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REVIEW ARTICLE

TOXICOLOGICAL ANALYSIS

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". . . Orfila first introduced and several authors have copied from him, a long methodical table of all poisons with their properties so arranged that a suspected substance might, by a successive comparison of its qualities, be referred to its proper head amongst them. But as in nineteen cases out of twenty the poison is presented to the medical jurist in so impure a state that this mode of examination is inapplicable; and as it is very doubtful whether an unknown poison is ever in practice found out in such a manner I have omitted the table altogether. By moral evidence and the consideration of the symptoms the particular poison is always indicated presumptively and the analysis directed by this presumption . . .".

So wrote Robert Christison in the preface to his *Treatise on Poisons* over 120 years ago. To-day the pendulum has been forced back to the Orfila philosophy. The cunning of modern poisoners is such that the tell-tale symptoms, especially of common poisons, are often well concealed. This is not the only factor with which the chemist has to contend. Since the nineteen-thirties the number of new compounds used in medicine, industry and the home has been increasing almost daily. The number of suitable substances readily available for the poisoner has correspondingly increased. Modern treatment also presents its own problems. As an example of this trend which illustrates the difficulties of the toxicologist, it is sufficient to quote the common occurrence of the injection of niketh-amide as a medullary stimulant in instances of poisoning. This compound is extracted by the analyst together with the poisonous alkaloids. Indeed, the number of basic, naturally occurring and synthetic, nitrogen-containing compounds in use in medicine presents, in itself, a formidable problem in analysis.

To meet this growing challenge the newer techniques of electrophoresis, paper and ion-exchange chromatography, and ultra-violet spectro-photometry have arisen as the major techniques in use to-day. Infra-red spectrometers and X-ray diffraction cameras are within the reach of most toxicologists thanks to the ready co-operation of the universities and industry. The simpler techniques, however, are naturally in considerably wider use and this review will be concentrated in their direction.

Yet part of Christison's prefacory comment holds good, for the analysis is still guided by the symptoms and by the moral evidence, but in many cases these are either not available or have been deliberately disguised. Dr. H. Lederer and the author have recently reported a fatality in which the ingestion of a large quantity of a liquid arsenite produced no obvious post-mortem changes to suggest its consumption[1]. In these circumstances it is apparent that a systematic approach to the problem is essential.

The way in which the author approaches the analysis of, for example, a stomach contents is shown in the diagram.

STOMACH CONTENTS

1/3rd	1/3rd	1/3rd
Distilled from acid solution		Reserve

1/3rd — Unstable compounds and those requiring special attention

2/3rds — Organic Poisons
(*a*) Phenolic in character
(*b*) Acidic in character
(*c*) Neutral in character
(*d*) Extracted from alkaline solution by ether
(*e*) Extracted from alkaline solution by chloroform
(*f*) Extracted from ammoniacal solution by chloroform / *iso*propanol

Volatiles (1)   Distilled from alkaline solution

Volatiles (2)   Metals   Poisonous heat-stable anions

The examination is conducted using the above general scheme: the results being noted on a standard printed form. Individual poisons are quoted by name on this form, the tests for the detection of the poisons being detailed on a "master plan". It is obvious that the master plan must be under constant review. Much work is done in an effort to find the most suitable and sensitive test for each poison or group of poisons. The application of the principles of operational research is an obvious necessity in any scheme of this type.

If the time that elapsed between the suspected ingestion of poison and death is known, this leads the analyst to his choice of organ to be examined first. If a sample of urine is available in sufficient quantity much useful information can often be gleaned by a process of spot testing. For example, a 30 ml. quantity of urine is sufficient to make tests for the following list of compounds: alcohol, acetone bodies, salicylates, chloral, chloroform, paraldehyde, formaldehyde, borate, bromide, iodide, chlorate, bromate, nitrite, phenacetin, sulphonamides, phenols and cresols, santonin, tetralin, naphthalene, arsenic and antimony.

Tests for these compounds are mainly old-established ones but for an up-to-date digest on laboratory tests for poisoning, the reader should consult the relevant section in von Oettingens book on *Poisoning* (Heinemann 1952). Tests of this type are of great value to the police toxicologist for he is usually required in a very short period of time to rule out any possibility of poisoning, or conversely, to produce positive evidence of poison. Thus it is of great value to the analyst to make use of simple rapid tests that can, in themselves, in a large number of instances, rule out poisoning by particular compounds. Positive evidence for the presence of any of a very large number of organic compounds can be obtained by extracting 5 ml. of urine with ether or chloroform from acid and alkaline solution and subsequently examining the extracts by ultra-violet spectrophotometry. The choice of solvents for this test is of importance.

Barbiturates, for example, will not show any characteristic inflections if ethanol is used as the solvent. A 0·5 N ammonia solution is therefore used by the author in the investigation of the "phenolic" extract from the urine. Thus with only a little over an ounce of urine it is possible to test for the majority of common poisons. The simplicity and high sensitivities of the tests ensure that there is the maximum chance of detecting poisoning.

The large number of investigations in which carbon monoxide and ethanol play not the dominant but the supporting role illustrate the need in all instances for the routine examination of blood samples for these compounds. Carbon monoxide determination by the reduction of palladium chloride have been extensively studied[2-4], while the examination of the spectra of blood samples at 540, 562 and 579 mμ also provides an accurate measure of carboxyhæmoglobin[5,6]. Simpson has shown[7], that if the Hartridge reversion spectroscope is used for the determination, then the sample need not be analysed with undue haste. Experiments by the author and Mr. S. S. Kind have shown that this does not appear to be true of blood samples from thin films— e.g. from blood-splashes. A lapse of a few hours is sufficient for great changes to be found. Simpson's results may be explained, either by a similar decomposition rate *in vitro* in bulk samples of both oxy-, and carboxyhæmoglobin, or to fortuitous spectral changes.

The determination of ethanol by dichromate oxidation methods has recently been studied by a panel of analysts of the Royal Institute of Chemistry[8]. Other chemical methods have also been reported[9-14], as has an enzymatic method for ethanol determination[15,16]. Perhaps the discovery with the widest medico-legal significance in the past decade has been that of the post-mortem production of many lower aliphatic alcohols in blood by fermentation with yeasts, fungi and bacteria[17-19]. Fortunately fluoride completely inhibits this alcohol production and this knowledge is now being put to practical use. Synergism and drug potentiation are growing problems for the toxicologist and emphasise the need for routine alcohol determinations. While the toxicity of methylpentynol is low, the increasing use of this drug and its alcohol type of action make it of particular significance. Analytical techniques for its determination have been devised[20,21] but its instability makes it one of the compounds for which a special search is necessary. Academic research has recently shown that the active constituents of water-hemlock and hemlock water-dropwort are also highly unsaturated alcohols[22]. The acetylenic links in these compounds are not able to form silver acetylides and although they possess characteristic ultra-violet absorption spectra it is probable that only the routine examination of stomach and intestine contents will give positive evidence for poisoning by these plants.

So it is that modern techniques, while making the task of the chemist easier, have not replaced the classical approach to the problem. The practical method of dividing poisons into the groups in which they are extracted from viscera is the approach that has guided the author in preparing this review. The first poisons to be considered therefore are those that can be separated from viscera by a process of distillation.

Poisons Volatile in Steam from Acid Solution

One of the main requirements in any search for poisons is that the test applied should be sensitive enough to detect the quantities of poison likely to be present in the organs. "Approved tests" for this class of compounds are to be found in most textbooks. Those described below are not meant to be comprehensive but follow the trend of the review which emphasises the most recent aspects of the subject.

Tests for Alcohols

In tests for methanol little new has emerged in the past few years apart from variations in technique. The satisfactory method of controlled oxidation to formaldehyde and the determination of this compound with Schiff's or chromotropic acid still predominates[23]. Conway type of diffusion flasks have come into wide circulation for the determination of volatile poisons on a micro-chemical scale. Methanol[24] and cyanide[25] estimations are typical examples. The identification of aliphatic alcohols by the paper chromatographic separation of derivatives has been described[26,27] and there is also a method based on spectrophotometric measurements after conversion of the alcohols to the corresponding nitrites[28]. The differing toxicological properties of various glycols makes their identification important[29] and the separation by paper chromatography of the glycols present in foods, beverages and toothpastes[30] is of interest. The separation of polyhydric alcohols by paper electrophoresis[31] may also have some toxicological application as for example with erythritol derivatives.

Fujiwara's Test for Halogenated Hydrocarbons[32]

In this test 1 ml. quantities of distillate, together with 1 ml. of 20 per cent. sodium hydroxide and 1 ml. of pyridine are heated in a boiling water bath for one minute. Colours seen in the pyridine layer indicate halogenated aliphatic hydrocarbons. As there are 22 various halogenated hydrocarbons listed in a recent Government publication entitled *Toxic Chemicals in Agriculture*[33] which are being employed in this country as insecticides the significance of this test will be appreciated. Chloral, chloroform, methyl chloride, Frigen 12 and methylene dichloride give positive tests as does also trichloroethylene and its metabolites[34]. Chloral and chloroform can be determined together using this method[35]. Carbon tetrachloride does not give a positive result and the isolation of this compound and many other non-reactive volatile compounds is best approached by fractional distillation and determination of micro-scale boiling points using the techniques pioneered by Gettler[36].

Methyl salicylate, halogenated xylenols, cresols, and camphor are detectable in very small quanities if concentrated in the direction of an experienced nose. This may be done by extracting a portion of the distillate with ether and then carefully evaporating. The paper chromatographic separation of various cresols[37,38] is of interest in view of the continued popularity of the lysol type of disinfectant as suicide draughts.

Rothera's Test

This test has been recently re-investigated[39] and acetone, acetaldehyde and paraldehyde are of sufficient importance to warrant its routine inclusion in the scheme. Acetaldehyde disappears rapidly from the blood. Stotz, reporting a method for its determination[40], notes a 50 per cent. loss in 14 hours in a sample of blood kept in a refrigerator. In deaths from alcohol-disulphiram this is of importance. Methods for the determination of acetaldehyde as its 2:4-dinitrophenylhydrazone have recently been reported[41,42].

Measurement of the Ultra-violet Absorption spectrum

Measurement of the ultra-violet absorption spectrum constitutes a very sensitive test, suitable for eliminating from further consideration a vast number of compounds. In the toxicology section of a recent book on *Legal Medicine*[43] a table is given relating wavelengths maxima and minima with poisonous volatile compounds. The author feels that this list is a little unrealistic including as it does camphor which has $E_{1\,cm.}^{1\,per\,cent.} = 3$ at a wavelength maximum of 280 mμ. The only useful addition recently has been that of parathion. This compound has maximum absorption at 276 mμ and is readily detectable in concentrations of 1 mg. per 100 ml. This is the level likely to be found in accidental poisoning by this compound. Peaks about 270–280 mμ are not uncommon in distillates from stomachs and intestines from which no poison can be found. Heating the distillate with sodium hydroxide however will detect parathion, the yellow colour of *p*-nitrophenol being apparent in very low concentrations. Biggs uses this method for the determination of parathion from viscera[44].

Special Tests

(a) *Test for cyanide*[45,46]. A 1 ml. quantity of the distillate is put in a Gutzeit apparatus with some zinc and sulphuric acid. The test paper is made by dipping filter paper in slightly acid ferrous sulphate solution and drying. Before use the paper is dipped in 10 per cent. of sodium hydroxide and the excess removed by blotting. After 30 minutes in the Gutzeit apparatus it is dipped in concentrated hydrochloric acid containing a little ferric chloride. A blue stain is visible with only 0·2 μg. of cyanide. Concentrations of 0–14 μg. per cent. of cyanide have been reported recently in biological material[25].

(b) *Test for phosphorus.* Phosphorus poisoning presents special difficulties. Three murder trials, involving phosphorus, in the past two years in this country illustrate the toxicological importance of this element. Phosphorus is frequently concentrated in one section of the intestines, its length of travel depending on the length of time between ingestion and death. 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. Distillation into silver nitrate traps the phosphorus and Kaye[47] claims a sensitivity of 10 μg. of phosphorus by converting this silver phosphide to phosphine in a Gutzeit apparatus and detecting it on a mercuric bromide paper. With the increasing availability of volatile organo-phosphorus

compounds the significance of the Mitscherlich test is increasing in importance.

While some indication of the general methods for detecting volatile poisons have been given, an example of a particular problem is not out of place. Small quantities of a lighter fuel had been added to a bottle of medicine. Chloroform and oil of anise in the medicine to a large extent interfered with the comparison, by classical methods, of control lighter fuel with that separated from the medicine. Vapour phase partition chromatography completely solved the problem as shown in Figure 1.
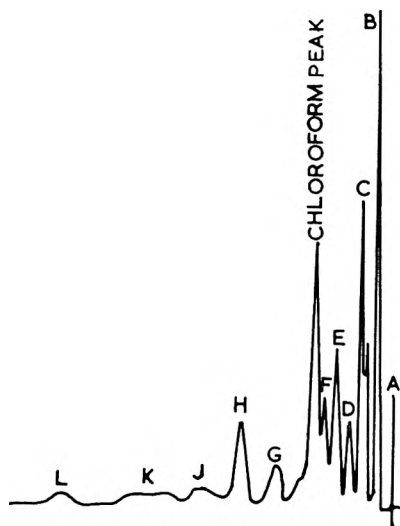
FIG. 1A.   Control lighter fuel with added chloroform.
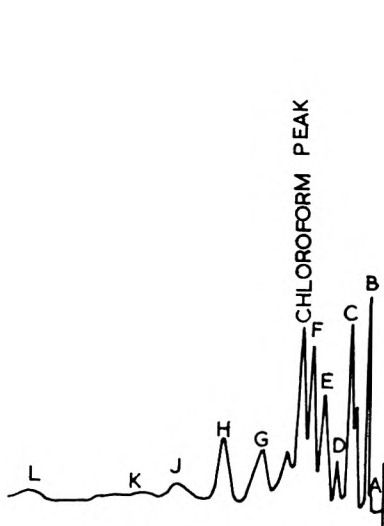
FIG. 1B.   Liquid from medicine.

POISONS VOLATILE IN STEAM FROM ALKALINE SOLUTION

Nicotine is the most frequently met poison in this fraction. Amphetamine is an example of one of the modern drugs that also may be found in it. Evaporation of the distillate made acid with 0·01 N hydrochloric acid gives the solution for test. Because of the possibility of confusing amphetamine with naturally occurring amines it is convenient to consider here some compounds that are not steam volatile but which are found in the "alkaloid" fraction. Wickström and Salvesen have separated many sympathomimetic amines by paper chromatography[48]. One of their solvent systems, the common butanol 4 : water 5 : acetic acid 1 system, is closely related to the system used by Schwyzer[49] for the separation of many primary amines, including cadaverine and piperidine. Both these authors used as a general chromogenic spray an indicator solution, a sensitivity of 1 μg. being claimed. Many colour reactions suitable for distinguishing the various aliphatic and aromatic amines likely to be found in biological media have also been published[48,50,54]. R_F values in a variety of solvents have been extremely well covered[51-53]. Tests of this type are very valuable where small quantities of these compounds are present, for

example in horse-, and dog-doping cases. The quantitative determination of amphetamine in biological material has also been described recently[55,56]. The ultra-violet absorption of amphetamine is so low that this technique is of little value. Nicotine has $E_{1\ cm.}^{1\ per\ cent.}$ about 300 at approximately 260 mμ and so measurements in the ultra-violet are useful for detecting and assaying this compound.

The main bulk of organic compounds, however, have to be separated from viscera by a process of solvent extraction. It is convenient at this stage therefore to consider recent advances in isolation techniques.

ISOLATION TECHNIQUES

The Stas-Otto alcohol extraction process for the isolation of organic, solvent soluble, poisons is under considerable criticism at the present time when the increasing complexity of the analysis demands a corresponding increase in the speed of working. Such work as has been published on the quantitative isolation of poisons by this process also suggests that it is not as efficient as had previously been hoped. The tendency at present is towards a direct extraction of the macerated tissue with organic solvent immiscible with the aqueous phase. Roche and Wright[57], for example, extracted buffered tissue extracts with chloroform and examined the extract by ultra-violet spectrophotometry, while a similar technique was used by Smith and Macdougal[58] who also published extensive data of the ultra-violet absorption curves of compounds of toxicological interest. Feldstein and Klendshoj[59] in an important paper on the general isolation of poisons removed proteins by heat treatment and extracted the filtrate from acid and alkaline solution in a liquid-liquid continuous extractor. In contrast to this approach, methods are still being sought for a suitable protein precipitant.

Berman and Wright[60] in the extraction of alkaloids before ultra-violet spectrophotometry used a tungstic acid precipitation for the final removal of protein. Valov[61] also used tungstic acid for protein removal in the isolation of barbiturates. The author has used a similar method for the extraction of salicylates and phenacetin and has found that after the extraction of the acid-aqueous solution by ether many basic compounds can be extracted by ether and chloroform after making the solution alkaline with ammonia. Some, however, for example methadone, are co-precipitated with the protein, but their extraction is ensured if this protein precipitate is macerated with ammoniacal chloroform. The absence of difficult emulsions and the general cleanliness of the extracts suggests that this technique could be profitably investigated. Experiments by Mr. H. Powell and the author have shown, however, that chlorpromazine is not extracted using this method. The ammonium sulphate, acid extraction method of Daubney and Nickolls[62] has recently illustrated its wide application by the recovery of cantharidin from organs in a recent medico-legal case[63].

Whatever process or combination of processes is used the first group of poisons will be extracted from acid aqueous solution by ether. These can be subdivided in the following way.

POISONS EXTRACTED FROM ETHER BY SODIUM BICARBONATE SOLUTION

The main bulk of toxicological specimens in this extract will be composed of aspirin metabolites. The colorimetric determination of salicylate has received its fair share of variations in the past few years[64-67]. Of interest to the toxicologist is the separation of aspirin metabolites by paper chromatography. Quilley and Smith[68] separated on Whatman No. 4 paper the compounds in Table I using a n-butanol 40:water 56:acetic acid 4 system.

TABLE I

R_F VALUES AND ULTRA-VIOLET DATA FOR SALICYLATE COMPOUNDS

Compound	R_F	Wavelength max. in mμ	Fluorescence in ultra-violet light
Salicylic acid	0·76	295	Violet
Salicyluric acid ..	0·65	320	Blue-violet
Gentisic acid	0·57	320	Intense blue
*Salicylamide	0·88	—	Blue-violet
*Gentisamide	0·75	—	Turquoise

* These compounds would not be found in this extract.

As individuals display a great variation in their sensitivity to aspirin it is important that the residues from a dose of 5 grains should be easily detectable. The paper chromatographic analysis, coupled with ultra-violet spectrophotometry, is capable of detecting microgram quantities of these compounds. The author has found, in agreement with other workers, that salicylic acid is normally the only acidic compound extracted from blood, brain and liver although the urinary excretion pattern on spraying with the ferric chloride, potassium ferricyanide reagent[69] shows a highly colourful picture. The determination of aspirin and salicylic acid in blood samples has recently been investigated[70]. Aspirin is the commonest poison in this group but many other compounds may also be found. One of these is diethylphosphoric acid from the hydrolysis of the insecticide tetraethylpyrophosphate. The author therefore puts an aliquot of this extract on a chromatogram, using a butanol-acetic acid system and detects by spraying with the Hanes and Isherwood[71] molybdate spray. The nitrophenols may also be found in this group, but by virtue of their colour are easily detected and determined. p-Nitrophenol is found as a metabolite of parathion. The dinitrophenols have caused many deaths of those accidently exposed to large concentrations; the observed dark colour of the urine of the victims has been exploited by malingerers. Picric acid, sometimes used in this way is readily identified by paper chromatography[72].

The naturally occurring organic acids sometimes cause difficulties of identification. The author has frequently isolated succinic acid from liver and intestines. The first time this compound was isolated its identification was delayed because of contamination with the anhydride after purification by a microsublimation method. Infra-red spectroscopy and paper chromatography finally led the way to its ultimate identification. A scheme for the identification of the common organic acids has recently been published[73] while organic acids in urine have been investigated by

ion exchange and paper chromatography[74]. Traces of hippuric acid in a urinary extract in an alleged case of poisoning recently aroused suspicions. The strong ultra-violet absorption showed its presence and identification was ultimately achieved by paper chromatography of the parent compound and of the glycine and benzoic acid formed by an acid hydrolysis. Hippuric acid is the normal excretion product after ingestion of benzoic acid and it is also found after exposure to toluene vapour. β-p-Hydroxy-phenylpropionic acid, i.e. deaminated tyrosine, is also known to occur in exhumed bodies[75,76]. Most of the acids occurring in this fraction have a characteristic absorption curve and Table II may be of use in interpreting peaks found in this extract.

TABLE II

ULTRA-VIOLET DATA OF SOME ORGANIC ACIDS

Wavelength max. mμ	Compound	Solvent	Approx. $E_{1\ cm.}^{1\ per\ cent.}$	Reference
225	Aspirin	Ethanol	455	57
226	β-p-Hydroxyphenyl propionic acid	Ethanol	460	
227	Benzoic acid	Water	666	57
229–31	Opianic acid	Water	586	132
234	Meconic acid	Water	715	132
235	Salicylic acid	0·5 N HCl	640	57
238	β-p-Hydroxyphenyl propionic acid	0·1 N NaOH	370	
269	Cinnamic acid	Ethanol	125	57
272·5	o-Nitrophenol	Ethanol	400	57
275	β-p-Hydroxyphenol propionic acid	0·1 N H$_2$SO$_4$	71	
279	β-p-Hydroxyphenyl propionic acid	Ethanol	103	
282–4	Opianic acid		657	132
295	β-p-Hydroxylphenyl propionic acid	0·1 N NaOH	94	
300	Salicylic acid	0·5 N NaOH	260	57
303	Meconic acid	Water	440	132
361	2:4-Dinitrophenol	Ethanol	800	57

POISONS EXTRACTED FROM ETHER SOLUTION BY SODIUM HYDROXIDE

When the strong acids have been extracted from the ether solution by sodium bicarbonate, the much weaker acids may be extracted from the neutral compounds by a wash with aqueous sodium hydroxide. Thereafter, acidification and re-extraction, followed by evaporation leaves the crude poison.

Approximately 45 per cent. of all deaths from poison in this country, at the present time, are the result of barbiturate ingestion. The toxicologist's main difficulties in these deaths used to be the very low level of drug remaining in the body after several days spent in coma, and the presence of metabolites preventing crystallisation of the unchanged barbiturate. The use of ultra-violet spectrophotometry has solved the former problem while the presence of metabolites, instead of being a problem, now facilitates the identification of the ingested compound. Raventos, in a comprehensive review on barbiturate metabolism[77] focussed attention on the large amount of work being done at the present time and especially on the use of paper chromatography in this type of work. The identification of pentobarbitone from its urinary metabolites in a medico-legal death was reported recently[78], while the author has reported a urinary pattern seen only in cases of phenobarbitone poisoning[79], the

"extra spot" in this pattern being due to the excretion of p-hydroxy-phenobarbitone[80]. While the isolation of metabolites is in this way helping the toxicologist, the discovery of the rapid N-demethylation of, for example, N-methylphenobarbitone to phenobarbitone shows that very careful interpretation of results is essential. This work has shown that colour reactions for the identification of barbiturates from biological material are unreliable. While on this subject, reference should be made to a paper by Krauss and Grund[81] who have critically studied the theoretical background to colour reactions. Goldbaum's ultra-violet method[82] must also be used with extreme caution. The differing ultra-violet absorption of phenobarbitone and its urinary metabolite described by the author elsewhere, emphasises this point. The discovery of an interfering compound, most probably naturally occurring and seasonal in origin, drew attention to the need for ultra-violet spectrophotometry *after* purification by paper chromatography[83]. Many paper chromatographic systems have been reported and many methods used for the detection of barbiturates on paper. Generally the systems resolve themselves into two classes.

(1) Organic solvents saturated with ammonia or diethylamine. For a general review, see Raventos[77].

(2) Paper buffered with a basic buffer system with an organic solvent as the developing agent[84-85].

Raventos, has also reviewed the methods for detecting barbiturates on paper chromatograms. These can be classified under general headings as

(a) Formation of an insoluble metal salts, for example, mercury and silver salts, and the subsequent colorimetric detection of these metals.

(b) Formation of complexes, like cobaltamines and copper and pyridine complexes.

(c) Methods making use of the high absorption of the barbiturates in the ultra-violet.

The author as a general rule uses the n-butanol saturated with the 5 N ammonia solvent system of Algeri and Walker[86]. For showing the position of the spots a contact print of the paper is taken on Ilford Reflex Paper No 50 using as the source of light a mercury ultra-violet lamp with no filters. This is the technique originally used by Markham and Smith[87] and Holiday and Johnson[88] for the detection of purines and pyrimidines on paper chromatograms. If the paper is exposed to ammonia vapour immediately before photographing then the increased absorption of the barbiturate at the more alkaline pH allows the ready detection of less than 10 μg. of barbiturate. The paper can then be cut and the spots eluted for quantitative ultra-violet spectrophotometry, or it can be dipped or sprayed to show the position of the spots as visible colours. The author prefers the mercury and diphenylcarbazone method and has used these techniques to separate phenobarbitone from diphenylhydantoin, compounds co-extracted, and frequently ingested together[89]. Using the modifications suggested by the author in this paper the background is made substantially colourless and the spots are stable for months and even years. Calculations which relate the ingested dose to concentrations in

the body at death are continually required of the toxicologist by coroners. For barbiturate drugs the accumulated experience acquired as a result of the very large number of suicidal and accidental overdose type of deaths encountered annually in such a laboratory as one of the Home Office Forensic Science Laboratories means that doses can be estimated in this way. It has been known, however, for a number of years that identical quantities of barbiturate given to different individuals will produce widely differing levels in the blood.

The identification of barbiturates by infra-red[90-92] and X-ray diffraction[93,94] analysis has also been reported.

The use of modern techniques for the analysis of barbiturate from viscera can be quoted as an example of a revolution in the last few years. One has only to investigate a death involving the simultaneous ingestion of two or three barbiturates and to develop the chromatograms, giving the distribution, and by elution and ultra-violet spectrophotometry the quantitative distribution, of each barbiturate in each organ to realise the tremendous analytical advances that these techniques have brought. It may well be that with the discovery of new drugs for the treatment of barbiturate poisoning and with the advent of improved hypnotics the phase of the barbiturates as the most common poisonous drug is passing.

It is unavoidable that so much of this section should be concerned with the barbiturates but the other compounds in this group must not be forgotten.

The application of paper chromatography to the detection and identification of hashish has been reported[95] and the ultra-violet absorption curves of the plant extracts before and after their extraction from viscera have been described[96]. The phenols derived from the anthraquinone type or purgatives may also be found in this fraction and paper chromatography is extremely useful for separating the complex mixtures of phenols found in these types of drugs. The author uses two systems, n-butanol-ammonia and butanol-acetic acid, for investigating these mixtures. Colours with ammonia vapour are striking but even more so are the fluorescent spots observed under ultra-violet light. Comparisons of samples of aloes, cascara, and rhubarb are simply made, using this technique.

Butazolidine, being a weak acid soluble in organic solvents, is extracted in this fraction. Methods for its determination have been published[97-99], and its distinctive ultra-violet spectrum ensures the detection and identification of very small quantities of this compound. The new rodenticide Warfarin also has a very characteristic absorption spectrum and the quantitative analysis of this compound using this spectrum has been reported[102]. Once the presence of Warfarin has been proved colorimetric methods for its determination are also available[100,101]. The ultra-violet spectrum of this compound is shown in Figure 2.

NON BASIC POISONS NOT EXTRACTED FROM ETHER BY ALKALI

The number of poisons found in practice in this group is not great, but the number potentially to be found is very large indeed. The author

has shown the presence of phenacetin in extracts from liver after ingestion of tablets containing aspirin, phenacetin and codeine. The only published work of which the author is aware for the detection of phenacetin poisoning refers to the detection of *p*-aminophenol in the urine. The detection of unchanged phenacetin by its characteristic 250 mμ peak in these extracts was therefore especially interesting. Apiol may also be found in this fraction and the investigation of this compound by paper chromatography has recently been reported[103]. The long chain acetylenic alcohols and related compounds from *Oenanthe crocata* have been referred to, as has the analysis of 3-methylpentynol. The chlorinated organic neutral insecticides are well represented in toxicological literature by dicophane (DDT). Levels of 3·6 mg. per cent. in the liver and 2·7 mg. per cent. in the kidney were obtained in a fatal case reported

FIG. 2.   Ultra-violet absorption curves for Warfarin 1·7 mg. per cent.

A in 0·1 N sodium hydroxide
B in ethanol.

by Luis[104]. The ultra-violet spectrum of this compound has been described[57] while its separation and identification on paper chromatograms has also received much attention. Mitchell and Patterson have dealt with this subject at length[105], while Mitchell has separated Prolan from Bulan[106], the isomers of benzene hexachloride[107], and aldrin, isodrin, dieldrin and endrin[108]. The determination of gamma benzene hexachloride from its absorption at 284 mμ has also been reported[109].

If an aliquot of this neutral extract is chromatogrammed and sprayed with the Hanes and Isherwood molybdate spray this ensures the detection of a number of neutral organic phosphorus insecticides. Methods for the detection and identification of these compounds are gradually being published; paper chromatography and ultra-violet spectrophotometry are again the main tools of this type of analysis[110,111,112,44]. While on the subject of insecticides reference must be made to review articles dealing with these compounds. Unfortunately the vast majority of the published methods of analysis are only of use when *the compound is known*. The main task of the toxicologist is to identify any abnormal analytical finding. The quantitative determination rarely presents difficulty. This is a fact very often not realised by those not actively engaged on this type of work.

The use in medicine of the substituted glutarimides in the treatment of barbiturate poisoning and as new hypnotics implies that these compounds may be found in this fraction. Already the isolation and identification and an urinary metabolite of bemegride (β-ethyl-β-methylglutarimide) has been reported[113].

Khellin, also found in this fraction, has been assayed from its ultra-violet spectrum and colorimetrically[114,115]. Santonin may also be conveniently detected and assayed by its ultra-violet spectrum[57] as may naphthalene[116], mephenesin[117], acetanilide[57], and phenacetin[57]. All these compounds show high absorption and are readily detectable in 10 μg./ml. concentrations.

While the identification of poisons is the main concern of the police toxicologist, there are many times when the identification of various pills and tablets assumes importance. The ready estimation and identification of the œstrogens and androgen preparations from ultra-violet spectra, coupled with paper chromatography, have led to the collection of control curves and patterns in the author's laboratory. So much work has been reported in the past few years in this field that it is obviously impossible in this short review even to begin to survey it. The toxicologist must have at least a passing acquaintance with the work of the clinical biochemist in this field. The publication of the *British Medical Bulletin* on Chromatography was especially useful[118]. The use of cortisone and hydrocortisone in horse- and dog-doping is a particularly pertinent example of the meeting of clinical biochemistry and toxicology. Methods for the differentiation of these two compounds have been reported[119,120]. The next group to be considered consists of poisons of basic character extractable from aqueous solution by ether.

POISONS EXTRACTED FROM AMMONIACAL SOLUTION BY ORGANIC SOLVENTS

The author prefers to extract first with ether. While the majority of alkaloids are successfully extracted using this solvent many other compounds, such as certain alkaloids, glycosides, aglycones and the rodenticide α-naphthylthiourea, are only extracted by chloroform or chloroform and ethanol mixtures. In this way an initial separation of these groups of poisons is possible. So much information has been of interest in the last few years that no review of this size can adequately cover even the outskirts of the field.

If a search for one, or a particular group of poisons, is requested it is a simple matter to turn to the relevant section of textbook and card index and perform the necessary analytical procedures that have been found to work for that particular compound or particular group of poisons. It is not unusual, however, to find that the deceased has had all the advantages of modern medicine before death, and consequently techniques that were suitable for academic studies on animals can not be used in the presence of, for example, procaine penicillin to quote only one commonly met antibiotic. In other instances the police may request a search for all poisons. It is at this stage that the author would echo Dr. Turfitt's sentiments in a similar review to this four years ago, "the actual problems of toxicological analysis are rarely appreciated except by those intimately connected with the field"[121].

The first stage in the analysis of poisons in this group is the separation of the poison from metabolites, from compounds that occur naturally in

the body and from any medicines administered in hospital. For analysis on a microchemical scale the author chooses paper chromatography. A system of n-butanol 100 : water 100 : citric acid 2, using Whatman No. 1 paper buffered with 5 per cent. sodium dihydrogen citrate was developed as a suitable system giving maximum resolution of poisons likely to be found in this group[122]. In passing it should be noted that Whatman No. 1 paper is essential for the production of really good spots. Whatman No. 4 paper gives only trails with many alkaloids. In the report of this work we gave a map reference for the poisons; by arranging them in order of ascending R_F it was possible to some extent to answer a single need of the police toxicologist—a rapid method of showing the absence of a vast number of poisons and at the same time concentrating attention on any abnormal finding.

After development the next stage is to show the position of the now separated compounds on the paper. To do this and at the same time cover as wide a field as possible, four methods are used by the author. Known alkaloids are put on the chromatogram as markers together with aliquots from the extract under investigation. If necessary, tests 1 and 4 can be carried out on one aliquot and tests 2 and 3 carried out in sequence on another aliquot.

The tests are as follows:

(1) The paper is photographed in ultra-violet light using the method as described under barbiturates. The sensitivity of this method for many compounds is high—10 μg. of many alkaloids being easily detectable. The paper is also inspected under filtered ultra-violet light. The fluorescence of quinine is particularly striking.

(2) The paper is sprayed with 0·2 per cent. solution of ninhydrin in acetone and heated at 80° C. for 3 minutes. This treatment ensures the detection of such compounds as ephedrine that have very weak ultra-violet absorption and do not react with the alkaloidal reagent in test 3.

(3) The paper is then dipped in a potassium bismuth iodide solution, or sprayed with potassium iodoplatinate. These general alkaloidal reagents detect 20 μg. quantities of most alkaloids.

(4) When the position of the spots has been revealed by one of the above tests quantitative ultra-violet spectrophotometry is carried out on relevant spots which are eluted from the paper and re-extracted.

The alkaloid complexes formed in test 3 can be cut from the paper, decomposed with ammoniacal chloroform, and the now pure alkaloid can be investigated by classical colour tests and micro-crystallography. Mannering and his colleagues in a paper on the use of paper chromatography applied to the detection of opium alkaloids in urine and tissues[123] give an excellent account of this type of scheme applied to a particular problem. Dependent on the type of isolation method used a "normal pattern" is soon established and any abnormality is cause for further investigation.

One disadvantage of the use of buffered paper is the decrease of sensitivity found with ninhydrin and Dragendorff reagents. While 30 μg. of ephedrine give an exceptionally good spot with ninhydrin using plain paper

and a butanol-acetic acid system only a faint spot is obtained with this quantity on citrated paper. In extracts from human viscera this degree of sensitivity is often acceptable. In other cases, however, especially if the analysis of a known compound is required, the advantage of the great resolving power of the citrated paper may not be required and by using systems not requiring buffered paper greater sensitivity is possible. The author obtained a very good ephedrine spot (approx. 10 μg.) using a butanol-acetic acid system for development and ninhydrin for detection of a known non-toxic dose, in an extract from only 2 ml. of urine. In this instance approximate quantitative measurement was made by the comparison of the ninhydrin colours with those from control quantities of ephedrine.

It is apparent that for test 4 to be of real value a great amount of relevant information about the ultra-violet absorption of compounds of toxicological interest has to be abstracted from the literature. The publication of logarithmic curves and those calculated from known molecular weights, coupled with the use of large number of various solvents, does detract from the value of some of this data. The amount of relevant information is now so large that for it to be of maximum value a card index system is really essential. Nevertheless, while the identification of ultra-violet peaks is often difficult, it is none the less rewarding. Even the apparent absence of absorption, in the presence of a positive Dragendorff reaction at a particular R_F, can point to the presence of a particular alkaloid, as for example, a member of the belladonna group.

Since the publication of the report of this citrate system a considerable number of additional compounds has been positioned on the map. Particularly useful is the way in which compounds like caffeine with their very high interfering ultra-violet absorption are separated one from another and from the majority of the common alkaloids.

There have been reports of confusion between the newer synthetic drugs and the alkaloids because of similar reactions with colour reagents, for example chlorpromazine with strychnine[124]. Paper chromatography was used to resolve this difficulty[125]. Morphine poisoning presents its own difficulties. Morphine has been found as a metabolite of codeine, while the use of nalorphine in the therapeutic management of morphine poisoning illustrates yet again the problem facing the toxicologist. With morphine and nalorphine an examination of the ultra-violet curves is of no avail, but X-ray, crystal formations and paper chromatography readily distinguish them[126]. In this last paper Pedley makes the very important point that while microcrystalline comparisons are readily reproducible on pure compounds in known concentrations, in the case of specimens extracted from viscera where the concentrations are not known and impurities are present completely different types of crystal formations can be, and are, obtained. This is a fact that has been very largely ignored in many publications and must to some extent detract from the value of this type of analysis. The same phenomena are repeatedly met with the barbiturate drugs.

So with this particular group of poisons, paper chromatography is

proving to be an invaluable tool. The great problem of separating microgram quantities of poison from the bulk of interfering material has been largely overcome. Obviously personal preference and experience have entered into the above description of the *modus operandi*. The foundation of this work is of course laid on the vast amount of work that has been published in the last few years on the chromatography of alkaloids, and especially on the work of Munier and his colleagues[127,128]. Once again the toxicologist is faced with the problem of abstracting from the whole field of chemistry, biology and medicine that which is particularly suited to his needs. It is with that problem in mind that the author has compiled Tables III and IV which summarise some of the more recent references that will be of interest in this field.

TABLE III

AIDS TO IDENTIFICATION

Compounds	Method	Reference
Selected alkaloids	Flavianic acid salts	129
Selected alkaloids	General crystal formation	130, 143
Narcotics	Reinecke salts	131
Narcotics	Ultra-violet	132
Narcotics	Infra-red	133
Narcotics	X-ray diffraction	134
Aliphatic amines	Colour reactions	50
Aromatic amines	Colour reactions	54
Local anæsthetics	Crystals	135
Local anæsthetics	Vacuum micro sublimation	136
Local anæsthetics	Paper chromatography	137
Antihistamines	H_2PtCl_6 crystals	138
Antihistamines	Colour reactions	139
Antihistamines	Picrate crystals	140
Antispasmodics	Colour reactions and crystals	141
Atropine and hyoscine ..	Infra-red	142
Methadone	Colour tests and crystals	143
Morphine and nalorphine	X-ray diffraction and crystals	126
Scopolamine	Crystals	145
Pethidine	Styphnate crystals	146
Opiates	Colour tests	147
Morphine	Mayer reagent	144

The extraction of the bulk of the alkaloids and synthetic basic compounds by ether does to some extent divide these from other alkaloids, the xanthines and other compounds only extracted to any real extent at the next stages. These are extracted by chloroform and chloroform-*iso*propanol. The author invariably uses for this last stage a liquid— liquid continuous extractor. While morphine and the xanthines are readily identifiable the detection and identification of the chloroform soluble glycosides present a rather more difficult problem. Current literature again shows that paper chromatography has proved invaluable for the separation of the various digitalis glycosides. Similarly the use of Carr Price reagent has been shown to give differential colours with various digitalis glycosides and their aglycones in concentrations of $0.5 \mu g./sq.cm.$[179]. The methods for detecting extremely small quantities of these compounds

are therefore available and, coupled with paper chromatography, are potent tools not only for the pharmacologist but also for the toxicologist. The literature on this topic is so extensive that there is not space to do justice to it here. The author was particularly grateful that some ultra-violet data on these compounds had been published because a successful detection of digoxin was initiated by a peak observed at 217 mμ.

TABLE IV

METHODS OF QUANTITATIVE DETERMINATION

Compound(s)	Methods used	Reference
Some alkaloids	Methyl orange reaction	148
Alkaloids	Chemical reactions, colorimetric	149, 150
Selected narcotics	Ion exchange	151
Local anæsthetics	Ion exchange and titration	152
Analgesics	Reinecke salt	153
Antihistamines	Ultra-violet	154
Belladonna	Colorimetric	155
Tropine alkaloids	Colorimetric	156
Brucine	Ultra-violet	157
Caffeine and theobromine	Ultra-violet	158
Cocaine	Ultra-violet	159
Codeine and morphine	Ultra-violet	160
Colchicine	Colorimetric	161
Ergot	Review	162
Hyoscine and hyoscyamine	Paper chromatography and colorimetric	163
Methadone	Reinecke salt	153
Morphine	Colorimetric	164, 165, 166
Morphine	Ultra-violet	157
Morphine	Paper chromatography and colorimetric	167
Nalorphine	Ultra-violet	168
Phenadoxone	Ultra-violet	169
Prantal	Colorimetric	170
Pilocarpine	Colorimetric	171
Procaine	Ultra-violet	172
Procaine	Ion exchange	152
Quinine	Ultra-violet	173
Quinine and strychnine	Ultra-violet	173
Strychnine	Ultra-violet	173
Strychnine	Colorimetric	174
Reserpine	Electrophoresis and ultra-violet	175
Veratrum	Infra-red	176
Strychine and brucine	Ultra-violet	177, 178, 157

IONISED COMPOUNDS THAT ARE NOT EXTRACTED FROM AQUEOUS SOLUTION BY SOLVENTS

This section is not devoted exclusively to inorganic compounds because the quaternary ammonium and quaternary pyridinium compounds are becoming increasingly important in medicine. Two main methods are available for the determination of these types of compounds. One is based on the extraction of dye-complexes[180,181] and the other on the formation of Reinecke salts[182,183]. The former is capable of high sensitivities suitable for biological media. To identify members of this group paper chromatography has been used[184] while the analytical properties of some methonium compounds[185] have been published as have those of some synthetic curarising agents[186]. The amino-acids fall into this chemical

group and of particular interest is the discovery of abnormal urinary amino-acid chromatograms in those with lead poisoning[187]. Blood cholinesterase determinations and their significance in poisoning, especially by the organic phosphorus insecticides, also underline the contribution of biochemistry to toxicology.

Activation analysis has been used to show the distribution of arsenic along the length of hair[188] and [75]As has been used to follow the uptake of arsenic by hair immersed in arsenical solutions[189,190]. This is of particular medico-legal interest.

Numerous poisons available to the general public are sold as rat poisons. Sodium fluoroacetate is one of the new rodenticides that is toxic to man. The detection of this compound and of inorganic fluoride has recently been reported by Goldstone[191]. Thallium compounds form the basis of other rat poisons and the detection of thallium by paper chromatography[192] and by radioactivation methods[193] again illustrates the application of modern methods to toxicology. The destruction of biological matter before analysis for metals has also received attention recently[194].

SUMMARY

This review has encompassed a wide range of analytical techniques. It will be noticed that instrumentation has entered into toxicology to a tremendous degree in the past few years. Only four years ago Turfitt wrote: "physical methods have been used only to a relatively limited extent in toxicology". In the author's laboratory the Unicam SP500 in the last year was in operation for nearly 400 hours.

While infra-red, ultra-violet and X-ray spectrophotometry are now accepted tools in this type of work the simpler techniques of paper chromatography, paper electrophoresis and ion exchange chromatography are playing an increasing role in the isolation, purification and analysis of poisons. Paper electrophoresis apparatus is very simple to construct and has yielded valuable analytical data on a wide variety of subjects in this laboratory.

Toxicological analysis is a specialised science in its own right, as the author hopes he has demonstrated in this review. While the only collected work on some modern methods to be published so far is in a textbook on *Legal Medicine* it is now certain that this subject merits greater attention than it has received in the past.

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SCIENCE PAPERS AND DISCUSSIONS

THE INTERFACIAL TENSION OF SOLUTIONS OF ARABIC ACID

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Introduction

Gum acacia has been used in medicine for many centuries[1] and is still valued in pharmacy particularly as an emulsifying and suspending agent for oral preparations. Nevertheless very little appears to be known about those properties of gum acacia which are responsible for its effectiveness as an emulsifying agent. According to the more recent theories of emulsions a substance must be adsorbed at the interface in order to act as an emulsifying agent, forming a coherent film the physical properties of which will determine the type and stability of the system.

Clark and Mann[2] studied the effect of the concentration of gum acacia on the surface tension of aqueous solutions and the interfacial tension with benzene using the drop-weight and drop-volume methods of determination. They found that the surface tension increased slightly with concentration up to 0·1 per cent. and as the concentration was increased further the surface tension was decreased giving a value of 61·49 dynes/cm. for a 10 per cent. solution. The interfacial tension however was reduced as the concentration of gum acacia increased and for the 10 per cent. solution a value of 22·87 dynes/cm. was reported. Banerji[3] determined the surface tension of solutions of gum acacia with Traube's stalagmometer allowing "a fairly long time" for the formation of the drops and found that an approximately 4 per cent. solution had a surface tension of 63·16 dynes/cm. compared with 69·69 dynes/cm. obtained by Clark and Mann for a 5 per cent. solution. Banerji also found that the addition of electrolytes depressed the surface tension.

The drop-volume method was also used by Matthews[4] who determined the effect of gum acacia on the interfacial tension between water and *cyclo*hexane. Both the crude gum and the gum purified by precipitation with ethanol were used. This work was more systematic than that of the previous workers since Matthews first determined the rate of formation of the drop below which a constant drop-volume was obtained with a

given tip. This maximum rate of formation corresponded to forming a drop in 2·5 minutes and under these conditions he assumed the surface would be in equilibrium. Further, by rapidly forming the drops to practically full size, allowing them to hang for varying lengths of time and then completing their formation at the rate of one drop in three minutes he concluded that the equilibration time was one minute. From the published graph he showed that the interfacial tension of water and *cyclo*-hexane was lowered uniformly as the concentration of the purified gum was increased (approximately 13 dynes/cm. by 3 per cent. of the gum) but that the crude gum showed a more rapid lowering up to about 1 per cent. concentration.

The ring method was used by Du Nouy[5] and by Boutaric and Roy[6], the latter showing that there was a decrease in the surface tension over a period of 4 hours, this decrease being greater if the solution was first boiled.

A review of the work on the chemical constitution of arabic acid has been made by Hirst[7]. The repeating unit in the molecule consisting of a branched chain.

The molecular weight of arabic acid was determined by Oakley[8] and Säverborn[9] who found it to be of the order of 290,000 and between 279,000 and 319,000 respectively. More recently Veis and Eggenberger[10] using a light scattering method found the molecular weight of arabic acid to be $1·00 \pm 0·05 \times 10^6$. With a substance of such large molecular weight it is to be expected that a comparatively long time would be necessary for the interface to reach equilibrium.

In the present work a comparison has been made of the drop-volume and the sessile drop methods for determining the effect of arabic acid on the interfacial tension of benzene and water and also the influence of time.

EXPERIMENTAL

Materials

Gum acacia. A sample was obtained from James Laing (London) Ltd. which was used throughout this work. According to the importers this material came from the province of Kordofan through the El Obeid market and was collected between November 1947 and "early 1948."

Distilled water. Distilled water from a Manesty still was re-distilled from an all-glass apparatus, a little potassium permanganate being added to the water in the still. The condenser and receiver were cleaned with chromic acid and the still was run for an hour before water was collected.

Benzene. Benzene of Analar quality was recrystallised twice immediately before use.

The purification of the gum acacia. 100 g. of the gum was dissolved in 150 ml. of water, strained free from insoluble matter and 300 ml. of 95 per cent. ethanol added gradually with constant stirring. After standing for 15 minutes the supernatant liquid was removed and the residue centrifuged to separate the precipitate. The gum was precipitated twice more and the purified gum dried in a vacuum desiccator.

The product had a moisture content of 5·71 per cent. and the pH of a 1 per cent. solution was 5·0.

The viscosity of a 2 per cent. solution was compared with that of the crude gum using a No. I B.S. viscometer of the Ostwald pattern. Crude gum acacia 2·060 per cent. w/v, viscosity (25° C.) 2·12 centipoise. Purified gum acacia 2·012 per cent. w/v, viscosity (25° C.) 2·15 centipoise. Purified gum acacia after 12 months storage 2·100 per cent. w/v, viscosity (25° C.) 2·18 centipoise.

These results indicate that the purification process did not materially affect the gum acacia and that it remained stable on storage. Taft and Malm[11] obtained a lower figure (1·6 centipoise for a 2·1 per cent. solution at 30° C.) but the above figures were in agreement with those of Riddell and Davies[12] (2·088 centipoise for a 1·994 per cent. solution at 20° C.).

The preparation of arabic acid. A solution containing about 12 per cent. of the purified gum acacia was passed through a column of cation exchange resin Zeo Karb 225 to remove the potassium, calcium and magnesium ions[13,14]. The arabic acid solution collected from the column was dried *in vacuo* over silica gel. When the initial foaming had subsided a high vacuum pump was connected and the solution frozen, the pumping being continued until a porous solid was obtained. The moisture content of the solid was determined by drying to constant weight at 100 to 110° C. and the concentration of solutions used in this work is expressed in terms of anhydrous material. The ash value showed some variation with different batches but was generally below 0·06 per cent. w/w.

Viscosity. The solution of arabic acid collected from the ion exchange column was diluted to about 2 per cent. and compared with a 2 per cent. solution prepared from the dried material. Solution from ion exchange column 1·896 per cent. w/v, viscosity (25° C.) 1·647 centipoise. Solution of the dried arabic acid 1·910 per cent. w/v, viscosity (25° C.) 1·793 centipoise.

A solution of arabic acid showed no change in viscosity after standing for 14 days at 25° C. Some change appears to take place in the arabic acid on drying[1] and the material was prepared at approximately 3 month intervals. One sample remained soluble for about 9 months after which time an increasing proportion swelled in water but did not dissolve.

The equivalent weight was determined by electrometric titration using a glass electrode. An approximately 10 per cent. solution of arabic acid was titrated against standard potassium hydroxide and four samples gave the following values for the equivalent weight, 1125, 1142, 1187 and 1201 (average = 1164). The shape of the curve indicating that arabic acid is a comparatively strong acid.

In a review of the literature Säverborn[9] shows that the reported values vary between 1000 and 1400, the lower results being obtained by heating the arabic acid with standard alkali and back titrating which indicates some of the carboxyl groups are combined as lactones.

Density of arabic acid solutions. A graph was constructed which showed the relationship between the density and concentration was linear. In the calculation of the interfacial tension the density of the solution was obtained from the graph.

The Determination of Interfacial Tension

The drop-volume method.   The method used by Gaddum[15] was adopted using an "Agla" micrometer syringe.   The tips were formed by grinding down a piece of "Viridia" tubing (bore 0·2 or 0·3 mm.) to give an external diameter of about 2 mm., in the manner described by Harkins[16] and the tubing was then fused on to the adaptor fitting into the syringe barrel. The glass apparatus was cleaned with chromic acid and well washed.   In order to prevent the aqueous solution from creeping up the sides of the tip it was treated with a 2 per cent. solution of Silicone DC 1107 in carbon tetrachloride to within 2 mm. of the end of the tip which was again cleaned with chromic acid.   The solutions and benzene were mutually saturated by shaking together, the separated liquids being used for the experiments.

The apparatus which was finally evolved is shown diagrammatically in Fig. 1 and was enclosed in a cabinet to protect from draughts, the water in the beaker K was circulated from a constant temperature water bath at 25° C.   When drops were allowed to hang from the tip for long periods of time it was found necessary to seal each end of the barrel of the syringe

TABLE I

DROP-VOLUME METHOD: THE EFFECT OF HANGING TIME ON THE INTERFACIAL TENSION
(25° C.)

Time drop allowed to hang	5 min.	30 min.	1 hour	19 hours
Water/benzene (dynes/cm.)	34·7	34·4	34·25	34·25
1·025 per cent. w/v arabic acid/benzene (dynes/cm.) ..	27·75 27·2	25·45 25·65	25·85	—
10·165 per cent. w/v arabic acid/benzene (dynes/cm.) ..	17·8 19·49 18·6 18·19	18·19 18·26 18·2	18·26 18·9	18·9 18·9

with a little liquid paraffin to prevent evaporation which otherwise would give a high result.   In preliminary experiments the micrometer head was turned by hand.   A drop was formed about ¾ of its probable final volume and after hanging for some time it was then increased by rotating the micrometer head three divisions (one division = 0·0002 ml.) every ten seconds until the drop disengaged.   The results in Table I show that the time the drop hung from the tip did not materially affect the value of the interfacial tension obtained.   The corrections of Harkins[16] were used in calculating the interfacial tension from the drop volume.   Harkins and Brown[17] found the interfacial tension between water and benzene to be 34·68 dynes/cm.

The variations in some of the results were probably due to the final additions to the drop not being made uniformly in each case and when drops were completed by larger increments at approximately 30 minute intervals the 1·025 per cent. arabic acid solution yielded drops which were approximately two thirds the previous size from the same tip.

It was concluded that the interface was not in equilibrium and in order to form drops at a uniform rate a synchronous motor (1 rev. per hour)

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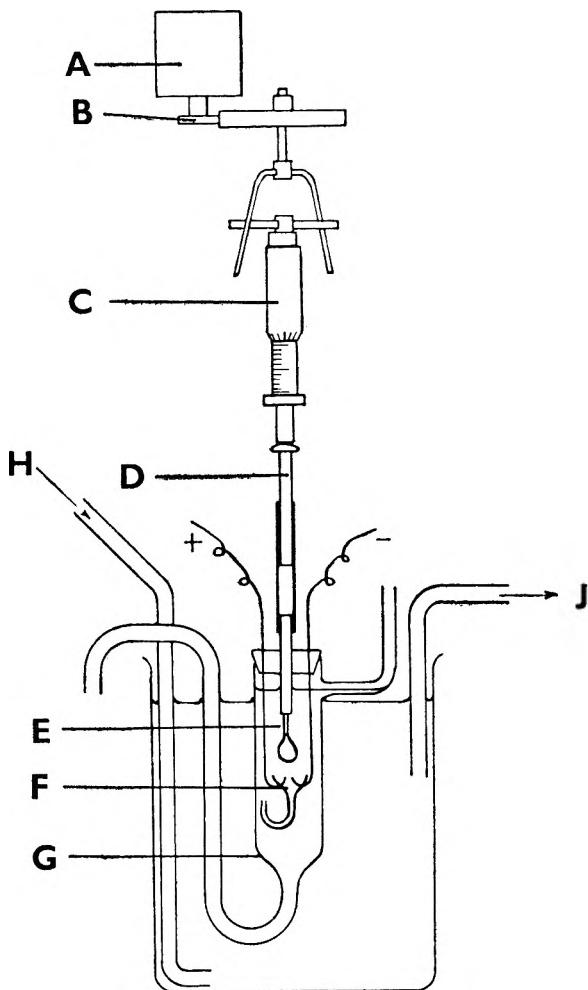
FIG. 1.   Drop-volume apparatus.

A.   Synchronous motor (1 r.p.h.).
B.   Gear drive.
C.   Micrometer screw gauge.
D.   Agla syringe (0·5 ml. capacity).
E.   Accurately ground tip.

F.   Glass funnel with Pt electrodes.
G.   Container for benzene.
H. and J.   Inlet and outlet for water at 25° C.

and gears were added to the apparatus as in Figure 1.   By this means it was possible to drive the micrometer syringe at speeds varying from 8 to 1/8 revolutions per hour corresponding to increments of 0·08 to 0·00125 ml. per hour.   When the drop fell into the small funnel F the circuit between the two platinum electrodes was closed and this caused a micro-switch to break the circuit of the synchronous motor which then stopped. The readings were then taken at a convenient time.   The results are given in Table II.

TABLE II

DROP-VOLUME METHOD: THE EFFECT OF THE RATE OF FORMATION ON THE INTERFACIAL TENSION

Solution per cent. w/v of anhydrous arabic acid		Rate of formation (revs. per hour)								
		By hand	8	4	2	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$
1·025	γ (25° C.)	27·5	—	22·0	20·4	19·1	17·8	17·7	17·5	17·2
	Time of formation	10 min.	—	2 hrs. 20 min.	4 hrs. 20 min.	7 hrs. 45 min.	14 hrs. 26 min.	28 hrs. 33 min.	42 hrs. 25 min.	55 hrs. 48 min.
5·265	γ (25° C.)	20·5	17·3	17·1	—	15·0	15·0	—	—	14·3
	Time of formation	5 min.	50 min.	1 hr. 35 min.	—	5 hrs. 32 min.	11 hrs. 5 min.	—	—	42 hrs. 12 min.
10·165	γ (25° C.)	18·27	—	—	15·9	15·2	—	14·8	13·6	14·0
	Time of formation	10 min.	—	—	2 hrs. 40 min.	4 hrs. 45 min.	—	18 hrs. 27 min.	25 hrs. 10 min.	34 hrs. 44 min.

In an attempt to shorten the time of the experiments drops of about $\frac{2}{3}$ of the final volume were formed immediately and completed at the rate of $\frac{1}{8}$ r.p.h. but the results exceeded those in Table II at $\frac{1}{8}$ r.p.h. by 3·5, 3 and 2 dynes/cm. for the 1, 5 and 10 per cent. solutions respectively.

The sessile drop method. In this method a drop of a fluid of suitable size is formed on a horizontal plane surface within the second fluid. The volume of any given drop is constant and changes in surface area take place only as a result of a change in shape. The shape of such a drop, which depends upon the interfacial tension, volume and the difference in density of the two phases, comes within the scope of Bashforth and Adams's treatment of surfaces of revolution. Porter[18] published a method of calculating the interfacial tension from the dimensions of a sessile drop shown in Figure 2 where r is the equatorial radius and h is the height of the vertex above the equator. The original paper should be consulted for the theory of the method.
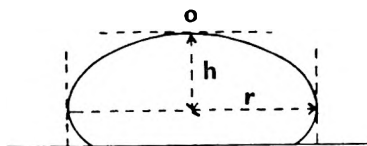
FIG. 2. Profile of the sessile drop.
r. radius of drop at the equator (maximum diameter).
h. height of drop from the equator to the vertex O.

The interfacial tension is obtained from the formula,

$$\frac{\beta^2}{r^2} = \frac{1 \cdot h^2}{2 \, r^2} + \Delta \quad \text{where } \beta^2 = \frac{\gamma}{g \, (\rho_1 - \rho_2)}$$

ρ_1 and ρ_2 are the densities of the two phases.

γ = interfacial tension in dynes/cm.

The correction Δ was obtained from a curve constructed from the values published by Porter.

When $\frac{h^2}{r^2}$ = 0·25 the correction is zero and the size of a drop was found for each solution that gave a value for $\frac{h^2}{r^2}$ between 0·20 and 0·30 after 96

hours since it is in this region that the correction is known with greatest accuracy.

The apparatus is shown diagrammatically in Figure 3 and for convenience it was mounted on an optical bench. The light from the mercury vapour lamp A was passed through two filters B, Chance ON16 and Ilford 807 so that the light of the mercury green line was transmitted and illuminated the ground glass screen D.
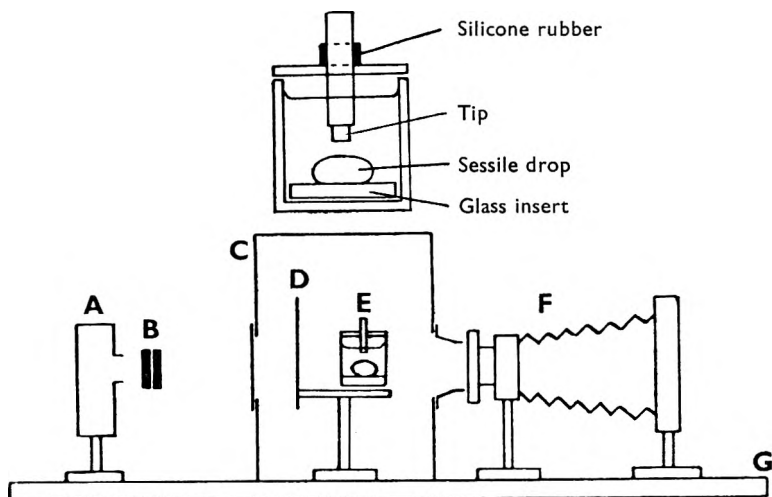
FIG. 3.   Apparatus for the sessile drop method.

A.	Mercury vapour lamp.	E.	Optical cell assembly.
B.	Filters: Ilford 807; Chance ON16.	F.	Camera.
C.	Thermostat cabinet at 25° C.	G.	Optical bench.
D.	Ground glass screen.		

The solution and benzene were mutually saturated at 25° C. by shaking together and allowing to stand overnight in the thermostatically controlled cabinet C. The drop was measured into the optical cell containing the benzene with a hypodermic syringe the apparatus having been maintained at 25° C. also.

The glass insert in the optical cell E was treated with a 2 per cent. solution of methyl chlorosilane in carbon tetrachloride and well washed with water to impart a water repellent surface to the glass which prevented the drop from spreading. This insert was cut from a concave mirror blank with a radius of curvature of one metre to locate the drop centrally in the cell.

The sessile drop was photographed in silhouette against the screen D through a hole in the side of the cabinet. An accurately ground tip of known external diameter was suspended in the benzene through the lid of the cell as shown so that the magnification of the image of the drop could be calculated and the actual dimensions of the drop found. Photographs were taken at intervals of 3 min., 1, 4, 8, 24, 48 and 96 hours and the resulting plates measured with a Cambridge Universal Measuring

machine which can measure in two dimensions at right angles. This machine is graduated to 0·001 cm. and estimates to 0·0002 cm. may be made without difficulty.

Figure 4 shows the effect of time on the shape of a drop and was formed by superimposing photographs of the same drop taken after 3 minutes and 96 hours. The results obtained for solutions of arabic acid are given in Figure 5 together with the results for the benzene/water interface. Facilities for conditioning and filtering the air in the laboratory were not available and manipulations were carried out as rapidly as possible to avoid extraneous contamination.

On a number of occasions contamination of the benzene occurred which resulted in a marked fall in the interfacial tension over a period of 1 hour and 4 hours as shown in Table III. These results have not been used. Recrystallisation of the benzene removed the contamination and the fall in the interfacial tension with time again followed the curves given in Figure 5. An investigation into the types of substances which can produce this effect will be undertaken later.

FIG. 4. The effect of time on the shape of the sessile drop. Photographs of a drop after 3 minutes and 96 hours have been superimposed to show the flattening of the drop after 96 hours.

Diameter of tip above drops is 0·4048 cm.

The solution contained 0·998 per cent. arabic acid.

TABLE III

SESSILE DROP METHOD: THE EFFECT OF CONTAMINATION IN THE BENZENE ON THE INTERFACIAL TENSION

Solution per cent. w/v of anhydrous arabic acid	Time after formation of sessile drop						
	3 min.	1 hr.	4 hrs.	8 hrs.	24 hrs.	48 hrs.	96 hrs.
0·0984	33·7	15·3	14·4	—	15·3	14·9	15·3
0·500	31·75	16·9	16·1	—	15·8	—	15·2
1·000	32·4	—	17·2	16·7	15·4	14·1	—
5·00	20·1	14·6	13·2	—	11·7	10·9	10·0

DISCUSSION

The ring method has been used for the determination of the surface tension of gum acacia solutions. The method was attempted in the present work using a chainomatic balance as described by Harkins[19] and although the results obtained for the surface tension of water (71·5 dynes/cm.) and of benzene (27·92 dynes/cm.) were in agreement with the

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FIG. 5. Sessile drop method. The effect of time on the interfacial tension of arabic acid solutions and benzene.

× 0·01 per cent. arabic acid. △ 1·0 per cent. arabic acid.
○ 0·1 ,, ,, ,, ,, □ 10·0 ,, ,, ,, ,,
—·— distilled water/benzene interface.

accepted values the interfacial tension for benzene and water was approximately 1·5 dynes/cm. too low. The correction factors of Harkins[19] and the equation of Zuidema and Waters[20] were used to calculate the results and both methods showed good agreement.

The difficulty most probably arises from the ring not being in one plane and parallel to the surface so that the interface broke away from the ring first at one point. This suggests that the liquid/liquid interface is more sensitive to the adjustment of the ring than is the liquid/air surface. Hutchinson[21] experienced similar trouble.

However, as the ring is raised there is a considerable extension of the surface so that the criticisms of the drop-volume method for the determination of the interfacial tension of arabic acid solutions will apply also to the ring method.

From Figure 5 and Table II it is evident that the change in interfacial tension with time is slow but the change shows the same general form by both methods. Since the values obtained by the drop-volume method are inversely related to the rate of formation of the drop and at the slowest rate gives greater values than those obtained by the sessile-drop method it is reasonable to suppose that the surface of the drop growing on the tip does not achieve equilibrium with the bulk of the solution. This is

supported too by the determinations in which the drop was partially formed rapidly and then completed at the slowest rate of growth yielded higher values than if the drop was grown completely at the slowest rate.

The results obtained by Clark and Mann[2] show a linear relationship between the concentration and the interfacial tension, the drop being formed over a comparatively short period of time. A direct relationship was obtained by Matthews[4] using *cyclo*hexane instead of benzene although Matthews' curve had a steeper slope. The determinations being carried out by the drop-volume method in both cases.

If the values which were obtained by the sessile drop method after the drop had aged for 3 minutes are plotted against concentration the relationship is seen to be linear also. This implies that the time allowed for the formation of the drop by Clark and Mann and by Matthews was insufficient for an equilibrium to be established and also insufficient to give an interfacial tension/concentration curve of the right shape. Banerji[3] whilst apparently recognising the problem of attaining equilibrium at the surface by stating that "a fairly long time was taken for the formation of the drops" made no attempt to determine the time necessary and his results for solutions of approximately 4, 2 and 1 per cent. gum acacia showed an almost linear relationship also.

FIG. 6. Sessile drop method. The effect of the concentration of arabic acid on the interfacial pressure between water and benzene.

A static method is therefore essential for the determination of surface or interfacial tension of solutions of such substances which attain equilibrium very slowly and the sessile drop or bubble method seems to be the method of choice. A difficulty arises with the hanging drop method as a drop formed initially which is suitable for measurement may detach itself after the surface has aged for a few hours.

Even after 4 days it was not certain that equilibrium had been reached by the sessile drop method and the interfacial tension obtained was plotted against the reciprocal of the time for each concentration and the curves extrapolated to infinite time. These values were deducted from the interfacial tension of benzene and water obtained in the same way and the resulting value for the surface pressure (π) was plotted against the con-

centratior in Figure 6. In this way any changes occurring in the cell were allowed for and the shape of the curve was unaltered.

From the data on this curve the approximate surface excess (Γ_2^v) has been calculated using the Gibbs equation for concentrated solutions.

$$-\frac{N_1 \cdot d\gamma \cdot N_2}{RT \, dN_2} = \left(\frac{N_1 + N_2 \cdot V_2}{V_1}\right)\Gamma_2^v$$

Where N_1 and N_2 are the mole fractions, V_1 and V_2 are the partial molar volumes of the components water and arabic acid and γ is the interfacial tension. By substituting values for N_1, V_1 and V_2, expressing N_2 in terms of the concentration C_2 and substituting $d\pi$ for $d\gamma$ the approximate expression

$$\frac{1}{RT} \cdot \frac{d\pi}{dC_2} \cdot C_2 = \left(1 + \frac{C_2}{150}\right)\Gamma_2^v$$

is obtained by assuming the solid arabic acid to have a density of 1·5 and a mclecular weight of 300,000.

As there are insufficient number of points on the curve in order to obtain $\frac{d\pi}{dC_2}$ the change in value for sections of the curve has been substituted, denoted by $\Delta\pi$ and ΔC_2 which should give results of the right order, C_2 being the approximate average for the corresponding section.

Concentrations per cent.	$\dfrac{\Delta\pi}{\Delta C_2}$	C_2 per cent. w/v	Γ_2^v moles/cm.^{2}
0–0·01	$\dfrac{3\cdot4}{0\cdot01}$	0·005	$0\cdot69 \times 10^{-10}$
0·01–0·1	$\dfrac{12\cdot1}{0\cdot09}$	0·05	$2\cdot7 \times 10^{-10}$
0·1–1·0	$\dfrac{6\cdot2}{0\cdot9}$	0·5	$1\cdot39 \times 10^{-10}$
1·0–10·0	$\dfrac{0\cdot5}{9}$	5·0	$1\cdot1 \times 10^{-11}$

The initial rapid rise in π and concomittant rise in Γ_2^v up to about 0·1 per cent. arabic acid is probably due to an increased adsorption of arabic acid at the interface. Further increases in concentration above 0·1 per cent. probably results in a rearrangement of the adsorbed layer and subsequently the building up of layers containing a high proportion of arabic acid immediately below the surface will account for the decrease in Γ_2^v above about 0·1 per cent. concentration. For convenience such a structure is referred to here as a "multilayer" and with a hydrophilic substance such as arabic acid it is probable that water molecules enter into its structure. This also offers an explanation of the interfacial tension/time curves given in Figure 5.

A highly concentrated multilayer such as this should exhibit a strong lateral attraction between the molecules and be very viscous or gel-like in character. It was observed that when a drop of the 10 per cent. solution was withdrawn from the cell with a hypodermic syringe after ageing for 96 hours the drop did not shrink uniformly but collapsed without

reducing the area of contact with the glass insert and the surface became wrinkled. This also supports the view that the surface layers have gel-like properties.

From his curve for gum acacia Matthews concluded that a vaporous film was formed at the interface but his curves for dextrin and for apple and citrus pectins were similar to Figure 6 for arabic acid which he interpreted as being due to the formation of a condensed film. In the same paper he mentions that in a preliminary experiment arabic acid also gives a curve of the condensed type implying a difference in behaviour of gum acacia and arabic acid. However, in some preliminary work with the potassium and calcium salts of arabic acid it was found that the shape of the curves are similar to Figures 5 and 6 which suggests that there is no great difference between the adsorbed layers of arabic acid and its simple salts. Further work on the salts of arabic acid is proceeding.

A Comment on the Emulsifying Properties of Gum Acacia

If it may be assumed from the preliminary work on the salts of arabic acid that gum acacia solutions exhibit the same structure as arabic acid at the interface a hypothesis can be given to account for the stability of acacia emulsions which is in agreement with the modern theories.

An interfacial film of the type suggested which has a gel-like structure should possess the necessary physical properties, such as lateral coherence and elasticity, for the stabilisation of an emulsion. In addition, with a strong acid, it may be predicted that the system would exhibit a marked interfacial potential which appears to be necessary for the formation of oil in water emulsions. In attempting to draw an analogy with the theory of emulsions put forward for soaps by Schulman and Cockbain[22] it must be remembered that the soaps having a strong hydrophobic group in the molecule will form a monolayer. The soaps are only effective as emulsifiers if a condensed film is formed and this is achieved by forming a complex at the interface by the addition of an oil souble components and it is-this complex condensed film which has these necessary physical properties for the stabilising of emulsions.

The lowering of the interfacial tension by gum acacia is not so great as that produced by the condensed complex film formed by the soaps and this would account for the greater amount of work necessary to bring about the subdivision of oil in acacia emulsions.

SUMMARY

1. The effect of arabic acid on the interfacial tension between water and benzene has been examined by the drop-volume and sessile drop methods. The change in interfacial tension with time has been found to be very slow and the sessile drop method is the method of choice since no change in volume is involved.

2. The shape of the interfacial tension/time and the interfacial tension/ concentration curves are interpreted as being due to the formation of an adsorbed "multilayer," containing a high concentration of arabic acid, possessing gel-like properties.

3. A film such as this should possess those physical properties, such as coherence, elasticity and surface potential, which would explain the emulsifying properties of gum acacia in solution.

I wish to express my thanks to Mr. R. Fitall for technical assistance.

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DISCUSSION

The paper was presented by THE AUTHOR.

MR. J. H. OAKLEY (London) asked whether the accepted 4:2:1 and 2:2:1 ratios were the most suitable for making a primary emulsion. Were these a compromise, and was there an optimum concentration in the final emulsion? It would be of interest to learn whether the author found much variation in the acacia available on the market, and whether allowance should be made for such variation.

DR. F. WOKES (Kings Langley) referred to the plotting of the results in Figures 5 and 6, and observed that the points in the early part of the curve were very close together and those in the latter part were spread out. He asked whether plotting against the log time or some other scale would have given a more linear curve, more suitable for extrapolation.

MR. VAN ABBÉ (Loughborough) said that he was interested in the property called "lateral coherence," and in the comparison of samples of acacia and gelatin as binding agents. The only method he knew involved the use of the Bloom gelometer, and it would be interesting to know whether the type of measurements carried out by the author was likely to be of use in evaluating coherence.

MR. H. D. C. RAPSON (Dorking) commented on the very long time taken for equilibrium to be reached, and said that there were three possible explanations. The first was the slow rearrangement of the multilayer at

the interface, the second, slow diffusion of the material to the interface, and the third, the final area of contact of the drop with the substrate compared with the initial area. It would be interesting to know whether the last affected the Porter method of calculating the interfacial tension.

DR. G. BROWNLEE (London) asked whether it was true that there were only two parameters to the curve, that is, was there only one rearrangement in the forces which reached equilibrium? Was the 1 per cent. arabic acid solution achieving equilibrium so the curve was flat thereafter, or was it true as the curve suggested that it was not really reaching equilibrium. Was there a new interfacial tension being introduced by the benzene?

MR. E. SHOTTON, in reply, said that he had not examined other samples of acacia, therefore he could say little new on the question of variation. It would be observed from the curves that the fall of interfacial tension of the 10 per cent. solution was much more rapid than in the weaker solutions. In the 4:2:1 ratio a highly concentrated solution was being used which should have sufficient lowering of interfacial tension. He had tried log plots but had discarded them because they did not produce straight lines and it appeared better to give the arithmetic plots. A reciprocal of the time against interfacial tension was plotted to obtain a figure for infinite time. With regard to the term lateral coherence, its use here was confined to the lateral coherence in the surfacelayer. It was the lateral coherence of the molecules in the surfacelayer which accounted for the physical properties. Gelometers and other instruments might be of some use in the bulk phase but not in the interfaces. It seemed at first that equilibrium was not established even with the sessile drop method, but one could never be sure that some contamination might not have got into the cell. In his view rearrangement did take place but it should not take so long. Matthews had suggested that the rate of diffusion which had been published for acacia should not account for a three minutes time-lag in achieving equilibrium in his case. Mr. Shotton's own conclusion was that it must be something else and that is why he referred to it as a multilayer.

DISSOCIATION CONSTANTS OF SOME COMPOUNDS RELATED TO LYSERGIC ACID

(β-Dimethylaminopropionic Acid, Dihydroarecaidine, Ecgonine and their Derivatives

By J. Chilton and J. B. Stenlake

From the School of Pharmacy, The Royal Technical College, Glasgow

It has been shown[1] that the dissociation constants of the dihydrolysergic acids are in agreement with the view that those dihydrolysergic acids in which the $C_{(8)}$ carboxyl is remote (equatorial) from $N_{(6)}$ are weaker bases than those in which the $C_{(8)}$ carboxyl is axial. Because of the small amount of material available it was necessary to measure these dissociation constants by the method of Craig, Shedlovsky, Gould and Jacobs,[2] in which the pH value of a solution half neutralised by the addition of the calculated amount of carbonate-free sodium hydroxide was recorded. Since this method is open to certain objections, the experimental work now described was undertaken to confirm the above conclusions by potentiometric determination of dissociation constants of analagous, but more readily available materials of known structure. Whilst this work was in progress, Stoll, Petrzilka, Rutschmann, Hofmann and Günthard[3] have published dissociation constants for the dihydrolysergic and dihydro-norlysergic acids, and other evidence which further substantiates our conclusions.

EXPERIMENTAL

Titrations were carried out in a specially constructed micro-cell thermo-statically controlled at 25° C., using a Doran alk-acid glass electrode in conjunction with a Cambridge Direct Reading pH meter. An Agla micrometer syringe was used as a burette. Carbon dioxide-free nitrogen was bubbled through the solution, which was stirred by means of a magnetic stirrer.

Solutions of 0·02 millimoles of the hydrochlorides or hydrobromides of the pure substances in carbon dioxide-free double-distilled water were titrated potentiometrically with 0·05N carbonate-free potassium hydroxide, the volume of solvent being calculated to yield a 0·005M solution at half-neutrality. The potassium hydroxide solution was obtained by the method of Steinbach and Freiser,[4] in which 0·05M potassium chloride solution is passed through a column of ion exchange resin IRA 400. The eluant standard alkali was fed directly to the micrometer syringe.

An experimentally determined correction for solvent effect was applied, based on the methods of Tague[5] and Harris[6] in the following way. A blank titration was carried out under the same conditions used in the test, whereby the material under test was replaced by an equivalent quantity of carbon dioxide-free hydrochloric acid. The volumes of 0·05N potassium hydroxide solution required to raise the pH value in the blank determination from 7·0 to various pre-selected values were determined accurately from a mean of eight titrations. From this it was possible to

determine solvent corrections for any desired *p*H value by interpolation. These corrections were subtracted from the observed titres in the test determinations. The blank determination was repeated for each fresh batch of distilled water.

Dissociation constants were calculated from three points on each of two or more titration curves. Values for all substances quoted were reproducible within ± 0.05 units (ecgonine ± 0.08 unit).

Preparation of Materials

Ethyl β-dimethylaminopropionate Hydrochloride.—Ethyl β-dimethyl-aminopropionate, prepared by the method of Adamson,[7] was dissolved in dry ether and treated with excess of dry hydrogen chloride. The product when recrystallised from ethanol-ether gave ethyl β-dimethyl-aminopropionate hydrochloride, m.pt. 134° C.

β-Dimethylaminopropionic Acid Hydrochloride.—Ethyl β-dimethyl-aminopropionate (1 g.) was refluxed for 3 hours with concentrated hydrochloric acid. The solution when evaporated to dryness and recrystallised from ethanol gave the required product, m.pt. 191° C. Gresham *et al.*[8] give m.pt. 191–192° C.

Arecoline hydrobromide.—Commercial sample recrystallised to m.pt. 172° C. The British Pharmaceutical Codex 1949 gives m.pt. 168–175° C.

Arecaidine Hydrochloride.—Arecoline was refluxed with concentrated hydrochloric acid for 3 hours. The solution, evaporated to dryness and recrystallised from ethanol-ether, gave arecaidine hydrochloride, m.pt. 263° C. (decomp.). Wohl and Johnson[9] give 262–263° C.

Dihydroarecoline Hydrobromide.—Arecoline hydrobromide (1 g.) in methanol (20 ml.) was hydrogerated at atmospheric pressure in the presence of a platinum catalyst. Hydrogenation was complete in 4 hours. The solution, after filtration, was evaporated and the residue crystallised from a mixture of methanol and ethyl acetate (1 : 1) to give dihydroare-coline hydrobromide, m.pt. 114° C. (after drying *in vacuo* over phosphorus pentoxide). Preobrazhenskii and Fisher[10] give m.pt. 115–116° C. The product was highly deliquescent.

Dihydroarecaidine Hydrochloride.—Arecaidine hydrochloride (0·3 g.) in ethanol (10 ml.) was hydrogenated at atmospheric pressure in the presence of a platinum catalyst. The solution, after filtration and concentration, yielded dihydroarecaidine hydrochloride, m.pt. 173–175° C. from ethanol-ether. Winterstein and Weinhagen[11] give m.pt. 175° C.

Ecgonine Hydrochloride.—Ecgonine $\{[\alpha]_D^{18}-44 \cdot 15°$ $(c = 4 \cdot 3$ in water); Henry[12] quotes $[\alpha]_D-45 \cdot 4°\}$ was dissolved in concentrated hydrochloric acid and the solution allowed to evaporate spontaneously to dryness *in vacuo* over potassium hydroxide. The resulting ecgonine hydrochloride had m.pt. 252° C. (decomp.). Liebermann[13] gives m.pt. 246° C.

Ecgonine Methyl Ester Hydrochloride.—Prepared by the method of Einhorn and Klein[14] m.pt. 216° C. (decomp.). Einhorn and Klein give m.pt. 212° C. (decomp.).

ψ-Ecgonine Hydrochloride.—Prepared by the method of Einhorn and Marquart,[15] m.pt. 236° C. Einhorn and Marquart[16] give 236° C.

ψ-Ecgonine Methyl Ester Hydrochloride.—Crude *ψ*-ecgonine hydro-chloride was dissolved in saturated methanolic hydrochloric acid and the solution refluxed for one hour. The solution was evaporated to dryness, the residue converted to base with aqueous sodium carbonate (10 per cent.) and the base extracted with ether. The base in the dry ether was treated with dry hydrogen chloride and the precipitated hydrochloride recrystallised from ethanol to give *ψ*-ecgonine methyl ester hydrocyloride, m.pt. 197° C. Mixed m.pt. with the hydrochloride from authentic *ψ*-ecgonine methyl ester, 197° C.

DISCUSSION

Dihydroarecaidine

Neuberger,[17] using ΔpK (pK'a_2 amino-acid–pK'a amino-acid ester) as a function of the distance between the centre of the negative charge of the carboxyl and the dissociating hydrogen of the $-NH^+$ group, established that aliphatic amino-acids exist in solution in the zig-zag open chain form (I) in which the two charged groups are remote, a conclusion which was supported by measurements of dipole distances.[18,19] By analogy it would appear therefore that the preferred conformation of dihydroarecaidine in solution should be that in which the two charged groups are remote as in (II) (COOH equatorial) rather than as in III (COOH axial). That such a conformation represents the preferred structure for dihydroarecaidine may also be deduced theoretically by analogy with similar *cyclo*hexane systems on the grounds that a single substituent will adopt the more stable equatorial configuration (the orientation of the N-methyl group may be ignored since tertiary nitrogen derivatives are not resolvable into optical enantiomorphs). On the other hand, such conformational analogies are of doubtful value when charged groups are involved, since it is known that both steric and electrical repulsion between ring substituents are factors which can affect confor-mational stability.[20]

(I)

(II)   (III)
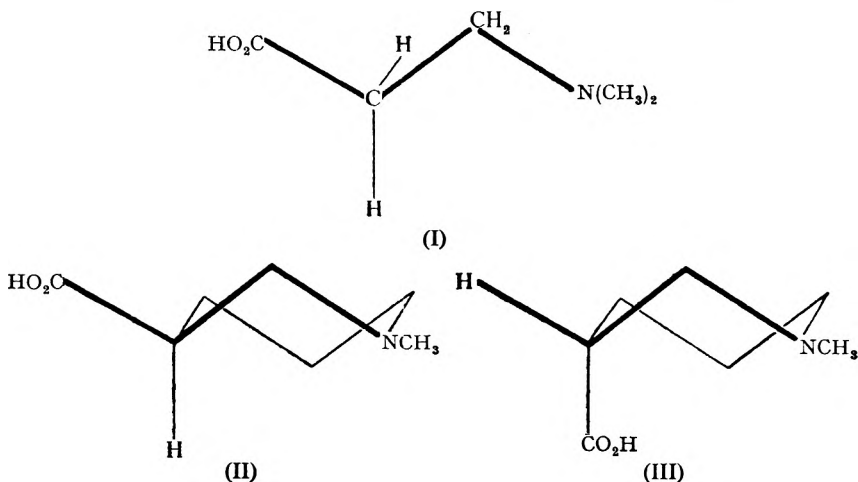
Introduction of a carboxyl group into the molecule of an aliphatic amine is base-weakening. The effect is influenced by the position of the carboxyl group in relation to the amino group.[2] Examination of molecular models reveals that there is identical chain and spacial separation of $-CO_2^-$ and $>NMeH^+$ groups in β-dimethylaminopropionic acid (I) and dihydroarecaidine provided the latter adopts the conformation (II). Consequently we should expect these two substances to have dissociation constants of the same order.

We have measured the second acid dissociation constants of these two amino-acids by potentiometric titration of their hydrochlorides, and the results are recorded in Table I. An experimentally determined blank was

TABLE I

Amino-acid							Acid pK'a at 25° C.	Ester pK'a at 25° C.	Δ pKa
β-Dimethylaminopropionic acid	..	..	..	..	..	..	9·85	8·60 (Et)	1·25
Dihydroarecaidine	..	..	..	..	..	..	9·70	8·45 (Me)	1·25
Arecaidine ..	..	..	..	..	..	..	9·07	7·70 (Me)	1·37
Ecgonine ..	..	..	..	..	..	..	10·91 11·15*	9·22 (Me)	1·69
ψ-Ecgonine	..	..	..	..	..	..	9·70	8·21 (Me)	1·49
Benzoylecgonine ..	..	..	..	..	..	..	11·80*	8·65 (Me)* 8·80 (Me)	

* Kolthoff.[17]

used to correct for solvent effect and applied as described by Tague[5] and Harris.[6] Second acid dissociation constants only have been measured, since these are more clearly indicative of the structural features concerned. Our measurements show that the pK' of β-dimethylaminopropionic acid (9·85) is distinctly lower than that of the corresponding primary amino-acid, β-alanine (10·36).[21] This is in agreement with the observations of Bredig[22] and Hall and Sprinkle[23] who showed that whereas the introduction of a single methyl group into a primary amine gives a small increase of pK value, introduction of a second methyl group to form a tertiary amine produces a marked fall in pK value. A similar lowering of pK value in passing from secondary to tertiary base has been observed with corresponding pairs of dihydronorlysergic and dihydrolysergic acids.[3] The concordance of pK' values for β-dimethylaminopropionic acid (9·85) and dihydroarecaidine (9·70) clearly demonstrates that the steric relationship of basic and acidic groups is the same (at least in solution) in both amino-acids. This not only supports the conformational assignment (II) for dihydroarecaidine, but also the validity of the general principles of conformational analysis to compounds of this type.

Ecgonine and ψ-Ecgonine

Findlay[24,25,26] assigned the structures (IV) and (V) to ecgonine and ψ-ecgonine respectively, and these conformational assignments have since been confirmed by Fodor, Kovács and Weisz.[27,28] We now report second acid dissociation constants for these two substances (Table I). The expected base-strengthening effect when the carboxyl group is in close spacial proximity to the ring-N is observed in the much higher value

(IV)   (V)
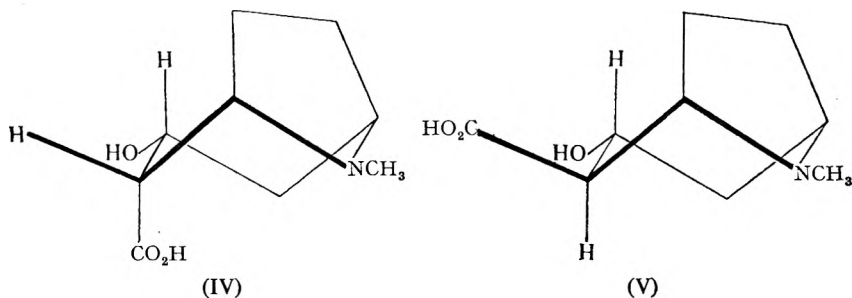
for ecgonine (IV; pK' 10·91) than in ψ-ecgonine (V; pK' 9·70). These results therefore provide a parallel which supports the configurational assignments proposed for the $C_{(8)}$ carboxyl in the dihydrolysergic and lysergic acids.[1] Whilst the agreement between the pK' for ψ-ecgonine (9·70) and dihydroarecaidine (9·70) would appear to provide further evidence for the assignment of an equatorial carboxyl in ψ-ecgonine (V), this agreement must be regarded as fortuitous, as it takes no account of either the methylene bridge or the C^4 hydroxyl in the latter substance. We have therefore examined differences in pK' value between acid-ester pairs in the two series.

(VI)   (VII)   (VIII)
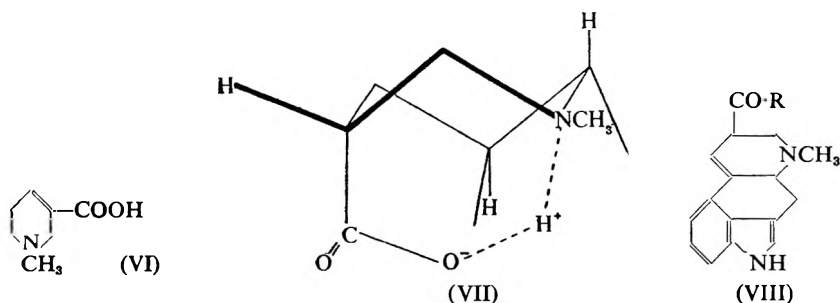
Acid-Ester Differences

It was established by Neuberger[17] that ΔpK (pK'a_2 amino-acid–pK' a amino-acid ester) provides a measure of the distance between the two charged groups of the amino-acid zwitterion, ΔpK increasing with increasing proximity of the groups. The assumptions made do not permit the use of dissociation constants for accurate measurements of such distances, but this in no way invalidates the use of ΔpK in a qualitative sense. The data in Table I shows that there is reasonable correlation between ΔpK values for dihydroarecaidine (II; ΔpK 1·25), arecaidine (VI; ΔpK 1·37) and ψ-ecgonine (V; ΔpK 1·49) in all of which the carboxyl is equatorial. β-Dimethylaminopropionic acid, in which there is a comparable spacial relationship between the -COOH and $\rangle$NMe groups, also has ΔpK 1·25. On the other hand, ΔpK for ecgonine (IV; ΔpK 1·69), in which the carboxyl is axial, is significantly greater.

The influence of structural features such as the ethylenic bond of

arecaidine is seen to be largely cancelled out when ΔpK values are considered. Both arecaidine and its methyl ester are weaker bases than the corresponding dihydro compounds by about 0·7 pK units, yet their ΔpK values are very similar (see Table I). This concordance of ΔpK values demonstrates that this cyclic double bond does not materially alter the space relationship of the carboxyl and ring-nitrogen groups, and supports the view that such unsaturated rings adopt the *cyclo*hexene-like semi-chair conformation.

The effect of the hydroxyl groups in the ecgonine series is obviously more complex. ΔpK for benzoylecgonine (calculated from measurements by Kolthoff[29]) is 3·15 compared with ΔpK 1·69 for ecgonine. This large difference could be ascribed to hydrogen bond formation between the adjacent hydroxyl and carboxyl groups in ecgonine and its methyl ester. Such a hydrogen bond would have opposite effects in acid and ester, and it is observed that whereas the pK' value of ecgonine (10·91) is less than that of benzoylecgonine (11·8), the pK' value of ecgonine methyl ester (9·22) is greater than that of cocaine (8·80).

Solvent Effects on the Dissociation of the Dihydrolysergic Acids

Michaelis and Mizutani[30] and Speakman[31] have shown that a decrease in pK'a value in changing from a mixed organic-aqueous solvent to water is typical of enols and acids. Conversely, an increase in pK'a value for a similar solvent change is consistent with the attachment of a proton to nitrogen in a base-conjugate acid. Comparison of the dissociation constants of the dihydrolysergic acids obtained in 80 per cent. cellosolve by Stoll *et al.*[3] with those obtained in aqueous solution by Craig *et al.*[2] and Stenlake[1] (Table II) reveals that solvent effects are appreciable.

TABLE II

Amino-acid				80 per cent.[3] cellosolve	Water[2] 24° C.	Water[1] 20° C.	Shift in pK'
Dihydrolysergic acid I.	pK'a₁	..	..	4·85	3·57	—	−1·28
	pK'a₂	..	..	7·85	8·45	8·50	+0·65
Dihydro*iso*lysergic acid II.	pK'a₁	..	..	4·97	3·60	—	−1·37
	pK'a₂	..	..	8·38	8·57	8·52	+0·14
Dihydro*iso*lysergic acid I.	pK'a₂	..	..	9·25	—	8·91	−0·34

Dihydrolysergic acid-I and dihydro*iso*lysergic acid-II (in which the COOH is remote from $N_{(6)}$) behave as most amino-acids in that the pK' value shows a negative shift on changing solvent from 80 per cent. cellosolve to water for the first dissociation constant (proton attached to carboxyl) while there is a positive shift for the second dissociation constant (proton attached to nitrogen). On the other hand the behaviour of dihydro-*iso*lysergic acid-I is anomalous in that its second dissociation constant shows a negative shift on changing solvent from 80 per cent. cellosolve to water. Such an effect is consistent with the axial orientation of the carboxyl group in dihydro*iso*lysergic acid-I which favours chelation of the

proton between the carboxylate ion and $N_{(6)}$ as in (VII), thus permitting behaviour typical of carboxylic acid ionisation.

Conclusion

The evidence cited above and elsewhere indicates that lysergic acid (VIII, R=OH) has the $C_{(8)}$ carboxyl remote from $N_{(6)}$ (equatorial carboxyl). It would appear that the physiologically active series of ergot alkaloids, which are related to lysergic acid, have a similar configuration. We are now investigating synthetic model substances based on this essential structural feature.

SUMMARY

1. Dissociation constants have been recorded for a number of substances structurally related to lysergic acid.

2. The dissociation constant of dihydroarecaidine is consistent with a chair piperidine conformation, with the carboxyl group equatorial.

3. ΔpK values between acid and ester for dihydroarecaidine, arecaidine, ψ-ecgonine, and β-dimethylaminopropionic acid are comparable and significantly smaller than for ecgonine, indicating that in stereoisomeric pairs of amino-acids of this type, the isomer in which the basic and acidic groups are remote is a weaker base than that in which the groups are in propinquity. These results also indicate that the double bond in arecaidine does not materially alter the conformation of the ring as compared with its dihydro derivative.

We wish to thank Messrs. T. and H. Smith, Ltd., for a gift of ecgonine.

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DISCUSSION

The paper was presented by DR. J. B. STENLAKE.

DR. F. HARTLEY (London) said that until there was more detailed knowledge of stereochemical structures no more than partial progress would be made in any attempt to correlate structure with action. In applying the principles which Barton had so successfully applied in steroids and triterpenes the authors were doing a very worthwhile job. Inevitably in order to sort out whether a group was axial or equatorial, Dr. Stenlake had made studies of the dissociation constants of closely related substances.

DR. L. SAUNDERS (London) said that the author had stirred his solution with a magnetic stirrer which he thought unwise when using a high resistance potentiometer such as the pH meter. He suggested that a stream of nitrogen was a better method. He wondered whether it would be more satisfactory if, instead of taking pH at half neutralisation and determining dissociation constants direct from titration curves, one used more precise techniques.

DR. A. H. BECKETT (London) said that his own results using nitrogen stirring agreed with the authors for the dissociation constants of ecgonine and pseudoecgonine. He felt that there were a number of complexities, for instance, in the case of tropine and pseudotropine one would expect that when the OH group was near the nitrogen the cation would be stabilised by hydrogen bonding and give a stronger base. However, that was not the case. He had reduced ecgonine and pseudoecgonine to primary alcohols, and where the OH group of the primary alcohol was near the nitrogen there was a completely predictable effect in terms of hydrogen bonding.

MR. H. D. C. RAPSON (Dorking) said he had experienced no trouble as a result of using a magnetic stirrer with a pH meter.

DR. J. B. STENLAKE, in reply, said that he was aware the magnetic stirrer affected the pH meter, but before making the measurements care was taken to switch it off. The danger of stirring with nitrogen was that the nitrogen could be contaminated with carbon dioxide. Owing to the small amount of material sometimes available it was necessary to effect a compromise by using a reasonably accurate method giving useful results. The question of salt effect on concentrations had been investigated. On the question of interference from other groupings, there were two opinions as to the correct interpretation of the dissociation constant of tropine and pseudotropine. He drew attention to the measurements by Cavalieri on adenylic and cytidylic acids which paralleled his own results with lysergic acid.

DIFFUSION MEASUREMENTS IN THE STUDY OF THE STRUCTURE OF SOAP SOLUTIONS

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INTRODUCTION

THE importance of soaps in the preparation of pharmaceuticals is widely realised, yet even to-day uncertainty exists as to the detailed structure of a soap in solution. This arises out of the fact that a soap can exist in the same solution in two forms which are in equilibrium, a simple ionic form and a colloidally dispersed micellar form. Experimental methods for investigating this association of soaps into micelles have varied widely; conductivity, solubilisation studies, light scattering, viscosity and other physical properties have frequently been reported. Many of these measurements involve the application of an external force, or additive to the soap solution. Such application of an external agent is liable to vitiate the results as far as calculation of the size, shape, number or other structure of the particles in the pure soap solution.

In the present studies, anionic soaps and other association colloids have been investigated by means of the recently revived Gouy interference method for studying diffusion[1,2,3]. This is a free boundary method, briefly described below. Other diffusion measurements using soap solutions have involved tagging with dyes[4], diffusion through porous discs[5,6] or the use of the Lamm scale-line method[7,8]. The Gouy interference method provides a sensitive, accurate means of determining diffusion coefficients without extraneous additives and without the limitations imposed by the use of glass discs or porous plates. Sodium dodecyl sulphate (NaDS) has been used here as an example of the type of investigation that has been attempted.

EXPERIMENTAL METHODS

Materials. Sodium dodecyl sulphate was prepared according to the method described by Dreger *et al.*[9], as modified by Burick[10]. The sulphur content of the pure material (two samples) after three re-crystallisations from water at 5° C. was 11·07 and 11·15 per cent. (theoretical 11·12). The sulphur content was determined by hydrolysis with 50 per cent. hydrochloric acid, ether extraction and precipitation with barium chloride. Potassium laurate was prepared by the neutralisation with carbonate-free standard alkali of pure lauric acid (Eastman Kodak) which had an equivalent weight of 200·5, determined potentiometrically, and a melting point of 42 to 43° C. Water used was double distilled from potassium permanganate. All sols were allowed to age for 24 hours before use.

Diffusion coefficients. The apparatus (see Fig. 1) consists of a two metre optical bench mounted on a steel beam supported on concrete pillars embedded in earth beneath a basement laboratory floor. A water cooled
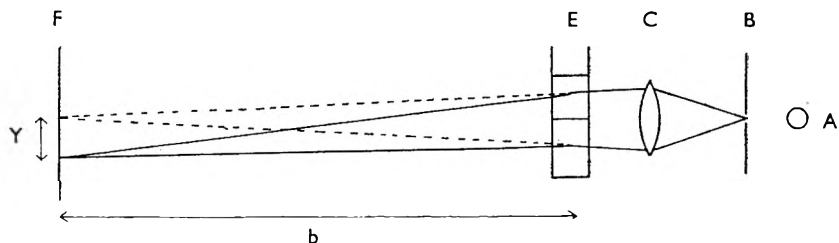
FIG. 1. The optical system of the Gouy interference diffusiometer.

A. Mercury vapour lamp fitted with filters (5461 A°).
B. Spectrometer slit.
C. Lens.
E. Cell.
F. Photographic plate.

Y. Displacement of the Gouy minima below the optic axis.
b. Optical distance between the centre of the cell and the photographic plate.

mercury vapour lamp, A, fitted with suitable filters to isolate monochromatic light (5461 Å) illuminates a horizontal spectrometer slit, B. A precision camera lens, C (Ross Express $f2.9$) of 13·5 cm. focal length focusses an image of the slit through the thermostat fitted with circular optically flat ($\lambda/2$) windows, on to a photographic plate, F, at the far end of the bench. The thermostat is supplied with constant temperature water from a larger tank, the supply being so arranged as to avoid any vibration of the thermostat.

The nickel coated brass cell, E, fitted with two optically flat ($\lambda/2$) windows, fits inside the thermostat. A 50 μ slit through which the two liquids are drawn to form the boundary is placed such that it lies exactly on the optic axis of the apparatus. The whole of the interior of the cell is coated with silicone by the hydrolysis of methylchlorosilane. The two liquids are introduced into the cell by means of motor driven syringes whose glass needles reach to the top of the cell windows. Mixing of the two solutions during boundary formation is prevented by pre-heating the solutions and by continually withdrawing the liquids through the slit. When sufficient solution has been introduced into the cell, the syringes are removed, the whole allowed to come to temperature equilibrium and a sharp boundary is formed by rapid flowing out through the slit and abruptly stopping flow. That a very sharp boundary is formed initially by this method is confirmed by the fact that the observed diffusion coefficients do not vary with time as has been previously reported in some cases. Longsworth[1] introduced an expression Δt which represented the time which an infinitely sharp boundary would take to reach the state of the boundary existing when flow was stopped. Since no Δt value was obtained in any of the measurements reported here, it is valid to assume that an extremely sharp boundary exists when flow is stopped. The boundary starts to spread by diffusion and the refractive index gradients produced cause a set of interference fringes to appear on the photographic plate. A very fast emulsion plate (Kodak P 2000) has been used to record the interference patterns after timed intervals.

After diffusion has spread beyond the cell windows, the cell is washed

out *in situ* and filled with distilled water. After 30 minutes, when temperature equilibrium is reached, a pair of stops is lowered in front of the cell window and a photograph of the interference pattern produced by the stops is recorded. This record enables the optic axis of the apparatus to be located.

The diffusion coefficient D is calculated by means of the method described by Kegeles and Gosting[11] and Gosting and Onsager[12]. The calculation is based on the determination of C_t which is obtained from Y, the displacement of each minimum in the Gouy pattern below the optic axis in cm. C_t is the ratio of the observed displacement of a minimum to the theoretical displacement. If the diffusing substance is homogeneous, C_t is constant for the outer minima of a given pattern (see Table I).

TABLE I

EXAMPLE OF THE CALCULATION OF C_t, THE DIFFUSION OF 64 MILLIMOLES/1. OF NaDS INTO 50 MILLIMOLES/1.
$t = 1805$ SECONDS

Gouy fringe number* j	Observed displacement below optic axis Y(cm.)	Theoretical displacement† $e^{-(z)^2}$	Ratio of observed to theoretical displacement C_t
1	0·4574	0·771	0·593
2	0·4125	0·693	0·595
3	0·3714	0·627	0·592
4	0·3361	0·565	0·595
5	0·3033	0·510	0·595

Mean $C_t = 0.594$.

* Fringe numbers are counted by giving the outermost minimum in the pattern the number zero.

† The theoretical displacement, $e^{-(z)^2}$ is calculated from j and j_m (the difference in optical path length. between the two liquids in the cell in wavelengths of light) by the theory of Kegeles and Gosting[11]. The maximum value of $e^{-(z)^2}$ for any pattern is unity.

D can be found from C_t by the application of Longsworth's[1] equation :—

$$D = \frac{b^2 \cdot j_m^2 \cdot \lambda^2}{4\pi \cdot C_t^2 \cdot t}$$

where j_m is the difference in optical path length between the two liquids in the cell in wavelengths of light and is determined from a count of the number of minima in the Gouy pattern. b is a constant for the apparatus and is the optical distance between the centre of the cell and the emulsion of the photographic plate; t is the time in seconds after flow has been stopped, at which the photograph was taken and λ is the wavelength of light in cm. The variation of diffusion coefficient of NaDS with concentration is illustrated in Figure 2 which shows Gouy patterns taken at the same time (1800 seconds) for different mean concentrations ($\bar{c}$) of NaDS. Δc, the difference between the lower and upper layer concentrations has been kept constant at 14 millimoles/1. The numbers below the Gouy patterns correspond to the numbers used in Table III.

If C_t is not constant, then the material diffusing is heterogeneous and another method of calculating D is applied. This method[13] is based on a measurement of the relative Gouy fringe deviations from Gaussian positions.
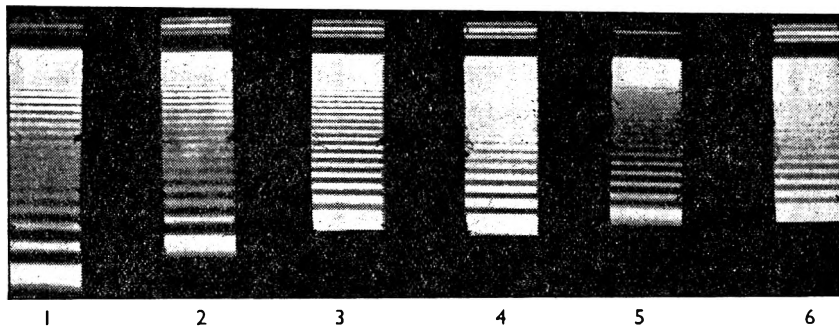
FIG 2. Gouy patterns for the semi-differential diffusion of sodium dodecyl sulphate in water, showing the effect of variation of mean concentration on diffusion coefficient. Numbers correspond to those in Table III. Intensity minima are dark. t = 1800 seconds.

RESULTS

The terminology of Stigter *et al.*[6] has been used throughout this communication, i.e., integral diffusion coefficients are measured by diffusing a solution into water, semi-differential coefficients by diffusing a solution of concentration c_a into c_b (where c_a is greater than c_b and c_b is greater than zero) and differential diffusion coefficient, the value of D when c_a and c_b merge, i.e., when Δc tends to zero. All diffusion coefficients are expressed in cm.²/sec. at 25° C., concentrations in millimoles/l. Diffusion coefficients are the average values determined from at least three Gouy patterns.

The pattern produced by the diffusion of 10 millimoles/l. of NaDS into water, when calculated gave an anomalous result, D′ quoted for this concentration in Table II is derived only from the penultimate minimum and not from several of the outer minima as is the case with the other values of D given. This anomaly is attributed to micelle formation which takes place at about this concentration.

TABLE II

INTEGRAL DIFFUSION COEFFICIENTS FOR SODIUM DODECYL SULPHATE

Concentration	j_m	$10^6 D_a$	$10^6 D′$
5	8·08	—	6·48
7	11·05	—	6·56
8	12·27	—	6·54
9	14·62	—	6·43
10	15·58	—	6·43
15	22·03	1·94	4·58
20	30·49	1·06	3·80
50	79·02	1·59	2·76

D_a = Height-area average diffusion coefficient.
$D′$ = Weight average diffusion coefficient.

Above the critical micelle concentration (CMC) the diffusion coefficient decreased rapidly with concentration. The intensities of the fringes in patterns obtained with concentrations of 15, 20 and 50 millimoles/l., of NaDS diffusing into water were anomalous. This was due to heterogeneity in the diffusing solution caused by the presence of soap micelles. The values of D given for these solutions in Table II have been calculated by Akeley and Gosting's[13] method.

Semi-differential diffusion coefficients in which the upper solution was of concentration greater than the CMC gave homogeneous diffusion patterns in which C_t was constant. The results of these measurements are shown in Table III.

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TABLE III

SEMI-DIFFERENTIAL DIFFUSION COEFFICIENTS FOR SODIUM DODECYL SULPHATE

Number*	Lower layer conc. c_a	Upper layer conc. c_b	Mean conc. $\bar{c}$	j_m	$10^6 D$
1	24	10	17	20·94	1·51
2	34	20	27	20·37	2·26
3	54	40	47	20·82	3·21
4	64	50	57	20·80	3·27
5	77	63	70	20·70	3·36
6	100	86	93	20·38	3·46

* These numbers correspond to the number of the Gouy pattern in Fig. 2.

Integral diffusion coefficient measurements therefore show that the diffusion coefficient decreases rapidly with increasing concentration above the CMC. However, semi-differential diffusion coefficient measurements show an increase of diffusion coefficient with increasing concentration to an almost constant value above 60 millimoles/l. There must therefore be a minimum in the overall diffusion coefficient/concentration (D/c) curve. Such a minimum has already been shown to exist in the case of potassium laurate[14]. The minimum has been found for NaDS and is very close to the CMC. The minimum was determined by differential diffusion coefficient measurements, these results are given in Table IV together with some differential diffusion coefficients for potassium laurate.

TABLE IV

DIFFERENTIAL DIFFUSION COEFFICIENTS FOR NaDS AND POTASSIUM LAURATE

Lower layer conc. c_a	Upper layer conc. c_b	Δc	j_m	$10^6 D$
Sodium dodecyl sulphate				
20	10	10	13·39	1·08
15	10	5	8·71	0·97
Potassium laurate				
250	210	40	62·87	4·56
240	220	20	30·34	4·13
235	225	10	14·86	3·55
232·5	227·5	5	7·50	3·26

The differential diffusion coefficient of potassium laurate at the minimum on the D/c curve has previously been reported[14]. In the case of NaDS, the differential diffusion coefficient cannot be taken any further than the results quoted in Table IV. This is due to the close proximity of this minimum to the CMC thus preventing any variation of Δc around the constant mean concentration at the minimum.

Theoretical diffusion coefficients have been determined for NaDS in dilute solution by the following calculation. As Δc is decreased, the differential diffusion coefficient of the soap is reached. Taking the lowest value of $9·7 \times 10^{-7}$ as being the true diffusion coefficient of micelles, a value of n the mean aggregation number has been determined by applying the Stokes-Einstein equation[15]:—

$$\text{At } 25° \text{ C., molecular volume of micelle} = \left[\frac{33·06 \times 10^{-6}}{9·7 \times 10^{-7}}\right]^3 \quad (1)$$

then $n = \dfrac{\text{molecular volume of micelle}}{\text{molecular volume of NaDS}} = 156.5$ .. .. (2)

This equation can be applied since as Δc tends to zero, effects due to any electrical potential difference between the two solutions are eliminated. For a solution containing two components, the weight average diffusion coefficient D' can be defined as follows[16]:—

$D' = \alpha_1 D_1 + \alpha_2 D_2$ .. .. (3)

where D_1, $D_2 =$ the diffusion coefficients of components 1 and 2, α_1, α_2 are the refractive index fractions of components 1 and 2.

Then $D' = (1 - \alpha_2)D_1 + \alpha_2 D_2$ .. .. (4)

Taking the results obtained for D', D_1 as the diffusion coefficient of simple soap and D_2 as the diffusion coefficient of micelles, α_2 can be evaluated. From a knowledge of the refractive index increment per unit concentration of simple and micellar forms, which can be derived from experimental values of j_m, α_2 can be converted to x, a concentration fraction. Assuming that all the NaDS in solution below the CMC is in the simple form, then values of c_1 and c_2, the concentrations of simple ions and micelles respectively, are found from x.

In this theoretical interpretation, the law of mass action without activity coefficients has been applied to the equilibrium between simple ions and micelles:—

$\log c_2 - n \log c_1 = \text{constant}$ .. .. (5)

This constant can now be determined since all of the other terms in equation (5) are known. From this basic equation, a table of c_1 and c_2 values can be set up as in Table V.

By means of this table, the theoretical diffusion coefficients for experiments carried out with solutions below 24 millimoles/l. were calculated using a reverse process to the one outlined above for finding the constant in equation (5). These theoretical values are compared with the experimental values in Table VI.

TABLE V

THE RELATIONSHIP OF C_1 AND C_2 FOR NaDS BASED ON THE EQUATION:

$\log c_2 - 156.5 \log c_1 = -155.801$

c_1	c_2	$c_1 + c_2$	x
9·80	0·208	10·008	0·021
9·90	1·02	10·92	0·094
10·00	5·00	15·00	0·333
10·03	7·98	18·01	0·443
10·04	9·23	19·27	0·479
10·05	10·67	20·72	0·515
10·06	12·76	22·82	0·559
10·07	14·76	24·83	0·594

TABEL VI

COMPARISON OF THEORETICAL AND EXPERIMENTAL DIFFUSION COEFFICIENTS FOR NaDS

Solution concentrations	Theoretical $10^6 D'$	Experimental $10^6 D'$
15/ 0	4·75	4·58
20/ 0	3·87	3·80
20/10	1·12	1·08

DISCUSSION

A minimum in the D/c curve for association colloids has previously been reported for some α-sulphonic acids[17] and for potassium laurate[14]. In both of these cases and in the present work, diffusion measurements have been made in the absence of any added electrolyte. Previous measurements of the diffusion coefficient of NaDS in the presence of added electrolyte show no minimum in the D/c curve, e.g., Miller and Anderson[18] in the presence of lithium ions, Hakala[19] in the presence of excess sodium chloride and Granath[20] in the presence of sodium carbonate and sodium bromide. The only other reported measurements of the diffusion coefficient of NaDS are those of Stigter et al.[6] who measured micellar self-diffusion both in the presence and absence of added electrolyte. These authors have used a porous glass disc apparatus and measured micellar self-diffusion by tagging the micelles with a dye, Orange OT. In view of the important effect of small additions of both polar and non-polar substances on the structure of soap solutions, we consider that the diffusion coefficients reported by Stigter et al.[6] are not the diffusion coefficients of pure NaDS. It seems difficult to believe that the porous glass discs used by these workers have no effect on the diffusion rate, either by adsorption of the surface active soap or by the modification of boundary potentials. Hartley and Runnicles[5] have pointed out that a free boundary method is essential for anionic soaps since these substances tend to build up a structure in porous discs.

Hartley and Robinson[21] were the first authors to suggest that only diffusion coefficient measurements carried out in the presence of a swamping amount of electrolyte were of use in the estimation of micelle size. However, it has been shown by Granath[22] that the diffusion coefficient of anionic soaps varies widely with the concentration of added cation, and also with the type of added anion even if the ionic strength is kept constant. Since this variation has such a large effect on D we have completely avoided the addition of electrolytes to the soap solutions and have only measured diffusion coefficients in salt-free media. We consider that the use of differential diffusion measurements in which Δc tends to zero and electrical effects also tend to zero, gives an estimate of the true micellar diffusion rate, and not an artificial rate in the presence of an excess of extraneous additive.

The value of the CMC taken here is between 9 and 10 millimoles/l. The authors are of the opinion that the CMC is not an exact concentration but is a small concentration range. It has often been found in the past that the CMC varies quite considerably with external factors such as temperature, added substances and particularly with the method of determination[23]. The value found here for NaDS is in general agreement with previously determined values at this temperature (25° C.), e.g., 9 millimoles/l. from conductivity measurements[24] and 8·1 millimoles/l. from conductivity, solubilisation and light scattering measurements[25].

Ekwall[26,27] has shown that the properties of dilute soap sols change abruptly at other concentrations besides the CMC. He terms these points concentration limits. In the case of potassium laurate, the

minimum in the D/c curve ccincides with Ekwall's fourth limit at 50 millimoles/1. A concentration limit for NaDS would seem to occur at approximately 12·5 millimoles/l., where the diffusion coefficient is minimal.

The agreement between theoretical and experimental diffusion coefficients as shown in Table VI would appear to justify the simple mass law interpretation that has been applied to these dilute solutions. The extremely rapid increase in the concentration of micellar form (c_2) with very small increase in simple form (c_1), see Table V, would account for the rapid falling off of diffusion coefficient with increasing concentration after the CMC, to a minimum value. The rise of diffusion coefficient after the CMC to a constant value above 60 millimoles/l. (see Table III) is a property of soap solutions that has until recently been seldom observed or commented upon. McBain[17] in an attempt to explain the minimum in the D/c curve for α-sulphonic acids which she obtained, has applied McBain's theory of the structure of association colloid solutions. Thus "neutral colloid" is supposed to predominate in the transition region where the diffusion coefficient decreases rapidly with increasing concentration, and "ionic micelle" in the higher concentrations where its mobility in the potential gradient accounts for the subsequent rise of diffusion coefficient. Hartley[28] has on the other hand stated that the minimum in the D/c curve can be explained on the basis of a single spherical ionic micelle. Thus, if the one kind of chargec micelle is present, it will not contribute as much in the transition region where it exists in excess of simple electrolyte (i.e., c_2 is less than c_1) as it will when it exists in much higher concentration than the simple ions from which it is formed (i.e., c_2 greater than c_1). Hartley therefore considers that the minimum in the D/c curve is not due to a change in the nature of the micelles in solution, but is due to a change in the electrical effect upon the micelles. We consider that the results obtained for potassium laurate shown in Table IV are corroboratory evidence for Hartley's theory. In these measurements the mean concentration ($\bar{c}$) of the two solutions has been kept constant while the small difference in concentration (Δc) between them has been varied. Consequently the same types of particles are present in all the solutions and the only variation is in the electrical potential differences between the pairs of solutions. The results shown in Table IV for potassium laurate indicate that D varies with Δc, but tends towards a constant value above 20 millimoles/l.

SUMMARY

1. Diffusion coefficients at 25° C. for pure sodium dodecyl sulphate have been measured by the Gouy interference method.

2. A minimum in the overall diffusion coefficient/concentration curve, occuring at a concentraticn limit close to the critical micelle concentration has been found.

3. An estimate of micellar size from the differential diffusion of pure sodium dodecyl sulphate has been obtained. A table of the relative concentrations of simple soap and micelles in dilute solutions is given.

4. Theoretical diffusion coefficients, showing general agreement with

experimental values, have been determined by application of the law of mass action.

5. Differential diffusion rates for potassium laurate in concentrated solution are reported and these indicate that the increase of D after the minimum point is due to electrical effects.

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DISCUSSION

The paper was presented by MR. N. BRUDNEY.

MR. P. ELWORTHY (Romford) referred to the authors' statement that previous measurements of the diffusion coefficients of NaDS in the presence of added electrolyte showed no minimum in the D/c curve. Those values were not quoted in the paper, and it would be interesting to know how they compared with the present measurements. Had the authors any information on the bactericidal activity of the solution?

MR. H. D. C. RAPSON (Dorking) asked whether the critical micelle size referred to agreed with the measurements made by conductivity methods. It would be very interesting to follow up the work by using

radio-active tracer techniques as had been done in the study of diffusion in molten metals.

Mr. N. Brudney, in reply, said that the values of previous measurements were not given because the main point for consideration was the fact that no minimum in the diffusion coefficient curve had been found, but the values generally obtained were close to the results shown. No endeavour had been made to correlate results with bactericidal activity. It might be considered in the future. Measurements agreed generally with previous measurements of micelle size of potassium laurate. The tracer technique had not been attempted as yet.

ANTI-INFLAMMATORY COMPOUNDS

Part I. The Activity of a Series of New Compounds Compared with Phenylbutazone and Cortisone

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From Smith and Nephew Research Limited, Hunsdon Laboratories, Hunsdon, Ware, Hertfordshire

Introduction

SINCE the discovery by Hench[1] that cortisone and adrenocorticotrophic hormone possess the property of relieving rheumatoid arthritis and other inflammatory conditions, many workers have sought compounds which might simulate the action of these hormones. It has been shown that other substances possess anti-inflammatory properties and their mode of action is as yet unknown. Our interest in the production of compounds of this type arose from a consideration of the possible reasons for the toxicity of phenylbutazone, introduced by Wilhelmi[2]. This substance has been shown to have an outstanding anti-inflammatory activity but its use has been associated with a number of toxic side effects. At the time no evidence was available about the route by which the drug (I) was metabolised but it seemed possible that in the body the drug might be decomposed to form hydrazobenzene (II) and that this, or its rearrangement product, benzidine (III), or its disproportionation product, aniline (IV), might be responsible for the toxic effects of phenylbutazone.
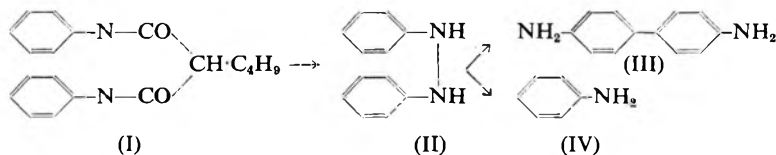
In an attempt therefore to obtain phenylbutazone derivatives with reduced toxicity, we planned to prepare a series of compounds which on similar decomposition would yield non-toxic derivatives of aniline, such as *p*-aminobenzoic acid and *p*-aminosalicylic acid. The above hypothesis is to be regarded solely as a theoretical possibility which provided some logical basis for the synthesis of the compounds described below. Burns *et al.*[3] investigated the metabolism of phenylbutazone but did not detect any of the possible decomposition products shown above.

Whilst our work on phenylbutazone derivatives was in progress, the report by Lecompte *et al.*[4] came to our notice, in which the anti-inflammatory effect of cysteinamine was described. Moreover, Cornforth and Long[5,6] suggested that cortisone and the methionine antagonist, ethionine, desensitised the guinea-pig to the tuberculin reaction by their interference with glutathione production in the tissues. It therefore appeared desirable to investigate the anti-inflammatory activity of sulphur-containing

compounds related to cysteinamine and ethionine. The compounds studied in this work have, therefore, fallen into two categories, namely phenylbutazone derivatives and amino-sulphide derivatives.

CHEMISTRY

1. Compounds Related to Phenylbutazone

A series of compounds of general formula I,

$$
RCH \Big\langle {\underset{CO-N}{\overset{CO-N}{}}} \Big\rangle
$$

(I)
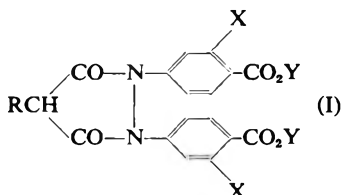
Where R = alkyl (methyl-hexyl)
X = H or OH
Y = alkyl or H

was prepared[7]. Furthermore, in order to study the effect on anti-inflammatory activity of small structural variations in the phenylbutazone molecule, the following types of compounds were studied:—

(a) A series of compounds of general formula II.
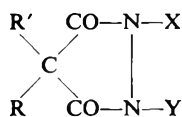
(II) (Phenylbutazone:—
$R = C_4H_9(n)$ $R' = H, X = Y = Ph.$)

(b) A group of cyclopentanediones (III) and cyclopentenediones (IV) derived from phenylbutazone by replacement of the ring nitrogens by carbon.

(III)   (IV)
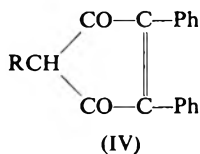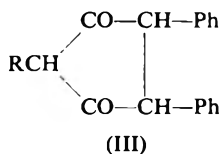
(c) Acyclic compounds (compounds 342 and 423).

(d) Compounds of general formula V in which two pyrazolidine rings are linked by a carbon chain.

(V)
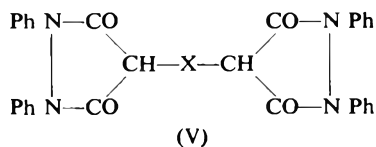
The compounds tested are given in detail in Table I.

TABLE I

ANTI-INFLAMMATORY ACTIVITY OF PHENYLBUTAZONE DERIVATIVES

Ref. no.	Name	Formula	Anti-inflammatory activity in ultra-violet erythema test	
			Dose mg./kg.	Activity
358	4-*n*-Butyl-1 : 2-di(4'-carboxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *n*Butyl; X = Y = H	3 × 60	+
361	4-*n*-Butyl-1 : 2-di(4'-carboxy-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *n*Butyl; X = OH; Y = H	3 × 60	−
389	4-*n*-Hexyl-1 : 2-di(4'-carboxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *n* Hexyl; X = Y = H	3 × 300	−
390	4-*n*-Hexyl-1 : 2-di(4'-ethoxy-carbonylphenyl)-pyrazolidine-3 : 5-dione	I. R = *n*-Hexyl; X = H; Y = Et	3 × 300	−
392	4-Ethyl-1 : 2-di(4'-carboxy-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = Et; X = OH; Y = H	3 × 50	−
393	4-*n*-Propyl-1 : 2-di(4'-carboxy-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *n*Propyl; X = OH; Y = H	3 × 30	−
394	4-*n*-Propyl-1 : 2-di(4'-ethoxy-carbonyl-phenyl)-pyrazolidine-3 : 5-dione	I. R = *n*Propyl; X = H; Y = Et	3 × 50	−
395	4-*n*-Propyl-1 : 2-di(4'-carboxy-phenyl)-pyrazolidine-3 : 5-dione	I. R = *n*Propyl; X = Y = H	3 × 50	+
396	4-*iso*Propyl-1 : 2-di(4'-ethoxy-carbonylphenyl)pyrazolidine-3 : 5-dione	I. R = *iso*Propyl; X = H; Y = Et	3 × 50	−
397	4-Ethyl-1 : 2-di(4'-carboxyphenyl)-pyrazolidine-3 : 5-dione	I. R = Et; X = Y = H	3 × 50	−
398	4-*iso*Propyl-1 : 2-di(4'-carboxy-phenyl)-pyrazolidine-3 : 5-dione	I. R = *iso*Propyl; X = Y = H	3 × 50	−
404	4-*iso*Propyl-1 : 2-di(4'-carboxy-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *n*Propyl; X = OH; Y = H	3 × 50	−
405	4-*n*-Hexyl-1 : 2-di(4'-carboxy-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *n*Hexyl; X = OH; Y = H	3 × 50	−
406	4-Methyl-1 : 2-di(4'-methoxy-carbonyl-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = Y = Me; X = OH	3 × 30	−
407	4-Ethyl-1 : 2-di(4'-methoxycarbonyl-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = Et; X = OH; Y = Me	3 × 30	+
408	4-*n*-Propyl-1 : 2-di(4'-methoxy-carbonyl-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *n*Propyl; X = OH; Y = Me	3 × 10	−
409	4-*iso*Propyl-1 : 2-di(4'-methoxy-carbonyl-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *iso*Propyl; X = OH; Y = Me	3 × 20	−
410	4-*n*-Butyl-1 : 2-di(4'-methoxy-carbonyl-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *n*Butyl; X = OH; Y = Me	3 × 50	+ +
411	4-*iso*Amyl-1 : 2-di(4'-methoxy-carbonyl-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *iso*Amyl; X = OH; Y = Me	3 × 50	−
412	4-*n*-Hexyl-1 : 2-di(4'-methoxy-carbonyl-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *n*-Hexyl; X = OH; Y = Me	3 × 20	−
421	4-Methyl-1 : 2-di(4'-carboxy-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = Me; X = OH; Y = H	3 × 4·5	−
422	4-*iso*-Amyl-1 : 2-di(4'-carboxy-3'-hydroxyphenyl)-pyrazolidine-3 : 5-dione	I. R = *iso*Amyl; X = OH; Y = H	3 × 5	−

TABLE I (*contd.*)

			\-\- Anti-inflammatory activity in ultra-violet erythema test	
Ref. no.	Name	Formula	Dose mg./kg.	Activity

Structure II:

$$R' \quad CO-N-X$$
$$\diagdown C \diagup \qquad \vert$$
$$R \quad CO-N-Y$$

II

Ref. no.	Name	Formula	Dose mg./kg.	Activity
466	4-Allyl-1 : 2-diphenylpyrazolidine-3 : 5-dione[16]	II. R = C$_3$H$_5$; R' = H; X = Y = Ph	3 × 50	+ + +
482	4-n-Propyl-1 : 2-diphenylpyrazolidine-3 : 5-dione[16]	II. R = nPropyl; R' = H; X = Y = Ph	3 × 50	+ + +
343	4-n-Butyl-1-phenylpyrazolidine-3 : 5-dione	II. R = nButyl; X = Ph; R' = Y = H	3 × 50	+
430	4-n-Butyl-1 : 2-di-p-tolylpyrazolidine-3 : 5-dione[16]	II. R = n-Butyl; R' = H; X = Y = p-CH$_3$C$_6$H$_4$-	3 × 50	+ + +
424	4-n-Butyl-4-methyl-1 : 2-diphenylpyrazolidine-3 : 5-dione	II. R = n-Butyl; R' = Me; X = Y = Ph	3 × 50	+
434	4-n-Butyl-4-methylpyrazolidine-3 : 5-dione	II. R = nButyl; R' = Me; X = Y = H	3 × 50	—

Structure III:

$$\qquad CO-CH\ Ph$$
$$RCH \diagdown \qquad \vert$$
$$\qquad CO-CH\ Ph$$

III

Ref. no.	Name	Formula	Dose mg./kg.	Activity
488	4 : 5-Diphenyl*cyclo*pentane-1 : 3-dione	III. R = H	3 × 10	—
486	2-n-Butyl-4 : 5-diphenyl*cyclo*pentane-1 : 3-dione	III. R = nButyl	3 × 10	—
415	2 : 4 : 5-Triphenyl*cyclo*pentane-1 : 3-dione	III. R = Ph	3 × 50	—
491	2-p-Methoxyphenyl-4 : 5-diphenyl-*cyclo*pentane-1 : 3-dione	III. R = p-MeOC$_6$H$_4$-	3 × 50	—

Structure IV:

$$\qquad CO-C\ Ph$$
$$RCH \diagdown \qquad \Vert$$
$$\qquad CO-C\ Ph$$

IV

Ref. no.	Name	Formula	Dose mg./kg.	Activity
487	4 : 5-Diphenyl*cyclo*pent-4-ene-1 : 3-dione	IV. R = H	3 × 10	—
485	2-n-Butyl-4 : 5-diphenyl*cyclo*pent-4-ene-1 : 3-dione	IV. R = n-Butyl	3 × 10	—
413	2 : 4 : 5-Triphenyl*cyclo*pent-4-ene-1,3-dione[16]	IV. R = Ph	3 × 50	—
490	2-p-Methoxyphenyl-4 : 5-diphenyl-*cyclo*pent-4-ene-1 : 3-dione	IV. R = p.MeOC$_6$H$_4$-	3 × 50	—

Structure V:

$$PhN-CO \qquad\qquad CO-N\ Ph$$
$$\vert \qquad CH-X-CH \qquad \vert$$
$$PhN-CO \qquad\qquad CO-N\ Ph$$

V

Ref. no.	Name	Formula	Dose mg./kg.	Activity
489	4 : 4'-Methylenebis-(1 : 2-diphenyl-pyrazolidine-3 : 5-dione)	V. X = CH$_2$-	3 × 50	—
492	4 : 4'-Trimethylenebis-(1 : 2-diphenyl-pyrazolidine-3 : 5-dione)	V. X = -(CH$_2$)$_3$-	3 × 50	—
510	4 : 4'-Ethylidenebis-(1 : 2-diphenyl-pyrazolidine-3 : 5-dione)	V. X = CH$_3$CH <	3 × 30	—

Ref. no.	Name	Formula	Dose mg./kg.	Activity
342	n-Butylmalondianilide[17]	Butyl CH< CONHPh / CONHPh	3 × 50	+
423	n-Butylmalondihydrazide[18]	Butyl CH< CONHNH$_2$ / CONHNH$_2$	3 × 50	—

Doses were determined by availability and toxicity of compound.

2. *Amino-sulphides*

Reports on the anti-inflammatory action of cysteinamine, and our own work with ethionine suggested the preparation of some amino-sulphides for screening as potential anti-inflammatory agents.

The compounds prepared are given below, and their chemistry will be described elsewhere.

$$X-\underset{Y}{\bigcirc}-\underset{SR}{CH}-CH_2NH_2 \quad (VI) \qquad \bigcirc-\underset{SR}{CH}\ CH_2CH_2NH_2 \quad (VII) \qquad RSCH_2CH_2CH_2NH_2 \quad (VIII)$$
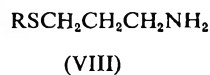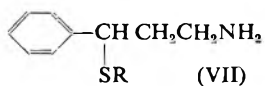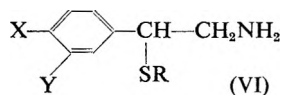
516 R=CH$_3$; X=Y=H
522 R=C$_2$H$_5$; X=Y=H
523 R=C$_2$H$_5$; X=CH$_3$O; Y=H
524 R=C$_3$H$_7$(*iso*); X=Y=H
525 R=C$_3$H$_7$(*iso*); X=CH$_3$O; Y=H
526 R=C$_3$H$_7$(*n*); X=Y=H
532 R=C$_3$H$_7$(*n*); X=CH$_3$O; Y=H
550 R=C$_2$H$_5$; X=Y=CH$_3$O

569 R=C$_2$H$_5$
571 R=C$_3$H$_7$(*iso*)

575 R=C$_2$H$_5$

EXPERIMENTAL METHODS

1. *Anti-inflammatory Activity*

The difficulty of inducing a clinical condition such as rheumatoid arthritis in experimental animals, together with the lack of real knowledge about the mechanism of anti-inflammatory activity, is reflected in the numerous methods suggested for screening possible anti-inflammatory compounds. Some of these have been recently enumerated by Wilhelmi and Currie[8] and additional methods have been described involving the production of granuloma by cotton pellets[9] or air pouches[10]. Three of these methods have been used to screen the compounds described above.

(*a*) Inhibition of the increase of capillary permeability by croton oil in the ear of the mouse[11], or by chloroform in the thoracic skin of the rat[4]. The former method was found difficult to manipulate and so was abandoned in favour of the latter. In this, 5 mg. of Pontamine Sky Blue was injected intraperitoneally into each rat exactly 2 hours before, and the drugs were injected intraperitoneally exactly 45 minutes before chloroform treatment. The experimental groups were compared with the control group for statistical significance. This is indicated in Table III by the sign, +.

(*b*) Inhibition of the production of inflammatory erythema by exposure of the depilated guinea-pig skin to ultra-violet light, using the Kromayer Lamp Model II and exposures of 20 or 30 seconds. Doses of test substances were given 45 minutes before, immediately before, and 30 minutes after, irradiation. This is a modification of the method used by Wilhelmi[2].

(*c*) Inhibition of the acute and chronic swelling of the rat's foot after the injection of 0·05 ml. of 10 per cent. mustard powder suspension[12]. This produces a gross œdema of the foot within thirty minutes (the "acute" stage) which slowly subsides by the next morning and which may reflect an increase in capillary permeability. During the days following, inflammatory tissue is laid down resulting in recurrence of swelling (the "chronic"

stage). The effect is measured daily for one week. The size of the foot before and after injection is measured by placing the foot on the platform of a dial micrometer and lowering the spring until very light contact is made with the surface of the most swollen point of the foot. The measurement of the normal foot is subtracted from each reading. The average percentage increase is compared with that of the control group, any difference being analysed for significance. Measurements are made after half an hour, and hourly for five hours after injection of mustard, then daily for the next five days. Doses are administered daily starting 3 days before the injection of mustard into the foot.

2. Toxicity

Acute and chronic toxicity of the compounds were determined by the usual methods in various species of experimental animals.

RESULTS

1. Phenylbutazone Derivatives

(a) *Anti-inflammatory activity.* For the assay of the anti-inflammatory activity of phenylbutazone derivatives it was decided to use the guinea-pig erythema test in view of the work by Wilhelmi[2] who had shown that phenylbutazone displays marked activity with this type of test.

The results are given in Table I.

Phenylbutazone in a dose of 3×10 mg./kg. intraperitoneally was used as a standard in each test with a $+++$ result in each case. It is interesting to record that cortisone, administered as a micro-crystalline suspension of the acetate in total doses up to 50 mg. with single or repeated injections, showed no effect whatever in this test, suggesting that cortisone and phenylbutazone have a different mechanism of action in this particular test. Since compound 358 was of particular interest in view of its chemical structure, it was decided to carry out other tests on it. The results showed that the compound had a definite anti-inflammatory effect when assayed by the croton oil test at a dose of 500 mg./kg. intraperitoneally. The same substance also showed some degree of inhibitory effect against both types of swelling when assayed by the rat foot test at a dose of 400 mg./kg. intraperitoneally. On the other hand, only a doubtful effect was produced in this latter test when compound No. 358 was administered orally. In each of these tests, however, effective doses for phenylbutazone were close to the toxic range and the therapeutic index was, therefore, small. Toxicity tests on compound No. 358 demonstrated that it possessed a considerably lower toxicity and a higher therapeutic index than phenylbutazone.

(b) *Toxicity of Compound No.* 358. *Acute toxicity to mice.*

	Oral	Subcutaneous	Intraperitoneal
		LD50 in g./kg.	
Phenylbutazone	0·73	0·23	0·23
No. 358	>8·0	>8·0	>8·0

Chronic Toxicity

TABLE II

MAXIMUM TOLERATED DOSES OF PHENYLBUTAZONE AND COMPOUND NO. 358

Species	Phenylbutazone			Compound No. 358		
	Oral	Subcutaneous	Intra-muscular	Oral	Subcutaneous	Intra-muscular
Mouse ..	0·025 g./kg. (2 months)	0·01 g./kg. (2 months)	—	0·25 g./kg. (2 months)	0·063 g./kg. (2 months)	—
Rat ..	0·04 g./kg. (4 months)	—	0·016 g./kg. (5 months)	>0·5 g./kg. (4 months)	—	>0·1 g./kg. (5 months)
Guinea-pig	<0·012 g./kg. (4 months)	—	—	0·05 g./kg. (4 months)	—	—

2. *Amino-Sulphides.*

(*a*) *Anti-inflammatory action.* None of these compounds was found to be active by the guinea-pig erythema test. The results for the rat foot arthritis test and the chloroform patch test are given in Table III together with the acute toxicity figures for mice.

TABLE III

ANTI-INFLAMMATORY ACTIVITY OF AMINO-SULPHIDES

Ref. no.	Name	Dose mg./kg. intra-peritoneal	Anti-inflammatory activity			Acute toxicity to mice LD 50 mg./kg. intraperitoneal
			Rat foot test		Chloro-form patch	
			Acute	Chronic		
516	β-Methylthio-β-phenylethylamine hydrochloride	100	3/6	1/6	+	160
522	β-Ethylthio-β-phenylethylamine hydrochloride	50	5/6	2/6	+	97
523	β-Ethylthio-β-(4-methoxyphenyl)-ethylamine hydrochloride	100	2/6	0/6	+	220
524	β-*iso*Propylthio-β-phenylethylamine hydrochloride	50	4/6	0/5	+	140
525	β-*iso*Propylthio-β-(4-methoxy-phenyl)-ethylamine hydrochloride	50	2/6	0/5	+	140
526	β-Phenyl-β-propylthioethylamine hydrochloride	35	3/6	1/5	—	97
531	*N*-Dimethyl-β-phenyl-β-propylthio-ethylamine hydrochloride	75	3/6	0/6	—	160
532	β-(4-Methoxyphenyl)-β-propylthio-ethylamine hydrochloride	75	0/5	0/5	—	120
550	β-3:4-Dimethoxyphenyl-β-ethyl-thioethylamine hydrochloride	100	0/6	0/6		250
551	Di-(α-phenyl-β-aminoethyl) sulphide dihydrochloride	100	0/6	0/6		310
567	1-Ethylthio-1-phenyl*iso*propylamine hydrochloride (m.pt. 201 to 203°)	50	6/6	0/6		120
569	γ-Ethylthio-γ-phenylpropylamine hydrochloride	50	0/6	0/6	+	140
	Cysteinamine	50	3/5	0/6	+	>230
	Ethionine	100	4/6	5/5		

Note.—Scores in the rat foot test columns refer to the number of significant inhibitory results obtained over the experimental period.

It will be seen that only a small number of compounds showed activity and this was mainly confined to the inhibitory effect on the acute swelling. Ethionine appeared to be the most active of the compounds. It is also interesting to note that both cortisone and phenylbutazone had a very marked effect in this test in doses of 40 mg./kg. and 150 mg./kg. respectively, in distinction to the results quoted above with the guinea-pig

erythema test. However, the difference in action between cortisone and phenylbutazone is again illustrated by the results of the chloroform patch test, in which cortisone showed a high degree of activity and phenylbutazone only a low activity.

DISCUSSION

The results reported here appear to fall into two divisions, namely those which relate chemical structure of the compounds to their anti-inflammatory activity and those which are of more purely physiological interest.

In the series of phenylbutazone derivatives, the results have shown that the basic structure has a high degree of specificity. Minor variations in the alkyl chain, for example, replacement of butyl by n-propyl (482), or by allyl (466), yield compounds with the same order of activity as phenylbutazone, but linking two diphenylpyrazolidinedione nuclei by an alkyl chain yields inactive compounds. This latter result may in part be explained by the fact that these bis compounds no longer give readily soluble neutral sodium salts. Replacement of the butyl group by H led to an inactive compound. Minor variations in the phenyl groups (430) likewise have little effect on activity but more radical substitution of the aromatic nuclei (formula I) yields compounds with a measurably smaller activity, although in these instances the toxicity also is reduced, and to an even greater extent. It is realised of course that this is in no way a proof of our original hypothesis, the validity of which can be tested only by comparative studies on the metabolism of phenylbutazone and its carboxy derivatives. Replacement of one of the phenyl groups by hydrogen led to a marked reduction in activity.

It appears that the enolisable 1 : 3-dioxo system is essential for activity, since on blocking this enolisation by substitution of the hydrogen on carbon atom 4 by methyl, activity was largely abolished. Replacement of both ring-nitrogens to give *cyclo*pentanedione or *cyclo*pentenedione derivatives (III, IV) yielded compounds which were completely inactive.

Investigation of compound No. 358 (4-n-butyl-1: 2-di(4'-carboxyphenyl)-pyrazolidine-3 : 5-dione), in particular, suggested that it might be of clinical value and trials of its action in rheumatoid arthritis are now in progress.

With the series of amino-sulphides, our results are as yet not sufficiently numerous to justify any extensive deductions about structure-activity relationships. Ethionine, $C_2H_5S\cdot CH_2\cdot CH_2CH\cdot(NH_2)\cdot COOH$, appears to be the most active compound so far tested with β-methylthio-, and β-ethylthio-β-phenylethylamine, probably the most active of this series of homologues.

Of the more purely biological implications of this work, perhaps the most interesting is the wide range of activity covered by the term "anti-inflammatory." A number of tests have been developed to detect this activity and the three methods employed in this investigation have yielded quite different results when used with cortisone and phenylbutazone. The former drug is known to have a marked anti-inflammatory effect in a number of clinical conditions, including rheumatoid arthritis, whilst the

latter appears to exert its anti-inflammatory effect mainly in rheumatic conditions. The comparative experimental effects of phenylbutazone and cortisone may be summarised.

Test	Phenylbutazone	Cortisone
Guinea-pig erythema	Active	Inactive
Rat foot arthritis	Active	Active
Rat chloroform patch	Low activity	High activity

It has been suggested that the beneficial action of phenylbutazone in rheumatoid arthritis is due solely to analgesia. However, using the technique previously described[13] we could demonstrate no analgesic activity in the rat, nor do the results above confirm this view of the mechanism of action of phenylbutazone.

A recent report[14] suggested that phenylbutazone had both a central and peripheral action and it is possible that cortisone may be acting only peripherally in those tests where differences were seen.

The anti-inflammatory activity of ethionine appears to parallel the observation by Cornforth and Long on the action of ethionine in inhibiting the tuberculin reaction. Ethionine is, of course, known to cause toxic effects in animals, due presumably to the metabolic disturbance produced by its antagonism to methionine, and it seems possible that the stress of this toxic effect may be responsible for the anti-inflammatory action. A more detailed investigation of this problem is now in progress.

SUMMARY

1. A series of phenylbutazone derivatives has been tested for anti-inflammatory activity, using the guinea-pig erythema test, the rat foot arthritis test and the rat skin chloroform patch test.

2. The phenylbutazone structure shows a high degree of specificity; changes, other than minor ones, result in complete loss of activity.

3. None of the compounds tested was more active than phenylbutazone, but compound 358 (4-*n*-butyl-1:2-di(4'-carboxyphenyl)-pyrazolidine-3:5-dione) has a somewhat better therapeutic index.

4. A number of amino-sulphides was also shown to have some anti-inflammatory activity when tested by the rat foot test and the chloroform patch test but none was active by the guinea-pig erythema test. Similar differences are found between the actions of cortisone and phenylbutazone.

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DISCUSSION

The paper was presented by MR. E. M. BAVIN.

MR. T. D. WHITTET (London) said that it would be useful if a less toxic derivative of phenylbutazone could be found. Cortisone and phenyl-butazone each had their advantages and disadvantages. At his hospital the dose administered was that which gave reasonable remission with a minimum of side effects.

DR. R. F. TIMONEY (Dublin) said it appeared that compounds with the pyrazolidone structure showed greater activity than those with the cyclopentane structure, so it seemed that the activity of phenylbutazone lay in the former. When the *cyclo*pentanediol derivatives were formed, and replacement of the ring nitrogens by carbon effected, the activity of the compounds was lessened considerably.

The CHAIRMAN of the Session (DR. DAVIS) asked whether the effect of phenylbutazone was local or systemic. Cortisone and some of its derivatives were being used locally as anti-inflammatory agents with some success. Was there an association between the local and systemic effects of these compounds?

MR. E. M. BAVIN, in reply, said that the locus of action of the drugs was by no means settled. A recent German paper showed that cortisone and salicylates had both a central and peripheral action. That had been shown experimentally by measuring the response of drugs in animals. It seemed clear from the results that a dual action with cortisone was obtained. It might be that something similar would occur with phenyl-butazone. As a means of trying to avoid side reactions blood levels of phenylbutazone could be determined, and American workers had shown that below a fairly sharp limit in the blood levels side reactions were unlikely. It was agreed that the specificity of the phenylbutazone mole-cule was a high one. It had been hoped that it would be possible to identify the activity with a particular grouping, but this had not been possible.

FURTHER OBSERVATIONS ON THE ANTIBACTERIAL ACTIVITY TO *MYCOBACTERIUM TUBERCULOSIS* OF A DERIVATIVE OF ISONIAZID, o-HYDROXYBENZAL *ISO*NICOTINYLHYDRAZONE (NUPASAL-213)

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INTRODUCTION

A previous report[1] described the *in vitro* tuberculostatic activity, and, in some cases, the toxicity and *in vivo* tuberculostatic activity, of a number of new isoniazid derivatives. Of these, compound HP.213 o-hydroxybenzal *iso*nicotinylhydrazone (I) by reason of its low toxicity and high activity, merited further examination, the results of which are now reported. The compound has been given the name "Nupasal-213."*

$$OC \cdot NH \cdot N : CH$$

I
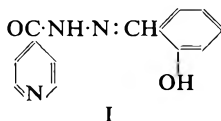
EXPERIMENTAL

Methods

(i) *In vitro* tests were made with the technique previously described[2], using initially a solution of 5 mg. HP.213 in 2 ml. propylene glycol and 18 ml. Dubos Tween albumin medium. The solution, after Seitz filtration, was successively diluted in the same Tween albumin medium. Inoculation of the tubes was carried out using 0·1 ml. of a 10 to 14 day old culture of *Myco. tuberculosis* H37Rv. Growth was finally recorded after 14 days incubation at 37° C.

(ii) *In vitro* tests on the development of resistance to *Myco. tuberculosis* H37Rv were carried out by a technique subsequently described by Steenken[3]. The technique was the same as described above. The tube containing the maximum concentration of the drug in which growth was apparent, for each set of dilutions, was used as the source of inoculum for the second set of dilutions. This represents the first transfer. Subsequent transfers were made in the same way.

(iii) *In vivo* activity was assessed by the corneal test in mice[4] using *Myco. tuberculosis* H37Rv. The intra-corneal injection was made using approximately 1000 organisms in suspension. The drug was administered in the diet immediately after infection. The infected eye was examined frequently for the development of lesions and 28 days after injection final examination of the eyes was made.

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* o-Hydroxybenzal-*iso*nicotinylhydrazone is available in America under the name "Salizid" and a preliminary account of tests with this material has been given by Barry and Conalty[7].

(*b*) *Toxicity*. Acute toxicity tests were made on male albino mice, weighing between 18 and 25 g. Chronic toxicity tests were made on groups of 10 male rats, of the Wistar strain, weighing from 90 to 110 g., and groups of 4 male and 1 female guinea-pigs weighing 150 to 270 g. The rats were fed on "diet 41" rat cubes, the guinea-pigs on "diet 18" pellets and green food. Doses from 100 mg./kg. to 2·0 g./kg. of HP.213 were given by stomach tube. In the chronic tests, administration was daily, with the exception of Saturdays and Sundays, in the form of a mucilage of acacia suspension. Comparative experiments using isoniazid in doses of 50 mg./kg. and 100 mg./kg. were made and control groups were given mucilage of acacia only.

(*c*) *Estimation in plasma*. Two methods have been used, (i) the cyanogen bromide method of Rubin *et al.*[5] which estimates all *iso*nicotinic acid derivatives present, and (ii) the naphthoquinone method of Short[6] which estimates free isoniazid. HP.213 is not estimated by the latter method, since the terminal amino-group is blocked. In the absence of a specific method for the estimation of HP.213 in plasma, the difference between the results of method (i) and (ii) measures the concentration of HP.213 together with the concentration of metabolites containing an *iso*nicotinic acid group.

RESULTS

Tuberculostatic Activity

(i) *In vitro*, the minimum inhibitory concentration (M.I.C.) of HP.213 was 0·09 μg./ml. The corresponding figure for isoniazid was 0·03 μg./ml. Against an isoniazid resistant strain, the results were HP.213 15 μg./ml. and isoniazid 7·5 μg./ml.

(ii) Results of experiments on the development of resistance by *Myco. tuberculosis* to HP.213, isoniazid, and mixtures of the two substances are given in the Table I.

TABLE I

DEVELOPMENT BY *Myco. tuberculosis* OF RESISTANCE TO H.P.213 ALONE AND IN COMBINATION WITH ISONIAZID

	M.I C. μg./ml. at start of experiment	M.I.C. μg./ml. after 2½ months (8 transfers)	M.I.C. μg./ml. after 6 months (13 transfers)
HP.213	0·06	7·81	not continued
Isoniazid	0·03	7·81	not continued
Isoniazid (0·01 μg./ml.) and varying proportions of HP.213	0·06 (HP.213)	0·48 (HP.213)	15·62 (HP.213)
HP.213 (0·01 μg./ml.) and varying proportions of isoniazid	0·03 (isoniazid)	0·24 (isoniazid)	7·81 (isoniazid)

TABLE II

CORNEAL TEST IN MICE USING *Myco. tuberculosis* H37RV SENSITIVE TO ISONIAZID

Compound	Per cent. in diet	No. of mice with lesions	Protection per cent.
Isoniazid	0·C02	4/9	55
Isoniazid	0·C04	0/8	100
HP.213	0·C04	5/8	38
HP.213	0·C08	0/10	100
Untreated controls ..	—	6/7	14

(iii) The results of *in vivo* tests on the tuberculostatic activity of HP.213 are given in Tables II and III.

TABLE III

CORNEAL TEST IN MICE USING A STRAIN OF *Myco. tuberculosis* RESISTANT TO ISONIAZID

Compound	Per cent. in diet	No. of mice with lesions	Protection per cent.
HP.213	0·1	4/8	50
HP.213	0·04	7/9	22
Isoniazid	0·02	5/6	17
Isoniazid	0·004	6/6	0
Untreated controls ..	—	8/8	0

Toxicity

(i) The acute oral LD50 to mice was found to be greater than 10 mg./g. The corresponding dose for isoniazid, determined simultaneously, was 0·14 mg./g.

(ii) The chronic oral toxicity tests were continued for three months. Chronic oral approximate lethal doses were found to be as follows.

	Rats	*Guinea-pigs*
HP.213	250–500 mg./kg.	500–1000 mg./kg.
Isoniazid	50–100 mg./kg.	50–100 mg./kg.

Weight curves of some typical groups of both rats and guinea-pigs during the chronic toxicity test are shown in Figures 1 (*a*) and (*b*), 2 (*a*) and (*b*). Examination of these curves shows that animals treated with 500 mg./kg. of HP.213 gained weight at approximately the same rate as those treated with 100 mg./kg. of isoniazid. During the course of the experiment, in both species of animals, neither HP.213 nor isoniazid produced any change in the red and white blood cell count or in the blood levels of prothrombin, glucose, urea and amino-acids. Histological specimens taken post-mortem after the termination of the chronic toxicity test showed no significant abnormalities compared with similar specimens taken from control animals.

Plasma Levels

Preliminary experiments on rabbits had shown that HP.213 after oral administration was broken down, at least in part, to form isoniazid. The average results from a group of 3 rabbits receiving 25 mg./kg. of HP.213 orally are given in Table IV. Plasma levels of isoniazid in human volunteers receiving a single dose of 600 mg. of HP.213 orally are shown in Figure 3.

TABLE IV

PLASMA LEVELS IN RABBITS OF ISONIAZID FOLLOWING ORAL ADMINISTRATION OF HP.213

Dose: 25 mg./kg. of HP.213

Time after administration, hours	Isoniazid, μg./ml. plasma
1	4·7
2	2·0
4	0·6
6	0

To test whether the formation of isoniazid from HP.213 occurs mainly in the gastro-intestinal tract, rabbits were given intraperitoneal injections of HP.213, and plasma levels of isoniazid and HP.213 were determined.
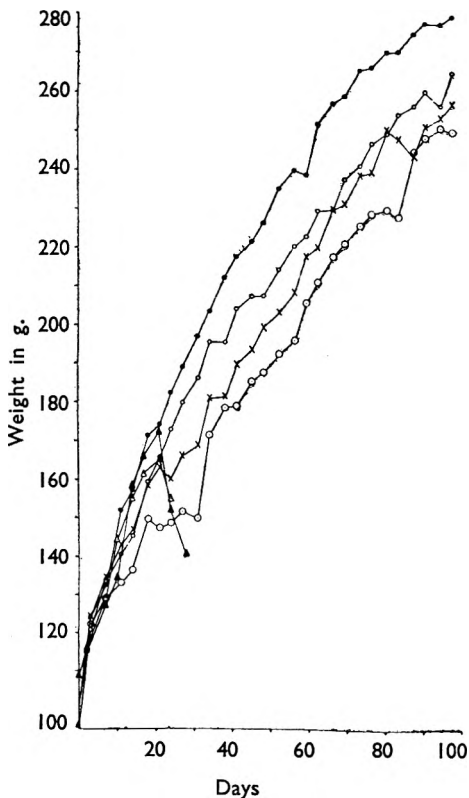
FIG. 1A. Growth curves of rats treated w:th HP.213.

FIG. 1B. Growth curves of rats treated with isoniazid.

●———●	Controls. Saline.
○———○	100 mg./kg. HP.213.
×———×	250 mg./kg. HP.213.
⊙———⊙	500 mg./kg. HP.213.
▲———▲	1·0 g./kg. HP.213.
△———△	2·0 g./kg. HP.213.

●———●	Controls. Saline.
△———△	50 mg./kg. isoniazid.
⊙———⊙	100 mg./kg. isoniazid.

The average results from a group of six rabbits receiving 20 mg./kg. of HP.213 intraperitoneally are given in Table V.

DISCUSSION

The foregoing results show that HP.213 has a tuberculostatic activity of the same order as that of isoniazid, with only one-fifth to one-tenth of the toxicity of the latter drug. Cross resistance has been observed between the two

TABLE V

PLASMA LEVELS IN RABBITS OF ISONIAZID AND HP.213 FOLLOWING INTRAPERITONEAL ADMINISTRATION OF HP.213

Dose: 20 mg./kg. of HP.213

Time after injection, hours	Isoniazid, μg./ml. plasma	HP.213, μg./ml. plasma
1	0	5·8
2	0	3·8
4	0	2·8
6	0	2·0
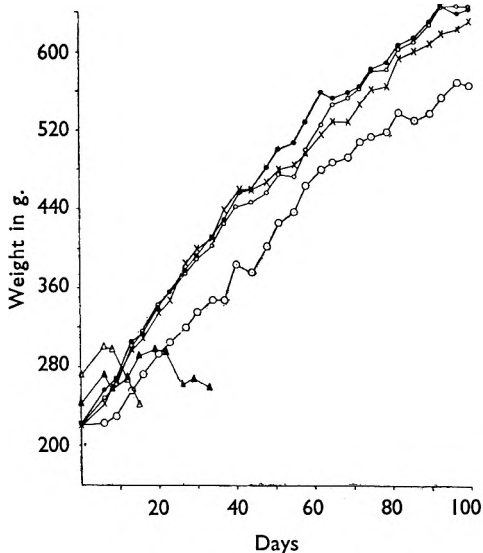
FIG. 2A. Growth curves of guinea-pigs treated with HP.213.

FIG. 2B. Growth curves of guinea-pigs treated with isoniazid.

●———● Controls. Saline.
○———○ 100 mg./kg. HP.213.
×———× 250 mg./kg. HP.213.
⊙———⊙ 500 mg./kg. HP.213.
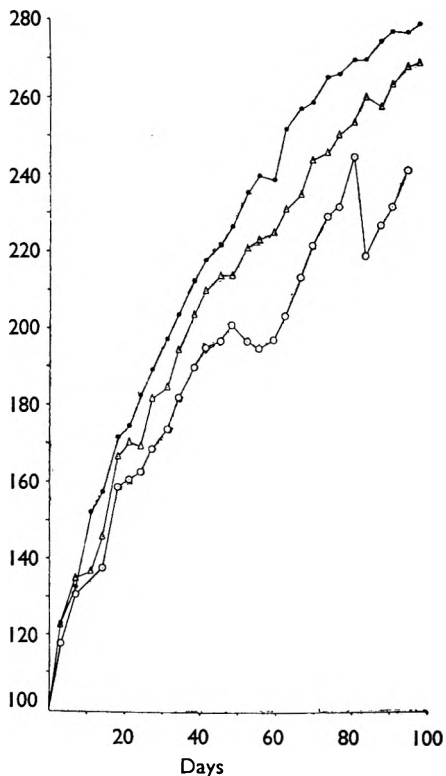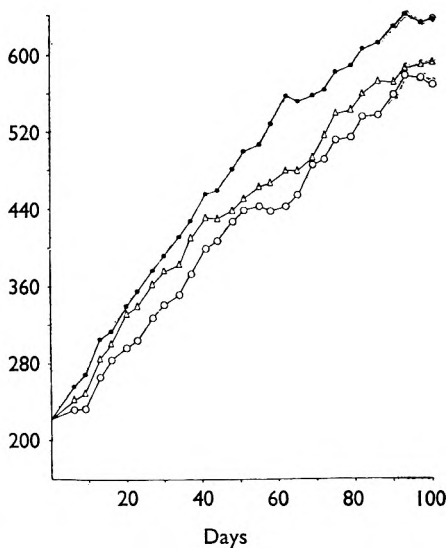▲———▲ 1·0 g./kg. HP.213.
△———△ 2·0 g./kg. HP.213.

●———● Controls. Saline.
△———△ 50 mg./kg. isoniazid.
⊙———⊙ 100 mg./kg. isoniazid.

compounds in the sense that strains of *Myco. tuberculosis* highly resistant to isoniazid were also resistant to HP.213. However, the low toxicity of the latter substance enables sufficiently high doses to be used *in vivo* to produce some effect against isoniazid-resistant organisms. Moreover, isoniazid and HP.213 appear to possess a mutual action on the emergence of resistance, inasmuch as each substance delays the development of resistance by *Myco. tuberculosis* to the other compound. While this work was in progress, similar results were reported by Steenken, Wolinsky and Montalbine[3]. Further work is now being carried out to determine whether a similar mutual effect can be reproduced *in vivo*.

Absorption experiments in rabbits show that, after oral administration of moderate doses of HP.213, isoniazid is present in the plasma, whereas no isoniazid was detected after the intraperitoneal injection of a similar dose of HP.213.

In a group of 12 mice receiving daily subcutaneous injections of 60 mg./kg. of HP.213 for 5 days, 2·7 μg./ml. of isoniazid was found in the plasma. These results suggest that HP.213 is much more easily decomposed to isoniazid by the gastro-intestinal tract than by tissue enzymes. This result is not unexpected since it is known that HP.213 is easily decomposed by dilute acids to form isoniazid and salicylaldehyde. Plasma levels of

isoniazid in human volunteers after a clinical dose by mouth of HP.213 were of the same order as those produced by an equivalent dose of isoniazid. Further work on the estimation of HP.213 in plasma and its metabolic products is in hand.

The question whether HP.213 owes all its tuberculostatic action to the formation of isoniazid, or whether it has an action *per se*, cannot be answered categorically at present. The facts that HP.213 has a marked tuberculostatic action *in vitro*, that HP.213 and isoniazid act mutually in the delay of resistance emergence, and that HP.213 is considerably less toxic than isoniazid strongly suggest that the properties of HP.213 are not solely due to isoniazid formed as a breakdown product.

The experimental results obtained, so far, with HP.213 indicate its possible value in the treatment of human tuberculosis, and clinical trials of the substance are in progress.
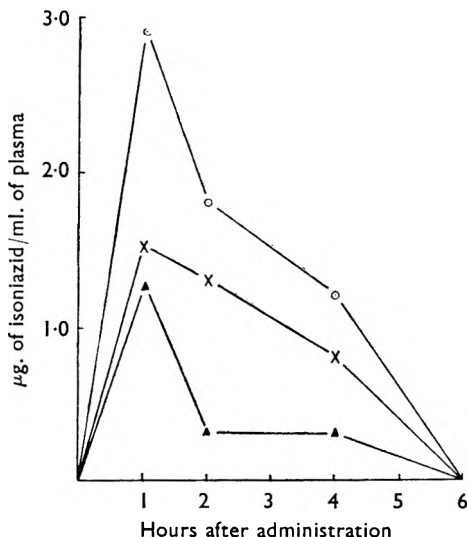

FIG. 3. Human plasma levels of isoniazid following administration of HP.213. Dose: 600 mg. orally of HP.213.

×———×	Subject J.H.
▲———▲	Subject H.Y.
○———○	Subject S.T.

SUMMARY

1. o-Hydroxybenzal *iso*nicotinylhydrazone ("Nupasal-213") possesses tuberculostatic activity of the same order as that of isoniazid.

2. Its chronic toxicity towards rats and guinea-pigs is about 1/5 to 1/10 of that of isoniazid.

3. Isoniazid and o-hydroxybenzal *iso*nicotinylhydrazone act mutually to delay the development of resistance by *Myco. tuberculosis* H37Rv to either substance.

4. o-Hydroxybenzal *iso*nicotinylhydrazone is decomposed, at least partially, in the gastro-intestinal tract to form isoniazid. This decomposition occurs to a much smaller extent when the substance is administered parenterally.

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DISCUSSION

The paper was presented by MR. E. M. BAVIN.

MR. H. GRAINGER (London) said that no information was given about the possible route of administration. It appeared that if any great advantage was to be derived clinically from the use of the compounds it would probably be by parenteral administration, because given orally the effect was largely that of the breakdown compound.

MR. G. SYKES (Nottingham) said it was apparent that even traces of one compound in the presence of the other delayed the development of resistance of the organism, and he wondered whether the authors had any idea of the mechanism of the combined effect. It seemed that the administration of Nupasal *in vivo* would be advantageous, because a mixture of the two compounds would be present and therefore, presumably, optimum conditions for delaying resistance to the organism.

DR. G. BROWNLEE (London) said that the capacity of the new substance to deal with strains resistant to isoniazid was of interest, and he suggested that it was necessary to have information about a number of strains shown to be resistant. The acid test would be an animal experiment in which strains resistant to isoniazid were shown to produce generalised infection which was adequately suppressed by the new substance.

MR. E. M. BAVIN, in reply, said that the compound was not intended for parenteral use, but orally it was much less toxic than isoniazid. It had been shown that it was possible to give up to 1·5 g. per day clinically without any trace of side reaction. Some breakdown to isoniazid did occur, but no method was available for identifying Nupasal specifically in the presence of isoniazid. It was not possible to give a detailed reply on the question of the mechanism of resistance. One could only hazard a guess that substances like isoniazid and Nupasal, which were similar chemically, did interfere mutually with each other's metabolic pathway in the tubercle organism.

THE CONFIGURATION OF ALPHAPRODINE AND BETAPRODINE

By A. H. Beckett and J. Walker

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Two diastereoisomeric forms (α and β) of 1:3-dimethyl-4-phenyl-4-propionoxypiperidine have been isolated[1] and shown to possess high analgesic activities, the β-isomer racemate possessing 5 to 6 times the activity of the α-isomer racemate[2]. The approved names of alphaprodine and betaprodine have been given to the hydrochlorides of the α-and β-isomers respectively.

The *trans* [*trans* Me/Ph≡ *cis* Me/propionoxy (see Beckett and Casy[3])] configuration has been assigned to the β-isomer and the *cis* (Me/Ph) configuration to the α-isomer[1,2], but these assignments were not rigidly established. They are stated to be dependent upon the easier breakdown of the α-isomer under hydrolytic conditions and upon the pharmacological results presuming that the more active β-isomer was more closely related to the analgesically active dihydrodeoxymorphine-D than the α-isomer as shown in Figure 1.

In an earlier communication Beckett and Casy[3], although presenting diagrams indicating that both isomers could fit their proposed "analgesic
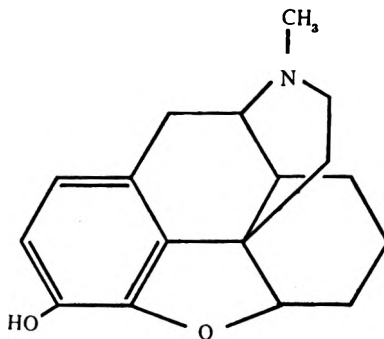
(I) Dihydrodeoxymorphine
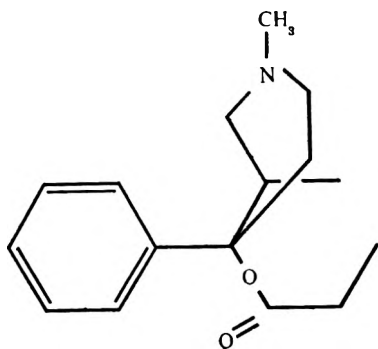
(II) *trans*(β-isomer?)

(III) *cis*(α-isomer?)

Fig. 1.

receptor site," stated that if these provisional configurational assignments were incorrect, then the most probable conformation of the more active β-isomer (IX) would represent a 3-dimensional arrangement closer to that of morphine (VII) than the most probable conformation of the α-isomer (VIII) (see Fig. 2.).
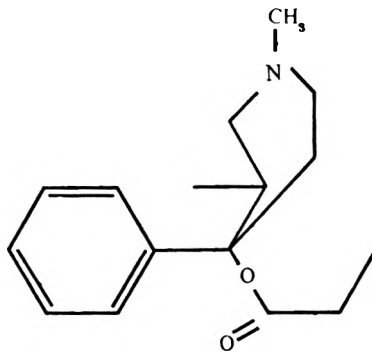
In the absence of strong electrostatic effects between groups, the most stable conformations of substitued *cyclo*hexanes are chair forms with the maximum number of equatorial substituents[4,5,6]. If one axial and one equatorial substituent be present, the molecule will assume the chair form in which the larger group is equatorial. Ignoring the conformation of the methyl group on the nitrogen atom, four possible chair forms of 1:3-dimethyl-4-phenyl-4-propionoxypiperidine are possible as shown in Table I, and applying the above generalisations to a piperidine ring leads to 1 and 3 as the most probable conformations of the *cis-* and *trans*-isomer respectively. This is even more likely as the phenyl and propionoxy groups do not differ greatly in size. The *cis*-isomer will thus possess an equatorial and the *trans*-isomer an axial propionoxy group.

TABLE I

	Configuration	4-Propionoxy	3-Methyl	4-Phenyl
1	cis (Me/Ph)	e	e	a
2	cis (Me/Ph)	a	a	e
3	trans (Me/Ph)	a	e	e
4	trans (Me/Ph)	e	a	a

It is known that carboxy esters of equatorial hydroxyl groups are more readily hydrolysed than the corresponding axial esters[4,5,6], and consequently the *cis* (Me/Ph) isomer would be expected to hydrolyse more readily than the *trans*-isomer.

The hydrolysis of alphaprodine and betaprodine in aqueous ethanolic alkaline solutions was examined under comparable conditions at a number of concentrations. Some of the results are presented in Table II.

TABLE II

COMPARATIVE HYDROLYSIS STUDIES OF ALPHAPRODINE AND BETAPRODINE

Initial concn. of alphaprodine and betaprodine (in g. mol.)	Initial concn. of NaOH (in g. mol.)	Time after mixing	Percentage hydrolysis	
			Alphaprodine	Betaprodine
0·023	0·036	20 hours	11·5	16·5
0·023	0·036	79 hours	21·0	32·0
0·067	0·450	12·5 hours	54·5	62·0
0·067	0·450	44 hours	80·5	98·0

There is a possibility that the determination of the acid liberated in the reaction might not be a true measure of the hydrolysis because of the possibility of the occurence of the competing elimination reaction also removing hydroxyl ions as outlined below. That the latter reaction does not constitute a serious complicating factor under the conditions used for the hydrolysis experiments was shown in the following manner. The ultra-violet absorption spectra of the α- and β-alcohols (V) will approximate roughly to the spectra of the parent esters (i.e., ϵ_{max}. *ca* 220 to 250

(IV) $\xrightarrow[\text{(elimination)}]{\text{OH}'\,\text{(hydrolysis)}}$

Structures:

(IV) — O·CO·C$_2$H$_5$, —CH$_3$, N—CH$_3$

(V) — OH, —CH$_3$, N—CH$_3$ $+$ C$_2$H$_5$COO$'$

(VI) — —CH$_3$, N—CH$_3$ $+$ C$_2$H$_5$COO$'$ + H$_2$O
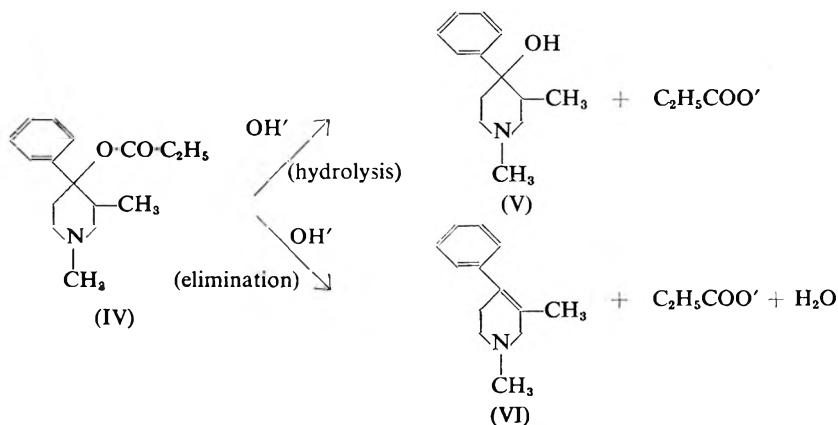
at $\lambda_{max.}$ ca 260 mμ e.g., ethylbenzene has $\epsilon_{max.}$ 220 at $\lambda_{max.}$ 261[7]) whereas compound VI in which the double bond is conjugated with the aromatic ring would exhibit an intense band (K band) ($\epsilon_{max.}$ ca 10,000 to 12,000 at $\lambda_{max.}$ ca 245 mμ e.g., β-methystyrene has $\epsilon_{max.}$ 12,600 at $\lambda_{max.}$ 244 mμ[8]) in addition to the B band ($\epsilon_{max.}$ ca 500 at $\lambda_{max.}$ ca 280). Ultra-violet absorption curves of the alkaline aqueous-ethanolic solutions of alphaprodine and betaprodine after standing and then neutralisation, although exhibiting an increased intensity of absorption in some of the solutions in the region 240 to 250 mμ (e.g., two-fold at 245 mμ in one run of betaprodine) showed the general pattern exhibited by the parent esters in freshly made solutions of comparable concentration, indicating the presence of negligible quantities of (VI) as compared with (V) in the hydrolysing solutions. An alcohol (V), identical in melting point with the α-isomer obtained by Ziering and Lee[1] from the mixture resulting from the reaction of 1:3-dimethyl-4-piperidone with phenyllithium, was also isolated in good yield from an hydrolysis experiment involving alpha-prodine; an isomeric alcohol m. pt. 77 to 78° C. was isolated from experi-ments involving betaprodine.

The results quoted in Table I can therefore be regarded as a measure of the hydrolysis of the isomers. They show that betaprodine hydrolyses more readily than alphaprodine and indicate that the former possesses an equatorial and the latter an axial propionoxy group. Betaprodine should therefore be allocated the *cis* (Me/Ph) configuration and alphaprodine the *trans* (Me/Ph) configuration, i.e., the reverse of the previous provisional assignments.

These new configurational assignments result in the thermodynamically most stable conformation of betaprodine (IX) showing a closer relation-ship than alphaprodine (VIII) to the 3 dimensional structure of morphine (VII) (see Fig. 2 for diagrammatic representation). The pharmaco-logical results[2] show that, at least in rats, alphaprodine is as active, and betaprodine 5 to 6 times as active, as morphine. It is possible to explain these results in terms of the analgesic receptor site (X) being fitted less closely by morphine than by IX, in which the projecting portion of the

(VII) Morphine

(VIII) Alphaprodine

(IX) Betaprodine
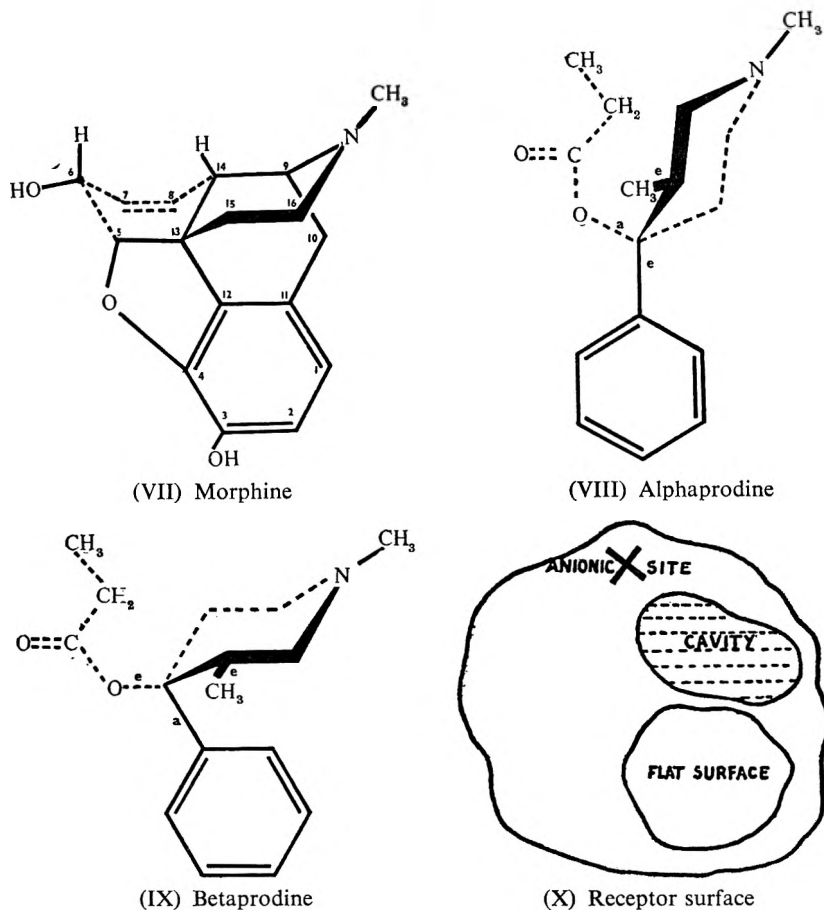
(X) Receptor surface

FIG. 2. Diagrammatic representation of the relationship of alphaprodine and betaprodine to morphine and the "analgesic receptor surface". The diagrams represent the lower surface of the drug and the upper surface of the receptor, i.e. complementary surfaces. In front of, behind, and in the plane of the paper are represented by ——————, - - - - - - , and ——————— respectively.

piperidine ring and the equatorial methyl group constitute a more bulky hydrocarbon moiety than that present in morphine. Alphaprodine will also "fit" the receptor site, but the distance between the centre of the aromatic ring and the basic group is greater than that in morphine or betaprodine.

<center>EXPERIMENTAL</center>

Hydrolysis Experiments

Reagents. Carbonate-free $0.1N$ *sodium hydroxide solutions,* prepared using the ion exchange resin IRA 400 (OH) by the method described by Davies and Nancollus[9]. The solution was stored in an automatic burette protected against entry of carbon dioxide. *Ethanol* (70 *per cent. w/w),* freshly boiled and cooled under reflux, the condenser being closed with a

soda-lime tube. *Ethanolic sodium hydroxide solutions* 0·05N and N solutions were prepared by dissolving freshly cut sodium in 70 per cent. w/w ethanol freshly boiled as described above. The solutions were stored in automatic burettes protected against entry of carbon dioxide.

Method. A typical hydrolysis experiment is described below. Other determinations were also performed in which the relative concentrations of alkali and samples were varied.

1 ml. portions of solutions of alphaprodine and betaprodine (4·004 g. per 100 ml. 70 per cent. w/w ethanol solution) were pipetted into Pyrex tubes fitted with ground-glass stoppers, the tubes being previously swept out with carbon dioxide free nitrogen. To each tube was added 1 ml. of N ethanolic sodium hydroxide, taking precautions against entry of carbon dioxide.

To the contents of 1 tube (for each isomer), 10 ml. of 0·1N hydrochloric acid was added, carbon dioxide free nitrogen bubbled through the solution for 10 minutes, 4 ml. of chloroform added to remove the organic base liberated in the subsequent titration, and the contents titrated with carbonate-free 0·1N sodium hydroxide solution using phenolphthalein as indicator.

The remaining tubes with their contents were immediately stoppered, the stoppers well sealed in position with Picien Wax to prevent entry of carbon dioxide, and placed in a water bath thermostatically controlled at 55° C. ± 0·1° C. Tubes were removed after the stated periods (Table II), the hydrolysis terminated by the addition of 10 ml. 0·1N hydrochloric acid and the procedure completed as described above. In all determinations, parallel sets of blank titrations were carried out, the contents of the tubes consisting of 1 ml. of N-ethanolic sodium hydroxide solution and 1 ml. of 70 per cent. ethanol containing hydrochloric acid equivalent to that present in the hydrolysis determinations due to the fact that alphaprodine and betaprodine are hydrochlorides.

(Preliminary experiments performed by dissolving known weights of alphaprodine and betaprodine in 70 per cent. ethanol, adding known volumes of standard 0·1N hydrochloric acid, and then back-titrating with carbonate-free 0·1N sodium hydroxide solution using conditions described above gave correct and reproducible equivalent weights.)

Examination of the reaction products. The hydrolysis was carried out using the precautions stated above. Samples (0·25 g.) of alphaprodine and betaprodine were dissolved in 6 ml. of 70 per cent. w/w ethanol and 6 ml. of N ethanolic sodium hydroxide solution added. The tubes were placed in the thermostatically controlled water bath for 70 hours to ensure complete hydrolysis. (After 20 hours, 1 ml. was withdrawn to check the course of the hydrolysis by the manner reported above.) The remaining solution was diluted to 20 ml. with 70 per cent. ethanol (Dilution A).

Ultra-violet measurements. 1 ml. of Dilution A (of both alphaprodine and betaprodine) was neutralised with 0·1N hydrochloric acid and diluted to 20 ml. with 70 per cent. ethanol and the ultra-violet absorption measured (1 cm. cell in a Unicam S.P. 500 spectrophotometer). The solvent cell

contained a solution prepared in a similar manner but omitting the ester samples.

The measurement was compared with those obtained by dissolving samples of the two esters in 70 per cent. ethanol.

Isolation of the products. 10 ml. of Dilution A was diluted to approximately 20 ml. with water and extracted with 3×5 ml. portions of chloroform. The combined chloroform extracts were washed with water, dried (anhyd. $MgSO_4$), and the chloroform removed under reduced pressure.

The alphaprodine solution yielded a solid product (53 mg. $\equiv$ 70 per cent. yield calc. as alcohol V). Recrystallisation from *n*-hexane gave α-1:3-dimethyl-4-phenyl-4-hydroxypiperidine (42 mg.) as white prisms, m.pt 101 to 102° C. (Found: C, 76·3; H, 9·3; N, 6·9 per cent. Equiv. 203. Calc. for $C_{13}H_{19}ON$: C, 76·0; H, 9·3; N, 6·8 per cent. Equiv. 205). (Ziering and Lee[1] report m.pt. 103° C.)

The betaprodine solution yielded a gum (61·5 mg. $\equiv$ 80 per cent. yield calc. as alcohol (V)) (Found: Equiv. 203. $C_{13}H_{19}ON$ requires Equiv. 205). The gum slowly solidified and recrystallisation from *n*-hexane gave β-1:3-dimethyl-4-phenyl-4-hydroxypiperidine as white needles, m.pt. 77 to 78° C. (Found: C, 76·2; H, 9·3; N, 6·95. $C_{13}H_{19}ON$ requires C, 76·0; H, 9·3; N, 6·8 per cent.) (Equivalent weights of the bases were determined by dissolving the samples in glacial acetic acid and titrating with 0·02N perchloric acid in glacial acetic acid using crystal violet as indicator.)

The authors thank Roche Products Ltd. for samples of alphaprodine and betaprodine.

SUMMARY

1. A study of the reactions of alphaprodine and betaprodine in alkaline solutions under comparable conditions has been made.

2. As a result of experimental observations and conformational considerations, alphaprodine is now allocated the *trans* (Me/Ph) and betaprodine the *cis* (Me/Ph) configuration, (i.e., the reverse of the previous provisional assignments).

3. The new assignments result in the observed differences in analgesic potencies of the isomers being more readily explicable in terms of the receptor theory proposed by Beckett and Casy[3].

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DISCUSSION

The paper was presented by DR. A. H. BECKETT.

MR. A. R. ROGERS (Brighton) asked whether the authors had made absorption measurements on the purified alcohols which were isolated and, if so, whether the data could be included in the published paper.

DR. J. B. STENLAKE (Glasgow) said that the paper provided definite evidence on rates of hydrolysis, and was another example of the successful application of configurational assignments to ring systems. The relation of configuration and resistance was fraught with several hazards. There was in addition to normal non-bonded interactions the possibility of electrostatic interactions between the basic group and other groups present. It was a pity that the differences in rates of hydrolysis which the authors had been able to establish were not more marked, and they might consider the use of dissociation constant measurements.

DR. A. H. BECKETT, in reply, said that the ultra-violet analysis of the purified alcohols had been carried out, and since the submission of the paper one of the alcohols had crystallised, so it would be possible to insert the actual analysis at a later stage. Dissociation constants had been considered, but a more suitable method would be that of elimination experiments which would give a cross-link with hydrolysis.

A NEW APPROACH TO STERILITY TESTING

By A. Royce and G. Sykes

Microbiology Division, Standards Department, Boots Pure Drug Co. Ltd., Nottingham

In carryirg out any aseptic operation, extraneous contamination may be introduced from the environment or from the operators, and many and varied precautions are practised in efforts to minimise it. The purpose of this paper is to describe a sealed screen technique which has proved successful in enhancing the precision of sterility testing; it can also be applied to other difficult small scale operations.

Such operations usually require specially designed "sterile" rooms which can be thoroughly disinfected before each work session. The rooms are often provided with airlocks and there is usually a flow of sterile air to keep the level of aerial contamination as low as possible. However, it must be accepted that complete elimination of all airborne contamination in a room occupied by even one or two operators is unattainable, in spite of the most careful preliminaries of "scrubbing up" and the wearing of sterilised gowns and other coverings. Therefore, as further precautions for insulating the work from such contamination and so preventing access of organisms to the material being handled, screens of various designs are employed, combined with careful flaming techniques, studied motions and minimum exposure of materials and containers to the open atmosphere.

Such methods used properly can result in negligible adventitious contamination. Nevertheless they are not absolute; the degree of contamination introduced is variable and depends on the sustained concentration and skill of the operators and of the difficulty of the operations concerned. For these reasons a detailed training scheme for all operators should be followed, such as that proposed by Coulthard[1], and the techniques employed need to be continually checked, as emphasised by Sykes[2], to assess the level of contamination introduced and to maintain the necessary high degree of asepsis.

The enclosed screen technique described below is an attempt to approach more closely and with greater certainty the absolute standard of asepsis required in sterility testing. It can be used for many other aseptic manipulations, but it is not universally applicable, owing to certain limitations mentioned later. The actual manipulations within the screen are more difficult and possibly slower to carry out because of restriction of movements imposed by the enclosed design. But set against these disadvantages are the distinct advantages of being able to dispense with sterile rooms and the usual scrubbing up and sterile dressing procedures. Consequently there is a considerable saving of time in the preliminary preparations resulting in even greater productivity where large numbers of tests for sterility have to be carried out. Moreover, the greater certainty of the method practically eliminates repeat tests made necessary from adventitious contamination introduced during testing.

The Sealed Screen

Design and construction. The screen consists basically of a sheet-metal sealed box, with a large removable hatch, and fitted with long-sleeved rubber gauntlets. The size can be varied according to requirements but it is limited by the reach of the arms within the screen, unless reaching tools, tongs, etc., are provided. The model illustrated in Figure 1 is approximately 2 ft. 6 in. long, 2 ft. deep and 1 ft. 6 in. high. It is built on an angle-iron frame to such a height as will enable an operator to sit
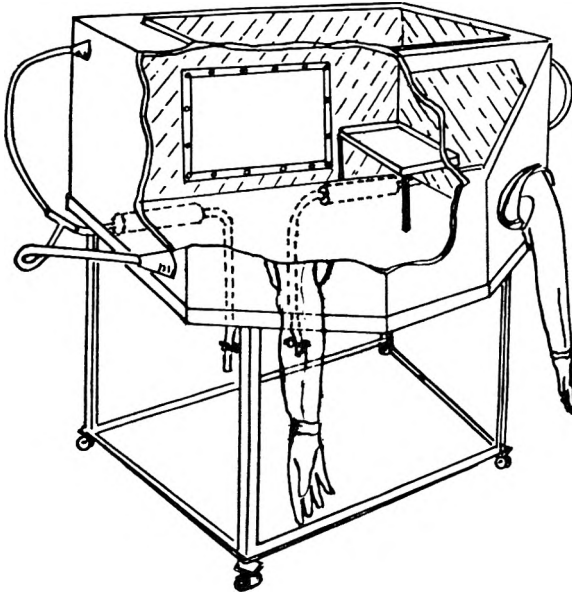

FIG. 1. Diagram of aseptic screen.

comfortably at the screen; it is mounted on wheels for mobility. All the joints of the screen must be well sealed with solder or other sealing compound so that it is practically gas-tight. It is provided with two perspex windows, one in the top to admit light and another in the sloping front to enable the operator to see inside. The removable hatch, through which the screen is loaded and unloaded, is built in the back; it is fixed in position by means of a number of wing nuts and sealed with a sponge rubber gasket. A removable tray is provided to take the remnants of samples, used syringes and other discarded materials. At diagonally opposite corners of each end of the screen are fixed short metal tubes joined by rubber tubing to air filters clipped to the back of the screen, the pair of tubes at one end being connected to one filter and those at the other end to a second filter. The filters are made from glass or metal tube about 1 ft. long and $1\frac{1}{2}$ in. in diameter and packed with non-absorbent cotton wool. A screw clip on a short length of rubber tubing at the distal end of each filter allows it to be closed off as required.

The screen is also provided with two oval, flanged arm holes to which the rubber gauntlet gloves are sealed; the oval shape allows a certain lateral arm movement. The holes are set on the angle faces of the front to give the most comfortable and efficient working position for the operator. The gauntlet arms are made of heavy gauge rubber and are wide enough at one end to fit the flange of the arm holes, to which they are sealed by means of a sponge rubber gasket and a metal band fitted with a tightening screw. The gauntlets are tapered down their length so that at the other end they can be sealed to the wrists of rubber gloves. Heavy domestic grade gloves are necessary; those of surgical quality are not sufficiently robust for this purpose as they are too easily ruptured. It is advisable to reinforce the rubber joints with an elastic adhesive band.

From the foregoing description it is seen that when the door is in position and the air filters are sealed off, the whole screen is, for all practical purposes, gas tight. Within the screen, any normal hand and finger manipulations can be carried out by an operator who is himself external to the system.

Operation of the screen. The principle of the operation of the screen is that the whole of the contents, including the surfaces of apparatus, bottles and other containers placed in the screen, can be sterilised in a suitable gaseous atmosphere—ethylene oxide is used for this purpose—after which any aseptic manipulations can be carried out within the screen without any possibility of contamination from the environment or operator.

Because the sterilising agent is a gas, it is obvious that it must not gain access to any of the materials being handled, neither must it be in contact with liquids in which it may dissolve or react. Therefore, all samples and culture media must be in sealed containers. Screw-capped containers for culture media are not novel; they were advocated some years ago[3], and they are in common bacteriological use to-day. For anaerobic media they are advantageous as the seal reduces to a minimum the rate of diffusion of oxygen into the sterilised media. For aerobic media an adequate air space must be left. In practice, a space equivalent to one-quarter of the total capacity of the container allows the free growth of strictly aerobic organisms.

The test samples, the necessary culture media, syringes and any apparatus required for measuring, weighing or redistributing into other containers, are first loaded into the screen. All requirements should be known and remembered at this stage, as any item forgotten cannot be put into the screen once it has been sterilised. Syringes, pipettes and other apparatus required for handling samples can be sterilised *in situ*. It is advisable, however, to treat them in the autoclave beforehand in suitable containers, so that the sterilising gas has then only to deal with a superficial surface infection and is not required to disinfect the inner surfaces of needles, syringe barrels and pipettes where diffusion of the gas may not always be adequate to sterilise in the time allowed.

A 12·5 per cent. (v/v) concentration of gaseous ethylene oxide is used for sterilising. Since it is liquid at temperatures below about 10° C.

it is most conveniently handled in this form. Therefore, the calculated quantity of the liquid in a chilled, screw-capped bottle is placed in the screen and the hatch is fastened in position. Immediately after closing the screen, the liquid ethylene oxide is poured on the floor of the screen. It immediately evaporates and the slight increase in pressure created in the screen is allowed to escape through the filters. When the balance is restored, the filters are sealed off by means of the screw clips and the sterilisation process is allowed to proceed overnight for 16 to 24 hours. After completion of this period the screen is flushed for about half an hour with sterile air introduced through one of the filters. The flushing must be such that it removes virtually all of the ethylene oxide gas. Subsequently, manipulations of any sort, including the opening of the bottles of culture media, can be carried out in the screen with impunity.

Two points should be borne in mind in connection with the sterilisation procedure. First, there is a small loss of ethylene oxide from the system when the excess pressure in the screen is allowed to escape. Secondly, there is a small loss by absorption of the gas by the rubber of the gauntlets and gloves. These losses can easily be balanced by including a slight excess of ethylene oxide in the first place, a 10 per cent. excess is adequate.

One objection to the technique is that owing to the enclosed nature of the screen a heavy contamination in one batch of a product, or even in one container of a batch, could easily cross-infect tests on other materials being examined at the same time. However, before materials are submitted for testing, they have usually been processed in such a way as to be reasonably certain that they are sterile. Most groups of samples examined are, in fact, sterile, or the contamination encountered is sufficiently light to render spread of infection highly improbable. The few products in which a heavy contamination may arise, due to the nutrient properties of the solution and the absence of a bacteriostatic agent, are known to the experienced operator and special isolating precautions can be taken. The simplest procedure with suspect material, including that which may have shown a contamination in a previous test, is either to put the tests on separately or to perform them last of a series in the screen.

The sterilising gas. Several gases might conceivably be chosen as suitable sterilising agents, but the majority have physical or chemical disadvantages. Thus chlorine and sulphur dioxide would attack the metal surfaces, and formaldehyde is difficult to flush out of the screen. For these reasons, ethylene oxide was chosen.

Ethylene oxide can be used as a sterilising agent both in solution[4], and in the gaseous phase. Its disinfectant action in the gaseous phase was first recorded in an American Patent[5] in 1936, since which time several further publications have appeared on the subject. It has been reported that glass, metal and paper surfaces and dry or wet rags infected with *Bacillus anthracoides* were easily disinfected in 8 hours by a concentration of 200 mg. per litre[6], and soils of different types were sterilised by the gas in periods ranging between 2 hours and 6 hours[7]. It is asserted also[8] that blankets and linens, soiled or laundered, are completely sterilised overnight by 10 per cent. of ethylene oxide in carbon dioxide. A useful review

of the subject was presented by Phillips and Kaye[9] who in subsequent papers[10,11] discussed the influence of time, concentration, temperature and moisture on the rate of disinfection of the spores of *Bacillus globigii*.

In our experience with the screen, we have found that a 12·5 per cent. gaseous concentration will sterilise glass and metal surfaces in 16 hours, provided the surfaces are clean and dry. If they carry any grease films or dried broth residues, etc., organisms may be protected from the action of the ethylene oxide and so remain viable. With the proper precautions taken, ethylene oxide has been uniformly successful in daily use for a period of over 3 years.

Attention must be drawn to certain of the undesirable properties of ethylene oxide. It boils at 10·7° C. and its vapour is toxic when inhaled. It is also explosive in mixtures with air between 3 per cent. and 100 per cent., but carbon dioxide quenches its explosiveness. Finally, in contact with the skin it can cause severe reactions. The amount absorbed by rubber is significant in this respect, so that unless due precautions are taken the operators are liable to suffer eruptions on the hands and arms. This has been studied in detail by Royce and Moore[12], who found that the danger could be obviated by adequately airing the gloves by hanging them in free air (see Fig. 1) for a minimum of 1 hour after flushing the screen.

DISCUSSION

The screen described can be applied to all types of tests for sterility where the material is packed in gas-tight containers. Thus it cannot be used in testing surgical dressings. On the other hand, it is valuable for carrying out many complicated aseptic manipulations with a greater degree of certainty. In this connection it can be used to advantage for carrying out the Davies and Fishburn[13] filtration test technique which otherwise is subject to the hazard of accidental aerial contamination. It is also useful for such operations as breaking down quantities of sterile bulk solids into smaller containers where perhaps weighings are involved, and for dispensing sterile media or other solutions which are heat labile or require the mixing of a number of previously sterilised constituents.

The principal virtue of the technique, however, is that in sterility testing it eliminates almost completely the risk of infection from outside sources, so that any growth occurring in a test must almost certainly have originated from the material under examination. The question does not arise, therefore, whether a contamination might have been introduced during testing. To illustrate the value and reliability of the technique in sterility testing, it has been subject to control testing since the screens were put into operation in 1951. A "control" test is one carried out under normal conditions but with test material such as water, saline or sodium chloride sterilised in their appropriate containers by a reliable process in the laboratory. They represent tests on the "bulk" stage of a manufactured product and on the "final container" stage in which 20 containers are examined in each test. Of some 800 such control tests carried out during the period, only one was contaminated.

SUMMARY

1. A sealed screen is described. It consists of a sheet-metal, sealed box with a large removable hatch and is fitted with long-sleeved rubber gauntlets.

2. The contents and the surfaces within the screen are sterilised by gaseous ethylene oxide after which any aseptic operations may be carried out free of contamination from environment or operator.

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DISCUSSION

The paper was presented by MR. A. ROYCE.

DR. H. S. BEAN (London) said that if a suitable technique on the lines proposed could be developed it would considerably simplify sterility testing. It would be interesting to learn how the authors obtained the sterile air which was passed into the screen. He asked why the authors resorted to a rather hazardous material such as ethylene oxide since there were other chemicals available. Aerosols which functioned in low concentrations would not be as dangerous. In his experience screw cap containers were not ideal for growing cultures, and he sometimes failed to grow *B. subtilis* in such bottles.

DR. R. M. SAVAGE (Barnet) said that it should be emphasised that what the test did was to reduce the contamination to a very low level and not to eliminate it altogether. In the last paragraph of the discussion it was stated that "in sterility testing it eliminates almost completely the risk of infection from outside sources . . ." but there followed the statement that "any growth occurring in a test must certainly have originated from the material under examination." It would be preferable to see the word "almost" inserted before "certainly" because the possibility of contamination had not been reduced to zero. One would also have liked to see some evidence that material which was very lightly contaminated had not had that light contamination reduced.

MR. A. ROYCE, in reply, said that the sterile air was developed *in situ* by blowing the air into the screen and withdrawing an equal volume of mixed air and gas through a cotton-wool filter. The aim was to eliminate all organisms in the screen; therefore it was felt that the method used was better than using an aerosol. In the screw-capped containers satisfactory

growths were obtained with organisms in solid and fluid media. When the air space was of the order of a quarter or one-third there was no difficulty with aerobic organisms. He agreed that the word "almost" should be inserted. It was felt that the present method gave conditions as near as possible to absolute sterility, but it was agreed that under special conditions it could break down. If anything did go wrong one was left in no doubt that it had because of the gross contamination, but this had only happened twice in some 20,000 tests over three years.

THE ESTIMATION OF VITAMIN B₁ IN PHARMACEUTICAL PRODUCTS

BY J. J. DOHERTY, NORA CANE and F. WOKES

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BIOLOGICAL assays[1] of vitamin B₁ were described in the first addendum, and fluorimetric assays in the seventh addendum, to B.P. 1932. The fluorimetric method has been shown to give satisfactory agreement with biological and microbiological methods when applied to unmalted and malted cereals, malt extract, malt and oil, yeast and meat extracts[2,3,4,5]. Nevertheless, in the B.P. 1953, the fluorimetric method was replaced by a chemical precipitation method[5] claimed to be more accurate. This precipitation method was shown to give satisfactory agreement with the fluorimetric method on 4 samples in which different degrees of destruction of the vitamin had been produced by heating. No comparison was made with biological, microbiological or spectroscopic methods. The precipitation method requires much larger quantities of material than the fluorimetric method, and further investigation of the latter seemed desirable before eliminating it from pharmaceutical analysis, especially as it has proved so successful in food analysis. Our fluorimetric results on B.P. injections of vitamin B₁ have been checked by a spectrophotometric method which has enabled us to detect changes in the vitamin molecule which are not directly shown by the fluorimetric method.

METHODS

The fluorimetric method[5] employed was that recommended by the Vitamin Sub-committee of the Society of Public Analysts (now the Society for Analytical Chemistry,) but more correct evaluation of results was secured by using a modified fluorimeter[7] and adopting precautions suggested in a recent treatise[8]. These precautions included more exact measurement of blanks, which the highly sensitive fluorimeter made possible, and use of cuvettes calibrated by means of cross-over experiments. They enabled us to improve the accuracy of our fluorimetric measurements, the coefficient of variation being reduced below 1·0.

The gravimetric (chemical precipitation) method was that of Bessot[9] as used by Adamson and Handisyde[6]. Spectrophotometric estimations were made using the Beckman DU Photoelectric Spectrophotometer, allowance being made for irrelevant absorption as described below. A number of microbiological assays, using *Ochromonas malhamensis*, were kindly carried out for us by Dr. J. E. Ford of the National Institute for Research in Dairying.

EXPERIMENTAL

Spectroscopy of vitamin B₁. Spectroscopic methods offer promise for the assay of vitamin B₁ injections in which little irrelevant absorption is

caused by the other substances present. Our tests on a number of commercial samples showed them to be free from such substances, although the possibility remained of other samples containing interfering substances. Allowance for these might be made by the Morton-Stubbs method, provided that the specific absorption band is not too much distorted. However, difficulties arose because of the susceptibility of the absorption spectrum to slight changes in pH, which may explain why no spectrophotometric method of assaying vitamin B_1 has received official recognition, in spite of its apparent advantages of simplicity and accuracy. A detailed study of pH effects was therefore undertaken.

Effects of p*H.* Since attention was first drawn[10] to this effect, various workers[10,11,12,13,14] have explored the changes in the absorption spectrum of vitamin B_1 when the pH of the solution is raised from 1 to 7. The single peak at about 245 mμ seen in solutions at pH 1 to 3 gradually changes to 2 separate peaks at approximately 236 and 265 mμ with considerably lower extinctions. The greatest drop in extinction occurs at approximately 245 mμ. On bringing the pH back to about 1, the 2 peaks revert to the single one, but the reversibility may not be quite complete, since a slight flattening of the peak may occur[13].

An attempt was made[15] in 1946 to allow for the pH effect by measuring the extinction at 5 different wavelengths—250, 255, 260, 265, 270 mμ— and statistical examination of the results indicated some improvement in accuracy. However, the method, which had been developed using photographic techniques, does not seem to have been adapted to the more rapid and accurate photoelectric techniques which have since come into general use.

Isosbestic point(s). Our investigations show that as the pH of a vitamin B_1 solution is changed from 1 to 7, significant alterations in extinction occur at all the above 5 wavelengths. The most marked are at about 245 mμ. There is, however, no change in extinction at approximately 273 mμ over the whole pH range 1 to 9, and this establishes the presence of an isosbestic point (see Figs. 1 and 2) at approximately 273 mμ. Indications of the occurrence of such an isosbestic point have been provided by data published by several workers[13,14] but none of these seems to have mentioned its existence or attempted to use it in spectrophotometric assays.

We have used this isosbestic point to detect any changes in irrelevant absorption which may occur when the pH of the solution is altered from 1 to 7. As will be seen from Figures 1 and 2, this alteration produces a marked change in E 245 mμ but no change in E 273 mμ for the pure vitamin. Any change in E 273 mμ which does occur in our extracts when the pH is raised from 1 to 7 must be due to irrelevant absorption, for which due allowance can be made, so that the corrected change in E 245 mμ becomes directly proportional to the concentration of the vitamin.

Spectrophotometric method. Our spectrophotometric assays of vitamin B_1 injections are made as follows. A stock solution containing about 0·25 mg./ml. of vitamin B_1 is prepared by diluting with 0·1N hydrochloric acid a known volume (about 1 ml.) from an ampoule to a known volume

(e.g., 100 ml. for 1 ml. of a 25 mg./ml. injection.) This stock solution is stored in a cool dark place and used on the day it is prepared. An aliquot of 2 ml. of this stock solution is diluted to 50 ml. with 0·1N hydrochloric acid, giving a dilution containing about 10 μg/ml., which in a 1 cm. cell is suitable for measurement of extinction in a photo-electric spectrophoto-meter.

Another aliquot of 2 ml. is diluted to 50 ml. with the B.P. phosphate buffer pH 7, and similarly examined. The extinc-tions needed for the assay are only those at 245 and 273 mμ, but it is helpful to take readings also at every 5 mμ between 240 and 290 mμ to elucidate the general shape of the absorption curves.

To calculate the vitamin B₁ content, it is first necessary to correct the observed change in E 245 mμ for any alteration in that extinction caused by the pH change on the irrelevant absorption, as indicated by a change in E 273 mμ. In our experience with B.P. injections of vitamin B₁, if there

FIG. 1. Absorption spectra of vitamin B₁ solutions (10 μg./ml.), at — pH 1, — — pH 3·9, ···· pH 5·4, — — — pH 6·9.

is any such correction needed, it is very slight. The corrected change in E 245 mμ is then directly proportional to the vita-min B₁ content, an in-crease of 0·1 in E 245 mμ produced by changing the pH from 7 to 1, being equivalent to approxi-mately 5 μg. anhydrous vitamin B₁ per ml.

Our spectroscopic data also indicate that under certain conditions, there may be another isosbestic point at approximately 236 mμ. Such an ad-ditional point could be of
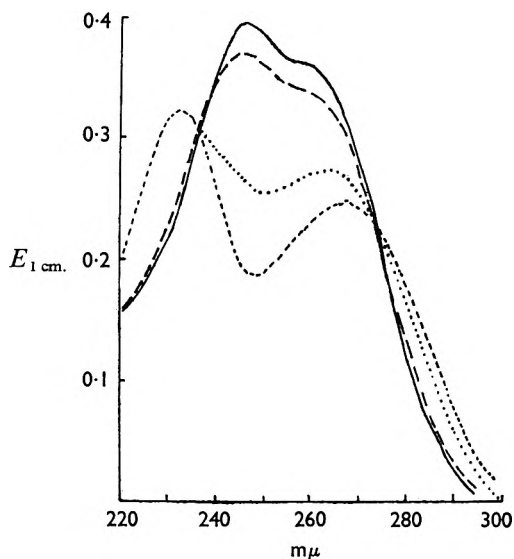

FIG. 2. Effect of pH changes on extinction of vitamin B₁ solutions (10 μg./ml.) at

····	250 mμ	— · —	265 mμ
— —	255 mμ	— ·· —	270 mμ
— — — —	260 mμ	———	273 mμ

great value in supplementing the check on irrelevant absorption provided by the other isosbestic point at approximately 273 mμ. However, we have not yet established this second isosbestic point with certainty. Some variations which we have observed in it are probably due to factors connected with pH changes which affect the molecular structure and may be responsible for slight distortions of the peaks at approximately 236 mμ and 265 mμ in neutral solution and at approximately 245 mμ in strongly acid solution.
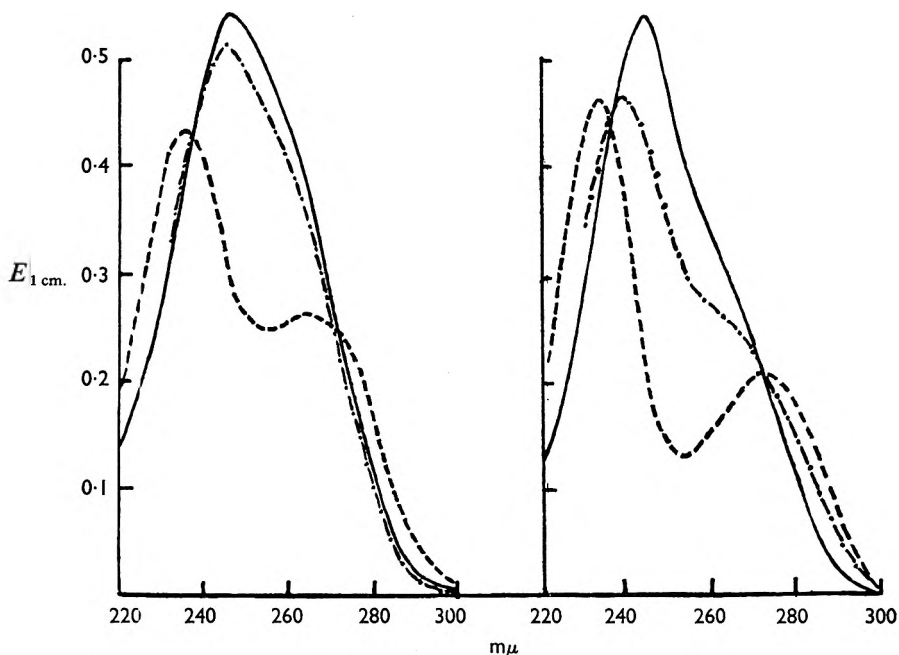

FIG. 3. Absorption spectra of pyrimidine derivatives, 2-methyl-4-amino-5-hydroxy-methylpyrimidine (A) and 2-methyl-4-amino-5-aminomethylpyrimidine dihydrochloride monohydrate (B), at ——— pH 1, — · — pH 5, - - - - pH 7. Aqueous solutions of 6·7 and 10·3 μg./ml. respectively.

If this spectrophotometric method is applied to materials in which the vitamin B_1 has been broken down into its pyrimidine and thiazole components, complications may arise because of the fact that the spectra of the mixed components closely resemble[13] those of the unaltered vitamin. Moreover, pyrimidine derivatives show with alterations in pH a series of changes similar to those given by vitamin B_1 (see Fig. 3.) However, the spectra of thiazole derivatives, whilst changing with pH, do not show an isosbestic point. (See Fig. 4.) We therefore do not consider our spectrophotometric method suitable for the assay of vitamin B_1 in preparations in which there has been extensive destruction of the vitamin, e.g., by excessive heating. However, in our experience, the method gives reliable results on injections subjected to any reasonable sterilisation or storage conditions.

Sulphite splitting of vitamin B_1. Whilst our work was in progress, a paper was published by Somogyi[13], describing a spectrophotometric method of assaying vitamin B_1 by measuring the increase in E 250 mμ produced by heating to 100° C. with sodium pyrosulphite. This method gave good agreement with the fluorimetric method when applied to simple aqueous solutions of vitamin B_1, but was not found to be completely reliable for more complex materials, possibly because the sulphite treatment had altered the irrelevant absorption at 250 mμ. It is of interest to note that Somogyi's published data showed isosbestic points at approximately 235 mμ and 265 mμ which might perhaps be used to correct for this action on the irrelevant absorption and thus improve Somogyi's method.

Using our spectrophotometric method, we have assayed vitamin B_1 in a number of B.P. injections. Our replicate assays show an average coefficient of variation of 0.51 ± 0.3 which compares favourably with that previously obtained with the M.R.C. spectroscopic method[15] when applied to solutions of pure vitamin B_1. We therefore used this spectrophotometric method, in comparison with fluorimetric and precipitation methods,

FIG. 4. Absorption spectra of 4-methyl-5-hydroxyethylthiazole at —— pH 1, —·— pH 5, — — pH 7. Aqueous solutions of 16·2 μg./ml.

in a series of experiments on the effect of heat and light on vitamin B_1.

Effect of heat on vitamin B_1. Experiments were carried out at 3 different temperatures—70° C. (in a hot air oven,) 100° C. (in a boiling water bath) and 112 to 115° C. (in an autoclave.) The vitamin B_1 solutions ranged from 1 to 30 mg./ml., but were all at the same approximate pH (3·7) and were contained in sealed ampoules such as are used for injections. The results are summarised in Figure 5, in which a logarithmic time scale is used to enable long term storage results also to be included, and the values are all shown as percentages of the respective initial strengths, which are indicated in the legend. As the mean fluorimetric result was 101 $\pm$ 1·9 per cent, and the mean spectrophotometric result was 105 $\pm$ 2·1 per cent. of the mean gravimetric method, we considered it justifiable to include in the figure results obtained by all 3 methods.
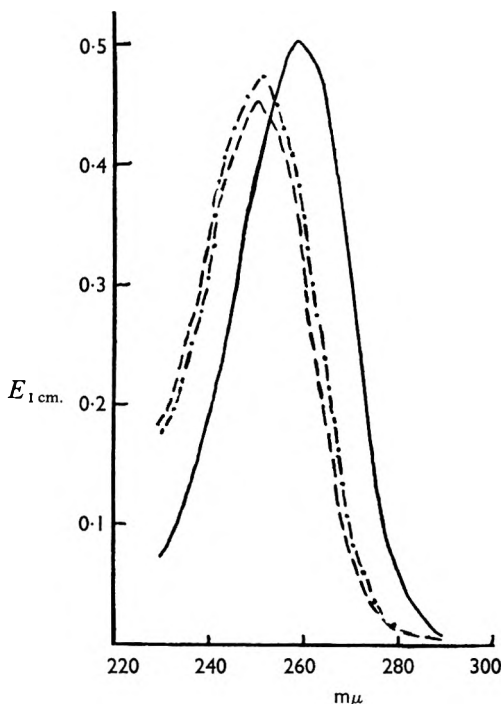
The rate of loss at 100° C. was slower than was observed by Adamson and Handisyde, perhaps because of a difference in pH or other experimental conditions. The pH of their solutions was not stated. However, our conditions, though they may have differed from those of these workers, were still such as might be encount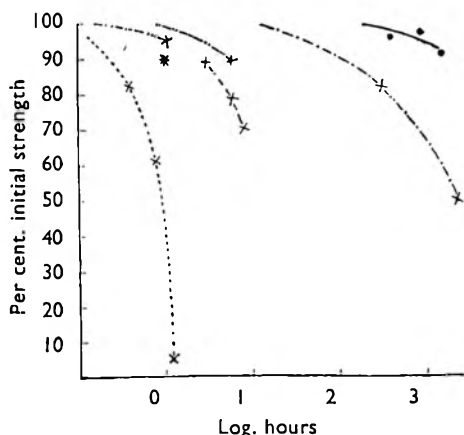ered in general practice, and hence we were glad to confirm their finding that the fluorimetric results agreed with the gravimetric results.

Comparison of the rate of loss at 70° C. with that at 100° C. indicated a very low temperature coefficient. However, the solution stored at 70° C. developed a yellow colour, which first became visible within 2 or 3 days storage, and steadily increased in intensity, being so marked at the end of a week that the ampoules would certainly not have been accepted for clinical use. As a loss of only 9 per cent. was indicated by the physicochemical methods, we had the injections assayed microbiologically which showed no larger losses. Thus, although the yellow colour suggested serious deterioration, this was not confirmed by any of the 4 assay methods employed.

FIG. 5. Effect of heat and light on vitamin B_1. Results on injection solutions at room temperature in sunlight indicated thus ●——●. Results on injection solutions at—

70° C. in dark indicated thus × — · — ×.
100° C. „ „ „ „ × —··— ×.
112 to 115° C. in dark indicated thus × —···— ×.
100° C. (Adamson and Handisyde) indicated thus × — — ×.
100° C. (Beadle *et al.*) indicated thus *.

Results on baked vitamin B_1 indicated thus × ------ ×.

Ordinates indicate vitamin B_1 content as per cent. of initial content. Abscissæ indicate duration of treatment in hours on log. scale.

Effect of light on vitamin B_1. Although the experiments at 70° C. had been carried out in a closed hot air oven, there was a possibility that during the prolonged storage light might have penetrated the solutions and caused development of yellow colour. We therefore carried out a number of experiments in which the ampoules were fastened by transparent tape to the upper part of a window facing south west and hence exposed to sunlight for much of the day. The results, which are also summarised in Figure 5, indicated a lower rate of loss than in the solutions stored at 70° C., but the difference was much less than would have been expected from the temperature coefficient between 70° C. and 100° C. This suggested some destructive action by light. Again there was marked development of yellow colour and also pungent odour, signs of definite deterioration.

Effect of baking on vitamin B_1. In the above experiments the losses of

vitamin B_1 were not very great, and in some instances were not much larger than the experimental error. In order to test the different assay methods more critically, we decided to subject the vitamin to the drastic conditions of baking, such as are applied to dietetic products in which it may be present. A diabetic bread, for example, may be heated to 180° C. to 190° C. for 60 minutes. A number of experiments were carried out in which a known quantity of the vitamin, dissolved in 0·1N hydrochloric acid, was thoroughly mixed with "filter aid,"* and the mixture placed in an evaporating basin covered with a clock glass and passed through an automatic gas heated oven for 25 minutes at approximately 230° C. The baked material, after cooling, was extracted with 0·1N hydrochloric acid, filtered through No. 3 sintered glass and then through No. 3 Whatman, and the vitamin in the filtrate was assayed by the 3 methods.

Control experiments on unbaked mixtures showed that extraction of the vitamin was not quite complete, the recovery ranging from 95 to 98 per cent. Due allowance was made for these small losses when calculating the percentage of vitamin destroyed by the baking. The net results thus obtained indicated by the fluorimetric method an average loss of 30·1 per cent. and by the gravimetric method an average loss of 34·8 per cent. in 4 experiments each of 25 minutes heating. The spectrophotometric method indicated an average loss of only 16·9 per cent. in the same experiments. The absorption curves revealed breakdown of the molecule which was probably responsible for the low results. In a further experiment with more prolonged heating there was good agreement between the average fluorimetric and gravimetric results, which indicated losses of 41·0 and 38·3 per cent. respectively, although the gravimetric result was now higher than the fluorimetric result, and the precipitate looked rather abnormal. Spectrophotometric results indicated a lower loss of 20·2 per cent. Finally, very drastic baking (3 periods of 25 minutes) was employed in an attempt to destroy most of the vitamin. The fluorimetric results indicated an average loss of 94·8 per cent., but the gravimetric results indicated an average loss of only 60·4 per cent., and the precipitate was quite abnormal, consisting of brown particles in place of the usual fine white powder.

Discussion

The main purpose of this investigation has been to compare the fluorimetric method of assaying vitamin B_1 with the present official gravimetric method. The accuracy and specificity of the latter is not disputed, as far as general pharmaceutical analysis is concerned, although its reliability may be diminished when it is applied to products which have been subjected to prolonged baking. The gravimetric method has, however, one serious disadvantage—that it requires a minimum of about 25 mg. of the vitamin for each assay, and therefore would not be applicable to single ampoules of low potency injections. The fluorimetric method is far more sensitive, measuring as little as 2 or 3 μg. of the vitamin, in a much shorter

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* Dicalcite special speedflow obtained from F. W. Berk & Co., Ltd.

time, provided that a carefully calibrated fluorimeter is available. Our fluorimetric results have shown good agreement with our gravimetric results on materials which have been subjected to different degrees of exposure to heat and light covering practically all possibilities in normal pharmaceutical practice, and we therefore think that the *specificity* of the fluorimetric method need not be questioned. The *accuracy* of this method must now be considered.

Adamson and Handisyde rejected the fluorimetric method in 1948 because of lack of accuracy, stating that it cannot be relied upon to give results of much greater precision than $\pm$ 5 per cent. As far as assays of vitamin B_1 tablets are concerned, this may well be true for the method published[16] from these laboratories in 1945, which as then described was less accurate, though more sensitive, than the gravimetric method described by Adamson and Handisyde in 1948. Its accuracy might indeed be considerably improved by recent advances in fluorimetric techniques, particularly for overcoming quenching difficulties.

Turning to fluorimetric assays of vitamin B_1 in solutions for injection, the accuracy of these was studied by the Aneurine Panel of the Society of Public Analysts (now the Society for Analytical Chemistry) by means of calibration experiments with quinine solutions against solutions of thiochrome obtained from aneurine, as well as by collaborative assays on aneurine solutions circulated to different laboratories. The results obtained in half a dozen well-known laboratories, specified in the Panel's report[5], indicated that the coefficient of variation in such assays usually lay between 1·0 and 1·5 and could be reduced to 0·5 to 1·0 by utilising cross-over tests to eliminate errors due to differences between different cuvettes. We therefore think that for assays of vitamin B_1 in injections, the fluorimetric method, with suitable modifications, might well be employed *not* to replace the gravimetric method, but as a supplement to it, especially for examining small amounts of material for which the gravimetric method is unsuitable. In many pharmaceutical laboratories use could thus be made of fluorimeters available for other purposes.

The alternative use of the spectrophotometric method, based on the decrease in E 245 mμ produced by lowering the pH of the solution from 7 to 1, might also be considered for the assay of vitamin B_1 in injections. The spectrophotometric results can thus provide a useful check on the gravimetric results, in instances where a fluorimeter may not be available.

Although spectrophotometric methods have at present only limited practical applications, they are of some theoretical interest because of the isosbestic points which have been revealed. These may help to elucidate the changes which take place when the vitamin B_1 molecule is exposed to heat and light.

Finally, our results provide a certain amount of new information on the effect of heat and light on the vitamin. When they are correlated, as far as is possible, with the findings of other workers, they help to make a more complete picture of this phenomenon which is of importance in pharmaceutical and food problems.

SUMMARY

1. A spectroscopic study of vitamin B_1 over the pH range 1 to 9 has revealed the existence of an isosbestic point at approximately 273 mμ which can be used as a check on the production or destruction of irrelevant absorption when the pH is lowered from 7 to 1. This lowering of pH produces a rise in E 245 mμ which for anhydrous aneurine is approximately 0·1 for 5 μg/ml., a factor employed in a new spectrophotometric assay method.

2. Results obtained by this new spectrophotometric method have been in good agreement with those obtained by the fluorimetric and gravimetric methods on B.P. injections of vitamin B_1 before and after subjection in various degrees to heat and light. The latter causes development of a yellow colour which may be due to breakdown of the vitamin, although this is not detected by the fluorimetric, spectrophotometric or gravimetric methods, or by the microbiological method using *Ochromonas malhamensis*.

3. Baking vitamin B_1 at about 230° C. produces breakdown of the molecule as evidenced by the distorted absorption spectra which invalidate the spectrophotometric assay. The fluorimetric and gravimetric results are in agreement for short period baking (e.g., 25 minutes,) but as baking is prolonged the gravimetric method gives too high results, that is, too small losses.

4. On the basis of these findings it is suggested that the present official vitamin B_1 assay for injections be supplemented by an optional alternative method, preferably fluorimetric or failing that, a spectrophotometric method as outlined above.

5. The results on the rate of destruction of vitamin B_1 by heat and light enlarge the picture obtained by previous workers.

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DISCUSSION

The paper was presented by DR. F. WOKES.

DR. G. E. FOSTER (Dartford) said that in the present method of estimating vitamin B_1 by means of silicotungstic acid, the factor varied with each batch of reagent, and he determined the factor for each batch of silicotungstic acid, using one particular batch of aneurine hydrochloride.

DR. D. C. GARRATT (Nottingham) asked whether the authors suggested that an alternative method should be included in the Pharmacopœia, because for the official preparations the silicotungstic acid method was sufficiently accurate.

MR. WHITTET (London) said that in his experience discolouration of vitamin B_1 did not appear to indicate loss of activity.

DR. F. WOKES, in reply, said that only one batch of silicotungstic acid was used. The work had shown that the gravimetric method was entirely satisfactory for the injections, even when they had been subjected to much more drastic storage and exposure to light than would occur in normal practice, but it had a serious disadvantage in that it required a minimum of 50 mg. of vitamin B_1. Despite the deep yellow colour produced in ampoules after exposure to sunlight for months, there was no evidence of decomposition of the vitamin itself. He was gratified that Mr. Whittet confirmed this finding.

RESEARCH PAPERS

THE POISONOUS PRINCIPLE AND HISTOLOGY OF *GLORIOSA VIRESCENS* LINDL.

By E. H. W. J. Burden, D. N. Grindley and G. A. Prowse

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IN recent years many instances of poisoning have been examined by these laboratories which have been received from the Police and Medical Authorities of Equatoria. In this Province of the Sudan, native witch-doctors are very active, and certain articles of their *materia medica* occasionally find their way into the hands of unscrupulous persons who use them for criminal purposes. From the viscera of several persons who died, the alkaloid colchicine has been isolated, and in connection with a recent death a specimen of plant material was submitted which was identified as a corm of *Gloriosa virescens* Lindl. Although the alkaloid of the related species *G. superba* has been isolated and identified as col-chicine, the active principle of *G. virescens* has hitherto been referred to as an unknown compound. We have now shown that the principal alkaloid present in this species is colchicine.

PHARMACOGNOSY

Gloriosa virescens Lindl, is an attractive Liliaceous plant widespread in southern Sudan, extending westwards to Darfur, and also found in the Red Sea Hills. In habit it is a climbing or semi-climbing plant, with long slender stems. The leaves are usually oblong to oblong-lanceolate, acuminate, with cirrhose tips which assist in climbing. The lower leaves may be verticillate or opposite, while the upper ones are often alternate. The flowers form a lax terminal corymb, with long pedicels, cernuous at the tip. The perianth is $1\frac{1}{2}$ to $2\frac{1}{2}$ in. in length, strongly reflexed, the segments lanceolate-cuspidate, scarcely at all crisped, and $\frac{1}{3}$ to $\frac{1}{2}$ in. broad at the middle. The colour is variable, bright red to yellow, or in the type specimens yellow tinged with green on the outside. The fila-ments of the stamens are about half as long as the perianth, the anthers linear, $\frac{1}{3}$ in. long. The style is 1 to $1\frac{1}{2}$ in. long, branched in the upper third. The capsule is 2 to $2\frac{1}{2}$ in. long. The species closely resembles *Gloriosa superba* Linn., from which it differs by being smaller in all its parts, with terminal corymbs, not axillary as in that species. The perianth segments of *G. superba* are markedly crisped, while those of *G. virescens* are scarcely so if at all.

The tubers, which are of importance in the present studies, are fleshy root tubers, 4 to 6 in. long, $\frac{3}{4}$ to 1 in. in diameter, tapering very slightly from the tip. Forking may occur in the upper 2 in., so that the root is bifid, or occasionally trifid. The tubers are free from secondary roots in the lower portions, but the remains of adventitious roots are frequently

to be found attached at the apex, as the growing plant appears to be stem rooting. The cuticle of the storage root is comparatively thin, but may thicken with age, while the colour of the freshly-dug tuber is pale yellowish-white, darkening slightly with age.

Anatomically the tubers show the features of a fleshy, monocotyledenous storage root. The ground tissue, forming the bulk of the tuber, consists
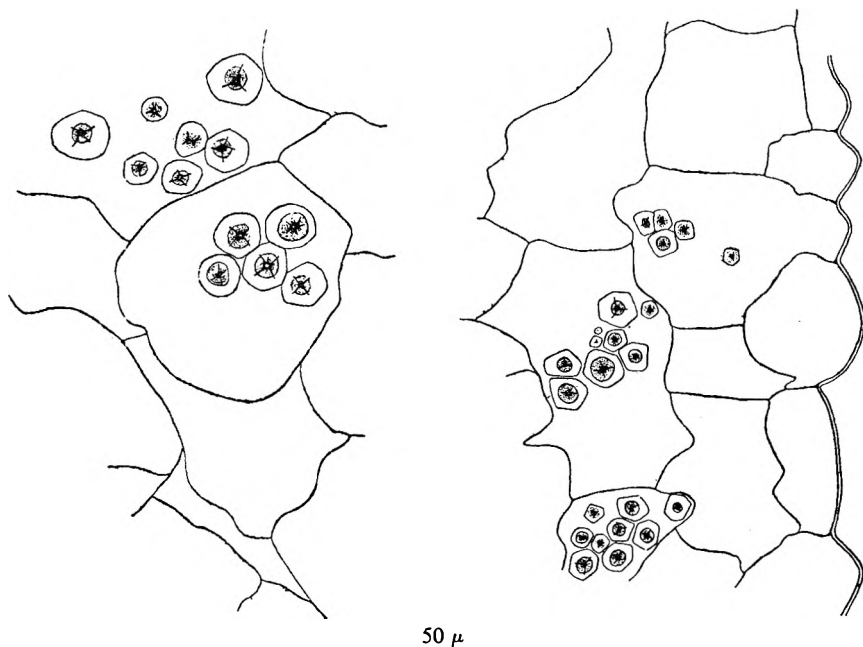

50 μ

FIG. 1A.   Transverse section of root tuber of *Gloriosa* showing ground tissue with laminated starch grain.

FIG. 1B.   Transverse section of the root tuber of *Gloriosa* showing epidermis and ground tissue.

of thin-walled, isodiametric, parenchymatous cells, many of which contain starch grains. (Fig. 1 A). These starch grains show a characteristic lamination and are especially frequent near the epidermis (Fig. 1 B) and near the vascular bundles (Fig. 1 C). It is in the ground tissue that food-storage takes place. The epidermal layer is more regularly rectangular in shape, and has a comparatively thin cuticle, but it is possible that in old tubers it may be much thicker.

The vascular bundles are scattered throughout the ground tissue in a manner typical of monocotyledons, but as the tubers are storage roots the bundles are widely spaced and reduced in structure. Vessels appear to be absent, and the number of tracheids per bundle is 1 to 3, rarely more. In longitudinal section these tracheids are seen to be annular or spiral, scalariform or reticulate pitting being absent (Fig. 2). This is as might be expected in a storage root, where the xylem elements are needed only to conduct foodstuffs to the young growing shoot, absorption of mineral nutrients from the soil being carried out by the adventitious roots as soon
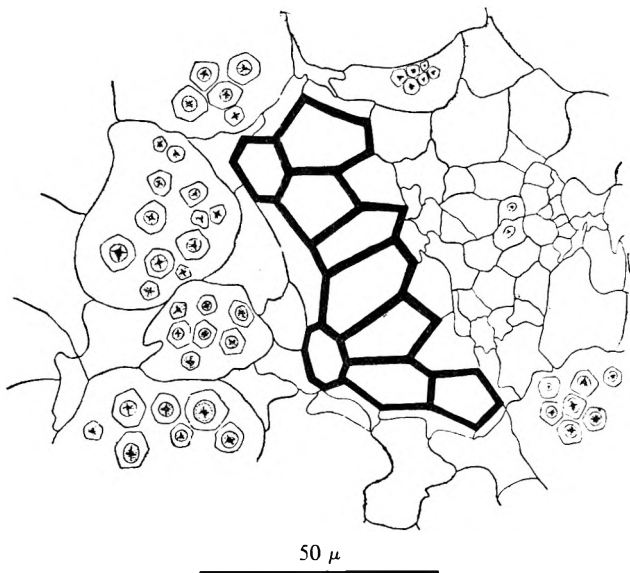
50 μ

FIG. 1C. Transverse section of root tuber of *Gloriosa* showing stele and starch grains.

as they appear. The phloem appears to be scanty, although a little more abundant than the xylem. The phloem cells are narrow, elongate and nucleated (Fig. 2), while wider sieve tubes have not been seen in any of the material examined. This again is what might be expected in a fleshy root tuber, where the distance through which built-up foodstuffs have to be carried is small.

CHEMICAL EXAMINATION

The specimens used for examination were collected by Mr. J. K. Jackson, Government Silviculturist, from Gilo, Imatong mountains, Equatoria Province, Southern Sudan.

Only a small quantity of the roots (about 25 g.) was available for examination. A part was extracted with ethanol and separated into fractions using the well-known Stas-Otto procedure, when the following results were obtained: (*a*) light petroleum extract from acid solution— no visible residue, (*b*) ether extract from acid solution—a minute amount of long needle crystals soluble in ammonia but giving no colour with ferric chloride solution. This probably contained an organic acid, (*c*) chloroform extract from acid solution—a yellow residue representing 0·12 per cent. of the roots was obtained which after purification gave the following reactions.

General alkaloidal reagents. Wagner's reagent, Sonnenschein's reagent, Dragendorff's reagent, and Meyer's reagent, all gave positive results. Picric acid reagent and Marmé's reagent gave negative results.

Colour reagents. (i) Concentrated sulphuric acid—orange to yellow, (ii) Mandelin's reagent—green, changing to violet, changing to brown, (iii) Marquis' reagent—orange, (iv) Mecke's reagent—green yellow

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changing to brown, (v) Fröhde's reagent—deep yellow brown, (vi) Vitali's test—violet in the cold, turning to brown on evaporating to dryness, giving a deep brown and violet with alcoholic potash, (vii) Sulphuric acid-potassium nitrate test—green, changing to blue and violet changing to bro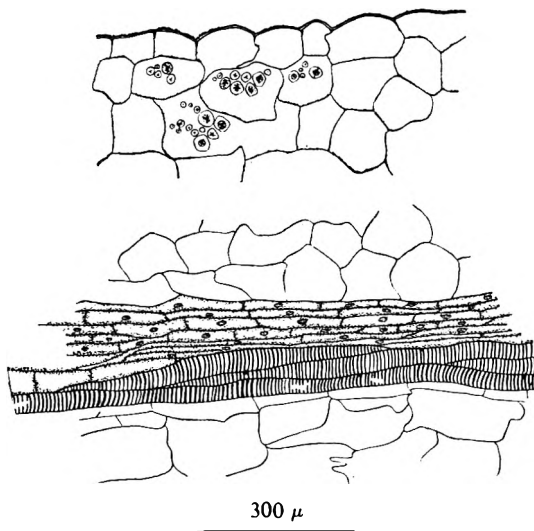wn then fading to colour-less. These reactions indicate that the alkaloid is colchicine. Unfortunately there was insufficient remaining to recrystallise from ethyl acetate to obtain a melting point. It should be noted that colchicine is most unusual if not unique among the alkaloids, in that it is extracted by chloroform from a solution acidified with sulphuric acid.

300 μ

Fig. 2. Longitudinal section of the root tuber of *Gloriosa*.

(*d*) Chloroform extract from a solution made alkaline with caustic soda—a yellow residue representing 0·014 per cent. of the roots which gave reactions similar to those given by the alkaloid in fraction (*c*). (*e*) Chloroform extract from ammoniacal methanolic solution—the slight residue gave negative reactions for alkaloids. (*f*) The aqueous solution remaining from the above extractions gave negative reactions for alkaloids.

The remainder of the roots was examined for colchicine content using the B.P. 1953 Assay for Colchicum, when 0·066 per cent. was found (equivalent to 0·22 per cent. on dried roots). When examined the roots contained 69·5 per cent. moisture.

It is clear from the above results that the toxic properties of the root reported in the literature is due to the presence of colchicine.

SUMMARY

An illustrated description of the plant *Gloriosa virescens* Lindl, is given, and the toxic principle is identified as colchicine.

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THE DETECTION OF APOATROPINE AND BELLADONNINE IN ATROPINE

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THE test for apoatropine in both the 1953 B.P. and the U.S.P. XIV depends on the formation of an immediate turbidity on adding ammonium hydroxide solution to an alkaloid solution of specified concentration. In the U.S.P. XIV, this is given as a test for "other alkaloids," and is supplemented by one with platinic chloride. Maeda[1] has criticised the ammonia test, claiming that no turbidity was produced in a sample of atropine sulphate which contained 3·9 per cent. of apoatropine, as measured by catalytic absorption of hydrogen. He recommended the adoption by the Japanese Pharmacopœia of a test with potassium permanganate solution, such as is given in both the 1953 B.P. and the U.S.P. XIV for detecting apohyoscine in hyoscine hydrobromide. It appeared desirable to compare the sensitivity of these tests for apoatropine, to investigate the possibility of using a bromine absorption method, and to extend the study to belladonnine, which is readily formed from apoatropine by dimerisation.

APOATROPINE AND BELLADONNINE

Apoatropine (under the name of atropamine) was isolated by Hesse[2] from a crude extract of *Atropa belladonna*, but was later considered by him to have been an artefact[3]. Belladonnine was reported by Hübschmann[4] as occurring in henbane berries. It is doubtful whether these compounds ever occur naturally in the plant, and they have not been found during the examination of about two hundred *Duboisia* extracts submitted to partition chromatography by the Division of Plant Industry of the Commonwealth Scientific and Industrial Research Organisation at Canberra[5]. Apoatropine is formed by the action of heat whilst concentrating the alcoholic percolate of the leaf. It is removed with the hyoscine fraction during the pH separation of the bases, but is produced in greater proportion by the action of alkali during the racemisation of (−) hyoscyamine to atropine.

Maeda[1] found that dehydration to apoatropine proceeds at about one-seventh of the rate of alkali racemisation, resulting in from 5 to 7 per cent. of apoatropine in the crude atropine, and his figures have been confirmed by the extraction of apoatropine as a pH 7 fraction from many samples of racemised solutions. Atropine itself, in alcoholic solution, produces apoatropine when treated with alkali hydroxide or ethoxide.

Pure apoatropine was prepared by the method of Willstätter and Hug[6], as modified by Maeda[1]. The purified product melted at 62–3° C., and its hydrobromide at 234° C. It was converted to belladonnine by the method described by Küssner[7], the base becoming partly crystalline on standing, and being unaffected by potassium permanganate solution,

since the dimer, belladonnine, lacks the unsaturated linkage present in the side chain of apoatropine.

The solubility of the two bases in a mixture of 10 ml. of water and 4 ml. of solution of ammonia, corresponding to the B.P. test solution was, apoatropine 0·05 per cent. w/v, and belladonnine 0·002 per cent. w/v. The conversion of apoatropine to belladonnine in alkaline solution was followed by titrating with decinormal potassium permanganate solution a series of aliquots withdrawn from a solution of 1 per cent. w/v apoatropine and 0·2 per cent. potassium hydroxide in 50 per cent. ethanol, each ml. containing 10 mg. of base.

TABLE I

DECREASE IN KMnO$_4$ CONSUMED WITH TIME. APOATROPINE IN ALKALINE SOLUTION

Time, hours	0·1 N KMnO$_4$ per mg. bases	Per cent. dimerisation
0	0·55	0
2	0·36	34
4	0·23	58
24	0·14	75

The endpoint was taken to a pink colour permanent for 5 minutes, as in the B.P. test for apohyoscine.

Hydrolysis over the 24-hour period was measured by extracting tropic acid from the acidified solution with ether, and titrating after removing the solvent. It amounted to 3 per cent. of the original base. In acid solution, at pH 3, the permanganate titre fell much more slowly, showing 33 per cent. conversion to belladonnine after 7 days, whilst in an aqueous solution of the hydrobromide, at pH 5, it remained unchanged over the same period.

0·5 ml. of the original 1 per cent. solution, diluted to 10 ml. and treated with ammonia solution as in the B.P. test, gave no perceptible cloudiness, but, as the less soluble belladonnine was produced, a slight turbidity appeared in the 2-hour sample, and became marked after 4 hours.

Sensitivity of the B.P. Ammonia Test

A 1·5 per cent. w/v aqueous solution of pure atropine sulphate of m.pt. 195° C. gave no turbidity in the B.P. test, and no reaction with decinormal permanganate solution. To 10-ml. portions were added increasing amounts of apoatropine and belladonnine sulphates, and each was tested by adding 4 ml. of solution of ammonia. With apoatropine, there was no turbidity until from 3 to 4 per cent. of the amount of atropine sulphate was present, but this became marked when the apoatropine content reached 5 per cent., these figures being in accordance with the observed solubility of apoatropine (0·05 per cent. w/v), which is reached when this impurity in the sample amounts to 3·3 per cent. When the impurity was belladonnine, turbidity appeared when it reached 0·4 per cent. of the atropine sulphate, and became marked at 0·5 per cent. These figures are virtually the same in the U.S.P. test, which uses a 1·67 per cent. atropine sulphate solution, a slight opalescence appearing at 3·5 per cent., and a marked turbidity at 5 per cent., of apoatropine in the sample.

The Platinic Chloride Test

This U.S.P. XIV test was applied to similar solutions, prepared according to the official instructions, and proved somewhat more sensitive than the

ammonia test. Two per cent. of apoatropine in atropine sulphate was just detectable, whilst 3 per cent. gave a marked cloudiness. With belladonnine, 0·1 per cent. was not detectable, but the reaction with 0·2 per cent. was definite.

Sensitivity of the Permanganate Test

The B.P. test for readily oxidisable substances in hyoscine hydrobromide requires that when 1 drop of 0·1 N potassium permanganate solution is added to 5 ml. of a 1 per cent. w/v solution of hyoscine hydrobromide in water, the solution is not completely decolorised in 5 minutes. This test was applied to 5-ml. quantities of a 1 per cent. solution of atropine sulphate, containing added apoatropine. The observations are given in Table II.

Titration with 0·1 N permanganate solution may be used as a means of estimating apoatropine in presence of atropine sulphate in a solution slightly acidified with sulphuric acid, although when much apoatropine is present, the solution develops a brownish colour which takes some time to disappear, and it is best to use another solution which has slightly less than the full amount of permanganate for comparison of the colour at the end-point. 20 mg. of apoatropine alone, dissolved in 1 ml. of dilute sulphuric acid, and diluted to 150 ml. required 10·9 ml. of 0·1 N permanganate to give a pink colour which did not fade for 5 minutes, whilst 4 mg. of apoatropine with 5 ml. of 1 per cent. w/v atropine sulphate and 0·5 ml. of dilute sulphuric acid in 150 ml. required 2·2 ml. of 0·1 N permanganate solution.

TABLE II
PERMANGANATE TEST FOR APOATROPINE IN ATROPINE SULPHATE

Per cent. apoatropine in atropine sulphate	0·1 N KMnO$_4$, drops	Stability of colour
0	1	Permanent over 15 minutes
0·05	1	Almost fades
0·10	1 2	Fades Stable 5 minutes
0·50	5	Fades in 3 minutes

Bromine Absorption Test

When an aqueous solution of bromine is added to a solution of atropine sulphate an additive compound is precipitated, which is converted to a black iodine addition compound by potassium iodide. This reacts slowly with sodium thiosulphate, dissolving gradually on shaking, and the ultimate blank titre is the same whether there is atropine present or not. Apoatropine absorbs two equivalents of bromine to saturate the double bond, but each ml. of decinormal bromate-bromide solution is equivalent to only 1·35 mg. If a 0·1 g. sample of atropine sulphate containing 5 per cent. of apoatropine is brominated with acidified 0·1 N bromate-bromide solution, treated with potassium iodide, and titrated with decinormal sodium thiosulphate, 10 ml. of the bromate-bromide are necessary to give an excess of bromine in solution, and the difference between the blank and the actual titre is only 0·36 ml. The method is obviously impracticable.

Relative Toxicities

Maeda[1] gave the toxicity of apoatropine to mice as twenty times that of atropine, and regarded it as a dangerous impurity. Pure samples of the sulphates of atropine, apoatropine and belladonnine were administered to mice by intramuscular injection at the Department of Pharmacology of the University of Melbourne. The LD 50, in mg. per kilo, calculated as the bases, was found to be, atropine 230 mg.; apoatropine 35 mg.; belladonnine 50 mg.

Although these figures do not suggest that a small proportion of apoatropine or belladonnine in atropine would have a very injurious effect, it is apparent that, whilst the existing tests in the B.P. and U.S.P. are adequate for controlling the belladonnine content, they will allow considerable proportions of apoatropine to be present. It is quite possible to prepare commercial atropine and atropine sulphate which comply with the permanganate test for oxidisable impurities, and the introduction of this test, or a modification of it, might be considered.

Injection of Atropine Sulphate

One per cent. w/v solutions of atropine sulphate, with and without added apoatropine and belladonnine, were heated to 98–100° C. for 30 minutes. They were tested before and after heating with ammonia, platinic chloride, and 0·1 N permanganate.

Atropine sulphate solution alone gave a stable colour with 1 drop of permanganate before heating, and 3 drops afterwards. A sample containing 0·5 per cent. apoatropine required 4 drops, increased to 6 drops after heating, and one with 0·5 per cent. belladonnine, 1 drop before, and 3 drops after heating. A sample containing 4 per cent. of apoatropine showed no visible difference in its response to the ammonia and platinic chloride tests after sterilisation, and its titre of 1·1 ml. of 0·1 N permanganate solution was not appreciably altered. It is concluded that there is a very slight production of apoatropine from atropine during sterilisation, but no conversion to belladonnine.

This investigation has been confined to the product from natural (−) hyoscyamine. It would be of interest to compare the extent to which apoatropine and belladonnine are formed during the manufacture of the synthetic alkaloid.

SUMMARY

1. It is improbable that apoatropine and belladonnine occur naturally in the plant, but both are formed during the manufacture of atropine from natural sources.

2. The B.P. and U.S.P. ammonia tests, and the U.S.P. platinic chloride test are sensitive tests for belladonnine, but allow the presence of appreciable amounts of apoatropine.

3. Belladonnine does not react with potassium permanganate, but this reagent is a sensitive one for apoatropine, and is capable of quantitative application.

4. Apoatropine and belladonnine are both more toxic than atropine.

5.   If apoatropine in amounts up to 3 or 4 per cent. is regarded as an undesirable impurity, a test with permanganate should be added to the B.P. monographs on Atropine and Atropine Sulphate.

The authors are grateful to Professor F. H. Shaw, of the Department of Pharmacology, University of Melbourne, who carried out the toxicity tests.

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A NOTE ON A URINARY METABOLITE OF PHENOBARBITONE

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RECENTLY a report of two spots forming a paper chromatographic pattern characteristic of acute phenobarbitone poisoning drew attention to the first metabolite of this compound found in human urine[1]. The material from the slower running spot has now been isolated and identified as *p*-hydroxyphenobarbitone. This is the same compound recently isolated from dog urine by Butler[2].

The paper chromatographic separation of phenobarbitone from its metabolite offered a ready, if tedious, method for isolating the metabolite. The first crystals were obtained by this method from several pooled samples of post-mortem urine. It was observed during these experiments that the metabolite was much less soluble in chloroform than was phenobarbitone. When recently nearly one litre of ante-mortem urine was submitted to the laboratory in a case of acute phenobarbitone poisoning it was decided to attempt to use this differing solubility as a means of separating the two compounds on a macro scale. The urine, acidified with dilute hydrochloric acid, was therefore extracted first with chloroform in a continuous extractor for seven hours and then four times with 1000 ml. quantities of ether. The chloroform was evaporated and the residue examined by paper chromatography. Both metabolite and phenobarbitone were present. In the ether extract, however, there was only the slower-running spot of the metabolite. Crystals were obtained by evaporation of an ether/light petroleum (40-60°) solution of this second extract. These, decolourised with charcoal and recrystallised from water, had a melting point = 222° C. (15 mg.). Found; C, 53·2; H, 5·3; N, 10·2 per cent. $C_{12}H_{14}N_2O_5$ requires C, 54·1; H, 5·3; N, 10·5 per cent. Melting point 222-3° C.

The crystals were examined by paper chromatography in the ascending, *n*-butanol saturated with 5N ammonia, system. No other ultra-violet absorbent material was present as shown by a contact print of the chromatogram on Ilford Reflex Contact Paper No. 50 using as the light source a mercury arc ultra-violet lamp, with no filters. The spot at $R_F = 0·17$ reacted with the ferric chloride/potassium ferricyanide spray reagent for phenols[3] to give a blue spot. It gave no reaction with 2:6-dichloro-quinone-chloroimide confirming the substituted *para*-hydroxy position.*

Observations in the ultra-violet are shown in Figures 1 and 2.

These show significant differences between phenobarbitone and this metabolite and are a valuable criterion for demonstrating phenobarbitone ingestion. The wavelength maxima and minima for the metabolite are:

*Note added in proof. Comparison of this compound with an authentic sample kindly provided by Dr. T. C. Butler, has confirmed its identity.

At pH 13, $\lambda_{max.}$ = 249 mμ, 290 mμ: $\lambda_{min.}$ = 231 mμ.

At pH 10, $\lambda_{max.}$ = 244·5 mμ, 290 mμ.

At pH 2, $\lambda_{max.}$ = approximately 273 mμ.

Apart from these frequency differences, in contrast with phenobarbitone, decreasing the pH from 13 to 10 does not significantly increase the maximum extinction reading. Because of the very small amount of material
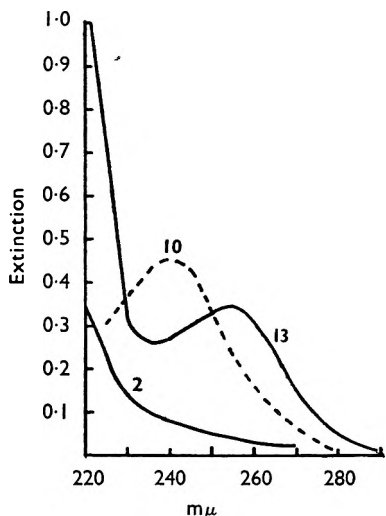

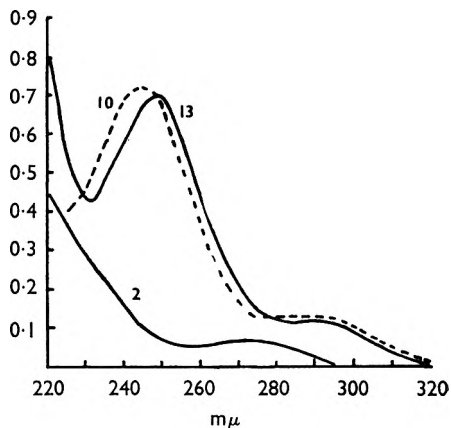FIG. 1. Phenobarbitone showing ultra-violet observations at pH 2, 10 and 13.

FIG. 2. p-Hydroxyphenobarbitone showing ultra-violet observations at pH 2, 10 and 13.

available, the extinction coefficient of p-hydroxyphenobarbitone has not been accurately determined. Solutions were made in 0·1 N sodium hydroxide and the pH lowered by the addition of micro drops of 6N sulphuric acid.

It was suggested by Butler that this compound was, in the dog, excreted in a conjugated form. After these first extractions the urine was therefore boiled with hydrochloric acid for six hours and then re-extracted with ether. No more p-hydroxy compound was obtained. This suggests that this metabolite is excreted in man in the free form.

Carbon hydrogen and nitrogen analyses were by Drs. Weiler and Strauss, Oxford.

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2. Butler, *Science*, 1954, **120**, 494.
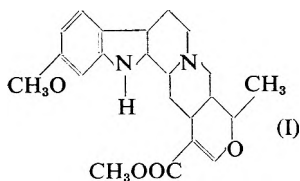3. Barton, Evans and Gardner, *Nature Lond.*, 1952, **170**, 249.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

3-Epi-α-yohimbine, A New Rauwolfia Alkaloid. F. E. Bader, D. F. Dickel, C. F. Huebner, R. A. Lucas and E. Schlittler. (*J. Amer. chem. Soc.*, 1955, **77**, 3547.) The isolation from *Rauwolfia serpentina* of a new alkaloid isomeric with yohimbine and identified as 3-epi-α-yohimbine is reported. It is demonstrated by degradative and synthetic evidence that 3-epi-α-yohimbine has the 3-epialloyohimbane configuration and represents the first recognition of this ring system in a natural product. The structure is confirmed by the isomerisation to α-yohimbine. Neither the alkaloid itself nor any of its synthetic esters resembled reserpine in general pharmacological activity. A. H. B.

Tetraphyllin and Tetraphyllicine, New Alkaloids from *Rauwolfia tetraphylla* L. C. Djerassi and J. Fishman. (*Chem. and Ind.*, 1955, 627.) Four alkaloids were obtained in a pure state from extracts of the roots of *Rauwolfia tetraphylla* L. The presence of additional ones is indicated by paper chromatography. Reserpine (*ca.* 0·03 per cent. yield) was separated easily by virtue of the chloroform solubility of its acetate. Chromatography of the remaining alkaloids on alumina gives 3 alkaloids which were eluted in the following order. Elution with benzene followed by crystallisation from methanol led to colourless plates of a new alkaloid named *tetraphyllin*, $C_{22}H_{26}O_4N_2$ m.pt. 220 to 223° C. (decomp.) $[\alpha]_D^{28}$ − 73° (chloroform) − 35° (pyridine). The ultra-violet absorption spectrum was essentially superimposable with that of reserpinine. Infra-red and other evidence suggests that tetraphyllin has the structure (I), and is thus a stereoisomer of reserpinine and *iso*reserpinine. With increasing polar solvents (benzene/chloroform 7:3) there was eluted another new alkaloid *tetraphyllicine*, $C_{20}H_{26}N_2$, which crystallised from acetone as needles, m.pt. 320 to 322° C. $[\alpha]_D^{27}$ + 21° (pyridine), pKa 8·5. The ultra-violet absorption spectrum is completely superimposable with that of ajmaline, and the infra-red spectra of the two alkaloids is similar. The most polar alkaloid obtained crystallised from methanol as bright yellow needles m.pt. *ca.* 270° $\lambda_{max.}$ in chloroform 5·84 and 6·18 μ. It was shown to be identical with serpentinine. A. H. B.

Veratrum viride, New Hypotensive Ester Alkaloids of. G. S. Myers, P. Morozovitch, W. L. Glen, R. Barber, G. Papineau-Couture and G. A. Grant. (*J. Amer. chem. Soc.*, 1955, **77**, 3348.) The isolation of the five hypotensive ester alkaloids *iso*germidine, germbudine, neogermbudine, desacetylneoprotoveratrine and veratetrine (neoprotoveratrine) from commercial *Veratrum viride* is described. *iso*Germidine is germine monoacetate-mono-α-methyl-butyrate. Germbudine is a diester of germine which gives germine, α-methylbutyric acid and the high melting diastereoisomer of αβ-dihydroxy-α-methylbutyric acid upon hydrolysis. Desacetylneoprotoveratrine is a known triester of protoverine which gives one mole each of acetic acid, α-methylbutyric

acid and the high melting isomer of $\alpha\beta$-dihydroxy-α-methylbutyric acid on hydrolysis. Veratetrine is shown to be a tetraester of protoverine and to be identical with the alkaloid neoprotoveratrine. All five ester alkaloids are powerful antihypertensive agents. In comparison with a mixed alkaloidal preparation from *Veratrum viride* Deravine which produced a 30 per cent. fall in the mean arterial blood pressure of the anæsthetised dog at a dose of 2 μg. per kg., administered intravenously over a 10 minute period, the relative activities of germbudine, neogermbudine, *iso*germidine, veratetrine and deacetylneo-protoveratrine are 0·8, 1·0, 0·1, 1·5 and 0·4, respectively. The infra-red spectra of the alkaloids are recorded.   A. H. B.

ANALYTICAL

Hyoscine Hydrobromide in a Tablet Mixture, Determination of. R. B. Scott, E. J. Schoeb and J. M. Vanderbelt. (*J. Amer. pharm. Ass., Sci. Ed.,* 1955, **44**, 377.) By the use of the following method it is possible to determine hyoscine hydrobromide with a mean recovery of about 97 per cent. from material containing only 0·25 per cent. of the alkaloidal salt admixed with diphenhydramine hydrochloride. The determination depends upon the infra-red absorption band of hyoscine at 11·82 μ. Interference due to the absorption by diphen-hydramine (maximum at 11·58 μ) is minimised by the use of a selective solvent and corrected by a geometric procedure. After a preliminary extraction of a sample of about 1·25 g. of tablet material with dehydrated ethanol, the residue is dried in a vacuum oven at 75° C. for 4 hours to remove the ethanol. The dry material is extracted with dimethylacetamide at 50° C., the excess of solid being separated by centrifuging. The solution is examined in an infra-red spectro-photometer between 11 and 12 μ, against a solvent blank. A tangent is drawn to the absorption minima at 11·02 and 11·82 μ and the distance from this to the maximum at 11·61 μ is a measure of the hyoscine hydrobromide content. The dimethylacetamide used must be saturated with sodium chloride and kept dry to avoid attack on the spectrophotometer cells. The method is applicable only to uncoated tablets; coated tablets give low results.   G. B.

Morphine, Elution from Ion Exchange Resins. C. H. Van Etten. (*Analyt. Chem.,* 1955, **27**, 954.) Experiments were made to find conditions under which morphine could be quantitatively removed from strong anion and cation exchange resins using micro ion exchange columns and samples of about 10 mg., and results are reported which show the effect of the degree of cross linkage of the exchange resin on the elution of morphine, and the effect of pH and ionic concentration of the elutriant. Dowex 50 (a strong, sulphonic acid, cation exchange resin) and Dowex 1 (a strong, quaternary ammonium anion exchange resin) of different degrees of cross linkage were used and complete elution was obtained from the 1, 2, and 4 per cent. cross-linked cation resins with either ammonium or sodium hydroxide, but incomplete elution was obtained with 8 and 16 per cent. cross-linking. Conditions for complete elution from the anion resin were more restricted, quantitative elution being obtained only for the 1 per cent. cross-linked resin with acetic acid as the elutriant. The elution of codeine and narcotine also was incomplete from resins having more than a certain degree of cross linkage. Factors affecting elution were the degree of cross linkage of the resin and the size of the ion, the pH of the elutriant especially in case of ampholytes, and the effect of the ionic concentration of the elutriant on volume changes of the exchange resin. Both lysine and aneurine were, however, incompletely eluted under all conditions examined.   R. E. S.

Neomycin, Turbidimetric Assay of. J. Dony and J. Conter. (*J. Pharm. Belg.*, 1955, **10**, 104.) Solutions are prepared in phosphate buffer solution, pH 8, containing 6, 9, 12, and 15 μg. of a standard preparation of neomycin. To 1 ml. of each solution is added 9 ml. of a nutrient broth medium (pH 7), inoculated with *Klebsiella pneumoniæ*. Six tubes are prepared for each concentration. The tubes are incubated for 3 to 3·5 hours at 37° C., after which growth is stopped by the addition of 2 drops of solution of formaldehyde. The turbidity of each suspension is measured photoelectrically and a standard curve is prepared. The same procedure is carried out with samples of the neomycin under test containing the equivalent of about 11 μg. of neomycin base per ml. in phosphate buffer, pH 8. The potency is calculated by reference to the standard curve. Reproducible results have been obtained with samples of neomycin sulphate, neomycin ointment and ointment of neomycin sulphate with bacitracin.   G. B.

Nitro-Nitrogen, Determination of. P. R. W. Baker. (*Analyst*, 1955, **80**, 481.) It was found that the sealed tube micro-Kjeldahl method of White and Long (*Analyt. Chem.*, 1951, **23**, 363) could be used for the reduction of nitro compounds by the addition of 50 mg. of thiosalicylic acid or glucose to the digest; 25 mg. were found to be insufficient. Results are given for a number of nitro-compounds. For compounds containing N–N linkages, glucose was slightly more efficient than thiosalicylic acid, but the results did not approach the theoretical values with either reagent. The temperature of 470° C. was found to be dangerously high and 45 minutes at 420° to 440° was sufficient. Nitro compounds with the structure $-C-C_6H_5-NO_2$, oximes, and N-oxides did not require reduction.   R. E. S.

Sulphate Ion, Colorimetric Determination of. J. L. Lambert, S. K. Yasuda and M. P. Grotheer. (*Analyst. Chem.*, 1955, **27**, 800.) A colorimetric procedure for determining sulphate ion in the range of 0 to 400 p.p.m. is described, which uses an insoluble thorium borate-amaranth dye reagent; dye molecules are released from the reagent in direct proportion to the concentration of sulphate ion and are determined from their absorption at 521 mμ. Bicarbonate, phosphate, and fluoride ions interfere by reacting with the thorium-dye reagent to release dye into solution. The addition of lanthanum ion removed the fluoride ion with little effect on the sulphate ion while bicarbonate ion could be eliminated by passing the sample solution through Amberlite IRC-50(H) weak acid ion exchange resin.   R. E. S.

Vitamin B_6, Assay of. N. A. Diding. (*Svensk farm. Tidskr.*, 1955, **13**, 321.) The paper describes the use of *Escherichia coli* (154–59 L, Dr. Davis' collection) in the microbiological assay of pyridoxine, pyridoxal and pyridoxamine. Details of the procedure and of the medium are given, the organism being incubated in tubes for 20 hours at 37° C.; growth is determined by measurement of the turbidity produced. Pyridoxal is a little more active than pyridoxine towards the organism, while for pyridoxamine a concentration about 10 to 20 times that for pyridoxine is necessary to obtain the same growth response; no growth is shown in blank tubes. Sterility is not absolutely necessary in performing assays, since the minimal medium used is too poor to permit growth from most contaminating organisms. The assay can be used for vitamin B_6 in multivitamin preparations as there is no growth response with aneurine, riboflavine, nicotinic acid or pantothenic acid although vitamin-free casamino acid caused a marked increase in response.   R. E. S.
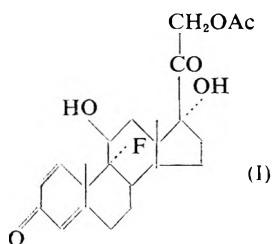
BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Catechol Amines, Inactivation of, by Liver Tissues. Chr. von Euler. (*Acta physiol. scand.*, 1955, **33**, Suppl. 118, 39.) When dopa is incubated with guinea-pig liver, dopamine, catechol acetaldehyde and catechol acetic acid may be demonstrated by chromatographic separation and formation of fluorescent condensation products with ethylenediamine. Noradrenaline, under the same conditions, forms catechol glycolic acid and catechol glycolaldehyde. Corbasil (3 : 4-dihydroxynorephedrine) forms no such similar products, indicating that it is not attacked by amine oxidase. The presence of large amounts of catechol acetic acid in normal urine suggests that this is formed by decarboxylation, deamination and subsequent ox.dation of dopa. M. M.

Catechol Derivatives, Biologically Inactive in Urine. Chr. von Euler, U. S. von Euler and I. Floding. (*Acta physiol. scand.*, 1955, **33**, Suppl. 118, 32.) The normally occurring biologically active catechol amines (adrenaline, noradrenaline and dopamine) fcrm only a small part of the total catechol compounds in urine. This is shown by a comparison of the biological activity with the strength of the fluorescence after condensation with ethylenediamine. It is suggested that it is catechol acetic acid, which is also present in urine, which gives an erroneously high value for the catechol content of urine when the ethylenediamine fluorescence reaction is used. This fluorescence method therefore is not suitable for the estimation of adrenaline and noradrenaline in urine. M. M.

1-Dehydro-9α-Fluorohydrocortisone Acetate: New Biologically Potent Steroid. R. F. Hirschmann, R. Miller, R. E. Beyler, L. H. Sarett and M. Tishler. (*J. Amer. chem. Soc.*, 1955, **77**, 3166.) A brief description of the preparation of 1-dehydro-9α-fluorohydrocortisone acetate (I) is given. It is the most potent glucocorticoid known; it possesses about 25 times the activity of hydrocortisone acetate in the mouse liver glycogen assay and in the rat systemic granuloma inhibition test. A. H. B.

Noradrenaline and Adrenaline, Free and Conjugated, Preparation of Extracts of Urine and Organs for Estimation of. U. S. von Euler and I. Orwén (*Acta physiol. scand.*, 1955, **33**, Suppl. 118, 1.) This paper describes a modification of Euler's original procedure for the extraction of catechol amines from urine and tissue extracts. It is simpler and quicker and gives as great an accuracy. The method involves the adsorption of the free catechols on to aluminium oxide at a *p*H of 8·5 and subsequent elution with acid. Sulphuric acid is used when the solution is to be assayed biologically (80 per cent. recovery obtainable) and with oxalic acid when a fluorimetric determination is employed. Estimation of the conjugated catechol amine content is made as above, after an initial hydrolysis with boiling normal hydrochloric acid. M. M.

BIOCHEMICAL ANALYSIS

Blood in Urine, a Tablet Test for. E. T. Watson-Williams. (*Brit. med. J.*, 1955, **1**, 1511.) The tablets each contained citric acid 50 mg., barium peroxide 35 mg., *o*-tolidine 12·5 mg., and sodium carbonate 2·5 mg. If the tablets are kept in a closed screw-capped container they retain their sensitivity for at least 12 months. One drop of the urine to be tested is placed on an inch square of Whatman No. 1 filter paper and allowed to spread. A tablet is placed on the centre of the paper and 2 drops of cold tap-water are allowed to flow over the tablet. A definite blue colour appears round the tablet within 2 minutes if the urine contains at least 50 red cells/c.mm. or the equivalent concentration of hæmoglobin (150 μg./100 ml.). If a smaller quantity is present a blue colour develops after 2 minutes; and if none is present the tablet and the filter paper remain colourless for at least 15 minutes. The test is equally sensitive throughout the temperature range 4–37° C. If the urine is heated to 60° C. or boiled the test is much less sensitive. Variation of urinary *p*H 5–8 has no significant effect on the sensitivity, which is however slightly reduced if the urine is either very alkaline or very acid. False negatives may be encountered if the ascorbic acid content of the urine is high. False positives occur if the urinary concentration of iodide is greater than 20 mg./100 ml.; in these cases the urine should be boiled for 1 minute and immediately retested; if the colour is due to iodide it will then be deeper, whereas if due to blood a negative will be obtained. Pus, bromides and sweat give negative results, but any oxidising agent will give a false positive. In no case has hæmaturia been found by the pathological laboratory when the tablet has given a negative result. The tablet has been found particularly valuable as corroboratory evidence in a clinical diagnosis of cystitis or pyelitis, and as a daily test to discover when known hæmaturia ceases. It is also useful as an additional safeguard to prevent overdosage with anticoagulant drugs.   S. L. W.

Chromium in Biological Media, Determination of. C. H. Grogan, H. J. Cahmann and E. Lethco. (*Analyt. Chem.*, 1955, **27**, 983.) A method is presented which consists of a wet or dry ashing of the sample, hypobromite oxidation of trivalent to hexavalent chromium, and spectrophotometric determination of the hexavalent chromium in the form of the red-violet complex formed on reaction with *sym*-diphenylcarbazide. The choice of wet or dry ashing depended on the type of material to be analysed; plasma or serum could be ashed equally well by either method; the wet ashing of filter paper was tedious, while the dry ashing proceeded smoothly; urine gave better results when wet ashed, as dry ashing yielded a difficultly soluble ash. Erratic results were obtained when egg albumin was dry ashed due to the transformation of the chromium present to refractory oxides or phosphates. The wet ashing process oxidation was performed with nitric acid and hydrogen peroxide; dry ashing was at 420° to 460° C. overnight. Recoveries of chromium, trivalent or hexavalent, added to human plasma ranged between 94 and 101 per cent.   R. E. S.

Hydrogen Peroxide in Biological Materials, Estimation of. W. A. Andreae. (*Nature, Lond.*, 1955, **175**, 859.) A sensitive method is proposed for the estimation of hydrogen peroxide which employs the disappearance of the fluorescent peroxidase substrate scopoletin (6-methyl-7-hydroxy-1 : 2-benzopyrone). At concentrations up to $2·5 \times 10^{-9}$ mole per ml., the intensity of fluorescence is proportional to the concentration of scopoletin. An aqueous solution of scopoletin is stable in diffused light and is not oxidised by either peroxidase or

hydrogen peroxide alone; with both present, however, oxidation with loss of fluorescence proceeds very rapidly. Results and graphs are given showing the quantitative relationship between the amount of hydrogen peroxide added and the disappearance of scopoletin using an aqueous extract of dehydrated horseradish; one mole of hydrogen peroxide was required for the oxidation of one mole of scopoletin, this simple relationship applying so long as 20 per cent. of scopoletin was present. Results are also given showing the rate of oxidation of scopoletin ($1 \cdot 2 \times 10^{-8}$ ml. per ml.) by pea juice (pH 6·8) with putrescine as source of endogenous hydrogen peroxide. Ascorbic acid, glutathione, and manganous ions interfered by competitively inhibiting the oxidation of scopoletin by peroxidase; esculetin and umbelliferone were also examined for fluorescence but scopoletin appeared to be the best fluorescent indicator for the peroxidative estimation of hydrogen peroxide. permitting the determination of amounts of endogenously produced hydrogen peroxide in the range of 10^{-10} mole.

R. E. S.

Noradrenaline and Adrenaline in Urine, Fluorimetric Estimation of. U. S. von Euler and I. Floding. (*Acta physiol. scand.*, 1955, 33, Suppl. 118, 57.) A method for fluorimetric estimation of adrenaline and noradrenaline in urine is described. The urine extracts are prepared by adsorption on to aluminium oxide at pH 8·5 and elution with 0·3 N oxalic acid. The catechol amines in the extracts are oxidised to adrenochrome and noradrenochrome at pH 3·5 and 6·0 with potassium ferricyanide. The reaction is enhanced by zinc sulphate at pH 3·5. After treatment with strong alkali and stabilisation of the fluorescent product with ascorbic acid the fluorescence intensities are measured. At pH 6·0 the sum of the adrenaline and noradrenaline is determined and at pH 3·5 adrenaline plus about 4 per cent. of the noradrenaline. From these data and the relative fluorescence of adrenaline and noradrenaline the amounts of adrenaline and noradrenaline can be calculated. M. M.

CHEMOTHERAPY

Isoniazid in the presence of Hæmin, Destruction of. R. Knox, A. Albert and C. W. Rees. (*Nature, Lond.*, 1955, 175, 1085.) When isoniazid (10^{-2}M) and hæmin (10^{-3}M) are shaken with air at 20° C. (pH 7·5) two substances are formed; di*iso*nicotinoylhydrazine (I) and *iso*nicotinic acid (II). Quantitative paper chromatography shows that after 24 hours half the isoniazid is unchanged and the other half is converted to equal parts of (I) and (II), (I) being an intermediate product in the formation of (II). Hæmin acts as a catalyst in the reaction; it also acts catalytically in very low initial concentrations of isoniazid; a concentration of isoniazid (10^{-4}M) falling to (10^{-7}M) in the presence of hæmin (10^{-4}M). Hæmin rapidly forms a hæmochromogen, with an excess of isoniazid or with less isoniazid plus a reducing agent—which is purple and distinct from other hæmochromogens. Solutions of this substance are stable only in the absence of air. Substance (I) slowly forms a hæmochromogen which may be the true catalyst as many hæmochromogens are more efficient oxidation catalysts than hæmin. The antagonism of hæmin to the action of isoniazid on *Myco. tuberculosis* was shown by adding hæmin to tubes containing isoniazid and *Myco. tuberculosis* in asparagine glycerol phosphate medium when the bacteria grow normally, but if its addition is delayed for a day growth is slow, and when addition is delayed for two or three days growth is entirely inhibited. Thus in three days the drug may reach a site in the bacillus inaccessible to hæmin or at too low an E_h to be oxidised, or isoniazid may irreversibly damage the cell in this period. G. P.

PHARMACY

NOTES AND FORMULÆ

Cetyltrimethylammonium Bromide, The Sterilisation of Blankets with. R. Blowers and K. R. Wallace. (*Lancet*, 1955, **268**, 1250.) Blankets were given a final rinse containing 0·036 per cent. cetyltrimethylammonium bromide (Cirrasol OD) after laundering with a non-ionic detergent (Lissapol N), which does not neutralise the bactericidal properties of the cationic surface-active agent. Before and after treatment the blankets were tested by pressing an inverted Petri dish firmly against each blanket and sweeping it across fairly rapidly and evenly so as to throw particles of dust and fluff on to the surface of the medium, and incubating the plate. The nutrient agar medium used contained 0·75 per cent. of Perminol COL to neutralise any cationic detergent in the sample. Before treatment large numbers of organisms were isolated from the blankets. After the treatment only a few organisms were detected, indicating that the process is an effective method of disinfection. Cetyltrimethylammonium bromide is active against *Staphylococcus aureus*, but is less effective against *Pseudomonas pyocyanea* than other organisms. The process is inexpensive to apply and does not damage the blankets. G. B.

Oil in Water Emulsions, Analysis of. T. Freen, R. P. Harker and F. O. Howitt. (*Analyst* 1955, **80**, 470.) A general method is given for the analysis, using ion exchange resins, of the constituents of emulsions stabilised by different types of detergents. Using columns of Zeo-Karb 225 in bead form mixed with powdered animal charcoal, percolation of soap solutions and soap stabilised emulsions gave clear liquors from which organic matter had been removed; percolation of the emulsion and subsequent elution with a series of solvents resulted in the isolation of the separate components. Emulsions stabilised by sodium cetyl sulphate were analysed successfully by this method; De-Acidite FF was used in the presence of cetyltrimethylammonium bromide. Using columns of Biodeminrolit (an intimate mixture of Zeo-Karb 225 and De-Acidite FF), percolation of lanolin—Lissapol N emulsions containing up to 1 per cent. of sodium chloride gave clear liquors, which on evaporation left no residue; columns containing powdered animal charcoal were equally effective but the Lissapol N was held too firmly for successful solvent elution. Lissapol N—soap-lanolin emulsions could be estimated accurately using a combination of the above methods. R. E. S.

PHARMACOLOGY AND THERAPEUTICS

Aldrin and Dieldrin, Autonomic Manifestations seen in Acute Poisoning with. C. W. Gowdey and G. W. Stavraky. (*Can. J. Biochem. Physiol.*, 1955, **33**, 272.) The parasympathomimetic actions of the insecticides aldrin (hexachloro-hexahydro-dimethano-naphthalene) and dieldrin (hexachloro-octahydro-epoxy-dimethano-naphthalene) were investigated in vagotomized and adrenalectomized cats under combined chloralose and urethane anæsthesia. Of the two, only aldrin had any peripheral effects, causing bradycardia and augmenting the effects of vagal stimulation on blood pressure and heart rate and of stimulation of the chorda tympani on blood pressure and salivary secretion from the decentralized sub-maxillary gland. Blood withdrawn five minutes after intravenous injection of aldrin showed a reduced rate of destruction of acetylcholine, assayed on the frog rectus abdominis muscle. These results are in accord with an anti-cholinesterase-like mode of action. Other effects included a potentiation of the excitability of spinal neurones to intra-arterial injections of acetylcholine, skeletal muscle twitches, potentiation of the crossed extensor reflex and

augmented transmission across the neuromuscular junction; large doses of the compound had a depressant action at these sites. More powerful actions common to both aldrin and dieldrin were stimulation of the vagal centres, hyper-reflexia and a convulsant action. G. P.

Chlorpromazine in Psychiatric Disorders. I. M. Cohen. (*Amer. J. med. Sci.*, 1955, **229**, 355.) The experiences reported in this study cover a period of 9 months and were drawn from a series of about 1000 patients suffering from psychiatric illnesses of various types. Treatment with chlorpromazine was shown to be most effective where there is evidence of agitation, anxiety or aggression. In approximately 60 per cent. of the patients improvement occurred, varying in degree from reduction in intensity of symptoms to complete resolution of illness. Dosage requirements vary widely and it is believed that many failures with the drug are attributable to the use of inadequate dosage. In this series effective dosage for neurotics was in the range of 200–600 mg. daily and for psychotics from 400–800 mg. daily; as much as 2250 mg./day was administered without toxic effects. With rare exceptions psychogenic nausea and vomiting responded favourably to the drug; it was also effective in nausea and vomiting due to carcinomatosis, pregnancy, acute alcoholic gastritis and dumping syndrome, and the nausea and vomiting sometimes accompanying insulin shock therapy and electro-convulsive therapy. The most common early subjective complaints noted were dizziness, weakness, lethargy, dryness of the mouth and nasal congestion; less common were a burning sensation in the œsophagus, nausea and vomiting and constipation. Objectively, the most impressive finding was fall in blood pressure, but there was no case of circulatory collapse. Late effects included excessive appetite, blurring of vision, intense pruritus, photosensitisation, rest tremor and increased sexual urge. Swelling of the breasts and secretion of a colostrum-like fluid from the nipples were sometimes noted in female patients. The most frequent complication was dermatitis which occurred in about 10 per cent. of cases and usually appeared 2–3 weeks after starting treatment; it usually cleared in 3 or 4 days, and in most cases treatment could be resumed without a further recurrence. Jaundice occurred in 6 patients, and occasionally Parkinsonism developed, clearing in about 5–14 days after withdrawal of treatment. Rare instances of leucopenia and eosinophilia occurred but in no case of agranulocytosis. S. L. W.

Frenquel Corrects Certain Cerebral Electrographic Changes. F. Rinaldi and H. E. Himwich. (*Science*, 1955, **122**, 198.) Lysergic acid diethylamide (LSD–25) and mescaline induced changes in the electro-encephalograms of curarised unanæsthetized rabbits, consisting of disappearance or diminution of slower waves and increased frequency and decreased voltage of the fast low-voltage activity. Frenquel, (α-4-piperidyl benzhydrol hydrochloride), in suitable doses reversed these changes. It had been shown previously that this drug blocked the psychotic states induced in normal human subjects by LSD–25 (Fabing, *Science*, 1955, **121**, 208.) In the rabbit there was a direct relationship between the dose of hallucinogen administered and that of Frenquel necessary to reverse the subsequent EEG changes. Frenquel given alone had no effect on the EEG. Dyflos, amphetamine and Meratran (α-2-piperidyl benzhydrol hydrochloride), like the hallucinogenic substances, evoked fast low-voltage activity EEG patterns in rabbits, but Frenquel had no corrective action on the changes induced by these drugs, although one of them, Meratran, is a positional isomer of Frenquel. The blockade of LSD–25 and mescaline actions by Frenquel appeared to be limited to the central nervous system, since the mydriasis associated with these drugs was not affected by the blocking agent. G. P.

Hydergine, the Effect of, on Uterine Action. T. N. A. Jeffcoate and J. K. Wilson. (*Lancet*, 1955, **268**, 1187.) Hydergine, despite reports to the contrary, had slight oxytocic activity in patients in normal labour at term. Uterine activity was observed by clinical signs and by the use of a Lorand tocograph. Where uterine contractions had been induced by oxytocin, hydergine had no inhibitory activity; in some cases the two drugs acted synergistically. During and after the third stage of labour an apparent inhibitory action of hydergine was observed, but this may have been due to withdrawal of the drug at full cervical dilatation; similar inhibition has been reported where ergometrine or other ergot alkaloids were used during labour.   G. P.

Hydrocortisone Ointment, A Valuation of. B. Russell, J. S. Pegum, N. A. Thorne and R. V. Grange. (*Lancet*, 1955, **268**, 1038.) The value of Hydro-Cortisyl ointment, a product containing 2·5 per cent. of hydrocortisone, in various skin conditions was assessed in comparison with concealed, alternating, self-contained controls, the patients themselves recording progress during 14 days. The controls used the vehicle alone, and the records were made on cards requiring an assessment of 4 grades of itching. Every patient was given the vehicle for a week and the medicated ointment for a week, the sequence being changed for each successive patient. In some cases, treatment was continued for several weeks. Some patients were given a 1 per cent. hydrocortisone ointment for comparison with the 2·5 per cent. preparation. The case notes and records were assessed by an independent observer. The vehicle itself, containing propylene glycol, polyethylene glycols and zinc stearate, was found to be remarkably effective as an emollient and antipruritic but its efficacy was far less than that of the medicated ointment in most conditions treated. Hydrocortisone ointment gave distinct or complete relief in 14 of 25 patients with anogenital pruritus, 10/21 with lichen simplex, 2/12 with infantile eczema, 3/22 with Besnier's prurigo, 9/23 with discoid eczema, 3/11 with idiopathic eczematous dermatitis of the hands, 7/11 with otitis externa, and 2/7 with itchy flexural and genital psoriasis. 1 patient complained of a burning sensation and in another infection increased during treatment. Only 1 patient was sensitive to the vehicle but relief was obtained with hydrocortisone in hydrous ointment.   H. T. B.

2(β-Hydroxyethylaminomethyl)-1 : 4-benzodioxan Hydrochloride, Pharmacology of. R. F. Banziger, T. S. Miya and L. D. Edwards. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 302.) Of a series of derivatives of 2-aminomethyl-1 : 4-benzodioxan and 2-aminomethylcoumaran, the most effective antagonist of the pressor effect of adrenaline appeared to be 2(β-hydroxyethylaminomethyl)-1 : 4-benzodioxan. The compound was soluble in water, and non-irritating. It was active in dogs, cats and rats when given by mouth or by injection, the activity being of fairly short duration (1 to 2 hours). Larger doses were needed to produce adrenaline blockade of isolated organs such as ileum, uterus and heart. Intravenous injection into cats caused respiratory arrest, but this could be avoided by giving 2 mg./kg. intraperitoneally. After an interval of 30 minutes, 5 mg./kg. could be given intravenously without toxic effects.   G. B.

5-Hydroxytryptamine, Action of, on Urine Flow and Chloride Excretion. V. Erspamer and P. Correale. (*Arch. int. Pharmacodyn.*, 1955, **101**, 99.) Further experiments are reported confirming that physiological doses of 5-hydroxytryptamine cause a significant reduction of diuresis in hydrated rats. Doses of 20 μg./kg. cause, over a period of 60 to 90 minutes, a 40 to 75 per cent. decrease in urine flow. A reduction in the absolute chloride content occurs but leaves unchanged the relative content. This is in contrast to the posterior pituitary antidiuretic principle which causes a conspicuous, absolute and relative increase in chloride excretion.   G. F. S.

Miltown, a New Tranquilising Drug. L. S. Selling. (*J. Amer. med. Ass.*, 1955, **157**, 1594.) Miltown (2-methyl-2-*n*-propyl-1 : 3-propanediol dicarbamate) has been shown to possess a selective blocking action on interneurons; it produces relaxation of skeletal muscles without affecting respiration and other vital functions, and has important effects on the brain. The present study was made on 187 psychiatric patients. The drug was supplied in 400 mg. tablets, the usual dose being one tablet after each meal and one at bedtime. As soon as possible the dose was reduced, and the drug finally withdrawn. All patients had regular sessions of psychotherapy in addition to treatment with the drug. Patients were treated for periods ranging from less than a month to more than 8 months. In about half the patients withdrawal from Miltown could be effected within a week; in the other half gradual withdrawal was necessary and placebos were sometimes substituted towards the end. Only 3 of the patients were allergic to the drug, and 5 complained of gastric discomfort. One patient who hoarded a supply and ingested about 100 tablets within 24 hours suffered no serious adverse effects. Miltown was of considerable value in anxiety and tension states; of 86 patients complaining of these symptoms all but 7 showed marked improvement. The tension state was usually relieved in 3 to 4 months. Related psychoneurotic conditions, such as behaviour problems and conversion hysteria, were also favourably influenced. In alcoholism, Miltown helped to avoid serious withdrawal symptoms and assisted in keeping alcoholics sober after withdrawal was completed. In frank psychoses the results were not so favourable. The most dramatic reports of recovery or improvement came from those patients whose chief complaint was tension headache. Of 27 patients with this complaint 23 either recovered or were greatly improved. Miltown was very effective in producing sleep, and by adjusting the dosage it was found possible to produce sleep in every patient except in those suffering from a true psychotic depression. Patients who had previously been taking phenobarbitone and were put on to Miltown preferred the latter. By producing in the patient a feeling of ease and relaxation Miltown was found a useful adjunct to psychotherapy. S. L. W.

Miltown, Effect on Psychiatric States. J. C. Borrus. (*J. Amer. med. Ass.*, 1955, **157**, 1596.) This study involved 104 patients all of whom had failed to respond to barbiturate and mephenesin therapy. The largest group (67) had anxiety reactions. Favourable results were reported in 71 of the patients: 24 showed complete recovery, 17 obtained very favourable results, 30 reported some favourable effect, and the remaining 32 showed no appreciable benefit. Complete blood cell counts and urinalyses taken prior to and two months after continuous use of Miltown failed to reveal any evidence of toxic effect of the drug, and, within the limits of the dosage used, one to six 400 mg. tablets daily, no serious side-effects or toxic manifestations were noted. The chief side-effect was drowsiness which occurred in about half the patients during the first 2 weeks of medication but gradually subsided as therapy was continued. There were no complaints of dizziness, vertigo, nausea, vomiting, diarrhœa or dermatological manifestations. The drug seemed most effective in patients with both acute and chronic anxiety reactions. The ability to obtain more restful sleep was a prominent finding; the other chief effects of the treatment were lessening of tension, more complete relaxation and ability to feel at ease in groups. The drug appeared less effective in psychoses, though it may be of value in ambulatory schizophrenics. It also offers promise for further investigation in such diseases as epilepsy and paralysis agitans. The antagonistic action of Miltown to leptazol-produced convulsions suggests that in the event of an accidental or deliberate (suicidal) overdosage leptazol might be used as counteractant. S. L. W.

Proguanil and its Metabolite, Action on Neuromuscular and Synaptic Transmission. M. J. Dallemagne and E. Philippot. (*Brit. J. Pharmacol.*, 1955, **10**, 147.) Proguanil and its active metabolite, triazine, blocked neuromuscular transmission in dogs and rats. Transmission through the superior cervical ganglion of cats was also blocked. At the neuromuscular junction the action appeared to be competitive, since the drugs antagonised block by decamethonium and enhanced tubocurarine block. Also their blocking action was reduced by neostigmine and by adrenaline. There was, however, a transitory potentiation of the decamethonium block, before reversal, by both proguanil and triazine. The ganglionic blockade also seemed competitive as there was no stimulation before blockade. Triazine was more effective at both the neuromuscular junction and the ganglionic synapse than was proguanil, except with the frog rectus, where proguanil antagonized acetylcholine contracture more readily. *In vivo* proguanil was rapidly transformed into triazine.   G. P.

Reserpine in the Treatment of Anxious and Depressed Patients. D. L. Davies and M. Shepherd. (*Lancet*, 1955, **269**, 117.) Of 67 outpatients, whose main symptoms were anxiety and depression, about half were treated with reserpine, 0·5 mg. twice daily, and the others with placebo. The trial covered a period of 6 weeks. Among the 54 patients completing the trial, those treated with reserpine showed more benefit than those receiving the placebo. No toxic effects were observed in any of the patients. In addition, 4 severely disturbed inpatients were treated with larger doses of the drug. They were given a 3-week course of reserpine by mouth, 3 of them receiving 10 mg. daily and 1 of them 15 mg. daily, for 3 weeks. Their condition remained unchanged by the treatment though all responded satisfactorily to subsequent electroconvulsion therapy.
                                                                        S. L. W.

Ethyl Pyrophosphate, Effect of Drugs in Antagonising the Toxicity of. J. R. Lewis, W. B. McKeon and A. M. Lands. (*Arch. int. Pharmacodyn.*, 1955, **102**, 371.) Tests are reported on a number of synthetic compounds and combination of drugs for the treatment of poisoning with organophosphorus compounds. The antidotal effects of the compounds were studied in mice and pigeons against ethyl pyrophosphate (TEPP). None of the compounds alone protected, even in large doses, but a combination of drugs gave more successful results. Atropine with Mytolon ($2:5\text{-}NH(CH_2)_3N(C_2H_5)_2 \cdot OCH_2Cl$) gave excellent protection. Scopolamine with Mytolon was even more effective, while atropine methylnitrate and (+)-hyoscyamine were ineffective. The muscle relaxing compounds curare and gallamine were ineffective alone but were effective with atropine. Decamethonium and mephenesin were ineffective even with atropine. Mytolon has muscle relaxing properties, and compounds related to Mytolon were also studied and their curarimetic potencies compared with their anticholinesterase activities *in vitro*. The amine analogue was ineffective as an antidote with atropine. Compounds in which the quaternary group included benzyl chloride had the greatest protective action and these compounds also had high curarimetic and anticholinesterase activities, but there was no correlation of these activities with their protective action. None of the central nervous system depressants gave protection alone, but a combination of phenobarbitone and atropine gave protection. A combination of magnesium sulphate and atropine was not effective, nor were the anti-epileptic drugs diphenylhydantoin and trimethadione. The ganglionic blocking drugs penta- and hexamethonium gave protection in high doses with atropine, and of the anticholinesterase compounds, physostigmine with atropine gave protection

while neostigmine was not effective. In pigeons, atropine alone injected 15 minutes before TEPP gave slight protection. Mytolon with atropine and physostigmine with atropine gave protection. G. F. S.

Tropine 4-Chlorobenzhydryl Ether Hydrochloride (SL-6057), a Potent, Long-acting Antihistaminic Agent. J. Y. P. Chen. (*J. Pharmacol.*, 1955, **114**, 192.) Originally investigated for antispasmodic activity, SL-6057 was found also to have a high degree of antihistamine and local anæsthetic activity. Single oral or intraperitoneal doses of the drug afforded better and much longer protection to guinea-pigs against fatal histamine sprays than did promethazine, tripelennamine or prophenpyridamine. The compound was similarly effective in reducing the vasodepressor action of histamine in dogs. Local anæsthetic action, measured in rats by sciatic nerve block and in guinea-pigs by the intradermal weal method, was more prolonged than procaine, but local irritation was produced with the higher doses used. SL-6057 had about one-tenth of the antispasmodic activity of atropine on the isolated rabbit duodenum and on the intact ileum of the dog. The antisialogogue and mydriatic actions in the rabbit and inhibition of chromodacryorrhœa in rats were also moderate compared with atropine. Anti-emetic activity in dogs was one-fourth of that of chlorpromazine. SL-6058, the quaternary methyl bromide of SL-6057, had similar activity, but was generally less potent than SL-6057, except for anticholinergic actions. Toxicity studies, acute in mice, rats and dogs and chronic in rats and dogs, showed SL-6057 to be relatively non-toxic. G. P.

WIN 8077 in the Treatment of Myasthenia Gravis. R. S. Schwab, C. K. Marshall and W. Timberlake. (*J. Amer. med. Ass.*, 1955, **158**, 625.) The compound WIN 8077, *NN'*-bis(2-diethylaminoethyl) oxamide bis-2-chlorobenzylchloride, is a white crystalline powder with a molecular weight of 608·5 and a melting point of 184·3° C. It is very soluble in water and can be sterilised by heat. It has been found as active an anticholinesterase substance as neostigmine, and has an anticurare effect 2 or 3 times greater than neostigmine and of longer duration. Since February, 1954, the drug has been used in 50 patients with myasthenia gravis, whose ages ranged from 15 to 74 years. Of this number, 41 are still taking it and feel that their adjustment is superior to that with either neostigmine bromide or Mestinon (a dimethylcarbamate of 3-hydroxy-1-methyl pyridinium bromide). The compound is given in the form of a syrup containing 12·5 mg. in 4 ml. The dosage requirements of patients vary from 2 to 4 mg., to 57–75 mg., the doses being given at 2 or 3-hourly intervals. Several patients reacted unfavourably to the drug, developing typical symptoms of overdosage by an anticholinergic substance. In muscarinic effect the drug seems usually to stimulate the upper gastrointestinal tract, so that nausea and vomiting are more prominent than with neostigmine. On the other hand, 17 of the patients still taking the drug report a reduction in the side-reactions as compared to previous medication, and another 20, still taking it, report a very definite prolongation of the effect as compared to previous medication. It is of interest that, in patients with tracheotomies, when larger doses of the drug caused increased secretions these were almost entirely due to salivation, with little, if any, increase in tracheal and bronchial secretions; this is of great benefit in the management of patients in respirators. The parenteral use of the drug suggests a 1/30 to 1 ratio to the oral dose, with a prolonged effect of nearly 3 hours, but experience in this type of administration is insufficient to recommend its use at present. S. L. W.

PHARMACOPŒIAS AND FORMULARIES

THE EXTRA PHARMACOPŒIA (MARTINDALE)*

INCORPORATING SQUIRE'S COMPANION

REVIEWED BY D. C. GARRATT

A bigger and better Volume II of the Extra Pharmacopœia has now been published. The ambition cf the Editor, Dr. Capper, and his collaborators to maintain the original concept of a comprehensive reference book and yet to expand it and add new sections has been successful. However, this success is not without its drawbacks, for although the publishers are to be congratulated on the production of a compact volume containing such a wealth of information, the user is given the task of reading almost microscopical print through some 1500 pages. The editorial staff must be complimented on the excellent proof-reading of such print.

The new Volume II has been completely revised and re-arranged and new sections added. The analytical addenda to chemicals and materia medica in Volume I includes valuable information from foreign pharma-copœias and other official publications but the abstracts from technical publications seem to be chosen quite haphazardly and the reader is given no indication of their value; for example, the sorting of oils by the iodine value of their unsaponifiable matter has been discarded as untenable for at least 20 years. The Editor disclaims in the Preface any pronouncement on the merits of the methods given but without some critical sorting the section loses its value.

This section is followed by others on physical techniques of analytical interest which have been revised to include recent applications. In a book of reference the elementary treatment of the theory of hydrogen ion concentration and the history of absorption chromatography from Tswett might well be deleted and it is difficult to understand why coulo-metric and dead-stop titrations should come under the general heading of Polarography. The section on titrations in non-aqueous media is new; this technique is proving wide application and the full treatment given should be of great value.

The Microbiological Assay of Vitamins, another new section, is a concise summary of a very extensive subject; a good feature is the various media for microbiological assay set out in tabular form to show their similarities.

In this edition, the former sections dealing with foodstuffs have been brought together under the general heading of Food Analysis; Water Analysis remains as in previous editions. Because of the fortuitous association of foods with drugs in the work of Hassell and their consequent incorporation in the Food and Drugs Acts it would need a courageous

reviser of the Extra Pharmacopœia to delete these sections on food and water analysis but, in fact, they are incongruous in a work of pharmaceutical interest. The associated section on Food Law, however, would be of value if allowed to remain.

The following section on Recognition of Organic Chemicals is a remarkable achievement and, if it fulfils in practice the possibilities held out on paper, it will be of immense value. It follows the usual approach of classification through the elements present but the re-arrangement and the correlation of derivatives and additional tests must have been a monumental task for which the authors can be congratulated.

The section on Nomenclature of Organic Compounds has of necessity been re-written and if after use of the information the reader is unable to name a chemical correctly from its structural formula it will not be the fault of the book. This section is followed by an absorbing review on structure-action relationships.

A new section on Compressed Tablets summarises the essential features of the methods of preparation of the various types of compressed tablets and the physical tests employed to study the pharmaceutical quality of the tablet produced. This section seems out of place in Volume II of the Extra Pharmacopœia and is more suited to a publication such as the *Pharmaceutical Pocket Book*.

The Bacteriological and Clinical Notes are very comprehensive; such a wealth of information makes Martindale so valuable as a reference book to clinicians. A more discriminate selection of media and staining methods would, however, save some unnecessary reading. Sterility testing has been well reviewed and most of the precautions necessary have been mentioned.

There is considerable information of practical value in the section on Disinfectants. Skin disinfection has been adequately dealt with but more space might have been given to air filtration for operating theatres and sterile rooms. The author tabulates Rideal–Walker coefficients of a typical black fluid against various organisms other than *Salmonella typhi*; by definition of the standard this is inadmissible. Dilution figures for Black and White Disinfectant Fluids are given without any mention of the germicidal value of the particular product to be used; without it the table is meaningless. With the advances in isotope work the section on Radiotherapy has been re-written.

Nutrition and Vitamins have been brought up to date. It is observed in the Preface that vitamins provide the most striking advances in nutritional research since the publication of the previous edition and that a vast amount of information has been accumulated but apart from vitamin B_{12} and vitamin C there is a disappointing paucity of information in this chapter for a book of reference.

The section on Chemical Tests and Microscopic Methods for the Examination of Urine, Blood, Fæces, Stomach Contents, etc., of the previous edition has been replaced by two entirely new sections, the first on Hæmatology, the other dealing with Clinical Biochemistry. The intention is to provide pathologists, pharmacists and workers in clinical

laboratories with details of the most useful of the published methods for the various examinations and determinations. The outcome has been almost a text-book of clinical biochemistry.

With the large amount of information packed in the confines of such a compendium it is essential to have a comprehensive index. Needless to say this part of the work is in keeping with the rest of the book.

The Extra Pharmacopœia Volume II is an encyclopædia of pharmaceutical information recommended to general practitioners in pharmacy and medicine as well as to the workers in specialised fields. The criticisms given above are small in relation to the fine quality of the publication; they are intended as constructive suggestions for the interest of the compilers. The Editor, his staff and collaborators must be given the highest praise for yet another Extra Pharmacopœia upholding the recognised high standard of the Pharmaceutical Society's publications.

BACTERIOLOGY AND CLINICAL TESTS

Tubercle Bacilli: Semi-solid Agar Media for Rapid Culture. R. Knox. (*Lancet*, 1955, **269**, 110.) Semi-solid agar media have certain great advantages over solid agar media for the growth of tubercle bacilli. Agar, in the concentrations of 1 to 2 per cent. necessary to give a gel firm enough for surface culture, is often highly inhibitory to tubercle bacilli but in the concentration of about 0·1 per cent. used in the semi-solid media it allows at least as rapid growth as the corresponding liquid media without the same risk of contamination or dehydration which so often spoils cultures of tubercle bacilli on the surface of agar plates; moreover the presence of a few contaminating organisms is not so disastrous as in liquid media. The semi-solid agar media used were those of Kirschner as modified by Mackie and McCartney and Dubos and Davis, to each of which was added agar in a final concentration of 0·1 to 0·125 g./100 ml. Horse serum (seitz-filtered) was added aseptically in a final concentration of 10 per cent. to Kirschner's medium, and bovine albumin fraction V (0·35 per cent.) and Tween 80 (0·05 per cent. to Dubos medium. For use, media were heated to melt the agar and cooled to 50 to 55° C. The medium was distributed aseptically into test-tubes, plugged with cotton wool, and kept in racks in a water bath at 48° C. ready for inoculation. The tubes were inoculated by means of dropping pipettes either with cultures of tubercle bacilli or directly with sputum concentrates, and were then incubated at 37° C. Inoculated tubes were examined after 2 days and at intervals thereafter. Profuse and rapid growth was consistently obtained. With large inocula, growth was easily visible in 2 or 3 days, while with small inocula discrete colonies could be counted in 7 to 10 days. The media have been used successfully for rapid primary cultures of tubercle bacilli from patients' sputa, for tests of drug sensitivity, for recording small numbers of viable organisms, for rapid viable counts, and for quantitative studies of the action of antituberculous drugs. S. L. W.

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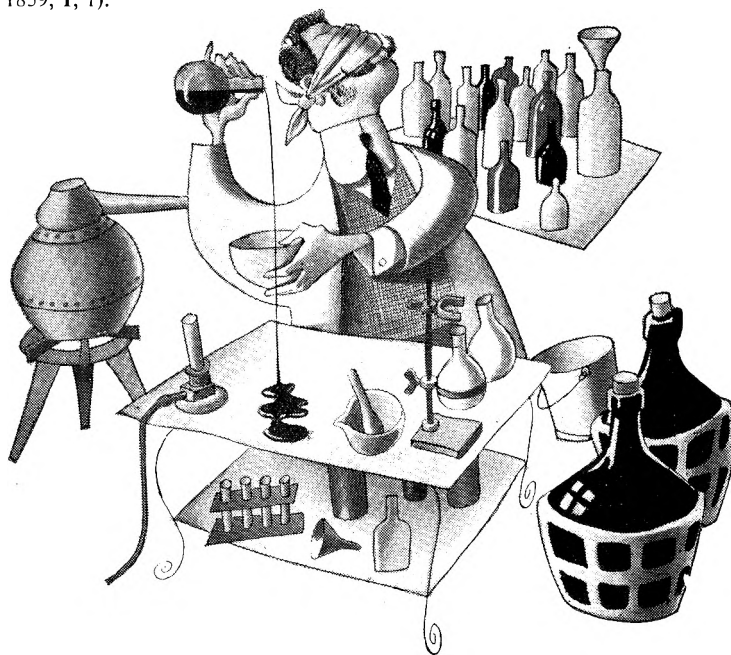
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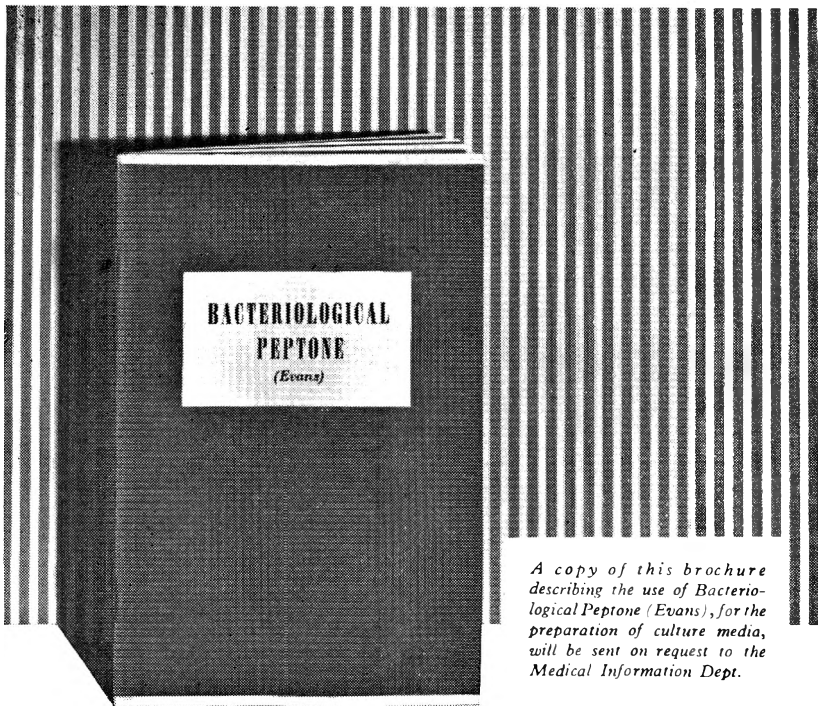
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