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Applications of partition chromatography

Nobel Lecture, December 12, 1952

This lecture is intended to depict some of the applications and consequences of the invention of partition chromatography. Before embarking on this, though, I should explain how I came to be taking part in the collaborative work which Dr. Martin has just described.

While still at school I heard tell of the science of biochemistry, which should draw together two aspects of nature into a common study. I had long been fascinated by living things (particularly the wild plants), and more recently by chemistry. This last was probably more for the appeal to the senses of the chemist's activities than for the knowledge of the nature of things which chemistry imparts. It was particularly reading in the newspaper Sir Frederick Hopkins' presidential address to the British Association for the Advancement of Science at Leicester in 1933 that impressed on me the idea that living things must have wonderfully precise and complicated working parts on the molecular scale and that the biochemist had the best chance of finding out how these are put together and do their work. As I was to begin studying natural science at Cambridge University in the same year, the ambition of studying in Prof. Hopkins' laboratory could easily be realized. Nevertheless, experiences in that laboratory in the elementary course taken by medical students did not give the impression of biochemistry being a refined or precise science. It was with a rather blind faith in the ultimate justification of Hopkins' ideas that, after several months of indecision, I embarked on the advanced course in his laboratory (Part II of the Natural Sciences Tripos). Here, immediately, one came in contact with exciting facts and exciting ideas. The latter outnumbered the former, usually. However, at an early stage in the course the student engaged in some quite rigorous isolative work under the guidance of Mr. N. W. Pirie. Pirie used to enliven the long hours at the bench with caustic anecdotes from the history of biochemistry which helped, quite as much as the isolative work itself and his comments thereon, to develop the critical faculty in those who possessed a rudiment of it. On graduating in 1936 I continued in the laboratory as a research student under

Pirie's supervision, and he suggested that I should make a chemical study of the glycoproteins, a class of substances then, as now, obscure in chemical nature and of great physiological interest. Soon I found my knowledge both of carbohydrate and protein chemistry was inadequate to the task, and began some synthetic work with Dr. D. J. Bell which involved preparing partially substituted derivatives of glucose. Among many useful things, I learnt from Bell the power of liquid-liquid extraction, with and without salting out, for separating methylated sugars according to the extent of their methylation. I returned to work on glycoproteins, with the idea of studying them by methylation. This brought me into contact with Dr. A. Neuberger, who was then working in Prof. C. R. Harington's laboratory at University College Hospital, London. Neuberger had developed an ingenious method for isolating the carbohydrate moiety from digested egg albumin by *N*-acetylating the digests and then removing *N*-acetyl amino-acids and peptides by exhaustive extraction with chloroform. He then *O*-acetylated the residue, whereupon the acetylated carbohydrates became extractable.

About this time, in 1937, Dr. Hedley R. Marston (then, as now, director of the C. S. I. R. Nutrition Laboratory at Adelaide, South Australia) came to the Biochemical Laboratory in Cambridge for a year as a guest. He was given bench space in the room where Pirie and I were working. He brought with him apparatus much more complicated than most workers in that laboratory were accustomed to, including an "artificial rumen" for microbial digestion of cellulose and a long fractionating column for distilling the esters of the resulting fatty acids. This by no means occupied his whole time, and we were impressed by the large number of letters which he wrote and by the frequent recurrence of his birthdays, which he celebrated by inviting numerous colleagues to very lively scientific discussions in a nearby public house. After about six months I began better to understand Marston's extramural activities when he told me that he was advising an organization called the International Wool Secretariat. This was given funds by the wool growers of Australia, New Zealand and South Africa for the purposes of publicity and research; they were worried both by the contemporary slump and by the advent of various synthetic fibres which it was then thought might prove to be effective substitutes for wool. Marston's advice was to apply some of their money to fundamental studies of the nature of wool, and he suggested that part should be given to me as a Studentship to study in detail the amino-acid composition of wool, beginning by improving the methods of amino-acid analysis. "If you work steadily at that for five years, you will revolu-

tionize the whole of protein chemistry" he said. The Studentship was on unusually generous financial terms, and as I also thought it would fit in with acquiring a more detailed knowledge of the protein side of the glycoprotein problem, I readily agreed. I began work in 1938 by studying the distribution of acetyl amino-acids between chloroform-water phases as a possible analytical procedure, directly suggested by Neuberger's experiments. The partition coefficients showed very encouraging differences, and I was advised from all sides to consult Dr. A. J. P. Martin as to how best to conduct the separations, since his bizarre-looking apparatus for purifying vitamin E at the Dunn Nutritional Laboratory had attracted widespread notice in Cambridge.

Dr. Martin has told you how our collaboration then developed. It might well have been effective in any case, but it was made enormously easier because Martin's appointment at Leeds had also been financed by the International Wool Secretariat and their scientific adviser, Sir Charles J. Martin, kept in close touch at all times with our work. Both during and after the time I was working in Cambridge I received much very valuable advice from Sir Charles and he made it easy for my Studentship to be transferred when it became desirable to join Archer Martin in the laboratories of the Wool Industries Research Association at Leeds.

I will discuss in most detail the application of partition chromatography to the study of proteins. Partly because of intense interest of biochemists in proteins and partly because the earliest work with the method was in this field, the results obtained with partition chromatography have been especially striking here, although the method has similar capabilities in other branches of biochemistry.

First, the method has given the possibility of exhaustive analysis of complete hydrolysates of proteins. For qualitative work the two-dimensional procedures using paper have been most convenient. For quantitative work the procedures using starch grains elaborated by S. Moore and W. H. Stein have permitted accurate and complete analyses on a few mg of hydrolysed protein (Fig. 1). Latterly these workers have found ion-exchange resins to have advantages over starch. Apart from the positive value to protein chemistry of having convenient methods for carrying out the fundamental operations, the use of partition chromatography has helped firmly to establish that the vast majority of proteins yield on hydrolysis only the well-known amino acids and that these account for the entire substance of the protein molecule. This generalization is fundamental for the future devel-

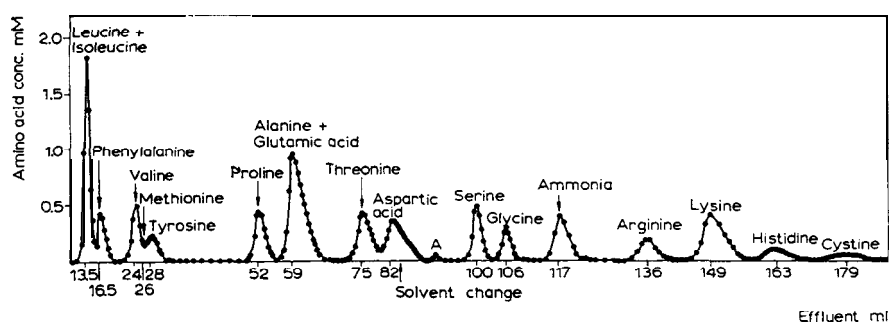


Fig. 1. Separation of a mixture of amino-acids on a starch chromatogram, as shown by analysis of the effluent. (S. Moore and W. H. Stein, *J. Biol. Chem.*, 178 (1949) 53.)

opment of the study of proteins and was too often taken on trust in the past.

In parallel with studies of amino acids in protein hydrolysates, partition chromatography has thrown much light on the free amino acids and related compounds found in living organisms and products derived therefrom. This work has shown how certain compounds are found in the tissue juices of all living organisms. Fig. 2 shows a two-dimensional chromatogram of potato juice, by C. E. Dent and colleagues. A number of new metabolites have been recognized and isolated by these means, and considerable light has been thrown on metabolic relationships, distribution of amino acids in different tissues and so forth. Unfortunately, the standards of identification used by many workers with paper chromatography have often been inadequate and the new meaning of the term "paper chemistry" is often not so far from the old one.

Partition chromatography has likewise been valuable for assessing the purity of amino acids and simple peptides and for studying the actions of enzymes so far as such simpler molecules are concerned. Previously unsuspected "transpeptidations" and syntheses have been revealed by this means.

The method has proved useful for the various procedures for allocating free functional groups within molecules of proteins or peptides; thus at present we have procedures for recognition of free amino groups by substitution with dinitrophenyl and other radicals, and of carboxyl groups by reduction to the corresponding alcohols or by enzymic hydrolysis of terminal amino-acids. These methods nearly always use partition chromatography as a final stage in the identification.

Finally, partition chromatography is of great importance for ascertaining the sequence of amino-acid residues in the peptide chains of proteins. Martin



Fig. 2. Two-dimensional paper chromatogram of an extract of potato, coloured with ninhydrin showing free amino-acids, etc. (C. E. Dent, W. Stepka, and F. C. Steward, *Nature*, 160 (1947) 682.)

and I had this use particularly in mind throughout our work. If a peptide chain is partially degraded to dipeptide and tripeptide fragments, etc. it should be possible, by identifying these, to recognize the original compound from which they are derived; thus in a simple case the peptide chain represented A-B-C-D-E will give rise to the fragments A-B, B-C, C-D, and D-E, permitting an unequivocal reconstruction. Martin and I, with R. Consden and A. H. Gordon, were able in this way, mainly using partition-chromatographic methods, to determine the amino-acid sequence in gramicidin-S, which is probably a cyclic decapeptide (Fig. 3). Subsequently F. Sanger and colleagues have elucidated by similar methods what may be the entire peptide sequences in the structure of ox insulin, the minimum molecule of which embodies 51 amino-acid residues. One of the two sequences established is shown in Fig. 4.

In the study of carbohydrates partition chromatography has played a very similar rôle. It can be used for determining ultimate hydrolysis products of polysaccharides qualitatively and quantitatively, it permits analysis of naturally occurring free sugars and their esters, and also the determination of the

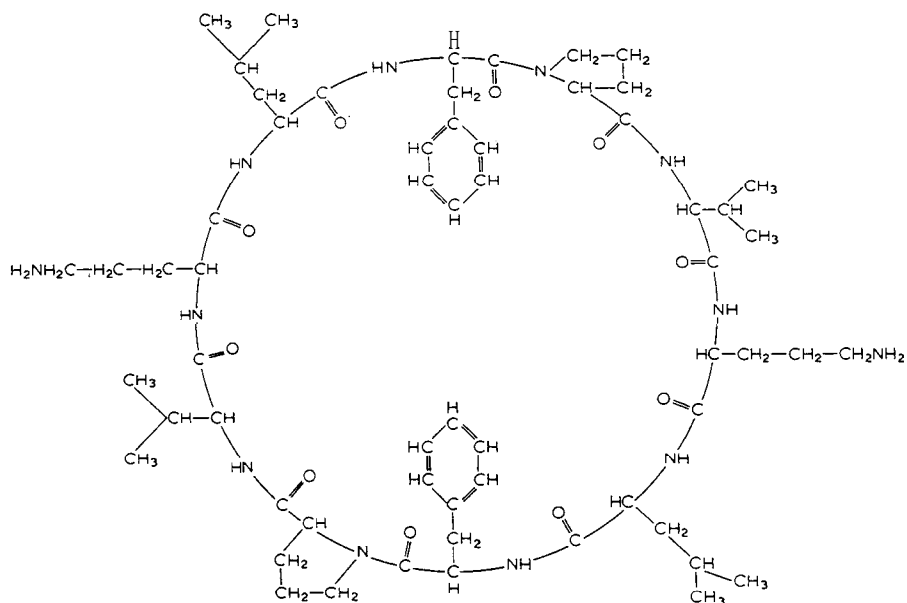


Fig. 3. Probable formula of gramicidin-S.

mode of linkage of the component parts of more complicated molecules by substitution methods or by partial hydrolysis methods. Special mention should be made of the discovery of series of complicated fructosan-like oligosaccharides in the juices of various plants by means of paper partition chromatography (cf. Fig. 5). Partition chromatography has also proved almost ideally adapted for analysing the hydrolysis products of methylated polysaccharides, and permits more accurate analysis with much smaller quantities of material, thereby greatly increasing the scope of the methylation method as a step in determining the structure of natural polysaccharides. It gives me great pleasure that my teacher D. J. Bell was the first to use the method for this purpose.

It is evident that partition chromatography has considerable application in the study of the nucleic acids and their breakdown products, the third great chemical family of biological importance. Here the method can fulfil a similar group of functions. However, owing to the ionic nature of so many of the compounds concerned, methods using electrical transport or chromatography on ion-exchange materials seem likely to assume greater importance with these compounds.

With the lipides there is not the problem of chemical study of giant mole-

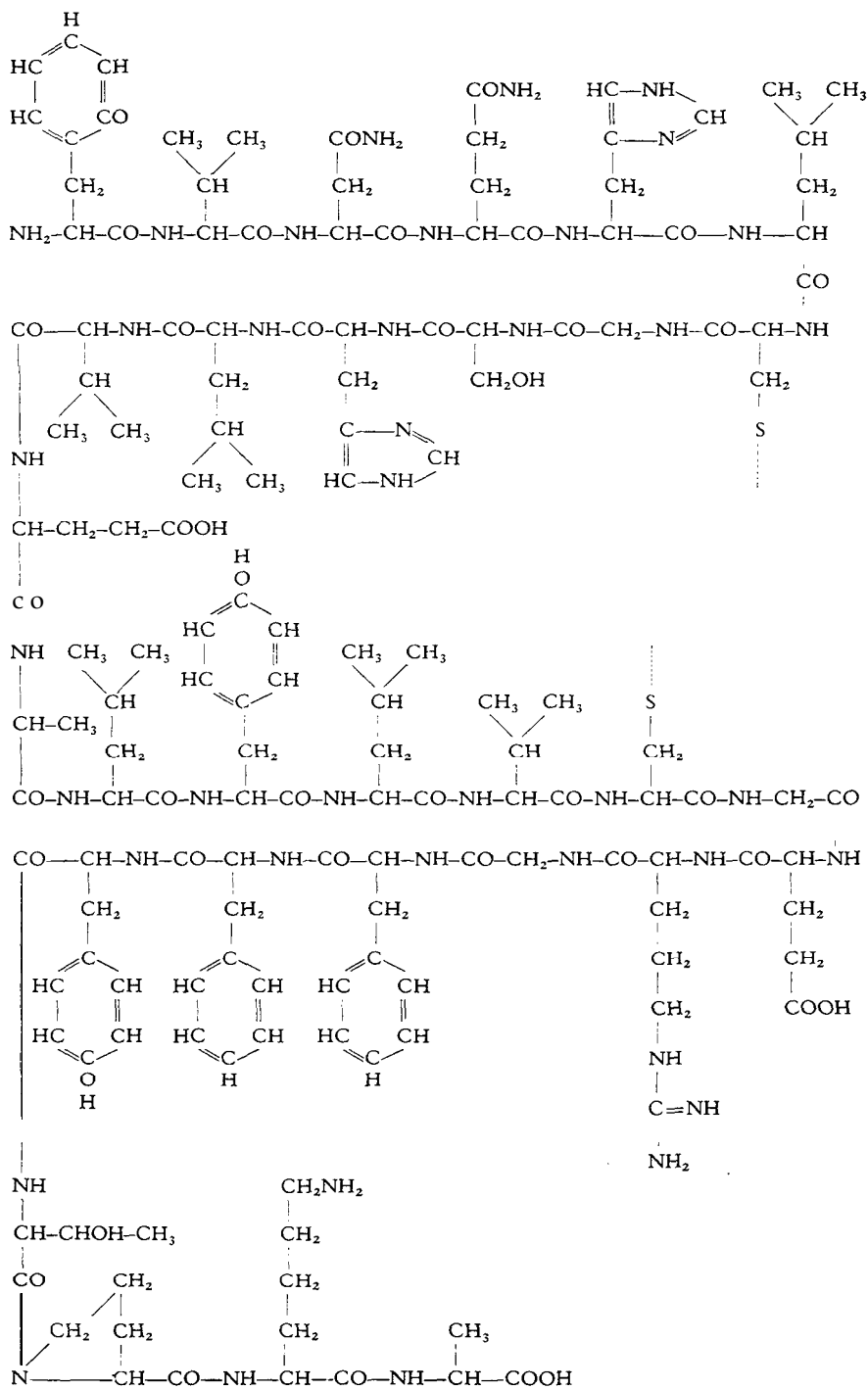


Fig. 4. Probable formula of the phenylalanyl chain in ox insulin.

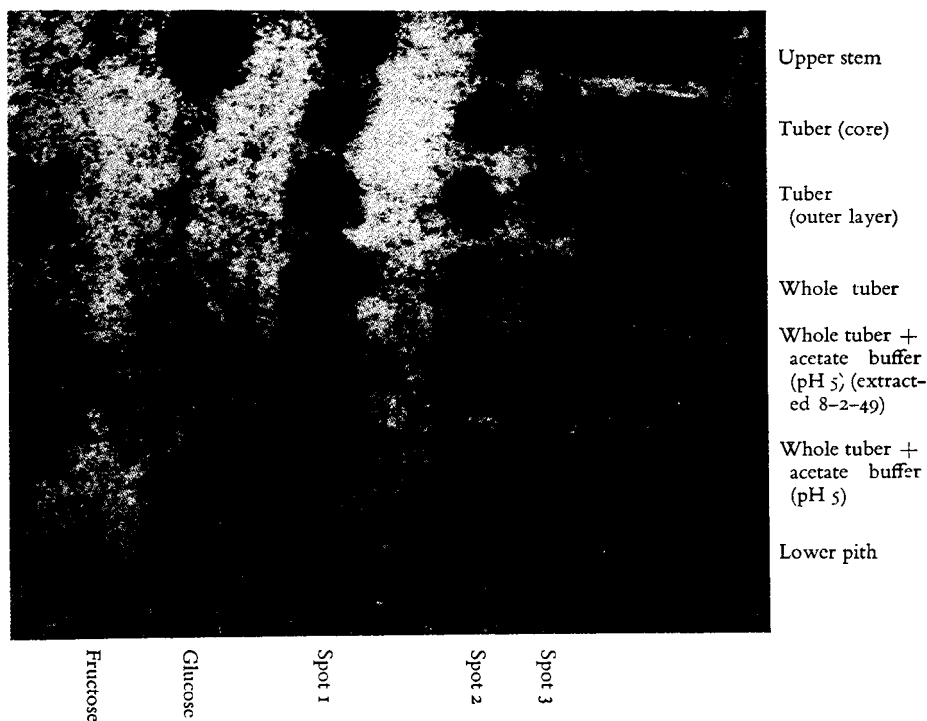


Fig. 5. One-dimensional paper chromatograms of extracts from the Jerusalem artichoke (*Heliunthus tuberosus* L.) showing fructosan-like oligosaccharides. (J. S. D. Bacon and J. Edelman, *Biochem. J.*, 48 (1951) 114.)

cules. However, partition chromatography is admirably adapted to effecting separations within homologous series, and the use of non-aqueous or multi-component two-phase liquid systems combined with the various procedures for determining which phase in the chromatogram shall be stationary have greatly increased the scope of the method for dealing with lipides in recent years. I would like here to pay tribute to the usefulness of gas-liquid chromatography as developed by James and Martin for analysing the lower fatty acids, which are the main nutrients absorbed by the ruminant animal from its intestinal tract. The possibility of rapid simultaneous analysis of all members of this group will help to increase our understanding of important problems in the physiology of these animals.

Partition chromatography has now been used for studying a wide variety of other substances of biological interest. Time prevents detailed discussion,

but mention should be made of alkaloids, glycosides, amines, alcohols, aldehydes, ketones, carboxylic acids and their derivatives, tannins and a wide variety of natural pigments, as well as many of the highly assorted chemical substances included in the groups coenzymes, vitamins, hormones and antibiotics.

Before leaving the biochemical uses of partition chromatography I would like to give an illustration of how the method can be useful in pharmacological and similar studies. R. A. Peters and colleagues have studied in detail the toxic effects of fluoroacetic acid on living tissues, and partition-chromatographic methods were used to show that fluoroacetic acid is transformed into another substance which acts directly on an enzyme system in the tricarboxylic acid cycle. The chromatographic behaviour further gave preliminary evidence of the chemical nature of the transformation compound (a fluorinated tricarboxylic acid) before this had been isolated in quantities suitable for chemical study. The fact that the method works just as well with ultramicro-quantities as on the ordinary scale implies that it has an important future for studying the mode of action and fate of substances of very high biological activity.

Outside biochemistry, it is probably the technological applications of partition chromatography that are most important. However, the method, particularly using cellulose, has proved useful for a wide variety of separations of metals and inorganic ions, and the fact that it can be used to handle minute quantities of carrier-free radioactive isotopes gives it considerable scientific importance. However, the main importance of the method seems to be towards making easier, cheaper and quicker the various analytical operations used in industrial research and routine control of production. The applications in metallurgy are obviously considerable, and the method is useful for control of intermediates in organic-chemical industry generally, whether concerned with dyestuffs, drugs, explosives, plastic materials, or other products. The gas-liquid chromatogram, representing fractional distillation pushed to its logical conclusion, obviously has an important future role in industrial research and control wherever mixtures of volatile compounds have to be handled, as in the fermentation, tar-distilling and petroleum industries. Here the method seems likely to compete with mass spectrometry. Except possibly with the most expensive drugs, partition chromatography has had little use as a method for actual production, on account of the large bulk of apparatus, chromatographic materials and solvents in relation to the substances being separated.

Partition chromatography facilitates in many obvious ways the work of those engaged in the control of foods and drugs, in clinical laboratories and generally in the safeguarding of the public health, while making more difficult the work of adulterators and other criminals. The dietitian and food technologist, as well as the pure biochemist, also gain by being able more cheaply and quickly to assay chemically a wide variety of foodstuffs for their essential constituents and to be able to ascertain the effects on these of processing, cooking and so forth. A striking recent use of the method was the isolation from flour processed with nitrogen trichloride ("Agene" process) of a toxic derivative of methionine, which was shown to be responsible for the "running fits" induced in dogs fed on this flour. For some reason governmental action towards prohibiting this treatment first occurred in our country when this chemical evidence became available. Irrespective of this particular case, one cannot help, in these days of increasing sophistication of foodstuffs, feeling proud at having aided the analyst in his increasingly complicated duties.

There are two new trends in analytical work which I must deal with briefly, although they are not essentially connected with partition chromatography. However, the fact that partition chromatography permits the separation on paper of such a wide variety of substances has made it of decisive importance for this work.

The first is the use of radioactive isotopes as tracers. Radioactive chromatographic zones are particularly conveniently studied on paper, either by scanning with a counter or by radioautographic contact printing. The use of radioactive tracers in combination with paper chromatography has enormously helped in discovering metabolic pathways in living organisms. The first studies of this kind were done with ^{131}I by R. M. Fink, C. E. Dent and K. Fink (1947). Since then a number of workers have elucidated the metabolism of this element in the thyroid gland, culminating with the recent observation by J. Gross and R. V. Pitt Rivers that triiodothyronine and not thyroxine may be the physiologically active form of the thyroid hormone. ^{14}C has of course a wider field of application in biochemistry; its most striking use so far in conjunction with chromatography has been the use by M. Calvin and colleagues of radioautographic printing of two-dimensional filter-paper chromatograms to follow the fate of ^{14}C from $^{14}\text{CO}_2$ during different time intervals after its assimilation by the photosynthesizing green plant. These observations, although not leading to any simple picture of the photosynthetic process, have provided a wealth of facts sufficient to disprove all

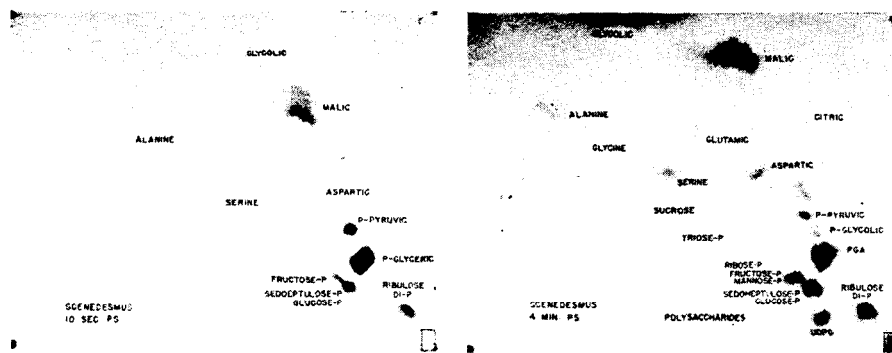


Fig. 6. Radioautographs of two-dimensional paper-chromatograms of extracts of *Scenedesmus* after 10 sec (*left*) and 4 min (*right*) photosynthetic assimilation of carbon dioxide labelled with ^{14}C . (*Courtesy of Prof. M. Calvin.*)

detailed theories of photosynthesis previously advanced and they suggest new directions for future experiments (Fig. 6).

Mention should also be made of the ingenious use of radioactive elements in a substituent grouping for extending the isotope dilution method of analysis to the ultramicro scale. A. S. Keston, S. Udenfriend and M. Levy substituted the unknown amino-acid sample with *p*-iodobenzenesulphonyl groups having radioactive I and added a known quantity of the synthetic derivative having radioactive S. Differential counting of ^{35}S and ^{131}I in a single spot on a paper chromatogram thus permitted accurate analysis for μg quantities of amino acid. The ratio of the activities at different regions of the spot provided rather rigorous evidence of the chemical identity of the compounds prepared.

The second special application of paper chromatograms is for locating substances affecting the growth of micro-organisms "bioautographically". Following the work of R. R. Goodall and A. A. Levi with the penicillins this procedure has been widely used in studying antibiotics and growth factors for normal and mutant organisms. An agar plate is impregnated with the test organism, and the developed paper chromatogram is laid on it. Diffusion of substances from paper to plate is allowed to occur at low temperature for a suitable time. The plate is then incubated and the different active substances are identified as zones of stimulated or inhibited growth (Fig. 7). Such procedures have helped quickly to reveal the complicated variety of chemical substances often responsible for a single biological effect. Striking examples are provided by recent research in the vitamin B_{12} and coenzyme A groups.

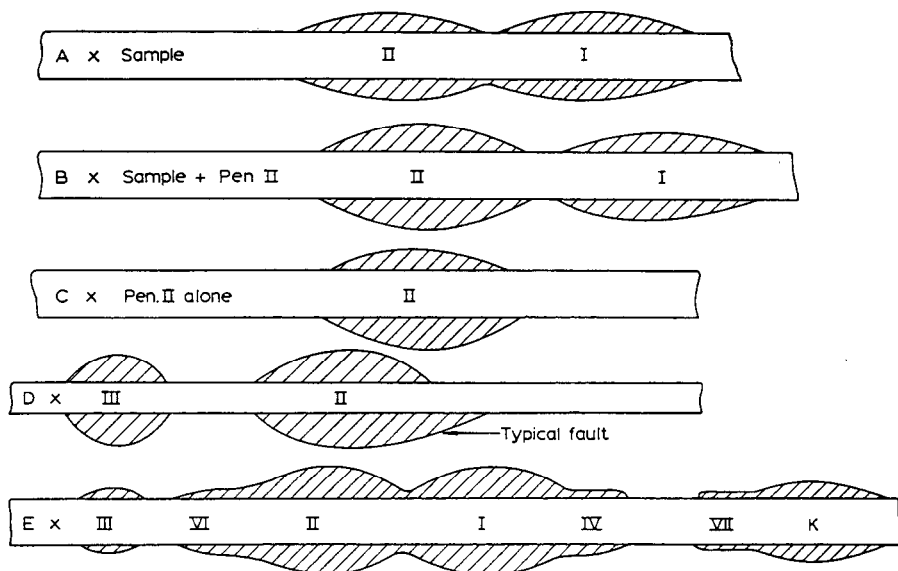


Fig. 7. Bioautographic detection in agar plates of zones of inhibition of bacterial growth due to penicillins separated on paper-strip chromatograms. (R. R. Goodall and A. A. Levi, *Analyst*, 72 (1947) 277.)

What I have said gives some idea of the scope and field of application of partition chromatography. But it does less than justice to the hundreds of workers who have helped in the past decade to explore rather thoroughly the possibilities of the method. One should bear in mind also the parallel development of bulk countercurrent distribution between two liquid phases for which L. C. Craig and colleagues have perfected the technique. While these procedures lack either the simplicity or else the resolving power of partition chromatograms, they will conveniently handle greater quantities of material and the form of the distribution curve can give a rather rigorous test of homogeneity. Craig's methods have another important advantage which I will deal with later.

It must further be remembered that these partition methods are only one group in a wide family of refined physicochemical separation procedures developed during the present century and especially during the past decade. Adsorption chromatography, using M. S. Tsvett's elution development and such agents as alumina and charcoal, is a very powerful method giving useful separations, in many cases where partition chromatography is not very effective. Its field of application has been considerably extended by the displace-

ment and carrier-displacement development procedures originated by A. Tiselius and colleagues. Further, it is only in recent years that the potentialities of the newer ion-exchange materials for chromatography have at all fully been realized. And leaving aside chromatography altogether, there are a variety of electrical-transport procedures which will effect refined separations of charged substances. With all these methods at his disposal it has become, for the analyst, essentially a routine matter to separate any pair of substances of molecular weight lower than, say, 500.

But biochemists, although they value these recently acquired powers, are not content with them. They are deeply concerned with purifying substances of still higher molecular weight and are finding the methods at present available to be increasingly inadequate to their requirements. Efforts to use partition chromatography for separating larger molecules have often been unsuccessful. Two main reasons for this are becoming apparent.

First, the larger molecules have either very high or very low partition coefficients in the simple two-phase two-component systems. The theoretical reason for this was clearly expounded by J. N. Brønsted in 1931. Consequently one must use more complicated, multicomponent systems with the larger molecules. This involves many trials and much error. Second, the solids most used hitherto for partition chromatography (cellulose, starch, silica gel, etc.) have pores of molecular dimensions which will not admit the larger molecules. Kieselguhr, where the spicules entangle droplets of liquid phase, is largely free from this defect, and it is surely no accident that the most successful separations of larger molecules by partition chromatography have been those of some simpler proteins by A. J. P. Martin and R. R. Porter using kieselguhr. The bulk distribution techniques of Craig and colleagues have also been successful with larger peptides and some smaller proteins. Here even the interface between the two liquid phases as well as the kieselguhr surface can very largely be eliminated as sites for adsorption.

However, despite these potentialities, it seems to me that the liquid - liquid methods are likely, when we come to these larger molecules, to be less easy of extension than the other techniques which I have mentioned. And there are also possibilities for new techniques based on diffusion, molecular-sieve effects and so forth. Irrespective, though, of the future importance to the biochemist of partition chromatography as an analytical technique, I think it will be found in future years to have had an influence on biochemistry which is not very widely appreciated at present, when so many biochemists are using it simply as an analytical method for skimming the cream of their

science. And here of course the method is giving to the biochemist something akin to what the microscope gave to earlier biologists, permitting a recognition of that characteristic interplay of diversity and uniformity that seems to be found in living things whether the student's measure is graduated in cm, μ or Å. But each R_f value or partition coefficient determined in each solvent system gives a new fact (or confirms a prediction) about the secondary-valence interactions of certain small molecules, and hence adds to the general body of knowledge of intermolecular forces. When the effects with small molecules are better understood we shall be much better able to understand from the structure of the larger molecules what is their function in terms of such intermolecular forces. Then chemistry really will begin to merge with biology as closely as it has already merged with physics. Partition chromatography may well be remembered for this contribution long after it is quite obsolete as an analytical method.