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A New Technique in Paper Chromatography

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Paper partition chromatography, originally introduced by Consden, Gordon, and Martin [1], has proved an effective method of separating chemical compounds from complex mixtures such as protein hydrolysates and of identifying them on a micro scale. A review of paper chromatography has recently appeared [2]. For several months we have used in this laboratory the original method of Consden, Gordon, and Martin for qualitative analysis of mixtures of amino acids. In practice certain technical improvements — to be reported in this paper — have suggested themselves.

By means of the original technique a complete separation of only a few amino acids from protein hydrolysates can be attained by *one-dimensional* chromatography. For example, when phenol is used as a solvent, only aspartic acid, and when s-collidine is used as a solvent, only valine is often separated as an individual spot whereas all the other amino acids partly overlap one another. Certain alcohols would have a good resolving power but the amino acids move relatively slowly with them. The R_f-values of the fastest moving amino acids in benzyl alcohol (phenylalanine), in *n*-butanol (leucine) and in *tert.* amylalcohol (leucine) are 0.36, 0.43, and 0.30 respectively. So, when the solvent frontier reaches the lower edge of the strip of filter paper, the amino acids have advanced only short distances forming a tightly packed chromatogram (Fig. 1, A) which contains no one-acid spots. For a more effective separation the chromatograms must be developed longer. This can be effected by the following, very simple »continuous developing technique» which gives chromatograms of any desired length.

THE NEW PROCEDURE

A thick pad of cotton wool, cellulose tissue, or other absorbing material is sewed or stapled to the foot of the strip of filter paper. This pad must be able to soak up an abundant amount of developing solvent. The development of

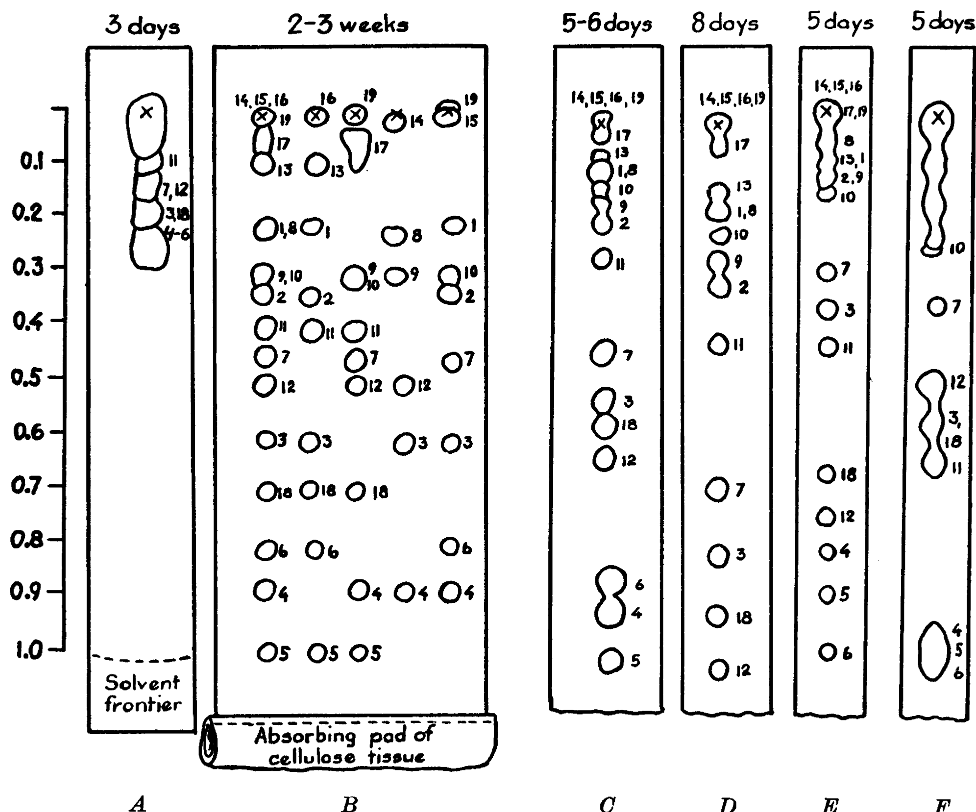

Fig. 1. Diagrams of one-dimensional paper chromatograms of mixtures of amino acids

1. Gly	*4. Ileu*	*7. Tyr*	*10. Hypro*	*13. His*	*16. Asp*	*19. $(CyS\text{-})_2$*
2. Ala	*5. Leu*	*8. Ser*	*11. Pro*	*14. Arg*	*17. Glu*	
3. Val	*6. Phe*	*9. Thr*	*12. Try*	*15. Lys*	*18. Met*	

(The symbols proposed by E. Brand [3])

A. A chromatogram of 19 amino acids, developed by the original technique with tert. amyl-alcohol. The solvent frontier has advanced abt. 50 cm in 3 days. The length of the chromatogram is abt. 13 cm. In experiments A—E the hydrolysate was treated with NH_3-vapour on the paper.

B. A chromatogram corresponding to A but obtained by the 'continuous developing technique' in 3 weeks. The length of the chromatogram is abt. 50 cm. It contains 9 one-acid spots.

C. A chromatogram of 19 amino acids obtained in 5—6 days with n-butanol and by the 'continuous developing'.

D. The same as in C after 8 days' development.

E. A chromatogram of 19 amino acids obtained in 5 days with benzyl alcohol and by the 'continuous developing'.

F. The same as in E, but this time the drop of hydrolysate was not neutralized with NH_3-vapour while applied to the filter paper.

the chromatogram is continued until the fastest moving compound has advanced near the pad. Using big sheets (60 by 60 cm) of Whatman no. 1 filter paper, the approximate developing times for amino acids are: in *tert.* amylalcohol 2—3 weeks, in *n*-butanol as well as benzyl alcohol 5—6 days. The corresponding chromatograms are to be seen in Fig 1.

The spots are identified best by known control mixtures. The rates of advance can be given, *e. g.*, by comparing the final distances of the compounds from the starting line with that of the fastest moving one; these values can be easily read by the use of the scale in Fig. 1, left, but even they — although considerably more consistent than the so-called R_f values — have not been found to be very reliable. The use of one 'complete mixture' containing all compounds that can be in question and of several 'partial mixtures' as controls on the very paper on which the unknown mixture is run eliminates all errors. Such a set of controls is in Fig. 1, B.

RESULTS

Tert. amylalcohol (Fig. 1, B) has the best resolving power. Eight amino acids are completely separated as individual spots (*leucine*, *isoleucine*, *phenylalanine*, *methionine*, *valine*, *tryptophan*, *tyrosine*, and *proline*). *Hydroxyproline* can be identified by its yellowish colour only in the absence of threonine. *Threonine* and *alanine* are often partially separated allowing a sure identification. When there is a marked predominance of either of them, an individual identification is impossible. *Glycine* and *serine* form one spot in common. *Histidine* is not usually completely separated from glutamic acid but if it is present in abundance, it can be identified with certainty, because of its nice grey colour with ninhydrin. We have identified even minute amounts of histidine by using a duplicate chromatogram and the Pauly reaction, a more sensitive colour reaction for histidine than that with ninhydrin [4, cf.2]; this reaction can be very easily made on filter paper and it is in this case quite specific, tyrosine being in another part of the chromatogram. Even *glutamic acid* can often be identified as in Fig. 1, B, but the other 5 amino acids form one complex spot.

n-Butanol (Fig. 1, C) has a resolving power nearly equal to *tert.* amylalcohol. After developing for eight days the first three amino acids have entered the pad (Fig. 1, D), the other amino acids being somewhat more effectively separated. This is advantageous especially if their quantitative determination [5] is in question.

When the original technique is used, one of the most effective solvent combinations in *two-dimensional* chromatography is perhaps phenol-collidine.

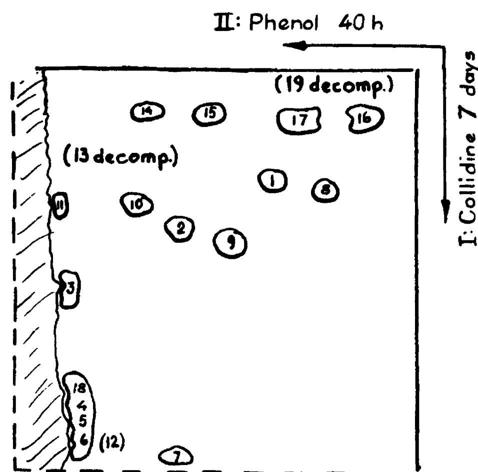

Fig. 2. Diagram of a two-dimensional chromatogram (abt. 50 by 50 cm) of 19 amino acids, obtained with phenol (40 h) and s-collidine (7 days). The absorbing pads torn away. Collidine run first.

By the new technique a somewhat more effective separation can be obtained in the direction of collidine when the development is continued for seven days. The leucines, phenylalanine, and methionine still form a complex spot but most of the other amino acids are completely separated from one another. Histidine and cystine are decomposed by collidine (Fig. 2.) This mode of technique is practical only if a one-dimensional test is made simultaneously with some alcohol and by the continuous development.

The new technique gives good possibilities for alcohols as solvents in two-dimensional chromatography. An example is given in Fig. 3. The location of some compounds not usually present in protein hydrolysates is marked by dotted lines in Fig. 3, A. — The use of alcohols as solvents is to be preferred especially in the quantitative paper chromatography [5] because they cause less decomposition of amino acids than solvents such as collidine, pyridine, *etc.*

GENERAL REMARKS

The great influence the 'reaction' of the system has on the flow of the amino acids has been shown already by Consden, Gordon, and Martin. For the sake of conformity in this respect the drops of hydrolysates and control mixtures have usually been put on the filter paper over an open Petri dish containing ammonia. Without this neutralization the chromatograms may have rather different structures. An example (benzyl alcohol) is to be seen in Fig. 1, E and F. The arrangement of the neutral amino-acids is changed. — In phenol lysine, arginine, and glutamic acid are the most sensitive ones.

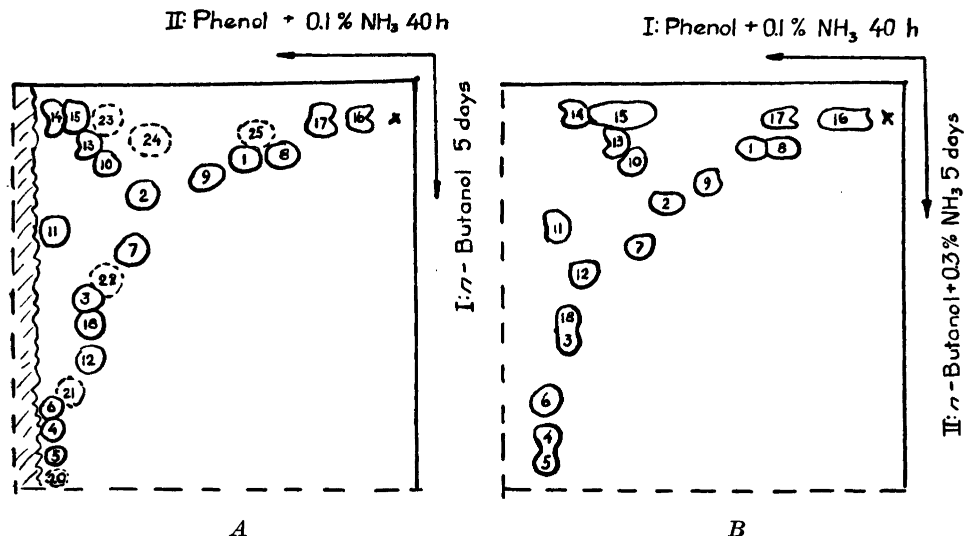

Fig. 3. Diagrams of two-dimensional chromatograms (abt. 40 by 40 cm) of amino acids obtained with phenol + 0,1 % NH_3 (40 h) and n-butanol (5 days). (A) Butanol run first; (B) butanol + 0.3 % NH_3 (5 days); phenol run first.

20. Norleucine	*22. a-Amino-isobutyric acid*	*24. Glutamine*
21. Norvaline	*23. Ornithine*	*25. Asparagine*

We have used exclusively Whatman no. 1 filter paper. The two-dimensional experiments have been made in a special constant-temperature room (20° C). Glass-sided boxes (aquaria) of 40 by 70 by 70 cm containing 4 throughs and automatic filling apparatuses have been used. The one-dimensional experiments have been made in stoneware drainpipes [1]; constant temperature is not necessary in this case. Ascending technique [6] has been found practical for preliminary experiments because it does not require much work. For instance, with isobutyric acid a short chromatogram is obtained in an over-night experiment giving a good idea of the complexity of the material to be analyzed.

SUMMARY

1. A 'continuous developing technique' in descending paper chromatography is described, in which a thick pad of cellulose tissue capable of absorbing a large amount of solvent is fastened at the foot of the strip of filter paper by stapling or sewing. The development is continued until the fastest moving compound nearly reaches the pad, or even longer. The identification is made by the use of several differently composed control mixtures.

2. Developing times for amino acids in various solvents and the structures of the corresponding chromatograms are given.

3. *Tert.* amylalcohol has the best resolving power: in 2 — 3 weeks 8 amino acids (leucine, isoleucine, phenylalanine, methionine, valine, tryptophan, tyrosine, proline) are completely separated from protein hydrolysates as individual spots.

4. Being relatively inert solvents the alcohols cause less decomposition than collidine, isobutyric acid, pyridine, *etc.*

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ADDITION TO PROOF.

Using Whatman no. 4 filter paper the developing times are reduced to half of those above, but the spots are sometimes less sharp.

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