REVIEW ARTICLE

TOXICOLOGICAL ANALYSIS

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Five years have elapsed since the last review in this series. Although the large number of published papers show that they have been highly productive years it is the intention here to record general impressions rather than produce a comprehensive list of references.

The years 1950–55 were years of immense advances in all branches of analysis. Toxicologists were not slow in utilising the advantages of the new techniques and the previous reviews[1,2], reflected some of these developments. By 1955 however, X-ray diffraction cameras, ultra-violet and infra-red spectrophotometers were in widespread use and the impact of the various forms of chromatography had been already felt: in contrast to these early formative years, the last five years have been ones of consolidation. In many instances duplication of work has produced a situation where the toxicologist is now able to choose from any one of a number of methods. Although this augurs well for the subject, there are many outstanding problems yet to be solved.

The systematic chemical approach to these analyses was discussed in outline in the previous review and is treated comprehensively by the author elsewhere[3]. The same general pattern will be followed here. There has been no major change in the proportional division of viscera in the last five years but the number of separate tests on blood, urine or small quantities of viscera for individual poisons has materially increased. There are two advantages in the use of "spot tests". Firstly, they provide a double check for the presence of a particular poison or drug, thus ensuring the chances of missing it are minimised and, secondly, because the whole series of them can be completed in a very short time, they provide a section, which can often reveal an early lead to the cause of death. It is inevitable in a general scheme where some compromise has to be made between sensitivity and specificity that for some poisons and drugs sensitivity of the general test falls short of requirements and a separate portion of blood or tissue has to be used in a special test. There are an increasing number of these special tests because the ingenuity of the industrial and drug chemist knows no bounds in devising new compounds with, to the toxicologist, inconvenient physical and chemical properties.

There has also been an increase in the number of biological and biochemical tests incorporated into the comprehensive analysis. The unreliability of post-mortem blood sugar determinations was again emphasised by Price[4] in an insulin murder. He obtained values up to 1 g. per cent in right heart blood specimens within a few hours of death. Not only does the glycogenolysis phenomenum, which occasionally occurs in peripheral parts of the body, mean that an apparently raised blood sugar

level may be found when hypoglycaemia existed *ante-mortem*, but terminal adrenal release may also increase the glucose concentration with a consequent chance of misinterpretation. The detection of insulin in buttock tissue in this case was an example not only of the importance of systematic chemical analysis coupled with biological methods in toxicology but also drew attention to the great help that the Home Office Forensic Science Laboratories receive from their colleagues in industry and the universities.

A number of homicides who have used organo-phosphorus compounds reflects their increased use as insecticides, and blood cholinesterase determinations must now be done routinely. This enzyme is fortunately stable *post-mortem* and the results are reliable. For those compounds which are direct inhibitors, stomach contents can also be tested and the enzymatic technique has been used to assay organo-phosphorus insecticide residues on plants[5]. The histochemical demonstration has also been described by Petty and Moore[6].

Heart blood transaminase concentrations have also been shown to rise rapidly after death[7]. Estimations of the concentration of this enzyme in the diagnosis of myocardial infarcts is well established in clinical medicine; these do not appear to be of great application in toxicology, unless values are obtained on peripheral serum and the clinical state was known to exist for more than 6 hours before death. Enzymatic methods have also been used widely for the determination of ethanol[8] and Lundquist has published a method for acetaldehyde[9].

The statement in the latest edition of Gonzales, Vance, Helpbern and Umberger[10] that fluoride is the poison most likely to be missed in a routine analysis means that special attention must be paid to it. An enzymatic method using liver lipase and ethyl butyrate has recently been published[11] and a semi-quantitative figure for fluoride in blood can be obtained reasonably quickly.

In the isolation of fluoride and oxalate from stomach and intestinal contents by dialysis it must be remembered that because of the presence of calcium ion, these anions must be looked for in the second dialysate, that is, after the one-ninth aliquot has been made acid. The concentration of the dialysate must however be done by evaporation from neutral solution. Goldstones modification[12] of the fluorosilicate crystal test is capable of detecting 0·2 μg. of fluoride ion. We have not had the success with fluoroacetamide that was claimed in the original paper and prefer sodium peroxide to sodium hydroxide for the destruction of the organic molecule. In this context it seems pertinent to enquire whether or not aconitase could be used in the investigation of deaths from fluoroacetates.

In the examination of intestinal contents for the amanita fungi there are several reported instances in which a diagnosis of the phalloides species has been made from the microscopic appearance of the isolated spores. They are said to be 8 to 11 μ by 7 to 9 μ while subgloboid bodies each with a central oil drop. In a case investigated by Dr. G. Roche and myself although almost intact "mushrooms" could be picked

from the contents of the colon, expert advice from a mycologist indicated that spore examination was unsatisfactory and an attempt was made to isolate phalloidin using as a guide the work published by Wieland[13]. Ethanol was used as the solvent for extraction, the products being examined by paper chromatography using methylethyl ketone : acetone : water (20 : 2 : 5) and ethyl formate : acetone : water (100 : 145 : 40) systems. Detection by chlorine : starch-iodide and by cinnamaldehyde : hydrochloric acid showed a spot at the R_F corresponding to phalloidin. Elution of a similar spot and its injection into a mouse caused the animal's death, although animals injected with eluates from other parts of the chromatogram survived. Unfortunately we had insufficient material to produce adequate criteria to confirm that the toxin was in fact phalloidin. The results are sufficiently encouraging to indicate an alternative approach to the purely botanical one.

Turning now to the subject of gases, poisoning by carbon monoxide continues to be a major cause of accidental and suicidal death. The spectrophotometric methods are reliable in fresh blood samples but not in blood from exhumed bodies that have undergone putrefaction. In aircraft accidents blood is often difficult to obtain and in these the gas chromatographic examination of the gases liberated from tissue is an elegant technique[14]. Rutter[15] has used the Siebe-Gorman carbon monoxide detector in a very rapid and theoretically sound method. In his method the gas is liberated from a 0·5 ml. sample of blood by potassium ferricyanide and drawn through the tube containing a yellow palladium salt. The length of the black stain of palladium gives a direct measure of the volume of carbon monoxide liberated from a known volume of blood. Lead acetate glass wool prevents sulphide interfering. There are two great advantages in this method, the apparatus is completely portable, so enabling values to be obtained in the mortuary in less than one minute, and, by using larger volumes of blood, accurate determinations can be made when the concentration is less than 1 per cent saturation of carbon monoxide. For highly accurate work haemoglobin determinations must be done and if, despite Simpson's work[16], the blood has been covered with liquid paraffin with no added anticoagulant, this can be annoyingly tedious.

A method has been published for the determination of halothane in blood[17].

Poisons Volatile in Steam from Acid Solution

Gas chromatography is the technique which has been developed most in the analysis of this fraction. The alcohols have been investigated in a quantitative manner[18] and the literature abounds with references to essential oils. It may be possible in the future to determine from an analysis of the viscera whether the victim was drinking "Scotch" or "Canadian Rye"! In investigating an allegation that oil of juniper was taken we found that the results of an infra-red examination of the oil obtained by evaporating an ether extract of the steam distillate were more easily interpreted than those from gas chromatography. The extract

from the intestines did not have a peak at 12·7 μ which was present in the spectrum from the control sample of juniper oil that had been extracted in the same way. The ferric chloride colour test for turpentine oil given by Taylor[19] was, however, positive, suggesting either that this particular test is not highly specific or that the bulk of oil of juniper undergoes extensive changes in the body. Feldstein and Klendshoj[20] have published a series of methods using microdiffusion for methanol, ethanol, isopropanol, formaldehyde, acetaldehyde, acetone, cyanide, sulphide, phenols, halogenated hydrocarbons and carbon monoxide: their work extends the trend previously noted of using micro tests on small volumes of blood. X-ray diffraction patterns of the xanthogenates of several alcohols have been reported[21] and the reaction of ethanol with dichromate: sulphuric acid mixtures has been fully investigated[22]. Quantitative methods for acetone[23] and carbon disulphide[24] have also been described. Curry, Rutter and Lim Chin-Hua[25], have elaborated the very important test first described by Schwartz and his co-workers[26] for yellow phosphorus. This new approach now modifies my previously expressed opinion[2] that "it is likely that if phosphorus is suspected then the whole of the intestine contents will have to be distilled if its detection is to be ensured". As little as 0·1 μg. of yellow phosphorus can now be detected and 20 g. samples of liver give excellent positive results for this common homicidal poison. In the Wilson case, Dr. Barclay obtained a positive result from the centre of a phosphorus victim's liver even though 14 months had elapsed between death and analysis. The finding of 3·8 mg. in the stomach and intestines of this body makes this, as far as we are aware, the longest period yet noted between ingestion and a successful analysis. Further experience has demonstrated that after the ingestion of yellow phosphorus there can be a phosphorus metabolite present in the liver which behaves as if it were a phosphide, that is, it liberates phosphine, at room temperature on treatment with acids. Although in the deaths of animals after ingestion of radioactive zinc phosphide Curry, Price and Tryhorn[27] showed that traces of phosphide could also be demonstrated in the liver, phosphine predominated. Furthermore, in cases of yellow phosphorus poisoning the application of the micro test to a small portion of the intestinal contents, or the wall of the intestine, distinguishes the two rodenticides—the unchanged phosphorus distils only on heating. Free phosphine, and that liberated by acid from any phosphide, are volatile at room temperature. In a 20 g. blood sample submitted by Dr. W. Stewart from a patient suffering from yellow phosphorus poisoning we were unable to demonstrate free phosphorus or metabolite. There is obviously an interesting and difficult field of enquiry open here to anyone who has the inclination to investigate yellow phosphorus metabolites.

Kaye and Goldbaum[28] have illustrated the Prussian-blue stains obtained when cyanide is liberated from small volumes of blood. This is another routine test which ensures that cyanide inhalation or injection deaths are not overlooked. Results in this laboratory agree very well with the illustrations and we agree that this is a simple and rapid test.

In the field of halogenated hydrocarbons the apparent discrepancies in the literature concerning the reaction of carbon tetrachloride in Fujiwara's test have been resolved by the work of Burke and Southern[29] who, using 10 ml. of pyridine and 0·4 ml. of 0·1N sodium hydroxide and heating at 100° for 15 minutes showed that 0·1 to 1·0 mg. of spectroscopically pure carbon tetrachloride gave excellent colours. Klondos and McClymont[30] use acetone (7 ml.) to precipitate serum proteins and extract any carbon tetrachloride in blood (3 ml.) and then use 7 ml. of pyridine, 2 ml. of the acetone extract, and 3·5 ml. of 20 per cent sodium hydroxide, heating at 100° for 2·5 minutes. They claim a lower limit of sensitivity of 4 μg./ml. of blood. Gas chromatography has been used to determine chloroform in aqueous pharmaceutical preparations and Dr. Toseland tells me he has used it successively in a fatality following the ingestion of carbon tetrachloride. When in blood or body tissues, chloral hydrate decomposes rapidly even in refrigerated storage and reports of body concentrations in fatal cases from poisoning are rare. Levels of about 10 to 20 mg. per cent coincide with very large doses under the conditions of time betwen ingestion and analysis prevailing in our area. A case of poisoning by methyl bromide has been described[31].

Paraldehyde is still widely used as a hypnotic and sedative and cases are known where old samples have extensively decomposed with subsequent injury to the patient. One sample we examined contained 40 per cent of acetic acid because of oxidative decomposition. The analysis of paraldehyde has been described and levels higher than about 50 mg. per cent in the blood indicate poisoning[32]. This is in agreement with earlier work.

As was noted previously parathion is now a common poison: reports of fatalities and analytical techniques has been reported from all parts of the world. Workers in Singapore showed that 2 mg. was a fatal dose for children aged 5 to 6 years[86].

Fiori[34], after precipitating the proteins with a trichloracetic acid: ethanol mixture, adsorbs the parathion on alumina from which it is eluted with ether. Using 5 per cent ethyl ether in water-saturated light petroleum, parathion has an R_F of 0·98, free *p*-nitrophenol running at R_F 0·30. By using two ultra-violet lamps emitting 254 and 360 mμ the two compounds, whose absorption maxima are at 278 and 320 mμ respectively, can be seen as absorbent spots. Otter[35] has also shown that although organophosphorus compounds do not normally react with an acid molybdate spray on paper chromatograms they will do if previously treated with *N*-bromosuccinimide. European workers have also very fully described instances of poisoning and the analytical work concerned in the detection of parathion[36,37].

Poisons Volatile in Steam from Alkaline Solution

There has been no great increase in the number of drugs to be found in this fraction. Propylhexedrine has replaced amphetamine sulphate in inhalers in this country and this compound gives a positive reaction with potassium bismuth iodide. Systematic analysis of this fraction

usually involves the paper chromatographic examination (butanol 40: water 50: acetic acid 10) of the base hydrochlorides. Detection is in three stages: firstly inspection in 254 mμ radiation; secondly, spraying with 0·1 per cent bromocresol green in ethanol; thirdly, a superimposed spray of potassium bismuth iodide. This order of sprays detects the bulk of compounds in this fraction. Further sprays of great use are ninhydrin, and the acetaldehyde: sodium carbonate: sodium nitroprusside spray[38]. These latter two are of special use for the secondary amines.

The fact that nicotine hydrochloride is more volatile than nicotine, recently manifested itself in an interesting problem. A paper chromatographic examination of alkaloids extracted from the urines of suspected dope addicts unaccountably gave a Dragendorff spot at the R_F of nicotine, a phenomena not previously noted. Two of these spots were sent to us and we were able to elute and obtain R_F values in other solvent systems and also ultra-violet absorption curves. Dr. E. G. C. Clarke succeeded in getting two microcrystal tests which also confirmed the identification as nicotine. The method of evaporation of the aqueous base hydrochlorides has been changed and this accounted for the unexpected appearance of nicotine in the urine: previously the hydrochloride had been lost at too high an evaporation temperature.

Isolation Techniques

There have been a number of papers published concerning the isolation of organic solvent-soluble poisons from viscera. Three types of approaches are discernible. In the first of these the poison or drug is extracted with an excess of organic solvent from small quantities (about 5 g.) of urine, blood or homogenised tissue in buffered aqueous solution. The compounds involved in these methods are almost invariably those whose high ultra-violet absorption characteristics make them suitable for detection and assay by this means. The second approach involves attempts to use solvents which show a less tendency to emulsify when shaken with tissue. Rieders[39] has used butanol from sulphuric acid homogenates which have been saturated with ammonium sulphate, and Abernethy[40] and his co-workers have used acetonitrile-ether (1 : 2). The third approach depends on the use of ethanol as the extracting solvent. Dybing, for example, has used it for both 3,3-diethyl-2,4-dioxotetrahydropyridine[41] and α-naphthylthiourea[42]. In many parts of the world, organs are submitted to the toxicologist preserved in ethanol because high temperature conditions make putrefaction a major problem. Curry and Phang[43] have devised an ethanol continuous extractor of very simple design working under reduced pressure which shortens the extraction time of the Stas–Otto process from days to 4 to 5 hours, gives excellent extracts, and whose efficiency has been demonstrated with such labile alkaloids as cocaine and atropine and such glycosides as digoxin and solanine. The device has also the advantage of economy in the volume of ethanol necessary for an extraction.

There has also been a realisation in toxicological analysis that when very small quantities of poison are being extracted by very large volumes

of organic solvent, the purity of the solvent may be of vital importance. The reaction of chlorobromomethane, which can be present in chloroform B.P., with strychnine is a typical example[44]. This type of reaction has also been examined by Williams[45]. The presence of peroxides in ether and their interference in analysis has been noted[46].

The treatment of tissue or urine samples to hydrolyse drug metabolites, so as to increase the amount of free drug which can be isolated, is now commonplace. Morphine in urine is a typical example. Mannering[47] makes the urine approximately normal with hydrochloric acid and autoclaves for 30 minutes under 15 lb. pressure. 5 N acid at atmospheric pressure is also used. In the isolation of chlorpromazine and promazine we tried all the usual methods with complete failure. It appears that only after an acid hydrolysis as recommended by Dubost and Pascal[48] can the phenothiazine derivatives be extracted.

Acidic and Neutral Poisons Soluble in Organic Solvents

In our hands Trinder's method[49] for the determination of salicylate in blood and tissue homogenates gives results in excellent agreement with those obtained by ultra-violet spectrophotometry. This is another example of a test which can give an accurate result in approximately one minutes' working time on only 1 ml. of blood.

Unfortunately there is still no simple, rapid, spot test for determining the barbiturates. It is now recognised that in order to determine the effect of a barbiturate upon an individual it is necessary to know, not only the concentration of barbiturate in the blood or tissues, but also the identity of the particular drug. Phenobarbitone and barbitone accumulate in the body when taken in therapeutic dosage over long periods and in these circumstances blood levels of 5 mg./100 ml. are to be expected. With the shorter acting barbiturates a concentration like this would certainly imply the consumption of grossly excessive quantities. The identification of the particular barbiturate is therefore of great importance. The discovery[50] that the anti-epileptic drug, mysoline, is converted in the body to phenobarbitone underlines the comments made in the previous review stressing that care must be taken in the interpretation of results.

For the isolation of barbiturates from blood or tissue my own preference is a tungstic acid (Folin–Wu) precipitation followed by ether extraction and separation of the weakly acidic fraction. If the digest is warmed on a water bath the protein is precipitated and is easily removed by filtration. Quantitative assay by ultra-violet spectrophotometry can be accomplished either by measuring the decrease in optical density at 240 mμ when acid is added to an ammoniacal solution at pH 10[51], or by Goldbaum's differential method[52] (see also Maher and Puckett[53] and Broughton[54]) which involves readings at eleven wavelengths all at two pH's. An immediate approximate value for the concentration is obtained using the former method because one mg. of all the 5,5′-disubstituted barbiturates dissolved in 45 ml. of solution gives an optical density difference of 1·0 ($\pm$ 15 per cent). Broughton's method, which includes an extension of Goldbaum's and Maher and Puckett's works, can be used for identification provided

a single, reasonably pure, barbiturate is present. This latter proviso can never be assumed and consequently the method is of very limited usefulness. For identification, paper chromatography is undoubtedly still the most revealing technique. Jackson[55] has written an excellent chapter on this subject in Ivor Smith's book on Chromatographic Techniques. For routine use the butanol : ammonia : water (2 : 1 : 3) system is most reliable. Despite the greater resolution claimed for buffered paper systems using halogenated hydrocarbon solvents my own experience has not been sufficiently rewarding to warrant, as yet, a change from the Algeri and Walker[56] system. Even 2 hours running time can adequately separate the slow from the quick acting barbiturates and as such it is of great use for the clinical biochemist. Cobalt salt sprays followed by exposure to ammonia or the mercury : diphenylcarbazone reagents are convenient colorimetric methods but inspection in a "chromatolite" emitting 254 mμ radiation is undoubtedly the quickest and most convenient method. The fact that the spots cannot be seen until ammonia is blown over the paper also directly indicates the pH : ultra-violet absorption changes which are particularly associated with the barbiturates.

Phenobarbitone, which absorbs slightly at this wavelength at neutral pH can be faintly seen before exposure to ammonia and so distinguished from barbitone which runs at a similar R_F. Barbiturates with an allyl, or other unsaturated radical in the molecule can be detected by a 0·1 per cent aqueous potassium permanganate spray and the β-bromallylbarbiturates by the copper catalysed reaction with peroxide and fluorescein give the pink spot of eosin[57]. In the butanol : ammonia solvent system, amylobarbitone, pentobarbitone and quinalbarbitone all run very close together. Complete differentiation of these three barbiturates either singly or in any mixture combination can be achieved by using the method published by the author[58], whereby the ultra-violet absorption spectrum is measured in ammoniacal solution after treatment with concentrated sulphuric acid at 100° for 1 hour. Only butobarbitone, R_F 0·69, and amylobarbitone, R_F 0·73, can be confused. In a less than 1 mg. sample submitted to us for our advice on this problem we found that infra-red analysis and Broughton's modification of Goldbaum's technique did not resolve the ambiguity. Micro sublimation of the eluates from the paper chromatogram did, however, give crystals and X-ray diffraction analysis showed that the compound was butobarbitone. Although the ready crystallisation of amylobarbitone coupled with an examination of crystal shapes are other useful diagnostic criteria, polymorphism in this series makes crystallographic studies unreliable unless combined with other techniques. Apart from this one instance, we have never had any difficulty in achieving identification using the combination of paper chromatography, a permanganate spray and ultra-violet spectrophotometry. Some clinicians use blood barbiturate concentrations as an aid in assessing the length of time the patient is likely to remain in coma. All the common barbiturates are excreted or metabolised at a rate corresponding to a drop of approximately 2 mg./100 ml. in the blood per 24 hours and consciousness can be expected at about 8 mg. per cent for barbitone,

5 mg. per cent for phenobarbitone and 2 to 3 mg. per cent for amylobarbitone and butobarbitone. Pentobarbitone and quinalbarbitone need to be almost completely destroyed before consciousness is regained. The fact that the smaller hospitals do not have an ultra-violet spectrophotometer prevents the more widespread use of this clinical aid. Addiction to barbiturates can also be confirmed by a consideration of the concentration of barbiturate in the blood. Normal therapeutic doses of the short or intermediate acting barbiturates do not lead to concentrations of more than 0·5 mg. per cent; in patients taking, say, 30 grains (2 g.) of amylobarbitone a day a level of 3 to 4 mg. per cent can be expected[60]. Sunshine and Curry[59] following Wright's work[60] have plotted liver: blood concentration ratios against the time interval between ingestion and death and have discussed the possible use of the ratio in assessing these time intervals and in distinguishing cases of poisoning from those of addiction. These workers stress the great importance of avoiding blood which has been taken from a part of the body near to the stomach. Analyses on blood samples in the same case taken from a body cavity and from a peripheral vein have been found to give results differing by more than a factor of 10.

Bemegride is widely used to combat barbiturate poisoning and can interfere in the examination by ultra-violet spectrophotometry unless adequate precautions are taken. Broughton[54] and Curry[61] have both suggested means whereby this can be done. Glutethimide, α-ethyl-α-phenylglutarimide, is a cyclic imide, whose ring is very easily opened in alkaline solution. Great care must be taken in the isolation of these imides to avoid alkaline conditions because even a few minutes in 0·5 N sodium hydroxide can hydrolyse them. Measurement of the rate of hydrolysis by observing the decrease in optical density at the absorption maximum with time provides a simple criterion of identity[62]. Investigations on the metabolism of glutethimide and reports of tissue concentrations in cases of poisoning have been reported[63]. The oral hypoglycaemic agent tolbutamide has been the subject of a paper by Bladh and Norden[64] who used column chromatography as the means of purification and the absorption maximum at 228 mμ as the method of assay. After a single dose of 4 g. to diabetic patients, maximum serum levels of about 15 to 20 mg. per cent were found 4 to 5 hours after ingestion.

The ready availability of the bromoureides, carbromal and bromvaletone, has meant that these drugs are often found in routine analyses. There is unfortunately very little known about the metabolism of either and because they cannot be detected unchanged other than in the alimentary tract interpretation of analytical results can be difficult. The consumption of bromoureides over a long period of time can lead to a clinical state requiring medical attention. Copas, Kay and Longman[65], describing this state of chronic intoxication, drew attention to the very high inorganic bromide levels that occur in the blood of such patients. Although levels in the region of 50 to 200 mg. per cent imply habituation, a very low blood bromide level does not exclude a single gross overdose. The determination of blood bromide levels, alone, does not therefore materially

assist in investigations of routine sudden deaths where bromoureides are concerned. Dr. Halstrøm tells me that a low blood bromide level coupled with an elevated concentration in the liver implies the recent consumption of an overdose. This is a most valuable observation. If alkali is used in the extraction of the bromoureides then loss of hydrogen bromide can occur to give, in the case of carbromal, α-ethyl crotonylcarbamate. The compound can be confused with apronal (Sedormid) but infra-red curves resolve any ambiguity. Cyanide is also a by-product of this reaction. Curry and Grime[120] have isolated diethylacetyl urea from the intestines of a person who was a carbromal-bromvaletone addict. This is presumably the ureide noted by Turner[119]. The tranquilliser, meprobamate, has been the subject of much attention, even the tritium-labelled drug has been used to follow its metabolism[66]. Bedson[67] has very fully covered the analysis from the forensic chemist's viewpoint. His methods for identification included paper chromatography, the determination of partition coefficients, and the measurement of the spectrum of the product obtained by heating meprobamate with concentrated sulphuric acid at 100° for 30 minutes. This treatment afforded a method for assaying the drug: the optical density difference between 440 mμ and 500 mμ being directly proportional to the concentration of meprobamate. Therapeutic dosing did not lead to blood levels much over 2 mg. per cent whereas in the case of coma observed by Bedson the concentration of drug in the blood was 21·5 mg. per cent.

The drug is isolated by extraction with ether after deproteinisation of the blood by a Folin–Wu precipitation. It is usual in a routine scheme to use this method on a 5 ml. sample of blood for the assay of any barbiturate in the weakly acidic fraction. The neutral fraction, after routine checking for ultra-violet chromophores can then be screened for meprobamate either by this treatment with concentrated sulphuric acid or by the chlorine-starch iodide treatment on a paper chromatogram. This latter method will also detect other carbamates. Petty and his colleagues[68] have emphasised the value of recording ultra-violet spectrophotometers in plotting inflexions in spectra in a case of diphenylhydantoin poisoning. This compound has no major absorption maxima but exhibits significant minor inflexions which would not normally be observed in a manual measurement.

Alkaloids

There is some difficulty in the nomenclature of compounds in this fraction. Naturally occurring nitrogen-containing compounds, the alkaloids, are not differentiated in the extraction from the synthetic compounds whose number now greatly exceeds that of the true alkaloids. The absence of a simple noun for the complete group is inconvenient and henceforth where alkaloid is mentioned it must be taken to include the toxicological group and not only one of the classically named compounds.

There has been no need to alter the general approach to the analysis of alkaloids outlined in the last review. This emphasised the use of paper chromatography in the analysis of this fraction as a means of purification

of the extract as well as concentrating the attention of the analyst on a particular spot or spots. Systematic spot testing on the chromatogram coupled with other tests on the eluates was the main approach. A method of eluting the μg. quantities of alkaloids from spots, and of concentrating the solutions, has been described by Curry and Tryhorn[69] and reviewed by Curry[70]. For general colorimetric methods of revealing the alkaloid spots on chromatograms the nitroprusside : acetaldehyde spray has been found useful as an ancillary test for secondary amines and potassium bismuth iodide, and potassium platinic iodide, are the preferred general spray reagents for tertiary amines. Modifications in these spray reagents have been fully reviewed elsewhere by the author[70] as has the systematic paper chromatographic examination of 55 alkaloids in the butanol : citrate system. The separation of alkaloids into those with significant ultra-violet absorption or fluorescence by inspection in 254 mμ light is most useful because compounds with a similar R_F value can often be completely differentiated by this simple visual inspection. The fluorescence of ergometrine is particularly striking, 0·1 μg. being easily visible on a normal sized spot[71]. As was indicated above, there are now so many alkaloids used in general medicine that one solvent system cannot hope to resolve them all. Dr. E. G. C. Clarke tells me he has a collection of over 400 alkaloids and obviously even two-dimensional chromatography is doomed to failure in attempting an identification on R_F values alone. However, choosing one solvent system and separating those alkaloids with similar R_F by ancillary physical and chemical tests is at the moment an adequate approach. The solvent system is obviously important because it must have a good general resolution so that no more than about 10 alkaloids need to be considered at a particular R_F. Many workers have described methods of this type dealing with up to about 60 alkaloids and Dr. Clarke's review[72], which I am told is much more comprehensive than any previously reported, is eagerly awaited. The use of paper buffered in strips of different pH's[73] was one device which attracted our attention and in the future, if the numbers of alkaloids continue to increase in numbers at a similar rate, it may be more advantageous than a paper buffered at a single pH. Perhaps naturally we have kept in the main to the sodium dihydrogen citrate system and it has, in conjunction with others been instrumental in many important separations. The use of nalorphine as a pharmacological competitive antagonist to morphine has reflected itself in the finding of both drugs in cases of poisoning. In one instance in which a baby accidentally ingested 10 mg. of morphine, 20 mg. of nalorphine were given by injection. At death, five days later, both drugs were detectable (about 10 μg. of each) in the contents of the alimentary tract but neither could be detected in the liver or brain. Morphine is only one drug which is commonly given by injection and the rate of absorption of drugs from injection sites has received very little attention. It is hoped that experimental work in this direction will not be long delayed because it could be a means of assessing the probable injected dose—a subject which in itself is demanding of close attention. Fortunately the practice of measuring concentrations of drugs in the

blood after therapeutic doses is increasing but in several cases at the present time only if an obviously excessive quantity is found in the viscera can dogmatic statements be made. In many cases when death is delayed for several days, interpretations on the analytical findings for the alkaloids are not as easy as for the barbiturates or aspirin. This period of enquiry and consolidation has only just begun.

In the last five years two techniques have been of great use to us in the investigation of this fraction. These are the classical colour tests performed *on* the paper chromatogram and the microcrystal technique devised by Dr. E. G. C. Clarke. In the history of toxicology colour tests based in the main on the concentrated sulphuric acid reagents have been to the fore. There has been built up over the years a most comprehensive literature concerning the reaction of alkaloids with Mecke's, Frohde's, Marquis' and Mandelin's reagents and it seemed a pity that this work appeared to be in danger of sliding into obsolescence with the advent of ultra-violet spectrophotometry and paper chromatography. The discovery that dried paper chromatograms, even those buffered with sodium dihydrogen citrate, stood up to concentrated sulphuric acid without charring or even colouring to the slightest degree has given the classical tests a new lease of life. A combination of the new with the old has revitalised the analysis. Previously the co-extracted fatty material gave deep brown colours with concentrated acid and this obscured traces of alkaloid and put a severe limit on the sensitivity of the tests. These interfering compounds usually run at completely different R_F values to the majority of the alkaloids and so the now pure alkaloid can be detected often in sub microgram quantities by the classical reagents. The reaction of the morphine alkaloids with Marquis' reagent is a typical example. 1 μg. of morphine and codeine in 200 μg. of impurity can be separated by paper chromatography and their different R_F values, coupled with the difference in shade of blue colours, give a very satisfying technicolour picture which is stable for several minutes and can be photographed. Even Vitali's reagent works well for the atropine alkaloids on a citrated paper chromatogram; it is possible to cut a small 2 to 3 sq. mm. piece of paper from the main spot and do the test on a white tile. The use of divided spots in this way increases the number of tests than can be applied. We have not had success with the dichromate: sulphuric acid reagent for strychnine on chromatograms but manganese dioxide: sulphuric acid appears to work quite well.

Coupled with the use of the classical reagents, systematic chemical analysis using reagents designed to detect functional groups in the molecule is also a most useful approach. The phenolic group in morphine provides an obvious example. There is one case which at the moment requires close attention and that is the pethidine series. Although several colour tests work well if relatively large quantities are present there is, to my knowledge, no reagent which will provide a good specific test for this group in the μg. region. The absence of high ultra-violet spectra in the aryl piperidines and the diarylalkoneamines underlines the difficulties of these particular analyses.

Other tests designed to reveal a functional group in the molecule include diazotised *p*-nitroaniline and *p*-dimethylaminobenzaldehyde, two classical reagents of sound theoretical validity. Another typical example would be the formation of a xanthogenate from the alcohol groups in aconitine followed by a iodide : azide spray to detect the sulphur linkages. Colour tests for aconitine are in general not very specific however and it seems probable that assessment of biological activity of eluates from chromatograms is still the best method. Dr. Dupré's and Dr. Stevenson's work in the Lamson case of 1881 must still be considered a masterpiece of toxicological analysis.

It is now possible by combining ultra-violet spectrophotometry after paper chromatography with these arbitrary and general colour tests to differentiate the majority of those alkaloids which run at similar R_F values. For those few cases in which ambiguity still exists and in all cases as additional criteria of identity, microcrystal tests are used.

Clarke's innovation of the use of capillary rods to convey micro-drops (0·1 μl.) of alkaloid solution and reagents to cover slips which are then inverted over ringed cavity slides has increased by many magnitudes the sensitivity of these tests. With strychnine and potassium mercuri-iodide, for example, a sensitivity of 0·001 μg. is claimed. The differentiation of the optical isomers of *N*-methylmorphinan is a particularly elegant and important example of the value of this type of test. It was shown[74] that only a racemic mixture of isomers could be induced to crystallise and used this fact to distinguish an unknown optical isomer. Because dextromethorphan is not subject to the provisions of the Dangerous Drugs Act although the laevo form is, the value of a test which will differentiate them with a sensitivity of 0·2 μg. is obvious. A saturated solution of trinitrobenzoic acid will also form crystals only with the racemate—another useful diagnostic criterion.

I used to be a sceptic of the value of micro-crystal tests and doubted their validity in assisting in an identification. Over the last few years, however, I have become increasingly enthusiastic and as a result of many examples where their use has been of first rate help I now recommend them as a technique which can materially help the harassed toxicologist. Dr. Clarke's papers include descriptions of tests on common alkaloids,[75] less common alkaloids[76], local anaesthetics[77], antihistamines[78], analgesics[79] and atropine-like drugs[80].

There have been several recent reviews on the subject of the toxicological analysis of alkaloids in which there are comprehensive reference collections. Table I covers only the latest work.

Other Poisons

Metallic poisons continue to form a substantial part of toxicological analysis and improvements in analytical technique have led to easier and quicker determinations. This has also meant that more data is available on normal values in tissue and consequent interpretation of results is becoming easier. Tompsett's method[81] for the determination of lead has given excellent results in our hands although purification of solvents still

continues to be a major time-consuming operation. The practice of keeping separate sets of glassware, one for each particular metal has been found to be practical and avoids extensive cleansing before every analysis. Accidental poisoning of young children from the ingestion of lead paint still occurs and an X-ray examination of the intestines often enables flakes to be easily localised for manual extraction.

The simple test for the excessive excretion of coproporphyrin III[82] in very small volumes of urine as an aid in the diagnosis of lead poisoning has been found to be reliable. Severe infection or liver damage also

TABLE I

RECENT REFERENCES TO TOXICOLOGICAL ANALYSIS OF ALKALOIDS

Paper chromatography and other methods for separation of the mixture	Identification techniques	Quantitative assay
Opium[100]	Colchicine[106,107]	Atropine[112]
Aconitine from brucine[101]	Morphine[108]	Chlorpromazine[113,114]
Papaverine from narcotine[102]	Opium[109]	Colchicine[106]
General methods[103,104]	Phenazocine[110]	Morphine[108]
Reserpine[105]	Solanine[111]	Papaverine[115]
		Quinine[116]
		Reserpine[117,118]

seems to produce this porphyrin in excessive quantities, and the test is of limited usefulness in a systematic search. Abnormal urinary amino acid patterns observed in cases of lead poisoning also have their parallel in other poisons which produce damage to the renal tubules such as mercury, cadmium, uranium, copper, lysol, oxalic acid and phosphorus[83]. Kawerau's comment that aminoaciduria is not a reliable criterion by which to judge a patient's condition is worthy of note by toxicologists. Poisoning by tetraethyl lead is still an occupational hazard; in a series of four cases reported by Boyd, Walker and Henderson[84], blood-lead levels were not raised (cf. 85) but lead concentrations in urine and faeces were higher than normal. In only one person was the coproporphyrin III urinary excretion raised.

It is not possible to detail the many variations in analytical technique or the multitude of new reagents introduced for all the toxic metals, indeed, in a systematic analysis for chemical evidence of poisoning the development of rapid screening tests can be as important as investigations into methods which will give a very high degree of accuracy for one particular metal. Routine arc spectroscopy of tissue has been implemented in this laboratory by a paper chromatographic examination. We find that the results are to hand in a shorter time by using paper chromatography and the extensive work required to read the photographic plate from the arc is avoided. Butanol: 3N hydrochloric acid is the solvent system most used and dithizone in chloroform the favourite dipping reagent. For the few metals that do not react with dithizone other selective reagents are used; titan yellow for magnesium being one example. Measurement of blood magnesium levels must be done routinely in toxicological investigations because large doses of magnesium sulphate are

commonly taken in attempts to procure an abortion. The administration to a baby of sugar contaminated with Epsom salts recently came within our experience. The clinical manifestations of magnesium deficiency have recently been reported and in the same paper[33] Henly and Saunders described slight modifications to the usual analytical method. Thallium is almost unknown in the united Kingdom as a poison but the presence of large numbers of Central Europeans in many cities in England demands its inclusion in the analytical scheme. A hydrochloric acid : hydrogen peroxide digest of 50 g. of liver tissue followed by extraction of the metal chloride into ether is the method of analysis favoured here; evaporation, treatment with nitric and sulphuric acid, reduction and precipitation with potassium iodide complete the screening test. Tissue levels above 0·5 mg./100 g. of tissue are to be expected in cases of poisoning[87].

An authoritative review on the estimation of zinc in biological material appeared fairly recently[88] and the analytical findings in a case of poisoning following inhalation of cadmium fumes were reported by Manley and Dalley[89].

A welcome sign of the times has been the investigation of isolation procedures using radiochemical techniques. Gorsuch's work[90] using radioactive isotopes in the recovery of trace elements in organic and biological materials is a most important example of the value of this type of approach. He investigated recoveries of lead, mercury, zinc, selenium, arsenic, copper, cobalt, silver, cadmium, antimony, chromium, molybdenum, strontium and iron using several methods of destroying the organic material and came to the conclusion that no comprehensive instructions could be given although nitric and perchloric acid digestion was most satisfactory with the single exception of mercury. In this context the recommendations of the Analytical Methods Committee of the Society of Analytical Chemistry on the handling of perchloric acid must be followed[91].

Arsenic has been the subject of many papers and Smith[92] has recently investigated the arsenic content of human hair using activation analysis. His use of the Gutzeit apparatus is particularly interesting as it is widely used in forensic toxicology.

Anions of interest include borate and fluoride, both readily obtainable by the general public. Borate will not dialyse from tissue homogenates, presumably because of the formation of sugar complexes and the measurement of the concentration of total boron in a sample of blood is essential. An easy, reliable, method has been published[93] and normal blood levels are said to be up to as high as 1 mg. per cent. Fluoride is a most difficult analysis and for routine screening the paper published by Gettler and Ellerbrook[94] in 1938 is still probably the easiest method. It can be done using as little as 5 ml. of blood, or even smaller volumes of urine, and in experienced hands takes relatively little working time. As was indicated above, the enzymatic test will give a semi-quantitative figure but for accurate work dry ashing of the tissue followed by distillation and titration cannot be avoided. A diffusion method[95] which uses a sealed polythene bottle as the container holds promise of an easier method however.

Two other classes of poisons must be considered in this fraction. They are the quaternary ammonium compounds and the water soluble physiologically active amines of which adrenaline is the most important. There has been no fundamental advance in the isolation of the former group from viscera and their identification follows the pattern established for the alkaloids. A paper by Lund and Møller[96] on blood levels of adrenaline after accidental injection of fatal doses must be studied in its entirety by forensic toxicologists. The concentrations, 12, 15 and 91 μg./l. speak for themselves in emphasising the extremely delicate and sensitive techniques required in this work. An authoritative review in Methods of Biochemical Analysis has been published[97].

Discussion

In the last ten years toxicological analysis has been but one example of the revolution experienced in analytical techniques. Although nomenclature now covers the range of macro to ultra-micro, 100 mg. to 100 μg., it is becoming necessary in toxicological analysis to consider quantities which in the case of adrenaline for example are present in tissue in about 1 part in 10^8. The volume of peripheral blood which can be obtained from a body sets the limit on the number of tests which can be performed on it. If the number of separate tests continues to increase at the present rate a radical improvement in the sensitivity of each test must be sought. The preservation of blood samples in itself is becoming a major problem. Although an anticoagulant is desirable and fluoride essential to prevent fermentation of blood samples, the presence of such additives can radically affect other analyses. Fluoride itself is a common poison and as a preservative it interferes in some enzymatic tests and in the determination of disulfiram. Heparin also affects disulfiram assay and Wright[98] noted substances in it which interfered in barbiturate analyses. The separation of the blood from a peripheral part of the body into discrete samples each with its own additive is yet another burden the toxicologist puts upon the pathologist and emphasises the very close liaison which must exist between them.

In looking to the future, the objective, that is, the establishment of the true cause of death or illness in all cases, must be kept firmly in view. Because poison in many cases does not radically alter the appearance of the body and poisoning is not even suspected until the analysis has been completed the close co-operation between pathologist and toxicologist must be further cemented. Cases of poisoning admitted to hospital, 4 per cent of all admissions in one series reported[99], are numerous and the training of and the instrumentation available to hospital biochemists should receive close attention.

The need to increase the sensitivity of certain tests because of the limited supply of blood and tissue that can be obtained from one body and because of the vast increase in the number of poisons available to the potential poisoner has already been noted. Because at the moment in many cases non-specific methods are used to measure blood concentrations in relating concentrations to effects of new drugs, the co-operation

of industry and the universities must be sought to encourage the development of tests suitable for use by the forensic toxicologist. Turfitt's words[1] in 1951 are still pertinent, "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 as 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 in number and in scope."

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