REVIEW ARTICLE

RECENT ADVANCES IN TOXICOLOGICAL ANALYSIS

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A MAJOR development of the present century has been the rapid growth of the science of chemotherapy, a development which has had inevitable repercussions in other branches of science. In toxicology the impact has been such that toxicological analysis can no longer be regarded as essentially the domain of the forensic analyst, but is becoming more and more a fundamental instrument of the hospital biochemist. With most of the newer groups of drugs introduced into therapeutic practice, as, for example, the methonium hypertensive agents, the margin between medicinal dose and toxic dose is often uncomfortably narrow, and the biochemist must exercise a strict analytical control during clinical administration of the drug. Recent trends, therefore, amply confirm that there can be no sharp segregation of chemicals and drugs into specific categories, poisons and non-poisons, but that the quantity rather than the quality determines whether a particular chemical be deemed poisonous or otherwise. Fortunately, for forensic purposes, the expert toxicologist is to some extent safeguarded by the comprehensive legal wording dealing with the offence in poisoning cases—"...shall unlawfully or maliciously administer to, or cause to be administered to or taken by any person, any poison or other destructive or noxious thing, so as thereby to endanger the life of such person, or so as thereby to inflict on any such person any grievous bodily harm...". Nevertheless it is desirable for a working definition of a poison to be available, a definition which, in the author's experience, should be sufficiently comprehensive to include the corrosives, and yet exclude such materials as powdered glass which owe their destructive qualities to purely mechanical action. With due attention to the quantitative aspect mentioned above a poison may perhaps be most satisfactorily defined as "any substance which, when introduced into the system, either directly or by absorption, causes destruction of the living tissues, or by its chemical activity prevents the normal functioning of the healthy body."

The actual problems of toxicological analysis are rarely appreciated except by those intimately connected with the field. Material isolated from viscera or body fluids may be residues of pharmaceutical extracts and infusions, the active ingredients of which have not been chemically identified. The toxic principles of many of the poisonous members of the vegetable and animal kingdoms are similarly completely uncharacterised. The problem presented by the newer synthetic drugs, on the other hand, is of a different nature. The chemistry and pharmacology of these materials are, of course, adequately known, but their metabolic fate, their detoxification *in vivo*, the nature of their breakdown products,

and their mode of excretion have in general been but briefly investigated. Their chemical nature, too, is generally complex, and tests for identity in the small amounts recovered from viscera are mostly non-existent.

Isolation Techniques

The pharmacological classification of poisonous materials into such groups as irritants, corrosives, convulsants and narcotics, is not without importance and diagnostic significance to the analyst. Since, however, the initial stages of any toxicological analysis are usually concerned with removing in crude form the poisonous drug or chemical from tissues or body fluids, medicinal products or other source, it is convenient to adopt a broad chemical classification, based upon this isolation process, into volatiles, metallic poisons, and non-volatile organic poisons.

Isolation of volatile materials by steam-distillation from both acid and alkaline solutions is readily accomplished and, apart from possible interference by neutral fats or fatty acids carried over by entrainment, subsequent analysis of the aqueous distillate presents little difficulty. In the case of metallic poisons, organic material is destroyed by a process of wet oxidation using either the classical Fresenius-v. Babo[1] technique or one of its modifications, or alternatively the nitric acid-sulphuric acid process of Gautier[2]. For general purposes these processes have not been superseded.

A far more complex problem is that of devising a process capable of removing any and all organic drugs from association with viscera. For this purpose the Stas-Otto method is, despite its inherent disadvantages of tediousness and in certain instances paucity of yield, still the method of choice. Attempts have been made by Stewart, Chatterji and Smith[3], using trichloracetic acid as protein precipitant followed by absorption of alkaloids upon kaolin, to evolve a satisfactory rapid method for the isolation of alkaloids. With the same object, Daubney and Nicholls[4,5] have used ammonium sulphate to precipitate proteins, alkaloids being removed from the filtrate by chloroform extraction. Valov[6], concerned with the isolation of barbiturates, precipitated proteins with phosphotungstic acid and extracted the acid filtrate with ether. These, and other similar recent methods, have certain advantages when a particular drug or group of drugs is being sought, but as a universal process for the isolation of an “unknown” poison the Stas-Otto principle is still to be recommended. A necessary prelude to any of the above methods is the obtaining of viscera in a suitable finely-divided state. Preliminary freezing of the organs, either in a refrigerator or in a Drikold-alcohol bath, undoubtedly materially assists the mincing process, but by use of the recently marketed high speed macerators of the Waring type an extraordinary fine state of subdivision may be obtained in a few seconds. For this purpose a top-drive instrument is preferable in order to facilitate transfer of material and subsequent cleaning. In practice viscera, cut into approximately one-inch pieces and just covered with faintly acid alcohol, are rapidly converted to the consistency of a

readily filterable gruel. There is thus achieved, not only the obvious saving in time, but more important, a much more thorough extraction and a consequently greater yield of drug.

PURIFICATION TECHNIQUES

The residue isolated from viscera is invariably in a more or less crude condition, and prior to any attempt at identification a preliminary purification must be effected. In the hands of even the most experienced analyst this operation may be expected to result in some loss of product, the loss being in general directly proportional to the final state of purity! Although this appears to introduce a complicating factor from the quantitative aspect, it is in practice customary to utilise an aliquot part of the isolated material for purification and establishment of identity, and to devise an appropriate quantitative technique for the examination of the remainder of the sample.

Fractional crystallisation is of somewhat limited application, chiefly owing to the micro-scale on which the work must be carried out. It may nevertheless be occasionally employed to advantage when dealing with synthetic drugs, especially when these are present in admixture.

In the case of such compounds as caffein which readily sublime, a most convenient and satisfactory method is clearly available. A great number of drugs, however, which do not sublime under ordinary conditions, readily do so on heating under reduced pressure, and this technique is easily applicable on a micro-scale. The isolation of barbiturates is frequently achieved by this method (Cohen[7]), and it may be further extended as a fractional procedure whereby mixed barbiturates may be separated (Hanson[8]). Whilst certain of the alkaloids do not yield crystalline sublimates even under reduced pressure, Janot and Chaigneau[9], were able to record definite sublimation temperatures and pressures for many of the more common of these bases.

The preparation of insoluble derivatives affords a further convenient method of purification. In the alkaloidal group purification of the crude residues is notoriously difficult, and Bamford[10] recommends that in particularly stubborn cases the crude residues be treated in hydrochloric acid solution with Mayer's reagent (potassium mercuric iodide solution); the resulting precipitate is filtered off, washed, suspended in dilute acid and decomposed with hydrogen sulphide, thereby leaving the alkaloidal salt in solution. The process inevitably entails some loss due to the slight solubility of the complex iodide in dilute acid, but the resulting product is usually in an appreciably purer condition. Silicotungstic acid may well replace Mayer's reagent for the same purpose, the alkaloid being liberated from the filtered and washed silicotungstate complex by treatment with dilute alkali. By virtue of the relative insolubility of their hydrochlorides in dry ether, bases may in general be prepared by passing dry hydrogen chloride into dry ether solutions of the free bases. This well-known preparative method is frequently utilised in toxicological analysis for purification of bases, especially when the free base is itself

a liquid; the experimental technique is slightly modified in such cases as procaine, where decomposition occurs under these conditions (Hucknall and Turfitt[11]). The reverse situation, of an acidic material which is itself relatively insoluble in water, may be treated in a similar manner. An impure barbiturate, for example, may be purified by dissolving the crude material in dilute alkali, precipitating with mineral acid, filtering and washing the barbiturate precipitate (Selwyn and Dark[12]). For the problem of mixed alkaloids a fractional extraction procedure based upon the differences in basicity of the mixed alkaloids is of value. A solution of the alkaloids in dilute acid is treated with successive portions of dilute ammonia, the liberated alkaloid being extracted with chloroform after each such addition. In the event of it being necessary to separate quaternary ammonium salts from alkaloidal bases, a method described by Auerbach[13,14] for the quantitative determination of quaternary ammonium salts may usefully be employed. The method, too, provides a general purification technique for alkaloids. In alkaline (sodium carbonate) solution sulphonphthaleins such as bromophenol blue and bromothymol blue react with quaternary ammonium salts, but not tertiary bases, to form dye complexes which may be removed from aqueous solution by immiscible organic solvents. In the case of tertiary bases an extractable complex is similarly formed in acid solution. Thus a separation of alkaloids and quaternary salts may be effected by extracting the complex of the former group from acid solution, and the latter after rendering the solution alkaline. In both cases the complex is split by reversal of the *p*H conditions, enabling the free base to be extracted with immiscible solvents.

The outstanding method of attack for purifying chemical entities and for separation of mixtures is undoubtedly chromatography. In recent years the principle has been developed and applied in a most extensive manner in all branches of organic and inorganic chemical practice, and there appear to be but few instances where it cannot be used to advantage. An excellent account of the subject and its potentialities is given in the textbooks of Zechmeister[15,16], whilst the more recent advances since 1947 are reviewed by Strain[17]. In the toxicological field the general utility of the method is shown by such detailed investigations as those carried out by Stolman and Stewart[18,19], Fischer and Goll[20] and other workers on the isolation from viscera, separation and determination of alkaloids, barbiturates and other drugs. For the examination of the various herbal extracts and tinctures used in pharmaceutical practice comparative chromatography frequently provides the only means of identification, and in toxicological analysis in general the importance of chromatographic methods can hardly be overestimated. A refinement of the parent process is paper chromatography, in which paper fibres replace the column as adsorbent. This development, the recent work on which is comprehensively reviewed by Clegg[21], is particularly suited to the specialised requirements of toxicological micro-analysis, and such works as that of Munier and Macheboeuf[22,23] on the use of paper

chromatography for the separation of alkaloids and other nitrogenous bases, are doubtless only a prelude to its application on a much broader scale.

IDENTIFICATION TECHNIQUES

The criterion of the degree of purity achieved by the above or other methods is, wherever the quantity of material permits, the sharpness and constancy of the melting point, the determination being made either by the capillary tube or heating-stage method. The extensive work of Kofler[24] on this subject, especially in connection with the phenomena of isomorphism and polymorphism, should be consulted. For the characterisation of a pure compound so obtained, information resulting from the method of extraction and from the reactions of the compound under appropriate chemical tests usually serves to allocate it within some specific group of drugs, if not to identify it as a particular member of the group. Wherever possible, conclusions as to identity are checked by a mixed melting-point determination.

In the author's laboratory a card index system is employed for reference purposes. The information here available is in no way exhaustive, but the records contain appropriate details of all official drugs, and, further, an attempt is made to include all synthetic drugs as they appear on the market. It has been found in practice that this scheme, even in its present embryonic state, assists very materially in the frequently difficult identification of organic residues; suitably modified, extended and adapted according to the dictates of experience, it should prove a most valuable and time-saving aid to all analysts concerned with the identification of organic drugs and poisons. For reference purposes five card-index files are maintained: (1) elements, (2) reference data, (3) chemicals contained in the reference collection, (4) "manufactured" drugs, i.e., tablets, capsules, pills, etc., contained in the pharmaceutical reference collection, and (5) melting points. A description of the practical use of these files is given below.

QUALITATIVE MICRO-ANALYSIS FOR ELEMENTS

The material isolated during the toxicological analysis of viscera or of medicinal preparations is frequently insufficient in amount to permit of the use of the standard methods of micro-analysis for the identification of elements. By the adoption of a suitable experimental technique, the Lassaigne test may be utilised on a micro-scale for the detection of nitrogen (Emich and Schneider[25]) and also of certain other elements. In addition to its inherent hazards, however, fusion with metallic sodium fails to give satisfactory results with all types of nitrogenous compounds, and the use of calcium oxide—copper powder (Emich and Schneider[26]), or calcium oxide—zinc dust (Johns[27]) is much preferable as the fusion mixture. A systematic procedure for the detection of nitrogen, halogens, sulphur, phosphorus and arsenic in a single 1 mg. sample following pyrolysis with a calcium oxide-zinc dust mixture has been described by Bennett, Gould, Swift and Niemann[28].

During toxicological investigations extending over a number of years the present author has utilised a calcium hydroxide-zinc dust fusion mixture as the basis of a systematic scheme for the micro-detection, in the presence of one another, of those elements other than carbon, hydrogen and oxygen, most frequently encountered in poisons and drugs of therapeutic importance, viz., nitrogen, chlorine, bromine, iodine, sulphur, phosphorus, arsenic, antimony and mercury. The detailed procedure described below is summarised diagrammatically in Table I. All chemicals employed should be analytically pure, and blank experiments should be performed in all cases as a check on the purity and efficacy of the individual reagents. The sensitivity of several of the reagents, especially Nos. 6, 12, 13, 15, 18, is seriously impaired on keeping, and therefore it is advisable for them to be prepared immediately prior to use.

Reagents

1. *Fusion mixture.* Calcium hydroxide—zinc dust, equal parts by weight.
2. *Silver nitrate solution.* 0·05N.
3. *Nitric acid.* 2N.
4. *Acetic acid.* 2N.
5. *Sodium nitrite.* A.R.
6. *Starch solution.* 0·5 per cent.
7. *Lead peroxide.* A.R.
8. *Fluorescein solution.* Saturated solution in alcohol.
9. *Hydrochloric acid.* 2N.
10. *Iodine-azide reagent.* 1 g. sodium azide, 1 g. sodium iodide, 15 mg. iodine, 3 ml. water.
11. *Copper wire,* 26 S.W.G., cleaned in nitric acid and washed with water immediately prior to use.
12. *Ammonium molybdate—tartaric acid reagent* (Vogel[29]). 5 g. of ammonium molybdate dissolved in 100 ml. of cold water, and the solution poured into 35 ml. of concentrated nitric acid; 20 g. of crystalline tartaric acid is then added. (Use of this modified ammonium molybdate solution renders the test specific for phosphate in the presence of arsenate or silicate.)
13. *Benzidine reagent.* 0·05 g. of benzidine dissolved in 10 ml. of glacial acetic acid, and diluted to 100 ml. with distilled water.
14. *Nitric acid.* 0·2N.
15. *Diphenylcarbazone solution.* Freshly prepared 1 per cent. solution in alcohol.
16. *Phosphomolybdic acid.* 5 per cent. aqueous solution.
17. *Zn dust.*
18. *Cuprous chloride solution.* Cuprous chloride dissolved in concentrated hydrochloric acid to give an approximately saturated solution, which is then diluted with an equal volume of distilled water.
19. *Gold chloride solution.* 1 per cent. aqueous solution.

The majority of the micro-tests used in this scheme are described in detail by Feigl[30].

TABLE I

1 mg. substance + 5 to 10 mg. Zn + Ca $(OH)_2$

Heat in ignition tube 2″ × ¼″
Test vapours with red litmus

Litmus blue = N

'Fuse' thoroughly, and plunge red-hot tube into 1 ml. water in 5″ × ⅝″ test tube. Filter off approx. ¾ of supernatant liquid.

Filtrate

To a drop in a m.pt. capillary tube add 1 drop $AgNO_3/HNO_3$. White turbidity = **HALOGENS.**

Transfer filtrate to a micro-crucible, and add 3 drops 2N acetic acid and approx. 1 mg. solid $NaNO_2$; cover crucible with filter paper moistened with starch soln., and warm on water bath. Blue colour on paper = **I.**

Continue warming until, on adding further acetic acid, test for I is negative. Add approx. 1 mg. solid PbO_2, cover crucible with filter paper impregnated with fluorescein soln., and warm on water bath. Red colour on paper = **Br.**

Continue warming until, on adding further acetic acid, test for Br is negative. Remove 1 drop of clear liquid into m. pt. capillary tube, and add 1 drop $AgNO_3$/HNO_3. White turbidity = **Cl.**

Residual Suspension

Add 0·5 ml. 2N HCl. (Remove 1 drop on glass rod and add to iodine-azide reagent in capillary tube. Bubbles rising up tube = **S.**) Add two 5 mm. lengths of bright Cu wire and heat 15 min. in boiling water bath. Remove Cu wires.

Cu Wires

If any discolouration wash well with water; dry between filter paper.

Wire 1

Introduce into capillary tube, and heat with micro flame. Examine any sublimate under microscope:—globules = Hg; octahedra or amorphous = As and/or Sb.

Dissolve sublimate in 0·2N HNO_3 in microbeaker, and add to a drop of 1 per cent. EtOH soln. diphenylcarbazone on test paper. Violet or blue colour = **Hg.**

Wire 2

If sublimate is obtained with wire 1, repeat sublimation with wire 2. Dissolve sublimate in few drops 2N HCl in 3″ × ⅜″ test tube. (Remove one drop of this soln., and add to a spot of 5 per cent. phosphomolybdic acid on test paper; expose to steam. Blue coloration = **Sb.**)

Add Zn dust, and quickly stopper tube as shown in Fig. 2. Blue stain on gold chloride paper after 5 to 10 min. = **As.**

Solution

Remove few drops on to spot test paper. Add 1 drop ammonium molybdate—tartaric acid reagent. After warming paper over hot gauze add 1 drop benzidine reagent; expose to ammonia vapour. Blue colour = **P.**

Procedure. Approximately 1 mg. of the test material is intimately mixed with 5 to 10 mg. of calcium hydroxide—zinc dust fusion mixture, and transferred to a 2″ x $\frac{1}{4}$″ test tube. The tube is heated gently in a small flame, and the issuing vapours tested with a moistened, pointed strip of red litmus paper; a change to blue is indicative of the presence of nitrogen in the original material. Heating of the tube is continued gradually until the contents are at red heat, when it is plunged into 1 ml. of water contained in a 5″ x $\frac{5}{8}$″ test tube. After allowing the insoluble matter to settle, approximately three-quarters of the supernatant liquid is filtered off, and the filtrate and the remaining portion of the suspension are analysed separately. This filtration is conveniently carried out by transferring the supernatant liquid by means of a teat-pipette (Fig. 1) to a second pipette the constriction of which has a loose plug of cotton wool or pulped filter paper. Pressure on a teat subsequently fitted to this pipette now forces the liquid through the filter, and a clear filtrate results. (*i*) *Filtrate:* A drop is removed on the end of a glass rod, and added to a drop of 0·5N silver nitrate, acidified with 2N nitric acid contained in an open melting-point capillary tube. The presence of halogens is indicated by a white turbidity especially evident on standing for a few moments.

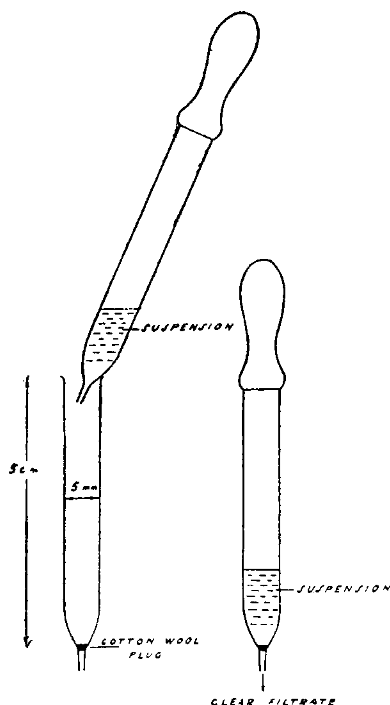

FIG. 1

If halogens are present the filtrate is transferred to a micro-crucible, 3 drops of 2N acetic acid and approximately 1 mg. of solid sodium nitrite are added, and the crucible, covered with a disc of filter paper moistened with starch solution, is gently warmed on a water-bath. A blue colour developing on the paper indicates the presence of iodine.

The crucible is warmed until no iodine is detected following addition of further acetic acid. Approximately 0·5 mg. of solid lead peroxide is added, and the crucible, covered with a filter paper impregnated with fluorescein solution, is again warmed on a water bath. A red colour on the test paper indicates bromine.

The crucible is allowed to remain on the water bath until, on adding further acetic acid, the test for bromine is negative. The original halogen test is repeated on a drop of the solution; a white turbidity is now indicative of chlorine in the original compound.

(*ii*) *Residual suspension:* To this suspension still in the 5″ × $\frac{5}{8}$″ test tube 0·5 ml. of 2N hydrochloric acid is added. A drop of the liquid is removed on a glass rod and added to a drop of iodine-azide reagent contained in an open melting-point capillary tube; bubbles rising up the tube are indicative of sulphur in the original compound.

To the remainder of the suspension, two 5 mm. lengths of bright copper wire are added, and the tube and contents heated for 15 minutes in a boiling water-bath. A few drops of the solution are removed on to a spot-test paper, and 1 drop of ammonium molybdate—tartaric acid reagent is added. The paper is warmed over a hot gauze, and one drop of benzidine reagent is added. The presence of phosphorus is shown by the development of a blue colour on holding the paper over the vapour of ammonia.

The remaining contents of the tube are placed in a white porcelain dish, and if there is any discolouration of the copper wires they are washed with a large excess of water, and dried between filter paper. One of the wires is placed in a melting-point capillary tube, one end of which is sealed, and heated cautiously in a micro-flame. Under these conditions mercury yields a sublimate appearing as shining globules when examined microscopically; arsenic usually appears in the form of octahedra, whilst the antimony sublimate is generally amorphous. The tube is cut, and the upper portion containing the sublimate is broken in a micro-beaker. After the sublimate has been dissolved in a few drops of 0·2N nitric acid, the solution is added to a drop of 1 per cent. alcoholic solution of diphenylcarbazone on spot-test paper; a violet or blue colour is a positive reaction for mercury. In this concentration of nitric acid the reaction is specific for this metal.

A sublimate is similarly obtained from the deposit on the second copper wire. The portion of the tube containing the sublimate is placed in a 3″ × $\frac{3}{8}$″ test tube, and a few drops of 2N hydrochloric acid are added. One drop of the solution thus obtained is added to a drop of 5 per cent. aqueous solution of phosphomolybdic acid on spot-test paper, and the paper steamed; a blue colour is indicative of antimony.

To the solution remaining in the tube approximately 10 mg. of zinc dust is added. A loose plug of cotton wool is introduced into the mouth of the tube (Fig. 2) to support a circle of filter paper moistened with cuprous chloride solution; this solution serves to obviate interference from hydrogen sulphide, phosphine or stibine. A bored cork, carrying a capillary tube in which is a strip of filter paper impregnated with a 1 per cent. aqueous solution of gold chloride, is placed in the mouth of the tube. After 5 to 15 minutes, the presence of arsenic is shown by the appearance of a reddish-blue stain on the gold chloride paper.

The above procedure, even in the hands of relatively inexperienced workers, is found to be satisfactory using quantities of approximately 1 mg. of the compound to be tested, provided the elements being detected form not less than 1 per cent. to 2 per cent. of the sample.

When the elements present have been determined in this manner

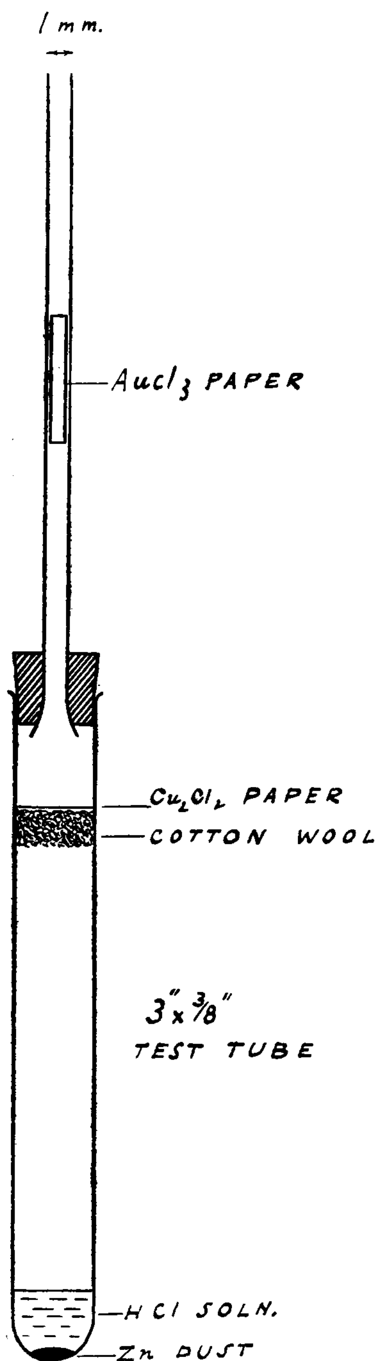

FIG. 2

reference is made to the " Elements " file. Each card in this index bears the name of a particular compound, together with its melting point or decomposition temperature; if the compound merely decomposes gradually on heating, the abbreviation " Mp. dec." is employed. The holes on the margin of the card* are keyed according to a standard card, each hole representing N, S, Cl, " ionisable Cl " or other element. An extension of the scheme is envisaged in which such physical characteristics as colour and sublimation, and perhaps chemical groupings may be represented. For any particular compound the holes representing the elements or other keyed features are clipped. In practice, therefore, if for example N and ionisable Cl have been detected, a needle-selector is first pushed through the file of cards at the nitrogen hole, when all cards of non-nitrogenous compounds will be retained on the needle; from the remaining batch the non-ionisable chlorine compounds are separated in a similar manner. There is thus obtained a relatively small number of likely compounds, from which a final selection is made on the basis of melting-point. Usually at this stage it is found that the choice has been narrowed to a single compound, and reference to this compound is then made in the " Reference Data " file. The cards here, labelled with the compound name and arranged in alphabetical order, contain the chemical constitution, collection number, a list of medicinal

* The right to produce this type of card is covered by patents held by the Copeland-Chatterson Co., of Exchange House, Old Change, London, E.C.4.

products containing the compound, and on the reverse side tests for identity. It may be at this stage unnecessary to apply identification tests, characterisation being sufficiently certain to warrant proceeding immediately to a mixed melting-point determination. The collection number on the card enables an authentic specimen to be immediately procured for this purpose from the reference collection. With identity thus established, the " Manufactured Drugs " index enables appropriate tablets, capsules or other products to be speedily located in those instances where comparison is required with the original medicinal product from which the compound was isolated.

The " Melting Point " file is a collection of cards representing each compound in the " Reference Data " file, and contains only the name of the compound and its melting point. The cards are filed in melting-point sequence, the " mp. dec." cards being arranged alphabetically at the back of the file. Liquids of definite boiling-point are included in sequence in this same file, such cards having the top left-hand corner punched for differentiation purposes. This file is not necessary for the routine identification described above, but as an obvious aid to rapid " sorting " it plays a significant role.

There are of course numerous instances where, by reason of the small amount of material available, a pure crystalline specimen cannot be obtained, and in such conditions the above scheme cannot be used in its entirety and is only of limited value. This situation is perhaps most frequently encountered in the alkaloidal group, where it is seldom possible to obtain a pure residue in sufficient quantity for a melting-point determination. Identification in these circumstances is usually accomplished by colour reactions, the systematic scheme of Bamford[31] being particularly noteworthy in this difficult field. The well-known " alkaloidal reagents " used in these colour and precipitation reactions have found extensive application in connection with other groups of compounds. Bamford[32] himself has utilised certain of them in the identification of barbiturates, whilst Haley[33] has employed them as the basis of a method for the differentiation and classification of sympathomimetic amines. Their general utility is in fact such that in developing tests for the newer nitrogenous drugs they are usually amongst the first reagents to be investigated. Colour reactions, derived from the presence of some reactive grouping in the organic molecule, have an undeniably valuable function in toxicological analysis, and there are few poisons for which colour tests have not been devised. The almost invariable disadvantage of the single colour test is, however, that it is not specific for an individual compound, but only for a group of chemically similar substances. Recent trends in chemotherapy have been responsible for introducing a considerable number of such groups, and for the differentiation of the individual members within the group, no single colour reaction suffices. The use of a series of colour tests in such a case is amply illustrated by the work of Chavez[34] and Sample[35] on the sulphonamides, of Cocking[36,37] and Smith and Turfitt[38] on the synthetic stilbene œstrogens, and of Haley[39]

on the antihistaminics benadryl and pyribenzamine. An example of the usual inadequacy of a single colour test for identifying an organic compound is afforded by the old-established Beam test for Indian hemp (*Cannabis sativa*). It is now known that this reaction, either in its original form or as one of its modifications, is due not to tetrahydrocannabinol but to the physiologically inactive cannabidiol. Not only is this reaction not always dependable, but it is not entirely specific, being given also by various resins and essential oils. A far more satisfactory reaction for identifying *Cannabis* resin is the acetaldehyde-vanillin test of Duqénois and Negm[40]. The reaction, which is in effect an extraction procedure followed by a colour reaction, is sensitive to 0·5 mg. of resin; in its complete form it is said to be specific, and was adopted by the League of Nations Sub-Committee on Cannabis (1939).

The microcrystalline form of chemical compounds or of suitable derivatives prepared under controlled conditions is frequently of great diagnostic value, and often constitutes a most useful supplement to colour reactions. A wide range of such crystal tests, with photomicrographs showing the crystalline form, is described by Rosenthaler[41]. Their most useful application, however, is perhaps as an aid to distinguishing individual members of a group such as the sulphonamides (Beck[42], Dodson and Todd[43], Hucknall and Turfitt[44]), the barbiturates (Pesez[45], Turfitt[46]), or the local anæsthetics (Sabon and Grignon[47], Hucknall and Turfitt[48]). An interesting variation of this method of identification is the technique of Saredo[49] in which solutions of alkaloids are seeded with crystals believed the same; in only 6 cases out of 400 tried did precipitation occur when the solution was seeded with chemically different crystals, and in these 6 cases the crystalline forms were modified. The diagnostic importance of these microcrystal tests is vastly increased if such optical properties as refractive index and appearance in polarised light are determined (Reimers[50]). The application of this technique to specific problems is illustrated by the publications of Keenan[51] on sulphonamide identification, of Haley and Keenan[52] on the thiodiphenylamine antihistaminics, and of Wickstrøm[53] on pethidine, nicotinamide and cinchophen.

The use of biological tests in the presumptive identification of pharmacological groups of drugs, or as confirmation of results obtained by chemical tests, has long been practised. A particularly important feature of such tests, which are described in standard text-books (Autenrieth[54], Webster[55], McNally[56]) is that the test material need not in general be obtained in the high degree of purity necessary for chemical tests. Whilst it is not the custom of toxicological analysts to resort to detailed pharmacological investigations in all instances, it is desirable for important forensic cases that the appropriate well-known biological tests should be applied for such alkaloids as strychnine, colchicine and those of the morphine and atropine groups. Recently Pick and Richards[57] have devised a quantitative method for the determination of curare and erythroidine alkaloids based upon antagonism of the morphine "tail reaction" in mice.

Physical methods for identifying organic compounds, so valuable as research tools, have been used only to a relatively limited extent in toxicological analysis. This in no way reflects upon the general usefulness of such methods, but is due almost entirely to the costly nature of the instruments and equipment. Two of the most valuable and informative of these methods are undoubtedly X-ray diffraction and absorption spectroscopy. In the former, a narrow beam of X-rays is passed through the suitably prepared test material, and the diffraction of the rays by the crystal lattice is recorded photographically. From the results so obtained identification is established by calculation and reference to standard tables, or alternatively by comparison of the records with standard photographs (O'Hara and Osterburg[58]). For toxicological purposes the method possesses certain advantages, in that it requires only small amounts of test materials which need not have been subjected to any really intensive purification; the material is not destroyed during the process, and a permanent photographic record of the crystal structure is obtained. Research investigations on the application of absorption spectroscopy to the identification of toxicologically important organic compounds are becoming increasingly numerous, but, as with X-ray diffraction, the high cost of equipment still tends to restrict its use in toxicological practice. The method consists essentially in recording the absorption of radiation by the test material solution at various wavelengths, the absorption curves so obtained frequently being highly characteristic of the compound under investigation. Elvidge[59] has recorded much useful data on the opium, ephedra, solanaceous, coca and other alkaloids, and also on the barbiturates. Numerous other references on the subject are to be found scattered throughout the literature, but it is evident that if the method is to be used for routine qualitative identifications a reference collection of absorption curves or records of extinction coefficients should be compiled. In operation the procedure is somewhat intricate, and for the practical details a treatise such as that of Twyman and Allsopp[60] should be consulted. The technique of absorption spectroscopy in the infra-red region and recent work on its application to the problems of analytical chemistry is comprehensively reviewed by Cropper and Hamer[61]. It is indeed fortunate that so useful a method for qualitative purposes should be especially valuable as a quantitative measure; this aspect is referred to in greater detail below.

Quantitative Techniques

The difficulties involved in employing gravimetric methods for quantitative determinations in toxicological work have already been indicated. In particular, the loss of material during purification is such that the choice lies between recording the weight either of an obviously crude residue or of an equally apparent low yield of purified material. In those instances where it is possible to prepare a derivative of known composition in quantitative yield, the purification of the derivative may be possible without the major loss inherent in the purification of the original

compound, and under these circumstances gravimetric determination may be used with success.

The standard techniques of volumetric analysis also find some application in cases where interference from extraneous material can be avoided. In the determination of cyanides, for example, the Liebig titration with silver nitrate gives excellent results with quantities of cyanide not less than 5 mg., the end point being particularly clear if diphenylcarbazide is used as an absorption indicator (Vogel[62]). Isolation from decomposing viscera, however, necessitates preliminary separation of the cyanide from hydrogen sulphide which otherwise interferes in this method. The chief virtues of volumetric methods are simplicity in operation and a high degree of accuracy, and this latter feature may be still further enhanced by employing absorption indicators or potentiometric methods to obtain a more crisp and certain end-point. In an extensive review Furman[63] summarises the vast amount of research which has been carried out in recent years in the potentiometric field, especially in its application to microtitrations, and acid-alkali and redox titrations. Potentiometry has already been used in the assay of sulphonamides (La Rocca and Waters[64]) and alkaloidal salts (Saunders and Srivastava[65]), and its extended use in toxicological investigations may confidently be predicted.

The present tendency, however, is an increasing reliance upon colorimetric determinations. It is relatively rare in toxicological analysis to encounter coloured compounds solutions of which may be compared directly with standards in visual colorimeters of the Dubosq type. The formation of a coloured product however by interaction of the test material with some appropriate reagent may frequently be accomplished, the colour intensity being then compared with standards by the Nessler or Dubosq methods. A survey of this field, with extensive bibliographies, is given by Allport[66], who rightly points out such inherent shortcomings as a somewhat low degree of precision, a general lack of specificity and interference by extraneous material. Nevertheless, these drawbacks are rarely prohibitive, and when visual standardisation is replaced by photoelectric measurement in the Spekker Absorptiometer or Fluorimeter or other similar instrument, the accuracy of colorimetric methods compares favourably with that of other analytical processes. Where the somewhat costly apparatus is available, spectrophotometric determinations are in general much preferable even to the refinements of photoelectric absorptiometry; absorption maxima may occur in the ultraviolet or infrared regions of the spectrum and thus the necessity for preparing coloured derivatives is obviated. Nicotine, for example, has been determined by Willits, Swain, Connelly and Brice[67] by measuring the extinction at 236, 259 and 282 mμ of an aqueous solution in 0·05N hydrochloric acid; the results obtained agreed with those by the gravimetric silicotungstate method to within ±2 per cent. The method is particularly valuable in the determination of the newer synthetic drugs, antihistaminics for example being determined spectrophotometrically in aqueous or dilute hydrochloric acid solution (Martin and Harrisson[68]), or in acetone solutions

of the reineckates (Bandelin, Slifer and Pankratz[69]). Bandelin[70] has also applied the reineckate method to the quantitative examination of alkaloids. In the determination of alcohol in blood or urine, the alcohol is commonly steam-distilled into a standard dichromate solution, unreduced dichromate being subsequently determined volumetrically. Delaunois and Casier[71] have proposed the interesting procedure of measuring the actual amount of reduced dichromate spectrophotometrically at 470 mμ, but for general purposes it is unlikely that this method will replace the much simpler and equally effective standard volumetric technique. Of the various methods suggested from time to time for the quantitative measurement of carbon monoxide in blood, perhaps the most frequently used is the Hartridge spectrum-reversion technique. This method is unfortunately somewhat insensitive with low concentrations of carbon monoxide, and under these conditions may well be superseded by a spectrophotometric method recently described by Bonneau[72] in which 2 to 3 per cent. of carboxyhæmoglobin is detectable. This selection from the wide range of recent publications on the applications of spectrophotometry clearly points to its immense possibilities in toxicological work.

In yet another sphere it has been found that the oxidation or reduction of both inorganic and organic substances takes place at specific electrical potentials characteristic of the reacting ion or radical. At potentials differing from the oxidation or reduction potential a solution of a substance passes a small current depending upon the conductivity of the solution; at the oxidation or reduction potential there is an increase in current proportional to the number of molecules of the reacting substance. Upon this principle depends the polarographic technique pioneered by Heyrovsky. A modern review of the apparatus and of the applications of the method in quantitative analysis is given by Sherrick.[73] Polarography, however, in spite of its wide industrial application as a routine method for metals and other ions and radicals, is still largely in its research stage so far as toxicological analysis is concerned. In view of its ready adaptability as a micro-method it should, however, constitute a useful instrument in this field, although the cost of the equipment may militate against its widespread employment.

Biological tests, mentioned above as providing useful confirmatory data in connection with identifications of drugs, are seldom employed on a quantitative basis in forensic toxicology, and then usually only when chemical or physicochemical methods are inadequate or the results indecisive. A typical example is the biological assay of tubocurarine, which is performed by measuring the percentage inhibition of contraction of the phrenic nerve diaphragm of the rat.

In a subject which is fundamentally a specialised application of analytical technology, progress is inevitably linked with research and development, and it is a regrettable but undeniable truth that, so far as this country is concerned, neither of these lines is receiving adequate attention. Until there is established a university department or some

research institute such as exists in various centres overseas, it is inevitable that developments here will be limited both in number and in scope.

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