QUANTITATIVE PARTITION CHROMATOGRAPHY AND THE COMPOSITION OF *E. COLI*

by

A. POLSON*

Laboratory of Physical Biology, National Institutes of Health, Bethesda 14, Maryland (U.S.A.)

Partition chromatography requires such small amounts of material that it can be used in the successful analysis of purified viruses and other biological complexes and proteins that can only be obtained in milligram quantities. This paper is a description of those modifications in the filter paper chromatographic techniques of CONSDEN, GORDON, AND MARTIN[1] which we have already shown, in the case of silk fibroin, to yield quantitative results[2]. Chromatography was begun in this laboratory as a means of gaining information about the relative composition of certain bacteriophages and their bacterial hosts; and therefore many of our quantitative procedures have been developed using bacteria for analysis. Results reached during this work on the composition of amino acids in hydrolysates of *E. coli* are included in this article.

METHODS

The essentials of the partition chromatographic methods are as follows. When a drop of a mixture of amino acids is dried on a sheet of filter paper and a solvent saturated with water allowed to creep past the spot, the different amino acids distribute themselves between the stationary water phase, saturated with solvent, in the paper, and the mobile solvent saturated with water phase that creeps through the paper. Due to their different partition coefficients between the solvents the amino acids are transported down the paper at different rates and thus arrange themselves in a characteristic way, which is visible after development with ninhydrin.

To obtain a complete qualitative analysis of an amino acid mixture, CONSDEN, GORDON, AND MARTIN[1] developed two dimensional chromatography. In this method a drop of protein hydrolysate or amino acid mixture is placed near one corner of a sheet of filter paper. One side of the paper near the spot is then immersed in a water-saturated solvent and the solvent allowed to creep down the paper while it is held in an atmosphere saturated with both solvent and water-vapor. When the first solvent has reached the bottom, the sheet is taken out, dried in an oven at 100° C, turned through 90° and another solvent used. The paper is again dried, sprayed with 0.1% ninhydrin solution in normal butanol and heated for 5 minutes at 100° C. After this treatment, colored spots appear on the filter paper wherever amino acids are present. They are distributed in a predictable fashion in positions that depend on the pair of solvents used for the resolution of the amino acid mixture. Suitable solvents for this purpose were found to be phenol saturated

* Special Fellow, National Institutes of Health, U.S. Public Health Service Permanent Address: Veterinary Research Institute, Onderstepoort, Union of South Africa.

References p. 581.

with water containing 0.3% NH_3 in the one direction and collidine saturated with water in the other direction. Phenol and collidine resolve all the amino acids except methionine, isoleucine, leucine and phenylalanine; and these can be separated using collidine and metacresol as solvent pair.

One Dimensional Analyses

After the qualitative composition of a hydrolysate has been established from a consideration of the one and two dimensional chromatograms it yields, the following procedures can be employed to estimate the concentration of each amino acid. The first is based on one dimensional analyses, using for standard comparisons a synthetic mixture containing 5 mg/ml of each amino acid present. It involves three steps described below under (a), (b) and (c).

a. Estimation of aspartic and glutamic acids, serine, glycine, threonine and alanine. A series of dilutions of the standard mixture ranging from full strength to 0.05 × full strength is applied in equal sized drops to a line drawn 12 cm from the edge of a square sheet of filter paper. The solution to be analysed is applied at several dilutions as drops along the same line. After neutralisation in NH_3 vapor of any hydrochlorides in the standard solution or in the hydrolysate, the edge of the paper is lowered in a water saturated meta-cresol solution containing 0.3% NH_3 and a one dimensional chromatogram is formed as previously described. At the end of the run, the paper is dried in a hot air oven at 100° C, sprayed with ninhydrin and again warmed for 10 minutes at 100° C. When held against a uniform source of illumination, comparisons can now be made between the intensities of the spots due to the unknown solution and those of the standard mixture. Since the concentration of amino acid in the standard is known a simple calculation will give the concentration in the unknown. For example, if dilution 0.3 of the standard matches with dilution 0.7 of the unknown and the concentration of amino acid in the standard is 5 mg/ml then the concentration in the unknown solution is $\frac{0.3}{0.7} \cdot 5 = 2.14$ mg/ml. Ordinarily a match can be obtained for each dilution of the unknown; their average is taken as the determined concentration.

Methods more elaborate than these visual comparisons were tried. For instance, an attempt was made to measure intensities of light reflected from the spots using a Beckman photometer. Neither this nor other procedures tried, however, gave better results than the simple visual comparisons.

b. Estimation of proline, tyrosine, methionine, valine, tryptophane, proline, leucine, isoleucine and phenylalanine. These amino acids can be determined from a similar chromatogram run in benzylalcohol saturated with water. The slow movement of the amino acids and the consequent low R_F values make it necessary to leave the paper in this solvent for at least 5 days. Glutamic, aspartic acid, serine, glycine, threonine and alanine as well as the basic amino acids are nearly stationary, but tyrosine, proline, tryptophane, valine, methionine, phenylalanine, leucine, isoleucine and tryptophane move out to form separate spots which can then be determined by color matching against standards prepared as described under (a) and run on the same paper as the unknown.

c. Estimation of the basic amino acids, arginine, lysine and histidine. The basic amino acids offered special difficulty because they are always associated with other amino acids whatever the solvent used. They can, however, be determined by the following composite procedure. A paper with the dilution is first placed in a solvent like

References p. 581.

meta-cresol plus 0.1% NH_3 or phenol plus 0.1% NH_3 that transports the bases as well as the other amino acids. The paper is then dried, turned through 180° and put in a solvent that will move all the contaminating amino acids away while leaving the bases stationary. Suitable solvents for this purpose are the organic alcohols amyl alcohol, butanol, isobutylalcohol and benzylalcohol. The whole procedure takes about a week to complete and the spots become somewhat diffuse during this period; nevertheless, this does not seriously interfere with a quantitative determination of the amino acids since standard mixture and unknown receive the same treatment.

Two Dimensional Analyses

Excellent quantitative results have been obtained by applying dilutions of the standard and the unknown to different sheets of filter paper and developing two dimen-

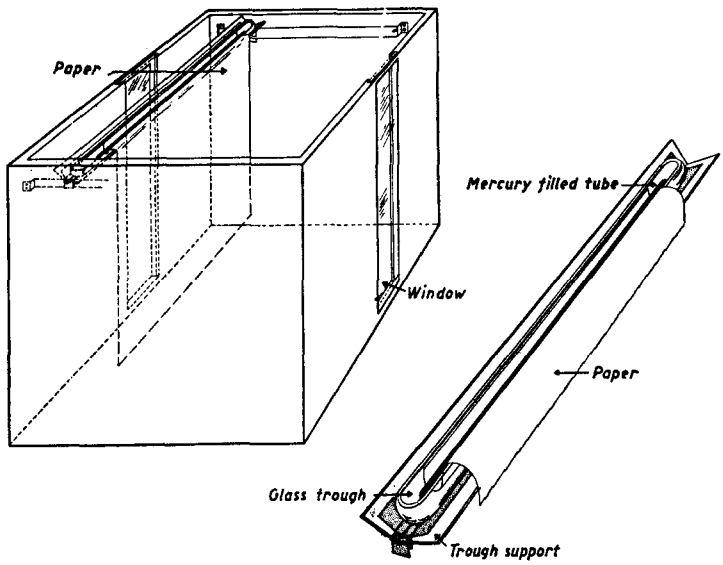

Fig. 1. A schematic drawing showing the construction of the metal tank for making chromatograms and the troughs used in supporting filter papers within this tank.

sional chromatograms from them simultaneously in a closed tank. For this purpose a large tank was constructed to hold eight glass troughs. Two sheets of filter paper were suspended from each trough and thus eight dilutions, each of unknown and standard, could be run under identical conditions.

The tank (Fig. 1) was constructed of angle iron and stainless steel sheets with two glass windows for observations and a glass lid that could be made air-tight by using plasticine. Glass troughs were supported by a metal trough and strips of glass as indicated. These metal troughs were held in position by two metal strips attached to the sides of the tank. Several pairs of solvents were used in making complete quantitative analyses. Phenol plus 0.3% NH_3-collidine gave complete resolution of all the amino acids except the group methionine, isoleucine, leucine and phenylalanine. These four acids were resolved in meta-cresol plus 0.3% NH_3 and collidine, or in collidine and an equal mixture of n-butanol and benzylalcohol.

References p. 581.

RESULTS

These methods of analysis have been applied to hydrochloric acid digests of *E. coli*. The organisms were grown for 18 hours on 0.5% casein digest agar slants, collected and washed four times by centrifugation and resuspension in saline. The final suspension in a small volume of saline was dialysed against a large volume of saline for two days at 90° C with frequent changes in the outer fluid and then against distilled water at 90°C to remove the salt present. Concentrated HCl to give a final concentration of 20% HCl was added to the final dialysate which was then sealed off in a tube and kept at 110° C for 24 hours, to provide complete hydrolysis. Excess hydrochloric acid in this digest was removed by repeated vacuum distillation. Addition of distilled water produced a dark-brown precipitate which was centrifuged off and discarded. After dilution to a nitrogen content of about 5 mg N/ml, this digest was ready for chromatography. Since acid hydrolysis destroys tryptophane, this amino acid was determined in another $Ba(OH)_2$ digest.

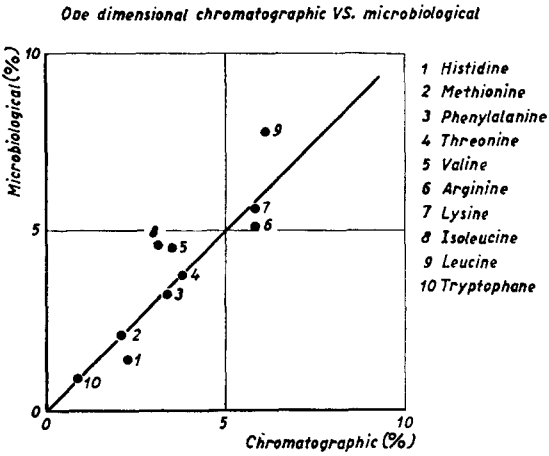

Fig. 2. A chart comparing results of one dimensional chromatographic analyses of *E. coli* with recent microbiological analyses for the same amino acids.

Results of one and two dimensional analyses of *E. coli* hydrolysates prepared in the foregoing fashion are collected in Tables I and II. In Figs 2 and 3 and in Table III they are compared with the recent microbiological assays of STOKES AND GUNNESS[3]. For the ten amino acids determined biologically, the agreement has been good, especially when two-dimensional chromatography was used.

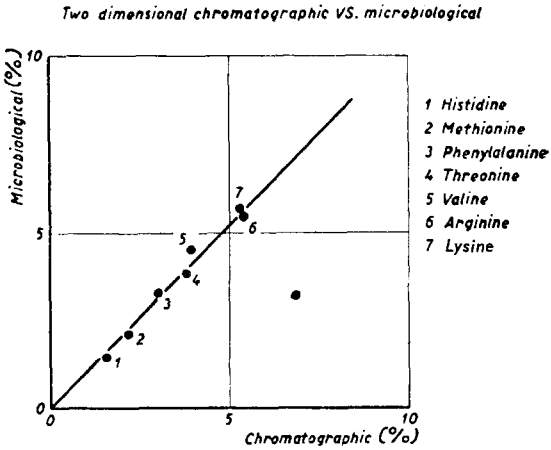

Fig. 3. A chart comparing results of two dimensional chromatographic analyses of *E. coli* with recent microbiological analyses for the same amino acids.

The slight disagreement between the one dimensional chromatographic method and the microbiological technique can most probably be explained by incomplete separation of the amino acids in one direction only.

In conclusion it can be remarked that when a choice of method of analysis is to be made, it must be borne in mind that apart from equal accuracy the chromatographic method described above is superior to the microbiological method in two respects. Firstly, the quantity of hydrolysate necessary for analysis by the chromatographic method is much smaller than

References p. 581.

TABLE I

ONE DIMENSIONAL ANALYSIS OF *E. coli*[*]

Digest	1	2	3	4	5	Average
	% of total protein					
Solvent = Meta-cresol 0.3 % NH_3						
Aspartic acid	—	6.7	—	6.82	—	6.76
Glutamic acid	6.69	6.73	6.9	7.2	6.32	6.77
Serine	3.76	3.34	3.76	3.0	3.21	3.45
Glycine	5.52	5.60	5.34	5.42	6.2	5.61
Threonine	3.60	3.87	4.16	3.60	3.51	3.75
Alanine	6.02	5.89	6.14	5.9	5.71	5.93
Solvent = Benzylalcohol						
Valine	3.3	3.28	3.16	4.74	3.2	3.53
Methionine	—	1.7	—	2.43	—	2.06
Phenylalanine	3.72	3.49	3.16	2.91	3.69	3.39
Isoleucine	3.30	3.47	3.3	—	3.00	3.27
Leucine	6.2	6.21	—	—	6.0	6.13
Tryptophane	—	0.91[**]	—	—	—	0.91
Proline	2.17	2.22	2.17	2.13	1.98	2.13
Tyrosine	2.98	3.17	3.07	2.90	3.16	3.06
Solvent = Meta-cresol plus 0.3 % NH_3 for one day in one direction then Benzylalcohol for four days in the reverse direction						
Arginine		5.56	5.82	6	—	5.80
Lysine		5.67	5.82	6	—	5.83
Histidine		2.29	2.42	2.2	—	2.30

[*] 5.2 mg N/ml (total protein 32.24 mg/ml). [**] Determined from $Ba(OH)_2$ digest of *E. coli*.

TABLE II

TWO DIMENSIONAL ANALYSES OF *E. coli*

Solvents = phenol plus 0.3 % NH_3 and collidine

	Digest		
	(1) %	(2) %	Average %
Aspartic acid	6.30	6.66	6.48
Glutamic acid	6.30	6.66	6.48
Serine	4.44	2.85	3.64
Glycine	4.44	4.73	4.58
Threonine	3.7	3.87	3.78
Alanine	5.18	6.02	5.60
α-amino butyric acid,	0.37	0.25	0.31
Valine	3.70	4.30	4.0
Proline	2.60	2.58	2.59
Histidine	1.48	1.77	1.62
Lysine	5.55	5.16	5.35
Arginine	5.55	5.37	5.46
Tyrosine	2.96	3.01	2.98
Methionine	—	2.15	2.15
Isoleucine[*]	—	—	—
Leucine[*]	—	—	—
Phenylalanine	2.96	3.08	3.02
Tryptophane	—	—	—

[*] Isoleucine and leucine were not separable in the pair of solvents used.

References p. 581.

TABLE III

COMPARISON OF THE RESULTS OF CHROMATOGRAPHIC AND MICROBIOLOGICAL ANALYSES OF *E. coli*

Amino Acid	Microbiological %	Chromatographic	
		One Dimensional %	Two Dimensional %
Tryptophane	0.90	0.91	—
Histidine	1.40	2.30	1.62
Methionine	2.10	2.06	2.15
Threonine	3.80	3.75	3.78
Phenylalanine	3.25	3.39	3.02
Isoleucine	4.60	3.27	—
Valine	4.50	3.53	4.00
Lysine	5.60	5.83	5.35
Arginine	5.20	5.80	5.46
Leucine	7.80	6.13	—

that required by the microbiological technique, and, secondly, where a single chromatographic run is usually necessary to establish the qualitative composition and approximate concentrations of the different amino acids, a considerable amount of work is necessary to gain this information by way of the microbiological method. However, a combination of the two techniques of analysis should provide a method of amino acid estimation which would be very difficult to surpass.

This work has been carried out in the laboratory of Dr RALPH W. G. WYCKOFF to whom I wish to express my gratitude for many helpful discussions.

SUMMARY

1. A quantitative chromatographic method of analysis on filter paper has been described. In essence it depends on comparing the intensities of spots of the amino acids of the unknown material with spots of a standard mixture of amino acids. This analysis was done in two different ways, *i.e.*, one dimensional and two dimensional.

2. In one dimensional chromatography aspartic acid, glutamic acid, serine, glycine, threonine and alanine can be determined by using as solvent meta- or para-cresol plus 0.3 % NH_3. Tyrosine, proline, valine, leucine and isoleucine can be estimated when benzylalcohol is used. The basic amino acids can be estimated by first separating the amino acids in meta- or para-cresol plus 0.3 % NH_3 on filter paper and by turning the filter paper chromatogram through 180° and by moving the contaminating amino acids away with benzylalcohol the bases can be separated and determined.

3. In the two dimensional chromatographic method different dilutions of the standard mixture and hydrolysate are placed on different filter papers and the papers run in the solvent pair-m- or p-cresol NH_3 and collidine, or phenol NH_3 and collidine. The amino acids are then estimated by comparing the spots on the unknowns with those on the standard mixture.

4. The methods of analysis have been applied to digests of *E. coli* in which the concentrations of the 17 amino acids present have been determined.

5. When the amino acid concentrations are compared with those determined by the microbiological technique on similar digests it was found that the agreement between the two methods was excellent.

RÉSUMÉ

1. Description de la méthode d'analyse quantitative par chromatographie sur papier. Cette méthode est basée sur la comparaison des taches correspondant aux acides aminés de l'échantillon à analyser avec celles correspondant à un mélange étalon des acides aminés; la chromatographie pouvant être uni- ou bidimensionelle.

2. Dans la chromatographie unidimensionelle, le dosage de: acide aspartique, acide glutamique, sérine, glycine, thréonine et alanine, se fait en utilisant comme solvant du méta- ou du paracrésol

References p. 581.

contenant 0.3 % de NH_3. Le dosage de: tyrosine, proline, valine, leucine et isoleucine, en utilisant l'alcool benzylique. Les acides aminés basiques peuvent être dosés en effectuant une première séparation à l'aide du méta- ou de paracrésol contenant 0.3 % de NH_3, puis en tournant le chromatogramme obtenu de 180°, et en achevant la séparation à l'aide d'alcool benzyline, ce qui permet alors le dosage.

3. Dans la chromatographie bidimensionelle, on place sur différents papiers des dilusions variables du mélange étalon et de l'hydrolysat à doser, puis on traite par les paires de solvants: m ou p-crésol, NH_3-collidine, ou phénol, NH_3-collidine. On détermine la concentration des acides aminés par comparaison des taches données par les solutions connues et inconnues.

4. Cette méthode a été appliquée à des hydrolysats de *E. Coli*, dans lesquels on a pu déterminer la concentration de 17 acides aminés.

5. Les résultats obtenus par cette méthode sont en excellent accord avec ceux fournis par la technique microbiologique appliquée aux mêmes hydrolysats.

ZUSAMMENFASSUNG

1. Eine quantitative chromatografische Analysemethode auf Filtrierpapier wurde beschrieben. In grossen Zügen beruht sie auf dem Vergleich der Flecke der Aminosäuren des unbekannten Materials mit Flecken einer Standardmischung von Aminosäuren. Diese Analyse wurde auf zwei verschiedene Weisen, und zwar ein und zweidimensional, ausgeführt.

2. Bei der eindimensionalen Chromatografie können Asparaginsäure, Glutaminsäure, Serin, Glykokoll, Threonin und Alanin bei Benutzung von meta- oder para-Kresol und 0.3 % NH_3 als Lösungsmittel bestimmt werden. Tyrosin, Prolin, Valin, Leucin und Isoleucin können bei Benutzung von Benzylalkohol bestimmt werden. Die basischen Aminosäuren können bestimmt werden, wenn man erst die Aminosäuren in meta- oder para-Kresol mit 0.3 % NH_3 auf Filtrierpapier trennt, dann das Filtrierpapier um 180° dreht und die verunreinigenden Aminosäuren mit Benzylalkohol entfernt.

3. Bei der zweidimensionalen chromatografischen Methode werden verschiedene Verdünnungen der Standardmischung und des Hydrolysats auf verschiedene Stücke Filtrierpapier gebracht und die Papiere mit den Lösungsmittelpaaren m- oder p-Kresol/NH_3 und Collidin, oder Phenol/NH_3 und Collidin behandelt. Die Aminosäuren werden dann durch Vergleichen der Flecke der unbekannten mit denen der Standardmischung bestimmt.

4. Die Analysemethoden wurden auf Verdauungsprodukte von *E. coli* angewandt, wobei die Konzentration der 17 vorhandenen Aminosäuren bestimmt wurde.

5. Die Ergebnisse dieser Methode stimmen ausgezeichnet mit denjenigen der microbiologischen Methode überein, wenn beide auf gleichartige Hydrolysate angewendet werden.

REFERENCES

1 R. CONSDEN, A. H. GORDON, AND A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224.
2 A. POLSON, V. M. MOSLEY, AND R. W. G. WYCKOFF, *Science*, 105 (1947) 603.
3 J. L. STOKES AND M. GUNNESS, *J. Bact.*, 52 (1946) 195.

Received July 27th, 1948