WATER CONTENT OF PAPER AS A VARIABLE IN PAPER CHROMATOGRAPHY

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(Received September 12th, 1963)

INTRODUCTION

In our long use[1,2] of the common two-dimensional papergram system, phenol–water followed by butanol–propionic acid–water[3], for the separation of various soluble, intracellular metabolic products, we have experienced only rare lapses of reproducibility; and most of these lapses we had crudely associated with the season of the year. HANES and his co-workers reported in great detail on the effects and the methods of control of the moisture content of chromatographic paper[4–6]. These reports suggested to us that moisture control might prove useful even in chromatographic systems less sophisticated than those developed in the HANES laboratory. The present report describes the simple procedure of moisture control which we have found satisfactory over the past two years.

EXPERIMENTAL

General procedure

Whatman No. 1 paper was used, twelve $18^1/_4 \times 22^1/_2$ in. sheets per cabinet, for descending chromatography.

The first (short) dimension of the paper was developed with a phenol–water mixture (722:278, w/w) (cloud point, $18^1/_2$ to $20^1/_2°$) at the controlled room temperature of 21 to 22°. When made with distilled phenol[7] and stored in the dark, this solvent remains colorless for over a month. As suggested by WADE *et al.*[5], there was no preliminary equilibration of paper with phenolic solvent.

The second solvent consisted of *n*-butanol–water (1770:119, v/v) and propionic acid–water (88:110, v/v) mixed in equal volumes, the cloud point being $18^1/_2$ to 20°. Commercial grade propionic acid (E.I. du Pont Co.)** was used directly, without redistillation. An arbitrary one hour of equilibration of paper with this solvent was allowed, following any preadjustment of the paper's moisture content.

Moisture adjustment

For most of the chromatographic experiments reported here, papers were stored for 24 h in the chromatographic cabinet with three 15 cm petri dishes containing thin

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** This material was stored for several years without any alteration of its miscibility in the solvent system. This stability was not characteristic of other commercial propionic acids (Cliffs Dow Chemical Co. and Celanese Chemical Co.) even after redistillation.

layers of various "constant humidity agents". These agents, together with the equilibrium relative humidities as measured on our instruments (see below) were: water (87 % R. H.); saturated aqueous ammonium sulfate, technical grade (72 % R.H.); sodium acid sulfate, technical grade crystals moistened with water (53 % R.H.); chromium trioxide, technical flakes moistened with water (42 % R.H.); potassium acetate, N.F. (24 % R.H.); and calcium chloride, anhydrous (10 % R.H.).

In experiments where equilibration time was a variable, the cabinet also contained a thermograph, and a very small fan (an 0.2 A tube-cooling fan available from radio supply stores). Ordinarily, we use this fan in conjunction with a proportionating timer (General Electric Co., TSA-14, 5-min cycle) set for 20 % on.

Relative humidity measurements were primarily by a humidigraph (Model 4033, The Bristol Co., Waterbury, Conn.), although a membrane hygrometer (Model 201, Serdex, Inc., Boston, Mass.) was included as a check and to provide a faster-responding instrument for short-term observations. Readings taken from a sling psychrometer (in the range of 25 to 75 % R.H.) were used to calibrate the humidigraph.

All instruments, the fan, and the dishes of humidity control agents were removed from the cabinet before the addition of chromatographic solvent.

RESULTS

Time required for equilibration

The relevance of humidigraph readings to water content of chromatographic paper was checked directly by using an external balance to weigh a pair of paper sheets suspended inside a cabinet containing ten other sheets and humidity control agent (potassium acetate or ammonium sulfate). Paper weights and humidigraph readings were both plotted against time, adjusting the graphic scales to cause coincidence of the curves at both their beginnings and their final equilibrium values (*e.g.*, Fig. 1). Under conditions of constant fan operation (except when weighing) the humidigraph responded somewhat faster than the paper; but in absence of fanning the paper responded somewhat faster. Discrepancy in the two responses never exceeded 8 units of % R.H. in either the humidifying or drying direction; and it was always 2 units or less by the time the paper weight had come to within 5 units of the final equilibrium value.

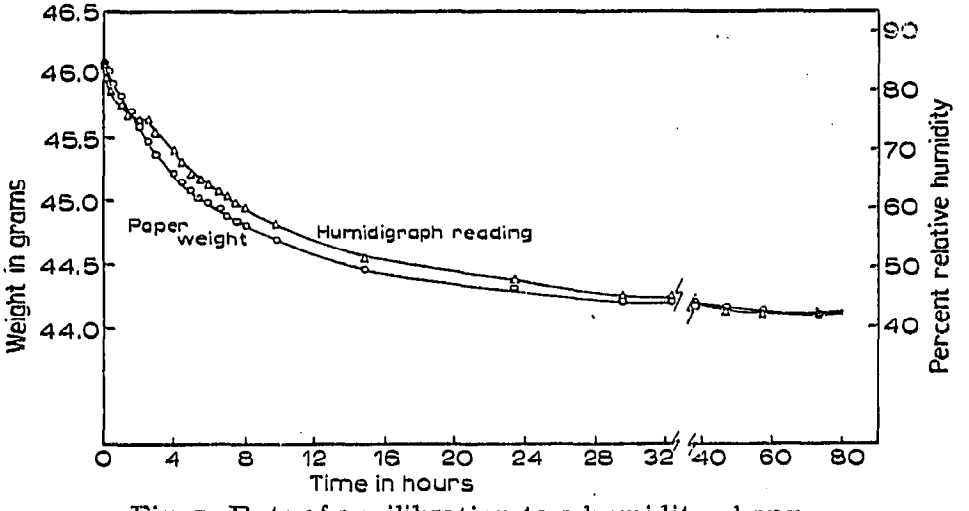

Fig. 1. Rate of equilibration to a humidity change.

Based on humidigraph readings only, using any of the humidity control agents*, 24 h were required for equilibration within 5 units of the final relative humidity. With the use of the circulating fan, this same extent of equilibration required only 9 h or 6 h, depending on whether the fan was used 20 % of the time or continuously. The use of intermittent fanning was devised primarily for insulated chromatographic boxes (Chromatocabs) in order to keep the temperature rise within the box from exceeding 0.5°.

These data have led to the evolution of the following routine procedure. When empty cabinets are available, the papers are stored in the cabinets with the sodium acid sulfate for at least 24 h. When cabinets are not available the papers are hung in the laboratory. Procedure with the latter papers varies according to the humidity of the laboratory. If the humidigraph shows that the relative humidity of the laboratory was within the chosen limits (50 to 75 % in our case) for the preceding 6 h, the papers may be placed in cabinets without any humidity control agent and stored there till ready for the addition of solvent. If the humidity record of the laboratory indicates the need for adjusting the moisture content of the papers, the papers are placed in a cabinet with an appropriate humidity control agent and a circulating fan.

Loss of equilibration upon opening of boxes

The literature on general chromatographic procedures leaves the impression that opening a box lid to permit the filling of solvent troughs (following equilibration of papers with solvent vapors) can cause a sufficient loss of solvent vapor to affect the chomatographic result. With regard to the special case of equilibration with water vapor, we find this impression to be misleading.

On days of especially low humidity we have tried opening boxes of high internal humidity (containing no papers) for various lengths of time. By comparison of the final equilibrium R.H. within the box to the initial and external R.H., we concluded that opening the box for 3 min permitted the interchange of only $^1/_3$ of the air volume of the box. To be sure, this did involve two precautions: the tray door near the bottom of the box was never opened when the top was open, and strong air currents were avoided by shutting off circulating fans in both room and box. What makes this result especially significant is the fact that the paper in a box contains several times the water content of the airspace in the box.

Effect of water content of paper on excursion values

For purposes of Figs. 2 and 3, sheets of known compounds were subjected to chromatography in only one dimension. The curves in Figs. 2 and 3 are chosen as typical. For both solvents a generality may be noted: excursion values are not appreciably affected by the water content of the paper, except that papers with very high water content produce a marked opening up of the chromatographic pattern near the origin together with some crowding in the remaining areas. Within this qualitative generality, some compounds differ markedly in the extent of their response to papers of very high water content. Glutamine in phenol–water solvent (Fig. 2) and lactic acid-^{14}C in butanol–propionic acid–water solvent (Fig. 3) illustrate that this quanti-

* After several days exposure to R.H. about 90 %, the humidigraph required several weeks to regain its former calibration.

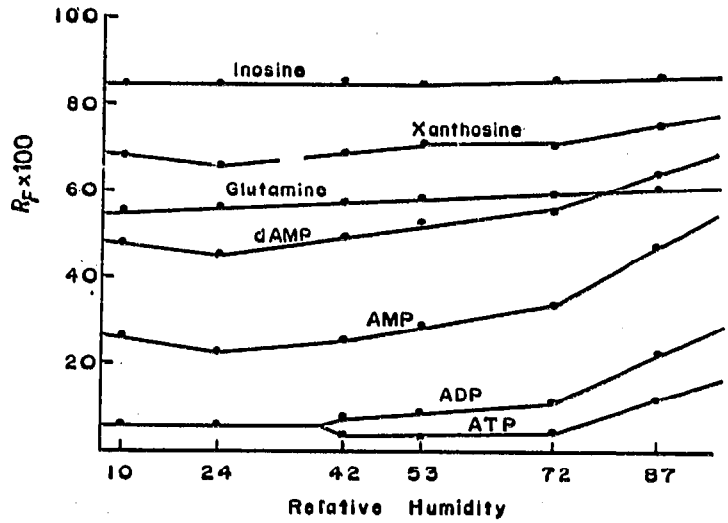

Fig. 2. Averaged excursion values of known compounds in phenol–water solvent. The merging of the ADP and ATP curves indicates that the compounds were no longer separated.

tative difference can result in a reversal of spot positions within the chromatographic pattern. Both of the above reversals had been observed on numerous occasions in our studies of mouse tissue and bacterial extracts before we resorted to controlling the water content of our papers. Additional reversals for the phenol–water solvent may be noted in Figs. 4 and 5 by comparing glutamic acid, aspartic acid, and malic acid with AMP, IMP, and ADP; also, (not illustrated) there is a similar reversal of glutamic acid and CMP. Indeed, it appears general for the phenol–water solvent, that the high moisture content of papers causes larger increases of R_F for purine and pyrimidine ribonucleotides than for aminoacids and hydroxyacids.

In the chromatography of larger amounts of tissue extracts, papers of low moisture content frequently lead to elongation of the fast-moving spots in the phenol–water direction. We take this to indicate that dry papers have less loading capacity.

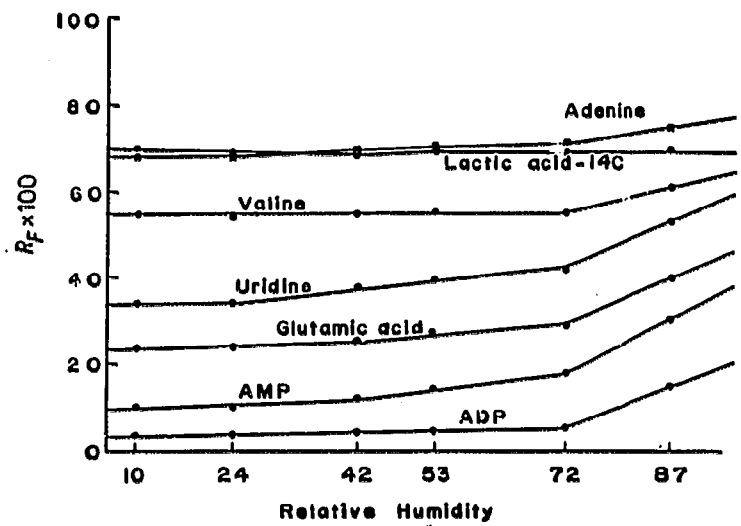

Fig. 3. Averaged excursion values of known compounds in butanol–propionic acid–water solvent.

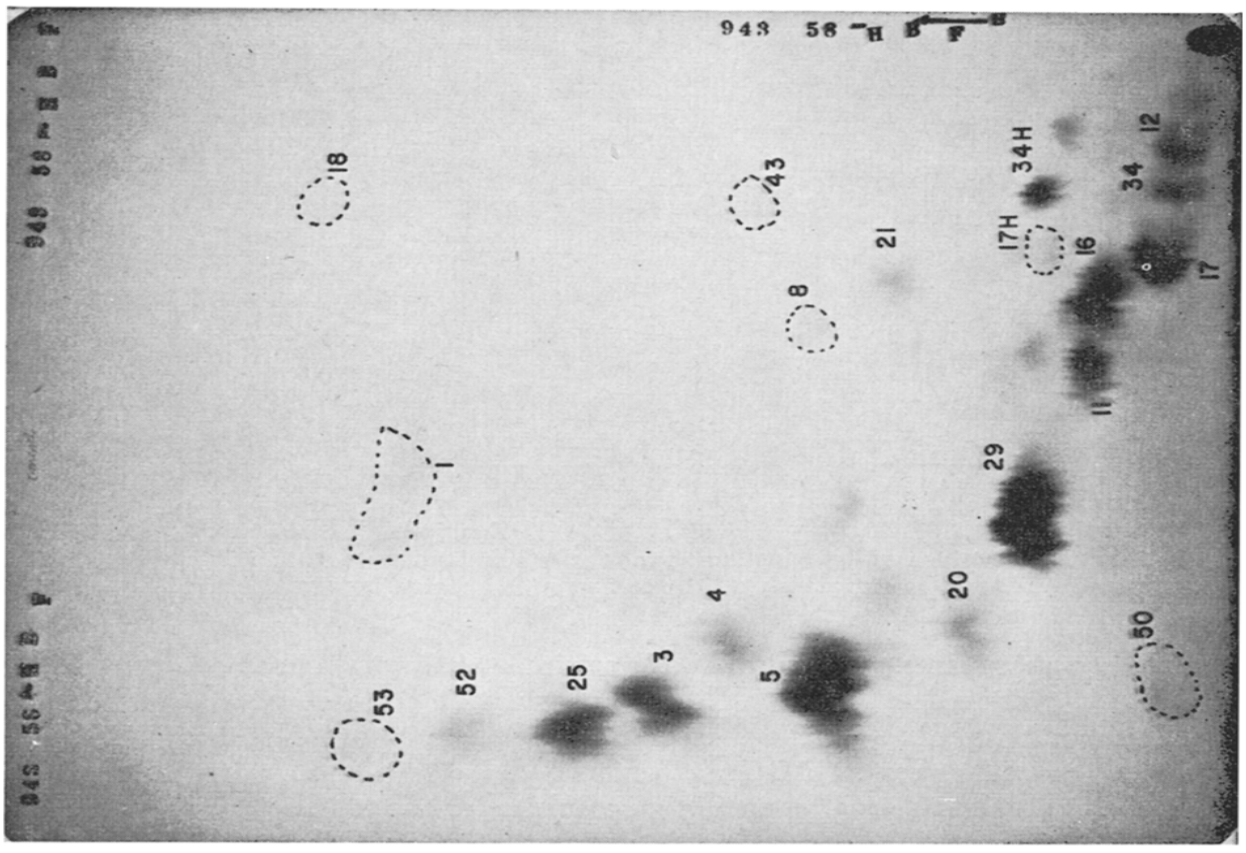

Fig. 5. Phenol–water solvent on a paper equilibrated to 87% R.H. Otherwise similar to Fig. 4. Identities of the spots are: 1, lactic acid; 3, hypoxanthine; 4, guanine; 5, inosine; 8, glutamic acid; 11, IMP; 12, GTP; 16, GMP; 17, ADP; 17H, hydrolytic artifact of ADP; 18, fumaric acid; 20, dAMP; 21, aspartic acid; 25, adenosine; 29, AMP; 34, ATP; 34H, hydrolytic artifact of ATP; 43, malic acid; 50, NAD; 52, adenine; 53, 5'-methylthioadenosine.

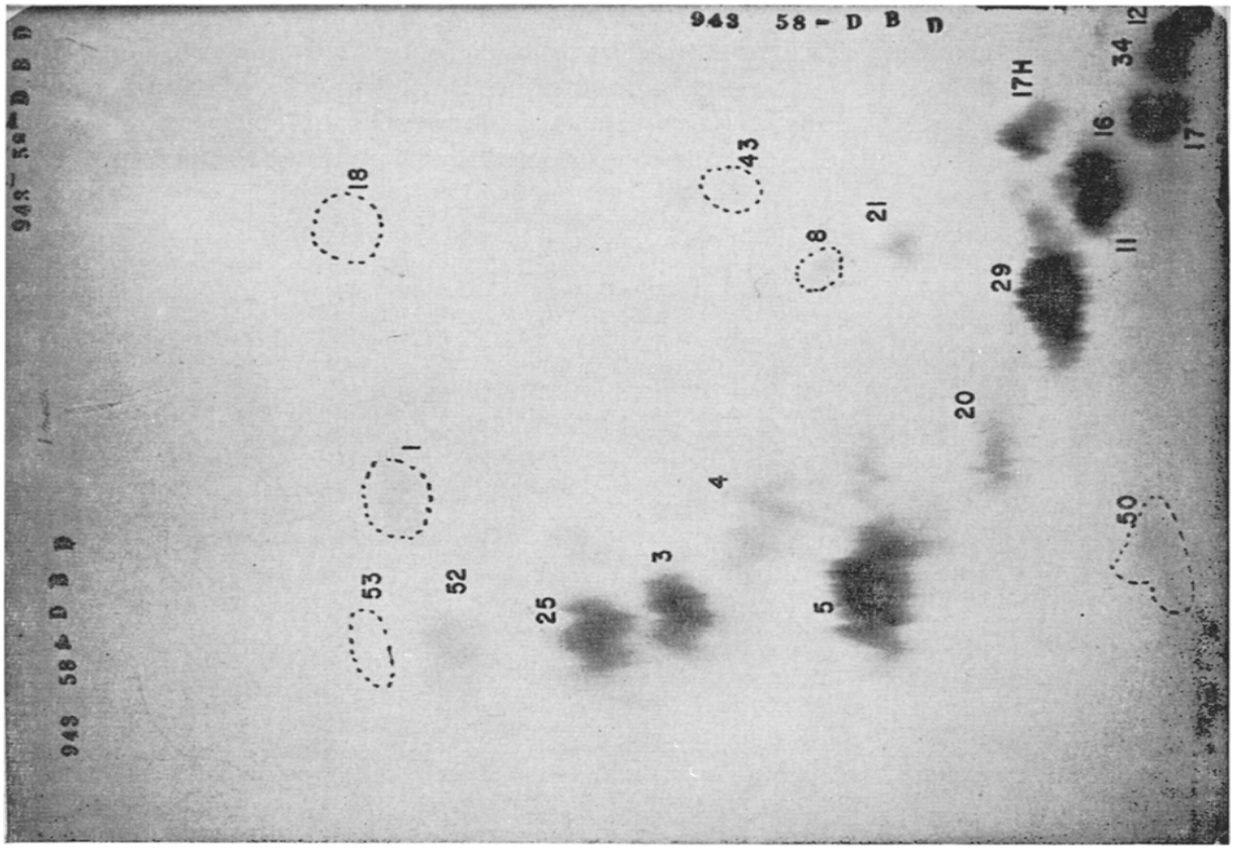

Fig. 4. Phenol–water solvent (right to left) on a paper equilibrated to 10% relative humidity; then butanol–propionic acid solvent (bottom to top) on paper equilibrated to 53% R.H. The photograph is from an autoradiogram after separation of the soluble fraction components from *Escherichia coli*[2] which had assimilated formate-^{14}C. Spot identies are as for Fig. 5.

DISCUSSION

Our main purpose in controlling the water content of chromatographic paper is to insure reproducibility of chromatographic patterns. We use this chromatographic system in testing for possible effects of various biological inhibitors upon the intermediary metabolism of purines (or pyrimidines). In deciding whether preliminary experiments warrant a more complete study, it is essential that individual spots in the normal pattern must be identifiable by visual inspection. Toward this end, the given procedure for control of water content of paper has been very useful. This has been true even though our use of the procedure is not so much for precise control as it is for the avoiding of harmful extremes.

Another purpose in controlling water content of paper might be the deliberate use of extreme conditions to produce some specially desired effect. In our work, we have occasionally resorted to very moist papers to effect difficult separations in the ribonucleotide area.

The use of a humidity meter whose sensing element consists of wood fibres provides a reasonably close analog of the water content of paper. In addition, the recorded time course of humidity variation is more pertinent to the moisture content of paper than a single humidity reading on a rapidly-responding instrument.

ACKNOWLEDGEMENTS

This work was supported by grants from the Alfred P. Sloan Foundation and the Charles F. Kettering Foundation.

SUMMARY

A simple procedure is described for improving the reproducibility of two-dimensional papergrams made by the solvent systems: phenol–water followed by butanol–propionic acid–water. The procedure involves control of the water content of the chromatographic paper prior to the addition of solvent. Some effects noted upon the variation of the water content of paper are described for some chromatographic patterns of biochemical interest.

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