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Effects of drying and equilibration of paper chromatograms of tritium labelled compounds

Experiments with ^{14}C-labelled compounds have shown that unequal drying of the two surfaces of a paper chromatogram may lead to unequal distribution of the solute throughout the thickness of the paper, both at the time of application[1,2] and after development[3]. This effect is more readily detected when ^{3}H is used as a tracer owing to the very soft β-emission of this isotope (maximum 18.5 keV) which has a range of only 0.006 mm in a medium of unit density compared with the range of 0.2 mm of ^{14}C; and we have found that chromatograms from which emission from one side of the paper is five times that from the other can be readily prepared.

25 μl of an aqueous solution of D-glucose-3-^{3}H (0.5 mC/ml, 77.6 μg/ml) was applied as one lot to Whatman No. 1 paper and immediately dried by a hot air blower directed perpendicularly to the front (top) face of the paper which was suspended horizontally. Each side of the spot was then scanned with a windowless gas flow proportional scanner and the paper developed in *n*-butanol–ethanol–water (104:66:30). The paper was then dried as above and again scanned. Control chromatograms were also prepared. These were dried in still air at room temperature both after application

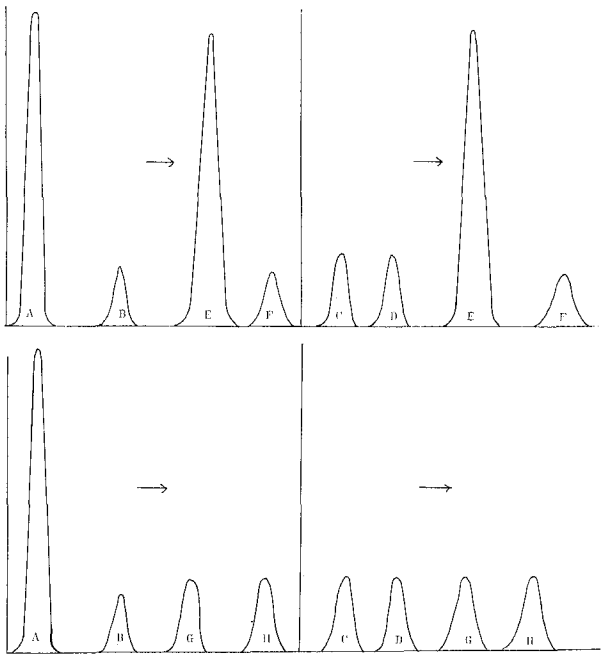

Fig. 1. Scans of peaks of paper chromatograms, D-glucose-3-^{3}H or L-leucine-4,5-^{3}H. (A) Front of paper dried with hot air blower before development, (B) back. (C) Front of paper dried in still air before development, (D) back. (E) Front of paper dried with hot air blower after development, (F) back. (G) Front of paper dried in still air after development, (H) back.

of the solute and after development. An aqueous solution of L-leucine-4,5-^{3}H (0.5 mC/ml, 2.9 μg/ml) was treated similarly. The results are summarized in Fig. 1 and clearly confirm the observations of DUNCOMBE[3] that the type of drying after development is the major factor determining the shape of the scan, since solute unequally distributed at the origin (A, B) is essentially evenly distributed at each surface of the paper (G, H) after development and remains so provided that uniform drying is applied to the paper. This behaviour should be general for water-soluble compounds developed with aqueous solutions, but compounds sparingly soluble in water do not always become evenly distributed during development. For example, DOBBS[4] has shown that benzoic acid and stearic acid having different concentrations on opposite sides of the same paper maintained this difference after a chromatogram had been run in ethanol–0.880 ammonia–water (16:1:3). We have on occasion observed very variable results when paper chromatograms of ^{3}H labelled compounds are dried in a fume cabinet and we suggest that drying in a draught free room is essential for the attainment of reproducible results.

We were recently informed[5] that the radioactivity of some ^{3}H labelled steroids supplied by us "disappeared" during paper chromatography under certain conditions. There was no evidence that the tritium in these compounds was labile or volatile, and we now put forward two possible explanations for this observation. It appears that the steroids are either concentrated at the surfaces of the paper at the time of application and are then essentially evenly distributed throughout the thickness of the paper after equilibration and/or development, or they are essentially evenly distributed on application and became concentrated at the centre of the thickness of the paper after equilibration. It is possible that microautoradiography of a section through the paper, as suggested by HAIS (see ref. 2), could determine which explanation is the more correct. We prefer the former explanation since the steroids had a lower R_F in the equilibrating solvent than in the solvent used to apply them (see below) and DUNCOMBE[3] reports that compounds with high R_F values tend to be more readily concentrated at the surface of the paper during drying. 15 μC aliquots of a benzene solution of progesterone-7α-^{3}H (2 mC/ml, 74 μg/ml) or oestrone-6, 7-^{3}H (1 mC/ml, 7.3 μg/ml) were applied to Whatman No. 1 paper as a line 4 cm long, and "run up" to a predetermined origin line according to the method of BUSH[6]. The papers were equilibrated for 18 h in a tank containing petroleum ether (b.p. 60–80°) and 90% aqueous methanol, and developed with petroleum ether (b.p. 60–80°). The papers were dried in still air after application of the spot, running up, and development. One paper of each steroid was removed from the tank and dried in still air after the equilibration stage. Scans of the chromatograms were made at each stage and are shown in Fig. 2. Scans of either side of each paper were virtually identical. The apparent loss of radioactivity at the equilibration stage was substantial and a decrease in peak area of about 90% was usual. After development, the area of paper containing the steroid was cut out and combusted to water using a Schöniger technique, and the water assayed by liquid scintillation counting in a scintillant of naphthalene (180 g), PPO (10 g), POPOP (0.1 g) in 2 l of xylene–dioxan–water (1:1:1). Recoveries of at least 14.5 μC tritium were obtained, the small loss of radioactivity being probably caused by "streaking" or irreversible adsorption at the origin of the chromatogram since no carrier steroid was added. We then spotted 0.25 μC samples of each steroid onto paper and assayed the spots before or after equilibration as above by adding the radioactive area of the

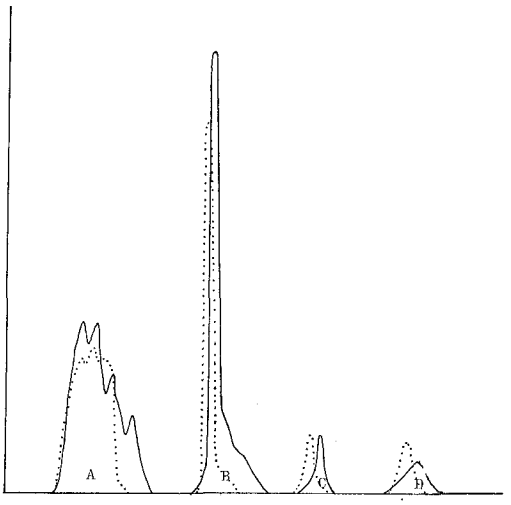

Fig. 2. Scans of peaks of paper chromatograms, (———) progesterone-7α-^{3}H, (· · · · ·) oestrone-6,7-^{3}H. (A) Origin, (B) after running up, (C) after equilibration, (D) after development.

paper directly to a scintillant of PPO (0.5 g) POPOP (0.01 g) in 100 ml of toluene. By this method about 80% of the tritium was detected before equilibration but only 20% after equilibration. Since this result appears to confirm that of the scanning, it is important to realise that absorption of the β-emission is again the cause of the spurious low result. This problem of self-absorption and other complications involved in the direct liquid scintillation counting of tritium labelled chromatograms has been reviewed by TURNER[7] and by DE BERSAQUES[8], and, as we have never been able to obtain satisfactory results in our laboratories by this method, we recommend that the combustion technique is used whenever possible.

If the direct method must be used we conclude that, in view of the effects of unequal distribution of the solution discussed above, complete solution of the solute into the scintillant is desirable if quantitative results are required.

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