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MODERN ANALYTICAL CHEMISTRY IN THE SERVICE OF PHARMACY AND MEDICINE

As with chemistry itself it is impossible to trace the origins of chemical analysis which must have slowly developed from ancient times, through the Middle Ages, and, as a result of the efforts of the founders of scientific chemistry, to have burst into full development during the nineteenth century. One of the first recorded analytical reagents, however, must surely be Pliny's[1] reagent for testing for iron in verdigris by using a papyrus soaked in extract of gall nuts. Although modified by numerous workers[2] Pliny's test has survived for 2,000 years and is still used in vinegar works for the detection of iron in vinegar. In literature and art there are many references to early chemical operations and the practice of iatro-chemistry (the study of chemical phenomena in order to obtain results of medicinal value) during the sixteenth century led workers in medicine and pharmacy to be closely associated with chemical investigations. Indeed, it has sometimes been possible to gain valuable knowledge on chemical procedures from works of art, illustrating early pharmaceutical laboratories. It is therefore not surprising that pharmacists have always been associated with analytical chemistry and particularly with the examination of drugs and their preparations. The names of some early pharmacists have found a place in chemical literature. Scheele (1742–1786), the Swedish pharmacist most famous for his discovery of chlorine, was also author of the method of obtaining hydrogen sulphide from iron sulphide and acid, a reaction which every schoolboy must associate with chemical analysis. The French pharmacist Baumé (1728–1804) has his name associated with the hydrometer and Mohr (1806–1879) is famous for his invention of the burette.

During the nineteenth century it was realised that many vegetable drugs contained active principles and much effort was expended in attempts at their isolation. Pharmacists played a prominent part in this work and achieved notable successes, of which the greatest were probably the isolation of morphine by Sertürner[3] in 1805 and of quinine by Pelletier and Caventou[4] in 1820. It was a natural development that estimation of the active principles in vegetable drugs should be undertaken and gradually pharmaceutical analysis emerged as a section of analytical chemistry. From these early beginnings has sprung the analytical control of pharmaceutical products, largely covered to-day by official and semi-official books of standards, such as the British Pharmacopoeia and the British Pharmaceutical Codex. Much may be learnt of the advances in drug analysis during the past fifty years by an examination of the various

editions of these publications. The B.P. 1898 consisted of monographs dealing with descriptions of official drugs and methods for making their preparations, little attempt being made to assay the products or even to test them for impurities. Some advance was made in the B.P. 1914, but it was in the B.P. 1932 that definite steps were taken to test official preparations for impurities, to standardise them by the inclusion of assay processes and to adopt limits for the content of active ingredients. This trend has continued until to-day the Pharmacopoeia uses appropriate methods covering a wide field of biology, chemistry and physics for the standardisation of its preparations.

While the establishment of classical pharmaceutical analysis was due to the achievements of nineteenth century workers, the present time at the middle of the twentieth century, is appropriate for reviewing the work of later analysts and assessing their contributions to pharmacy and medicine. A close study of modern analytical techniques shows that their great contribution to chemistry has been their increased sensitivity resulting in a reduction in the amount of material required for analysis. This goal has been reached by the development of a number of distinct analytical procedures and it is some of these, having great influence on pharmaceutical progress, that I wish to discuss in the present address.

The Rise of Microchemistry

Like many other activities the development of microchemistry has experienced a series of sporadic and disconnected advances due to the efforts of forceful personalities who brought their ideas before the scientific world. In the case of microchemistry this has given rise to some confusion as to what the term means. Nearly all the early recorded attempts to devise microchemical methods were associated with the microscope, which was used to examine small samples, as an aid to their identification, or for the observation of chemical reactions between drops of solutions on a microscope slide. Thus microchemistry was often regarded as that part of analytical chemistry concerned with the study of chemical reactions with the aid of a microscope. This field, however, is what we would to-day describe as chemical microscopy.

It was the invention of the microbalance early in the present century which set the scene for the great growth of what is now universally known as microchemical analysis. The names of Emich[5], Kuhlmann and Pregl[6] will for ever be associated with this advance. It was when Pregl saw a microbalance constructed by Kuhlmann in Emich's laboratory that he realised that slight modification would provide him with a balance suitable for organic microanalysis. Soon Kuhlmann had made a balance capable of taking a load of 20 g. and weighing 1 microgram. This enabled Pregl to develop his system of microchemical analysis, employed particularly for the determination of carbon, hydrogen and nitrogen in organic compounds. His system was an immediate success and became used in laboratories all over the world.

There have been many modifications in technique since Pregl did his fundamental work and these have been described in terms which have caused no little confusion regarding the size of the sample used. The Committee on Nomenclature[7], Analytical Division of the American Chemical Society, has recommended that the prefixes *macro-*, *semimicro-*, *micro-* and *ultramicro-* should be differentiated according to the size of the sample. *Macro-* methods use samples of 100 mg. or more, *semi-micro-* methods use samples of less than 100 mg. and more than 10 mg., *micro-* methods use samples of from 10 mg. to 0·1 mg. and *ultramicro-* methods use samples of less than 0·1 mg. These recommendations have been widely accepted.

Some Applications of Microchemical Analysis

Progress in medicine and the allied profession of pharmacy is intimately associated with advances in organic chemistry, which has undergone such tremendous growth within living memory. From 1900 until the end of the first world war organic chemists added much to our knowledge of natural products such as alkaloids, sugars and proteins; the chemical constitutions of many of these were established and some were synthesised. Substantial quantities of these materials could readily be isolated and subjected to analysis by *macro*-methods. In the period between the two world wars a new note was struck and the organic chemist became increasingly interested in natural products of a different type. The hormones and vitamins became major topics of investigation and in every case these substances occurred in minute amounts in nature and much effort was needed in order to isolate even a few milligrams before chemical research on composition and structure could commence. Many of those who are familiar with the B.P. 1953 and are used to handling grams or kilograms of aneurine hydrochloride, ascorbic acid, riboflavine, progesterone, testosterone, oestrone and related products may not realise that 30 years ago these were available only in small quantities or had not yet been isolated. Those were stimulating days and I can well remember the hundredweights of yeast and rice polishings worked up for vitamin B_1, the crates of oranges and lemons for vitamin C and the gallons of urine, which arrived in milk churns from maternity homes, for extraction of sex-hormones. Some idea of the yields obtained can be judged from the fact that about 5 grams of vitamin B_1 was isolated from a ton of rice polishings. How then was it possible for the compositions and structures of these products to be determined and their syntheses achieved? It was the development of microchemical analysis which enabled such work to be carried out with the extremely small amounts of materials available and, as a result, these compounds, obtained by total or partial synthesis are now found in hospitals, surgeries and pharmacies throughout the world. Microchemistry is unlikely to make a more useful contribution to pharmaceutical chemistry than it did during those inter-war years.

Microchemistry consists of ordinary chemistry carried out on a small scale and its great value depends upon this factor. Some drugs are very expensive, particularly when first introduced into medicine, and

analytical control of such products may on this account be very costly. Microchemical analysis enables the drug needed for analysis to be reduced to about 1/20th of that required by *macro*-methods and a substantial reduction in the cost of analysis is thereby achieved. The advantages of micro-methods were quickly appreciated by the British Pharmacopoeia Commission, for in the monograph on Ergotoxine Ethanesulphonate, included in the B.P. 1932, it is stated under one test requiring 1 gram of drug that methods of microanalysis, if of equivalent accuracy, may be substituted for this determination. Similar concessions have now become generally accepted in other books of standards. Although not strictly analysis, the use of a microbalance to weigh International Standard Preparations for use in biological assays effects a considerable saving of valuable materials.

The scope of microchemistry in pharmacy is emphasised by Table I, in which are summarised the human doses of a number of potent drugs.

TABLE I

Drug	Human dose
Adrenaline	0·1 to 0·5 mg.
Methadone hydrochloride ..	5 to 10 mg.
Aneurine hydrochloride	2 to 5 mg.
Atropine sulphate	0·25 to 1 mg.
Carbachol	0·25 to 0·5 mg.
Vitamin B_{12}	50 to 100 μg.
Digoxin	0·25 to 0·5 mg.
Ergometrine maleate	0·5 to 1 mg.
Menaphthone	1 to 5 mg.
Picrotoxin	0·6 to 6 mg.
Stilboestrol	0·5 to 2 mg.
Physostigmine salicylate	0·6 to 1·2 mg.
Glyceryl trinitrate	0·5 to 1 mg.

Generally one dose will be the amount of drug contained in one tablet or a single dose ampoule of injection and it is immediately obvious that a large number of products will be required if analysis is to be attempted by *macro*-methods. In cases where only single tablets or ampoules are available microchemical techniques afford the means of satisfactory analysis. Analysts in the control laboratories of pharmaceutical houses are frequently faced with problems of this nature arising from customers' complaints, the activities of drug addicts and incidents due to overdosage of drugs. Early in the second world war two tablets, found on a German prisoner, were referred to us in the hope that we might be able to identify them. By microchemical analysis, and some good fortune, we were able to state that the tablets consisted of Amphetamine Sulphate, 10 mg. A rather interesting application was the careful removal of a deposit, which had developed on the surface of some tablets returned to us, and its identification by microanalysis as cocoa butter, a constituent of the tablet base.

Without perhaps realising it, workers engaged on pharmaceutical analysis make considerable use of microchemical methods. The well known limit tests for lead and arsenic are obvious examples, while the use of organic reagents for the detection and estimation of trace metals is not uncommon in the B.P. and B.P.C. Diphenylthiocarbazone is used

in the separation of lead in many limit tests for this element, for the detection of mercury in dihydroxyanthraquinone (B. Vet. C.) and for the estimation of zinc in insulin preparations. Traces of chromium in menaphthone are detected by the use of diphenylcarbazide; methyltrihydroxyfluorone is employed for testing titanium dioxide for contamination with antimony while the use of thioglycollic acid for the identification and estimation of traces of iron is well known. In all cases the amounts of metals detected are well within the *ultramicro-* range; in some the sensitivity is very great and of the order of 1 part in 5 million.

In the alkaloidal field volumetric and gravimetric *macro*-methods are used largely for the assay of vegetable drugs and only in special circumstances are colorimetric or spectrophotometric methods, capable of estimating milligrams or less of active ingredients, employed. For the

TABLE II

Alkaloid	Reagent	Approximate weight needed for estimation
Morphine	Radulescu[56] (formation of nitroso derivative)	0·2 to 0·5 mg.
Atropine	Vitali[57] (treatment with nitric acid followed by alcoholic potash)	0·025 to 0·15 mg.
Strychnine	Malaquin[58] (reduction and treatment with sodium nitrite)	0·02 to 0·1 mg.
Ergot alkaloids.. ..	M. I. Smith[59] (*p*-dimethylamino-benzaldehyde)	0·05 to 0·1 mg.
Tubocurarine	Folin-Ciocalteu[8] (lithium and sodium molybdophosphotungstate)	0·05 to 0·1 mg.

assay of alkaloidal preparations containing small doses, however, microchemical techniques depending on specific colour reactions are often used. Table II includes a selection of well-known alkaloids, the reagents employed for their colorimetric estimation and the weight of each needed for a determination. Little difficulty is experienced in obtaining accurate results by these methods when tablets and injections containing pure alkaloidal salts are examined, but they are equally applicable to crude products if the alkaloids are first separated in a form suitable for analysis. The potentialities of these micro-techniques were well illustrated when a single ampoule containing 10 mg. of tubocurarine chloride in 1 ml. was used[8] to determine the strength of the solution by measurement of its optical rotation and by colorimetric assay, after which sufficient injection remained for a biological assay by the rat phrenic nerve-diaphragm method. A further example is afforded by the work of Silber and Bischoff[9], who carried out a very extensive investigation of the alkaloidal content of single sclerotia from crops of ergot, cultivated under varying conditions.

The accurate filling of ampoules is important and Scott[10] has described an apparatus for the precise measurement of the volume of an injection in a single dose container, usually holding 1 ml. of solution. The basis of the method is the direct measurement of the volume by use of a calibrated capillary tube, the liquid from 10 determinations being collected

in a burette for the purpose of assessing the average volume per ampoule. As a final example, reference may be made to the problem of filling insulin zinc suspensions large volumes of which have to be maintained in a state of uniform suspension during filling operations. In this case the variation in the suspension filled may be checked by taking samples of filled vials during the entire filling operation of the batch and carrying out nitrogen determinations by the micro-Kjeldahl method.

MICROBIOLOGICAL ASSAY

For many years pharmacologists have standardised biologically drugs for which no established chemical or physical assays existed and, for this purpose, experimental animals such as rabbits, guinea pigs and especially rats and mice have been used. In more recent times, however, it has been found that, like animals, many micro-organisms need certain nutritional requirements for their growth and by the choice of suitable micro-organisms these requirements may be exploited for analytical purposes. These new analytical tools have indeed earned the description of "micro" both on account of their physical size and for the *ultramicro*-quantities of antibiotics, vitamins and amino acids which may be estimated by their aid. Microbiological methods possess many advantages; no expensive animal houses are required, the costs of animals and their feeding stuffs are eliminated and quite simple apparatus such as is found in a bacteriological laboratory is all that is needed. The time factor is an important consideration. For example, the assay of a vitamin using rats may take six weeks; the same assay may sometimes be carried out microbiologically in as many days and it is understandable that, where possible, microbiological assays have replaced the earlier animal techniques. The procedures of importance in pharmaceutical analysis fall into two categories, concerned with antibiotics and essential growth factors respectively, according to whether the substance examined inhibits or stimulates the growth of the test micro-organisms. It is convenient to refer to them under separate headings.

Antibiotics

The clinical importance of antibiotics depends upon their ability to combat the growth of bacteria and assay procedures are designed to measure this antibacterial effect. The potency of an antibiotic is usually estimated by comparison with that of a standard preparation to which an arbitrary potency in units is allotted. Standard preparations of important antibiotics are available from the Department of Biological Standards, National Institute for Medical Research, London. The essential requirements for an assay are a strain of bacteria highly sensitive to the antibiotic under test together with a culture medium in which the micro-organisms readily grow. Portions of the culture medium inoculated with bacteria under standard conditions are treated with varying, but accurately measured, amounts of antibiotic and incubated; the extent of bacterial growth in the resulting cultures may be used to assess the potency of the antibiotic. This seemingly simple procedure

has proved difficult to control in practice and many modifications have been described. In fact, no one modification is satisfactory for all purposes but three have become established and are widely used. These are (i) dilution methods, (ii) turbidimetric methods, (iii) diffusion methods.

Dilution methods are based upon the standard bacteriological procedure for examination of the effect of antiseptics upon micro-organisms. Falling dilutions of the antibiotic to be assayed in suitable nutrient medium are set up in tubes or plates and inoculated with equal amounts of the test organism. Controls containing no antibiotic are included and these together with the dilutions are incubated and inspected at intervals for bacterial growth. The minimum concentration of antibiotic which inhibits growth is recorded and by carrying out simultaneous tests upon the standard preparation and the unknown the potency of the latter is estimated. A full account of the assay of penicillin by the serial dilution technique has been given by Pope and Stevens[11].

The turbidimetric method is similar in design but the inhibition of bacterial growth is not estimated by an "all or none" end point but by a graded diminution in growth which extends over several tubes, in which it is usual to use liquid nutrient medium. Turbidities, due to bacterial growth in the medium, are measured and a curve is drawn relating turbidity to concentration of antibiotic. A calibration curve, constructed from readings obtained similarly using the standard preparation, is employed for assessing the potency of the sample tested. Bond and Davies[12] have given a critical account of this method of assay.

A solid nutrient medium inoculated with the test organism either in bulk or on the surface is used in diffusion methods of assay. A solution of the antibiotic is applied to a small area of the medium, which is incubated at once or after remaining at a low temperature for some hours. Growth of the organisms in the vicinity of the area of application is inhibited and the distance which the inhibition extends from the latter is related to the concentration of the antibiotic. The seeded medium may be held in test tubes, measured volumes of the antibiotic solution being placed on the surface and allowed to act by vertical diffusion. The assays are, however, more frequently carried out by horizontal diffusion, in which case the antibiotic solution is applied to a seeded plate and a circular zone of inhibition obtained. The height of the column of inhibition or the diameter of the circular zone is directly related to the logarithm of the antibiotic concentration. The latter method was developed extensively by the Oxford workers under Florey's leadership and they have published a very full account of the technique[13]. For purposes of assay the solution under test may be placed in cups cut in the agar medium with a cork borer or it may be filled into small open glass or porcelain cylinders standing upright on the agar. Some workers use filter paper discs or simply apply measured drops to the surface of the medium. It must be remembered that the diameter of the zone of inhibition is related to the concentration of the antibiotic in the case of the cup and the cylinder procedures; with the paper disc or the drop method it is the actual amount of antibiotic which is the determining factor.

These assay methods have been applied to many antibiotics; Table III gives a selection and also indicates the test organisms used.

Essential Growth Factors

Williams[14], in 1919, suggested that the growth of yeast might be used for the assay of "vitamine", but it was not until recent years that the nutritional requirements of micro-organisms were used for the assay of vitamins, especially those of the B group. For this advance we are indebted particularly to Barton-Wright[15] and to Snell[16], who have given detailed descriptions of assay procedures.

The principle of the assay is similar to that used for antibiotics excepting that stimulation instead of inhibition of growth is measured. A suitable culture medium, containing all essential growth factors excepting that to be assayed, is inoculated with the test organism. Aliquots of the seeded medium are placed in a series of tubes to which suitable amounts of the solution of substance under test are added, the mixtures adjusted to standard volume and incubated. After incubation the bacterial growth in each tube is measured. A calibration curve is prepared by carrying out a similar experiment using a solution of the standard preparation instead of the substance to be assayed and the potency of the unknown is calculated from the readings.

TABLE III

Antiobiotic	Method of assay	Test organism*
Bacitracin	Cylinder plate	*Micrococcus flavus* (N.C.T.C. 7743)
	Turbidimetric	*Staphylococcus aureus* (A.T.C.C. 10537)
Chloramphenicol	Cylinder plate	*Sarcina lutea* (Strain known as P.C.I. 1001)
Chlortetracycline	Cylinder plate	*Bacillus pumilus* (N.C.T.C. 8241)
	Turbidimetric	*Micrococcus pyogenes* var. *aureus* (A.T.C.C. 6538-P
Dihydrostreptomycin and Streptomycin	Cylinder plate	*Bacillus subtilis* (N.C.T.C. 8236)
	Turbidimetric	*Klebsiella pneumoniae* (A.T.C.C. 10031)
Erythromycin	Cylinder plate	*Bacillus pumilus* (N.C.T.C. 8241)
Neomycin	Cylinder plate	*Bacillus pumilus* (N.C.T.C. 8241)
	Turbidimetric	*Klebsiella pneumoniae* (A.T.C.C. 10031)
Penicillin	Cylinder plate	*Bacillus subtilis* (N.C.T.C. 8236)
Polymyxin B	Cylinder plate	*Brucella bronchiseptica* (N.C.T.C. 8344)
Tetracycline	Cylinder plate	*Bacillus pumilus* (N.C.T.C. 8241)

* N.C.T.C. = National Collection of Type Cultures.
A.T.C.C. = American Type Culture Collection.

Assessment of bacterial growth may be carried out by the turbidimetric and diffusion methods. The latter when performed by the cup-plate or cylinder-plate modifications affords circular zones of growth or "exhibition"[17] instead of inhibition. By far the most popular methods, however, are acidimetric and these are made possible by the use of micro-organisms, such as strains of Lactobacillus, whose growth is accompanied by the production of lactic acid. Under favourable conditions the amount of lactic acid formed is proportional to the bacterial growth, which may be assessed by titration of the acid. Higher organisms, such as fungi and yeasts are sometimes used, and the dry weight of the organism produced used as the response. Table IV illustrates some of the micro-biological assays which are now established.

Besides vitamins microbiological assays have been employed for the determination of amino acids[15] and trace metals[18], but these are at present little used in pharmaceutical analysis.

Some Application of Microbiological Assays

The moment a new antibiotic is discovered microbiological assay becomes an essential tool for development of the discovery. It is most valuable for following the extraction of the antibiotic from the crude fermentation liquors and for controlling the various stages of purification until it is finally isolated in a chemically pure form; it is indispensable for the assay of its pharmaceutical preparations and for studying their stability and it may be used for investigation of the distribution, destruction and excretion of the product when administered to animals and man

TABLE IV

Vitamin	Method of assay	Test organism*
Vitamin B_1 (Aneurine) ..	Turbidimetric	*Lactobacillus fermenti* (A.T.C.C. 9338)
Vitamin B_2 (Riboflavine) ..	Acidimetric	*Lactobacillus casei* (A.T.C.C. 7469)
	Turbidimetric	*Lactobacillus casei* (A.T.C.C. 7469)
Vitamin B_6	Turbidimetric	*Saccharomyces carlsbergensis* (A.T.C.C. 9080)
Vitamin B_{12} (Cyanocobalamin)	Turbidimetric	*Lactobacillus leichmannii* (A.T.C.C. 4797)
Biotin	Acidimetric	*Lactobacillus arabinosus* (A.T.C.C. 8014)
Folic acid	Acidimetric	*Streptococcus faecalis* (A.T.C.C. 8043)
Nicotinic acid	Acidimetric	*Lactobacillus arabinosus* (A.T.C.C. 8014)
Pantothenic acid	Acidimetric	*Lactobacillus arabinosus* (A.T.C.C. 8014)

* A.T.C.C. = American Type Culture Collection.

The technique has played an outstanding part in the development of chemotherapy as an aid to screening antibiotics against a wide spectrum of bacteria. The phenomenal growth of the antibiotics industry during the last ten years has certainly resulted in the diffusion method of assay becoming one of the most widely used analytical tools and hundreds of thousands of such assays must be performed yearly.

The microbiological assay of vitamins has not enjoyed such widespread application but has some notable successes to its credit. That *p*-aminobenzoic acid, inositol, nicotinic acid, pantothenic acid and folic acid were essential for the growth of some micro-organisms was known before their need in animal nutrition was appreciated. The story of liver extract affords a good example of the value of microbiological tests. The introduction of liver therapy for the treatment of pernicious anaemia by Minot and Murphy[19] 30 years ago was followed by the manufacture of liver extracts consisting of crude hygroscopic powders or equally crude liquid preparations. There was no means of assay excepting the haemopoietic response in patients suffering from pernicious anaemia, and, in fact, this was the only guidance available to the many workers who attempted to isolate the active principle. Liver therapy was so effective that suitable patients for testing purposes became scarce and for the next decade progress was extremely slow although purified extracts, suitable for injection, became available and largely replaced oral preparations. In 1940–41 Peterson and co-workers[20,21] reported the presence in liver extract of a factor active in promoting the growth of *Lactobacillus casei* and this led to the employment of microbiological assay as a research tool

in this field. Liver extracts were found to contain several growth stimulating factors for strains of lactobacillus. Shorb[22] reported the presence of a growth factor for *L. lactis* Dorner, and later[23] announced that vitamin B_{12} possessed similar activity of a high order. There is no doubt that this work contributed substantially to the identification of folic acid as the *L. casei* factor and of vitamin B_{12} as the principle active in the treatment of pernicious anaemia. At the same time an accurate assay of liver extracts for vitamin B_{12} was provided.

The discovery that the bacteriostatic action of sulphanilamide is due to its interference with the utilisation of *p*-aminobenzoic acid by susceptible organisms[24] gave rise to the conception of antimetabolites and to a new approach to chemotherapy. It was evident that any substance which prevented the use of an essential growth factor by a pathogenic organism had potentialities as a chemotherapeutic agent. Microbiological assay techniques can readily be applied in the search for antimetabolites. The observation that some pyrimidine derivatives resemble proguanil in being folic acid antagonists[25] led to the suggestion that these compounds might also possess antimalarial activity. Of three hundred compounds examined 2:4-diamino-5-*p*-chlorophenyl-6-ethyl-pyrimidine proved the most active and has become established as an antimalarial drug under the official name of pyrimethamine. Microbiological studies also showed 6-mercaptopurine to be an antagonist of adenine and hypoxanthine[26] and indicated the possibility of using the compound to interfere with the enzyme systems of cells as a basis for the treatment of malignant disease. This work resulted in the use of 6-mercaptopurine in the treatment of chronic myelogenous leukaemia[27,28].

CHROMATOGRAPHY

The application of analytical techniques, including *micro*-methods, is very largely dependent upon the successful preliminary separation of the substance to be determined from other ingredients of the sample under examination. Of modern procedures of separation none has made a greater contribution to analytical chemistry than has chromatography, used 50 years ago by the Russian botanist Tswett[29] in his studies on chlorophyll. It was not until 1931, however, when Kuhn and his co-workers[30] used it for the separation of carotenoids that the importance of chromatography became generally recognised. The extreme simplicity of the method may be illustrated by a brief description of Tswett's experiment. A light petroleum extract of green leaves was poured on to the top of a column of powdered calcium carbonate held in a vertical glass tube. By slowly adding light petroleum the extract was washed down the column and as it descended it was observed to separate into a number of different coloured zones. The components could be separated by breaking up the column. Chlorophylls "a" and "b" may be separated in this way although they are very similar in chemical structure; it is this great ease with which closely related compounds are separated that makes chromatography of such value to the analyst. In order to effect separation a laboratory column can only deal with a small load of material.

On the other hand, there is practically no lower limit to the amount of material examined for it is the sensitivity of the method of detection which determines the amount used; this is often in the microgram range. For the purposes of detection chemical, biological and physical methods have been employed and it is true to say that advances in chromatography have gone hand in hand with improved methods of detection. To-day chromatography enjoys an extensive literature which can no longer be followed in detail. The procedures in general use may be classified as (1) adsorption chromatography, (2) ion exchange chromatography, (3) partition chromatography, and (4) gas-liquid chromatography.

Adsorption chromatography. This depends upon the varying adsorptive power of some solids for the substances under examination. Columns have been built of many materials; aluminium oxide, keiselguhr, calcium carbonate, Fuller's earth and magnesium oxide being amongst those most favoured. Early separations were achieved by cutting up the columns between the zones after development but this technique is difficult to carry out with colourless substances and it is more usual to wash the column with pure solvent and fractionally collect the solution, described as the eluate, emerging at the bottom. For analytical purposes it is sometimes convenient to obtain a continuous record by measurement of some physical property, such as the refractive index, of the eluate leaving the column; this is known as frontal analysis. Tiselius[31] found it was advantageous to develop the column with a solution of a substance more strongly adsorbed than those to be separated; this method is known as displacement development. Another device for increasing the efficiency of column separations is gradient elution, in which the composition of the solvent entering the column is gradually changed.

In the pharmaceutical field adsorption chromatography has been used very extensively for the separation of natural products, an outstanding example being the part it played in the discovery of vitamin B_{12}[32,33], which caused such world wide interest in 1948. The use of adsorption chromatography in quantitative analysis is illustrated by the U.S.P. procedure for the assay of digitoxin and the separation of vitamin D_2 from its preparations prior to its colorimetric determination[34].

Ion exchange chromatography. Base exchange, or cationic exchange, has been the subject of extensive investigation since the middle of the nineteenth century and has mostly concerned the zeolites, consisting of aluminium silicates of complex composition. Synthetic zeolites are used in water softening, carried out by filtration of water containing calcium ions through a bed of cationic exchanger, in the sodium form. In this way the calcium ions are replaced by sodium ions. This property of exchangers clearly has analytical potentialities and developments have led to the preparation of a range of synthetic resins, having free acidic or basic groups in their molecular structures. These have wide use in the field of analytical chemistry known as ion exchange chromatography. Columns of ion exchangers are used similarly to those in adsorption chromatography and are employed especially for separating acid and basic components from mixtures. Ion exchangers can be regarded as

ion filters of selective permeability, a cationic exchanger being permeable only to cations, and an anionic exchanger only to anions.

Schultz[35] has reviewed the analytical applications of ion exchangers and a more detailed account has been given by Samuelson[36]. Although the technique has been much used in inorganic analysis, it has also been successfully applied in the organic field which is of particular interest in pharmaceutical analysis. A solution of alkaloidal salt may be passed through a column of cationic exchanger, in the "H" ion form, and the alkaloid held on the column while the eluate contains an equivalent amount of acid which may be titrated. If necessary, the alkaloid may be removed from the column by washing with a suitable solvent containing ammonia; this procedure is valuable for the separation of alkaloids from crude extracts prior to analysis. When vitamin B_1 is determined in natural products it is removed from the preliminary extracts by adsorption on a column of Decalso F, from which it is subsequently recovered in sufficiently pure form to permit its estimation fluorimetrically by the thiochrome reaction.

Partition chromatography. It is well known that when an aqueous solution is shaken with an immiscible solvent until equilibrium is attained the ratio of the concentrations of the solute in the two phases will be a physical constant, known as the partition coefficient of the substance concerned. The difference between the partition coefficients of amino acids was utilised by Martin and Synge[37] to effect their separation when shaken with a battery of extractors containing two immiscible solvents. This procedure with solvent-solvent extractors was further developed by Craig[38] in his counter-current distribution machine. It was found by Martin and Synge[39] that more efficient solvent-solvent extraction could be achieved by use of columns of silica gel holding about 50 per cent of water, placing the sample under test on the column and developing with an immiscible solvent. Other materials, such as starch and cellulose powder, have been used for holding the stationary phase of water and the process has become known as partition chromatography. Later it was discovered that sheets of filter paper[40] could be used to support the stationary phase and this gave rise to paper partition chromatography, which is probably the most versatile method for analytical work on a micro-scale, and it has been used very extensively for the solution of problems in both pharmacy and medicine.

Monographs on paper chromatography have appeared[41], and the technique has been applied in a broad field, including the antibiotics, alkaloids, amino acids, carbohydrates, dyestuffs, glycosides, steroids and vitamins. Much ingenuity has been shown in locating the substances on the paper after development of the chromatogram. Coloured materials present no difficulty; colourless substances are usually located by spraying the paper with a reagent which will afford a coloured reaction product with the compounds under test. Ninhydrin[42] and Dragendorff's[43] reagents are popular sprays for location of amino acids and alkaloids respectively and sometimes, as with ergot alkaloids,[44] examination of the paper in ultra-violet light will reveal the compounds as fluorescent spots. Antibiotics

may be detected by laying the damp paper on an agar plate, seeded with suitable micro-organisms, and subsequent incubation of the plate when zones of inhibition on the agar coincide with the positions of the antibiotics on the paper. Compounds containing radioactive tracers may be detected by bringing the paper in contact with a photographic plate which is then developed. Besides the identification of components of mixtures, paper chromatography has been used for quantitative work by measurement of the intensities of the spots upon the paper or by their removal from the paper by elution with a solvent and use of micro-chemical analysis. Applications in the pharmaceutical field have been extensive and typical examples have been in the study of the glycosides of digitalis[45], the identification of the polymyxins[46], the detection of biologically active fractions of liver extract[47] and the separation and identification of the different penicillins[48]. Perhaps the greatest achievement of paper chromatography, however, was the part played by it in establishing the structure of the insulin molecule[49].

Gas-liquid Chromatography

Martin and his co-workers[50] have extended their work to include liquid-gas systems in which the stationary liquid phase, as a substance of high-boiling point and good stability, is supported on a column of inert solid carrier, such as keiselguhr, and the mobile phase is a gas. The sample under test is blown along the column by an inert gas and the substances, separated according to their volatility, are detected by a suitable device at the end of the column. It has been found advantageous to maintain the column at elevated temperatures. Volatile organic acids and bases[51,52] may be detected by use of an automatic titrimeter at the exit but sensitive physical detectors, giving a continuous record of the thermal conductivity or density of the emergent gases, are now widely used. This new technique has been described as gas-liquid chromatography, vapour-phase chromatography or gas chromatography, and a full description is given in the report of a recent symposium on the topic[53].

As with other types of chromatography, the procedure is of great sensitivity; a sample of no more than a few milligrams is required and as little as 0·1 microgram of test material in 1 ml. of carrier gas can often be detected. There is little doubt that gas chromatography has many analytical advantages in terms of time, expense and information which it makes available and it may well replace analytical distillation. So far the technique has been used little in the pharmaceutical field, although it was recently employed for the detection of impurities in Chloroform B.P.[54]; its use in the examination of essential oils is an obvious application.

SOME GENERAL CONSIDERATIONS

Any review of modern analytical chemistry would be incomplete without some reference to instrumentation. The thermionic valve and photo-electric cell have extensive applications in the design of scientific instruments and any chemical reaction capable of producing a suitable signal

affords scope for instrumentation, using servo-mechanisms to record chemical and physical changes with a precision unknown 25 years ago.

Full use has been made of instrumental methods in the pharmaceutical industry where the control laboratories employ spectrophotometers, covering the visible, ultra-violet and infra-red regions of the spectrum, pH meters, polarographs, flame photometers, fluorimeters and many other instruments. This has resulted in greatly increased sensitivity and a corresponding decrease in the size of samples examined. For example, when it was standard practice to use visual colorimeters for comparing the colours of solutions the experimental error was of the order of ± 5 per cent; to-day the use of photoelectric spectrophotometers has reduced the error of such determinations to $\pm 0{\cdot}1$ per cent. Radioactive elements have been used widely as tracers for metabolic studies in biochemistry and medicine[55] but, so far, the technique has been little used in pharmaceutical analysis. It is to be noted, however, that solutions of Sodium Radio Iodide (^{131}I) and Sodium Radio Phosphate (^{32}P), requiring the use of a Geiger-Muller counter for their standardisation, are included in the B.P. 1958.

Microchemical analysis, microbiological assay and chromatography, in conjunction with instrumentation, embrace much of modern analytical chemistry. If analysts of a past generation laid the foundations of pharmaceutical analysis when the general analytical method was "dry, ignite and weigh", the analysts of to-day can justly claim that they have not missed their opportunity to apply modern analytical developments to the solution of pharmaceutical problems. The pharmaceutical industry consists essentially of research leading to the discovery of a new drug, the purchase of raw materials needed for its manufacture, pharmaceutical development resulting in its satisfactory presentation, the production of the product and its preparations, advertising its availability and finally its commercial distribution. In the pharmaceutical field no organisation can operate successfully without the support afforded by the analytical chemist; no research worker, no production manager, no buyer, no publicity manager and no salesman could face his task to-day without the backing of a reliable analytical laboratory. That this Conference is fully aware of the importance of analytical chemistry in pharmacy I have no doubt; this has been shown by your election of an analyst to be your chairman this year. I do not feel this is an exclusive personal honour but prefer to consider it as a tribute to that army of analysts, particularly the young laboratory assistants upon whom the burden of much routine work falls, who ceaselessly labour in the control laboratories of our pharmaceutical factories. It is they who safeguard the interests of the prescriber, the pharmacist and the patient and do so much to keep British products in the vanguard of pharmaceutical progress.

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