RECENT ADVANCES IN PHARMACEUTICAL ANALYSIS*

INFRA-RED SPECTROSCOPY

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Methods based on the measurement of the absorption of infra-red radiation by substances have up till now been used comparatively little in pharmacy. The reasons are many. Firstly, the equipment needed is expensive and available only in large laboratories or in big manufacturing units. Secondly, the work which must precede a determination is often rather tedious. Pharmacy with its broad spectrum of substances and complicated mixtures is, however, a natural field for the application of the infra-red technique. If one looks through some of the scientific pharmaceutical journals, one notices also in recent years a distinct trend to increased use of infra-red spectroscopy.

The fundamental advantages of the technique are two: (1) it is possible to obtain a fool-proof identification of a substance, and (2) it is usually possible to construct assay methods for quite complex mixtures. Where only a single substance is involved in a quantitative determination infra-red spectroscopy seldom has any special advantages over visible or ultra-violet spectrophotometry.

Infra-red technique is especially useful in basic work with a new substance, for example in the control of methods of syntheses. Another application which often is well worth investigating is the application of the technique to routine assays. In the first example the method often gives the best criteria of identity, and in the second example it is often the cheapest way to solve difficult or tedious analytical problems.

No attempt has been made in this survey of infra-red spectroscopy to cover the literature completely. On the contrary, only some special applications have been singled out; perhaps too many according to personal opinion and experience. Extensive literature reviews have been published during the last few years. Those of Gore[1–4], Price[5], Carol[6] and Larsson[7] deserve a mention. Recently some new books have appeared, for example, Bellamy[8], Brügel[9], Dobriner[10]. Older standard texts are by Randall[11] and by Barnes[12].

The modern instrumentation is well covered in the above cited literature. The instruments may be either single or double beam instruments. All commercial instruments are to-day based on some form of recording, usually coupled with a servo mechanism and are sturdy enough to allow robust use. The interpretation of the data, however, remains the difficult part of the technique. Good schemes covering typical group absorptions have been published and may be found in the literature cited above.

On the following pages some recent applications of infra-red spectroscopy to pharmaceutical problems will be discussed.

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IDENTIFICATION OF UNKNOWN SUBSTANCES

One of the most embarrassing problems for an analyst is to identify a single member in a large group of similar substances, for example from among the barbituric acids, antihistamines, derivatives of isoniazid or derivatives of cyanoacetic acid hydrazides.

The usual way to solve such a problem is to record a number of spectra of known substances and to compare the spectrum of the unknown

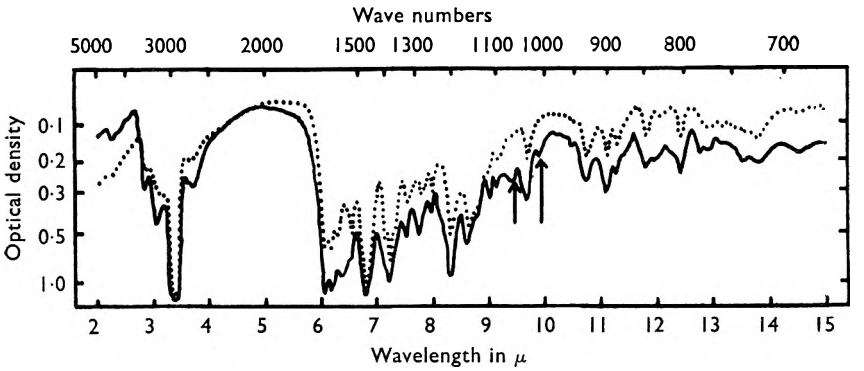


FIG. 1. Infra-red spectra of flavaspidic acids in paraffin mull. Note the difference at 9·44 μ (short arrow) and 9·92 μ (long arrow).

compound with the spectra of known compounds. If identical spectra can be found, identification is obtained. However, in practice the problem usually is a little more difficult. Often the commercial substances are not pure enough to give an unquestionable identification. A barbituric acid may contain a small quantity of an isomer. A very useful method in such cases is to record a differential spectrum. The method is well-known from ultra-violet spectroscopy, but not until recently has it been applied to infra-red spectroscopy by McDonald[13]. The method requires a double-beam instrument with sufficient energy from the source to allow the instrument to operate on a favourable signal-to-noise ratio. The method consists essentially in adding to the compensating cell the known compound in a concentration equivalent to that in the sample cell. The spectrum is recorded and, if

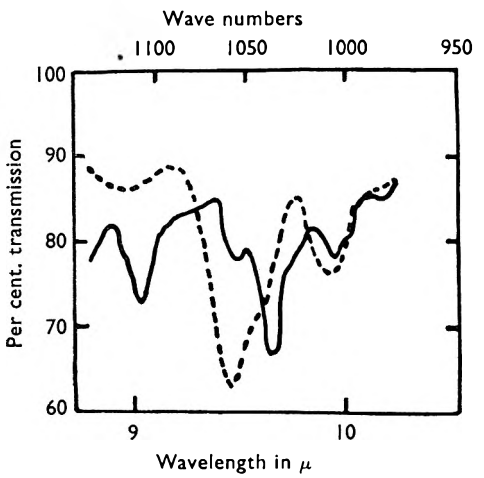


FIG. 2. Broken line = Differential spectrum of the two flavaspidic acids in Fig. 1.
Unbroken line = Amyl alcohol.

nothing but the same compound is present in both cells and in the same concentration a straight line is obtained. If an impurity is present in the unknown substance a rough spectrum of that compound is recorded. As an example I have included the infra-red spectrum of pure flavaspidic acid in a paraffin mull together with the spectrum of an impure acid. (Fig. 1.)

As seen, the spectra are very alike but some minor differences may be noticed at 9·44 and 9·92 μ. The differential spectrum in carbon disulphide, however, unveils immediately the presence of a second compound. (Fig. 2.)

If you compare that spectrum with the spectrum of amyl alcohol the impurity is identified. This method is very useful, especially when the purification of a new compound is being followed.

As a second example I have included a new morphine antagonist, recently introduced to medicine by Shaw *et al.*[14,15], 2:4-diamino-5-phenylthiazole (amiphenazole). (Fig. 3.)

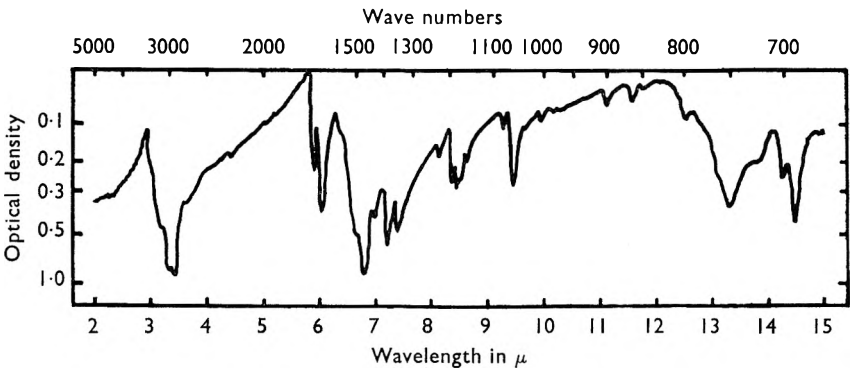
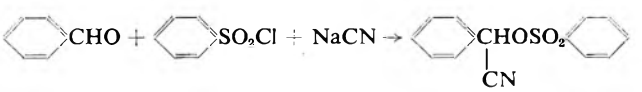
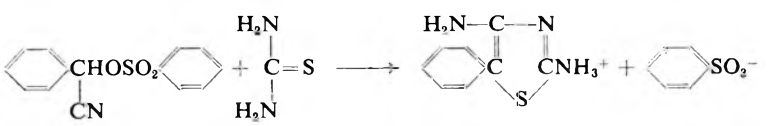


FIG. 3. Infra-red spectrum of 2:4-diamino-5-phenylthiazole hydrochloride in paraffin mull.

The substance can be synthesized by different routes. One way is to react benzaldehyde, benzene sulphochloride and sodium cyanide to give α-cyanobenzyl benzenesulphonate.

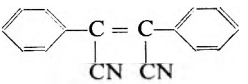


This compound reacts with thiourea:—



which can be transformed into the hydrochloride in the usual way. The reaction is in practice not as simple as the above scheme indicates. There are several possibilities for side-reactions. After running a few test syntheses we discovered that our final product was not a single substance. A small quantity of an unknown compound was present.

After some discussion we made a rough guess and the expected stilbene compound was shown to be the impurity:



The compound was synthesized. It was soluble in chloroform. Our impure 2:4-diamino-5-phenylthiazole hydrochloride was extracted with chloroform and the extracted compound was shown to be identical with the expected one. (Fig. 4.)

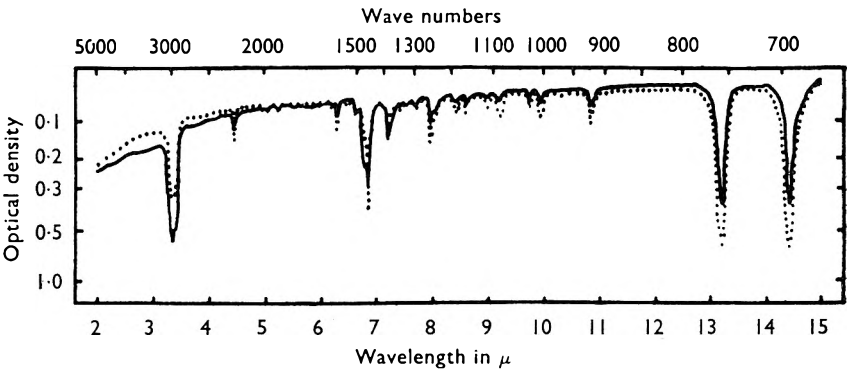


FIG. 4. Infra-red spectrum of αα′-dicyanostilbene.

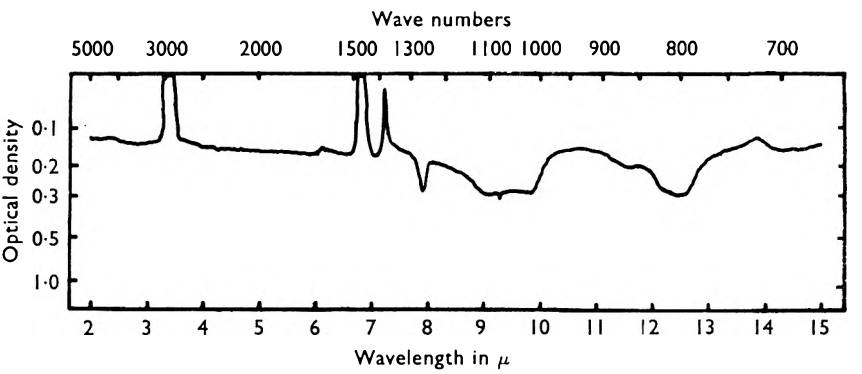


FIG. 5. Infra-red spectrum of extracted silcone-grease.

With this rather tedious method we were able to show the presence of side-reactions in the selected method of synthesis. One might have expected that the stilbene compound would have given a pronounced peak in the infra-red spectrum at 4·48 μ where the CN group has a strong absorption. This is not the case. However, a differential spectrum between the impure compound and the purified compound shows the presence of the stilbene compound.

When substances have been extracted in separators by solvents some caution has to be exercised in the interpretation of the recorded spectrum.

One particular spectrum was a peculiar one which introduced some unexpected difficulties until it was identified as the infra-red spectrum of the silicon-grease used on the stopcocks. (Fig. 5.)

An example may illustrate the usefulness of infra-red methods in stability tests. 2:4-Diamino-5-phenylthiazole is in aqueous solution very sensitive to hydrolysis. The amino group in the 4-position is easily replaced by an OH-group. If an aqueous solution is autoclaved, the resulting bases extracted and the infra-red spectrum of the mixture recorded, the spectrum is only slightly different from that of the parent compound. The spectrum of the pure 2-amino-4-hydroxy-5-phenylthiazole is given in Figure 6.

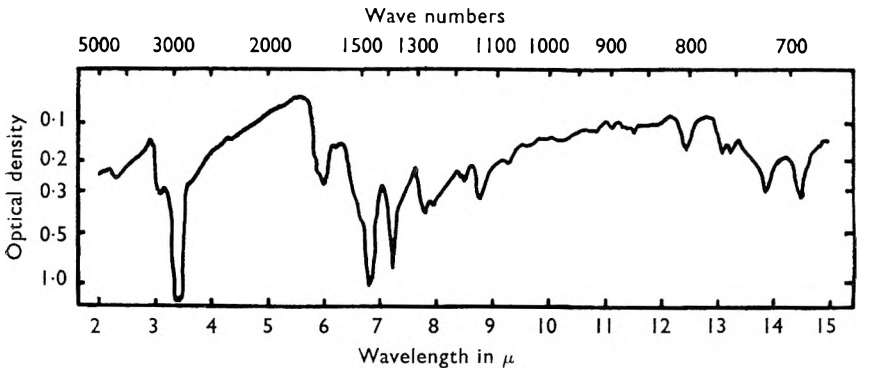


FIG. 6. Infra-red spectrum of 2-amino-4-hydroxy-5-phenylthiazole.

Usually not less than one mol. per cent. of an impurity can be seen on an infra-red spectrum. However, if an appropriate amount of the parent compound is added to the reference cell, it is possible to evaluate the degree of decomposition from the differential spectrum.

In a similar way it is possible to evaluate the decomposition of barbituric acids in alkaline solution.

QUANTITATIVE MEASUREMENTS

As previously mentioned, one of the greatest advantages of the technique is its application to assays of multicomponent systems. The first step is to choose a suitable technique for the preparation of the sample. The main problem is to choose between recording the spectrum of the solid substance (or mixture) or that of a solution of the substance (or mixture). When working with solids one can choose between the mull technique and the pressed potassium bromide disk technique. The latter is a recent contribution which eliminates some of the difficulties of the mull technique. The concentration in the pressed disk is fairly easy to estimate and disturbing absorption is not present as when working with paraffin mull. The pressed disk technique is elegant but requires a new tool and a heavy press.

The main part of the quantitative measurements on multi-component systems will in the future be made on solutions. One is not so much

limited by the choice of solvents as by the recording of suitable curves for identification. In the latter instance an essential point is to record as broad a spectrum as possible, while in the former, one can select narrow ranges where suitable peaks are located. The most used solvents for the recording of identification spectra are carbon tetrachloride, carbon disulphide and chloroform, quantitative measurements can, however, be made in polar solvents such as acetone and dioxane. Aided by the numerous tables of infra-red spectra of solvents, the analyst has little difficulty in choosing a suitable one. In this connexion it may be mentioned that acids like phenylacetic acid in carbon disulphide have a pronounced tendency to attack the amalgamated lead spacer in fixed-thickness cells, with the result that lead phenyl acetate is precipitated on the sodium chloride plates. The precipitate has a strong absorption and as a consequence the cell must be dismounted and cleaned.

When the solvent has been chosen, absorption curves of the substance or substances under test must be recorded and suitable bands selected for measurement. If only one substance is involved, the task is comparatively easy. However, if more than two, and as many as six substances are present, the problem of locating test points is much more complicated. Seldom is it possible to find test points for three substances all of which are free from interference of the other compounds and to which the Beer-Lambert equation may be applied. If polar solvents like dioxane or acetone are used, hydrogen bridges may introduce considerable difficulties. Usually it is necessary to introduce the old technique of correcting the values first obtained, then to start again with the corrected value, and go on until a repetition of the calculation gives values which are close or identical. The evaluation of a four component system can involve a remarkable amount of calculation. Special machines for this type of calculation have been constructed.

In recent years the isotope dilution principle using deuterium has been applied to a number of problems. The best known method is that of determination of penicillins in brews. The determination depends on the specific absorption of proteophenylacetic acid at 14·37 μ which deuterophenylacetic acid lacks. The technique presupposes deuterobenzylpenicillin. This compound is produced by fermentation, deuterophenylacetic acid being used as precursor. The latter is obtained from proteophenylacetic acid which is dissolved in deuterosulphuric acid which in turn is produced from SO_3 and D_2O. One obtains a compound with the approximate composition $C_6D_5CH_2COOH$.

Similar methods have been constructed for the determination of the γ-isomer of benzene hexachloride. A recent application is to tropic acid derivatives[16].

As mentioned above, it is usually not possible to estimate less than one mol. per cent. of an impurity by means of infra-red spectroscopy. A recent technique enables, however, such small amounts as 0·1 to 0·01 per cent. of an impurity to be determined. The method depends on the use of fractional crystallization[17]. Certain requirements must be fulfilled. The solubility of the main substance in the solvent chosen

should be of the same order of magnitude at or below room-temperature as the impurity. The solubility of the main substance shall increase with temperature, and further, the impurity shall not have a tendency to be occluded by the main substance during crystallization.

Further to increase the sensitivity of the method the differential method discussed above was used. The method was used for the determination of small quantities of catechol and resorcinol in hydroquinone. The substance to be tested was crystallized from acetonitrile, and the mother liquid which contained the impurities was worked up. The method seems to be generally applicable.

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GAS-LIQUID CHROMATOGRAPHY

A Method of Separation and Identification of Volatile Materials

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Most workers concerned with the separation of closely related compounds are familiar with chromatographic techniques involving the use of a stationary solid or liquid and a moving liquid. Less widely known however are chromatographic techniques utilising a stationary solid or liquid and a moving gas. The absorption chromatography of gases (gas-solid system) has been studied by many workers, in particular Claesson[1] and Janak[2] but until recently has not come into general use owing to the frequent occurrence of non-linear distribution isotherms with consequent impairment of separations. Gas-liquid chromatography is a recent innovation, suggested originally by Martin and Synge[3] and developed by James and Martin[4] for the separation of volatile fatty acids.

Since the mobile phase of the chromatogram is a gas, it is possible to use long thin columns and to obtain high rates of flow of mobile phase impossible with liquid-liquid chromatograms of similar shape. In all chromatograms the main factor limiting the efficiency is the rate of diffusion in the two phases concerned. In the gas-liquid system, diffusion in the gas phase is virtually instantaneous and the elevated temperatures at which the columns are generally run increases the rate of diffusion in the stationary liquid phase. For these reasons gas-liquid columns have very high efficiencies even at high rates of flow so that refined separations may be carried out more rapidly than with any other chromatographic technique. In addition it is generally much easier to detect low concentrations of vapours in gas streams than it is to detect low concentrations of solutes in solvents.

The columns are constructed of lengths of glass or metal tube into which is packed by vibration a suitably inert solid, usually kieselguhr, over whose surface is distributed a thin layer of the stationary liquid, a thermally stable substance of high boiling point. The column is held in a heated jacket at the desired temperature. The substances to be separated are applied to the top of the column packing with a micropipette and are then blown down the column by a stream of permanent gas, usually nitrogen, from a constant pressure source. The substances separate according to their relative volatility in the stationary liquid chosen and are detected as they leave the column in vapour form in the stream of permanent gas.

There are many theoretically possible methods of detecting vapours in gases but so far only three have been widely used. The first method is limited to those substances capable of ionising in solution or which can be caused to produce ions by rapid chemical reaction in solution. The gas stream from the column is led into the base of a small cell

containing water or other solvent. The acids or bases are extracted as the nitrogen bubbles through the solvent and can be continuously titrated. James and Martin[4] devised a simple automatic recording burette to carry out the titration continuously so that after loading the column and turning on the gas stream the whole process was automatic, the final result being recorded on a moving drum (see Fig. 1). The method was

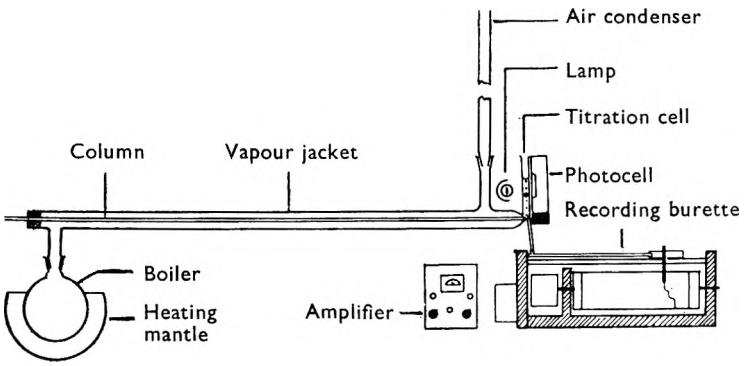

FIG. 1. Schematic lay-out of apparatus for separating volatile acids or bases, using titration as the method of detection and estimation.

sufficiently sensitive to allow the detection of 0·02 mg. quantities of acids such as acetic acid or of 0·002 to 0·01 mg. quantities of volatile amines such as ethylamine.

The records produced by the automatic burette show a series of steps (see Fig. 2, lower curve) rather reminiscent of the types of diagrams

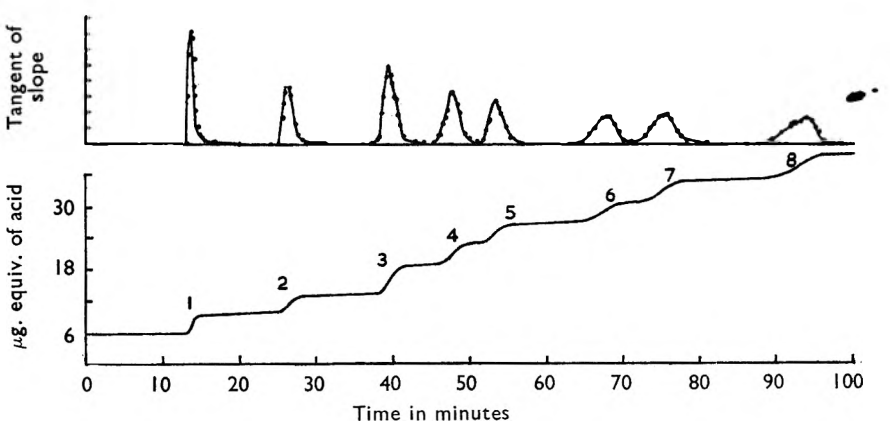

FIG. 2. The separation of acetic (1), propionic (2), *iso*butyric (3), *n*-butyric (4), trimethylacetic (5), *iso*valeric (6), methylethylacetic (7) and *n*-valeric (8) acids, showing complete resolution of all acids.

Upper curve: Differential of experimental curve.

Lower curve: Experimental results.

Column length, 11 ft.; stationary liquid phase, DC550 silicone fluid containing 10 per cent. (w/w) stearic acid; temperature, 137° C.: rate of flow of nitrogen, 18·2 ml./min.; pressure of nitrogen, 74 cm. Hg.

used to follow fractional distillations. When only carrier gas is leaving the column a horizontal line is drawn by the automatic burette and the emergence of titratable material is denoted by a step in the curve, the height of the step being a direct measure of the amount of material titrated. The machine is recording in effect a measure of the total amount of material that has emerged from the column. By plotting the differential of the experimental curve there result the peaks so familiar to those who have used chromatographic techniques (see upper curve, Fig. 2).

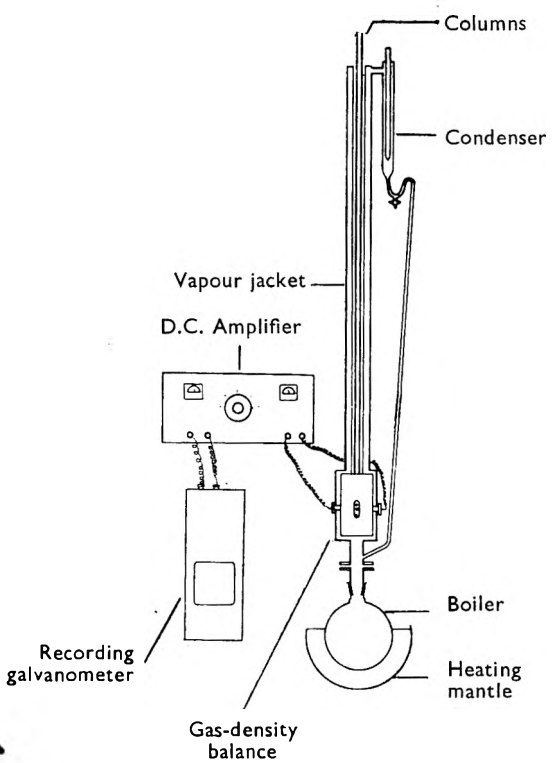

FIG. 3. Schematic layout of gas-liquid chromatograph using measurement of gas density as the detecting technique. Applicable to all volatile compounds.

Wider application of the gas-liquid chromatogram necessitates a detection technique that does not depend on the presence of particular types of chemical grouping in the molecules to be detected, so physical methods are indicated.

The second method used is to measure the thermal conductivity of the gas leaving the column and has been chiefly exploited for this purpose by Ray[5], and by Phillips and his colleagues[6]. The apparatus (the catharometer) consists of a platinum wire heated electrically and fixed along the axis of a tube through which is passed the gas stream. The composition of the gas stream determines the rate of loss of heat of the wire and hence its electrical resistance. By using two cells, one for the gas stream leaving the column and the other for the gas stream entering the column, and connecting the two wires in a bridge circuit, any change in resistance of the wire in the chromatogram gas can with the aid of a suitable amplifier and recorder be plotted automatically. Whilst having the advantages of simplicity and cheapness the method suffers from two main defects: (*a*) not very high sensitivity and (*b*) a high sensitivity of its zero to changes in rate of flow of gas through the cells.

The third method utilises two columns, one being the chromatogram,

the other acting as a reference source of carrier gas, any volatility of stationary phase being thus compensated for. The densities of the two gas streams are continuously compared in an instrument known as the gas density meter developed by Martin[7]. In general the emergence of a substance from the chromatogram increases the density of the carrier gas. The instrument consists of a series of tubes bored in a copper block, maintained at the same temperature as the columns in order to prevent any condensation of the vapours, and connected in a manner analogous to a Wheatstone bridge. Adjustable throttles are placed in some of the channels so that when the instrument is first set up, resistance to flow in the channels can be adjusted until no pressure difference is generated across the network when the rate of flow of either gas stream changes. Two sets of channels, one for each gas stream, are connected by a cross channel in such a way that any difference in density between the two gas streams causes a small flow of gas through the cross channel. This channel contains a flow detector consisting of a small filament, heated electrically, arranged below and equidistant from two connected thermojunctions; any cross-flow of gas causes the stream of hot convected gas to be diverted to one or other of the thermojunctions, heating one and cooling the other. The resultant thermo e.m.f. is fed to a D.C. amplifier whose output is led to a recording galvanometer. The galvanometer deflection is linearly related to the density difference of the two gas streams making quantitative measurement relatively simple. The

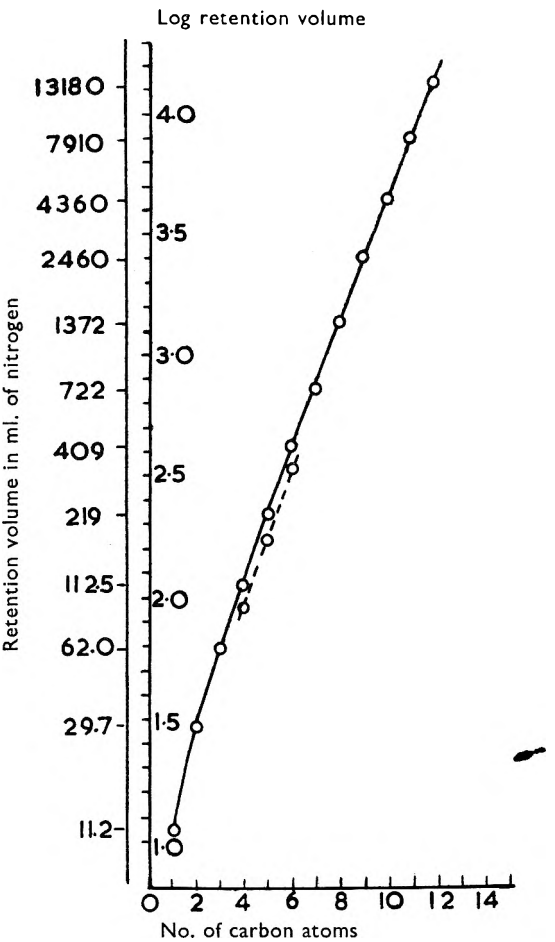

FIG. 4. The relationship between the retention volume at zero pressure difference, $V^{\circ}r$, and the number of carbon atoms in the lower fatty acids. Data are for a 4-ft. silicone-stearic acid column at 137°C. ○—○. *n*-acids; ○- - -○ *iso*-acids.

instrument is highly sensitive, density differences such as those caused by the presence of one molecule of amyl alcohol in 50,000 to 100,000 molecules of nitrogen being easily detectable. Its zero is relatively insensitive to changes in rate of gas flow and its response is determined only by the molecular weight of the substance being detected. As with the other two methods described, the whole process is automatic once the column has been loaded and the carrier gas stream started. A schematic diagram of the whole apparatus is shown in Figure 3.

SOME APPLICATIONS OF THE TECHNIQUE

The separation of fatty acids and their esters

In Figure 2 is shown a separation of acetic, propionic, *iso*butyric, *n*-butyric, trimethylacetic, *iso*valeric, methylethylacetic, and *n*-valeric acids using a column 11 ft. long, arranged in a hairpin shape so that its overall length is only 4 ft., at 137°C. The total time for the separation is only 100 minutes. All the acids are well separated as is demonstrated in the upper curve, the differential of the lower experimental curve. The four isomeric valeric acids are difficult if not impossible to separate in such small amounts by any other technique. This result was obtained by using the automatic burette to detect the zones leaving the column.

The columns give highly reproducible results and can be used some hundreds of times without exhaustion. The time at which a given substance emerges from a column depends on the temperature, the flow rate of nitrogen and on the nature of the stationary phase in the column. At a given temperature and with a particular stationary phase it is found that there exists a simple relationship between the retention volume (the volume of nitrogen passing through the column before the centre of the peak emerges) and the number of carbon atoms in the molecule for the members of any homologous series. In Figure 4 is shown the result obtained with fatty acids when log. retention volume is plotted against the number of carbon atoms in the molecule. The straight line produced demonstrates that the free energy of solution of the CH_2-group added to the molecule in ascending the series is constant and independent of its position in the chain. This regularity of chromatographic behaviour enables one to identify an acid from its time of appearance relative to a standard acid; this relative retention volume can be considered as a physical constant as useful as an R_F value or melting point or boiling point for identification purposes.

The range of acids capable of being detected is limited by the titration technique because it is not possible to raise the column temperature to a value high enough for a reasonable rate of movement of the longer chain fatty acids. Recently the range has been extended to include the C_{18} acids, using methyl esters instead of the free acids, with the gas density meter[8] as the detector. In Figure 5 is shown the separation of a variety of saturated and unsaturated methyl esters carried out with a 4 ft. column at 200° C. The range covered here is from methyl-*n*-heptanoate to methyl stearate in a time of 100 minutes. With this type

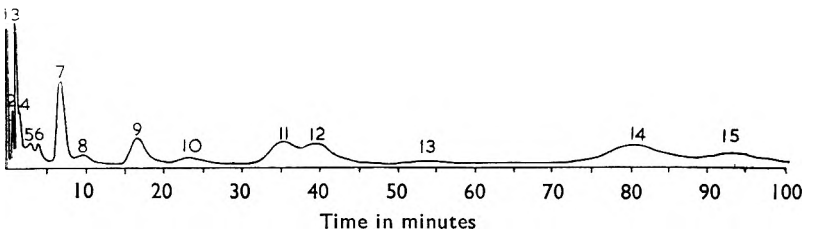

FIG. 5. The separation of methyl esters of some branched- and straight-chain saturated acids C_8 to C_{18} and also palmitoleic and oleic acids on a 4 ft. column with Apiezon M vacuum grease as stationary phase at 197°C. Nitrogen pressure 76°5 cm. mercury, nitrogen flow rate 98 ml./min.

Peaks in order of appearance:—

1. Air
2. Methyl-*n*-heptanoate
3. Methyl-*n*-octanoate
4. Methyl-*n*-nonanoate
5. Methyl-*n*-decanoate
6. Methyl-8-methyldecanoate
7. Methyl-n-dodecanoate
8. Methyl 10-methyldodecanoate
9. Methyl-*n*-tetradecanoate
10. Methyl-10-methyltetradecanoate
11. Methyl-*cis*-palmitoleic
12. Methyl-*n*-hexadecanoate
13. Methyl-14-methylhexadecanoate
14. Methyl oleate
15. Methyl-*n*-octadecanoate

of column acids differing in chain length by one carbon atom can be separated, (cf. an earlier attempt by Cropper and Heywood[9], an *iso* or anti-*iso* acid can be separated from its straight chain homologue, and mono- and di- unsaturated acids can be separated from the corresponding straight-chain saturated acids.

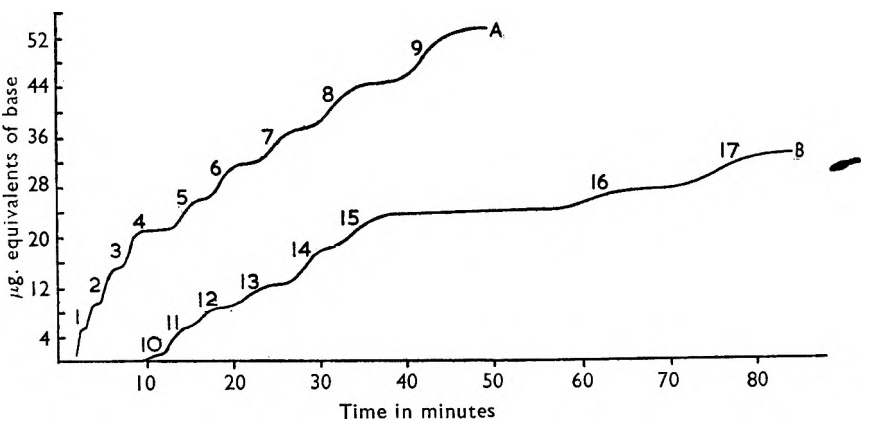

FIG. 6. The separation of 15 aliphatic amines on a 4 ft. column, with liquid paraffin as the stationary phase, at 100° C.

Curve A: Rate of flow of nitrogen, 5·7 ml./min.; pressure of nitrogen, 7·5 cm. Hg.

Curve B: Rate of flow of nitrogen 18·7 ml./min.; pressure of nitrogen, 22·5 cm. Hg.

1. Methylamine
2. Ethylamine
3. *iso*Propylamine
4. *n*-Propylamine
5. *iso*Butylamine
6. *n*-Butylamine
7. Di*iso*propylamine
8. *iso*Amylamine
9. *n*-Amylamine
10. *iso*Amylamine
11. *n*-Amylamine
12. Di-*n*-propylamine
13. *iso*Hexylamine
14. *n*-Hexylamine
15. Di-*sec*.-butylamine
16. *n*-Heptylamine
17. Di-*n*-butylamine

The separation of volatile bases

The bases dealt with so far by this technique have been ammonia and the three methylamines[10], higher aliphatic amines and substituted pyridines[11] and aromatic amines[12]. In all cases titration was used for detection and estimation. The aromatic amines, because of their weak basicity, were titrated in glacial acetic acid solution with perchloric acid. An example of a separation of aliphatic amines is shown in Figure 6, and an example of a separation of aromatic bases in Figure 7.

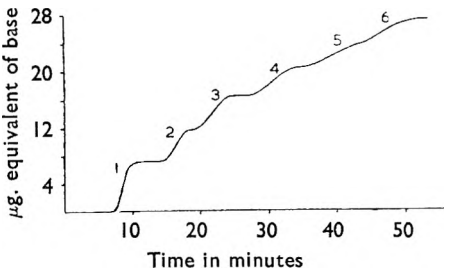

FIG. 7. The separation of aromatic bases. Column length, 4 ft.; stationary phase, liquid paraffin; temperature, 137°C.; rate of flow of nitrogen, 75 ml./min.; pressure of nitrogen, 47·2 cm. Hg; titration carried out in glacial acetic acid with perchloric acid.

1. Aniline
2. Methylaniline
3. Dimethylaniline
4. *m*-4-Xylidine
5. Dimethyl-*o*-toluidine
6. Diethylaniline

The separation of other types of compounds

Two further examples of some typical separations are shown in Figures 8 and 9. Figure 8 shows a range of alcohols from methanol to *n*-pentanol, the separation being completed in only 32 minutes. The separation of ethanol and *iso*propanol would be improved if the column were run more slowly. Figure 9 shows the separation of five ketones carried out in less than thirty minutes.

FACTORS INFLUENCING THE SEPARATIONS

At a constant temperature and a constant flow rate of mobile phase the relative times of emergence of a mixture of compounds are dependent on the differences in their free energies of solution in the stationary phase. In solvents such as paraffin hydrocarbons the forces involved in solution are Van der Waals forces, which vary with molecular weight, configuration

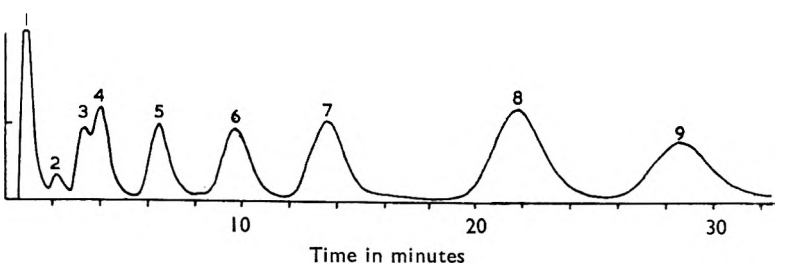

FIG. 8. The separation of alcohols. Column length, 4 ft.; stationary phase, benzyldiphenyl; temperature, 100°C.; rate of flow of nitrogen, 12 ml./min.; pressure of nitrogen, 13 cm. Hg.

1. Air
2. Methanol
3. Ethanol
4. *iso*Propanol
5. *n*-Propanol
6. *iso*Butanol
7. *n*-Butanol
8. *iso*Amyl alcohol
9. *n*-Amyl alcohol.

etc., and not with the polarity of the solute molecule. In such paraffinic stationary phases therefore separations are based on differences in molecular weight, etc.

In more polar stationary phases such as polyethers, long-chain alcohols, aromatic hydrocarbons etc., polar forces such as hydrogen bonding are also involved. Separations in these phases can therefore take advantage of differences in polarity of the solute molecules. Aliphatic amines for

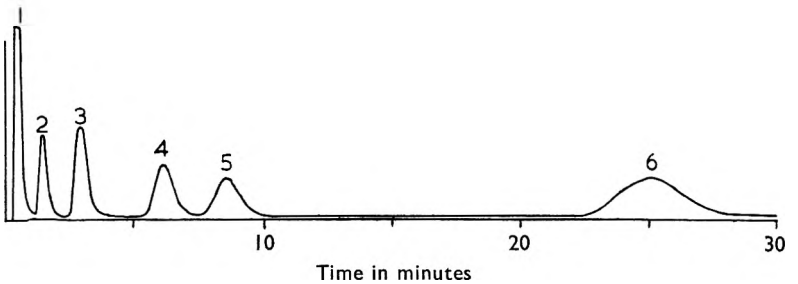

FIG. 9. The Separation of ketones. Column length, 4 ft.; stationary phase, paraffin wax; temperature, 100°C.; rate of flow of nitrogen, 24·8 ml./min.; pressure of nitrogen, 17·8 cm. Hg.

1. Air
2. Dimethyl ketone
3. Methylethyl ketone
4. Diethyl ketone
5. Methyl*iso*butyl ketone
6. Methyl-*n*-amyl ketone

example fall into three types, primary, secondary and tertiary. Primary and secondary amines possess an -NH group and are therefore capable of hydrogen bonding with a suitable acceptor molecule such as an alcohol or an ether. Tertiary amines possess no -NH group and cannot hydrogen bond in the same way. Thus methylamine, dimethylamine and trimethylamine emerge in order of molecular weight from paraffin columns and in order of hydrogen bonding power (i.e. the reverse order) from columns with substances such as glycerol as stationary phase. A study of the behaviour of different types of amine on two types of column, one with a paraffin hydrocarbon stationary phase and the other a polyether stationary phase showed that plotting relative retention volumes, relative that is to a standard substance, in one stationary phase against the corresponding values obtained in the other gave rise to a family of straight lines of different slope. Each line corresponded to a different type of amine, i.e. primary, secondary, tertiary, diamino, hydroxyamine, so that an unknown amine could be classified by running it on both types of column and comparing the values obtained with the standard values from the graph. The different slopes refer to different ratios of Van der Waals solution forces to Van der Waals and hydrogen bonding solution forces[11].

A similar study carried out with a wider range of substances, comparing behaviour in the paraffin hydrocarbon stationary phase with that in an aromatic hydrocarbon stationary phase gave rise to the result shown in Figure 10. Again substances of similar chemical type fall along one of a family of straight lines. In practice it was found that aliphatic

hydrocarbons, alkyl chlorides, bromides, iodides, amines, alcohols, methyl ketones, nitriles and nitro compounds could be readily differentiated. In this manner a great deal of information can be obtained as to the structure of an unknown compound.

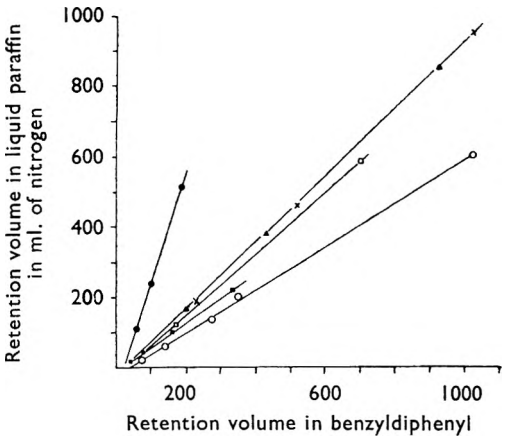

FIG. 10. Graphical representation of relative interaction forces in two types of stationary phase, liquid paraffin and benzyldiphenyl.

●—● *n*-Aliphatic hydrocarbons
▲—▲ Aromatic hydrocarbons
×—× *iso*Alkyl iodides
□—□ *n*-Akyl iodides
■—■ Alcohols
○—○ Ketones.

To conclude, the advantages of gas-liquid chromatography can be seen to be: (1) highly refined separations can be carried out very rapidly; (2) the technique can be used with quantities of material ranging from micrograms to grams; (3) a study of the chromatographic behaviour of an unknown substance can give valuable information as to its structure; (4) it is applicable to all substances capable of being distilled, and (5) suitable commercial apparatus is already available in the United Kingdom and the United States. Indeed the advantages of the technique over analytical distillation in terms of efficiency, time and convenience are so great that in time it will replace the older technique.

I should like to thank the Editors of the *Biochemical Journal* and the *British Medical Bulletin* for permission to reproduce material already published.

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ELECTROMETRIC TITRATIONS

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Introduction

The electrometric titrations used in pharmaceutical assays form a special section of the important field of electrometric determinations in chemical analysis, and their development is so closely linked with that of general methods that it is not always easy to separate them. This fact cannot be overlooked when describing recent progress, all the more so because those methods not particularly designed for pharmaceutical preparations may easily be applied to them or, at least, serve as a useful starting point.

The usual reservations must also be made about applying an existing method for assaying a certain substance to all cases. Analytical methods cannot be applied generally; this is even truer in pharmaceutical determinations, where working requirements necessitate the addition to drugs of other substances such as antoxidants, stabilizers, buffers and colouring agents as well as various excipients such as sugar, fats, starch and talc, so that the methods described for the analysis of the pure substance are often of little help to the pharmaceutical analyst, who must solve the problem of detecting, identifying and estimating often extremely small quantities of active substance.

Descriptions of assays taken from pharmaceutical literature and sometimes from general literature will therefore be given.

Except for a few cases, this survey refers to works published since 1951 and, naturally, must not be considered as complete. It does not contain any details of the theoretical principles of electrometric methods, for which the reader is referred to specialized articles. On the other hand, it is well to mention briefly the well-known advantages offered by these methods. First of all, they do not depend on the operator's judgment of the end-point, this always being the most subjective factor affecting the result of an analysis. Secondly, they permit the application of certain methods to highly coloured or turbid solutions, and other cases in which it is not practically possible to use indicators. Furthermore, they can be used in titrating very dilute solutions, with great accuracy, for example graphical methods or simple calculations indicate the end-point much more precisely than the volume of a drop from a burette.

In this way, it is possible to carry out titrations in which as little as 0·03–0·04 ml. of solution are used, with obvious advantages. Finally, the end-point shown by the indicator is not always unaffected by other substances present in the solution for titration, apart from the fact that certain reactions, such as the simultaneous titration of more than one halogen, can be rapidly carried out only electrometrically. The advantages largely compensate for the disadvantages of making numerous burette readings, and also for the necessity for slightly more complicated equipment which may however be adjusted to suit the purpose.

Potentiometric Titrations and Dead Stop

This section summarizes potentiometric methods and the so-called dead stop end-point, which are dealt with together, because they are often applied concurrently.

No description will be given of the basic apparatus usually employed, but it may be stated that this is continuously being improved. The mercury electrode has been suggested as a reference electrode and has been used coupled with the glass electrode. Descriptions have been published of a new mercury-mercurous acetate indicator electrode[1], a modern automatic titrator and a semi-microautomatic apparatus for semi-microanalysis. In order to interpret the results obtained with equipment registering the potential-quantity curves of a solution, apart from the usual methods, the use of the method of concentric arcs has been suggested. A series of concentric arcs is inscribed on a template which is used to determine the centres of curvature of the two parts of the graph on either side of the end-point: the centres are joined by a straight line and the point at which this intersects the graph indicates the end-point.

Much information about the results and methods may be obtained from the literature and reviews, each containing a large number of references. Some of these surveys refer to electrometric methods for pharmaceutical products[3,4] but many more, unfortunately, only to general methods. Particular care has been paid to the discussion and classification of potentiometric methods and equipment for the pharmaceutical analytical laboratory has been catalogued[5]. In order to give an idea of the thoroughness of this survey and its evident interest, *Analytical Chemistry* divided its April 1955 number into two sections, one containing only the surveys and consisting of 116 pages, with almost 3000 references.

Aromatic amines. Up to 5 years ago, only a few attempts[6] had been made to titrate aromatic amines electrometrically using sodium nitrite, and fully satisfactory results had not been obtained. The problem was solved by many investigators between the end of 1950 and the beginning of 1951. We, ourselves, reported[7] excellent results using Pinkhoff's method, the dead stop end-point method, the bimetallic couple method and the normal potentiometric one, and emphasized the advantages of a method that abolishes the use of starch-iodine paper and the consequent indicative tests, and of ice, and, above all, allows the reaction to be carried out even when the liquid is coloured or becomes coloured during the reaction. The method is applicable to many pharmaceutical substances and not only to normal primary amines, but also to other substances of which the NH_2 group reacts with nitrite, for example, sulphonamides, novocaine and *p*-aminobenzoic acid can be titrated by this method.

At almost the same time[8–10], and later[11], other authors, mostly working outside the pharmaceutical field, obtained equally good results, confirming that the method could be used for titrating compounds such as derivatives of aniline, naphthylamine, and amino-anthroquinone, amino-azo dyes and diphenylamine. Each author gives working details of the methods which are rapid, easy[12, 13] and of very wide application.

Aliphatic amines. Aliphatic amines have also been titrated potentiometrically even in mixtures of amines[13].

Halogens. These form one of the groups of ions that most often have to be assayed under widely differing conditions, so that the methods used are very numerous. Some methods have been given of the titration of traces of chlorine[14]. Bromide and thiocyanate in the presence of each other may be determined in acetone at a concentration of 80 to 90 per cent. as the reaction cannot take place in aqueous solutions[15]. Iodides and bromides present in the same solution in the proportion of 1 : 5000 may be titrated argentimetrically, using platinum electrodes in the presence of barium.

Iodides can be titrated with silver nitrate using a hydrogen electrode, and the silver with potassium iodide, both in the presence of *p*-ethoxychrysoidine[16].

Even small quantities of chloride present in solutions of high ionic strength[17], or in organic compounds[18] can be titrated, so that it is possible to combine Pregl's technique with electrometric titration. Some methods now simplify those previously used for chlorine and show that chlorine and bromine can be titrated using amalgamated gold electrodes[19]. Many others are suitable for routine assays[20], for small quantities[21], or for use with automatic apparatus[22]. Even the dead stop end-point method can be used for assaying bromine and iodine present in the same mixture[23].

Free or combined acids. Acids whose salts give an acid reaction on hydrolysis such as aluminium sulphate can be titrated with alkali. Pentavalent phosphoric acids and small quantities of oxalic acid can be titrated using the dead stop method, a potential difference of 200 mV. being applied to the electrodes. Acetic, formic, benzoic, cinnamic, tartaric acids, as well as many alcohols are first oxidized and the excess of oxidizing reagent is then titrated potentiometrically. Under certain conditions, the sulphate ion may be directly titrated with the barium ion. Sodium gentisate may be potentiometrically titrated with iodide solutions, using platinum-calomel electrodes[24].

Alkaloids. Nicotine is titrated[25] with acetic acid and some of its derivatives; morphine and opium[26] by oxidation and very many other alkaloids by precipitation with picrolonic acid[27].

Miscellaneous substances. Vanillin, *p*-hydroxybenzaldehyde, benzaldehyde formaldehyde, furfurol, urea and many other similar products, sulphonamides[28], hydrazine, *iso*nicotinic acid, hydroxylamine[29], hydroquinone, cardiotonic glycosides[30] and tri-(diethylaminoethyloxi-) 1 : 2 : 3-benzene triethiodide[31] have all been satisfactorily titrated.

Dead stop end-point

In view of the wide use of this method, many explanations[32] and clarifications of the principle have been reported[33–35].

Antimony and Arsenic. The dead stop method has been used for the assay, by oxidation with potassium bromate, of important products of pharmaceutical interest, such as the arsenious acid in Fowler's solution

and the antimony in Seignette's salt[36]. Even ascorbic acid has been successfully assayed[36].

Methods in Non-aqueous Media

In recent years, the great possibilities of electrometric methods have been extended to the wide and extremely fruitful field of non-aqueous titrimetry developed from Brønsted's theory; in fact, as has elsewhere been stated, indicators often have only a limited use[37]. Very many basic compounds may be titrated with perchloric acid: sodium derivatives of barbituric acids[38], papaverine[39], alkaline metal salts of aliphatic acids[40], sodium *p*-aminosalicylate[41,42], and caffeine[43, 44]. A good indication of the possibilities of the method is given in a paper[45] reporting the results obtained on 65 different compounds such as alkaline or alkaline earth salts of organic acids, primary, secondary and tertiary amines, amino-acids, amino alcohols, derivatives of pyridine hydrazine and hydrazide, as well as hydrochlorides of various bases the titration of which is made possible by addition of mercuric acetate.

Other investigators[46] have titrated many of these, and other products, such as urea, caffeine, antihistamines[47] present in the form of hydrochlorides, stovaine and lignocaine[48].

Amidopyrine and barbituric acids in the same solution[49], proguanidine[50] isoniazid[51] and certain antihistamines[52], have all been satisfactorily titrated and a general method for antihistamines has been evolved[53]. Titration in non-aqueous media may also be applied to injections in aqueous solution[54], and are especially useful for the active principles of suppositories[55].

Excellent results[56] were obtained for *p*-aminosalicylic, perchloric, formic, phenolic[57] and hydroxybenzoic acids[58]. The possibility of utilizing a wide range of basic solvents permits good differentiation of even extremely weak acids[58]. The effects of the substances being titrated on the slope of the titration curve have been illustrated[59], by over 75 examples; even fatty acids and many of their substitution products can be satisfactorily titrated[60].

Oxidation-reduction titrations can also be performed in non-aqueous media and have been described for various compounds ranging from bromides to permanganate, and hypobromites to sulphonamides[61,62].

Conductimetric Titrations

The application of this method does not seem to be spreading, nevertheless, it is very useful in certain cases: since all the ions present influence the result, the reaction of an ion which produces no potential and without a definite polarographic wave can be studied by conductimetry, and this method often enables ions not taking part in the reaction to be used for qualitative purposes.

Of the latest applications, mention should be made of acid-base titrations in water-organic solvents mixtures[63], the titration of weak organic acids[64] and of weak bases[65] and of hydrochloric and sulphuric acids and their mixtures, in non-aqueous solvents[66]. Barbituric acids (diethyl,

butylethyl, methylphenyl and phenylethyl), their sodium salts and thiopentone, (even mixed with carbonate)[67] as well as amidopyrine[68]—the latter two substances together[49]—and, finally, thiouracil and some derivatives[69], can be titrated conductimetrically.

POLAROGRAPHY

The importance of polarography in metallurgical analysis has long been known. During the last few years, particularly after the Prague Congress (1951), where the wide possibilities of this method were fully emphasized, even organic polarography has been enormously extended.

The increased interest in this field is shown by the work dealing with its theoretical principles and describing increasingly improved apparatus[70] and the new techniques studied for special cases.

A good deal of this development is due, among other things, to studies carried out with anhydrous or mixed solvents which sometimes allow operation in more favourable conditions than those given by aqueous solutions, and also permit the polarographic investigation of numerous organic substances insoluble in water.

The progress of polarographic analysis has widened the horizons but applications to pharmaceutical analysis, although numerous, appear to have only touched the fringe of the subject, if the possibilities of the method are considered.

Metals. First of all, it is necessary to mention the many determinations on metals, these being the most simple ones carried out by the polarographic method, that may be related to, or possibly connected with, some pharmaceutical problems.

Traces of metals have been determined in commercial gelatin[71] biological material, and as heavy metal impurities in organic substances of pharmaceutical interest such as dried yeast, carboxymethylcellulose[72], and in the glass of containers for injections.[73] Polarographic methods have been proposed even for arsenic and are based on the oxidation of the element in alkaline solution, or its reduction in acid[74, 75].

Organic substances containing metals. It is possible to assay polarographically metals present in an organic molecule, e.g., the mercury in phenylmercuric nitrate[76], the cobalt in vitamin B_{12}. This subject has been studied by various investigators, who have reached interesting conclusions on the possibility of distinguishing the various forms of the vitamin[77–80], Renal elimination of cobalt administered as vitamin B_{12} or as the chloride[81], has also been followed.

Vitamins. The polarographic method continues to be successfully used in assaying vitamins. Many studies have been carried out on the various forms of vitamin K, even in non-aqueous solvents, and also on the behaviour of other vitamins: vitamin D_2, analysed in tablet form and in solutions for injection, and whose photolytic decomposition has been followed, with results agreeing with those obtained spectrophotometrically[82]. Vitamin C may be determined in the combined form in vegetable extracts[83] even in the presence of similar substances accompanying it during the vegetative period of the plant, and in the various parts of the

latter. The behaviour of this vitamin in both reduced[84] and oxidized[85] forms has also been reported.

The polarographic behaviour of folic acid has been known for some time, and a method has been put forward for its assay in whale liver oil[86], where its presence has been detected by this same method. The existence of the S-S form along with -SH in aneurine has been demonstrated, and the relative amounts of the two determined[87]. This vitamin has even been assayed in pharmaceutical preparations, within limits of $\pm$ 3 per cent[88].

Research on the behaviour of nicotinic acid and its isomers[89] is still in progress; polarography preceded by separation using ion exchange resins has been used to assay nicotinamide present in extremely small quantities in pharmaceutical preparations and vitaminized powdered milk[90].

Steroids. Further studies have been carried out on the indirect analysis of steroids, using Girard's reagent[91], in order to find a method for their assay in biological fluids. An already known method of determination in aqueous dispersion[92] has been used for directly reducible compounds. The behaviour of some steroids has recently been studied[93], and the polarographic results compared with the colorimetric ones[94].

Alkaloids. Very many analytical methods have been proposed for alkaloids. A direct determination is based on measuring the catalytic current produced by the heterocyclic rings present in the molecule or the diffusion current due to possible reducible groups—as in the case of nicotine[95], the alkaloids of veratrum viride[96,97], and many others. Of all the direct determinations of non-reducible alkaloids, that of scopolamine after nitration[98] and of morphine after treating with nitrous reagents[99], are extremely interesting, as very small quantities can be assayed, the latter even in the various parts of the plant during growth.

Proteins. The characteristic behaviour of the proteins in producing catalytic waves in ammoniacal cobalt solutions continues to be studied, in view of the importance of the assay and properties of proteins in medicine[100]. For example, the catalytic wave method has been used to assay proteins in whey[101]. Furthermore, the possibility of introducing a polarographically reducible group[102, 103] into the molecule has been used for assay, and other indirect methods of analysis have also been applied[104–107]. Substances of high molecule weight, such as proteins, may also be distinguished by the anodic waves they form in suitable base electrolytes[108] and by the reduction or oxidation waves caused by particular functional groups[109, 110].

Antibiotics. The research carried out on penicillin[111] is important, especially for investigating the decomposition processes. New experiments have been carried out on streptomycin, and the tautomerism of its salts has been studied[112]. Analytical methods applicable even to pharmaceutical preparations have been put forward for aureomycin and terramycin[113–115]. Finally, chloramphenicol, which is easily reduced at the electrode because of the presence of the nitro-group, can be assayed polarographically in a series of widely varying pharmaceutical preparations in which it occurs either alone or mixed with various other active

substances; both high and very low concentrations can be determined[116]. Still dealing with analytical control of antibiotic pharmaceutical preparations, mention must be made of the assay of citric acid present in the buffer solution of sodium penicillin products[117], this easily allows the citric acid content of 5 per cent., to be controlled without separation.

Sulphonamides. Sulphonamides may sometimes be determined polarographically, not because of the sulphonamide group, but because of the presence of some other functional group reducible at the electrode, or for their properties as weak acids[118,119], while diaminodiphenylsulphone and its derivatives are easily determined with the polarograph, because the sulphone group is directly reducible[120].

Barbiturates. The special properties of barbituric derivatives leading to the formation of anodic waves have been utilized for the analysis of even extremely low concentrations[121–123]. Thiobarbituric acid derivatives[124], as well of those of thiourea and of 2:3-dimercaptopropanol[125] behave similarly, while the thioketones are reducible at the ketonic grouping[126].

Isoniazid. Isoniazid is another much studied compound of great pharmaceutical interest. It is reduced by the electrode reaction involving four electrons that allow it to be quantitatively assayed, and also permit its degradation by alkaline hydroylsis[127] to be followed.

Iodides and bromides. Indirect methods applicable to the iodate and bromate ions have been described for the halogens and are extremely interesting as they enable traces of these elements contained as impurities in various salts, water and plants to be assayed[128–131].

Sulphur-containing acids. Of all the sulphur compounds, only the sulphites can be directly determined, and it is of interest to report the direct determination of SO_2 in the atmosphere; sulphates may be indirectly determined by a method based on precipitation in the form of lead sulphate and measurement of the diffusion current produced by the excess of ionic lead[132, 133].

Aldehydes. New processes have been worked out for various aldehydes[134, 135], e.g., acrolein in glycerol[136] and chloral hydrate in the presence of chloro- and dichloro-acetaldehyde[137].

Peroxides. Even the study of the various oxidation products has continued to attract attention; the most interesting of these is a study on hydrogen peroxide[138] and another on ether peroxides[139].

Miscellaneous compounds. Some investigators have studied human saliva polarographically for diagnostic purposes[140], and still others, allergenic pollen[141]. Methods for assaying saccharin[142] and salicylic acid[143] have been reported and a method for determining ethylenediaminetetra-acetic acid[144], which is used as a diagnostic agent, in urine; the same compound is also used as a complexing agent[145,146] in other polarographic determinations.

Attempts have been made to solve the problem of assaying *m*-aminophenol in *p*-aminosalicylic acid[147]. As the acetophenone group is easily reducible, ephedrine, acetylsalicylic acid and atropine, which give acetophenone derivatives on acetylation, can be quickly determined by this

method[148]. It is of interest to mention some methods for assaying weak acids which are sometimes linked with questions of a pharmaceutical nature. The indirect oxalic acid method is based on the formation of an acid salt of europium and polarographic determination of the excess metal[149]. The anodic wave produced by gallic acid at a platinum electrode is utilized for assay[150] and a polarographic wave that appears to be caused by kinetic current[151], is employed in the case of boric acid. Even citric acid can be assayed sufficiently accurately, after being transformed into pentabromoacetone[152].

It can be seen that this method has already given great promise in pharmaceutical studies and assays, and promises still further interesting results.

Oscillographic polarography

The polarographic curves given by the oscillograph, represent the current-voltage phenomena for a single drop of mercury, or the potential-time phenomena seen at an electrode when periodic impulses are applied.

Numerous and detailed reports have been published by research-workers specialized in this particular branch of polarography[153–158]; these are mainly useful for the kinetic study of extremely fast reactions occuring at mercury drop, jet or other electrodes[159].

One of these, designed by Heyrovsky, the founder of the method, is of great importance because it deals with oscillographic polarography in pharmacy[160].

All the reactions causing passage of current at the electrode are translated into the characteristic step of the potential-time curve whose graphic representation is known. Apart from the normal curve, by using differential circuits it is possible to obtain curves representing the derivative of the potential in respect to the time, as a function of the time, and these show the electrode reactions more sharply. An oscilloscope of this type has recently been proposed by Heyrovsky for rapid qualitative polarographic analysis, approximately quantitative analysis, control of the purity of organic compounds and the separation of isomers. He also describes a method, carried out with the same apparatus, for determining CS_2, H_2S, HCN and SO_2 in industrial premises[161] and a study on the decomposition of penicillin[162], based on measuring the interval of time elapsing between the first and last steps on the oscillographic curves.

Sometimes the oscillographic method solves problems that cannot be tackled by the classical method, such as the determination of heterocyclic substances of like formulæ for instance when several *iso*nicotinic acid derivatives are contained in the same preparation[163].

Reports have also been published on substances of specific pharmaceutical interest such as vitamin B_1[164], and so have some preliminary communications promising greater development of oscillographic polarography in quantitative analysis, for example the article putting forward the possibility of using the oscillograph as a means of indicating the end-point of a titration[165].

ELECTROMETRIC TITRATIONS

AMPEROMETRIC TITRATIONS

The amperometric method, which is directly related to polarography, may be applied in all cases where constant diffusion current and a given voltage occur; it does not matter whether these are due to the reagent, to the substance under examination, or to both, so that even non-depolarizable compounds may be titrated with a reagent producing a diffusion current. In practice, the variations in current occurring at a rotating metallic electrode to which a suitable constant voltage has been applied, and due to the addition of an appropriate reagent, are carefully followed. In special cases, two equal electrodes are used without a reference one and this case is identical to the dead-stop end-point mentioned earlier.

The possibility of measuring extremely small diffusion currents extends the application of amperometric technique to microanalysis—e.g., chlorine in concentrations as low as 5×10^{-5} normal[166], halogens and other elements in organic substances.

The assay of *p*-aminosalicylic acid with potassium bromate[167] can be carried out using a rotating platinum electrode to which the constant voltage of + 0·2 V. (S.C.E.) is applied.

p-Diazobenzenesulphonic acid, which is polarographically reducible, may be used as the titrating agent for numerous substances such as sulphonamides and alkaloids, present in quantities of only 2 to 50 mg.[168] with an error of $\pm$ 3 per cent. Lead nitrate titration has been used for assaying glycerophosphates in solutions for injection and even for the assay of tartrates, while phenazone and irgapyrine can be titrated by precipitation with mercuric perchlorate, measuring the current due to the excess of reagent at a dropping mercury cathode without applied potential.

The titration of metallic ions in pharmaceutical preparations has been carried out by methods based on the formation of complexes with ferricyanide ions or on precipitation with dodecylmercaptan and titration of the excess of reagent with silver nitrate; this second method has also been used for assaying micrograms of heavy metals in pharmaceutical specialities.

The choice of reagents producing large diffusion currents is of importance for amperometric titrations. Silicotungstic acid has been used for alkaloids (nicotine, nornicotine); sodium hypobromite has been utilized in titrating even traces of ammonia, and the same method can be used for determining organic nitrogen.

The property of ethylenediaminetetra-acetic acid to form anodic waves has been utilized in assaying metals, giving good results for Mn^{++}, Co^{++}, Ni^{++}, Cd^{++}, Hg^{++} and Zn^{++}, and less satisfactory ones for the alkaline earths[169]. In the case of calcium, still using the same reagent, it is advisable to apply a constant voltage of $-1{\cdot}7$ V. to the electrode, the end-point being when the diffusion current of the calcium finally disappears[170].

Finally, the property of forming complexes with mercury, has been utilized in the amperometric assay of barbiturates.

COULOMETRIC METHOD

Of all the electrometric methods, coulometry is the best able to provide results having an absolute value. It does not depend on the individual

judgment of the operator, nor upon the accuracy with which the strength of a reference sample has been determined because the measurement in coulombs replaces the sample of known strength.

The method may be carried out in two ways: 1) the substance reacts at the electrode, and the number of coulombs required for the electrolytic reaction is measured; 2) a substance suitable for reacting with the one to be assayed is produced at the electrode. In this case, the generating electrode may be immersed in the same titration solution, or in another from which the reagent is run into the solution being assayed.

When the current intensity is kept constant, it is sufficient to measure the time for completion of the reaction, calculate the coulombs from the product of time and the current and, hence the exact quantity of the substance under examination that has been electrolysed, or that of the reagent produced reacting with that under assay.

The advantages of the coulometric method are most evident when the whole operation is completely automatic, as when automatic current-recording equipment is used.

This method gives accurate macro determinations, but the chief advantage over other conventional methods lies in its application to micro-assay, either by direct electrolysis[171, 172] or where the reagent is electrolytically prepared. It is possible, by choosing the right intensity of the current, to prepare as little as 10^{-12} and 10^{-17}g. of reagent, as well as to determine the end-point of titrations carried out with such small quantities, by means of precise potentiometric[173] or, better, amperometric measurements[174], using suitable circuits. As little as 0·01μg./ml. of ferrous iron[174] and 0·001–0·0005 μg./ml. of manganese may be assayed in this manner.

Coulometric methods have been proposed for neutralization reactions[175, 176], with external or internal preparation of the reagents[177].

A method for the assay of sodium thiosulphate has been proposed, based on the electolytic preparation of iodine from potassium iodide[178]; the chlorine produced from hydrochloric acid by electrolysis has been used in titrating *iso*nicotinic hydrazide[179] and long chain unsaturated fatty acids. Similarly, salicylic acid[180] and arsenious ions may be assayed by producing the bromine reagent electrolytically.

The possibility of titrating iodide ions with electrolytic silver ions has been reported, the end-point being determined by means of two silver indicator electrodes, as for the dead-stop end-point[181]; another useful method for assaying chlorides, bromides, iodides, and, in general, all those cases requiring silver ions, makes use of a potentiometric circuit for determining the equivalent point, allowing errors of only $\pm$ 0·005 mg. for quantities ranging from 10 to 0·2 mg.[182].

By allowing thiourea to react with solutions of silver bromide and potentiometrically titrating the bromine liberated with silver ions produced electrolytically, it is possible to assay microgram quantities of thiourea, either alone or in mixtures with other sulphurated compounds[183].

The coulometric method has wide possibilities and numerous new procedures are being proposed by investigators. A new indirect method of analysing mixtures of halogens has been discovered, by combining

coulometric measurement with the weighing of deposited mixtures of silver chloride and bromide[184].

Other investigators have proposed following the course of coulometric titrations by photometric measurements carried out in parallel with them. A photoelectric cell for use with the Beckmann spectrophotometer has been designed for this purpose[185]. Automatic photoelectric apparatus for the determination of the end-point has been suggested[186] and even a differential photometric apparatus has been designed, to overcome the formation of air bubbles at the generating electrode[186].

High Frequency Titrations

Electrometric analysis has recently been extended by a new method utilizing changes in the dielectric constant, the magnetic susceptibility and conductivity at high frequency on altering the composition of the medium. For measuring the dielectric constant, the vessels containing the solution for assay are connected to two metallic surfaces, acting as condensers; the magnetic susceptibility is measured by introducing the vessel with the solution into a solenoid.

General, theoretical and critical articles have been published on this subject[188, 189], as well as those describing the various types of instrument in use and the theoretical principles on which they are based. Others examine experimental data on various solutions, as a function of the various factors[194-198].

Although this method is very recent, several applications have already been described: neutralization[191, 198, 199], oxidation[191] and precipitation titrations[200–203] have all been reported.

Dilute solutions of metallic ions have been titrated with oxime solutions[204] and even the most recent forms of titration, using non-aqueous solutions have been successfully carried out using high frequency apparatus[205, 206].

This method, which has already found some applications in medical and pharmaceutical analysis[206], has certain disadvantages since many factors must be kept constant. Nevertheless, present experience has shown its wide range of possible application, particularly in solving special problems.

For reasons of space the paper as presented at the F.I.P. has been abbreviated and the references have been reduced. The authors will be pleased to give the full references to any who write for them.

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RESEARCH PAPERS

PHARMACOLOGICAL BLOCKING ACTIONS OF ATROPINE AND CERTAIN ATROPINE-LIKE COMPOUNDS

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Atropine is known to antagonise the responses of the guinea-pig ileum to 5-hydroxytryptamine (HT), when it is used in higher concentrations than those which antagonise the responses to acetylcholine (ACh). This has been explained by the theory that HT acts on nerve cells, and that atropine counters this effect by blocking the ACh liberated by these cells. An alternative theory is that atropine acts on both ACh and HT receptors. If the latter were so, some other drug might have a more specific action on ACh receptors than atropine itself; it was in the hope of finding such a drug that these experiments were done.

The drugs studied were four synthetic compounds supplied through the courtesy of Dr. A. C. White of the Wellcome Research Laboratories. They had been shown to be similar to atropine by various tests. Some notes of the comparative activities of each are given below.

Quantitative studies were made of the antagonism between atropine and HT, and also between atropine and ACh, and atropine and histamine. Similar experiments were made with the atropine-like compounds. In each case the pA_{10}, at equilibrium, of each drug/antagonist pair was found.

The Atropine-like Compounds

3:3-*Diphenyl propan*-3-*ol diethylamide methiodide* (186C47). This is "Compound 22" of White, Green and Hudson[1]. Comparisons by these workers of 186C47 and atropine showed that both had very similar activities against carbachol, pilocarpine and histamine. 186C47 was 0·7 to 1·0 times as active as atropine in tests on the same strip of isolated rabbit ileum, stimulated by carbachol or pilocarpine. In various tests, including one on mydriasis and another on histamine-induced asthma, the activity of 186C47 was found to be not less than half of the activity of atropine. (Information supplied by Dr. White.)

3-*Pyrrolidino*-1-*phenyl*-1-cyclo*hexyl propan*-1-*ol hydrochloride.* (54C50; procyclidine, Kemadrin.)[2] In the antagonism of ACh spasm in the isolated guinea-pig ileum, 54C50 was about 0·14 times as active as atropine. It was active also against carbachol in the guinea-pig ileum, and against both these activating drugs in the rabbit ileum. In higher concentrations, 54C50 could abolish contractions due to histamine and to barium. In tests involving salivation and mydriasis, the activity of

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54C50 was about 0·04 times that of atropine, and its effect was of shorter duration. (Information supplied by Dr. White.)

3-*Pyrrolidino*-1-*phenyl*-1-cyclo*hexyl propan*-1-*ol methiodide* (377C50, methiodide of procyclidine). This methiodide, 377C50, had a somewhat greater atropine-like activity than 54C50. It had about half the potency of atropine against ACh on isolated guinea-pig ileum. Also, in a mydriasis test in mice, it was about half as active as atropine. (Information supplied by Dr. White.)

1 : 1-*Diphenyl* 3-*piperidino propan*-1-*carbonamide hydrobromide* (182C52). The atropine-like activity of 182C52, and of the base from which it is derived, were equal, on a molar basis in a test of mydriasis in mice; being about 0·7 times as active as atropine. The base was about 1·5 times as active as atropine in the antagonism of ACh on isolated guinea-pig ileum. (Information supplied by Dr. White.)

Other investigations on this base were made by Schaumann and Linder[3]; it is their "compound 9980." They stated that it was about 0·16 times as effective as atropine in the reduction of the response of the isolated guinea-pig colon to histamine, but in a test of salivation in kittens, it was twice as active as atropine.

Experimental Method

Small guinea-pigs (150 g.), were fasted overnight, and killed by a blow on the head. Portions (1·5 to 2 cm. long) of the ileum, were taken from within 10 cm. of the cæcum, and were suspended in Tyrode's solution at 36° to 37° C. The organ bath was closely connected by a two-way tap to either of two warming coils and reservoirs. Regular responses were obtained to a sub-maximal dose of activator, while the tissue was bathed by Tyrode's solution. Then, without interruption of the dosage regimen, Tyrode's solution containing a certain concentration of an antagonist was allowed to fill the bath, and a 10-fold dose of activator was given at regular intervals. Responses were recorded with a light frontal writing lever, having a magnification of 10 to 15 times.

When the pilot experiments were done for the measurement of the pA_{10} of atropine and HT, the responses to the 10-fold dose in the presence of atropine diminished rapidly. If the response at a particular time, say 10 minutes, after the introduction of the atropine was measured in relation to the unantagonised response in each experiment, there were inconsistencies in the results. It appeared that certain portions of the ileum were especially liable to develop tachyphylaxis to stimulation by HT. Tachyphylaxis of this tissue to this drug has been noted elsewhere[4,5]. If dosing with HT was continued, there was some recovery of the responses, perhaps because the atropine had reached its full effect, and tachyphylaxis was no longer having its influence. In later experiments with higher concentrations of atropine, and in all the experiments with the atropine-like compounds, the antagonist rapidly reached its full effect, and the responses to HT rapidly reached an equilibrium level without tachyphylaxis.

The height of the steady response to the 10-fold dose was expressed as

a percentage of the response to the single doses of activating drug before the introduction of the antagonist. (See Fig. 1.) The regression line was calculated, and the pA_{10} determined.

This index (pA_{10}), was different from that of Schild[6] only in that Schild expressed the height of all responses in terms of the maximal response obtainable in the absence of antagonist, whereas here responses are measured in terms of a steady sub-maximal response (see Reuse[7]).

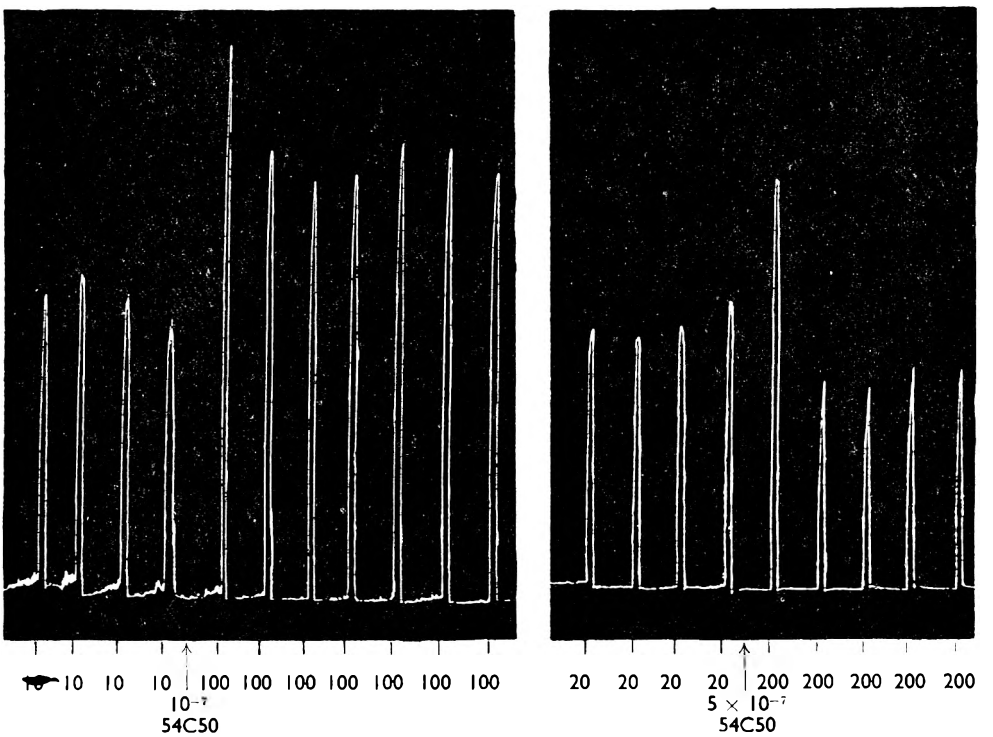


FIG. 1. Guinea-pig ileum in Tyrode's solution at 37° C., 2 ml. bath. First tracing: Responses to 10 ng. HT; from ↑, 10^{-7} 54C50 present in Tyrode's solution until the end of the experiment and the doses of HT are 100 ng.

Second tracing:
Fresh portion of ileum in Tyrode's solution. Responses to 20 mg. HT; from ↑, 5×10^{-7} 54C50 present in Tyrode's solution and the doses of HT are 200 ng. Concentration corresponding to pA_{10} lies between these two values.

Also, Schild's index was always qualified by the length of contact of the antagonist and the tissue, but here dosing was continued until the response reached a steady level (equilibrium response in 20 to 25 minutes).

Despite the difference in method, the effective concentrations for atropine/acetylcholine, and for atropine/histamine antagonism are of the same order.

The effects of atropine and the atropine-like compounds on the responses to HT were compared in a short series of experiments with their effects

on responses to nicotine. Doses of nicotine and HT, which caused similar steady responses were found, and then given alternately throughout the rest of the experiment. A particular concentration of one of the antagonists was introduced, and allowed to remain until constant responses to the constant doses were obtained (8 to 20 minutes). The antagonist was then removed, and the doses of the activating drugs were continued until recovery from the inhibition was complete.

TABLE I

COMPARISON OF AUTHOR'S RESULTS WITH THOSE OF SCHILD[6]

	Schild's pA_{10} at 14 minutes	Author's results pA_{10} equilibrium
Atropine/ acetylcholine	8·05	8·59
Atropine/ histamine	4·60	4·88

RESULTS OF THE ESTIMATIONS ON ATROPINE AND THE ATROPINE-LIKE COMPOUNDS

For each of the antagonists, the pA_{10} was found for HT, as well as for acetylcholine and histamine.

The equilibrium response to the 10-fold dose in the presence of a given concentration of antagonist, was compared with the response to the single dose in the absence of the antagonist. All the estimates were made on guinea-pig ileum.

Effect of Atropine

Under the experimental conditions which were used here, fairly high concentrations of atropine (10^{-7}), were necessary to cause inhibition of the response of the isolated guinea-pig ileum to the 10-fold dose of HT. In fact, the atropine concentration corresponding to pA_{10} for HT, was more than 100 times greater than that for ACh. For histamine, the atropine concentration was more than 10 times greater than that for HT.

Attempts to measure the effects of atropine, in concentrations less than 10^{-7}, on the 10-fold dose of HT failed because of apparent tachyphylaxis, (see method).

In a single experiment, where responses to HT and nicotine were matched, atropine (10^{-6}) was introduced into the Tyrode's solution. The responses to both nicotine and HT were similarly reduced and recovered in parallel on removal of the atropine.

Effect of the atropine-like compounds

All the four atropine-like compounds were more active in the antagonism of ACh responses than in the antagonism of HT responses. Their activity against histamine was much less (see Table II).

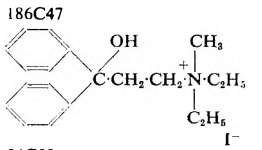
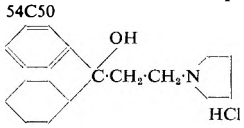
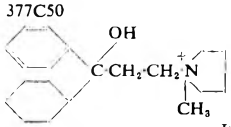
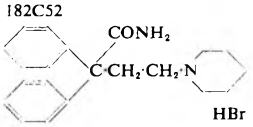
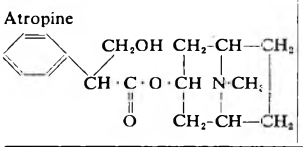
If the dose ratio is calculated by dividing the dose in the presence of the antagonist, by the dose having a similar effect in its absence, then in the presence of 186C47 (10^{-7}), the dose ratio for HT was ten, while that for ACh was fifty, and that for histamine was one[8].

The pA_{10}'s of the two quaternary compounds, 186C47 and 377C50, for HT are almost the same. These values are only a little less than the

pA_{10}'s of these compounds for ACh. The other two antagonists, 54C50 and 182C52, both show a greater difference between the pA_{10} for ACh and that for HT—but whereas with 54C50 (Fig. 2) there is a wide scatter of the estimates of effect, with 182C52 (Fig. 3) the estimates lie close to the calculated lines.

TABLE II

pA_{10} ESTIMATES FOR ATROPINE AND THE ATROPINE-LIKE COMPOUNDS

	Acetyl-choline	5-Hydroxy-tryptamine	Histamine
186C47 	7·87 (3)	7·43 (10)	4·97 (3)
54C50 	6·80 (4)	5·81 (11)	5·22 (3)
377C50 	7·86 (4)	7·25 (8)	4·63 (2)
182C52 	8·61 (4)	7·28 (8)	5·0 (2)
Atropine 	8·60 (5)	6·31 (6)	4·88 (2)

The figure in brackets indicates the number of experiments on which each pA_{10} is based.

The concentration-effect curves for histamine in the presence of 186C47 and 54C50 are shown in Figure 2. No antagonism was recorded for histamine responses by 377C50 in concentrations less than $10^{-4\cdot6}$ (Fig. 2). Spontaneous responses were recorded in the presence of 10^{-4} molar 182C52, and this made estimation of the pA_{10} impossible.

The effect of these atropine-like compounds on responses to HT and to nicotine were found to be very similar. Matching responses to nicotine and HT were equally reduced by a given concentration of any one of these compounds, and, on removal of the antagonist, the responses to both drugs recovered in parallel.

In most cases, the small spontaneous contractions occurring while Tyrode's solution was the bathing fluid, were reduced, or eliminated, by the antagonist solution.

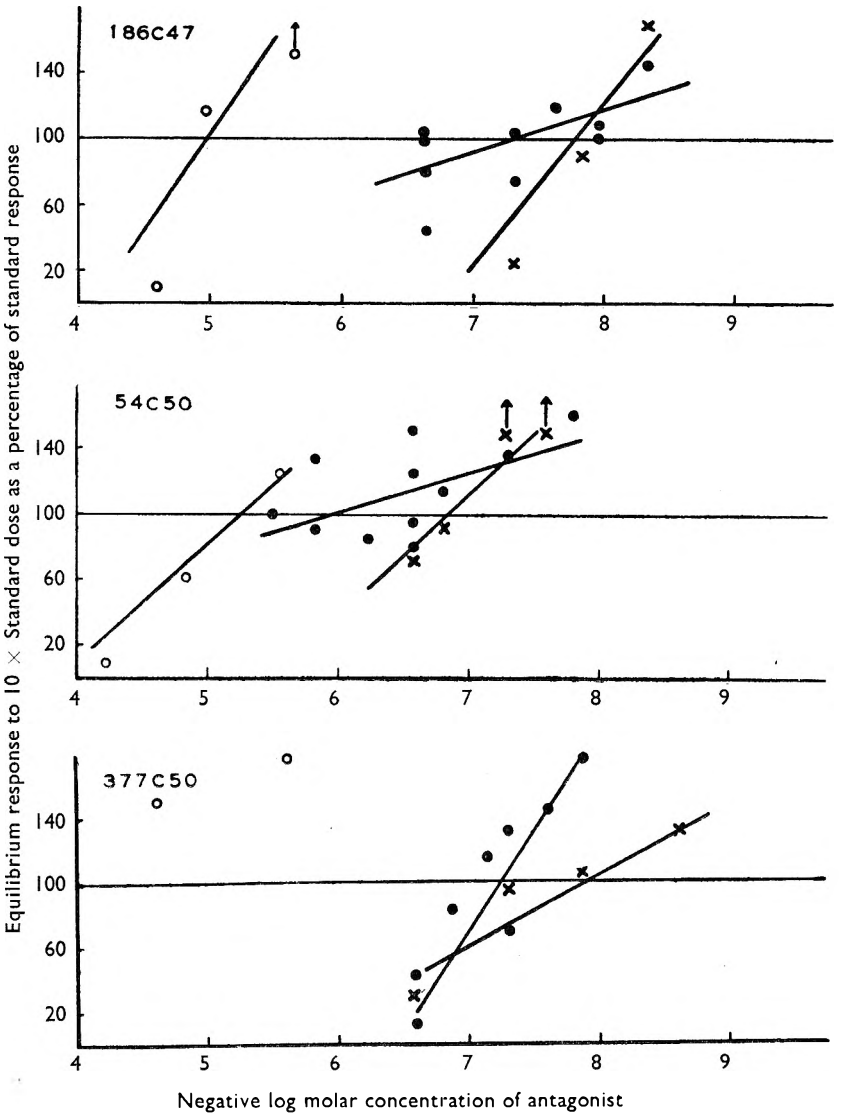


FIG. 2. The graphs show the calculated regression lines for various activator/antagonist pairs.

○ Histamine. ● 5-Hydroxytryptamine. × Acetylcholine.

DISCUSSION

Atropine was found to be more than 100 times more effective against ACh than it was against HT; and, more than 10 times more active against this drug than against histamine. These results agree with those reported by Rapport and Koelle[9].

Graphical comparison (Figs. 2 and 3) of the effect of the various

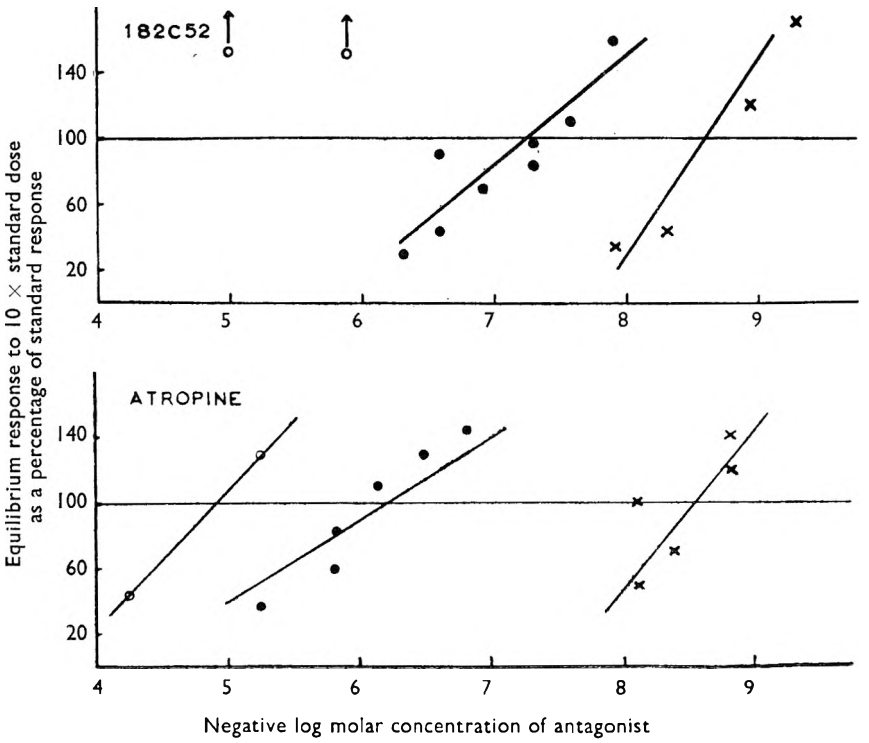


FIG. 3. The graphs show the calculated regression lines for the various activator antagonist pairs.

○ Histamine. ● 5-Hydroxytryptamine. × Acetylcholine.

atropine-like compounds and those of atropine on the spasmogenic drugs illustrated the following points.

(*a*) Widely different concentrations of both atropine and 182C52 were required to antagonise ACh, HT and histamine.

(*b*) Although the pA_{10} values of 54C50 antagonism of ACh and HT were themselves separated by one unit, the wide scatter of the observations reduces the significance of these estimates.

(*c*) More significance may be attached to the pA_{10} values of 186C47 and 377C50 for ACh and HT although they were separated by less than 0·5 units because the results of the individual estimations were less scattered.

(*d*) The activity of 182C52 on ACh responses was very slightly greater than that of atropine against ACh. The corresponding activity of the other compounds was less than that of atropine.

(*e*) 186C47, 377C50 and 182C52 were all considerably more active against HT responses than was atropine, 54C50 was less active.

(*f*) The activities of the compounds, where measurement was made, were of the same order with reference to the histamine response.

From these results, it was interesting to note that though 186C47, 377C50 and 182C52 are not more active anti-acetylcholines than atropine,

they are all more active anti-hydroxytryptamines than atropine. This suggests that they are not preventing HT spasm by antagonism of the effects of ACh, released by the HT stimulation. This conclusion could however be reversed were it shown that these drugs penetrate more readily to the site of released ACh action than does atropine. Alternatively, they may have a more specific blocking effect than has atropine on HT receptors.

The difference between the activities of 54C50 and 377C50 may be attributed to the quaternary ammonium radicle in the latter; 186C47, whose activity closely follows that of 377C50, also bears a quaternary ammonium grouping. This radicle cannot, however, be essential for activity in molecules of this type, for it is absent from the most active member of the series, 182C52. The nitrogen atom in this compound is contained in a piperidine ring. This latter compound has, also, an amide group in the position in which the other compounds carry an hydroxyl group.

Summary

1. The quantitative comparison of anti-acetylcholine and anti-hydroxytryptamine activities of atropine and certain atropine-like compounds, supplied through the courtesy of the Wellcome Research Laboratories, showed that three of them had more anti-hydroxytryptamine effect than has atropine.

2. All the compounds were, however more potent anti-acetylcholine than anti-hydroxytryptamine agents.

I wish to thank Professor J. H. Gaddum for the guidance and advice he gave throughout this study.

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A NOTE ON THE DETERMINATION OF SODIUM PHOSPHATE

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The British Pharmacopœia assay for Sodium Phosphate has the requirement that 6 g., dissolved in 100 ml. of water, should be titrated with 0·5 N hydrochloric acid, using a mixture of equal parts of solution of bromocresol green and solution of methyl red as indicator. The end-point is a grey colour indicating pH 4·5. In practice, a neutral grey colour was not found with this indicator, the colour change being from pink to blue, through a series of 'shaded' greys. We have examined the reaction between hydrochloric acid and sodium phosphate to determine the end-point, so as to indicate it precisely.

Method

The experimental work fell into three parts.

1. The colour change of various mixtures of bromocresol green and methyl orange was examined. Phthalate buffers of the British Pharmacopœia, ranging from pH 3·6 to pH 5·6 were prepared, and to 5 ml. of each buffer solution was added 0·1 ml. of the mixed indicator. The composition of the indicator varied from bromocresol green 1 : methyl red 1 to bromocresol green 6 : methyl red 1. The resulting solutions were examined both in daylight and in artificial light. The colour changes, recorded in Table I, show a 3 : 1 or a 4 : 1 mixture to cover the range about pH 4·5.

TABLE I

The colour change of mixtures of bromocresol green and methyl red

Composition of Indicator Bromocresol green : Methyl red	pH Range over which Indicator changes colour		Colour at pH 4·5
	Daylight	Artificial light	
1 : 1	4·2–5·4	4·5–5·6	pink
2 : 1	4·2–5·0	4·2–5·4	purple/grey
3 : 1	4·2–5·0	4·0–5·2	grey
4 : 1	4·2–5·0	4·0–5·0	grey
5 : 1	3·6–5·0	3·8–5·0	blue/grey
6 : 1	3·6–5·0	3·6–5·0	blue/grey

TABLE II

Titration of 100 ml. portions of 0·1 N sodium phosphate solutions with 0·5 N hydrochloric acid. (Consolidated results)

Volume of added 0·5 N hydrochloric acid (ml.)	Glass-electrode pH	Colour (artificial light) Indicator		
		1 : 1	3 : 1	4 : 1
0	9·12	blue	blue	blue
18·0	5·90	"	"	"
19·0	5·60	grey/blue	"	"
19·5	5·25	blue/grey	"	"
19·6	5·17	purple/grey	grey/blue	"
19·7	5·03	"	"	"
19·8	4·91	pink/purple	purple/grey	grey/blue
19·9	4·70	purple/pink	grey	"
20·0	4·48	pink	pink/grey	grey
20·1	4·30	"	orange/pink	pink/orange
20·2	4·13	"	"	orange/pink

2. Sodium phosphate solutions were titrated with hydrochloric acid. pH changes were recorded potentiometrically using a glass electrode, and at the same time the colour of the added indicator was noted. The results are shown in Table II.

3. Accurately weighed amounts of pure anhydrous sodium phosphate were determined by titration against 0·5 N hydrochloric acid using 1 ml. of the mixed indicator:—(*a*) 1 part bromocresol green : 1 part methyl red; (*b*) 3 parts bromocresol green : 1 part methyl red; (*c*) 4 parts bromocresol green : 1 part methyl red.

Finally, the determination was carried out using the 4 : 1 indicator, with buffer solutions of pH 4·4, 4·5 and 4·6 (120 ml.) each containing 1 ml. of the mixed indicator.

The results are shown in Table III.

TABLE III

THE DETERMINATION OF PURE ANHYDROUS SODIUM PHOSPHATE

	Percentage of Na_2HPO_4 found	
	Operator A	Operator B
Indicator 1 : 1 titrated to the closest approach to a grey colour	97·6 97·8 98·3	98·3 98·2 97·8
Indicator 3 : 1, titrated as above	100·6 100·6 100·3	98·8 99·8 100·1
Indicator 4 : 1 titrated as above	100·3 100·2 100·3	99·1 99·2 99·0
Indicator 4 : 1, using *p*H 4·5 buffer containing indicator for comparison	100·0 100·1 100·1 100·0 100·2 99·8	99·7 100·1 100·1 100·1 99·7 99·7

CONCLUSION

It is suggested that the following method of assay for Sodium Phosphate is more suitable than the present official method:

"Dissolve about 6 g., accurately weighed, in 100 ml. of water and titrate with 0·5 N hydrochloric acid using 1 ml. of a mixture of four parts of solution of bromocresol green and one part of solution of methyl red as indicator, and titrating until the colour of the solution matches that obtained by adding 1 ml. of the above indicator to 120 ml. of buffer solution of pH 4·5."

SUMMARY

1. The end-point of the B.P. 1953 assay of Sodium Phosphate is unsatisfactory.

2. A new indicator system employing four parts of solution of bromocresol green and one part of solution of methyl red is recommended.

3. Readings should be matched with a standard prepared by adding 1 ml. of the new indicator mixture to 120 ml. of buffer solution of pH 4·5.

THE REPRODUCIBILITY OF EXTINCTION TIME ESTIMATES

PART I.—VARIATIONS IN RESISTANCE OF TEST ORGANISMS AND VIABILITY OF TEST SUSPENSIONS

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THE most pertinent criticism which has been levelled at extinction methods of estimating bactericidal activity in general, as distinct from phenol coefficient determinations in particular, has been the alleged unreliability of the tests for sterility on samples of the bacteria-bactericide reaction mixture. Supposedly inconsistent and anomalous distributions of culture samples showing growth or absence of growth were observed soon after the introduction of the methods[1–4]; and it was generally recognised that the closer the sampling intervals, the greater were the anomalies. The type of result was often described as showing "skips" or "wild plusses". The explanation of these results is to be found in the survival of very small numbers of bacteria of greater than average resistance and which are to be found towards the end of the disinfecting period, as shown graphically by Chick[5], Withell[6], Jordan and Jacobs[7] and Berry and Michaels[8]. Where the number of survivors is very small—on the average one or no organism in each sample volume—there must be some negative samples in a number of replicate samples removed. This was realised by Thaysen[9] and by Cade[10], who approached the problem statistically, using the analysis of Halvorson and Ziegler[11] for the calculation of the most probable number of organisms in the parent reaction mixture from a knowledge of the sterility or fertility of the samples. This Cade was able to relate with counts of surviving organisms. He suggested that lack of reproducible results from extinction data could be ascribed largely to sampling variations and he advocated the employment of much more extensive replication than had been customary in obtaining such data.

The extinction method of Berry and Bean,[12] overcomes the errors inherent in previously described extinction methods, for it provides sufficiently extensive replication with adequately short sampling intervals for the determination of a highly reliable estimate. If the loglog analysis described by Mather[13] be used, the magnitude of the sampling variations and the standard error of an estimate may be computed. The reliability of the extinction times (expressed as mean single survivor times) so derived has been proved by Cook and Wills[14], who demonstrated a relation between the intervals of time required for different percentages survival and the mean single survivor times.

It is evident that estimates of extinction times are subject to two components of variation: that 'between estimates' and that lying 'within estimates' or between replicates. The reliability of an estimate is improved by narrowing the contact time intervals, i.e. increasing the number

of samples removed in a given time, which results in a progressively larger increase in the within-estimate variability; therefore it follows that the extinction method of choice will be that which gives the highest within-estimate variance with the provision that variations due to other sources are small compared with random chance variations. In terms of Mather's analysis, the method must yield a highly significant regression with a maximum of degrees of freedom. Berry and Bean[12] gave details of the "within-estimate' variability attached to their method, but were able to give little attention to the magnitude and sources of 'between-estimates' variability. It is the object of this communication to describe investigations into the reproducibility of estimates of extinction times. Bearing in mind that Berry's extinction method is the only method yet described which takes account of sampling variations, it follows that the investigations described here will provide probably the first attempt to adequately assess the sources of variability between extinction time estimates. Previous investigators must have attributed sampling variations to other sources.

Experimental Details

Bactericide. The bactericide solutions were prepared by diluting with sterile distilled water a 5 per cent. aqueous solution of phenol A.R., which, stored in well-closed containers, was found to be stable.

Organism, Bacterium coli, (*Escherichia coli*), type I, 44° C.-positive, laboratory strain, formerly N.C.T.C. No. 5933.

Media. The medium used for testing the sterility of samples of the reaction mixture was that described by Berry and Bean[12]. The solid medium used for cultivation of the test organisms contained 1 per cent. "Oxoid" peptone and 0·5 per cent. sodium chloride, was solidified with 2 per cent. Davis bacteriological agar and was adjusted to pH 7·2. The procedure adopted by Berry and Bean was as follows. At monthly intervals, a freeze-dried sample of culture of the organism was opened and transferred to a "master" slope of peptone agar, the 24 hour growth from which was used to propagate 4 "sub-master" agar cultures. Each day for 14 days, slopes were inoculated by successive subculture from one of the "sub-master" slopes. These daily slopes were used in experiments from the fourth to the fourteenth day, after which a fresh "sub-master" slope was introduced. When all four "sub-master" slopes had been used, a fresh "master" culture was generated from another specimen of freeze-dried culture.

Experimental Technique. The method adopted for the cultivation of the test organisms was similar to that described by Berry and Bean[12]. Test suspensions were adjusted to a density equivalent to 2×10^9 per ml. by the use of a clinical photoelectric colorimeter (Baird and Tatlock, model ZTA 7530). Variations in the viable counts of suspensions from day to day were estimated from two series of data: colony counts by the method of Miles and Misra[15], which gave an estimate from 39 counts of a mean count of $1{\cdot}948 \times 10^9 \pm 0{\cdot}099 \times 10^9$ per ml. ($P = 0{\cdot}95$);

and 10 counts by dilution series, as described by Cook and Wills[14], from which was obtained a mean estimate of $2{\cdot}211 \times 10^9 \pm 0{\cdot}237 \times 10^9$ per ml. ($P = 0{\cdot}95$). The design of the extinction method and the analysis of the results followed the scheme described by Cook and Wills[14]. All experiments consisted of between 15 and 20 replicate determinations, each with at least six different contact times.

A trial series of determinations was undertaken in which extinction times were measured on exposure of *Bact. coli* to solutions of phenol of concentration varying from 1·1 per cent. to 1·5 per cent. Of the eight determinations carried out, all estimates of 'mean single survivor time' obtained from the regression line best fitted by inspection easily fell within the limits of error of estimates derived from both first and second calculated approximations to the regressions lines. Hence, the reading of mean single survivor times from regression lines best fitted by inspection in subsequent experiments was considered to be justified. A highly statistically significant regression was demonstrated between logarithms of mean single survivor times and logarithms of phenol concentrations, this finding being in agreement with the observations of many other workers, see e.g. Berry and Bean[12], Phelps[16].

TABLE I

EXTINCTION TIMES OF *Bacterium coli* ON EXPOSURE TO SOLUTIONS OF PHENOL AT 20° C.

Concentration of phenol (per cent. w/v)	Mean single survivor times (minutes)	
	First series of determinations	Second series of determinations
1·10	39·0	50·2
1·15	24·0	32·7
1·20	16·4	20·2
1·25	10·6	13·8
1·30	6·39	9·54

Later, a further series of experiments was undertaken in which the mean single survivor times to five strengths of phenol were redetermined. The results shown in Table I indicate a considerable apparent increase in resistance of the test organism, the new values being about 20 per cent higher than those which were first determined. The components of the experiment which could have varied between the performance of the first and second series of determinations were:

(i) The organism, which had been generated from separate freeze-dried samples. The first series had been completed just before expiry of a monthly "master" culture.

(ii) The batch of medium used for reviving unkilled cells at the end of the reaction period. A new batch had been commenced for the second series of experiments.

It was decided to investigate both of these possible sources of variability, and in addition to investigate the viability of the test suspensions from day to day and within each day. This first communication will describe investigations into the resistance of test organisms and viability of test suspensions.

A Comparison of Methods of Maintaining Cultures

The method of maintaining cultures by limited series of subcultures from freeze-dried cultures of the organism had been adopted in these laboratories in view of the variations in resistance on repeated subculture reported by Chick and Martin[17] and Withell[18]. It was decided to compare variations in phenol resistance of organisms maintained by the monthly freeze-dried method of Berry and Bean[12] with those obtained by repeated daily subculture.

Procedure. One "submaster" slope of the then current freeze-dried culture was maintained in continuous daily subculture over a period of more than two years. This will be referred to as the "C" series of subcultures. A "submaster" slope of the succeeding culture was similarly maintained and was known as the "B" series. At varying intervals during the following two years, the mean single survivor times to 1·10 per cent. phenol were determined for both "B" and "C" series and for the corresponding culture in normal current use.

TABLE II

Mean Single Survivor Times of *Bact. coli* when Exposed to 1·10 per cent. Phenol, the Organism being Maintained in Culture by Different Methods

Extinction times of cultures maintained from monthly freeze-dried samples (minutes)		Culture "C"		Culture "B"	
		Serial number of subculture	Extinction time (minutes)	Serial number of subculture	Extinction time (minutes)
50·2	39·8	12	50·2	7	35·9
44·4	33·5	52	49·5	12	29·3
35·9	25·8	96	38·0	121	23·0
29·3	31·4	103	28·5	257	26·4
40·1	29·6	117	26·0	305	28·3
32·3	33·4	130	26·8	639	23·4
36·4	28·4	233	28·8		
41·4	29·0	370	22·3		
33·2	26·3	420	29·6		
		645	24·8		
		757	17·1		

Method of propagation of culture	Degrees of freedom	Variance	Standard error	Mean single survivor time (minutes)	95 per cent. Confidence limits of means.
1. Monthly series	17	43·35	1·552	34·47	± 3·27
2. Series "C"	10	112·29	3·195	31·05	± 7·12
3. Series "B"	5	22·49	1·936	27·72	± 4·97

Results and Analysis. The results, together with a summary of their statistical treatment, are shown in Table II. Comparison of variances 1 with 2 and 2 with 3 gave ratios of 2·59 and 4·99 respectively which, with the appropriate degrees of freedom, corresponds to a probability level slightly below 0·05 in each case. Comparison of variances 1 with 3 yields a ratio of 1·93 which is not statistically significant at the 5 per cent level. Similar comparisons of means give values for t of 1·077, 0·723 and 2·305 respectively, so that a significant difference exists only between means 1 and 3.

Inspection of the results for the ordinary monthly cultures reveals that one estimate, 50·2 minutes, lies well above the remainder. This culture was used for propagation of the "C" series of daily subcultures. On the other hand, the value 35·9 minutes, obtained for the culture used to propagate the "B" series, lies close to the mean of the monthly culture estimates. Both "B" and "C" series show a declining resistance during the course of subculture: with the "B" series the decline has been sufficient to reduce the mean result significantly from that of the ordinary series and the variance is smaller, but not significantly so. In the "C" series the decline from a high initial resistance to a steady resistance roughly the same as that of the "B" series has greatly increased the variance to an extent significantly greater than the variances of the other two series, and this large variance leads to a mean which is not significantly different from the means of the other series. If, however, the results from the "C" series up to and including the 52nd subculture are omitted, the remaining values have a variance not significantly greater than the "B" series ($F = 1{\cdot}44$ with 8 and 5 degrees of freedom) and a mean not significantly different from that of the "B" series ($t = 0{\cdot}298$ with 13 degrees of freedom) but which is significantly smaller than that of the monthly series ($t = 2{\cdot}95$ with 25 degrees of freedom).

Discussion. These findings indicate that cultures of *Bact. coli* derived from individual samples of a freeze-dried culture may vary considerably in phenol resistance, from limits of 50·2 minutes to 25·8 minutes extinction times on exposure to 1·10 per cent phenol. It appears that on continued daily subculture of a sample of the freeze-dried culture, using our medium, the resistance soon begins to decline to reach a reasonably steady resistance which is maintained for several hundred subcultures. Thereafter there may be a further decline. Over the period of reasonably constant resistance, estimates of extinction times may be more reproducible than with the use of the monthly method of propagation, although a statistically significant difference could not be detected. It is concluded that daily subculture has advantages over limited numbers of subcultures from freeze-dried specimens in experimentation lasting for little more than one year.

At six monthly intervals, samples of "B" and "C" series of subcultures were subjected to microscopical and biochemical examination. Normal characters were maintained with the exception of an increasing capacity of the organism to grow anærobically as demonstrated by reduction in catalase activity and enhanced methylene blue reduction. The tendency to anærobiosis was reversed by growth in peptone water containing 2 per cent. whole blood with 10 μg. per ml. riboflavine or 50 μg. per ml. nicotinamide. The change may therefore be associated with a metabolic disability after growth for long periods on poor media.

Examination of the data for the monthly series of cultures showed that apparently the distribution was predominantly single-tailed. However, a test for normality by computation of the four moments, employing correction for continuity, revealed that the distribution of resistances did not depart seriously from normality and was symmetrically platykurtic.

REPRODUCIBILITY OF EXTINCTION TIME ESTIMATES. PART I

Variations Between and Within Monthly Cultures

The results for extinction times of the ordinary monthly cultures given in the previous section did not differentiate between variations between "master" (monthly) cultures and variations within "master" cultures (or between "submaster" cultures). A comparison of variances due to these sources was desirable, since it would show whether each monthly culture would give rise to a short series of subcultures of more constant resistance than would be encountered in a random series of observations spread over several monthly cultures.

TABLE III

Extinction Times of *Bacterium coli* when Exposed to 1·20 per cent. w/v Phenol at 20° C.

Values of t are mean single survivor times in minutes determined for subcultures of serial number n. A,B,C,D denote the four "submaster" cultures of the "master cultures" 1,2 and 3.

"MASTER" 1		"MASTER" 2				"MASTER" 3	
n	t	n	t	n	t	n	t
A 9	18·44	A 4	15·39	C 10	17·21	A 2	15·02
B 4	11·42	A 5	15·10	C 11	16·98	A 4	19·87
C 6	13·83	A 6	16·26	C 13	18·45	A 7	18·42
C 15	15·03	B 4	19·05	C 15	16·54	A 9	15·70
D 8	16·38	B 10	17·53	D 5	16·23	A 11	17·67
D 9	17·58	B 13	16·70	D 8	15·02		
D 10	18·04	C 5	16·75	D 10	14·13		
D 11	18·40	C 6	17·40	D 13	12·20		
		C 8	17·42				

Procedure. Data for the mean single survivor times of *Bact. coli* when exposed to 1·20 per cent. phenol were obtained over a short period. The determinations were made during the use of all four "submaster" cultures derived from each of two "master" cultures and during the introduction of the first "submaster" culture of a third "master" culture.

Results and Analysis. The results are recorded in Table III. Since the experimental structure was both unbalanced and incomplete, an analysis of variance could not be undertaken. Instead, variances were computed as follows:—

(i) Between "submaster" cultures; within "masters" and replicates. The variances for blocks 1, 2 and 3 were calculated.

$\bar{x}_1 = 16{\cdot}14$ $S(\bar{x}_1 - \bar{\bar{x}}_1)^2 = 44{\cdot}9845$ $s_1^2 = 6{\cdot}427$ (7 degrees of freedom)

$\bar{x}_2 = 16{\cdot}37$ $S(\bar{x}_2 - \bar{\bar{x}}_2)^2 = 43{\cdot}2114$ $s_2^2 = 2{\cdot}701$ (16 degrees of freedom)

$\bar{x}_3 = 17{\cdot}34$ $S(\bar{x}_3 - \bar{\bar{x}}_3)^2 = 15{\cdot}7481$ $s_3^2 = 3{\cdot}937$ (4 degrees of freedom)

Comparison of the largest variance estimate, s_1^2, with the smallest, s_2^2, gives a ratio of $F = 2{\cdot}380$ ($N_1 = 7$, $N_2 = 16$), corresponding to a probability level between 0·2 and 0·1. Hence there is no evidence of significant heterogeneity among variances between "submaster" cultures.

(ii) Between "master" cultures; within "submasters" and replicates. The variances for groups A,B,C, and D were computed.

$\bar{x}_A = 16{\cdot}87$ $S(x_A - \bar{x}_A)^2 = 24{\cdot}9940$ $s^2_A = 3{\cdot}124$ (8 degrees of freedom)
$\bar{x}_B = 16{\cdot}18$ $S(x_B - \bar{x}_B)^2 = 32{\cdot}9873$ $s^2_B = 10{\cdot}996$ (3 degrees of freedom)
$\bar{x}_C = 16{\cdot}62$ $S(x_C - \bar{x}_C)^2 = 15{\cdot}4104$ $s^2_C = 1{\cdot}926$ (8 degrees of freedom)
$\bar{x}_D = 15{\cdot}99$ $S(x_D - \bar{x}_D)^2 = 31{\cdot}5125$ $s^2_D = 4{\cdot}502$ (7 degrees of freedom)

Comparing the highest variance, s^2_B, with the lowest variance, s^2_C, $F = 5{\cdot}709$ ($N_1 = 3$, $N_2 = 8$), corresponding to a probability level of between 0·05 and 0·01. The homogeneity of variance must therefore be tested, using the general form of Bartlett's test, which gives an estimate of $\chi^2 = 4{\cdot}82$ with 3 degrees of freedom. This corresponds to a probability of approximately 0·2. A significant heterogeneity therefore does not exist between these variances. An average variance of each of the two sets of variances (i) and (ii) was computed as 3·850 (27 degrees of freedom) and 4·035 (26 degrees of freedom) respectively. The variance ratio does not attain significance at the 5 per cent. level.

Conclusions. Definite conclusions are difficult to draw. Almost as much variability in extinction times is found on repetition of results for a single "submaster" culture and for other "submaster" cultures within the same monthly culture as would be found on performance of a few experiments on a "submaster" culture in one month and then passing on to the corresponding "submaster" culture on the following month.

Variations in Viability of Test Suspensions

The following factors could presumably affect the reproducibility of extinction time estimates:

(i) Variations in viability of suspensions of the test organism which had been adjusted to the same density using the photoelectric colorimeter.

(ii) Variations in viability of suspensions on storage for periods during a day on which extinction time determinations would be carried out.

(iii) Variations in viability and phenol resistance of slope cultures of the organism incubated over varying periods. Each of these factors was investigated, and a comparison made between variations in viability from day to day and variations within days.

Procedure. The counting method used was that described by Miles and Misra[15]. The method was selected to economise in apparatus and time and to reduce colony counting errors. The test suspension was serially diluted by addition of five drop samples to required volumes of sterilised water to give four final dilutions for plating which contained about 5, 10, 15 and 20 organisms per drop. Dilutions were made in screw-capped bottles, which were well shaken before removal of samples. The plates, which contained 20 ml. overdried peptone agar, were divided into eight sectors and one drop of each dilution placed on each sector. Each dilution was plated 20 times and the colonies were counted after incubation at 37° C., for 12–15 hours. Counts for each dilution were

subjected to χ^2 tests for homogeneity, and all results giving a value of χ^2 corresponding to a probability of less than 0·4 rejected. Counts were performed on suspensions prepared on 7 different days, performing them at varying times—from 1 hour to 15 hours after preparation. At least one dilution always gave a count of acceptable homogeneity; generally the results discarded were those with the lowest mean counts.

TABLE IV

VIABLE COUNTS (x 10^{-9} PER ML.) OF SUSPENSIONS OF *Bacterium coli* PREPARED ON SEVEN DIFFERENT DAYS, STORED FOR VARYING PERIODS AND DETERMINED FROM FOUR DIFFERENT DILUTIONS

Age after preparation	Dilution	Suspension 1	2	3	4	5	6	7	Means
A	1	1·5069	2·3933	2·6592	—	—	—	—	2·1865
less	2	1·8327	2·2350	2·3691	—	—	—	1·6092	2·0115
than	3	1·7957	2·1821	2·2275	1·7729	—	1·7502	1·5684	1·8828
2 hours	4	—	—	2·0662	1·4910	1·9488	1·6201	1·5849	1·7422
	Means	1·7118	2·2701	2·3305	1·6320	1·9488	1·6852	1·5875	1·9230
B	1			—		—	—		—
Between	2			2·5926		—	—		2·5962
2 and 4	3			2·1954		1·9093	—		2·0524
hours	4			2·0545		1·8901 2·0310	1·5732 1·4792		1·8056
	Means			2·2808		1·9435	1·5262		1·9657
C	1	2·2160					—		2·2160
Between	2	1·5645					—		1·5645
4 and 8	3	1·9321					1·8411		1·8866
hours	4	1·9019					1·9606		1·9313
	Means	1·9036					1·9009		1·9027
	1	—	—					2·3933	2·3933
D	2	—	—					1·7880	1·7880
Over	3	2·1594	1·7957					2·0230	1·9927
8 hours	4	2·0662	2·0075					—	2·0369
	Means	2·1128	1·9016					2·0681	2·0333
MEANS		1·8862	2·1227	2·3092	1·6320	1·9448	1·7041	1·8278	1·9484 ± 0·0986 (p=0·95)

Results and Analysis. Table IV shows the mean viable counts per ml. of suspensions as determined from four dilutions at different storage times on each of seven days. It is at once seen that there is a much greater variability between days than between storage times. However, these variations may be considerably influenced by variations between dilutions and the following tests were used to assess this influence.

(a) Variances between storage times for each dilution were calculated as $s_1^2 = 0{\cdot}0125$, $s_2^2 = 0{\cdot}197$, $s_3^2 = 0{\cdot}0069$, $s_4^2 = 0{\cdot}0173$, with 2,3,3 and 3 degrees of freedom respectively. Only one variance ratio, s_2^2/s_3^2, attains significance at the 0·05 level and a significant difference exists between only one pair of means, $\bar{x}_A$ and $\bar{x}_D$. The pooled estimate of variance is 0·0625 with 11 degrees of freedom.

(b) The variations between dilutions for each storage time were calculated as $s^2_A = 0{\cdot}0357$, $s^2_B = 0{\cdot}164$, $s^2_C = 0{\cdot}0712$, $s^2_D = 0{\cdot}0633$, with 3,2,3 and 3 degrees of freedom respectively. No significant difference exists

between any pairs of variances or means. The pooled variance estimate is 0·0944 with 11 degrees of freedom.

(c) Taking the mean counts over all dilutions within each storage time, the variances within each storage time were computed as $s^2_A = 0{\cdot}0955$, $s^2_B = 0{\cdot}143$, $s^2_C = 0{\cdot}000004$, $s^2_D = 0{\cdot}0124$, with 6,2,1 and 2 degrees of freedom respectively. A significant heterogeneity exists between these variances. The pooled variance estimate is 0·0803 with 11 degrees of freedom. The pooled variance estimates for (a), (b) and (c) are not significantly different.

Conclusions. It is concluded that variations between storage times of the suspensions up to 15 hours are certainly no greater than variations in viability of suspensions from day to day. Variations due to different dilutions are of similar magnitude. Generally, higher dilutions gave a larger estimate of viable density than the lower dilutions, a finding which might be explained either as an overcrowding effect when many organisms are exposed to a small surface area of medium or in terms of errors in counting confluent colonies where the numbers are considerable.

Variations in Viability and Extinction Times on Prolonged Incubation of Slope Cultures

Table V records the mean viable counts and the corresponding mean single survivor times to 1·10 per cent. phenol for suspensions prepared from slope cultures incubated for varying periods from 12 hours to 15 days. All the slopes had been inoculated at the same time from a

TABLE V

VIABLE COUNTS AND EXTINCTION TIMES TO 1·10 PER CENT. PHENOL OF *Bacterium coli* (SUBCULTURE C771) AFTER VARYING PERIODS OF INCUBATION

Age of slope culture (hours)	Viable count (x 10^{-9} per ml.)	Mean single survivor time (minutes).
12	1·540	18·8
25	1·588	17·4
50	1·738	18·6
75	0·973	16·4
170	0·281	11·7
195	0·294	11·1
335	0·287	10·3

serial subculture of the organism which had been previously maintained in successive daily subculture "C". Viability and extinction times remain reasonably constant for the first 50 hours. After 75 hours, the count has dropped by more than 30 per cent. and the extinction time has been reduced by about 10 per cent. But between 75 hours and 170 hours after inoculation, the count has fallen by more than 80 per cent. and the killing time has been reduced by about 40 per cent. Incubation for a further week is seen to result in no appreciable change in viability, although the extinction times continue to fall slightly.

In order to furnish confirmation of these results, two viable counts and extinction time determinations were carried out using suspensions

prepared from 24 hour and 170 hour slope cultures propagated from the culture in current use at that time and which was prepared from a monthly freeze-dried culture. The results were as follows:

24 hours. Viable count: $1{\cdot}632 \times 10^9$ per ml. Extinction time: 29·0 minutes

170 hours. Viable count: $0{\cdot}274 \times 10^9$ per ml. Extinction time: 15·8 minutes.

It is observed that, although the extinction times are higher than those obtained with the continuously subcultured organisms, the decrease in both extinction time and viable count are comparable to those determined in the first experiments. The steep fall in viability during the first week of incubation on a slope culture should be contrasted with the observations of Cook and Steel[19], who found that the viability of aqueous suspensions of the same organism remained remarkably constant for periods of over 6 weeks.

The results obtained here show that the extinction times of cultures are dependent upon their viability. But we conclude that viability is probably not related to phenol resistance since we have been unable to demonstrate any change in the phenol resistance of organisms with greater viability on an agar slope over those with less.

Summary

1. Attention has been drawn to the inadequacy of all extinction methods, except that of Berry and Bean[12], for the investigation of factors affecting the reproducibility of extinction times.

2. Reproducibility of extinction time estimates has been shown to be influenced by variations in resistance of freeze-dried samples of the test organism.

3. Methods of maintaining cultures of the test organism have been compared.

4. Variations in viability of test suspensions of the organisms on storage are probably smaller than day-to-day variations in the viability of suspensions.

5. Prolonged incubation of slope cultures of *Bacterium coli* results in decrease in both viability and extinction times.

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A NOTE ON THE X-IRRADIATION OF SEEDS OF *DATURA TATULA* WITH SPECIAL REFERENCE TO ALKALOID PRODUCTION

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The specific effects of X-rays on alkaloid production in plants appear to have received little attention. That radiations produce fragmentations, translocations and segmental interchange as well as gene mutation in *Datura stramonium* has been known for a considerable time and utilised for the isolation of prime types in this genus[1]. As an attempt to induce in *D. tatula* var. *inermis* mutations which might be of value in the study of alkaloid biogenesis the following experiments were performed.

TABLE I

Plants from irradiated seeds

Plant	General Morphology	Hyoscine : Hyoscyamine per cent. dried plant
1	Small and retarded with one immature fruit. Leaf abscission occurred early and plant not collected	
2	Resembled controls	0·06 : 0·20
3	Somewhat contorted but generally resembled controls ..	0·02 : 0·12
4	Stunted in early growth, branching not typically dichasial, one shoot tending towards excessive development at the expense of the other. Lower leaves often incomplete. The first four flowers and fruits produced showed six divisions to their structure, the remainder four	0·07 : 0·19
5	Resembled Plant 4 but no mature fruits produced. Two immature fruits possessed 6 carpels with a few immature seeds	0·06 : 0·23
6	A stunted plant which produced no really mature fruits ..	0·04 : 0·27
7	Resembled controls	0·05 : 0·30

Four groups, each of 50 moistened dormant seeds were subjected to X-ray doses of 5000, 10,000, 15,000 and 20,000 r respectively and the same day sown in seed compost, together with untreated seeds as controls. In those groups receiving 10,000 and 20,000 r, no seeds germinated and in the 15,000 r group one seedling developed but did not survive. In the 5000 r group many seeds germinated over a prolonged period. Most died before the cotyledons had fully expanded and others were chlorophyll deficient or badly mottled and gradually died. Seven seedlings survived, and when sufficiently large, were transplanted into open ground. Each plant was harvested at the flowering and fruiting stage and all excepting the seeds and roots analysed for hyoscine and hyoscyamine by the method of Evans and Partridge[2]. The separated alkaloids were in each case characterised by measurements of the optical rotations of the sulphate titration liquors, by the R_F values of paper chromatograms and by the melting points and mixed melting points of the picrates with authentic compounds. The observations on these parent plants are summarised in Table I.

In the subsequent year, the first generation of the above plants was raised together with control plants. Only two seedlings could be raised from the largely immature seeds of Plants 5 and 6 (Table I). Few seeds of Plant 7 were available but their germination was good and numerous seedlings of Plants 2, 3 and 4 were obtained. At the flowering stage the plants were harvested and where available, twenty from each parent were tested for hyoscine and hyoscyamine by paper chromatography. A further selection were analysed quantitatively, the separated alkaloids being identified by the melting points of their picrates. The results are recorded in Table II.

TABLE II

PLANTS OF SECOND GENERATION

Parent plant	General morphology of X_2 plants	Paper chromatography of extracts of X_2 plants	Hyoscine : Hyoscyamine as per cent. dried X_2 plants
2	33 plants; all resembled controls	20 plants tested. 19 contained both hysocine and hyoscyamine and one a trace of hyoscine only. With one exception the hyoscyamine content appeared below average	0·06 : 0·09; 0·03 : 0·08; 0·04 : 0·07; 0·06 : 0·09; 0·08 : 0·11
3	44 plants; two showed abnormal branching and the remainder resembled the controls	20 plants tested. All contained hyoscine and hyoscyamine in proportions in keeping with the quantitative estimations except for three instances in which the hyoscyamine content was very low	0·03 : 0·09; 0·07 : 0·25; 0·1 : 0·15; 0·05 : 0·11; 0·01 : 0·09; 0·06 : 0·11
4	54 plants; all more or less resembled the controls except for three with abnormal branching	20 plants tested. All contained hyoscine and hyoscyamine in apparently normal proportions	0·06 : 0·10; 0·01 : 0·05; 0·13 : 0·21; 0·04 : 0·14; 0·07 : 0·12; 0·04 : 0·09; 0·05 : 0·14; 0·08 : 0·19; 0·04 : 0·12; 0·06 : 0·03; 0·04 : 0·10; 0·02 : 0·03; 0·04 : 0·10
5	One plant; resembled the controls		0·006 : 0·01
6	One plant; very small but of normal appearance		0·0 : 0·006
7	10 plants; all shorter than the controls with a denser foliage	Four plants tested. All appeared to contain more alkaloids than the controls	0·03 : 0·28; 0·004 : 0·25; 0·07 : 0·18; 0·04 : 0·28; 0·04 : 0·24; 0·003 : 0·09
	Control plants. Hyoscine : hyoscyamine As per cent. dried plants	0·07 : 0·14; 0·07 : 0·11; 0·02 : 0·12; 0·08 : 0·13; 0·06 : 0·15; 0·03 : 0·15; 0·06 : 0·10; 0·04 : 0·13; 0·06 : 0·18; 0·05 : 0·18; 0·02 : 0·08; 0·03 : 0·12; 0·02 : 0·09; 0·02 : 0·08.	

It is apparent that irradiation of seeds with 5000 r of X-rays produced mostly lethal effects of the types recorded with other plants. Among the surviving plants distinct morphological differences were often obvious although the nature of the alkaloids was unchanged. The total alkaloidal content of these plants exceeded that of the controls but this may have been without significance since the two groups matured under different environmental conditions due to their different rates of growth.

Similarly in the second generation, no new alkaloids were encountered although in isolated instances only one alkaloid could be detected. The variations in the relative proportions of the individual alkaloids were

not however, without interest. All the X_2 offspring from Plant 7, with one exception, gave relatively high hyoscyamine yields, those from Plant 2 possessed average quantities of hyoscine but consistently low amounts of hyoscyamine and those from Plants 3 and 4 contained varied total alkaloid contents with hyoscine : hyoscyamine ratios in most cases similar to the controls.

Such results have obvious implications in the selective breeding of desirable strains of *D. tatula* but further investigation would be required to establish whether variations of the type reported here are significantly different to those which have been isolated by conventional breeding methods[3,4]. It is probable that in the present instances, no mutations directly involving alkaloid biogenesis were involved but since alkaloid content and ratios of individual alkaloids are known to vary throughout the life-cycle of *D. tatula*[5], mutations involving many factors may indirectly affect the ultimate alkaloidal content.

We are indebted to Dr. M. W. Partridge for his help and advice and to Dr. F. H. Cross for arranging the radiation of the seeds at the General Hospital, Nottingham.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Adrenaline and Noradrenaline Solutions, Analysis of. L. H. Welsh. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 507.) Solutions of adrenaline salts were assayed by converting the amine to its triacetyl derivative with the aid of acetic anhydride and sodium bicarbonate, extracting the acetyl derivative with chloroform, removing the solvent and weighing. By determining the optical rotation of a solution of the triacetyl derivative, the amount of racemisation which had occurred in the adrenaline solution was estimated and taken into account in calculating the potency of the preparation. Good recoveries were obtained by this method and the results agreed well with potencies determined by the U.S.P. XIV biological assay, using dogs. The method was also applicable to noradrenaline. For the determination of noradrenaline present as an impurity in solutions of adrenaline, the triacetyl derivatives were separated by partition chromatography on Celite columns, using water as the stationary phase and benzene as the mobile phase. The separated triacetyl derivative of noradrenaline was hydrolysed to the base which was estimated colorimetrically. G. B.

Barbiturates, Chromatography of. E. Hjelt, K. Leppänen and V. Tamminen. (*Analyst*, 1955, **80**, 706.) Barbiturates are separated by ascending-paper chromatography on Whatman No. 1 paper impregnated with M potassium nitrate, *n*-butanol–pentanol–ammonia being used as the solvent. The paper is kept in a solvent atmosphere for at least 6 hours, the chromatography time usually being 18 hours; mixtures are kept for 12 to 14 hours in a solvent atmosphere. After development, the dried paper is sprayed with a 1 per cent. solution of cobalt nitrate in absolute ethanol, dried again and then held in ammonia vapour when 50 μg. quantities of barbiturates show as a violet or reddish colour. For smaller quantities (5 to 10 μg.) the paper is sprayed with a solution containing 200 mg. of copper sulphate, 2 ml. of pyridine and 20 mg. of quinine per 100 ml. of water and the dried paper is then held in hydrochloric acid vapour. In ultra-violet light, the barbiturates are visible against the fluorescent background either as dark blue spots or circles; thiopentone is indicated by a yellow-green colour in visible light. Treatment with potassium permanganate then indicates unsaturated barbiturate derivatives. Using *n*-butanol–pentanol–ammonia not more than 5 days old, at 22° C., the following R_F values were found: barbitone 0·33, phenobarbitone 0·42, allobarbitone 0·49, cyclobarbitone 0·57, hexobarbitone 0·71, amylobarbitone 0·80, pentobarbitone 0·80, and thiopentone 0·83. R. E. S.

Digitoxin and Digoxin, Identification from their Acetyl Compounds by Means of Paper Chromatography. S. Rohatgi. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 428.) Digitoxin, α-acetyldigitoxin and β-acetyldigitoxin were separated by paper chromatography using the descending technique with propylene glycol as the stationary phase and a mixture of 9 parts of benzene with 1 of chloroform as the mobile phase. α-Acetyldigitoxin moved the most rapidly and could be separated by running the chromatogram for about 24 hours. For the separation of digitoxin and β-acetyldigitoxin about 48 hours was required.

In an alternative separation which was completed in about 5 hours, a mixture of equal volumes of chloroform and benzene was used as mobile solvent, with formamide as the stationary phase, but in this case the spots were not so well defined. Complete separation of digoxin and α-acetyldigoxin was effected in about 20 hours, using formamide as the stationary phase and a mixture of 3 parts of benzene with 7 of chloroform as the mobile phase. The spots were made visible by spraying the paper with *m*-dinitrobenzene solution, drying and spraying with sodium hydroxide solution. Digitoxin, α-acetyldigitoxin, digoxin and α-acetyldigoxin gave purple spots, whilst those due to β-acetyldigitoxin were deep blue. G. B.

3 : 5-Dinitro-*o*-cresol, Determination of. M. L. Fenwick and V. H. Parker. (*Analyst*, 1955, **80**, 774.) The method of determination of 3 : 5-dinitro-*o*-cresol in which an alkaline ethyl methyl ketone extract, containing the yellow sodium salt of DNOC, is compared photometrically with a similar extract of a standard solution of DNOC, has been found inadequate, in that β-carotene, also, is partly extractable by ethyl methyl ketone. Two ways of overcoming this difficulty are described. In the first, for cow's blood, the extraction procedure is that of Parker (*Analyst*, 1949, **74**, 646), the method depending upon the fact that, whereas the sodium salt of DNOC is bright yellow, the free acid is almost colourless in ethyl methyl ketone; the optical density of β-carotene at 430 mμ, however, is the same in acid or alkaline solutions. This method was not applicable to the determination of DNOC in locusts because of the production of highly stable emulsions after extraction with ethyl methyl ketone. In this method, the locust is homogenised in a mixture of chloroform and trichloroacetic acid. An aliquot of the chloroform extract is shaken with sodium carbonate solution, which extracts the DNOC but not the carotene. A portion of the carbonate extract is shaken with ethyl methyl ketone in the presence of sufficient sodium chloride to "salt out" the DNOC. The optical density at 430 mμ of the ketone solution is then compared with a standard curve. R. E. S.

Reserpine, Analytical Methods for. W. H. McMullen, H. J. Pazerda, S. R. Missan, L. L. Ciaccio and T. C. Grenfell. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 446.) The following methods were employed successfully for the assay of reserpine in tablets and other pharmaceutical preparations. (1) A sample of about 10 mg. was dissolved in chloroform and extracted with 0·01M hydrochloric acid to remove reserpic acid, followed by a 1 per cent. solution of sodium bicarbonate to remove trimethoxybenzoic acid. The ultra-violet absorption of the solution was determined at 295 and 268 mμ, after further dilution of the solution with chloroform. The ratio of the absorbancies at these wavelengths (1·83) served to identify the solute as reserpine. The concentration of reserpine was calculated by reference to a standard curve. The quantity of reserpine in the preparation, which had decomposed into trimethoxybenzoic and reserpic acids was calculated from the absorbancies of the sodium bicarbonate and hydrochloric acid solutions. (2) A sample of about 1 mg. was dissolved in chloroform and extracted with a 1 per cent. solution of sodium bicarbonate. The chloroform solution was evaporated to small bulk, and the remaining chloroform removed after the addition of M acetic acid. The solution was heated with concentrated sulphuric acid, cooled and the light absorption determined at 380 mμ. The quantity of reserpine was calculated by using a standard curve based on measurements with a standard solution of reserpine in M acetic acid, similarly treated. Reserpine was separated from serpentine, ajmaline, ajmalicine, yohimbine, reserpic acid hydrochloride and other sub-

stances by paper partition chromatography. A system employing 2 per cent. of acetic acid in propylene glycol as the stationary phase and equal quantities of benzene and *cyclo*hexane as the mobile phase yielded sufficient quantities of reserpine for absorption measurements. G. B.

Reserpine in Tablets, Identification and Determination of. D. Banes. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 408.) Reserpine is feebly basic and can be separated from more strongly basic alkaloids by extraction from acidic solutions with chloroform. Dilution of the chloroform solution with *iso*octane enables the reserpine and other weak bases to be extracted with a dilute solution of citric acid. The quantity of reserpine in this solution may be determined by adding vanillin and sulphuric acid and measuring the light absorption at 532 mμ at intervals until it reaches a maximum. The result is calculated by comparison with the absorption of a solution of a standard preparation of reserpine, similarly treated. Reserpine may also be assayed by hydrolysis followed by determination of the reserpic and trimethoxybenzoic acids produced. Reserpic acid is estimated colorimetrically after treatment with vanillin and sulphuric acid (as for reserpine) and trimethoxybenzoic acid is determined by ultra-violet spectrophotometry at 262 mμ using a standard solution of trimethoxybenzoic acid as a basis of comparison. Samples of reserpine for analysis must be freed from reserpic or trimethoxybenzoic acids occurring as impurities or high results will be obtained. When the result of the trimethoxybenzoic acid assay is significantly greater than that of the vanillin colorimetric assay, this indicates the presence of decomposed reserpine or recanescine in the sample. The method is suitable for the assay of reserpine tablets. G. B.

Surface Active Agents, Analysis of. V. W. Reid, T. Alston and B. W. Young. (*Analyst*, 1955, **80**, 682.) The identification of surface active agents can be simplified by the determination of ultra-violet absorption between 210 to 350 mμ followed by tests to show ionic character and the presence or absence of nitrogen; examination also for the presence of sulphur or phosphorus is sometimes necessary. Details are given of the absorption characteristics of surface active agents in four groups: anionic compounds with nitrogen absent including alkylbenzenesulphonates, alkylnaphthalenesulphonates, tetralinsulphonates, arylbenzenesulphonates, alkyl sulphates and conventional soaps; non-ionic compounds with nitrogen absent including phenols and cresols, naphthols, resin acids, fatty acids and alcohols; anionic compounds containing nitrogen including aniline derivatives and alkylolamine soaps; cationic compounds containing nitrogen including quaternary ammonium compounds, quaternary base with a pyridine nucleus, quaternary base with an *iso*quinoline nucleus and aliphatic amino compounds. When mixtures of surface-active agents are being examined greater emphasis should be placed on the characteristics of the ultra-violet spectrogram since chemical tests are confusing. R. E. S.

ORGANIC CHEMISTRY

Collagen, Polypeptide Chain Configuration of. P. M. Cowan, S. McGavin and A. C. T. North. (*Nature, Lond.*, 1955, **176**, 1062.) The main features of the polypeptide chain configuration of collagen are indicated by consideration of the high-angle X-ray diffraction pattern, the amino-acid composition and sequence analysis, and the structure of poly-L-proline. The high-angle X-ray diffraction pattern is consistent with a system of hexagonally packed rods, consisting of atom sets which may be one or more amino-acid residues. The changes in the pattern when the fibre is stretched suggest a helical structure.

Consideration of the general form of the intensity distribution to be expected from a number of simple helical structures, and comparison with the observed high-angle X-ray pattern shows that in collagen the single helical form with a period of 28·6 Å containing either three or seven turns, and ten atom sets is the most likely. The measured density of dry collagen fibres shows that there are probably three amino-acid residues in the 2·86 Å atom set, and analysis of the X-ray diagram suggests that the observed helix form could arise from a unit of three chains, each with one residue in 2·86 Å, and coiled round one another. Examination of the peptides derived from collagen by hydrolysis suggests that there is not a rigid repeated sequence of amino-acids, though the sequence proline-hydroxyproline-glycine has been observed. All three amino-acids occur in other sequences, though hydroxyproline is always associated with glycine. The amino-acids are found in the proportions glycine (1/3 of total), proline (1/8) and hydroxyproline (1/10). The hypothesis is put forward that the prolyl-hydroxyprolyl-glycyl sequence is concentrated in one of the three chains conferring a special configuration, with the remaining two chains crystallising about the first. Poly-L-proline also has a helical structure in which the amino-acid residues are related by an exact threefold screw axis, with repeat spacing of 3·12 Å, which suggests that the chain has a similar configuration to that of collagen. The high specific *lævo*-rotation of both suggests that the minor helixes of these structures are left-handed. It is suggested that the three chains are linked together by NH . . . O, and possibly OH . . . O, hydrogen bonds. J. B. S.

α-Corticotrophin, Amino-acid Sequence of. C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon. (*Nature, Lond.*, 1955, **176**, 687.) The complete amino-acid sequence of α-corticotrophin is proposed, based on data from the structure of peptide fragments isolated from enzymatic and partial acid hydrolysates of the hormone. From the results of limited digestion with crystalline pepsin followed by chromatographic separation of the peptides, the following sequence was deduced for the carboxyl end of α-corticotrophin: asp.glu.ala.ser.glu.ala.phe.pro.leu.glu.phe. The following peptides were isolated by means of paper chromatography and paper electrophoresis from the tryptic digest of α-corticotrophin: ser.tyr.ser.(met.glu.his.phe)arg; try.gly.lys.pro.val.gly.lys.; lys.arg; lys.arg.arg; arg.pro.val.lys; and val.tyr.pro. ala.gly.glu(asp_2.glu_3.ala_2.ser.phe_2.pro.leu). Other peptides were obtained from the products resulting from the action of chymotrypsin on α-corticotrophin and from the partial acid hydrolysis of the DNP-derivative of this substance. The complete sequence is ser.tyr.ser.met.glu.his.phe.arg.try.gly.lys.pro.val.gly.lys.lys. arg.arg. pro.val.lys.val.tyr. pro.ala.gly.glu.asp.asp.glu.ala.ser.glu.ala.phe.pro.leu. glu.phe. A. H. B.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

6-Aminonicotinamide, a Potent Nicotinamide Antagonist. W. J. Johnsonand J. D. McColl. (*Science*, 1955, **122**, 834.) A potent nicotinamide antagonist, 6-aminonicotinamide, was discovered during the course of investigation into the inhibition of sulphonamide acetylation. The LD50 for mice is 35 mg./kg. as compared with 305 mg./kg. of 3-acetylpyridine. Nicotinic acid (50 mg./kg.) increased the LD50 of 6-aminonicotinamide eight-fold although it was ineffective against 3-acetylpyridine. Tryptophan also afforded some protection against the new antagonist. Liver homogenates from mice injected intraperitoneally

72 hours previously with 100 mg./kg. of 6-aminonicotinamide and 25 mg./kg. of nicotinic acid had only 30 per cent. of the oxygen uptake of homogenates from untreated controls. The addition of either DPN or oxidisable substrate greatly increased the rate of uptake and addition of both together returned the rate almost to normal. No appreciable change in oxygen uptake was observed when 6-aminonicotinamide was added to normal liver homogenates *in vitro*. It seems probable that the toxicity of 6-aminonicotinamide may be due to the formation of an inactive DPN analogue with consequent depletion, in certain tissues, of DPN. Involution of the spleen, one of the pathological changes seen with 6-aminonicotinamide poisoning, may be related to the high rate of analogue formation by this organ. G. P.

BIOCHEMICAL ANALYSIS

Oxytocin and Vasopressin, Partition Chromatography of. P. G. Condliffe. (*J. biol. Chem.*, 1955, **216**, 455.) Separation of natural beef and hog oxytocin and vasopressin to a pure state by counter-current distribution requires material from the pituitary glands of some several thousand animals. Development of a chromatographic method has reduced the number of glands necessary for a suitable yield of the purified hormones to about 50 to 100 beef or 200 to 400 hog glands. Partition columns with diatomaceous earth as supporting phase and 2-butanol-dilute acetic acid as solvent were used. Distribution of peptide material in the column effluent fractions was determined with modified Folin phenol reagent. Ultra-violet absorption at 275 mμ of the fractions was compared with oxytocic activity assayed on chicken blood pressure and pressor activity on anæsthetised rats injected with dibenamine. The purity of the hormones obtained by this method was not as great as with counter-current distribution, but the method offers a practical solution to the problem of separation and characterisation of human posterior pituitary hormones. G. P.

Plasma Bilirubin, Determination of. J. H. Graham. (*Amer. J. med. Sci.*, 1955, **230**, 633.) The method utilises heparinised capillary tubes to collect blood from the toe or heel, and is particularly suitable for children. Sufficient plasma is obtained for direct reading as well as total bilirubin and simultaneous estimations of the hæmatocrit are possible. The method can also be used for the collection of plasma for the determination of plasma electrolytes. Heparinised capillary tubes 75 mm. long are used. They are filled approximately to two-thirds with blood and sealed at one end with a microburner. After centrifuging for 4 minutes at 11,000 r.p.m. the tubes are broken at the junction of the plasma and packed cells, and the plasma is blown into a small watch glass. To 0·1 ml. of plasma add 0·9 ml. of distilled water, and transfer 0·1 ml. of the diluted plasma to each of two microcuvettes of a spectrophotometer. To each microcuvette add 0·5 ml. of methanol, 0·1 ml. of a solution of sulphanilic acid (1g. dissolved in 15 ml. of conc. hydrochloric acid and diluted to 1 litre with water), and 0·1 ml. of freshly prepared diazo reagent. Allow to stand for 30 minutes and read the percentage transmission at 540 mμ and obtain the bilirubin values from a standard calibration curve. G. F. S.

Urinary Indoles, Paper Chromatography of. J. P. Jepson. (*Lancet*, 1955, **269**, 1009.) The method described can be applied directly to urine without any preliminaries and has been used during the past three years to study cases of carcinoid, phenylketonuria, mental and nervous disorders, and Hartnup disease, and is suitable for the routine screening of urine. The urine, applied at an origin on a 10-in. square paper on a frame is submitted to two-way chromatography:

firstly, in an ascending basic solvent (*iso*propanol-ammonia) and secondly in the right-angle direction in an acidic solvent (butanol-acetic acid). Indoles are revealed on the dried paper by dipping it through a modified Ehrlich reagent (*p*-dimethylaminobenzaldehyde in hydrochloric acid-acetone). The position of the resulting spots, their rate of appearance and fading and their differing colours serve to identify the indoles and give a rough guide to the amounts. The amount of urine needed to obtain an adequate chromatogram or assay must be found by trial but the method is such that 100 μl. of normal urine will show very little in the way of Ehrlich-reacting substances except urea and indican. This volume of urine from a case of metastasing carcinoid will show an immense reaction for 5-hydroxy-indolyl-acetic acid, a slight reaction for its sulphate ester, and traces of other unidentified indoles; similarly urine from a phenylketonuric will react strongly for indolyl-acetic acid and indolyl-lactic acid. S. L. W.

CHEMOTHERAPY

Albomycin, Recent Studies on. G. F. Gause. (*Brit. med. J.*, 1955, **2**, 1177.) Albomycin, a new antibiotic, has been manufactured during recent years by the pharmaceutical industry of the Soviet Union. It was obtained by Dause and Brazhnikova in 1951 from cultures of a new species of streptomycetes, *Actinomyces subtropicus*. Albomycin is a basic substance and forms salts with various acids. Chemically pure sulphate of albomycin is in the form of an amorphous red powder, easily soluble in water, slightly soluble in methanol, but insoluble in other organic solvents. It is effective against a variety of organisms and particularly against staphylococci resistant to other antibiotics; its action is about ten times as strong as penicillin. Albomycin is pharmacologically inactive; intravenous injections of large doses in animals do not affect the heart, blood pressure or respiration. It is devoid of cumulative toxicity and pyrogenic action and no local reactions occur when it is injected subcutaneously or intramuscularly. Intrathecal injection is safe and is not accompanied by any side-reaction. Complete absence of toxicity of the drug for man has been proved by extensive clinical practice over some years. A remarkable feature is the formation of a reversible complex between albomycin and serum proteins which facilitates its circulation in the body. Albomycin has proved effective in the treatment of pneumonia, especially in young children, in the septic complications of dysentery and measles, and in meningitis due to penicillin-resistant pneumococci. It has also been used in the treatment of peritonitis and other surgical infections and for penicillin-resistant prostatitis and gonococcal urethritis. It has been found very effective in the treatment of relapsing fever due to *Sp. sogdianum*, the dose being 3 million units intramuscularly twice daily for 7 to 12 days. Animal experiments indicate that albomycin is synergistic in combination with penicillin or streptomycin. S. L. W.

Filipin, an Antifungal Antibiotic: Isolation and Properties. G. B. Whitfield, T. D. Brock, A. Ammann, D. Gottlieb and H. E. Carter. (*J. Amer. chem. Soc.*, 1955, **77**, 4799). A new potent antifungal antibiotic, filipin, was obtained from the mycelium and culture filtrates of a previously unreported actinomycete, *streptomyces filipinensis*, found in a sample of Philippine soil. It is yellow neutral compound whose analysis best fits the empirical formula $C_{30}H_{50}O_{10}$. It is non-aromatic and has been characterised as a conjugated polyene. It is susceptible to autoxidation and particularly so when exposed to light. It is stable under nitrogen even when exposed to direct sunlight, and is stable in the dark in air at refrigerator temperatures. Filipin is relatively stable in very dilute solutions in ethanol. Fusion studies indicate that it exists in two

solid modifications; it undergoes transition to a second form at 147° C. and this second form melts at 195 to 205° C. with decomposition. It has a specific rotation of $[\alpha]_D^{22} - 148{\cdot}3$ (*c* 0·89 in methanol) and gives a positive Molisch test and negative ninhydrin, Benedict, anthrone, ferric chloride and 2:4-dinitrophenylhydrazine reactions. When concentrated methanolic or ethanolic solutions of filipin are stood at 4° C., a transition to a white crystalline substance ($C_{30}H_{50}O_{11}$) takes place. This product has no antifungal activity, but it is still a polyene as evidenced by its ultra-violet absorption spectrum and hydrogenation studies. A. H. B.

Sarcolysine (DL-*p*-Di(2-chloroethyl)aminophenylalanine), Anti-tumour Activity of. L. F. Larionov, A. S. Khokhlov, E. N. Shkodinskaja, O. S. Vasina, V. I. Troosheikina and M. A. Novikova (*Lancet*, 1955, **269**, 169.) Sarcolysine was prepared by reducing acetamido-*p*-nitrobenzylmalonic ester with hydrogen and Raney nickel. The amino compound was treated with an excess of ethylene oxide in aqueous ethanol in sealed tubes at 85 to 90° C., the product being acetamido-*p*-di(2-hydroxyethyl)aminobenzylmalonic ester. This was treated with thionyl chloride in chloroform and converted to DL-*p*-di(2-chloroethyl)aminophenylalanine hydrochloride by heating under reflux with hydrochloric acid. The following analogues were also prepared:—DL-*p*-di(2-bromoethyl)aminophenylalanine, DL-*p*-dimethylaminophenylalanine and DL-*p*-diethylaminophenylalanine. Sarcolysine was tested in rats infected with the transplantable rat sarcoma 45, a strain developed from a tumour induced by 9:10-dimethyl-1:2-benzanthracene. Three injections of 5 mg./kg. given intraperitoneally at intervals of 72 hours, or a single injection of 15 mg./kg. were sufficient to cause complete regression of the tumours. No toxic effects were observed unless the dosage was increased to 10 mg./kg. repeated at intervals of 72 hours. Sarcolysine also caused inhibition of the growth of another rat spindle-cell sarcoma (strain M1) and of mammary adenocarcinoma in mice (strain RSM), but complete regression did not occur with these tumours. Sarcolysine is also being studied for its action against neoplastic diseases. G. B.

PHARMACY

NOTES AND FORMULÆ

Ergot, Preparation and Stabilisation of Extracts. F. Gstirner and H. O. Müller. (*Arch. Pharm. Berl.*, 1955, **288**, 393.) By using ethanol (70 per cent. by volume) containing 0·5 to 1 per cent. of tartaric acid it is possible to extract 90 per cent. of the alkaloids from ergot by using a quantity of a menstruum only double that of the drug. Thus a 1:2 fluid extract can be prepared with no need for any working up of after-runnings. Such extracts, prepared from non-defatted drug, contain up to 1 per cent. of fat according to the strength of the ethanol used, but this does not interfere with the stability of the product. Stabilisation tests showed that the decomposition of the alkaloids is independant of the hydrogen ion concentration. The addition of 1 per cent. of ascorbic acid gave an extract which is stable for 3 months, with 2 per cent. for 4 months. An extract prepared with 45 per cent. ethanol and containing 1 per cent. of ascorbic acid, and gassed with nitrogen, was stable for 10 months, but in the case of a similar experiment with 70 per cent. ethanol the stability was quite low. In the latter case 2 per cent. of ascorbic acid and bubbling with nitrogen gave a stability of 7 months. Cysteine hydrochloride (1 per cent.) had a stabilising action only in 96 per cent. ethanol, and even without nitrogen treatment there was no appreciable change in strength after 10 months. G. M.

Emulsification with Ultrasonic Waves. H. M. Beal and D. M. Skauen. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 487, 490.) Emulsions were prepared experimentally by submitting the ingredients to the effects of ultrasonic vibrations in several exposure chambers. The emulsions were examined microscopically and evaluated by means of globule counts. Good results were obtained by the use of an exposure chamber consisting of a pyrex test tube the bottom of which had been replaced by a threaded brass collar, the tube being closed by a diaphragm of aluminium, copper, cellulose acetate or brass. Similar results were obtained with a flat-bottomed tube of cellulose acetate with a wall thickness of 0·5 mm. The best results were obtained by placing the exposure chamber so that the bottom was a definite number of half-wavelengths from the crystal generating the ultrasonic frequency. Under these conditions the maximum energy entered the chamber. Solutions of polyethylene glycol 400 monostearate, tragacanth and hard soap showed significant losses in viscosity when submitted to ultrasonic waves, and this would appear to be a disadvantage when they are used for the preparation of emulsions by this method. The viscosity of solutions of acacia, sodium lauryl sulphate and polysorbate was not affected by ultrasonic vibrations. Emulsions of light liquid paraffin, liquid paraffin, cottonseed oil and oil of turpentine were prepared by exposing a mixture of oil, water and emulsifying agent in a suitable exposure chamber. The best results were obtained by using hard soap, followed by polyethylene glycol 400 monostearate, polysorbate 80, acacia and sodium lauryl sulphate. Tragacanth was the least effective emulsifying agent, apparently because it acts by increasing the viscosity of emulsions and this property is affected by ultrasonic waves. The maximum exposure time employed in these experiments was 30 minutes, and the size of the oil globules appeared to decrease with increase in exposure time up to this limit. G.B.

Ophthalmic Solutions, An Evaluation of Chemical Preservatives for. C. A. Lawrence. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 457.) Several chemical preservatives were tested for bactericidal activity against 26 strains of *Pseudomonas æruginosa* and 4 species of *Proteus*. The chemicals were allowed to remain in contact with the organisms for definite periods of time from 30 minutes to 6 days, after which a loopful of solution was transferred to a suitable culture medium and tested for sterility. For thiomersalate and phenylmercuric nitrate, Brewers' thioglycollate medium was used to inactivate the antiseptic, while benzalkonium chloride was inactivated by using beef extract broth containing 0·1 per cent. of Asolectin and 0·7 per cent. of Tween 20. All substances tested were more active against *Proteus* than against *Pseudomonas* cultures, and benzalkonium chloride was the most effective agent studied. Phenylmercuric nitrate was more active than thiomersalate against *Pseudomonas æruginosa*, and phenylethyl alcohol appeared to be relatively inactive against these organisms. Similar results were obtained by testing the substances in distilled water and in solutions of ophthalmic drugs such as atropine sulphate, homatropine hydrobromide, pilocarpine nitrate and penicillin (potassium salt). G. B.

PHARMACOLOGY AND THERAPEUTICS

Antibiotics and Aluminium Hydroxide, Incompatibility of. A. Albert and C. Rees. (*Brit. med. J.*, 1955, **2**, 1027.) The administration of aluminium hydroxide with the tetracycline group of antibiotics to counteract the digestive upsets which often follow their oral use is condemned as an undesirable practice. The authors carried out the following experiment: aluminium hydroxide gel 8 ml.

was added to chlortetracycline hydrochloride 0·25 g. in water 250 ml. From the pH (6·48) and from the data yielded by potentiometric titration in the presence of known concentrations of aluminium cations they calculated that all the drug was firmly bound to the aluminium hydroxide, and it is unlikely that it would be liberated to any extent during the passage of the aluminium complex along the intestinal tract. The abolition of digestive upset is therefore explained by the fact that the drug is no longer present. The other tetracyclines behave similarly and it is suggested that milk should replace aluminium preparations as a protective agent for the gastro-intestinal tract when these antibiotics are administered. S. L. W.

Chlorpromazine Hydrochloride in the Treatment of Tetanus. A. C. E. Cole and D. H. H. Robertson. (*Lancet*, 1955, **269**, 1063.) In the belief that chlorpromazine hydrochloride is an antagonist to some central convulsant drugs and that it potentiates the action of hypnotics, it was given in conjunction with phenobarbitone or chloral hydrate to six African patients with tetanus. The drug was given in doses of 50 mg. either intramuscularly, diluted to 10 ml., or intravenously, diluted to 20 ml. The tetanic spasm was relieved dramatically; the abdominal and back muscles were moderately or completely relaxed, convulsive seizures were inhibited and a lazy sleepiness supervened. The simultaneous injection of 3 grains of soluble phenobarbitone prolonged the effect for up to 12 hours. For children, chloral hydrate was given orally instead of the phenobarbitone. Insufficient cases have been treated to show whether there is any effect on mortality. Oral treatment with 10 or 25 mg. of chlorpromazine hydrochloride was less effective but was useful when the patients were recovering. H. T. B.

Chlorpromazine, Reserpine, and Isoniazid in Mental Disorder. J. K. Hewat, P. W. W. Leach and R. W. Simpson. (*Brit. med. J.*, 1955, **2**, 1119.) This is the report of a pilot trial to assess the toxic effects of these three drugs used in combination in psychiatric conditions. Sixteen patients with chronic psychoses were treated for 3 weeks by the oral administration of a compound tablet containing chlorpromazine 25 mg., reserpine 1 mg., and isoniazid 50 mg. Beginning with one tablet, the dose was increased every day until 4 tablets daily were being given. The outstanding result was the unexpectedly high incidence of extrapyramidal signs. Parkinsonism occurred in 6 patients, tremor in 4, drowsiness in 11, insomnia in 1, dizziness in 2 and headache in 1. Other side-effects included a fall in blood pressure, nasal congestion, dryness of mouth, pallor, flushing, increase in weight, pains in trunk and limbs, malaise and weakness, and shivering. A further trial on another series of 16 patients was carried out, using half the dosage—namely, chlorpromazine 50 mg., reserpine 2 mg., and isoniazid 100 mg., daily for 4 weeks. The side-effects were markedly reduced. Parkinsonism was entirely absent, tremor occurred in 1 patient, drowsiness in 3, and giddiness in 2. The hypotensive and bradycardic trends were similar to those in the first trial. In both trials all side-effects cleared rapidly when treatment was stopped. These preliminary observations, over a short period on a small number of patients, did suggest that the combination of the drugs had a definite effect on the mental state. After the first trial 7 were worse, 5 unchanged, and 4 were better. After the second trial 2 were worse, 3 unchanged, and 9 were better (1 uncompleted). Where the effect was beneficial the trend was towards a quieter, more co-operative and more relaxed patient. Although Parkinsonism is known to occur with chlorpromazine and reserpine when used

singly in large doses its occurrence with small doses in combination was unexpected and suggests that these two drugs may have a potentiating action. This action is perhaps not entirely undesirable since it may have therapeutic significance. The authors conclude that these preliminary trials did not reveal any contraindication to proceeding with large-scale trials they are planning to undertake. S. L. W.

Cortisone, Influence on Connective Tissue, Epithelial Relations in Wound Healing, Hair Regeneration and the Pathogenesis of Experimental Skin Cancers. T. Gillman, J. Penn, D. Bronks and M. Roux. (*Nature, Lond.*, 1955, **176**, 932.) Cortisone diminished, through suppression of the usual connective tissue responses, wound healing in rabbits and hair regeneration after plucking, and tumour development after painting the skin with methylcholanthrene, in mice. In the case of tumour development, neoplasia within the epidermis itself were not eliminated, but the incidence of papillomata and the subsequent neoplasia in such benign tumours was decreased after cortisone. Histochemical findings indicate that cortisone induces its effects by acting on ribose nucleic acid and/or mucopolysaccharide metabolism of the dermis, thereby influencing epidermal responses to various stimuli. G. P.

Cortisone in Ulcerative Colitis. S. C. Truelove and L. J. Witts. (*Brit. med. J.*, 1955, **2**, 1041.) A therapeutic trial of cortisone in non-specific ulcerative colitis was carried out on a series of patients in 5 hospital regions. Of the 210 patients concerned 109 were treated with cortisone, the usual dose being up to 100 mg. a day for 6 weeks, and 101 received an inert preparation. At every stage of severity of the illness, and in both first attacks and relapses, the cortisone-treated patients did better than the control patients. Cortisone was particularly beneficial in first attacks. Patients treated with cortisone were more likely to suffer from pyogenic complications and when using this treatment it is probably wise to employ penicillin or sulphonamides in addition. Follow-up information is available for an average period of 18 months for 205 of the patients. Nine months after the trial period patients treated with cortisone in the first attack preserved a clear advantage over the corresponding control group. On the other hand, relapse cases treated with cortisone had lost the initial advantage they showed at the end of the trial period. At the end of the follow-up period, essentially the same pattern existed as at nine months, but with a slight worsening of the general picture. About one-fifth of the original group had been treated by ileostomy by the end of the study; of these 44 patients, 14 were dead at the end of the study. There would seem to be good grounds for advising that early cases of ulcerative colitis should promptly be brought under treatment with cortisone, which should, if necessary, be given in considerably higher dosage than used in this trial. Where treatment is successful, as it is likely to be in a substantial proportion of cases, the patients should be kept under careful supervision so that treatment can be promptly resumed if symptoms recur. Once irreversible damage has been done to the colon and the patient has persistent troublesome symptoms, it is wisest for him to submit to ileostomy, which is nowadays usually combined with colectomy. While it is true that cortisone greatly increases the chances of remission or improvement in all grades of chronic ulcerative colitis, nevertheless it is not a specific, and its effect is far from permanent, especially in the established disease. S. L. W.

***p*-(Di-2-chloroethylamino)-phenylbutyric Acid (CB 1348) in Malignant Lymphoma.** D. A. G. Galton, L. G. Israels, J. D. N. Nabarro and M. Till.

(*Brit. med. J.*, 1955, **2**, 1172.) This compound is a water-soluble aromatic nitrogen mustard which has been shown to be a powerful inhibitor of the transplanted Walker rat tumour. Ninety-three patients suffering from advanced carcinoma and from lymphomas have been treated with the compound since September 1952. This report concerns 62 cases of malignant lymphoma; these include 23 of Hodgkin's disease, 20 of lymphocytic lymphoma, 11 of reticulum cell sarcoma, 6 of follicular lymphoma and 1 each of generalised exfoliative erythrodermia and mycosis fungoides. Administration was usually by mouth, the dose ranging from 2 to 20 mg. a day (0·03 to 0·34 mg./kg.); in most cases it was either 0·1 or 0·2 mg./kg. daily. A course of treatment usually lasted 3 to 6 weeks, but the drug was given daily for 8 to 16 weeks on 13 occasions and for 6 to 12 months on 3. Eighteen patients had more than one course of treatment; 15 were given 2 courses at intervals of 3 to 27 months; 2 had 3 courses; and 1 had 6. Treatment was stopped when satisfactory improvement had been obtained. If there was no response treatment was usually continued for 6 weeks before concluding that the patient was resistant to the drug. In a few cases the drug was given by intravenous injection, the sodium salt being freshly made up in isotonic sodium bicarbonate. Single doses varied from 10 to 20 mg.; in one case single doses of 70 mg. were given. The injections were well tolerated and did not damage the veins or cause vomiting. Striking remissions were obtained in 4 cases of Hodgkin's disease, 7 of lymphocytic lymphoma, 4 of chronic lymphocytic leukæmia, and 5 of follicular lymphoma. The drug is relatively free from gastro-intestinal side-effects and has proved less damaging to haemopoietic tissue than cytotoxic agents hitherto available for the treatment of malignant lymphoma. It is safer than tretamine, especially when repeated courses are required. The dosage of CB 1348 likely to cause bone marrow damage almost always lies well outside the therapeutic range, at any rate for first courses. S. L. W.

Ecolid: A New Hypotensive Agent. R. D. H. Maxwell and T. J. G. Howie. (*Brit. med. J.*, 1955, **2**, 1189.) Ecolid is 4:5:6:7-tetrachloro-2-(dimethylaminoethyl)-*iso*indoline dimethochloride. It is a ganglion-blocking agent which has been shown in animal experiments to be several times as active by parenteral dosage as hexamethonium and twice as active as pentolinium. In oral dosage its activity is the same as pentolinium but the duration of action is appreciably longer. Vagal activity is affected less than sympathetic. Twelve patients suffering from severe hypertension were treated with ecolid. It was determined that the initial dose should not exceed 25 mg. and treatment consisted of 25 to 200 mg. administered before breakfast with a dose also during the evening if required. The treatment has been used in this series of patients over 4 months and it has been found possible to lower the blood pressure to a satisfactory level in every case. The severity of side effects depended to some extent upon the dosage used. Mydriasis was a constant finding and blurring of vision commonly occurred during initial treatment but was less severe with maintenance dosage. Precautions were necessary to overcome development of constipation and in 3 cases treatment had to be stopped owing to nausea, vomiting, and obdurate constipation. A combination of reserpine with ecolid was used in the maintenance treatment of 6 of the patients, reserpine, 2 mg. by mouth, being given in the evening. Reduction in the maintenance dose of ecolid was found possible and side-effects were fewer. During the period of observation tolerance to ecolid has not developed and in some cases reduction of dosage has been possible. The response was good in 8 cases and fair in 1, the drug being stopped in the remaining 3 owing to side-effects. S. L. W.

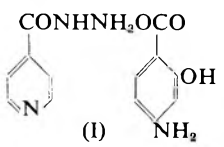
Noradrenaline in the Treatment of Acute Cor Pulmonale. J. de Swiet. (*Brit. med. J.*, 1955, **2**, 1253.) The use of (−)-noradrenaline in the treatment of the shock and hypotensive state following acute myocardial infarction suggested its use in combatting the profoundedly hypotensive condition resulting from massive pulmonary embolism. A case is described in which pulmonary embolism occurred after partial gastrectomy. Treatment with aminophylline, heparin and pethidine was started immediately, and a course of ethyl biscoumacetate was also begun. After an hour, the blood pressure still being extremely low, an intravenous infusion of 4 ml. of Levophed, containing 4 mg. of (−)-noradrenaline, in a litre of 0·2 N saline was begun at a rate of 30 drops/minute, the rate being varied from 25 to 35 drops/minute according to the blood pressure. A further 4 ml. of Levophed was added after 1 hour, and thereafter 4 ml. half hourly for 6 additions, at which time the drip was delivering about 45 μg./minute of noradrenaline. For the following 4 days, the drip contained 64 ml. of noradrenaline, delivering 90 μg./minute. Subsequently the patient twice collapsed but recovered when the noradrenaline dosage was increased to 150 μg./minute and thereafter the concentration was gradually reduced to zero. The total dosage was 798 mg. during 11 days. Profuse sweating was the only evidence of any toxic effect. H. T. B.

Noradrenaline, Skin Necrosis Following Intravenous. J. Humphreys, J. H. Johnston and J. C. Richardson. (*Brit. med. J.*, 1955, 2, 1250.) Extreme local vasoconstriction resulting in gangrene of the skin and subcutaneous tissues occurred 5 times in 3 cases following the administration of noradrenaline into a superficial vein. The patients had respectively undergone a subtotal colectomy for ulcerative colitis, panhysterectomy and abdomino-perineal resection for carcinoma, and urethral dilatation for stricture. In the first case, 2 mg. of noradrenaline in 0·5 l. of 5 per cent. dextrose solution was given into the left internal saphenous vein in the ankle and after 150 ml. had been given a mottled blue and white appearance of the leg was noticed. The infusion was stopped and given into the other ankle but the same effect was immediately produced. Necrosis followed in both legs. The second patient received a total of 450 mg. of noradrenaline in 7 different veins over about 3 weeks and skin necrosis occurred over 2 of the 3 veins where a tied-in cannula was used but not where the 4 percutaneous infusions were given. In the third patient signs of tissue necrosis appeared 46 hours after starting a drip infusion into an internal saphenous vein. The authors consider that the concentration of noradrenaline in the infusion is not significant but that the important factor is the concentration of the drug in the vein lumen. If given through a needle inserted percutaneously into a large vein it is rapidly diluted by the circulating blood, whereas with a tied-in cannula the recipient vein contains only the solution until it is joined by a large tributary. If the veins are collapsed and a cut-down is necessary, it is suggested that the drug be given through polythene tube passed far enough proximally to enable its tip to lie in a vein considerably greater in diameter than its own. [*Abstractor's Note.* M. T. Harrison (*Brit. med. J.*, 1955, **2**, 1502) reports two cases of necrosis in spite of the passage of a polythene cannula a long distance up the long saphenous vein.] If the skin overlying a vein receiving the drug shows colour changes, the administration must be stopped at once. Early injections of procaine and papaverine into the vein may possibly relieve the vasoconstriction. H. T. B.

Organic Phosphates, Pharmacological Effects of. R. E. Bagdon and K. P. Dubois. (*Arch. int. Pharmacodyn.*, 1955, **103**, 192.) The pharmacological actions of *p*-nitro-*m*-chlorophenyl dimethyl thionophosphate (Chlorthion),

(S-[1 : 2-dicarbethoxyethyl]*OO*-dimethyl dithionophosphate (malathion) and tetrapropyl dithionopyrophosphate have been studied and compared with the highly active organic phosphate 2-mercaptoethyl thionophosphate (systox). Intravenous injections into normal dogs caused increased urination, diarrhœa, copious salivation and deepened respirations. High doses showed the characteristic effects of poisoning by anticholinesterase agents. In anæsthetised dogs the compounds caused a fall in blood pressure, bradycardia and potentiated the actions of acetylcholine. The dose of systox causing death in dogs was 100 to 240 times less than the other compounds. The isolated rabbit heart was resistant to chlorthion, malathion, tetrapropyl dithionopyrophosphate and systox. Atropine in the perfusion fluid gave no protection. Tetrapropyl dithionopyrophosphate and systox caused a rise in tone of the isolated rabbit intestine and high doses spastic contractions. The peristaltic response was blocked by atropine or nicotine. Chlorthion and malathion stimulated the intestine, but in high doses caused a depression of tone. G. F. S.

PAS Salt of Isoniazid: Clinical Trial in Pulmonary Tuberculosis. W. J. Clegg. (*Brit. med. J.*, 1955, **2**, 1004.) The drug employed in this trial, called GEWO 339, is an additive compound between PAS and isoniazid to which the formula (I) is attributed. The substance is said to inhibit the growth of ordinary strains of tubercle bacilli at a concentration of 0·1 μg./ml. of medium. It is also claimed that strains resistant to isoniazid and PAS either alone or in combination, though not as sensitive as normal strains to GEWO 339, were still sensitive at therapeutic levels. Strains which grow normally on media containing 100 μg. of isoniazid and also media containing 100 μg. of PAS were inhibited by 5 μg./ml. of the new compound. For the purposes of the trial 17 patients were treated, all of whom were suffering from bilateral chronic pulmonary tuberculosis with cavitation. Each patient received a daily dose of 600 mg. of the compound in three doses of 200 mg. given as 100 mg. tablets, the treatment continuing for a period of 12 weeks. The most striking change following the treatment was the remarkable lowering of the positivity of the sputum. In only 1 case was there no significant alteration in the character of the sputum; in 10 cases there was a marked drop in both the volume and the bacillary content of the 24-hour samples, and in 6 cases sputum conversion occurred, none of whom, in spite of long periods of antibacterial treatment, had previously had sputum conversion. In only one of the cases did partial resistance develop during treatment. The drug was well tolerated in all cases. Most of the patients had previously taken large doses of PAS, isoniazid, and streptomycin, and the great majority expressed their appreciation of the ease with which the new compound could be taken in comparison with the nausea associated with large doses of PAS. Since the drug proved active *in vivo* and drug resistance did not develop quickly, the author states that it is hoped to organise a full-scale trial. S. L. W.



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Phenoxymethylpenicillin; Comparison with Benzylpenicillin on Oral Administration. W. W. Wright, A. Kirshbaum, B. Arret, L. E. Putnam and H. Welch. (*Antibiotic Med.*, 1955, **1**, 490.) This paper discusses the potency of phenoxymethylpenicillin (Penicillin V) when tested by various methods, and the serum and urine concentrations obtained following oral administration. When tested against numerous strains of *Micrococcus pyogenes* var. *aureus* it was found superior to benzylpenicillin against both penicillin-sensitive and

penicillin-resistant strains. In the latter cases its superiority was not sufficiently great to say that a significant number of benzylpenicillin-resistant strains are sensitive to phenoxymethylpenicillin. Thus, in spite of the greater over-all activity of the latter no clinical advantage would be expected from its use if it were present in the blood in the same amount as benzylpenicillin. Controlled experiments on 30 normal subjects showed that phenoxymethylpenicillin does produce higher blood concentrations than benzylpenicillin. When given orally, this is probably due to its greater stability in an acid environment. This is reflected also in the urinary excretion of active phenoxymethylpenicillin in quantities double those obtained with benzylpenicillin. In the light of these findings the authors consider that the use of phenoxymethylpenicllin may be advantageous in oral penicillin therapy. S. L. W.

Prednisone and Prednisolone in Rheumatoid Arthritis. F. D. Hart, C. J. M. Clark and J. R. Golding. (*Lancet*, 1955, **269**, 998.) Ten rheumatoid patients were transferred from cortisone to either prednisone or prednisolone (15 to 30 mg. daily) and back to cortisone, and 4 from prednisolone direct to cortisone. Assessment was by the patients' own estimation of pain and stiffness, by finger swelling, tenderness over joints, erythrocyte-sedimentation rate, simple function tests, and power of grip. The length of treatment with prednisone or prednisolone (the two substances appear identical in action) varied from 4 days to 10 weeks. The results of this short-term investigation showed that either of these drugs is a more effective agent than cortisone in four or five times the dose in relieving the symptoms and signs of rheumatoid arthritis. In no single detail in any of the 14 cases did cortisone give a better assessment figure. Of the 14 patients, 9 preferred prednisone or prednisolone to cortisone, 5 were indifferent, and none preferred cortisone. Over-all improvement took place in 10 of the 14 cases on transferring from cortisone to prednisone, whereas 8 deteriorated somewhat on returning to cortisone. No toxic effects of the new products were observed. S. L. W.

Prednisone, Clinical and Metabolic Effects of. J. D. N. Nabarro, J. S. Stewart and G. Walker. (*Lancet*, 1955, **269**, 993.) The clinical effects of prednisone, in a dose of 30 to 40 mg. daily, were observed in 5 cases of lymphatic leukæmia, 2 cases of lymphoma and 1 case of Henoch-Schönlein syndrome. In addition, prednisone (30 mg. daily) was given to a normal subject during a balance study, and varying amounts were given to 2 adrenalectomised women to study the effect on steroid excretion. Seven of the patients had been receiving cortisone in large doses (100 to 150 mg. daily) for periods up to 12 months, and in 6 of the patients this had produced a dramatic improvement in the general condition and well-being. When prednisone was substituted for cortisone the improvement was maintained. An eighth patient, in whom steroid therapy was initiated with prednisone, also showed an excellent response to treatment. Of the 7 patients who had been receiving cortisone 4 had had œdema, 1 severe dyspepsia and osteoporosis with vertebral collapse, 1 had steroid diabetes, 1 was euphoric and 1 had developed a moon-face. Transfer to prednisone abolished the œdema and improved salt tolerance but was without effect on the other complications; 2 patients developed diabetes while on prednisone. It would appear that prednisone is about five times more active than cortisone in maintaining the hæmoglobin level and reducing the size of lymphomatous deposits. This therapeutic ratio is similar to that observed in rheumatoid arthritis and asthma. The sodium-retaining activity of prednisone is not increased in parallel with the therapeutic activity and most patients are able to take prednisone (30 to 40 mg. daily) with a normal sodium intake. Prednisone causes adrenal sup-

pression and patients receiving long-term treatment will be in a state of adrenal deficiency if the steroid is abruptly withdrawn. The fact that prednisone can be given without restriction of salt intake and with little risk of œdema developing may encourage prolonged administration in high dosage, but the other and more serious complications of steroid therapy are just as easily produced and it is essential to keep the dose as low as possible. S. L. W.

Radioactive Iodine in the Treatment of Thyrotoxicosis. G. W. Blomfield, J. C. Jones, A. G. Macgregor, H. Miller, E. J. Wayne and R. S. Weetch. (*Brit. med. J.*, 1955, **2**, 1223.) The authors give a detailed analysis of the results obtained with radioactive iodine in 106 female and 34 male cases of thyrotoxicosis followed up for at least a year. Most of the patients received a single dose of ^{131}I calculated to deliver to the gland a dose of 6000 to 8000 rads (1 rad = 100 ergs/g. of gland tissue), the calculation being based on the effect of a preliminary tracer dose. Patients needing rapid control of the thyrotoxic state were treated with an antithyroid drug before treatment with ^{131}I. 36 patients received more than one dose, one of them having a resistant post-operative recurrence which was treated with 5 doses over a period of 2 years. Results were assessed on clinical grounds. At the time of assessment 118 patients were euthyroid and 17 were hypothyroid, while 4 were still thyrotoxic although one felt so well that further treatment was refused. The average time for achieving euthyroidism in single-dose cures was about $4\frac{1}{2}$ months. Of the hypothyroid cases, 9 needed only 0·1 mg. of L-thyroxine sodium per day to maintain thyroid balance. Two patients unwittingly received treatment while pregnant but both pregnancies proceeded normally to full term and the babies have developed normally. The authors conclude that the risk of radiation-induced neoplasms in the thyroid gland is slight, although the final answer cannot be known for another decade. As a precaution they suggest that, in general, the treatment should be given only to those whose life expectation does not exceed 20 years. A few patients complained of transient aching over the front of the neck for a few days after the dose. Two developed urticaria which needed prolonged antihistamine therapy before it abated. Thrombocytopænia occurred temporarily in one patient but may not have been due to the treatment. 15 patients complained of rheumatic symptoms, either articular or non-articular, but this also may not have been due to the isotope. The main problem in treatment is the determination of dosage. Scintillation counting techniques are of value in determining the size of the gland but there is a discrepancy between the actual uptake of ^{131}I and the uptake indicated by the tracer dose. H. T. B.

Rauwolfia in Hypertension. J. H. Moyer, E. Dennis and R. Ford. (*Arch. intern. Med.*, 1955, **96**, 530.) This report is a study of rauwolfia in the treatment of hypertension, its object being to compare the clinical results obtained with single pure alkaloids of rauwolfia (reserpine and rescinnamine) with those obtained with preparations containing multiple active alkaloids. The latter included whole root, Alseroxylon (a standardised alkaloidal extract containing reserpine, rescinnamine and other hypotensively active alkaloids not yet characterised) and Roxinil (an extract containing multiple alkaloids of both reserpine-like and serpentine-like material). A total of 346 cases of hypertensive vascular disease was treated. Most of the patients were treated as outpatients and were observed for a control period of from 1 to 3 months before treatment. The criterion for inclusion in the study was an average control blood pressure greater than 150/100. All patients received placebos during the control period. If the systolic or diastolic pressure fell below this level under placebo therapy the patient was discarded from the study. The criteria for responsiveness to

drug therapy was a fall of at least 20 mm. Hg. in the average upright mean blood pressure, or a fall below 150/100 mm. Hg. which was considered the normotensive level. The patients were divided into 5 groups according to the drug they received. The post-treatment blood pressure in each case was taken as an average of all recordings made after the first 6 weeks of treatment with one of the preparations. The study showed that there was no significant difference, quantitatively or qualitatively, in the hypotensive action of the 5 products. The hypotensive activity of 1 mg. of reserpine is estimated to be about equal to that of 250 mg. of crude root or 4 mg. of Alseroxylon. The side-effects of the 5 products were qualitatively similar but quantitatively there were minor differences. Reserpine produces more weakness, fatigue and sedation than either rescinnamine or extracts containing multiple alkaloids. Reserpine produced mental depression in a number of cases and this was not observed with rescinnamine and was much less frequent with Alseroxylon or crude root. All the preparations tested were bradycrotic but rescinnamine appeared somewhat less so than the others. All the preparations had a slow onset of action but reserpine appears somewhat more rapid than the others. S. L. W.

Reserpine and Rauwolfia in the Treatment of Psychoneuroses. A. Folkson and A. R. May. (*Brit. med. J.*, 1955, **2**, 1121.) In this investigation the effects of reserpine and rauwolfia were studied in 75 psychoneurotic patients. The patients were assessed in two separate groups. In group A (consisting of 30 patients) each patient received the active drug or a placebo in alternate courses lasting a month each. In group B (45 patients) the patients acted as their own controls. Most of the patients were out-patients and all were treated by the oral route. Sixty-seven patients received reserpine in a dose of 0·25 mg. three times daily initially rising to 0·5 mg. three times daily where this could be tolerated. Seventeen patients received rauwolfia in a dose of 2 mg. twice daily, and of these 9 patients subsequently received reserpine. The duration of treatment in both groups varied between 2 and 6 months. The investigation failed to confirm the results claimed in a number of psychiatric disorders. In group A no improvement occurred with either drug. In group B 6 patients benefited from reserpine of whom 3 were hypertensive. The authors suggest that these results indicate a possible discrepancy in the methods of assessment and the need for adequate control in such investigations. Side-effects in this series were infrequent and slight in nature. S. L. W.

Vitamin K_1 Intravenously, Effect on the Action of Phenindione. P. Dawson. (*Brit. med. J.*, 1955, **2**, 1427.) This paper records the results of tests performed on 24 volunteers receiving phenindione who were given intravenous injections of a stable emulsion containing 20 mg. of vitamin K_1/ml. Doses of 10 to 20 mg. of vitamin K_1, thus administered, were found to return the prothrombin activity to 100 per cent. within 24 hours in subjects continuing to take phenindione. These doses were also found effective within 24 hours in correcting a moderate deficiency of prothrombin itself. A dose of 5 mg. caused a rise of between 50 and 100 per cent. prothrombin activity within the same period. In more than half the experiments an appreciable rise of prothrombin activity occurred within 3 hours. In the treatment of hæmorrhage due to phenindione an intravenous dose of 10–20 mg. is likely to be effective. If vitamin K_1 is given merely to raise a dangerously low prothrombin activity to within therapeutic range the evidence suggests that even 5 mg. may be too large a dose if anticoagulant resistance in the subsequent days, with its danger of further thrombosis, is to be avoided. No toxic effects were observed during 40 intravenous injections of the vitamin K_1 emulsion. S. L. W.

BACTERIOLOGY AND CLINICAL TESTS

Influenza Virus Vaccines: Effects of Saline and Oil Adjuvants. (Second Progress Report by the Medical Research Council's Committee on Clinical Trials of Influenza Vaccine.) (*Brit. med. J.*, 1955, **2**, 1229.) Tests were carried out on 399 volunteers of the reactions and serological responses to three types of influenza vaccines—A: a water-in-oil emulsion of mouse-adapted virus, in doses of 0·25 ml. containing 2000 hæmagglutinating units; B: as A, but using egg-adapted virus; and C: mouse-adapted virus containing 20,000 hæmagglutinating units per dose of 1 ml., in saline to which 10 mg. of aluminium phosphate had been added. Each vaccine also contained 0·01 per cent. of thiomersal in the aqueous phase. A and B were given intramuscularly and C subcutaneously. There was no significant difference in the incidence of general reactions such as pyrexia, malaise, lassitude and nausea; they occurred in relatively few instances. Erythema and swelling, either alone or together, were found chiefly in volunteers receiving vaccine C. Induration at the site of inoculation occurred in 43 volunteers in the C group, 19 in the B group and 13 in the A group. In some cases it persisted, the figures at 6 months being 8, 2 and 1 and at 1 year 4, 2 and 0. In comparing the difference in the incidences of induration account must be taken of the greater difficulty in detecting it with A and B vaccines, which were given intramuscularly. Two volunteers, one given vaccine A and one given vaccine B, had unusually persistent and extensive local reactions, suggesting abscess formation although neither led to liquefaction or cyst formation. The peak in antibody titre was obtained at 2 weeks with vaccines C and 3 months with A and B, and the responses to A and B were greater and more prolonged than the response to C. After one year the mean titre in the C group was about 5 times the initial level, in the B group 7 times and in the A group 11 times. It is not certain that the differences in the results obtained with the A and B vaccines are significant since factors such as small antigenic differences and differences in the avidity for antibody may be concerned. H. T. B.

Organic Mercury Compounds, Bacteriostatic and Bactericidal Effect of. O. G. Clausen (*Medd. Norsk. Farm. Sels.*, 1955, **17**, 313.) In tests for the bacteriostatic effect of thiomersalate and phenylmercuric acetate and nitrate, 13 ærobic organisms and 1 anærobe (*Clostridium welchii*) were used. Three 'natural inocula' were also used in the tests, suspension of normal fæces, sputum suspension and dust suspension. These were chosen as representing microbial contamination more severe than is likely to be encountered during the preparation of solutions for injection and eye drops. Thiomersal was effective in the highest dilution, 1 in 50,000 being effective as a bacteriostatic against all the organisms and materials studied. The corresponding dilution for phenylmercuric acetate was 1 in 20,000, and for the nitrate 1 in 25,000. The organisms most resistant to thiomersalate were *C. welchii* and *Pseudomonas æruginosa*, those most resistant to phenylmercuric acetate were *Ps. æruginosa*, *C. welchii*, *Klebsiella pneumoniæ* and *Escherichia coli*, and those most resistant to the nitrate were *C. welchii*, *Ps. æruginosa and K. pneumoniæ*. Bactericidal tests were performed by allowing the antiseptics to act for 10 minutes at 37° C., and then inoculating a sample into thioglycollate medium which served as an inactivating agent. Owing to the comparatively slow bactericidal action of mercurial antiseptics, phenol coefficients could not be accurately determined, but using *Micrococcus pyogenes* var. *aureus* as test organism the phenol coefficient of thiomersalate was less than 10, and that of the phenylmercuric compounds was less than 25, whereas using *E. coli* the coefficients were less than 12·5 and less than 31·25. G. B.