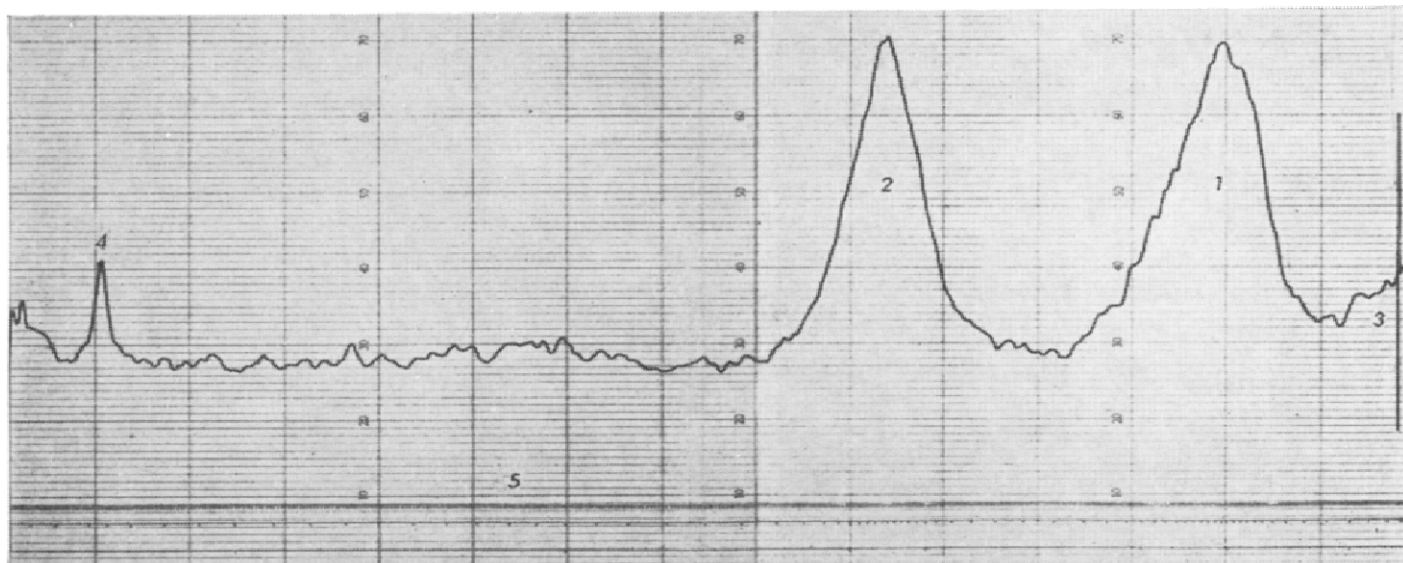


Fig. 14. Calibration curve (a) and typical scanning record (b) for dehydroepiandrosterone determined by the phosphomolybdic acid reagent carried out by the machine. Starting temperature: 95.5° . Steady-state operating temperature, 94.5° . Chromatograms on Whatman No. 2 paper, run 3.5 h with the system light petroleum-toluene-methanol-water (67:33:85:15, by vol.) at 25° . 1 = Androsterone; 2 = dehydroepiandrosterone; 3 = impurities at front; 4 = pencil line at origin; 5 = digital integral record (units, tens, hundreds, thousands from top to bottom). The recorder was run with minimal damping during this experiment in order to emphasize background fluctuations. Androsterone was too close to the front material for precise estimation.

to obtain quantitative estimation of hydroxylic steroids which is almost as precise as any other colorimetric method carried out by the scanning method.

A typical run is shown in Fig. 14. The starting temperature was 95.5° and the operating temperature during the run was $94.7^{\circ} \pm 0.7^{\circ}$. The calibration curve is linear up to $25 \mu\text{g}$ and the background optical density, while showing the usual short-length (1-3 mm) irregularity typical with dry filter paper (BUSH⁷), did not vary by more than ± 2 small divisions of the chart record from the mean over a 45 cm long strip. In another similar run with a train of 12 strips, each 50 cm long, the difference in the backgrounds of the strip with the highest and the strip with the lowest background optical density was 3 small divisions on the chart record. The scanner was run at moderate sensitivity and minimum damping (see paper 2 of this series) to accentuate small and rapid fluctuations in optical density, and a $25 \mu\text{g}$ zone of dehydroepiandrosterone (5 cm wide) gave a peak height of 34 small divisions of the chart. During this period the drift of the scanner and light source produced a change in optical density of 2-3 small divisions on the chart recorder over periods of 50 sec (the time needed to scan one strip) and a ripple of ± 0.5 small divisions at approximately 2 c.p.s.

Strips treated manually over the same period (dipping over a watchglass and heating before an open electric heater (BUSH⁷)) showed a minimum background variation (with the same sensitivity of the scanner) on any one strip (*i.e.* the "worst" strip) of ± 5 small divisions, and the differences between the background optical density of the strips with highest and lowest mean backgrounds was 20 small divisions on the chart.

Similar but less extreme results have been obtained with the Zimmermann reaction for 17-ketosteroids (alkaline *m*-dinitrobenzene, BUSH AND MAHESH⁹). Manually treated strips usually require constant readjustment of the background-setting potentiometer during scanning to cope with the wide variation (± 8 small divisions) in backgrounds of successive strips. With machine-treated strips little or no readjustment is required during the scanning of trains of 20-30 strips (see paper 2).

Other uses of the machine

Apart from its main use, which is the rapid and reproducible achievement of color and fluorescence reactions on paper chromatograms, the machine can be used for many other treatments of filter paper strips which are useful in paper chromatography. Thus, it can be used to pretreat filter paper with solvents or buffer solutions necessary for some types of paper chromatography (see *e.g.* LEVY AND CHUNG²⁴). The reproducibility afforded by the continuous, mechanized, process is greatly superior to spraying or dipping by hand, and is especially so when drying or heating to a precisely controlled degree is desired. Similarly, the pretreatment of chromatograms after they have been run is often a valuable preliminary before certain color reactions are carried out. Thus, the final elimination of traces of the solvent system is often only achieved by "steaming" (*e.g.* ISHERWOOD²⁵) or other similar procedures (*e.g.* BUSH⁷, Chaps. 3 and 4 and later papers in this series). In such cases the reproducible application of a liquid solvent or reagent and its controlled removal by evaporation is crucial to the achievement of satisfactory results, and can be done by the machine much more reliably than with manual methods, ovens, hair-driers and the like.

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SUMMARY

A machine is described for the reproducible treatment of paper chromatograms with liquid chemical reagents. Strips of filter paper 5 cm wide are passed through the machine by two pairs of belts which grip the edges of the strip leaving the central area exposed on both sides. A film of liquid reagent is fed on to the undersurface of the paper strip by means of a roller and trough which is fed from a simple constant level device. The reagent-soaked strips pass immediately into a straight tunnel into which a stream of hot air is introduced by ports opening from manifolds running along the sides of the tunnel. The air stream is passed out of the top of the tunnel by means of an exhaust tube and fan connected to its distal end. The strips are passed out of the machine by disengagement of the belts which are returned to the starting point of the machine after being washed, and dried on their return passage through the hot air tunnel. Operating temperatures up to 150° can be achieved and are adequate for producing quantitative and complete reactions with the majority of color reagents that are useful for the quantitative estimation of substances by direct scanning of paper chromatograms (*e.g.* Zimmerman reagent, ninhydrin, phosphomolybdic acid). The mechanization of the treatment of paper chromatograms with color reagents improves greatly the reproducibility of the results, the evenness and reproducibility of background, and enables the successful use of color reactions which cannot be controlled sufficiently by manual methods to allow their quantitative estimation. It also enables the treatment of very large numbers of chromatograms: the present apparatus is capable of treating 40 cm long chromatograms at a rate of up to 88 per h or over 500 per day. The development, important features of design, and method of operation of the machine are described.

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