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The development of partition chromatography

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The subject of partition chromatography has now an extensive literature and I feel on an occasion such as this it is better to describe the development of the method rather than to summarize the present position or to attempt to put forward any of the more recent advances, which have been described elsewhere. Perhaps indeed, if enough histories, written while the ideas are still fresh in the minds of the people concerned, are available for a variety of discoveries or inventions, it may eventually be possible to lay down some of the principles required to facilitate the obtaining of fruitful results in scientific research in general. Clearly also the background of knowledge at the time the advance was made will be best understood if the history is as recent as possible.

Partition chromatography resulted from the marrying of two techniques, that of chromatography and that of countercurrent solvent extraction. All of the ideas are simple and had peoples' minds been directed that way the method would have flourished perhaps a century earlier. In fact the minds of laboratory workers seem to have been closed to countercurrent procedures, which were adopted in industry, e.g. in distillation and lixiviation, long before they were generally used in the laboratory. In industry the use of the countercurrent principle led to great economies in heat and solvents, and its value was obvious: in the laboratory such economies are of comparatively little importance and the more subtle point that by its aid a vast improvement in certain difficult separations could be effected was not appreciated. Indeed it is only within the last twenty years that high efficiency distillation apparatus has been available in the laboratory and twenty-five years elapsed after Tsvett's classical work on chromatography before the latter method was put to general use. Synge and I were fortunate to work at a time when the need for new methods was apparent to many people, so that our methods have gained general acceptance in ten years.

I was fascinated by fractional distillation as a method while still a school-boy, and built in the cellar of my home, which was my combined workshop and laboratory, distillation columns, packed with coke of graded size, some

five feet in height. They were made from coffee tins (obtained from the kitchen), with the bottoms removed, soldered together! Experience with them served me in good stead and by the time I graduated I had a good understanding of the problems of fractional distillation.

When later I began to do research at the Dunn Nutritional Laboratory on the attempted isolation of vitamin E, I applied similar ideas to the problem of extracting into ether the unsaponifiable matter from saponified wheat germ oil. The apparatus consisted of a twenty litre aspirator bottle placed on the flat roof of the building, connected at its bottom tubulure by means of a half inch glass tube with a twenty litre bottle. The bottom vessel was filled with ether, the top with the soap solution. This was set up in the afternoon and by the next morning the liquids had interchanged their position. Experiment showed that there was an extraction efficiency corresponding to about ten theoretical plates.

The next stage followed the reading a single sentence at the end of an American paper on the purification of Vitamin E, that a countercurrent fractional extraction machine was giving an improved product. Until then it had not occurred to me that an extraction column could be used to separate two substances of rather similar partition coefficient. I can still vividly remember the satisfaction of working out how this could be done using a long column with the material to be analysed injected in the middle, the two halves of the column, as it were, opposing each other, the ratio of rates of flow of the two solvents being chosen to lie between the reciprocals of the partition coefficients of the substances to be separated, so that the two substances left the column, one at one end and the other at the other end. I worked out the theory of the columns, unaware because of my deficient reading, that it had mostly been done before. I recognized that it could be done in a succession of separating funnels, but the labour involved was prohibitive for any large number.

It was then I think I discovered what I might call Martin's principle of scientific research, viz. "Nothing is too much trouble if somebody else does it". Now this is a very difficult principle to apply, particularly if one is a Ph.D. student. Since, however, it is immaterial whether the work is done by assistants or a machine, I decided to build a machine equivalent to an array of about 200 separating funnels and to use essentially the same arrangement as I had used in the extraction of Vitamin E from the soaps. This required in essence a tube some 60 metres long, and I devised a method of breaking up the tube into a number (45) of vertical sections each united to its neighbours

by two pairs of small-bore glass tubes, each containing a ball valve. Fig. 1 shows a diagram of a single unit.

A diaphragm pump with adjustable valves pumped into the column the two phases, one at either end and also provided an alternating flow which circulated the liquid from the bottom of one main tube to the top of the next through the small-bore tubes. Fig. 2 shows a picture of this machine and the author at the time. Using methanol and 60°-80° boiling point petroleum ether this machine had an efficiency of about two hundred theoretical plates. It was, however, like the American machine due to Cornish (details of which were published before I had finished mine), before its time. I could not obtain support to continue work on it and after using it for a Vitamin E purification, in the course of which I obtained the first evidence of the multiple nature of Vitamin E, the machine was never used again. Since I failed also to publish my Vitamin E results the whole of the effort put in this work appeared to have been wasted.

The next two years I spent looking after pigs on diets deficient in the B₂

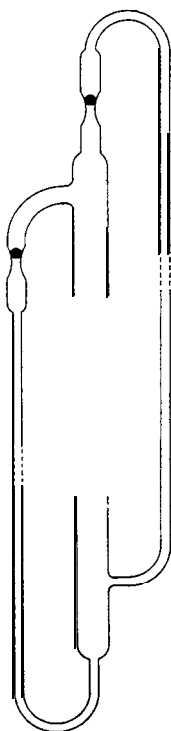


Fig. 1.

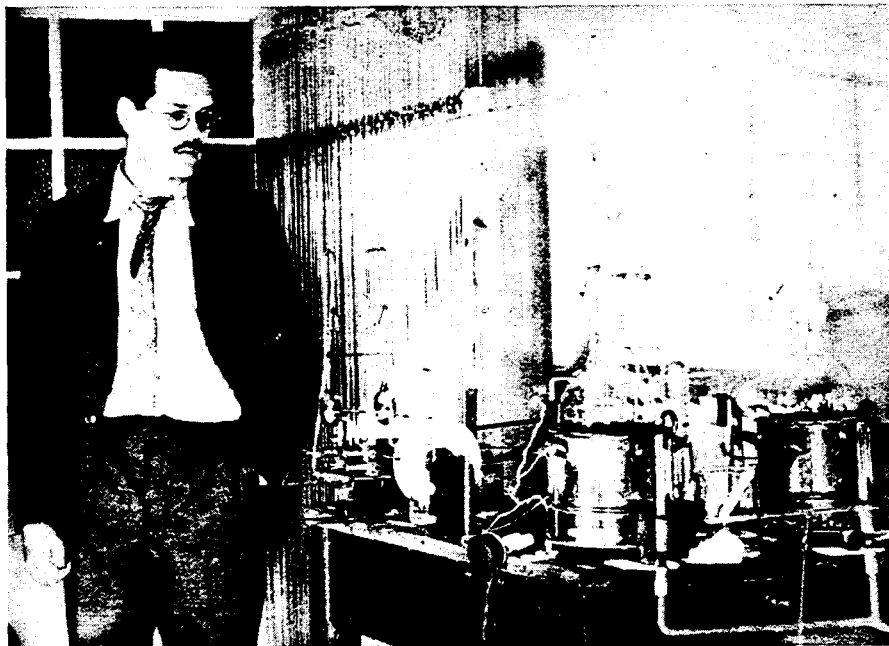


Fig. 2.

group of vitamins. This work was done under the close supervision of Sir Charles Martin, whose guidance has been of inestimable value to me. It was he who suggested that I should meet Synge and perhaps collaborate on the problem of separating acetyl amino acids.

Synge had already shown that the acetyl amino acids had partition coefficients between chloroform and water, which were different enough to permit of good separations if a high efficiency countercurrent extraction machine were available. Unfortunately the machine I had built was not suitable for chloroform and water, and methanol and petrol ether were not very good for the acetyl amino acid separation.

We decided, therefore, to build another apparatus for the purpose. I built a rough three plate machine of entirely different type, with stirring and settling compartments, as a model for a forty plate machine, which we decided would suffice for our needs. Synge had the forty plate model constructed in the Biochemical Laboratory at Cambridge. Meanwhile I had moved to the Wool Industries Research Laboratories at Leeds. The machine when complete needed modification, so Synge moved to Leeds so that we

could work on it together. After a lot of work we were able to determine the more fatty amino acids in wool, with much greater accuracy than had been done before. The machine, however, was very troublesome to use and required nursing day and night for several days at a time.

A better machine was needed before the method could be of general application, and I made dozens of new designs, which I put up in turn to Synge, who became very tired of listening to me. But none of them seemed easy to make, and no construction was undertaken.

I then had an idea of a radically different kind. This was to pack a tube with a mixture of wool and cotton, with the fibres parallel to the axis of the tube. Now cotton is wetted preferentially by water and wool by chloroform. By putting chloroform above and water below I hoped that I should obtain a large number of parallel streams of the two solvents with a large area of contact. When I tried it out I was very disappointed to find that large domains went over to one solvent or the other and the arrangement was actually less efficient than an unpacked tube.

Up to this time my thinking had been dominated by the idea of moving the two phases in opposite directions simultaneously. But in considering the failure of the cotton and wool experiment I realized that it should be comparatively simple to hold one phase stationary and move only one. The arrangement would then be essentially a chromatogram. I put this idea to Synge and he was full of enthusiasm, and we decided to work on it at once.

We decided to try first silica gel, of the type used to dry balance cases, suitably ground up, to hold the water, and having packed a chromatogram tube with this, to run chloroform down the column. We tried first the separation of acetyl alanine and acetyl leucine. We were then faced with the task of determining what was happening and how to decide whether separation had occurred or not. We quailed at the thought of evaporating down and titrating a large number of cuts and the idea occurred to us of including in the water in the silica gel an indicator with an appropriate range to show the presence of the acetyl amino acid. We chose methyl orange and the first column we made up showed the separation we looked for, as bright red bands on an orange column.

We were greatly encouraged by this and resolved to do the experiment with more care. We carefully purified our chloroform. Then the red bands stuck at the top of the column, as a result we presumed of adsorption on the silica gel; indeed addition of alcohol to the chloroform made the column

behave as before. Alcohol was, of course, present as a stabilizer in the commercial chloroform we used in the first experiment.

I would like to take this opportunity to record here my belief that one should take a minimum of care and preparation over first experiments. If they are unsuccessful one is not then discouraged since many possible reasons for failure can be thought of, and improvements can be made. Much can often be learned by the repetition under different conditions, even if the desired result is not obtained. If every conceivable precaution is taken at first, one is often too discouraged to proceed at all.

We soon found that a batch of commercial precipitated silica gave better columns, particularly after some fatty material had been extracted with alcohol. When, however, this batch came to an end we were unable to make the new batch work at all. The colour of the methyl orange was too red to show any change in the presence of the acids, and if the colour was made more yellow by addition of a base, though well-defined bands were seen at first, their intensity decreased as they moved down the column, and a large part of the acetyl amino acid was found to remain on the column. We then attempted to prepare our own precipitated silica by as many methods as we could think of, and finally arrived at an empirical method for its preparation from sodium silicate, which was reasonably satisfactory. It is of interest to note that in this method we found it necessary to carry out the precipitation in the presence of methyl orange. Dickey has since shown that an increased affinity for methyl orange can result from this; which was perhaps of assistance to us in preventing the methyl orange from being leached from the column by certain solvents. We found it was necessary to "age" the silica by standing with dilute hydrochloric acid for a day or two, in order to obtain a colour of column on which the acid zones were observable, but this ageing process could not be continued too long without so increasing the adsorptive power, that separation of the fast moving acids was unsatisfactory.

This method however has not been found to be satisfactory with other batches of sodium silicate, and an empirical approach has still to be made to the preparation of precipitated silica for any particular use.

It is of interest to consider how the method would have developed had we not thought of using an indicator on the column. The indicator enabled us to work rapidly and do many experiments, which were needed in the early stages, but most of our difficulties were due to the need to obtain a silica which gave a suitable colour with the indicator, and at the same time satisfactory separation of the acetyl amino acids. Had we thought of the titration

techniques such as are used at present, where hundreds of titrations can be carried out without undue labour, the problems to be solved would have been simpler, and the more extensive information gained of the behaviour of the column would have greatly improved the final method. I think it is indeed possible that the acetylated amino-acid method might have had a permanent place in analysis had we started with this technique. But at the time it seemed an unnecessary waste of labour to titrate hundreds of cuts from a single column when we thought we could collect each band separately.

About the same time at least two other investigators conceived essentially the same idea as we had had for this "partition chromatography". I refer to Sverre Stene in Norway and Van Dyck in Holland. They had, however, no problems they were immediately able to solve with its aid and consequently their work has passed comparatively unnoticed.

Since we had approached the partition chromatogram from the direction of fractional extraction, it was natural to apply an analogous type of theory to its operation. Once the concept of the height equivalent to a theoretical plate (H.E.T.P.) had been clarified (somewhat similar to that introduced by Peters for distillation in packed columns) it was easy to give the theory of the chromatogram for the case where the partition coefficient remained constant, and to guess qualitatively the result where the partition coefficient, because of ionisation, association or absorption on the silica, depended on the concentration.

Table 1.

	<i>R</i>	α <i>Direct</i>	α <i>Column</i>
Acetyl proline	0.37	9.5	9.4
Acetyl phenyl alanine	1.07	1.3	1.4

(Solvent CHCl_3 + 1% butanol.)

Synge and I were able to show that to a first approximation, the mono amino mono carboxylic acids behaved, under proper conditions in accordance with theory. They were not appreciably adsorbed by the silica, and the water in the silica had approximately the solvent powers of ordinary water. Table 1 shows the comparison of the partition coefficients deduced from the behaviour on the column and those measured directly for acetyl proline and acetyl phenyl alanine.

Gordon, Syngé and I developed the method for the analysis of the seven more fatty mono amino mono carboxylic acids using two types of column (Table 2).

Tristram continued with further work on this and his recoveries are shown in Table 3.

Gordon, Syngé and I were then unable to extend the method to other amino acids, and turned our attention to the separation of the amino acids them-

Table 2.

	Butanol Chloroform	Cyclohexane Propanol
Original mixture	→	→ Phenylalanine
	→	→ Leucine + isoleucine
	→	→ Tryptophan
	→	→ Valine
	→	→ Methionine
	→	→ Proline
	→	→ Tyrosine
	→	→ Alanine

Table 3. Percentage recovery of acetylamino acids from mixture of amino acids resembling hydrolysate of insulin (Tristram).

	<i>24-hours hydrolysis</i>	
	<i>before</i>	<i>after</i>
Phenylalanine	99 ± 2	96 ± 2
Leucine + isoleucine	102 ± 2	94 ± 2
Valine	98 ± 2	98 ± 2
Proline	93 ± 3	93 ± 3
Alanine	102 ± 2	104 ± 2
Tyrosine	95 ± 2	95 ± 2

selves, and not their acetyl derivatives. Here indicators could not be used on the column and a method of running a sewing thread beneath the column, (so that it was wetted by the effluent) and then winding it on to a slowly rotating screwed rod was tried. On treating the thread on the screwed rod with ninhydrin a record of the behaviour of the column was obtained. Using butanol and water, as suggested by the work of Dakin, and England and Cohn, we found that great absorption of the amino acid by the silica in-

terfered with the separations. The adsorption was of the Freundlich type causing a long tail to the bands.

Since, however, we could no longer use an indicator we were able to try again those substances as supports for water, which we had previously abandoned because we were unable to obtain a suitable change of colour of the indicator. We were thus led again to try cellulose as the water-holding medium.

I was already familiar with the use of filterpaper chromatograms as used by the dyestuff chemists and adopted at first their technique. A ten centimetre circular paper was cut to a semicircle with a three centimetre tail about one centimetre wide at the centre. On this tail near the centre was placed a drop of a solution of valine and leucine. The tail was put in butanol saturated with water contained in a Petri dish surrounded by water saturated with butanol in a glass jar. The semicircle of paper rested on the edge of the Petri dish. The top of the jar was covered with a glass plate. When the butanol had reached the edge of the paper it was taken out and dried and dipped in a solution of ninhydrin in ether. On heating two semicircles of colour were observable, and it was evident the method was a success. It was found quicker and more convenient to hang strips of paper from troughs containing the solvents, and this method was adopted as a routine. There followed a period in which we tried all the solvents we could lay our hands on, and logged the positions of the various amino acids in each of these. Experiment showed that the theory we had put forward for the silica columns applied equally well to paper chromatograms. It was indeed surprising with what closeness the solvent power of the water in the paper followed that of free water.

No one solvent, however, was able to resolve a mixture of all the common amino acids, and I tried to separate into acidic, neutral and basic fractions by ionophoresis, prior to separation in a direction at right angles by chromatography. The method adopted was rather clumsy. Each end of a square paper was treated with paraffin wax so that a centimetre wide strip of untreated paper was left, nearer one end than the other. This strip was moistened with acetate buffer and a drop of amino-acid mixture put in the middle. Copper wires were applied to either end of the strip and a potential of two hundred volts applied. After a couple of hours the paper was dried, the paraffin wax removed with petroleum ether and the paper developed as a chromatogram with water saturated butanol. A two dimensional separation of the amino acids was revealed on spraying with ninhydrin and heating. The

separation however of the neutral group of amino acids was not satisfactory. Attention was therefore concentrated on chromatography successively in two directions at right angles.

This two dimensional development of paper chromatograms, of the classical not the partition type, had already been done by Liesegang, but I was unaware of this until just lately.

At this stage Synge left us to go to the Lister Institute and I continued work with Consden and Gordon. At first finding good systems for two dimensional chromatograms was our main preoccupation. One problem caused us much worry. With butanol, phenol or benzyl alcohol, but not with isobutyric acid or collidine, the amino acids, particularly the faster moving ones, disappeared as they moved down the paper. As well as a purple spot, a continuous streak, starting from it but moving faster, of pale pink became visible after ninhydrin treatment. Sometimes the purple spot disappeared altogether. We thought this must be the result of oxidation or some other decomposition. Excluding oxygen did not improve matters. Then we found that phenol with ammonia was a particularly useful solvent, but here the paper rapidly blackened. We found in the literature that this was due to catalytic oxidation in the presence of copper. It was readily possible by including in the solvent or the atmosphere substances which formed complexes with copper, to eliminate this blackening completely. Simultaneously the "pink front" phenomenon disappeared. Thereafter we used a beaker of potassium cyanide in the bottom of the chromatogram box, or used a box which could be completely filled with coal gas.

There is enough copper in most papers to be able to show the effect, but in our case a large amount of copper was present in the atmosphere of the laboratory as we were using a motor in the drying cabinet, whose commutator sparked badly. (The copper complexes of the α -amino-acids do not give compact spots on paper and separation of the copper complexes is much more difficult than the separation of α -amino-acids. Dent, however, has shown that this complex formation on paper affords a ready method of distinguishing a from other amino acids.)

The high sensitivity of the ninhydrin reaction made it possible to do a chromatographic analysis with a few micrograms of any amino acid. So little labour was involved that it was possible for hundreds of analyses to be done simultaneously. Even in the case of unknown substances, something could be inferred of their nature from their behaviour in different solvents.

We were convinced that the method was by no means confined to amino

acids. Partridge spent a day or two in our laboratory and a few days after his return to Cambridge, wrote to us and told us that he had been able to separate reducing sugars, and detect them with silver nitrate. He found that practically the same solvent systems were satisfactory for sugars and amino acids. Later he worked out the methods for carbohydrates as completely as we had done for amino acids.

We found also that the method worked well for the simpler peptides, and decided to attempt to learn something of the structure of keratin, by the examination of partial hydrolysates of wool. This involved developing techniques for preliminary group separation of the peptides before the application of chromatography, since the mixture to be separated was of such extreme complexity. We developed the method of ionophoresis in silica jelly for this, preferring it to a chromatographic method because the fractions obtained would not then be chromatographically similar. We had also to work out the technique of washing peptide spots from the chromatogram, hydrolysing and returning to another chromatogram. We were able to do this without very serious losses using capillary tubes as vessels and a sheet of polythene, on which a drop of liquid stays, compact for evaporation and solution. We were able also to de-aminatate the terminal NH_2 group of a number of simple peptides to permit determination of nature of the end amino acid.

Synge at this time was working on the structure of gramicidin-S which he showed to be a cyclic polypeptide with one each of five different amino acids. This was an excellent test material for these methods; in collaboration we were in fact able by them to determine the order of the acids. Confirmation of this order was obtained using Sanger's dinitrophenyl end group method.

Sanger, in his recent remarkable achievement of the complete determination of the order of linking the amino acids in insulin, has shown how these methods may be developed for the much more difficult problem of the structure of a protein.

Elsden and Synge showed that potato starch could be used to pack a column and gave essentially the same separations of amino acids as did paper sheets. Moore and Stein showed that with careful control a complete quantitative analysis of a protein could be performed on starch columns using a ninhydrin calorimetric method. They also introduced the use of automatic fraction collectors which are now so widely used. Later the starch columns were superseded by ion-exchange resins. Their methods now provide the best means of protein analysis.

Many independent attempts have been made to put paper chromatography on a quantitative footing. Various sources of error have been discovered but so far the accuracy is inferior to the Moore and Stein method, though it may suffice for many purposes.

I will now return to the silica columns. For the acetylamino acids we used water as the stationary phase. So also did Elsdon, who adapted it for the lower fatty acids. Isherwood used sulphuric acid instead for the separation of hydroxy and dicarboxylic acids, since it was desirable to suppress their ionization. Levi used a buffer for penicillin separations, in this case to obtain a given proportion of the penicillin in the ionized form. These penicillin columns were widely used and kieselguhr rather than precipitated silica was found satisfactory in America. Its great advantage was that it was available commercially and was less variable than precipitated silica, and less likely to cause trouble due to adsorption. To get good columns, however, a new technique of packing the columns was required. This technique is not, however, difficult and I consider that for most purposes precipitated silica is now obsolete. Kieselguhr has an advantage in another respect. The pore size of precipitated silica is small enough to preclude its use for very large molecules, whereas that of the kieselguhr is relatively almost infinite. In fact, Porter and I have shown that it is possible to separate ribonuclease into two different fractions on a partition chromatogram using kieselguhr as support. Admittedly this is a small and atypical protein but Porter has extended its use to a few other proteins.

The surface of silica is wetted preferentially by the more polar of a given pair of liquid phases in equilibrium. So that in the usual column the mobile phase has to be the less polar one, and only those substances whose partition coefficient is in favour of the less polar phase, can be satisfactorily separated. By treatment of the silica with a silicone or silane the surface becomes preferentially wetted by the less polar phase. A "reversed phase" column thus becomes possible, suitable for the separation of substances which favour the less polar phase.

The first "reversed phase" chromatogram was a paper one loaded with rubber latex, and was due to Bolding. He also used columns packed with powdered rubber, for the separation of the higher fatty acids. Paper alone or silane treated, will hold a liquid hydrocarbon and give satisfactory chromatograms, with say aqueous alcohols as the moving phase.

Finally, I would like to mention gas-liquid chromatography. Synge and I suggested the possibility of this ten years ago in our first paper on the parti-

tion chromatogram. No one seems to have taken it up until James and I did so recently, to try and encourage ourselves after a very unsuccessful attempt to put fractional crystallization on a column basis. These chromatograms differ from the usual ones only in that the mobile phase is a gas instead of the usual liquid. They are suitable of course only for volatile substances.

Because gas is so mobile compared to liquid it is feasible to use much longer and thinner columns, and since diffusion is so much more rapid, to move the gas rapidly without increasing the H.E.T.P. It is thus possible to use columns with a very high separating efficiency. So far our work has been confined to substances which can be titrated, acids and amines. A more general method of detecting vapours in the gas issuing from the chromatograms should, however, make this type of column available for use with all types of substances that can be distilled at a pressure of a few millimetres of mercury. Whereas, with distillation, apparatus has at most several hundred theoretical plates, gas chromatographic columns with one to ten thousand plates are readily possible, and the quantity of material required for analysis is milligrammes instead of grammes.

It is very easy with the chromatographic apparatus to measure the free energy of solution of a given volatile substance in the stationary phase. I hope it will be possible to learn with its aid a great deal about the molecular forces involved in solution.