# 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 $\mu$g. 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 $\mu$ by 7 to 9 $\mu$ 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 $\mu$ 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 $\mu$g. 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 $\mu$g./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 m$\mu$ the two compounds, whose absorption maxima are at 278 and 320 m$\mu$ 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 m$\mu$ 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 m$\mu$ 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 $\beta$-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 $\mu$g. 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 m$\mu$ 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 $\mu$g. 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 $\mu$g. 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 $\mu$g. of morphine and codeine in 200 $\mu$g. 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 $\mu$g. 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 $\mu$l.) 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 $\mu$g. 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 $\mu$g. 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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# RESEARCH PAPERS

## THE OXIDATION OF SOLUBILISED AND EMULSIFIED OILS

### PART II. SOLUBILITY OF BENZALDEHYDE, CYCLOHEXENE AND METHYL LINOLEATE IN POTASSIUM LAURATE AND CETOMACROGOL SOLUTIONS

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The solubility of benzaldehyde, methyl linoleate and cyclohexene in solutions of potassium laurate and cetomacrogol is reported. A marked increase in the solubility of these water-insoluble materials was observed in solutions of potassium laurate above the critical micelle concentration (CMC). In cetomacrogol, the CMC could not be detected from inspection of the solubility curves, since the solubility was a function of cetomacrogol concentration over the entire concentration range studied. The solubility of methyl linoleate and cyclohexene was directly proportional to cetomacrogol concentration. The solubility curve of benzaldehyde showed a steep rise at low concentrations of cetomacrogol, and the possibility of this being due to a water soluble complex between benzaldehyde and cetomacrogol is discussed. The effect of temperature and the presence of sodium chloride on benzaldehyde solubility is reported.

IN a previous paper results of the oxidation of emulsified and solubilised benzaldehyde were presented[1]. These studies have been extended to cover the oxidation of methyl linoleate[2,3], and cyclohexene[3] in potassium laurate and cetomacrogol. As a preliminary to the study of their oxidation, the solubility of these "oils" in the two soaps was determined. The effect of temperature and addition of sodium chloride, on the solubility of benzaldehyde was also studied.

## EXPERIMENTAL

### *Materials*

*Potassium laurate.* Prepared from lauric acid and carbonate-free potassium hydroxide. Recrystallised from ethanol. *Cetomacrogol* (Texofor AIP, Glovers Chemicals Ltd.). Melting point 45° to 46°. Acetyl value 41·3. Other physical characters of this batch have been described[1]. *Polyethylene glycol* "1300". Prepared by melting together proportionate amounts of polyethylene glycols 600 and 1500 to give a product of average molecular weight 1300. *Benzaldehyde.* Analar benzaldehyde, redistilled under reduced pressure in an inert atmosphere was used, as in previous work[1]. *Methyl linoleate.* Prepared from cotton-seed oil by a bromination : debromination process, followed by esterification with methanol.[4] The ester was fractionated under reduced pressure and further purified by low temperature crystallisation from acetone. Wij's iodine number 263·6; iodometric peroxide value 30. The pure methyl linoleate used in oxidation experiments was found to have the same solubility

as this prepared material. *Cyclohexene.* Redistilled Analar material was used from which the peroxide had been previously removed with acidified ferrous sulphate.

*Methods*

*General method.* Known quantities of organic liquid were weighed into ampoules and the required quantity of surface active-agent solution

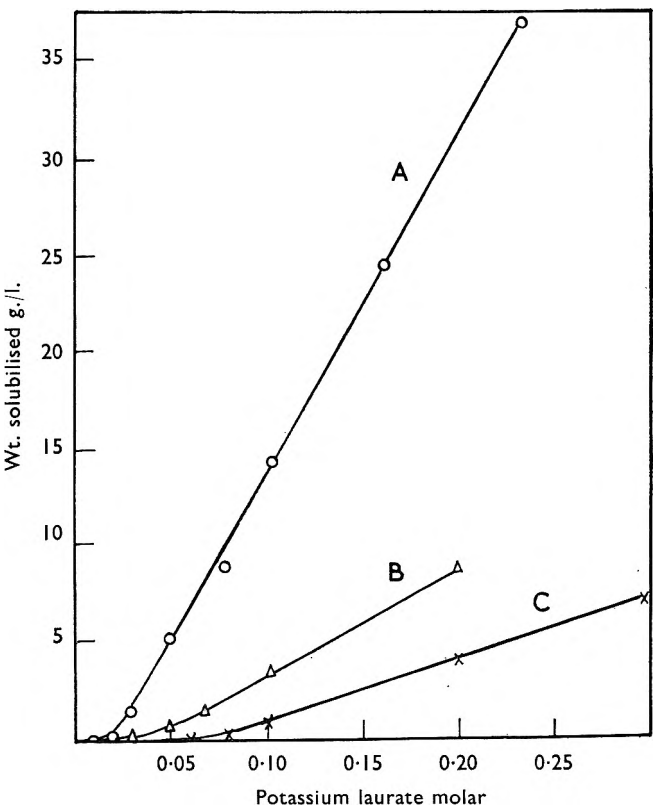


FIG. 1. Solubility* of benzaldehyde, cyclohexene and methyl linoleate in potassium laurate solutions at 20°. A, benzaldehyde; B, cyclohexene; C, methyl linoleate.

* Corrected for solubility in water.

and distilled water added from a burette. The ampoules were sealed by fusion of the glass and rotated for 5 hours at room temperature. They were then placed in a water bath at the desired temperature $\pm 0{\cdot}05°$ for at least a further 2 hours before reading the end point, which was taken as the average between an under-saturated and an over-saturated solution. For low concentrations of soap, 100-ml. flasks closed with ungreased glass stoppers, were used instead of ampoules.

*Determination of the solubility of methyl linoleate in potassium laurate.* Methyl linoleate was rapidly soluble in concentrated solutions of potassium laurate. This allowed the preparation of stock solutions which

TABLE I

THE SOLUBILITY OF BENZALDEHYDE, METHYL LINOLEATE AND CYCLOHEXENE IN POTASSIUM LAURATE AND CETOMACROGOL SOLUTIONS AT 20°

| Soap | Organic liquid | Concentration of soap M | Wt. solubilised g./l.* | Moles organic liquid/Moles soap |
|---|---|---|---|---|
| Potassium laurate | Benzaldehyde | 0·01 | 0·04 | 0·04 |
| | | 0·02 | 0·16 | 0·07 |
| | | 0·03 | 1·56 | 0·49 |
| | | 0·05 | 5·32 | 1·00 |
| | | 0·07 | 8·98 | 1·21 |
| | | 0·10 | 14·44 | 1·36 |
| | | 0·225 | 36·76 | 1·54 |
| | Methyl linoleate | 0·03 | 0·02 | 0·002 |
| | | 0·06 | 0·11 | 0·006 |
| | | 0·08 | 0·28 | 0·012 |
| | | 0·10 | 1·15 | 0·040 |
| | | 0·20 | 4·29 | 0·073 |
| | | 0·30 | 6·95 | 0·079 |
| | Cyclohexene | 0·01 | 0·05 | 0·01 |
| | | 0·02 | 0·14 | 0·09 |
| | | 0·03 | 0·32 | 0·13 |
| | | 0·05 | 0·72 | 0·17 |
| | | 0·07 | 1·47 | 0·25 |
| | | 0·10 | 3·51 | 0·43 |
| | | 0·20 | 8·91 | 0·54 |
| Cetomacrogol | Benzaldehyde | 0·000051 | 0·53 | 98 |
| | | 0·000103 | 0·82 | 76 |
| | | 0·00103 | 2·72 | 25 |
| | | 0·00253 | 3·83 | 14 |
| | | 0·00507 | 4·72 | 8·8 |
| | | 0·0126 | 6·69 | 5·0 |
| | | 0·0439 | 12·77 | 2·7 |
| | | 0·1013 | 24·86 | 2·3 |
| | Methyl linoleate | 0·0028 | 0·16 | 0·19 |
| | | 0·0086 | 0·43 | 0·17 |
| | | 0·0207 | 0·94 | 0·15 |
| | | 0·0470 | 2·43 | 0·17 |
| | | 0·0998 | 5·23 | 0·18 |
| | Cyclohexene | 0·0005 | 0·04 | 1·1 |
| | | 0·001 | 0·16 | 2·0 |
| | | 0·005 | 0·59 | 1·4 |
| | | 0·01 | 1·06 | 1·3 |
| | | 0·05 | 5·27 | 1·3 |
| | | 0·10 | 10·47 | 1·3 |
| Polyethylene glycol 1300 | Benzaldehyde | 0·005 | 0·25 | 0·47 |
| | | 0·01 | 0·51 | 0·48 |
| | | 0·03 | 1·65 | 0·51 |
| | | 0·06 | 3·25 | 0·51 |
| | | 0·10 | 6·05 | 0·56 |

* Corrected for the solubility in water.

could be diluted with further soap solution and distilled water to contain known varying amounts of methyl linoleate. These were then treated in the same manner as in the general method.

*Determination of the solubility of methyl linoleate in cetomacrogol solutions.* A constant weight of cetomacrogol was weighed into a number of 250-ml. glass stoppered flasks and varying quantities of methyl linoleate added. The stopper was inserted, the contents of the flask melted at 50° and gently shaken to disperse. The calculated weight of distilled

water was then added and the flask left in the constant temperature bath overnight. The end point was determined as previously.

## Results

The solubility of benzaldehyde in distilled water was found to be 3·45, 3·49 and 3·97 g./l. at 20°, 25° and 30° respectively. The solubility of cyclohexene was 0·13 g./l. at 20°. The water solubility of methyl linoleate was so low that it could not be detected.

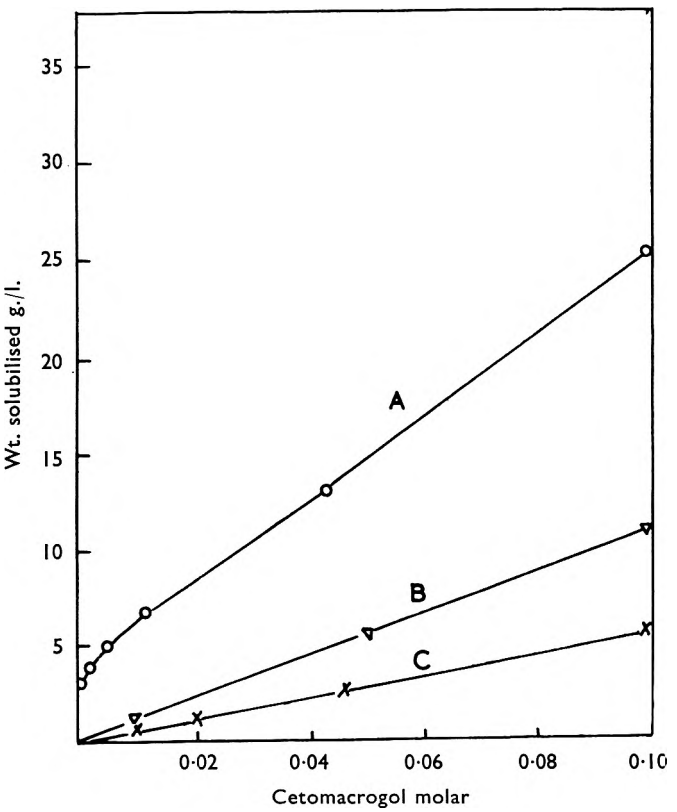


Fig. 2. Solubility* of benzaldehyde, cyclohexene and methyl linoleate in cetomacrogol solutions at 20°. A, benzaldehyde; B, cyclohexene; C, methyl linoleate.

* Corrected for solubility in water.

In solutions of potassium laurate the solubility of all three organic liquids began to increase at a soap concentration of approximately 0·03M and quickly became proportional to soap concentration throughout the range of concentrations studied (Fig. 1, Table I).

Within experimental error the solubility of methyl linoleate and cyclohexene was directly proportional to cetomacrogol concentration (Fig. 2). The increase in the solubility of benzaldehyde with increasing concentrations of cetomacrogol was greater at low concentrations but became

proportional to cetomacrogol concentration between 0·05 to 0·10M. In polyethylene glycol "1300" the solubility of benzaldehyde increased in direct proportion to the polyethylene glycol concentration.

The effect of temperature on the solubility of benzaldehyde is shown in Table II. In potassium laurate solutions the increase in the amount

TABLE II

THE INFLUENCE OF TEMPERATURE ON THE SOLUBILITY OF BENZALDEHYDE IN POTASSIUM LAURATE AND CETOMACROGOL SOLUTIONS

| Soap | Concentration of soap M | | 0·01 | 0·03 | 0·07 | 0·10 | 0·225 |
|---|---|---|---|---|---|---|---|
| Potassium laurate | Solubility* g./l. | 20° | 0·04 | 1·56 | 8·98 | 14·44 | 36·76 |
| | | 25° | 0·07 | 1·35 | 8·64 | 14·25 | 37·26 |
| | | 35° | 0·10 | 1·49 | 9·21 | 15·02 | 37·34 |
| Cetomacrogol | Soap concentration Molar | | 0·002 | 0·005 | 0·013 | 0·044 | 0·101 |
| | Solubility* g./l. | 20° | 3·83 | 4·72 | 6·69 | 12·77 | 24·86 |
| | | 25° | 4·12 | 5·17 | 7·62 | 15·03 | 30·62 |
| | | 35° | 0·01 | 0·08 | 2·73 | 9·88 | 29·74 |

* Solubility corrected for solubility in water at the appropriate temperature.

TABLE III

THE INFLUENCE OF SODIUM CHLORIDE ON THE SOLUBILITY OF BENZALDEHYDE IN POTASSIUM LAURATE AND CEOTMACROGOL SOLUTIONS

| Soap | Concentration of soap M | Solubility* g./l. in | | |
|---|---|---|---|---|
| | | Water | 0·1M NaCl | 1·0M NaCl |
| Potassium laurate | 0·05 | 5·3 | 9·3 | 10·0 |
| | 0·08 | 10·2 | 16·1 | 17·0 |
| | 0·10 | 14·4 | 19·8 | 21·5 |
| | 0·20 | 33·5 | 41·5 | 44·0 |
| Cetomacrogol | 0·005 | 4·8 | 4·9 | 5·0 |
| | 0·01 | 5·3 | 5·4 | 6·2 |
| | 0·02 | 7·8 | 8·0 | 9·3 |
| | 0·05 | 14·2 | 15·2 | 17·8 |

*Corrected for the solubility of benzaldehyde in the appropriate salt concentration.

dissolved is almost accounted for by the increase in the water solubility at the higher temperatures. In cetomacrogol the changes in solubility with temperature are slightly more complex. At 25° the amount of benzaldehyde solubilised increased at all concentrations of cetomacrogol but at 35° the initial rise in solubility was absent and the solubility did not rise above that in water until a concentration of approximately 0·004M cetomacrogol was reached. At higher concentrations of surface-active agent the increase in solubility was proportional to soap concentration and the slope of the curve was greater than that of the 25° solubility curve.

Sodium chloride was found to depress the water solubility of benzaldehyde. In 0·1M sodium chloride the solubility fell to 2·85 g./l. and in 1·0M sodium chloride it was 2·80 g./l. at 20°. However, the presence of salt caused an increase in the amount of benzaldehyde solubilised by a given soap concentration. This increase was much larger in potassium laurate than in cetomacrogol, Table III.

## Discussion

From the experimental results it will be seen that considerable amounts of benzaldehyde, cyclohexene and methyl linoleate are solubilised by the anionic and non-ionic soaps.

The solubility curves of the three materials in the anionic soap, potassium laurate, exhibit the form normally associated with solubilisation measurements. At low concentrations of soap the solubility is approximately the same as in water and it is only after passing the critical micelle concentration (CMC) that there is a rapid increase in solubility as is shown by the increase in the ratio moles solubilisate : mole soap. At soap concentrations of 0·07M and higher, this ratio approaches, but does not reach, a limiting value. This has been interpreted by Hartley[5] to indicate a continued increase in micellar size from the CMC to the point of almost constant molar ratio. At higher soap concentrations he envisaged an increase in the number of micelles with little further increase in size. The present results bear interpretation in this manner.

In solutions of cetomacrogol the solubility curves of methyl linoleate and cyclohexene are straight lines passing through the zero, but with benzaldehyde there was a rapid increase in solubility at low soap concentrations which decreased until it was proportional to cetomacrogol concentration. It will be seen, Table I, that the molar ratio benzaldehyde : cetomacrogol increases rapidly at low concentrations instead of falling as with anionic soaps. As pointed out by Stearns and others[6], molar ratio curves of this type are usually due to a failure to correct for the solubility of the organic liquid in water but in this work this correction has been applied.

Valko and Epstein[7] have reported an increase in the solubility of tripropyl phosphate below the CMC of sodium decyl sulphate. They suggested that this was due to the formation of mixed micelles, the increase in the solubility of tripropyl phosphate commencing at the CMC of the latter. There was no break in the solubility curve in the region of the CMC of sodium decyl sulphate. In the present studies the increased solubility occurred immediately soap was added but the break in the curve still occurred in the region of the CMC of the soap. Mixed micelles cannot therefore readily account for the present solubility results for benzaldehyde in cetomacrogol.

It has been suggested by Wurzschmitt[8] that non-ionic surface active agents exist either as a cationic form or as an equilibrium between a cationic and non-ionic form. He suggests that the cationic form could be written:

$$\left\{ \begin{matrix} & \overset{\displaystyle H}{\vdots} & \\ ROCH_2[CH_2\overset{\displaystyle \cdot}{O}CH_2]_x[CH_2OCH_2]_yCH_2OH & & \end{matrix} \right\}^{+} \quad [OH]^{-}_{x}$$

Wurzschmitt also found that cationic soaps form addition compounds with phenol and a number of instances have been recorded in the literature of complex formation between phenols and non-ionic soaps[9,10]. Mulley and Metcalf[10] consider that the acidic nature of the phenolic hydrogen accounts for the strong hydrogen bonding tendency of the phenolic

hydroxy group. The ultra-violet absorption method they used to show hydrogen bonding, is inapplicable in the present studies because the dilution required to produce a suitable benzaldehyde solution would render the aldehyde water-soluble, and changes due to hydrogen bonding with the soap would be masked. The hydrogen of the carbonyl group of benzaldehyde is much less acidic in nature and hydrogen bonds formed through it should be weaker than those with phenol.

A satisfactory interpretation of the results appears to be that in the non-ionic soaps, benzaldehyde forms a complex with the soap, this complex being more soluble than the benzaldehyde. Consequently there is a marked increase in solubility at low concentrations. When enough uncomplexed soap is present to form micelles the benzaldehyde is preferentially solubilised into the interior of these and a break in the curve results. The change-over does not take place suddenly but gradually as one mechanism of solubilisation takes over from the other. Further evidence for the formation of a complex is found in the increase in solubility of benzaldehyde in polyethylene glycol 1300 solution. This material does not form micelles and might be expected to show a normal solvent effect. Low concentrations have only a slight influence on the water solubility of benzaldehyde, but benzaldehyde solubility was found to increase proportionately to polyethylene glycol 1300 concentration as would be expected if a water soluble complex was being formed.

The effect of temperature on the solubilisation of benzaldehyde also indicates the possible formation of a weak hydrogen bonded complex. The slope of the solubility curve in cetomacrogol increases at higher temperatures, but at 35° the initial rise in solubility found at lower temperatures is absent and solubilisation begins at approximately 0·004M. The disappearance of the initial increase in solubility would be expected if it was due to a weak complex because an increase in temperature would split the complex so that no increase in the solubility of benzaldehyde would result until micelles are present. A concentration of 0·004M is higher than the CMC of cetomacrogol, $1 \times 10^{-6}$M, reported earlier[1]. Sjoblom[11] has found that the solubilisation of oestrone in the non-ionic Tweens does not begin until relatively high concentrations are reached, much higher than those needed to solubilise methylcholanthrene. Thus it appears that solubilisation in non-ionic soaps does not necessarily begin at the CMC, which is often indefinite, but at some higher soap concentration.

The presence of salts was found to increase the amount of material solubilised. Sodium chloride lowers the repulsive forces between the ionised soap molecules thus allowing formation of micelles at lower soap concentrations[12], and results in increased solubilisation. It is difficult to see how this argument can be applied to the increase in solubilisation in non-ionic systems unless the non-ionic soaps can exist in a cationic form.

Whilst observations of electrolyte effect on non-ionic soaps are rare, similar increases have been reported by McBain[13]. The increase in solubility with non-ionic soaps is smaller than with anionic soaps possibly

indicating an increase in size of micelle as was suggested by Hutchinson[12] and Kushner[14].

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# THE OXIDATION OF SOLUBILISED AND EMULSIFIED OILS

## PART III. THE OXIDATION OF METHYL LINOLEATE IN POTASSIUM LAURATE AND CETOMACROGOL DISPERSIONS

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Oxidation rates of aqueous dispersions of methyl linoleate emulsified and solubilised by potassium laurate and cetomacrogol, have been measured manometrically at 40°. Emulsions of methyl linoleate oxidised at a greater rate than solutions. The relation between concentration of surface-active agent and oxidation rate of emulsions is complex, and appears to depend on the relative amounts of emulsified and solubilised oil present. Poor agreement was obtained between oxidation rates calculated from peroxide values, chemical and polarographic, and those calculated from oxygen uptake. The results are discussed. An increase in light absorption at 234 mμ in the early stages of oxidation was observed. An uptake of 2·5 mol. oxygen per mol. of methyl linoleate was estimated to occur during the primary oxidation reaction in the presence of cetomacrogol, and 1·5 mol. oxygen per mol. of methyl linoleate in potassium laurate dispersions. The possible mechanism of the reaction and the nature of the oxidation by-products are discussed.

THE use of aqueous solutions of surface-active agents for the solubilisation of essential oils, steroids and oil soluble vitamins, is becoming of increasing importance. The stability of solubilised vitamin A has been reported by Kern and Antoshkiw[1] and Coles and Thomas[2] to be greater than the corresponding oily solution. In our previous paper[3] we reported the stability of benzaldehyde solubilised with potassium laurate and cetomacrogol. Solubilised benzaldehyde was found to be more resistant to atmospheric oxidation than emulsified benzaldehyde.

The autoxidation of methyl linoleate in the absence of water has been extensively studied and detailed mechanisms of oxidation have been suggested[4]. The primary products of autoxidation are conjugated hydroperoxides. The formation of these conjugated products can be detected by spectrophotometry since they show characteristic absorptions in the 230–236 mμ region. An accurate estimation of the amount of conjugated diene peroxide is impossible unless the relative proportions of the geometric isomers are known, since the *cis-trans* and *trans-trans* conjugated hydroperoxides have widely different extinction coefficients.

The oxidation of fat emulsified by gelatin and soya bean phosphatides for use by intravenous alimentation has been reported by Gayer and others[5]. They found that emulsification lowered the rate of oxidation of the fat, except when gelatin was used. They suggested that possible mechanisms for the lower oxidation rate were retardation of oxygen diffusion, an outer network of groupings able to scavenge metal ions and so prevent catalysis or the prevention of an optimal spacial arrangement for oxidation of the oil. They suggested that only materials which are

soluble in both the water and oil phases could act as a protective film. No evidence was given for the mechanisms suggested.

The rate of oxidation of irradiated methyl linoleate emulsions has been estimated[6] from chemical peroxide values and the conjugated diene content. The experimental details for this latter method were not specified and an attempt to correlate the degree of oxidation obtained from the two values showed wide discrepancies. The presence of up to 50 per cent water has been shown to retard the onset of autoxidation, but not the final rate attained[7] and a peroxide differing from the normal conjugated hydroperoxide has been reported by Bergstrøm and others[8] for the oxidation of sodium linoleate in water.

In this paper it is shown that emulsions of methyl linoleate oxidise at a greater rate than solutions. The ratio of emulsified methyl linoleate to solubilised methyl linoleate appears to play a predominant role in determining the rate of oxidation.

The solubility of methyl linoleate in potassium laurate and cetomacrogol solutions is reported elsewhere[9].

## EXPERIMENTAL

### *Materials*

*Methyl linoleate* was obtained from the Hormel Institute. The stated characters of this material were as follows. Wij's iodine number was 261·4. The theoretical value is 260·4. Infra-red analysis showed the compound to be completely *cis-cis* in character. Conjugated constituents, as methyl esters of $C_{18}$ fatty acids per cent were; dienoic, less than 0·05, trienoic, a trace; tetraenoic, a trace. Peroxide was absent.

*Surface-active agents.* The potassium laurate and cetomacrogol are those used and described in previous studies[3].

### *Methods*

*Preparation of dispersions.* Potassium laurate dispersions were prepared by dissolving the methyl linoleate in concentrated solutions of the soap and then diluting with water and catalyst solution to produce the required concentration. Dispersions in cetomacrogol solutions were prepared by adding the methyl linoleate to the molten cetomacrogol and shaking to disperse, before adding the water and catalyst solutions.

*Measurement of oxygen uptake.* The oxygen uptake was measured at 40° in the Warburg apparatus using the technique previously described[3].

*Measurement of iodometric peroxide values.* This was based on the method of Boehm and Williams[10] modified as follows. 1 ml. of the oxidising dispersion was added to 10 ml. of a mixture of 3 volumes of glacial acetic acid and 2 volumes of chloroform in a glass stoppered flask, the atmosphere of which was replaced by nitrogen. 1 ml. of a saturated solution of potassium iodide was added and the flask placed in the dark for 10 minutes. The solution was then titrated with 0·005 N sodium thiosulphate solution using 1 ml. of a 1 per cent starch solution as indicator. Blank determinations on the reagents were also made. The

results were calculated as milli-equivalents of oxygen per kg. of methyl linoleate.

*Measurement of polarographic peroxide values.* 1 ml. of the oxidising dispersion was diluted with 4 ml. of lithium chloride:lithium acetate buffer solution[11] and made up to 10 ml. with 96 per cent ethanol. 3 ml. of this solution was placed in the polarographic cell and the remainder

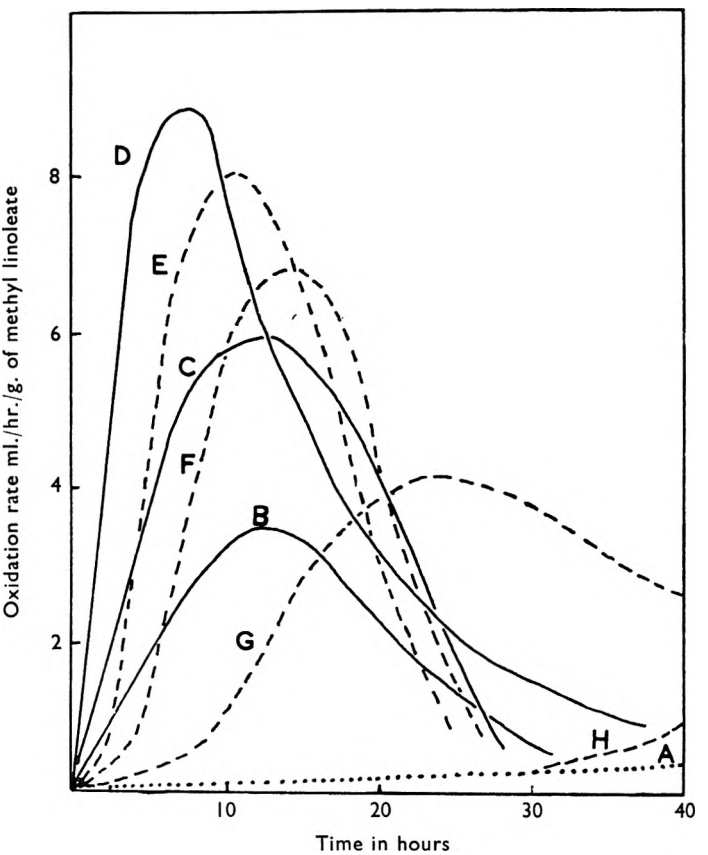



FIG. 1. The oxidation of dispersions of methyl linoleate in potassium laurate solutions at 40°. Methyl linoleate, 3·2 mg. ml. Catalyst $1 \times 10^{-5}$ M $CuSO_4$. Potassium laurate: A = 0; B = 0·01; C = 0·04; D = 0·07; E = 0·1; F = 0·2; G = 0·6; H = 0·8 molar.

.... Suspension. —— Emulsions. – – – Solutions.

in the second of two wash bottles, the first one of which contained 96 per cent ethanol. The cell and wash bottles were connected, placed in a water bath at 20° ± 0·1° and nitrogen passed through the cell for 10 minutes to deoxygenate. The current voltage curve between 0 and −2 volts was measured with a manual polarograph (Electrochemical Laboratories) and the polarographic peroxide value was calculated from the formula given by Willits and others[11].

*Measurement of ultra-violet absorption spectra.* A Unicam SP500 spectrophotometer modified by Morton[13] was used for all ultra-violet determinations. 1 ml. samples were diluted with water, or in the potassium laurate dispersions, absolute ethanol to give a suitable solution. The results were plotted as $E$ (1 per cent, 1 cm.).

## Results

### *Oxygen Uptake*

The uptake of oxygen by dispersions of methyl linoleate is shown in Figures 1 and 2. Figure 1 represents the results of two series of experiments. During the overnight periods the taps of the Warburg apparatus were left open so that the total uptake was unknown, and only the rate of oxidation could be measured after the first day.

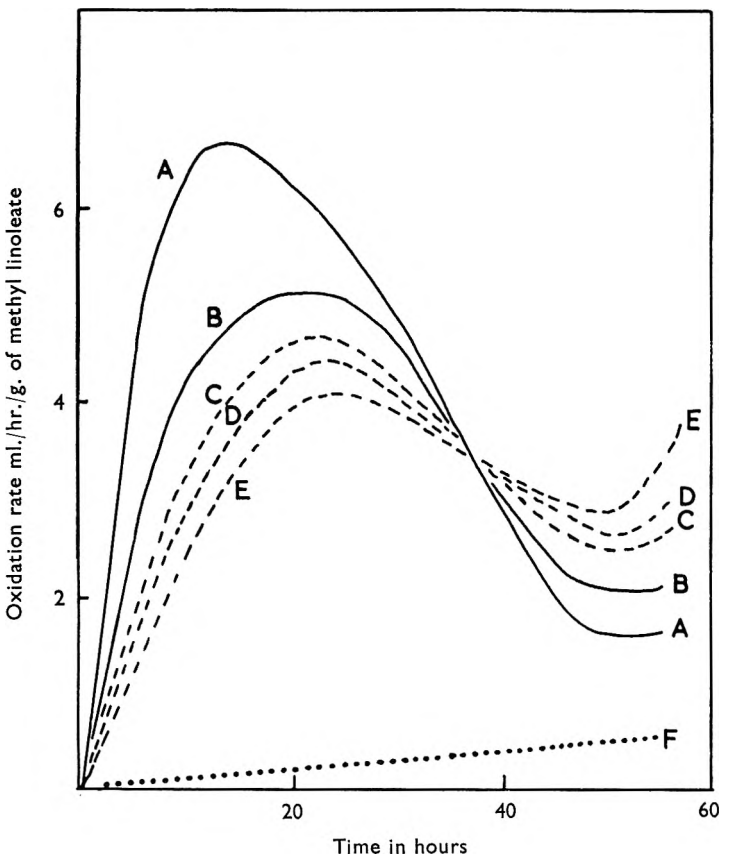



FIG. 2. The oxidation of dispersions of methyl linoleate in cetomacrogol solutions at 40°. Methyl linoleate, 2·43 mg./ml. Catalyst $1 \times 10^{-5}$ M $CuSO_4$. Cetomacrogol: A = 0·01; B = 0·03; C = 0·047; D = 0·065; E = 0·09; F = 0 molar.

.... Suspension. —— Emulsions. – – – Solutions.

Suspensions of methyl linoleate in water in the absence of surface-active agents oxidised at a very low rate. The emulsions formed by the addition of potassium laurate oxidised at a higher rate, which increased with soap concentration. When sufficient potassium laurate was added to solubilise the oil, the rate of oxidation fell. Reference to Figure 1 shows the pattern of oxidation rates as the oil is emulsified or solubilised with different amounts of potassium laurate. It can be seen that a reduction in the concentration of soap in the solutions resulted in a decreased induction period and a higher maximum rate of oxidation. Emulsions showed no induction period.

In cetomacrogol dispersions the maximum rate of oxidation fell with increasing soap concentration irrespective of the state of the dispersion (Fig. 2). Solutions showed no marked induction period as did the potassium laurate dispersions.

It also appears from Figure 2 that a second reaction takes place in cetomacrogol dispersions, although a noticeable velocity is only reached as the rate of the primary reaction decreases to a low value. The secondary reaction was more rapid in the solubilised systems.

The concentration of potassium laurate was maintained at 0·32M and that of cetomacrogol at 0·065M whilst studying the effect of methyl linoleate concentration on the oxidation rate. The rate of oxidation of all the dispersions passed through a maximum. The oxidation rates after four hours showed a sigmoid curve, the higher rates occurring with emulsions of methyl linoleate. In cetomacrogol systems it was found that concentrations of methyl linoleate greater than 9 mg./ml. resulted in a lower oxidation rate per gram of oil probably because of diffusion of oxygen becoming a limiting factor. When the concentration of oil was reduced to 0·19 mg./ml the oxidation rate was found to be 48 ml./hour/g. of methyl linoleate, but a tenfold increase in linoleate concentration caused a fall of oxidation rate to only 5 ml./hour/g. The rapid oxidation of dilute solutions of olefinic materials has been previously reported[14] and it was suggested that it proceeded by a mechanism differing from the normal chain reaction.

Methyl linoleate added directly to dilute cetomacrogol solutions formed an emulsion and no solubilisation could be detected even after several months of storage. It was found that these dispersions oxidised at a rate of 1·95 ml./hour/g. of methyl linoleate irrespective of the concentration of cetomacrogol used, over the range 0·02 to 0·08M. Thus in the

TABLE I

THE TOTAL OXYGEN UPTAKE DURING THE PRIMARY OXIDATION REACTION OF METHYL LINOLEATE IN CETOMACROGOL

| Concentration of cetomacrogol M | Concentration of methyl linoleate M | State of dispersion | Mol. of oxygen/ mol. methyl linoleate |
|---|---|---|---|
| 0·01 | 2·40 | Emulsion | 2·80 |
| 0·03 | 2·44 | Emulsion | 2·57 |
| 0·047 | 2·44 | Solution | 2·50 |
| 0·065 | 2·43 | Solution | 2·43 |
| 0·09 | 2·44 | Solution | 2·48 |

absence of solubilised oil the amount of soap does not affect the oxidation rate of these emulsions.

The estimation of total uptake of oxygen by methyl linoleate was obtained graphically from Figures 1 and 2, by measuring the areas under the curves, after extrapolation of the curves to the time axis. This method would tend to overestimate the amount of oxygen used in the primary reaction occurring in cetomacrogol dispersions, due to the overlap of the secondary reaction; a total uptake of approximately 2·5 mol. of

TABLE II

THE INFLUENCE OF CATALYST CONCENTRATION ON THE RATE OF OXIDATION OF EMULSIONS AND SOLUTIONS OF METHYL LINOLEATE

| Soap | Concentration of soap M | Concentration of methyl linoleate M | Concentration of copper sulphate M | State of dispersion | Oxidation rate* ml./hr./g. |
|---|---|---|---|---|---|
| Cetomacrogol | 0·045 | 0·0085 | 0<br>$1 \times 10^{-6}$<br>$1 \times 10^{-5}$<br>$5 \times 10^{-5}$<br>$1 \times 10^{-4}$ | Solution | 0·5<br>0·7<br>0·8<br>0·9<br>1·0 |
| Cetomacrogol | 0·046 | 0·0165 | 0<br>$1 \times 10^{-6}$<br>$1 \times 10^{-5}$<br>$5 \times 10^{-5}$<br>$1 \times 10^{-4}$ | Emulsion | 4·2<br>6·1<br>6·6<br>7·3<br>7·5 |
| Potassium laurate | 0·32 | 0·0211 | 0<br>$1 \times 10^{-6}$<br>$1 \times 10^{-5}$<br>$5 \times 10^{-5}$<br>$1 \times 10^{-4}$ | Solution | 4·2<br>4·7<br>4·8<br>4·9<br>4·2 |
| Potassium laurate | 0·32 | 0·0394 | 0<br>$1 \times 10^{-6}$<br>$1 \times 10^{-5}$<br>$5 \times 10^{-5}$<br>$1 \times 10^{-4}$ | Emulsion | 9·2<br>9·7<br>10·1<br>10·8<br>11·8 |

* Measured after four hours.

oxygen per mol. of methyl linoleate was found (see Table I). In potassium laurate dispersions where no secondary reaction interferes, approximately 1·5 mol. of oxygen per mol. of methyl linoleate was found. Bergstrøm and others[8] reported finding a total uptake of 2 mol. of oxygen per mol. of linoleate with aqueous solutions of sodium linoleate.

Table II shows the effect of copper catalyst concentration on the oxidation of emulsions; the rate being measured after four hours. Increases in catalyst concentration produced only a relatively small increase in the rate of oxidation.

*Peroxide Values*

The iodometric and polarographic peroxide values of the emulsions and solutions of methyl linoleate appear in Table III, together with the corresponding values of the oxygen uptakes of the systems. There was no apparent correlation of either of these two peroxide values and the oxygen uptake. In a further series of experiments, Table IV, of longer duration, the iodometric peroxide value reached a maximum and then began to fall, although the oxidation rate continued to increase; a similar

## TABLE III

CHANGES IN THE PEROXIDE VALUE DURING THE OXIDATION OF EMULSIONS AND SOLUTIONS OF METHYL LINOLEATE

| Soap | Concentration of soap M | Concentration of methyl linoleate M | State of dispersion | Time hours | Oxygen uptake mol. $O_2$/ml. linoleate | Chemical peroxide value m-equiv. $O_2$/kg. linoleate | Calculated oxygen uptake from chemical peroxide value mol. $O_2$/mol. linoleate | Polarographic peroxide value m-equiv. $O_2$/kg. linoleate | Calculated oxygen uptake from polarograph peroxide value mol. $O_2$/mol. linoleate |
|---|---|---|---|---|---|---|---|---|---|
| Potassium laurate | 0·08 | 0·0096 | Emulsion | 0 | 0 | 30 | 0·002 | 10 | 0·000 |
| | | | | 10 | 0·72 | 270 | 0·019 | 280 | 0·020 |
| | | | | 25 | 1·69 | 410 | 0·030 | 510 | 0·037 |
| | | | | 36 | 2·48 | 480 | 0·035 | 870 | 0·064 |
| Potassium laurate | 0·20 | 0·0105 | Solution | 0 | 0 | 30 | 0·002 | 10 | 0·000 |
| | | | | 10 | 0·46 | 280 | 0·021 | 150 | 0·011 |
| | | | | 25 | 1·12 | 350 | 0·026 | 440 | 0·033 |
| | | | | 36 | 1·41 | 390 | 0·028 | 480 | 0·035 |
| Cetomacrogol | 0·02 | 0·0093 | Emulsion | 0 | 0 | 20 | 0·001 | 0 | 0·000 |
| | | | | 10 | 0·36 | 1,200 | 0·088 | 270 | 0·019 |
| | | | | 25 | 0·99 | 5,200 | 0·383 | 680 | 0·050 |
| | | | | 36 | 1·45 | 5,960 | 0·439 | 590 | 0·044 |
| Cetomacrogol | 0·06 | 0·0091 | Solution | 0 | 0 | 30 | 0·002 | 10 | 0·000 |
| | | | | 10 | 0·23 | 1,140 | 0·084 | 240 | 0·017 |
| | | | | 25 | 0·58 | 3,210 | 0·237 | 470 | 0·035 |
| | | | | 36 | 0·83 | 4,870 | 0·358 | 890 | 0·066 |

TABLE IV

CHANGES IN THE IODOMETRIC PEROXIDE VALUE OF METHYL LINOLEATE OXIDISING IN POTASSIUM LAURATE AND CETOMACROGOL DISPERSIONS

| Soap | Concentration of soap M | Concentration of methyl linoleate mg./ml. | State of dispersions | Time hours | Oxidation rate ml./hr./g. | Peroxide value m-equiv./kg. |
|---|---|---|---|---|---|---|
| Potassium laurate | 0·2 | 3·10 | Solution | 0 | 0 | 20 |
| | | | | 3 | 0·3 | 220 |
| | | | | 9 | 1·6 | 410 |
| | | | | 27 | 4·6 | 360 |
| | 0·04 | 3·16 | Emulsion | 0 | 0 | 10 |
| | | | | 2 | 1·6 | 280 |
| | | | | 12 | 5·8 | 410 |
| | | | | 27 | 4·2 | 380 |
| Cetomacrogol | 0·06 | 2·54 | Solution | 0 | 0 | 0 |
| | | | | 35 | 4·1 | 3860 |
| | | | | 82 | 5·4 | 6750 |
| | | | | 99 | 6·5 | 6270 |
| | 0·02 | 2·57 | Emulsion | 0 | 0 | 0 |
| | | | | 27 | 3·7 | 5130 |
| | | | | 53 | 4·9 | 6270 |
| | | | | 70 | 7·2 | 4930 |

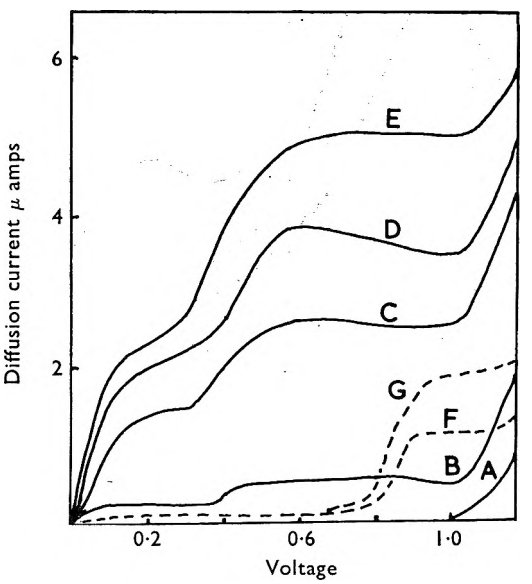



FIG. 3. Polarogram at 20° of oxidising methyl linoleate.

—— Cetomacrogol dispersions.

Cell concentration of methyl linoleate 0·32 mg./ml. Cell concentration of cetomacrogol 0·006 M. Oxygen uptake ml./g. of methyl linoleate: A = 0; B = 4; C = 17; D = 38; E = 61.

- - - - Potassium laurate dispersions.

Cell concentration of methyl linoleate 0·46 mg./ml. Cell concentration of potassium laurate 0·01 M. Oxygen uptake ml./g. of methyl linoleate: A = 0; F = 48; G = 86.

sequence of events occurs during the oxidation of anhydrous methyl linoleate[4].

Comparing the chemical with the polarographic peroxide values the following points are evident. In the potassium laurate systems, the polarographic values are higher than the iodometric, whilst in the cetomacrogol systems the iodometric values were from 5 to 8 times greater than the polarographic values.

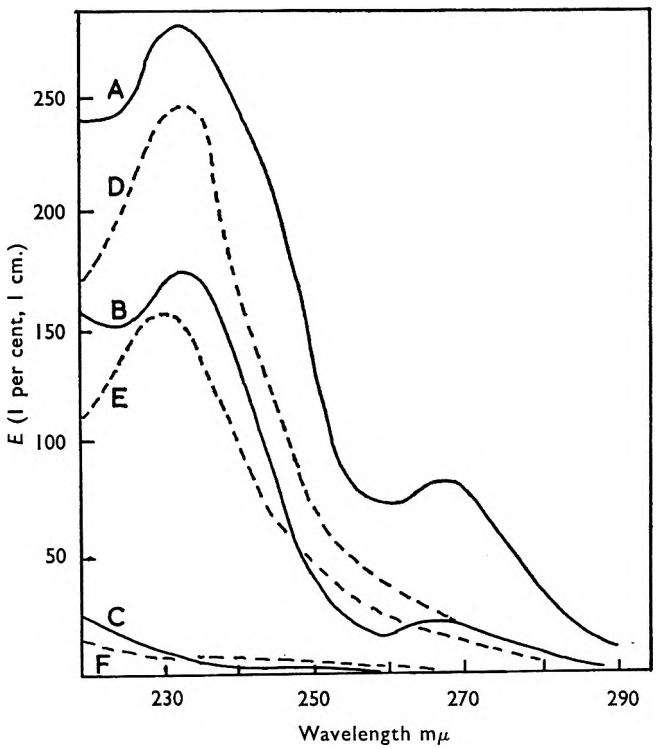



FIG. 4. The ultra-violet absorption spectrum of oxidising methyl linoleate. Oxygen uptake ml./g.: A = 138; B = 58; C = 0; D = 111; E = 33; F = 0.

—— Oxidised potassium laurate dispersions.
- - - - Oxidised cetomacrogol dispersions.

The polarographic waves of the oxidised methyl linoleate in potassium laurate and cetomacrogol differed from one another. In cetomacrogol dispersions two steps in the wave were observed, half-wave potential ($E_{\frac{1}{2}}$) of the first step was −0·04 volts and of the second step, which began at −0·35 volts, the $E_{\frac{1}{2}}$ was −0·55. This second step would appear to be due to the hydroperoxide although it is lower than previously reported values[12,15]. It does however come within the range of $E_{\frac{1}{2}}$ for hydroperoxides found by Willits and others[15]. The step, $E_{\frac{1}{2}}$ −0·04 volts corresponds with the small wave found by Willits and others[12], who suggested that it was due to a small amount of some other unidentified

peroxide. Kalbag and others[17] have also reported finding a similar preliminary wave. Typical polarographic curves appear in Figure 3.

In potassium laurate dispersions the polarographic wave had an $E_{\frac{1}{2}}$ of $-0{\cdot}82$, which agreed closely with the published $E_{\frac{1}{2}}$ of methyl linoleate[12,15].

*Ultra-violet Absorption Spectra*

The formation of conjugated material during the oxidation of the methyl linoleate dispersions was detected by the appearance of an absorption peak at 233–234 m$\mu$ (Fig. 4). In potassium laurate dispersions, at higher oxygen uptakes, an absorption peak in the region of 265 m$\mu$ was also found. This indicates the possible formation of a diketone such as is normally found as a secondary reaction product[16]. This peak did not appear in the cetomacrogol dispersions. Diketones give intense yellow coloration in alkaline solution and it was noted that potassium laurate emulsions became yellow on oxidation, which also suggests that ketone formation was occurring.

## Discussion

The oxidation of methyl linoleate proceeds by a chain reaction in which the free radicals produced by oxygen attack on the $\alpha$-methylene group propagates the reaction[18]. The mechanism for anhydrous methyl linoleate oxidation may be summarised as follows.

| | | | |
|---|---|---|---|
| $RH + O_2 \longrightarrow$ | $RO_2\cdot + H$ | Initiation | |
| $RO_2\cdot + RH \longrightarrow$ | $RO_2H + R\cdot$ | Propagation | |
| $R\cdot + O_2 \longrightarrow$ | $RO_2\cdot$ | Propagation | |
| $RO_2\cdot + RO_2\cdot \longrightarrow$ | Inactive product | Termination | |
| $RO_2\cdot + R\cdot \longrightarrow$ | Inactive product | Termination | |
| $R\cdot + R\cdot \longrightarrow$ | Inactive product | Termination | |

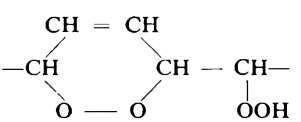
The addition of copper catalyses the reaction by breaking down the $RO_2H$ into further free radicals $RO\cdot$ and $RO_2\cdot$[18]. A similar chain reaction for benzaldehyde has been reported in our previous paper[3].

In all examples studied the rate of oxidation of methyl linoleate dispersed in soap solutions was higher than for suspensions in water. The relation between oxidation rate and soap concentration is a complex one as it appears that the proportion of solubilised to emulsified methyl linoleate greatly influences the rate. In suspensions there is no dissolved oil in the aqueous phase but when soap is included to form an emulsion some of the methyl linoleate will be solubilised. The solubilised methyl linoleate appears to increase the rate of oxidation. When sufficient soap is present to solubilise all the methyl linoleate then the rate of oxidation is below that of the emulsion. This shows that oxidation takes place most rapidly in the emulsion droplets although the need for solubilised oil to be present can be inferred from the above results.

It is suggested that the oxidation initiation stage commences in the micelle and the free radicals formed are transferred to the emulsion droplet where propagation is fast due to the large number of oil molecules available for oxidation. The slower rate of oxidation of micellar material could be due to the small number of methyl linoleate molecules resulting

in a greater chance of radical-radical termination reactions. In the droplet more methyl linoleate is available for oxidation once the reaction has been initiated and the chance of radical-radical termination would be reduced because of the relatively large number of oxidisable molecules in the droplet.

Because of the very small quantities of methyl linoleate present it was found impossible to isolate any of the reaction products. In both surface-active agents the formation of conjugated material having a peak density of 234 m$\mu$ indicates that the initial radical formation occurs by reactions similar to the anhydrous oxidation[4]. The height of this peak does not parallel the oxygen uptake after the initial stages of oxidation, and in cetomacrogol dispersions decreases in intensity with time, showing that the conjugated system is being broken down. In potassium laurate dispersions, the peak at 265 m$\mu$ indicates the possible formation of a diketone which is normally found as a secondary reaction product[16]. Its presence is unlikely to be detected by the polarographic method since diketones have polarographic waves with a similar $E_{\frac{1}{2}}$ to hydroperoxides[15]. The iodometric peroxide values of methyl linoleate in cetomacrogol are the same as those formed during the oxidation of the anhydrous oil[12]. The large difference between the high iodometric value and the polarographic value could be due to the presence of a peroxide form which is not polarographically reducible or which possesses a polarographic wave with markedly different characteristics from that of methyl linoleate hydroperoxide. Willits and other[15] have shown that ether linked peroxides have a half-wave potential of lower than $-0{\cdot}2$ volts which enables one to differentiate between this type of peroxide and hydroperoxides which have a half-wave potential of not lower than $-0{\cdot}5$ volts. The preliminary reduction wave in the polarograms of oxidising methyl linoleate in cetomacrogol dispersions, is in the region expected of the ether type of peroxides and it is possible that a diperoxide of the type suggested by Bergstrøm and others[8] (I) is formed. He found that sodium linoleate in solution ceased to oxidise at an oxygen uptake of 2 molecules of oxygen per molecule of linoleate.




(I)

This diperoxide contains both an ether-linked and a hydroperoxide grouping. The formation of such a compound would account for the fall in ultra-violet peak density as it possesses no conjugation. Bergstrøm found that his oxidising dispersions exhibited a maximum ultra-violet density at an oxygen uptake of 1·0 mol. of oxygen per mol. of linoleate and a similar observation was made in the present study. The reaction $RH \longrightarrow RO_2H$ would require an overall oxygen uptake of 1·0 mol. of oxygen per mol. of methyl linoleate. From the data in Figure 2 the oxygen uptake at the end of the primary reaction was approximately 2·5 mol. of oxygen per mol. of linoleate compared with the figure

of 2 found by Bergstrøm and others. It therefore appears probable that a diperoxide of the type suggested by Bergstrøm and others is formed during the oxidation of methyl linoleate dispersed in cetamacrogol. In potassium laurate dispersions an uptake of 1·5 mol. of oxygen per mol. of methyl linoleate was found. The increased oxygen uptake over and above the theoretical hydroperoxide formation is possibly due to the formation of secondary decomposition products as indicated previously.

Whilst the final products of the oxidation of emulsions and solutions of methyl linoleate appear to vary considerably from those normally found in the oxidation of the anhydrous oil, the general mechanism of the oxidation appears to be unchanged. Thus a conjugated hydroperoxide appears to be the initial oxidation product as in the oxidation of the anhydrous oil, but in emulsions and solutions it is rapidly broken down to secondary products.

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# A NOTE ON THE PRESENCE OF NORADRENALINE AND 5-HYDROXYTRYPTAMINE IN PLANTAIN (*MUSA SAPIENTUM*, VAR. *PARADISIACA*)

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Noradrenaline, 5-hydroxytryptamine and dopamine are present in the fruit of *Musa sapientum*, var. *paradisiaca* (Plantain), which forms a staple food of many inhabitants of West Africa. The amounts of 5-hydroxytryptamine and noradrenaline are highest when the fruit is ripe. An estimated daily intake of 10 mg. of 5-hydroxytryptamine by West Africans has little apparent effect on the normal functioning of the intestinal tract.

THERE has been correspondence in the literature on the presence of biologically active substances in edible fruits, notably tomato[1,2] and banana[1]. The banana-like fruit known as plantain, a staple food of many West Africans, has been examined for the presence of catechol amines and indole derivatives, especially 5-hydroxytryptamine (5-HT). Parts of the plant also were examined in various stages of growth to study the functions and biosynthesis of the amines in nature. When these experiments were nearing completion a brief report on the presence of catechol amines and tryptamines in the "matoke" banana was published by Marshall[3]. In samples obtained from East Africa he showed that both noradrenaline and 5-HT are present in the peel and pulp of the fruit of the "matoke" in amounts similar to those found in the common banana. Botanically both "matoke" banana and plantain are *Musa sapientum* var. *paradisiaca*, though in West Africa several varieties of plaintain are under cultivation. As the possibility arises that the time taken for the transport to England of bananas and "matoke" bananas affects the amounts of amines present, we used only fresh material obtained from locally grown plants.

## METHODS

### *Extraction and Estimation of* 5-*Hydroxytryptamine*

The plant tissues were cut into small pieces and extracted with 5 parts (w/v) of acetone for 24 hours. The solvent was then decanted and the residue washed with a little acetone. The acetone in the combined extracts was removed by evaporation in air at room temperature (25° to 29°). The dried extracts retained their activity for some days when stored at 0°. When required for biological assay the extract was dissolved in 0·9 per cent (w/v) saline to give a solution of 1 g. of original material in 50 ml.

These solutions were assayed on the isolated uterus of the rat in oestrus or the rat isolated colon, or both preparations, using an aerated 10 ml. bath of Jalon's fluid containing atropine ($10^{-7}$) at 28° for the uterus and at 25° for the colon. The standard 5-HT was used as the creatinine sulphate, and specificity of action was checked using bromolysergic acid diethylamide (brom-LSD).

*Extraction and Estimation of Catechol Amines*

Small pieces of tissue were extracted with 0·1 N hydrochloric acid for 24 hours. The samples were then assayed for noradrenaline on the rat blood pressure. Rats were anaesthetised with urethane, 0·75 ml. of 25 per cent solution subcutaneously, and the blood pressure recorded from the right carotid artery using a Condon manometer. Brom-LSD, 1 mg./kg., was injected intravenously to abolish the effect of 5-HT, which is also extracted by the hydrochloric acid.

*Paper Chromatography*

Extracts made with acetone or 0·1 N hydrochloric acid were chromatographed on Whatman No. 1 paper using as solvent systems: (i) 8 per cent sodium chloride in distilled water[4]; (ii) butanol-acetic acid-water mixture (4 : 1 : 5); (iii) phenol saturated with water and sulphur dioxide[5].

With systems (i) and (ii) ascending chromatograms were run for 4 and 16 hours respectively in an air conditioned room at 21°. Chromatograms using system (iii) were run for 24 hours at 26·5° to 27·5°. System (iii) separated noradrenaline ($R_F$ value 0·39) from adrenaline ($R_F$ value 0·61) and dopamine ($R_F$ value 0·54). The spray developers used were 1 per cent potassium iodate or 0·44 per cent potassium ferricyanide in a phosphate buffer of pH 7·8 for catechol amines and Ehrlich's reagent for indoles.

## RESULTS

*Estimation of 5-Hydroxytryptamine*

Plantain fruits, in various stages of ripening, were obtained from trees surrounding the College compound or from local traders. After extraction, the 5-HT content was estimated. The results are shown in Table I. These values should be compared with those obtained for two very small

TABLE I

5-HT CONTENT (μG./G., CALCULATED AS BASE) OF PARTS OF THE PLANTAIN FRUIT AT DIFFERENT STAGES OF MATURITY

| Stage | Part of fruit | Individual results | | | | | Mean |
|---|---|---|---|---|---|---|---|
| Unripe | Skin | 28·8 | 10·9 | 6·5 | 21·1 | 20·8 | 17·6 |
| | Pulp | 59 | 54·5 | 24 | 74 | 38·2 | 49·9 |
| Ripe | Skin | — | 32·7 | 17·7 | — | 73·4 | 41·3 |
| | Pulp | 52·5 | 52 | 39·2 | 39·2 | 101 | 56·7 |
| Over-ripe | Skin | 6·5 | 4·8 | — | — | — | 5·6 |
| | Pulp | 11 | 13 | — | — | — | 12 |

fruits (pulp and skin combined) of 2·6 and 11 μg./g. It is apparent that the amount of 5-HT in both pulp and skin increases during ripening. In another experiment a bunch of unripe fruits was cut from a tree and left to ripen in the laboratory. Every few days a fruit was removed and its 5-HT content estimated. The results (Table II) again indicate that

during the ripening process the content of 5-HT in both pulp and skin increases.

Smaller amounts of 5-HT are present in other parts of the plant. Thus only traces were detected in leaf and stem (0·12 to 0·5 μg./g.) and slightly more in buds (0·26 to 3·3 μg./g.) and flowers (1·3 μg./g.).

Plantains are nearly always eaten after being cooked, either boiled, or, more often, fried in groundnut oil. It was important therefore to deter-

TABLE II

THE 5-HT CONTENT (μG./G., CALCULATED AS BASE) OF PLANTAINS DURING RIPENING

| Parts of plantain | Days after removal from tree | | | |
|---|---|---|---|---|
| | 1 | 3 | 7 | 15 |
| Skin .. .. .. | 7·5 | 12·5 | 22·5 | 24 |
| Pulp .. .. .. | 27·5 | 63 | 76 | 44 |

mine whether these conditions destroy any 5-HT. Whole ripe fruits so subjected were extracted as described and tested for the amine. Values of 13·7 and 47 μg./g. respectively were obtained for the skin and pulp. No evidence was obtained for the presence of tryptamine, 5-hydroxytryptophan or 5-hydroxyindoleacetic acid in any plantain extract.

*Estimation of Catechol Amines*

In the rat anaesthetised with urethane, dopamine, 2 to 20 μg., produced a rise in blood pressure. This activity was 20 to 50 times less than that of noradrenaline. From the chromatography results it was estimated that

TABLE III

AMOUNTS OF NORADRENALINE (μG./G., CALCULATED AS BASE) IN SAMPLES OF PLANTAIN FRUIT

| Colour of sample | Pulp | Skin |
|---|---|---|
| Yellow-green (unripe) .. .. | — | 5·1 |
| Yellow-green .. .. .. | — | 5·1 |
| Yellow (ripe) .. .. .. | 2·5 | 6·2 |
| Yellow-black (over-ripe) .. | 10·1 | 15·2 |

the plantain skin contained dopamine, 25 μg./g., which means that no less than 80 per cent of the pressor effect of the acid extract of plantain is due to noradrenaline. An attempt was made to seperate dopamine from noradrenaline using chromatographic system (iii). The dopamine area[5] was eluted and assayed on the blood pressure of the guinea pig anaesthetised with urethane[6,7].

The amounts of noradrenaline found in extracts of plantain fruit are shown in Table III. Dibenamine, 1 mg./kg., reduced the response to both standard and extract by a similar amount.

*Estimation of 5-Hydroxytryptamine in other Tropical Fruits*

A few experiments were made to supplement the results of Waalkes and others[8] and of West[1], who have demonstrated large amounts of 5-HT, noradrenaline and dopamine in both the pulp and peel of the banana fruit. No 5-HT was detected in extracts of banana root, stem or leaf, although it was shown to be present in very young fruits up to 3 inches long, in amounts from 1·65 to 7·1 μg./g. of the peel and pulp combined.

Other plants investigated included pineapple (*Ananas comosus*), mango (*Magnifera indica*), passion fruit (*Passiflora foetida*), and papaw (*Carica papaya*), all of which produce edible fruits. Small amounts of 5-HT were shown to be present in papaw (1·1 to 2·1 μg./g.) and in passion fruit (1·4 to 3·5 μg./g.), but none was detected in the pineapple or mango.

## Discussion

These results show that the distribution of noradrenaline and 5-HT are similar in the banana and the plantain fruit. Thus in both, 5-HT is concentrated in the pulp, and its concentration increases as the fruit grows and ripens, the amount decreasing when the fruit is left to overripen. Only traces are present in other parts of the plant.

No evidence was obtained for the presence of 5-hydroxytryptophan, 5-hydroxyindoleacetic acid or tryptamine.

The plantain forms a major part of the diet of the West African. In Yoruba country, plantains are usually fried and eaten as a vegetable, an average of 6 to 15 being eaten each week. A plantain fruit can weigh over 300 g., and according to our results is estimated to contain at least 12 mg. of 5-HT, so that the oral ingestion of 5-HT in plantain may be from 70 to 180 mg. a week. In the villages more plantains are eaten, as there is less variety of food, so that the figures for 5-HT may be raised to over 200 mg. per week.

To study the effects of the regular ingestion of these amounts of 5-HT, students in the College were questioned, and with few exceptions they stated that the usual intestinal effect after a cooked plantain meal was constipation. This seemed interesting in view of the fact that increased intestinal motility and diarrhoea are frequently seen in patients with carcinoid tumours which secrete large amounts of 5-HT. It would not be wise to place too much significance to the questionaire but at least the present enquiry shows that the ingestion of such large amounts of 5-HT regularly in the form of plantains has little effect on the average Nigerian. While it may be that through prolonged ingestion the Nigerian has become resistant to the toxic effects of 5-HT, many resident Europeans also eat plantain fruits without ill effects.

The ingestion of plantain fruits may lead to the erroneous diagnosis of phaeochromocytoma and of carcinoid tumour by increasing the urinary excretion of noradrenaline and 5-HT and their metabolites While the cooking process would almost certainly destroy the noradrenaline, some of the 5-HT may be absorbed and excreted as 5-hydroxyindoleacetic acid in the urine, and this might lead to erroneous diagnosis of carcinoid tumour. This possibility is being further investigated.

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# *PHYTOLACCA ACINOSA* ROXB., AN ADULTERANT OF INDIAN BELLADONNA

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Pharmacognostical data of the leaves and roots of *Phytolacca acinosa* Roxb. has been collected and compared with that of *Atropa acuminata* Royle ex Lindley.

Roots and leaves of *Phytolacca americana* have long been used as an adulterant[1] of belladonna in European and American markets. Roots and leaves of *P. acinosa* have been reported[2] to have formed an adulterant in certain consignments of Indian belladonna shipped abroad. Over several years, we have repeatedly come across samples of Indian belladonna leaves and roots adulterated with the leaves and roots of *P. acinosa*. A detailed study of the adulterant was therefore undertaken to distinguish it from true Indian belladonna.

*Phytolacca acinosa* Roxb. (Family Phytolaccaceae) is a glabrous shrub with succulent hollow stem. It bears oblong, dark green alternate leaves. The flowers are pale green, arranged on a racemose inflorescence. The plants possess a thick fleshy branched tap root. It is a plant originating from China and it is found wild or cultivated in the temperate Himalayas from Hazara and Kashmir to Bhutan at an altitude of 5,000 to 9,000 ft. It is called "matazor" in Hindi and "sarangun" in Urdu. It contains[3] a bitter toxic substance, phytolacca toxin. No detailed chemical investigation of the plant has been reported. In the indigenous system of medicine, the plant is described as narcotic and the oil from the root is used to allay pain in joints. The leaves are cooked and eaten by the village folk as a vegetable. It is supposed that the toxic principles are destroyed during cooking.

## Material and Methods

Between 1953 and 1959, three samples of roots and leaves were obtained from different drug dealers under the name of Indian belladonna. These samples were found not to belong to either *Atropa acuminata* or *Atropa belladonna*. In 1959, an authentic specimen of *Phytolacca acinosa* Roxb. was collected from Kashmir and was found to be identical with the adulterant. For macroscopical descriptions the commercial drug was used whereas for histological work commercial as well as fresh authentic specimens preserved in 50 per cent formalin : acetic acid : ethanol (5 : 5 : 90) were used. The usual methods of paraffin embedding and sectioning on a rotary microtome were followed. Staining was with saffranin and fast green.

## The Root

### *Macroscopy*

The individual pieces (Fig. 1) in a drug sample vary greatly in their appearance. Those from the root crown (Fig. 1A) are irregular, knotty

and show occasionally the remains of aerial shoots. Such pieces are up to 10 cm. long and 3 to 5 cm. thick. The roots are frequently longitudinally cut and are twisted or curved. They are 1·5 to 3 cm. wide and 5 to 11 cm. long. The transversely cut and smoothed end of such roots (Fig. 1B) shows characteristic concentric rings of xylem which become more prominent on treatment with phloroglucinol and hydrochloric acid reagent. The longitudinally cut surface (Fig. 1C) exhibits vertical

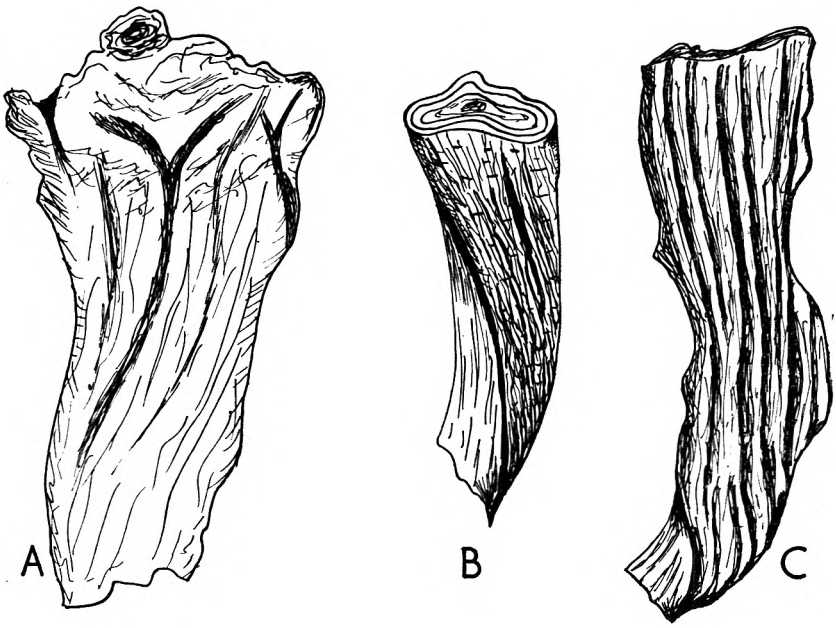


FIG. 1. Commercial roots × ¾. A, root crown; B, root showing longitudinal wrinkles, lenticels, twisted appearance and concentric rings of xylem at the transversely cut end; C, longitudinally cut view of root.

lighter coloured ridges. The external surface is light brown in colour and shows longitudinal wrinkles and transversely elongated lenticels. The roots have a short fracture and an indistinct taste and odour. A comparison with *A. acuminata* is made in Table I.

*Microscopy*

The root in transverse section (Figs. 2–3) shows from without inward, the cork, the secondary cortex and an abnormal stele composed of open collateral vascular bundles arranged in concentric rings.

The cork (Fig. 4) consists of 5 to 7 layers of rectangular, suberised and slightly lignified cells. The radial walls of these cells are wavy and measure T, 17–**42**–70 $\mu$; R, 11–**14**–28 $\mu$. They appear rectangular to polygonal in surface view. The phellogen is a single layer of thin-walled cells measuring T, 38–**63**–105 $\mu$; R, 6–**9**–25 $\mu$.

The secondary cortex is composed of about 15–16 layers of parenchyma cells which show well marked intercellular spaces. The first 5 to 6 layers

of cells are much more elongated tangentially and are rectangular in outline. The cells measure T, 35–**71**–157 $\mu$; R, 29–**39**–53 $\mu$. The next 10 or 11 layers of cells are smaller in size measuring T, 38–**61**–109 $\mu$;

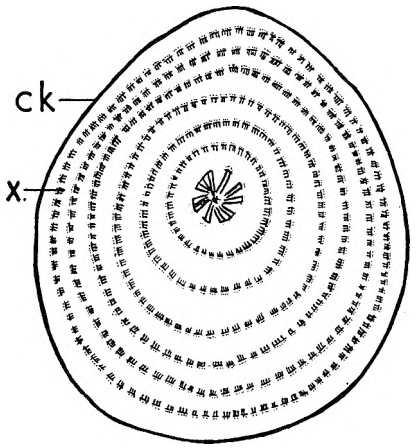


FIG. 2. T.S. root (diagrammatic) original size. ck, cork; x., xylem.

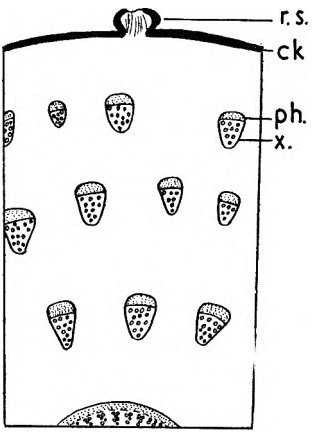


FIG. 3. A sector of the root in t.s. (diagrammatic) × 5. ck, cork; ph., phloem; x., xylem; r.s., root scar.

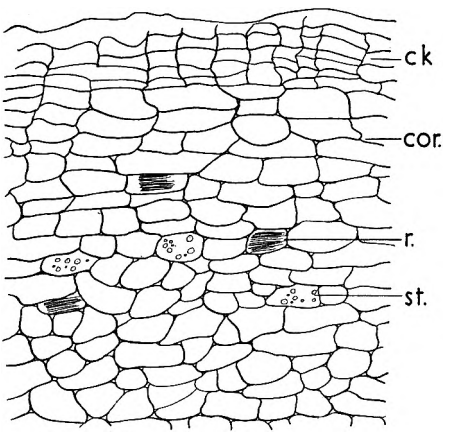


FIG. 4. T.S. root showing cork and secondary cortex × 60. cor., cortex; ck, cork; r., raphides; st., starch grains.

R, 32–**49**–73 $\mu$ and are arranged irregularly. Some of these cells contain starch grains and raphides of calcium oxalate.

The vascular bundles (Fig. 5) arranged in concentric rings are open and collateral. The phloem consists of sieve tubes, companion cells and phloem parenchyma. The cells of the phloem parenchyma are thin-walled and are devoid of intercellular spaces. Beneath the phloem there is a single layer of thin-walled cambium cells.

TABLE I

DISTINGUISHING FEATURES OF THE ROOTS OF *Phytolacca acinosa* AND *Atropa acuminata*

| Character | *P. acinosa* | *A. acuminata* |
|---|---|---|
| Form .. .. .. .. | Variable—mostly longitudinally cut and twisted | Variable—mostly transversely cut and cylindrical |
| Outer surface .. .. .. | Prominent lenticels and longitudinal wrinkles | Lenticels few and wrinkles less prominent |
| Transverse surface .. .. .. | Concentric rings of xylem | Radiate xylem |
| Longitudinally cut surface .. | Marked light coloured longitudinal ridges | No longitudinal ridges |
| Taste .. .. .. .. | Indistinct | Bitter |

The stele is traversed by multiseriate vascular rays, the cells are thin-walled, parenchymatous, radially elongated and contain abundant starch grains. The cells measure T, 27–**47**–66 $\mu$; R, 45–**60**–105 $\mu$. The xylem is formed of xylem vessels and xylem parenchyma. It is devoid of tracheids and fibres. The xylem vessel segments show reticulate thickening and have porous end walls. In macerated preparations, they measure 65–**150**–240 $\mu$ in length and 37–**93**–111 $\mu$ in breadth.

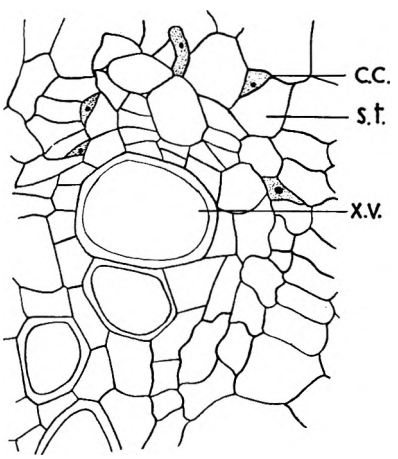


FIG. 5. T.S. portion vascular bundle × 148. c.c., companion cell; s.t., sieve tube; x.v., xylem vessel.

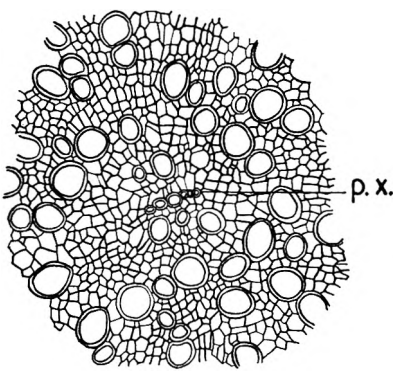


FIG. 6. T.S. root showing primary xylem × 72. p.x., primary xylem.

The primary root (Fig. 6) is diarch and shows normal growth, thus forming secondary xylem and secondary phloem.

Starch consists of simple and 2 to 5 compound grains. They are mostly circular but sometimes distorted in outline and show a dotted or cleft hilum in the centre. They measure up to 45 $\mu$ in diameter. Calcium oxalate crystals are found in all the parenchymatous tissues in the form of raphides which measure T, 35–**42**–87 $\mu$; R, 7–**11**–18 $\mu$.

## THE LEAF

*Macroscopy*

The leaves are oblong (Fig. 7A) as compared to belladonna leaves (Fig. 7B) which are ovate in outline. The medium-sized leaves measure

about 15 cm. in length and 5 cm. in breadth. However, in some samples, leaves up to 30 cm. in length may be seen. The margin is slightly wavy and toothed towards the upper half of the lamina. The apex is acute with a distinct apiculus and the base is asymmetrical. The lateral veins arise from the midrib at an angle of about 60° and anastomose near the margin. The commercial samples of leaves occur in a crumpled state

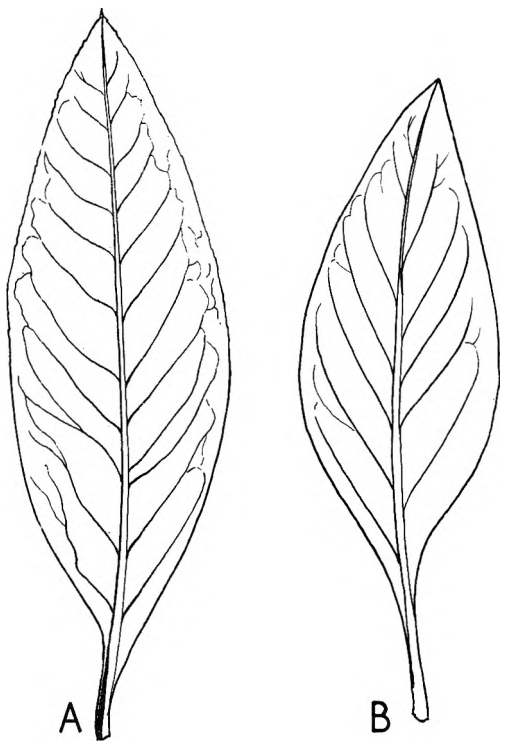


FIG. 7. Leaf × ½. A, *Phytolacca acinosa*; B, *Atropa acuminata*.

and have a thin membranous texture. If flattened leaves are held against the light numerous tiny scattered specks representing raphides may be visible to the naked eye. Odour and taste are indistinct.

*Microscopy*

The midrib (Fig. 8) in transverse section shows an arc-shaped meristele. The meristele is composed of 5 to 6 groups of open collateral vascular bundles which do not appear separate when the section is cut near the apex. The endodermis and pericycle are not very clear. The rest of the midrib is filled with thin-walled polygonal parenchyma cells except the upper and lower hypodermal regions which have a layer of collenchyma 2 to 3 cells thick. The cells of the parenchyma contain raphides of calcium oxalate.

The upper epidermal cells are rectangular to squarish in outline in a transverse section. The cells show straight anticlinal walls, but the outer tangential walls of some cells show papillose outgrowth which is much more prominent in the region of the midrib and lateral veins. These papillae contain some granular matter which is insoluble in chloral hydrate

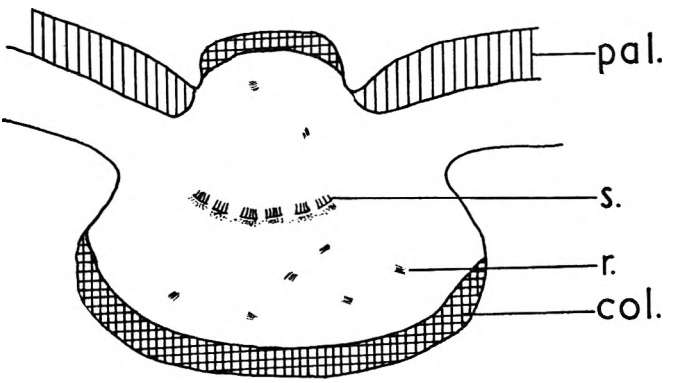


FIG. 8. T.S. leaf (diagrammatic) × 45. col., collenchyma; pal., pallisade cells; s., stele; r., raphides.

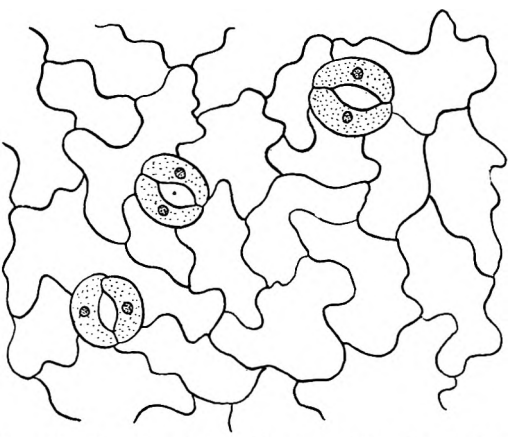

FIG. 9. Surface view of lower epidermis × 380.

and concentrated hydrochloric acid. The upper epidermis has an indistinct cuticle and rarely shows stomata, which if present are of the anomocytic type. In surface view the upper epidermal cells are polygonal with straight walls. The palisade layer is single cell thick and is discontinued at the midrib. The spongy parenchyma is 5 to 7 cells thick and shows intercellular air spaces. The lower epidermis also has an indistinct cuticle. The cells show papillae similar to those found on the upper epidermis, and there are numerous anomocytic stomata present. The cells show wavy anticlinal walls in surface view (Fig. 9).

The values of quantitative microscopic determinations taken from ten readings are as follows.

| | |
|---|---|
| Vein islet Number .. .. | 1–**1·25**–2·5 |
| Stomatal Index .. .. | 14·7–**14·9**–15·2 |
| Stomatal Number .. .. | 28–**48**–54 |
| Palisade Ratio .. .. | 1·7–**2**–3 |

The vein islet pattern of *P. acinosa* and *A. acuminata* was also found to be a distinguishing feature. These quantitative values have been compared (Table II) with those of *A. acuminata* reported by Wallis[4].

TABLE II

COMPARATIVE STUDY OF THE LEAVES OF *P. acinosa* AND *A. acuminata*

| Character | *P. acinosa* | *A. acuminata* |
|---|---|---|
| *Macroscopy* | | |
| Size .. .. .. .. .. | 15–30 cm. long<br>5–7 cm. broad | 6–20 cm. long<br>3–11 cm. broad |
| Outline .. .. .. .. | Oblong | Ovate |
| Apex .. .. .. .. | Acute, distinct apiculus | Acute, no apiculus |
| Margin .. .. .. .. | Slightly toothed | Entire |
| Texture .. .. .. .. | Papery | Tough |
| Angle of lateral veins .. .. | 60° | 45° |
| Trichomes .. .. .. .. | Absent | Present |
| *Microscopy* | | |
| Stele of the midrib .. .. | Collateral | Bicollateral |
| Crystals .. .. .. .. | Raphides | Microsphenoidal |
| Cuticle .. .. .. .. | Indistinct | Distinct |
| *Quantitative values* | | |
| Vein islet number .. .. .. | 1–1·25–2·5 | 3–4·2–5·1* |
| Stomatal index .. .. .. | 14·7–14·9–15·2 | 16·7–17·6–18·8 |
| Stomatal number .. .. .. | 28–48–54 | 62·5–93–174 |
| Palisade ratio .. .. .. | 1·7–2–3 | 4·0–8·0–13·5 |

* The vein islet number of *A. acuminata* has not been reported previously and was determined by the authors.

## CONCLUSION

On the basis of the description of *P. acinosa* outline it is clear that this adulterant can be easily distinguished in commercial samples. The distinguishing features are presented in Tables I and II.

*Acknowledgement.* We thank Dr. K. N. Gaind, Head of the Department of Pharmacy, Panjab University for facilities and encouragement.

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# THERMAL STABILITY OF INSULIN MADE FROM ZINC INSULIN CRYSTALS

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A commercial insulin preparation was stored for 2 years, its normal shelf-life, at temperatures from 2° to 36°. Full potency was retained for 2 years when the sample was kept at 2°. However, the activity of the insulin decreased with time as the storage temperature was increased. After one year at room temperature (20° to 25°) the activity of the insulin sample was 20 per cent below that stated on the label. Between 2° and 20° there was a consistent but not significant drop in activity. In the initial period the loss in potency may be essentially a reaction of zero order.

The stability of commercial insulin preparations is important, not only to the manufacturer, but also to the physician and in particular to the diabetic patient. Krogh and Hemmingsen[1] were the first to make a systematic study of the relation between temperature, time, and the destruction of amorphous insulin in a sterile aqueous solution. Their results suggested that the inactivation of insulin at a constant temperature followed a first order reaction, the rate of destruction at any moment being proportional to the concentration. In addition, it was reported that the optimal stability of an aqueous solution of insulin occurred between pH 2 and 4. Sahyun, Goodell and Nixon[2], working with a low-ash insulin preparation, revealed that the addition of $Zn^{++}$ to the aqueous medium improved the stability significantly. However, Lens[3] could not confirm this stabilising effect of $Zn^{++}$, and reported that the stability of crystalline insulin in aqueous solution was unpredictable. Lens concluded that the inactivation was not due to hydrolysis of the insulin, but could be attributed to denaturation or heat precipitation which was usually followed by an irreversible oxidative process.

These investigations[1–3] were carried out for short periods at elevated temperatures. There is a scarcity of information about the stability of commercial insulin preparations when kept at temperatures encountered under ordinary storage conditions for the expected shelf-life of the product. Consequently an experiment was designed to determine the rate at which insulin made from zinc insulin crystals loses activity when stored for a period of two years at temperatures of 2° to 36°.

## Materials and Methods

Insulin Toronto, made from zinc insulin crystals, Lot 942–1, 40 International Units per ml., was used in this study, and was prepared from Master Lot of zinc insulin crystals No 910 which had an activity of 26·2 International Units per mg. on a moisture-free basis. Insulin Toronto, Lot 942–1, contained 5·8 mg. of nitrogen and 0·22 mg. of zinc

for each 1000 International Units of insulin. The diluent consisted of 1·27 per cent (v/v) of glycerol and 0·15 per cent (w/v) of phenol in an aqueous medium which was adjusted to pH 3·0 with hydrochloric acid.

The material was filtered through sterilizing filter pads and, when known to be sterile, was filled into vials made from Type 3 Glass[4]. The vials were stoppered with rubber closures routinely employed in the Connaught Medical Research Laboratories for insulin preparations. This closure was secured by a three-piece aluminium seal.

A sufficient number of vials from Lot 942–1 were obtained and distributed to the collaborating laboratories where they were placed in storage at various temperatures. The stored vials were placed on their side allowing contact of the liquid with the rubber closure. In one of the laboratories the storage temperatures were 2 ± 1°, 11 ± 1°, 20 ± 1°, and 36 ± 1°; while in the other, the temperatures were 2 ± 1°, 14 ± 1°, 24 ± 1°, and 36 ± 1°. One of the 36°-incubators allowed a sudden rise in the temperature one week-end between the 18th and the 21st month of the test.

At intervals of 3 months during the 2-year study, samples were removed from storage and suitable dilutions for the bioassays were made with acid water containing glycerol and phenol. The dilutions from each of the samples were assayed for relative potency against dilutions of the material stored at 2°. To reduce the between assay variation, the three samples stored at temperatures above 2° were assayed at the same time against the reference standard in each individual assay. Several assays, sufficient to give standard errors of approximately ±6 per cent were combined to provide a weighted mean potency ratio for each of the samples. At the beginning of the experiment, as well as from time to time during the study, samples stored at 2°, the reference standard, were removed and assayed against the International Standard.

In both laboratories the insulin activities were determined using mice. The methods have been described elsewhere[5,6]. The individual potency ratios were estimated by means of the angular transformation method[7] in one of the laboratories, and by probits[8] in the other. Satisfactory agreement was found between the two procedures.

## Results and Discussion

At the beginning of the experiment, the potency of Insulin Toronto, Lot 942–1, was determined by assaying it against the International Standard. At other times during the experiment similar assays were performed on vials removed from storage at 2°. The results of these assays are indicated in Figure 1. The assay values and their 95 per cent confidence limits are shown as a percentage of the estimate of potency found in each laboratory at the beginning of the experiment. No indication was given of a drop in insulin activity in the material stored at 2°.

The results of the comparative assays of the biological activity of the material in the vials stored at various temperatures against the activity of the sample kept at 2° for each 3-month period up to 2 years are given in Table I. The 95 per cent confidence limits are included in the Table.

TABLE I

PER CENT RELATIVE POTENCY OF INSULIN STORED AT VARIOUS TEMPERATURES

| Temp. °C | Storage time | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 months | 6 months | 9 months | 12 months | 15 months | 18 months | 21 months | 24 months |
| 11 ± 1 | 108 (92–128) | 103 (91–117) | 114 (101–129) | 96 (81–113) | 73 (64–84) | 86 (76–98) | 92 (83–104) | 92 (83–103) |
| 14 ± 1 | 98 (89–111) | 100 (85–116) | 98 (87–111) | 97 (85–111) | 93 (83–104) | 89 (79–100) | 92 (80–106) | 91 (85–100) |
| 20 ± 1 | 94 (80–111) | 85 (75–97) | 88 (78–99) | 81 (69–96) | 78 (68–89) | 77 (68–88) | 92 (81–103) | 80 (72–89) |
| 24 ± 1 | 102 (90–114) | 87 (73–104) | 92 (83–103) | 79 (69–90) | 74 (66–83) | 74 (66–83) | 73 (63–84) | 74 (68–81) |
| 36 ± 1 (Lab A) | 82 (70–97) | 70 (61–79) | 67 (59–75) | 60 (51–71) | 52 (46–60) | 51 (45–59) | 56 (50–63) | 53 (47–58) |
| 36 ± 1 (Lab B) | 97 (86–109) | 87 (73–103) | 78 (70–88) | 56 (49–63) | 50 (44–56) | 47 (42–53) | 28* (24–32) | 22* (18–26) |

* The 36°-incubator allowed a sudden rise in temperature during a week-end.

The results from both of the laboratories for the 36°-storage condition are shown separately.

The effect of the sudden rise in temperature of one of the 36°-incubators is clearly seen by the lower values indicated for the 21st and 24th months.

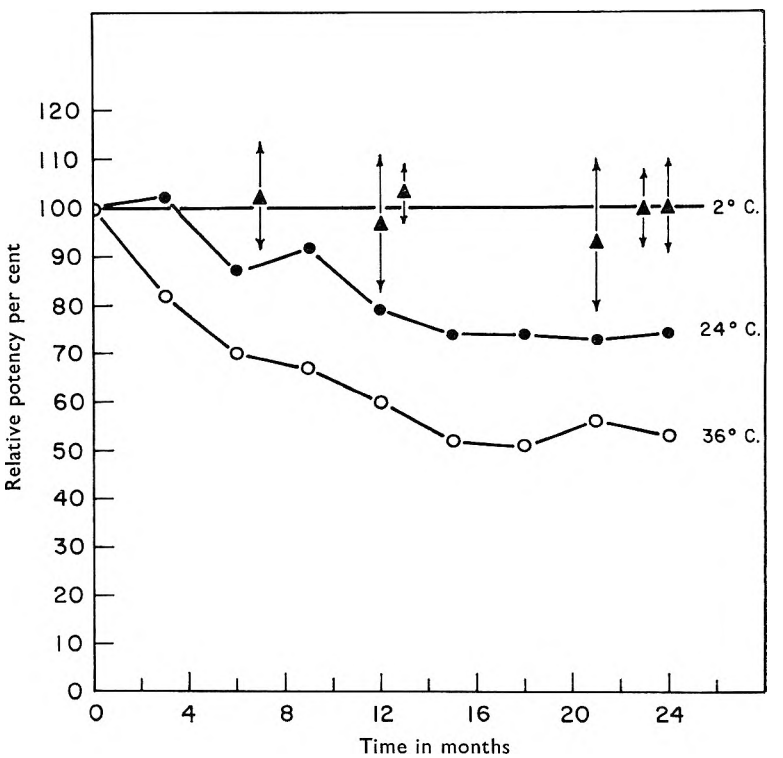


FIG. 1. The relative potency of insulin stored at 2°, 24° and 36° for a period of 24 months.

The relative potencies for two of the temperatures (24° and 36°) have been plotted in Figure 1.

The results of this stability study show that at room temperature (20°) and above, a significant drop in the potency of the commercial insulin preparation has occurred over the 2-year test period. At the other temperatures (11° and 14°) which have been defined as characteristic of a cold place,* the relative potencies do not differ from that of the material stored at 2° at the end of the 2-year period. It is worthy of note, however, that after twelve months of storage, the estimates of the relative potency are consistently below 100 per cent. This suggests that insulin should be stored in a refrigerator, and that the colder the refrigerator without actually freezing, the more stable the insulin.

Attention should be drawn to the relative potency at 11° for the 15th month period; 73 per cent with the 95 per cent confidence limits of 64 to 84 per cent. The significant loss in potency indicated by the assay at this time period was not confirmed at later periods. Apparently this finding can be attributed to between vial difference in stability.

Although the experiment was not primarily designed to investigate the kinetics of insulin inactivation in acid solution, some information is available from the data, concerning the relationship between the loss of biological activity and time, at the various temperatures.

Krogh and Hemmingsen[1] established the reaction to be of the first order. Examination of our data, excluding the experiment at 11° discussed in a previous paragraph, reveals that in certain cases deviation from the straight line between log relative potency and time occurs in excess of the estimated error in the determination of log potency. In other cases, even though such a straight line relationship is not definitely excluded, there is evidence of significant curvature.

An adequate description of the inactivation, according to our results, is given as a straight line between relative potency and time, for the first part of the inactivation, up to approximately 15 months. For this first period the reaction may be of zero order. It is however, followed by a marked levelling off of the rate of inactivation. These observations are in agreement with the work of Lens[3].

If the relationship had been more accurately determined by more precise bioassays and by limitation of the between vial variation, it is possible that they would have served to predict the stability of insulin in aqueous solution for various temperatures and for various periods of storage by the methods outlined by Garrett and Carper[9] and by Garrett[10,11].

Vials stored at 36° or below exhibited neither precipitation nor gel formation over the 2-year period.

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* Pharmacopoeia of the United States XV.

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# A NOTE ON ROYAL JELLY

## A Critical Evaluation

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Royal jelly is a secretion of the honey bee which is essential for the development of queen bees. Its composition is not yet completely known. It contains proteins, lipids, carbohydrates, fatty acids (one of which is unique) and vitamins. The biological effects of the jelly are disputed. It has antibiotic power; it affects the adrenal cortex and can cause hyperglycaemia; and it has an antitumorigenic effect in mice. There is no good evidence that it has oestrogenic activity, or that it affects the growth, longevity or fertility of animals. Clinically it has been used as a general tonic, to ward off the effects of old age and to ease sufferers from chronic degenerative diseases. It seems likely that the novelty of the treatment and not the substance itself has been responsible for whatever successes it has had in these conditions.

Royal jelly is a milky white highly viscous secretion from the paired salivary glands of the worker (western) honey bee (*Apis mellifera* L.)[1–3]. For the first 3 days of life it is the sole food of all bee larvae. After 3 days, future worker bees are weaned onto honey and nectar, while the future queens continue to be fed on royal jelly, which is in some way responsible for their development into mature female insects. Royal jelly has been known and studied for many years, but its effects on bees, other animals and man have been studied only recently. It contains a number of unique compounds, some with unusual biological properties, and it has been used clinically to relieve the effects of old age and various degenerative diseases. Several new discoveries have been made since the subject was last critically reviewed[1].

### *Collection, Stability and Composition of Royal Jelly*

Repeated attempts have been made to analyse royal jelly since von Planta's original work in 1888, but owing to the small quantities available and to its instability, some of the compounds responsible for its biological activities have yet to be identified. Methods of collection are often not stated. Weaver and Kuiken[4] placed young female larvae in cups in a queenless colony. After 2 to 3 days the cups were removed, the larvae separated and the jelly scraped out. Townsend and Lucas[5] and Goillot[6] removed queen cells from ordinary colonies and squeezed out the jelly.

Microscopic examination shows only a few pollen grains and apparently no other solid contaminants in royal jelly. This is important because even quite small amounts of pollen could greatly alter the composition of the jelly. For instance the vitamin content of honey is attributed to such contamination[7].

If royal jelly is stored at room temperature it changes slowly from a milk white jelly to a slightly yellow gum, and finally, after some weeks,

to a brittle amber solid. The taste is altered from that of a "seasoned ragout" to "rather acid"[1]. In the laboratory, queen bees can be reared from larvae only if they are fed on jelly which is not more than 2 hours old[8]. Goillot[6] has shown that there are complex changes in its conductivity shortly after secretion and Chauvin and Lavie[9] have pointed out that the antibiotic and hyperglycaemic effects are not demonstrable until the jelly has been stored for some hours. The experiments and therapeutic trials with royal jelly have been done on specimens stored for at least several weeks, and the results may well be due to substances developing during the ageing of the jelly. The instability of the jelly makes comparison between fresh jelly from the hive and the usual laboratory material difficult, and probably explains some of the contradictory experimental findings.

Published analyses of royal jelly have been in quite good agreement although done on composite specimens. The samples have been stored after drying, *in vacuo* over $P_2O_5$ or by lyophilisation, and have been kept at various temperatures for periods up to several months. The composition of several commercial preparations has been described and criticised as weak and impure[10].

The main classes of compounds found in royal jelly are shown in Table I, where it may be seen that the fresh material has a high moisture content and the solids are largely proteins and carbohydrates.

TABLE I

OVERALL COMPOSITION OF ROYAL JELLY[1,5,11,12]

| Constituent | Per cent weight of fresh specimen (pH = 5) |
|---|---|
| Moisture | 65–70 |
| Protein | 15–20 |
| Carbohydrate | 10–15 |
| Lipid | 1·7–6 |
| Pollen | Trace |
| Ash: | 0·7–2·0 |
| P | up to 0·5 |
| S | up to 0·6 |
| Na, K, Fe, Cu, Mg, Mn, Ca | Trace |
| Undetermined | up to 3 |

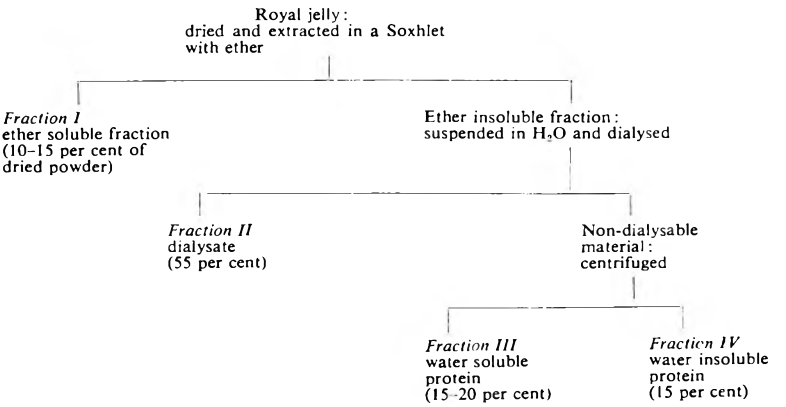
Fractionation of royal jelly, following the scheme of Townsend and Lucas[5], has involved the separation of ether-soluble, water-soluble and dialysable constituents (Table II). Fraction I (Table III) is a pale yellow semi-crystalline solid, which contains phenols, beeswax, sterols and fatty acids. The chief fatty acid has the formula $C_{10}H_{18}O_3$. It was isolated by Abbot and French[13] and later shown to be 10-hydroxy-$\Delta^2$-decenoic acid[14,15]. As it is not found in either pollen or nectar it is presumably synthesised by the bee. Its purpose is unknown.

Fraction II, the water soluble and dialysable constituents, contains 20 per cent of uronic acids, some unidentified nitrogenous compounds, and 50 per cent of various reducing sugars. These are mainly glucose, fructose, ribose and saccharose[5,16]. Fraction III consists of water soluble proteins in the proportion of two-thirds of albumin to one-third of

globulin, and it is only feebly antigenic[13,17–19]. The content of free and combined amino acids is shown in Table IV. The presence of hydroxyproline, histidine and tryptophan is disputed[4,5,20]. Taurine is found in fairly large amounts as is common in invertebrate tissues. de Groot[21]

TABLE II

FRACTIONATION OF ROYAL JELLY BY THE SCHEME OF TOWNSEND AND LUCAS[5]




has shown that the amino acid content of this mixture of proteins is sufficient to fulfil the requirements of honey bees and also that it is readily digested and metabolised by rats. The water insoluble protein of fraction IV dissolves only in alkalis and gives positive colour reactions for tryptophane, tyrosine and arginine.

TABLE III

COMPOSITION OF FRACTION I[5]

| Constituent | Per cent weight of fraction |
|---|---|
| Acids | 80 |
| Phenols | 4–10 |
| Waxes | 5–6 |
| Sterols | 3–4 |
| Phospholipids | 0·4–0·8 |

Royal jelly contains a number of vitamins[1,7] of which pantothenic acid is present in a higher concentration than in any pollen, honey or other natural source (Table V). The low content of vitamin C is almost certainly due to vitamin C oxidase activity[22].

Several other compounds have been isolated from the jelly, viz: a unique pteridine of unknown function, 2-amino-4-hydroxy-6(1,2-dihydroxypropyl) pteridine[23]: acetylcholine, 800 $\mu$g./g. of fresh material[24]: a non-specific cholinesterase and an acid phosphatase[16].

*Biological Effects of Royal Jelly*

Almost the only undisputed biological action of royal jelly is in producing the differentiation of queen bees from workers. The responsible substances are unknown, but there has been speculation about the

importance of the high concentration of pantothenic acid. There is independent evidence of the importance of minute amounts of hormones in the structure and activities of bee colonies, so it is possible that the differentiating substance will be found in such minute quantities as to make analysis difficult[3]. Apart from this physiological activity on bees, royal jelly has been reported to have divers effects on living organisms.

TABLE IV

FREE AND COMBINED AMINO ACIDS OF ROYAL JELLY[4,18]

| Amino acid | As free compound | As protein constituent |
|---|---|---|
| Alanine | + | + |
| Arginine | + | + |
| Aspartic acid | + | + |
| Cystine | – | + |
| Glutamic acid | + | + |
| Glycine | + | + |
| Histidine | – | ? |
| Hydroxyproline | – | ? |
| Isoleucine and/or leucine | + | + |
| Lysine | + | + |
| Methionine | – | + |
| Phenylalanine | – | + |
| Proline | + | + |
| Serine | – | + |
| Threonine | – | + |
| Tyrosine | + | + |
| Tryptophan | – | ? |
| Valine | + | + |
| B-Alanine | + | |
| Glutamine | + | |
| Taurine | + | |

TABLE V

VITAMIN CONTENT OF ROYAL JELLY[1,1]

| Vitamin | Concentration μg./g. of fresh material |
|---|---|
| A | nil |
| B | |
| Thiamine | 2 |
| Riboflavine | 10 |
| Pyridoxine | 2 |
| Nicotinic acid | 75 |
| Biotin | 2 |
| Folic acid | 0·3 |
| Inositol | 100 |
| Pantothenic acid | 250 |
| C | 3–5 |
| D | trace |
| E | trace |

*Oestrogenic and gonadotropic activity.* This was first suggested by Heyl[25] because royal jelly was known to stimulate the ovaries of bees. He found that the injection of aqueous or pyridine extracts into immature female rats caused premature formation of graafian follicles. This was not confirmed by Melampey and Stanley[26], Townsend and Lucas[27] or Hinglais and others[28]. Abbot and French[13] observed no effect when the ether-soluble fraction (fraction I) of royal jelly was injected into spayed rats. In normal rats injections interrupted the oestrus cycle. Chauvin and Herbert[29] noted great stimulation of the seminal vesicles of rats in one series of injections of royal jelly, but this has not been confirmed by them

or by other workers. There is therefore no evidence of any oestrogenic or gonadotropic activity of royal jelly in rats or mice, and presumably there is no such action in other mammals.

*Fertility.* More rapid maturation and a 60 per cent increase in the rate of egg laying was noticed when an ether extract of royal jelly was fed to a colony of Drosophila[27]. This was only a preliminary report, and no further evidence has been obtained. Earlier suggestions of a high vitamin E content have been disproved[30].

*Longevity.* Queen bees live considerably longer than workers and attempts have therefore been made to isolate "longevity factors" from royal jelly. It was shown that Drosophila flies fed on a diet with added royal jelly lived longer (17 days) than those receiving "normal" diet (13 days). However, if extra pantothenic acid was added to the "normal" diet the difference disappeared, suggesting that the "normal" diet was deficient in this substance[31]. The greater life span of the queen is possibly due to her lower energy expenditure[1].

*Adrenal cortical actions.* It was noticed[26] that a series of injections of royal jelly in rats caused involution of the thymus and lymphoid tissues. These findings have been confirmed[29] in mice receiving a larger dose (about 5 mg./mouse). Other changes, which were similar to the effects of corticosteroid injections, have been shown in the adrenal cortical enzymes and the hepatic glycogen content. Similar experiments in guinea pigs[19] produced haemorrhage and congestion of the adrenal glands 24 hours after injection of royal jelly, but there was no change in their ascorbic acid content. There was a slight neutropenia and reticulocytosis. This was explained as an anti-ACTH effect to which the animal had responded by a so-called "reaction of alarm". Aged solutions of royal jelly (stored for a week at 1°) produced hyperglycaemia when injected into mice[32]. The blood sugar one hour after injection was double the control blood sugar level. 6 mg. of jelly counteracted the effects of 5 units of insulin. There is evidence that royal jelly has effects on the adrenal glands, on blood sugar concentration and on lymphoid tissue of various animals, but it is not yet possible to say how they are caused. The only reported human trial in which urinary 17-ketosteroid excretion was measured was inconclusive.

*Antibacterial activity.* Royal jelly does not show any bacterial or fungal growth when kept at room temperature in non-sterile containers[33,34]. There is now evidence of antibiotic powers against a range of organisms including *Micrococcus pyogenes*, *E. coli* and *M. tuberculosis*[28,35–38,39]. The antituberculosis fraction was found to be soluble in water and ethanol[28]. 10-hydroxydecenoic acid has been investigated as the compound responsible for the antibacterial and antifungal activity of royal jelly[15], but it is probably too weak, having only one-quarter of the activity of penicillin against *M. pyogenes*, and less than one-fifth of that of chloramphenicol against *E. coli*[10]. The antibiotic activity is found only after the jelly has been stored for some time[9].

*Antitumorigenic action.* Very recently it has been found that mice injected with a mixture of viable leukemia cells, lymphosarcoma cells and

royal jelly developed no tumours[41]. This effect appears to be due to 10-hydroxydecenoic acid, but the mechanism of its action is unknown.

*Miscellaneous effects.* Royal jelly was found to have no significant effect on the rate of growth of young rats nor on their resistance to fatigue[37]. When tested on homogenates of rat diaphragm, constituents of royal jelly were found to increase oxygen uptake. This is suggested to be due to an effect on oxidative phosphorylation[42].

*Clinical and Therapeutic Uses of Royal Jelly.*

Royal jelly has been used mainly on the continent of Europe as a non-specific tonic for people who do not feel quite fit, or who have some mild chronic discomfort. It has been given to sufferers from rheumatism[43] because it "lessened their discomfort and produced a definite sense of well-being so that the patient was better able to live with his disease and his fellow men". It is claimed that it can relieve such effects of senility as apathy, loss of intelligence and personality degeneration. It is also claimed to help those patients who complain of inability to concentrate, neurasthenia and easy fatigue due to over-work and strain[44]. Some other effects of ageing are said to be prevented and even reversed[19,45–49]: "the cells are rejuvenated and the glands restored to a balanced state"[48], and arthritis, neuralgia and vascular insufficiency are greatly relieved[50]. There have been several claims that royal jelly can accelerate the growth of premature infants[51–55], but the reports are of very few cases and the increases in weight so small that it is impossible to show any definite effect. It has even been said[48] that royal jelly "helps the body to combat cancer by aiding reactive connective tissue formation", but no evidence is given to support this statement.

In all these reported treatments very small quantities of royal jelly have been used, and doses given were about the same as those employed in mice by Ardry[19] and Chauvin[29]. It is usually administered as some form of lyophilised preparation orally, sub-lingually or parenterally. Ardry[19] states that oral administration has no effect but others[44,48] have claimed good results by this route. Royal jelly has also been given by iontophoresis for certain joint conditions and has been used topically as a hair lotion for scurf[50]. The commercial preparations of royal jelly have been described as weak and impure mixtures[10].

One case of "poisoning" has been reported[56] in which a man swallowed about 5 g. of royal jelly. For the next few days he felt full of vitality and energy and was unable to sleep. He felt warm although his temperature was not raised. After a few days he made a complete recovery and did not show any ill effects during the next 4 years.

Apart from the confusion about how to give royal jelly and how much to use, it must be pointed out that the reports of its effectiveness are based on small numbers of short-term case histories of patients with just those symptoms which are relieved by any new therapy. From the information given it is impossible to decide whether royal jelly has had any effect, and it seems likely that its value was psychological and due only to the novelty and glamour of the treatment.

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# BOOK REVIEW

*AN ELECTRONIC OUTLINE OF ORGANIC CHEMISTRY.* By S. Horwood Tucker. Pp. xvi + 478 (including Index). University of London Press, Ltd., London, 1959. 63s.

The publication of yet another text-book of Organic Chemistry probably requires some word of explanation for its appearance and the following from the author's own preface is perhaps the best apologia. "*The book herein presented has been written for students* (reviewer's italics) and its aim is to present in small bulk, as much theory as is essential for a working understanding of the reactions of organic compounds. Whilst electronic theory is its main theme, for didactic reasons the time-honoured order of presentation of factual knowledge is retained." After a brief introduction on the structure of the atom and the types of bonds found in organic chemistry, there follows a chapter on the basic concepts of electronic theory. Thereafter the normal topics follow in order, viz. alkanes, olefins, acetylenes, alcohols, ethers, aldehydes and ketones, acids and esters, aromatic compounds, electrophilic and nucleophilic substitution amines and finally heterocyclic compounds. Throughout the text in appropriate places, the various substitution and displacement reactions, rearrangements and migrations are discussed.

The student would best approach the text already equipped with an elementary knowledge of functional group reactions. Given that basis, the general clarity of writing and arrangement seem well suited for teaching purposes. In providing a student text, there is a general tendency to overestimate the amount of material which a student can usefully read and digest: indeed, we have all suffered from "the heavy matrix of comprehensive fact." In making his selection of material the author has had this fact continually in mind. The result is a selection of the more important aspects of organic chemistry—considered throughout against the background of electronic theory.

Individual readers may, of course, find that favourite topics have been omitted and the reviewer personally regrets that there is no mention of conformational analysis.

Of necessity somewhat condensed in style, there are sections where clarity is sacrificed and the section on synartesis (p. 327) suffers thus. The solvolysis and rearrangement or bornyl and isobornyl chlorides is not easy to follow and could well be expanded. The author makes frequent reference to "Ingold" and adoption of the formulae for these epimeric chlorides given on p. 515 of this reference, rather than the ones actually used, would aid in the understanding of this phenomenon.

Inevitably in teaching one tends to adopt a somewhat dogmatic style. Thus on p. 149 the author deals with the formation of ethers and olefins from alcohols, arguing persuasively as to the mechanism: the student might be forgiven for concluding that the mechanism was securely based on reliable experimental evidence. The symbolism of p. 150 suggests that a carbonium ion is involved as an intermediate whereas there is no direct evidence upon which to decide this point and whether or no the mechanism is mono or bimolecular. It would have been preferable to make this point more clearly. These, however, are isolated instances which the present reviewer has noted and a welcome must be given to the book as being genuinely within the scope of the undergraduate for whose further reading copious references have been provided.

The standard of production is high and the result is a book which is as elegantly bound as it is pleasingly printed. D. W. M.