REVIEW ARTICLE

THE APPLICATIONS OF ULTRA-VIOLET ABSORPTION SPECTROPHOTOMETRY IN PHARMACEUTICAL ANALYSIS

By R. E. Stuckey, Ph.D., B.Sc., Ph.C., F.R.I.C.

The British Drug Houses, Ltd., London, N.1

Introduction

In any study of absorption spectrophotometry it is helpful to define some of the fundamental units of measurement which are commonly used. The *wavelength*, λ, is expressed in millimicrons mμ (10^{-6}mm.), or in Ångstrom units (1Å. = 10^{-8}cm.). The *wave-number* is the number of waves per unit length, i.e. the reciprocal of the wavelength, and is commonly expressed in cm.$^{-1}$. The curve relating wavelength and intensity of absorption is characteristic of a substance and is often loosely described as "light absorption curve" or "light extinction curve"; conversely the absorption may be plotted as "transmittancy" against wavelength in which case a reciprocal type of curve is obtained. There is unfortunately a great lack of uniformity in the recording of results, particularly in cases where spectra in the ultra-violet and visible region are plotted together.

Absorption measurements can be made in different parts of the spectrum. The infra-red region involves wavelengths greater than about 1000 mμ; the visible spectrum extends from approximately 800 mμ down to 400 mμ; while the ultra-violet is usually taken as extending from 400 mμ to the sudden air absorption at 185 mμ. Shorter wavelengths than 185 mμ are sometimes referred to as being in the Schumann region (185 to 125 mμ) or simply as the "far ultra-violet."

When radiant energy is absorbed by any substance it may be utilised for different purposes according to the wavelength of the energy available. Absorption of energy in the infra-red region (wavelength greater than 1000 mμ) involves relatively small energy changes and causes excitation of the modes of vibration of the molecule. Absorption in the visible and near infra-red region corresponds to larger energy changes causing excitation and changes in the vibration and rotation of the molecule.

When radiation of wavelength 200 mμ to 400 mμ is absorbed the energy is used for the excitation of the electronic energy levels, involving the displacement of valency bonding electrons, these being associated as before with simultaneous vibrational and rotational changes. The spectrum produced with some liquids and vapours is therefore of the complex band type and is difficult to interpret. In the liquid phase, as is the case with solutions and pure liquids, intermolecular forces are present causing disturbances, and the fine structure disappears producing the single wide bands usually observed for solutions of organic compounds.

Mathematical treatment of these molecular processes is available for some simple molecules on the basis of quantum mechanics; it is, however, complex and need not be considered for the practical purposes of analysis. Nevertheless it is of value for the analyst to have a broad

appreciation of the class of compounds to which ultra-violet absorption spectrophotometry can be usefully applied.

The ultra-violet absorption spectra of inorganic compounds have been used to a relatively small extent in general analysis, particularly for pharmaceutical purposes. Results indicate that those cations which have complete electronic structures of the inert gas type are transparent in the region 185 to 700 mμ; ions with an incomplete inert gas electronic structure show absorption in the visible or in the ultra-violet region. Thus ultra-violet absorption measurements have proved to be of value in the study of the rare earth elements and for complex ions; solutions of nitrates and dichromates have been examined in detail and potassium nitrate and potassium dichromate have been widely used as pure salts having known light absorption properties suitable for the calibration of spectrophotometers; the absorption spectra of the halogen elements in various solvents have also been used for their analysis.[1]

It is in the organic sphere, however, that ultra-violet light absorption measurements have been of inestimable value. A considerable amount of practical data has made possible an empirical approach which ascribes absorption of certain definite wavelengths to particular organic groupings or "chromophores." Theoretical treatment indicates that when electrons are more mobile or loosely bound the frequency of absorption is lower, i.e. the wavelength of the absorbed light is higher. Compounds which are unsaturated and are known to exhibit resonance will absorb light within the ultra-violet and visible regions, whereas saturated and non-resonating compounds are transparent. Saturated hydrocarbons are therefore transparent in the ultra-violet and visible regions, although it is often difficult to purify them to such an extent that all extraneous absorption has been eliminated. Aromatic hydrocarbons on the other hand exhibit ultra-violet absorption,[2] benzene derivatives absorbing in the region 250 to 280 mμ according to the substituent groupings; the spectra in a homologous series such as the alkyl benzenes are very similar, the absorption being mainly due to the benzene nucleus and suffering slight modification only due to the substituent groupings.

Among aliphatic compounds many chromophoric groupings, e.g. a simple ethylenic linkage C = C, absorb in the 185 to 200 mμ region although this is of little value for analytical purposes. The carbonyl group = CO is associated with absorption *ca.* 280 mμ as shown for acetone and for many of the steroids.[3] Where two chromophores are conjugated, i.e. are separated by a single bond, a new type of absorption arises with increased intensity of absorption at longer wavelengths. Thus conjugation of one ethylenic bond with a second or with a carbonyl, carboxyl, acetylenic or nitro-group results in high intensity bands in the region 200 to 230 mμ. A lengthening of the conjugated chain to produce compounds containing two or more groups in conjugation further increases the intensity of absorption; when the number of conjugated groups reaches five or six, $\lambda_{max.}$ approaches the visible region of the spectrum and a yellow colour results.

The ultra-violet light absorption of isocyclic systems generally resembles

that of aliphatic compounds containing the corresponding number of alkyl groups; isocyclic systems of particular pharmaceutical interest are encountered in the sterols.[3] Aromatic and heterocyclic compounds generally absorb light and their ultra-violet absorption spectra have been the subject of extensive study. A number of useful reviews have been published dealing with the ultra-violet absorption spectra of organic compounds from an empirical viewpoint, detailing the characteristics of a wide range of chromophores both separate and conjugated. An excellent review by Braude[4] can be consulted, or articles by Lewis and Calvin[5], and by Ferguson[6]; the detailed study of aromatic compounds by Jones[7] is also useful.

Experimental Methods

In absorption spectrophotometry we have to consider the fate of light falling on the absorbing medium. It may be reflected, transmitted, or absorbed, though the effect of reflection is usually eliminated by means of a comparison cell. Quantitative absorption measurements are based on relationships derived from two fundamental laws. *Lambert's Law* states that the proportion of light absorbed by a substance is independent of the intensity of the incident light. *Beer's Law*, derived from quantitative work on the absorption of red light by aqueous solutions, states that the absorption is proportional to the total number of molecules in the light path. Combining the algebraic form of these two laws we obtain the expression

$$\log_{10} I_0/I = \epsilon c.l.$$

where I_0 is the intensity of the incident light, I is the intensity of the emergent light, ϵ is the molecular extinction coefficient, c is the concentration in g. mol./l., and l is the length of absorbing medium in cm. The molecular extinction coefficient ϵ is most frequently used in fundamental studies on the structure of organic molecules. More commonly used in analysis is the notation E^{c}_{l}. By this is meant the value of log I_0/I for a layer l cm. thick, of concentration c. Most modern spectrophotometers have a scale graduated in terms of log I_0/I and this method forms a convenient way of expressing, for example, the empirical absorption of a substance of uncertain or unknown molecular weight, c in this case being measured in g./l. In practice interrelationships used are

$$\epsilon = E^{1\,\text{per cent.}}_{1\,\text{cm.}} \times \text{Mol. wt.}/10 \text{ and}$$

$$\text{Log T} = 2 - \log I_0/I$$

where T is the percentage transmission, equal to $100\ I/I_0$.

Beer's Law is usually regarded as being valid at least in dilute solutions; where deviations occur these can usually be explained by a change in molecular species due to association, ionisation or other phenomena. Published curves of light absorption are usually plotted of ϵ against λ, log ϵ against λ, or more commonly in analytical studies of $E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ against λ in mμ. The latter notation, $E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ is used throughout the B.P. 1948.

Several methods are available for the determination of ultra-violet

absorption spectra. Glass absorbs strongly at wavelengths below 300 mμ, and prisms of quartz are usually used in conjunction with an optical mechanical or photoelectric device for comparing light intensities.

Older methods were based on Hartley's work, thicknesses being plotted against wavelength, thus giving the curves of the early literature which are angular and atypical when compared with those produced to-day. Henri, between the years 1910 and 1919, made advances on this method, but the bulk of the work done prior to 1941 was carried out with photographic instruments such as the rotating sector and "Spekker" photometers. These instruments depended finally on the matching of two adjacent spectra on a photographic plate, and great accuracy was not possible. With a number of determinations, however, relatively good results were obtained, an accuracy of the order of ± 3 per cent. being obtainable using a number of replicate determinations. Photographic measurements with a continuous light source are also useful for determining fine structure spectra. Detailed studies are available of the determination of absorption spectra using photographic instruments.[8,9,10]

The introduction, in 1941 by Cary and Beckman, of an accurate photoelectric spectrophotometer which soon became commercially available, changed the whole field of ultra-violet absorption studies. The intensity of the incident and emergent light were compared directly by photoelectric means after dispersion through a quartz prism; details of the instrument are available in the original paper[11], or as a Technical Bulletin issued by the makers. Two instruments of similar type have been available commercially in Great Britain for some years, the "Unicam" manufactured by Unicam, Ltd., Cambridge, and the "Uvispek" manufactured by Hilger & Watts, Ltd., London. The ready availability of instruments on which an absorption curve can rapidly and accurately be determined has revolutionised the whole field of ultra-violet spectra and during the past decade an immense amount of accurate information on the ultra-violet absorption spectra of organic compounds has been published.

Quantitative Analysis

The accuracy to be obtained with photographic instruments varies considerably, depending more on individual performance than is the case with photoelectric instruments. In general the reproducibility to be obtained with any one instrument can be as good as ±3 per cent., although greater variations would be expected between instruments in different laboratories.

With regard to photoelectric instruments, at first sight it would appear that a relatively high degree of accuracy can be obtained; thus sources quote limits of error ±0·1 per cent.,[12] ±0·22 per cent.,[13] ±0·4 per cent.,[14] and ±0·5 per cent.[15] It is fairly certain that a reproducibility of the order of ±0·5 per cent. can be obtained on any one instrument after a reasonable attention to detail and on a solution of a pure substance in water, i.e. where there is no likelihood of error due to lack of homogeneity. Comparisons between different instruments, however, soon show greater discrepancies as the results obtained on a single aqueous solution of potassium

nitrate and of potassium dichromate in the collaborative trials organised by the Photoelectric Spectrophotometry Group show. In addition there is still some doubt as to the correct value to be assigned to the solutions of potassium nitrate and potassium dichromate used as standard, so that it is not easy to calibrate a particular instrument which deviates from the standard at certain wavelengths.

In order to obtain results of the highest degree of accuracy, considerable attention must be paid to detail. Such factors as stray light, slit width, wavelength calibration, cell thickness, and phototube response must be studied; an extremely useful discussion of these and other factors is reported in the early Bulletins of the Photoelectric Spectrometry Group[16] and these sources should be consulted when making a detailed check of any particular instrument.

Analysis of Mixtures. If there is no interaction between the various absorbing entities in a solution containing a number of components, the total light absorption of all the components may be additive; the amounts

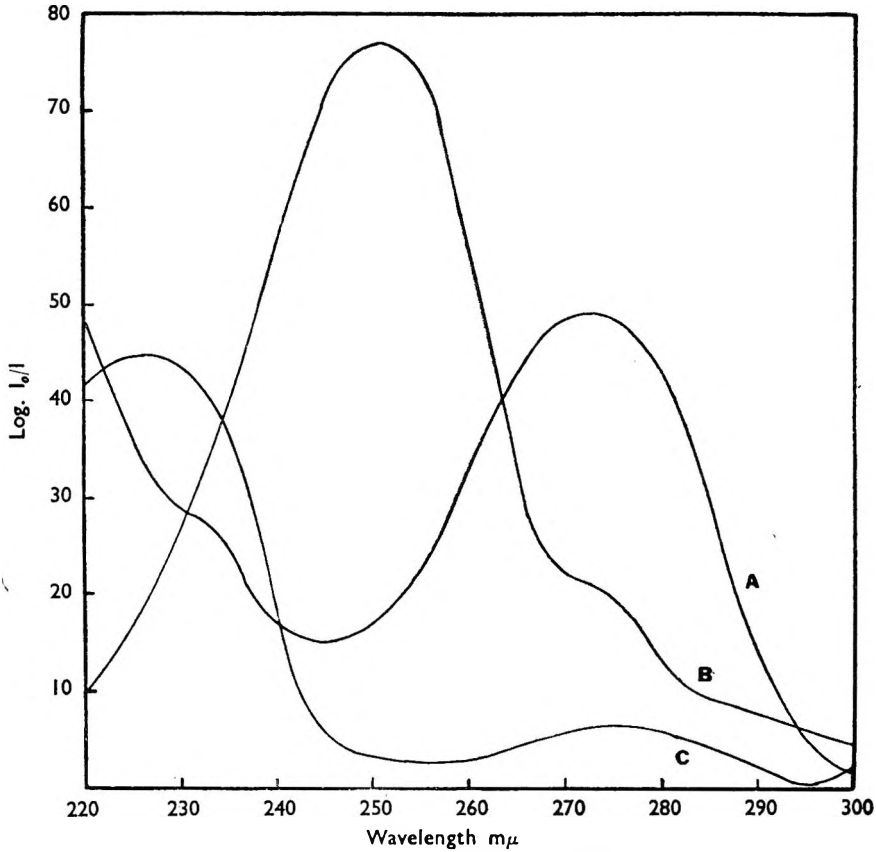


FIG. 1. Absorption spectra of aspirin, phenacetin and caffeine in ethanolic solution: A, Caffeine; B, phenacetin; C, aspirin.

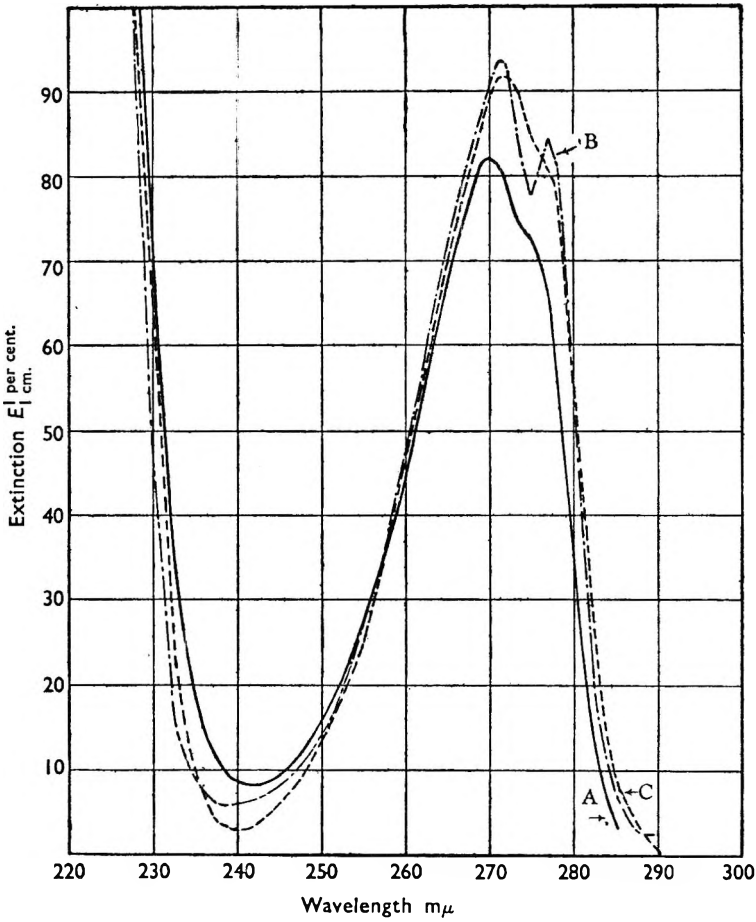


FIG. 2. Absorption spectra of mephenesin. A, in water; B, in *cyclo*hexane; C, in *iso*propanol.

of the individual members present may then be determined by the solution of a number of simultaneous equations obtained from data at varying wavelengths. In the Beer's Law equation $\log I_0/I = \epsilon.c.l.$, if a constant cell thickness is used the product $c.l.$ can be denoted by a factor a. If, for example, three materials are present the equation becomes:

$$\log I_0/I = a_1 c_1 + a_2 c_2 + a_3 c_3$$

where a_1, a_2, and a_3 are the factors for the three components, and c_1, c_2, and c_3 are the respective concentrations.

In order to perform a successful analysis of mixtures by means of such simultaneous equations, a number of requirements are necessary. (1) the data for the pure components must be known, (2) one component must absorb more strongly than the others at the particular wavelength chosen

for that component and (3) a high degree of instrumental accuracy must be obtained if the final results are to be of real value.

This method has been applied with some success to the aromatic hydrocarbons toluene, benzene, ethylbenzene and the xylenes. It can equally well be applied to mixtures of substances of pharmaceutical interest and Hernandez and Mattocks[17] have, for example, published such a method for the analysis of caffeine and sodium benzoate preparations. One of the most interesting pharmaceutical uses of this technique is in the analysis of mixtures of aspirin, phenacetin, and caffeine; Mattocks and Hernandez[18] have worked out a method depending on three simultaneous equations using ethanolic solutions and absorption measurements at the wavelengths 226, 250, and 272 mμ. The absorption curves are shown in Figure 1.

A further method available for the spectrophotometric analysis of mixtures was devised by Morton and Stubbs[19] for the determination of anthracene in petroleum oils and for vitamin A estimation. The method

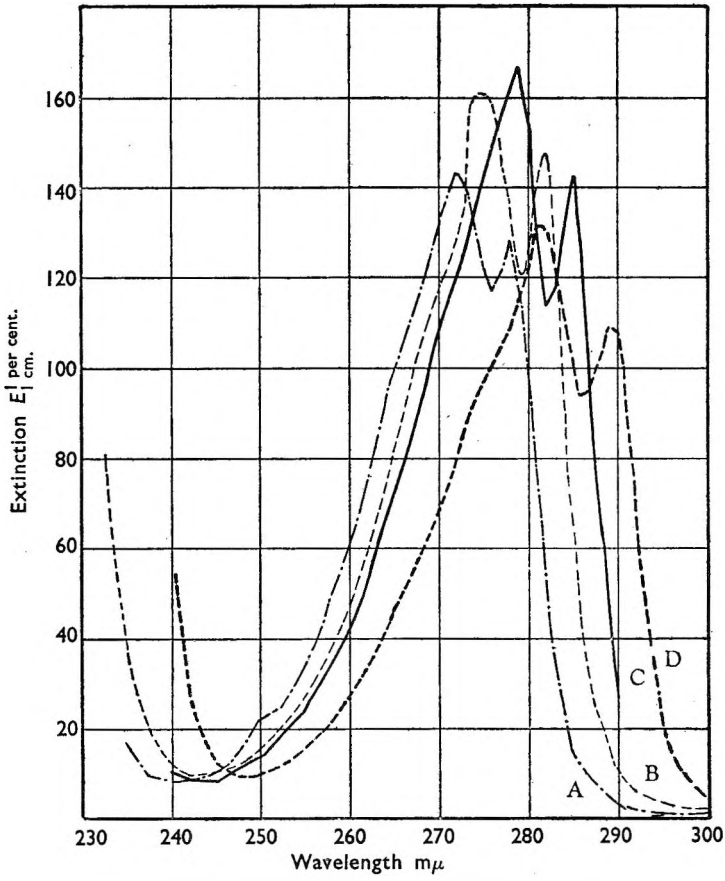


FIG. 3. Absorption spectra of phenyl ethers in *cyclo*hexane. A, *o*-cresyl ethyl ether; B, *o*-chloroanisole; C, *p*-cresyl methyl ether; D, *p*-chloroanisole.

depends on the assumption that the absorption of one component or the total absorption of the impurities is essentially linear over the relevant wavelengths. This method has been used extensively for the estimation of vitamin A and numerous papers and articles relating to its accuracy and precision have appeared.[20,21,15] For a more comprehensive study of the application of spectrophotometry to the analysis of mixtures an account by Lothian[8] can be consulted.

The Effect of *p*H and of Solvent

Various changes in ultra-violet absorption spectra are noted when a single substance is examined in different solvents. Such changes may be divided into two classes: (1) relatively minor solvent alterations presumably due to interaction between the solute and solvent molecules, and (2) fundamental changes due to ionisation.

As an example in the first class, simple phenols examined in ethanolic solution show a shift towards longer wavelenghts in comparison with the spectrum measured in a non-polar solvent such as a paraffin hydrocarbon. This applies to benzene derivatives generally and is seen in the absorption spectra of mephenesin ($\alpha\beta$-dihydroxy-γ-(2-methylphenoxy)-propane)[22] shown in Figure 2. It is interesting to compare the alterations in spectra due to solvent with the changes brought about by the introduction of different substituents in the benzene nucleus (*see* page 351) shown in Figure 3.

Ionisation causes even greater and more fundamental changes. Almost all polar substituted aromatic compounds show large shifts of absorption maxima when examined in solutions of different *p*H values; many nitrogeneous compounds in general show similar shifts. The spectra of barbituric acid, the parent compound of many hypnotic derivatives, show a variation with *p*H to a marked degree[23,24]; such changes are due to tautomerism following ionisation, the presumption being that enolisation to one of the possible tautomeric forms (I, II, III) occurs.

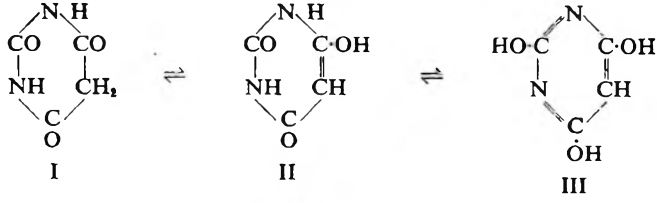


Figure 4 shows the variations in $E_{max.}$ with change in *p*H.

Pharmaceutical Applications

It is difficult to give a comprehensive account of the application of ultra-violet absorption spectrophotometry to pharmaceutical analysis. With the advent of photoelectric instruments the increase in the amount of such work published during the past decade has been phenomenal. In many research laboratories the determination of absorption spectra has

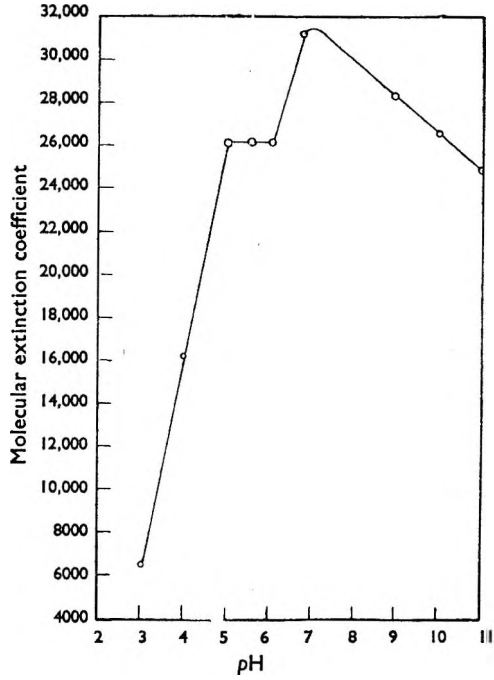


FIG. 4. Variation of absorption maximum of barbituric acid with change in *p*H. (Loofbourow and Stimpson).

become a property almost as important as melting-point for the determination of the purity of a compound and considerably more important for the elucidation and confirmation of molecular structure. Any selection of material from the mass of information available can only be arbitrary; preference will, however, be given to those compounds already in use in pharmacy, especially if they illustrate any special method of general interest in the application of ultra-violet absorption spectrophotometry.

In routine analysis ultra-violet absorption measurements are of special importance in the examination of solutions intended for injection and in the examination of tablets after the extraction of the active principle; in addition measurements of an empirical nature can also be used. The absorption characteristics can be used as a criterion of purity of the sample, e.g. calciferol, and for the determination of the pure substance in a simple solution; processes can also be worked out, as indicated above, for the analysis of mixtures of drugs found in liquid or solid pharmaceutical preparations.

In considering the various classes of compound to which ultra-violet absorption spectrophotometry can be applied the general principles given above (page 346) can be used and for any new substance a consideration of its organic structure will quickly indicate whether or not it is likely to possess selective absorption in the ultra-violet. It will be convenient,

however, from a pharmaceutical point of view to consider the applications under a number of headings as follows:

(1) Vitamins.
(2) Hormones.
(3) Antibiotics.
(4) Urea derivatives and sulphonamides.
(5) Alkaloids.
(6) Miscellaneous pharmaceutical applications.

Vitamins

Most water-soluble and oil-soluble vitamins possess chromophoric groupings and absorb light in the ultra-violet region and, in fact, for many of the vitamins spectrophotometry is the main method of assay. In some cases it can be adapted for the estimation of the vitamin in pharmaceutical products and in foods, in addition to being of great value in the determination of the purity of the parent compound. Many accounts on the subject have been written and some of the assays have been officially adopted by various organisations. Two text-books on the subject are noteworthy, one by Morton[3] summarising the position to 1942, the other published by the Association of Vitamin Chemists.[25]

Vitamin A. This vitamin has probably been the subject of more published work relating to its ultra-violet absorption than any other single compound. The method is used extensively and is now virtually the sole process for the final evaluation of vitamin A. The subject has been adequately reviewed by Morton[20] and will not therefore be covered here.

Aneurine hydrochloride. The study of the absorption spectra of degradation products of this compound materially helped in the elucidation of its structure[26] and in the subsequent synthesis. The compound shows a peak *ca.* 270 mμ in water, the addition of acid displacing the peak towards the ultra-violet and at the same time increasing the extinction, giving a value of $E_{1\text{ cm.}}^{1\text{ per cent.}} = 450$ at 247 mμ in 0·005N hydrochloric acid.[27] The property can be used for the determination of the substance in tablets (after suitable extraction) and injections.[28]

Riboflavine. Much work has been done on the spectrum of riboflavine and several authors have reported peaks at slightly differing wavelengths and intensities.[29,30,31,32] Wokes[33] in examining the position showed that the absorption curve was materially affected by the *p*H of the solution and published graphs showing in detail the alterations to be expected. Solutions of *p*H 3 were recommended for spectrophotometric estimation, such solutions exhibiting peak absorption at 223, 267, 375 and 444 mμ; if the peaks at 267, 375 and 444 mμ only were used the *p*H range of the solution could be allowed to vary between 3 and 7 since only the 223 mμ peak was greatly affected.

Other members of the vitamin B complex group also show well-defined ultra-violet absorption spectra. Nicotinic acid shows a maximum at 261·5 mμ, which is greatly affected by a change in *p*H[34]; Hughes *et al*[35] record the variations over the *p*H range 1·3 to 13 and confirm the validity of Beer's Law when applied to aqueous solutions at any definite *p*H value.

Pyridoxine shows two well-defined peaks changing in alkaline and acid solution; in 0·1N sodium hydroxide $\lambda_{max.} = 244$ and 309 mμ, in 0·1N hydrochloric acid $\lambda_{max.} = 291$ and 324 mμ. Folic acid absorbs throughout the visible and ultra-violet range and the light absorption is of value in its identification and in the estimation in injections and tablets. In 0·1N sodium hydroxide solution, i.e. as "folate" ion, the characteristic absorption shows $\lambda_{max.}$ 256 mμ $E_{1\text{ cm.}}^{1\text{ per cent.}}$ about 570, $\lambda_{max.}$ 283 mμ $E_{1\text{ cm.}}^{1\text{ per cent.}}$ about 560 and $\lambda_{max.}$ 365 mμ $E_{1\text{ cm.}}^{1\text{ per cent.}}$ about 200; these requirements are included in the monograph for folic acid in the B.P.C. 1949.

Vitamin B_{12} (*Cyanocobalamin*). For this substance the light absorption data are unique in that they form almost the only real criterion of purity for the parent compound. Following upon the isolation of the substance the absorption curve was published in 1949 by Ellis, Petrow and Snook,[36] three peaks at 278, 361 and 548 mμ being observed. Brink *et al*[37] subsequently published data on the absorption spectra and the U.S.P. XIV now uses spectrophotometric measurements for the characterisation and asay of the compound in the official monograph.

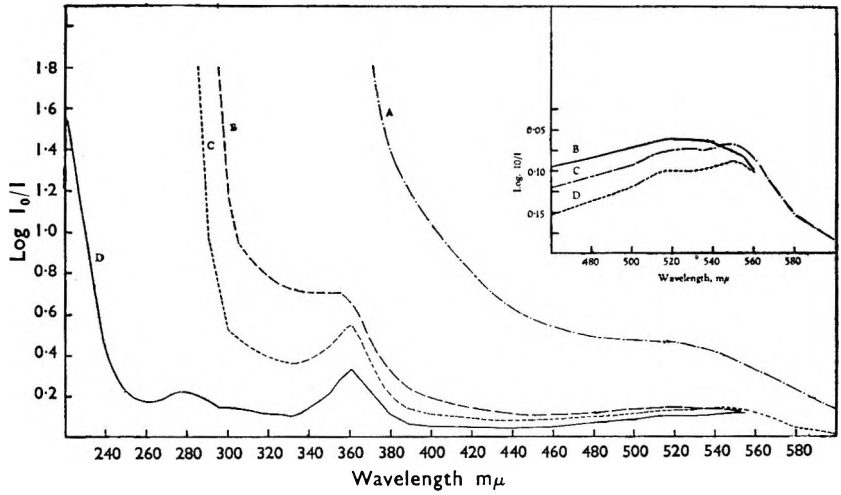


FIG. 5. Absorption spectra of vitamin B_{12} of varying purity. A, containing approximately 0·6 per cent.; B, containing approximately 2 per cent.; C, containing approximately 5 per cent.; D, pure vitamin B_{12}. The curves are adjusted so that they relate to the same total amount of vitamin B_{12} in each case.

Hartley *et al*[38] have made a study of pharmaceutical aspects of vitamin B_{12} and show clearly the manner in which the absorption spectrum is altered by the presence of impurities. Figure 5 shows the type of spectra obtained with impure samples, the curves being adjusted so that they relate to the same total amount of vitamin B_{12} in each case. Curve A represents the spectrum of the solution prepared from a concentrate containing about 0·6 per cent. of vitamin B_{12}; it shows no peak at 278, 361, or 548 mμ, since the presence of relatively highly absorbing impurities masks the characteristic vitamin B_{12} spectrum, leaving a slight shoulder only *ca.*

520 to 550 mμ. A solid concentrate containing approximately 2 per cent. of vitamin B_{12} (Curve B) begins to show the peaks at 361 and 584 mμ, while for a solid concentrate containing 5 per cent. of vitamin B_{12} (Curve C) these peaks are more apparent and the divided peak can be seen in the visible region. It is necessary to have a much purer sample before the peak at 278 mμ can be realised, and even with samples having a purity of approximately 10 per cent. the value of $E_{max.}$ at 278 mμ is relatively high when compared with the curve for pure vitamin B_{12}; the peaks at 361 and 548 mμ, however, approximate to those shown by pure vitamin B_{12}. Thus a suitable criterion exists for establishment of the purity of a sample of vitamin B_{12} in that the three peaks at 278, 361 and 548 mμ should be in a definite ratio and the U.S.P. XIV has taken advantage of this property in fixing a limiting range for the ratios:—

$E^{1\ per\ cent.}_{1\ cm.}$ 361 mμ/$E^{1\ per\ cent.}_{1\ cm.}$ 278 mμ of 1·62 to 1·88, and for
$E^{1\ per\ cent.}_{1\ cm.}$ 361 mμ/$E^{1\ per\ cent.}_{1\ cm.}$ 548 mμ of 2·83 to 3·45.

Limits for wavelength values are also specified allowing maxima within ±1 mμ at 278 and 361 mμ and within ±4 mμ at 548 mμ, allowance being made in the latter case for the broad maximum shown in the visible region. Results obtained in spectrophotometric studies of deterioration of aqueous solutions at various *p*H values indicated that a *p*H within the range 4·0 to 7·0 was necessary for maximum stability. The spectrum did not change significantly with *p*H, but decomposition occurred below *p*H 2 and above *p*H 7.

Ascorbic acid, Calciferol and α-Tocopherol. Ascorbic acid shows a peak absorption at 245 mμ in acid; solutions do not obey Beer's Law, presumably due to the change in *p*H and ionisation at relatively high concentrations. Dehydroascorbic acid does not show a similar peak and this property can be used in the determination of ascorbic acid in biological fluids, the absorption at 245 mμ being determined before and after oxidation.[39]

The light absorption characteristics of calciferol are of value in determining the purity of the compound itself, but not of great value in the analysis of pharmaceutical preparations owing to general interference shown at the relatively low peak wavelength of 265 mμ. Bacharach *et al*[40] recorded values of $E^{1\ per\ cent.}_{1\ cm.}$ 265 mμ ($\lambda_{max.}$) ranging from 460 to 500; the figure of 460 has since been adopted as a minimum limit for the purity of the substance in the B.P. 1948.

Vitamin E, α-tocopherol, shows an absorption peak at 292 mμ, which helped in determining the structure of the compound as isolated from natural sources, but has not been used to any great extent in analysis owing to the fact that it is of low intensity and does not give a satisfactory distinction from other tocopherols. The B.P.C. 1949 includes an ultraviolet absorption requirement, $E^{1\ per\ cent.}_{1\ cm.}$ 285·5 mμ of 45 to 50 in *cyclo*hexane, as a criterion of purity. Stern *et al*[41] discuss the absorption spectra of α, β, γ and δ tocopherols in considering their biological and anti-oxidant properties.

The vitamin K substitute, 2-methyl-1:4-naphthoquinone (menaphthone) and its diacetate (acetomenaphthone) both absorb strongly,[42] the

former with $\lambda_{max.}$ at 250 mμ and 331 mμ, the latter with $\lambda_{max.}$ 220 to 227 mμ 272 to 277 mμ.[43]

Rutin. This substance can be considered here as belonging to the general class of compounds having "Vitamin P-like" activity. It has been isolated from a number of plants and has been characterised by its spectrum[44,45,46,47] The hydrolysis product, quercetin, also absorbs in the ultra-violet.

The spectra of rutin and quercetin are shown in Figure 6. When dissolved in ethanol (95 per cent.) containing 1 per cent. of 0·02N acetic acid, both rutin and quercetin obey Beer's and Lambert's laws. As shown in Figure 6 rutin exhibits absorption maxima at 259 mμ and 362·5 mμ; quercetin exhibits maxima at 257 mμ and 375 mμ. The maxima at 362·5 mμ and 375 mμ are suitable for the determination of rutin and quercetin in mixtures. Owing to the similarity in the values of $\lambda_{max.}$ for rutin and quercetin, the determination of small amounts of quercetin in rutin presents some difficulty. A method devised by Porter *et al*[44,48] to overcome this difficulty uses the ratio $E^{1\ per\ cent.}_{1\ cm.}$ 375 mμ/362·5 mμ. A formula is given from which the quercetin content can be calculated assuming that the actual value for the ratio for pure rutin is 0·875.

Hormones

The many naturally occurring steroid hormones have been the subject of extensive investigation and the spectra of numerous such compounds and related steroids have been published. A review by Morton[3] gives much of the work done on this subject and although chiefly of interest from the organic structural viewpoint records many absorption spectra for hormones and related compounds. Of the more common pharmaceutical substances œstrone and œstradiol have been the subject of investigation and, more recently, the highly potent substance ethinylœstradiol; the latter possesses a phenolic grouping and the peak at 281 mμ in ethanol shifts to 300 mμ in aqueous alkali, a peak at 242·5 mμ becoming apparent, which is not realised in ethanolic solution.

An interesting method due to Hilmer and Hess,[49] based on the determination of the spectra of the 2:4-dinitrophenylhydrazones, has been applied to the determination of androsterone and testosterone. The spectra were examined in solution in 0·1N ethanolic potassium hydroxide, the hydrazine of androsterone having $\lambda_{max.}$ 430 mμ, the hydrazine of testosterone $\lambda_{max.}$ 460 mμ. By combining chromatographic methods with photoelectric spectrophotometry it was possible to separate the androgens from pharmaceutical mixtures containing œstrone and progesterone.

Progesterone has been determine by Haskins, Sherman and Allen[50] in oily solution following paper chromatography by determination of $E^{1\ per\ cent.}_{1\ cm.}$ 240 mμ correcting for oils which interfered; deoxycortone acetate and testosterone propionate could be determined similarly. Ethinyl testosterone[51] showed $\lambda_{max.}$ 238 mμ $E^{1\ per\ cent.}_{1\ cm.}$ 580.

The synthetic hormones stilbœstrol, stilbœstrol dipropionate and hexœstrol all possess absorption properties which can be applied to pharmaceutical analysis, and a useful study of the subject has been made

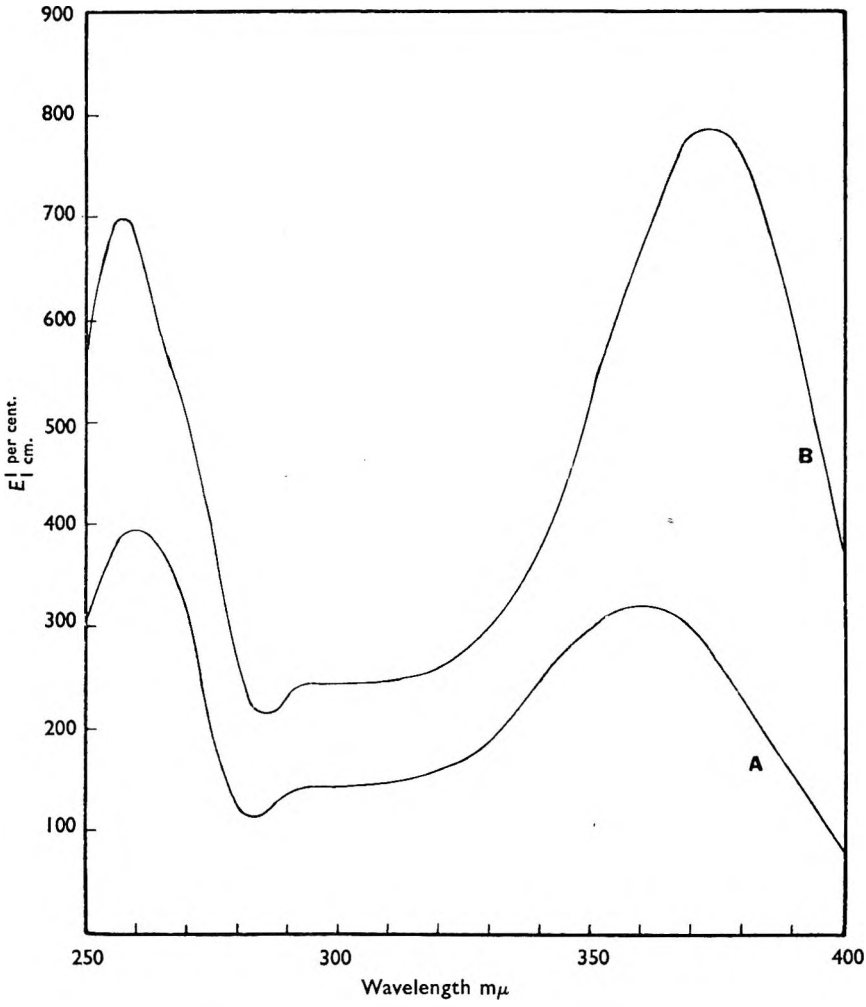


FIG. 6. Absorption spectra of rutin and quercetin in 95 per cent. ethanol containing 1 per cent. of 0·02 N acetic acid: A, rutin; B, quercetin.

by Elvidge[52] together with a report on the general determination of œstrogens in pharmaceutical preparations. Requirements for ultra-violet absorption are given in the B.P 1948 and Addendum 1951 for ethinyl œstradiol,[53] dienœstrol, ethisterone, progesterone and testosterone.

ANTIBIOTICS

Benzylpenicillin and its salts show in general smooth curves with only weak inflexions of little value for analytical purposes. Other penicillins absorb more strongly and there is comprehensive information in the literature on their absorption spectra.[54] Degradation products of penicillin possess well defined absorption spectra and spectrophotometry after

various chemical treatment has been recommended for the estimation of penicillin.

The ultra-violet absorption curves of aureomycin and terramycin[55] show multiple maxima and minima between 220 and 400 mμ. For analysis aureomycin preparations are hydrolysed by boiling with dilute sulphuric acid before measuring the absorption at 274 and 350 mμ; the aureomycin content is proportional to the difference in absorption at these two wavelengths. Terramycin can be determined similarly after hydrolysis and examination at 249 and 572 mμ. The processes have been applied satisfactorily to capsules, troches and ointments containing these antibiotics.

Chloramphenicol, the first generally used, orally active, antibiotic to be synthesised, contains a nitro-group the presence of which in the molecule was originally forecast from a consideration of its absorption spectrum. The monograph for the pure substance in the Addendum 1951 to the B.P. 1948 contains the requirement that $E^{1\text{ per cent.}}_{1\text{ cm.}}$ 278 mμ shall be between 289 and 307, this property also being of value for the determination of chloramphenicol in pharmaceutical preparations.

Urea Derivatives and Sulphonamides

Certain groups, when substituted for hydrogen attached directly to a chromophoric system show selective light absorption in the visible region of the spectrum and bring about an increase in $\lambda_{max.}$. Thus the colour of dyes is deepened and displaced towards the red by co-ordinatively unsaturated substituents in certain positions, such substituent groupings being frequently termed *auxochromes*; the group $-NR_2$ is a typical example.

In addition to any auxochromic effect due to the presence of the groups NH_2 or $-NR_2$ in the molecule the urea groupings possesses a special significance in that when suitably "activated" the tautomerism:

$$-NH-\underset{\underset{O}{\|}}{C} \rightleftharpoons -N=\underset{\underset{O^- + H^+}{|}}{C}-$$

can occur. Such tautomerism, following on ionisation, materially affects the spectrum so that in alkaline solution strongly selective absorption is often shown by urea derivatives. Another ionisation effect which influences the spectrum is the formation of substituted ammonium salts in acid solution, e.g.

$$-NR_2 + H-Cl \rightleftharpoons \underset{\underset{H^+ + Cl^-}{|}}{-NR_2}$$

Hydantoin derivatives show a shift in absorption to longer wavelengths in alkaline solution, compounds of pharmaceutical interest in this class including 5:5′-diphenylhydantoin or phenytoin.[56] The acyl ureas show a similar shift and increase in absorption, acetylurea, bromvaletone and carbromal exhibiting spectral curves which can be used for analytical purposes.

Barbituric acid derivatives[57] can be determined in solutions, tablets

and pharmaceutical preparations generally from their absorption spectra. Numerous publications have also appeared on their determination similarly in biological fluids, based on the differences between the spectra

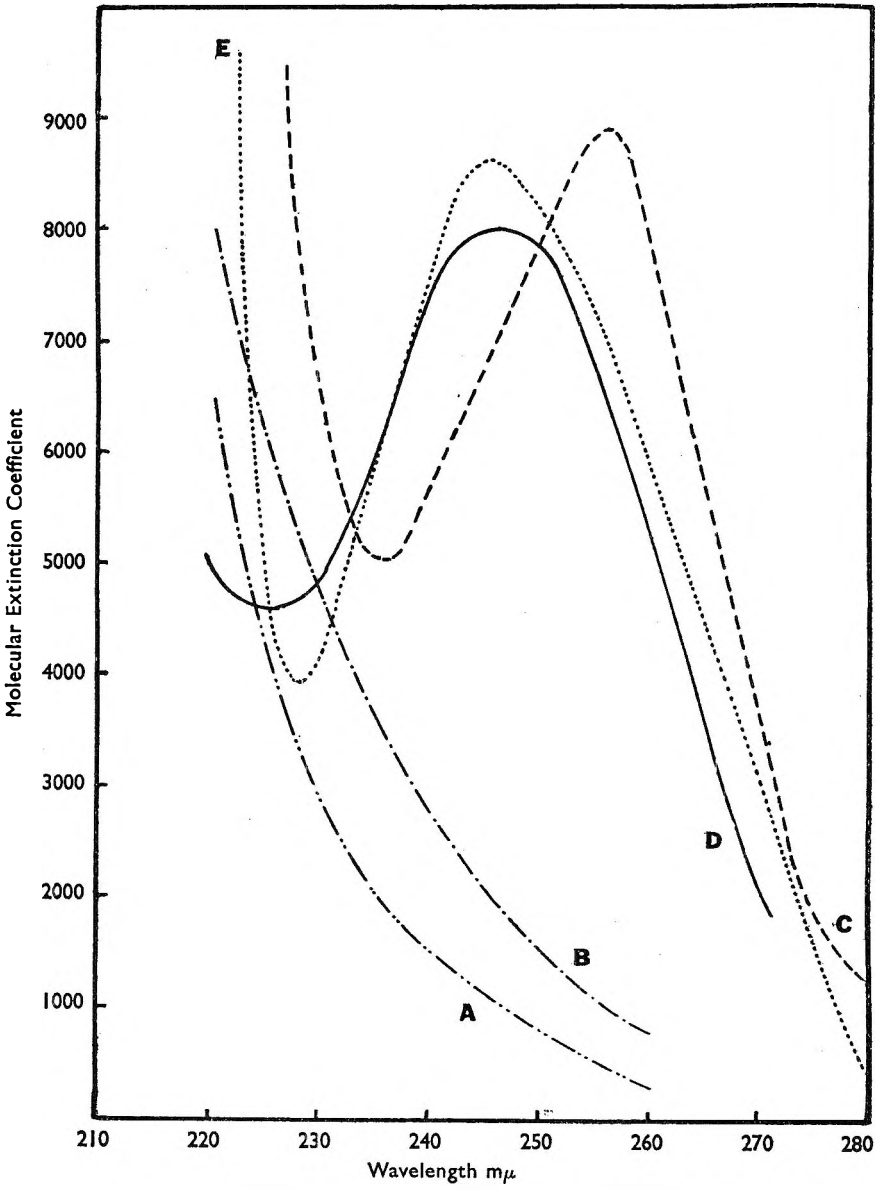


FIG. 7. Absorption spectra of barbituric acid derivatives:
A, phenobarbitone in 0·1 N hydrochloric acid;
B, phenobarbitone; 0·00025 M in water;
C, phenobarbitone in 0·1 N sodium hydroxide;
D, 5:5′-*iso*amylethyl barbituric acid in 0·1 N sodium hydroxide; E, barbitone in 0·1 N sodium hydroxide.

in acid and in alkaline solution. The ionisation occurring in alkaline solutions produces tauteromeric forms yielding a peak absorption suitable for analytical purposes. Stuckey[58] gives data for 13 derivatives which have molecular extinction coefficients (ϵ) ranging from 6500 to 9000, $\lambda_{max.} = 245$ to 253 mμ. The similarity in the peak absorption values renders this property of little use for diagnostic purposes, although the spectrum differences between acid and alkaline solutions give a useful function for the determination of any one barbituric acid derivative in mixtures (*see* Figure 7).

Numerous members of the "sulphonamide" group have been investigated in ethanol, in water and in sodium hydroxide solutions[59]; they show pronounced peaks in each solvent. Among the compounds examined were sulphanilamide, sulphapyridine, sulphathiazole and sulphacetamide; the absorption spectra are markedly altered with change in *p*H. Thiosemicarbazones, including *p*-aminobenzaldehyde and *p*-acetylaminobenzaldehyde thiosemicarbazones can be determined either separately or together by means of their absorption spectra; the estimation in biological fluids can be accomplished according to Spinks[60] by extracting with chloroform and reading the optical densities at 320 and 342 mμ, followed by the solution of simultaneous equations. *p*-Aminobenzaldehyde semicarbazone can be estimated directly by reading the optical density at $\lambda_{max.}$ 330 mμ.

Ureides with a structure similar to caffeine have been examined spectrophotometrically both with regard to their analysis and also as reference compounds in organic structural studies. Caffeine itself has been the subject of much work, although the early work of Hartley [61] is of interest only; more recent studies[62,63], in particular due to Ishler *et al*[64], have applied the absorption spectra to the determination of caffeine in crude products including coffee, interfering impurities being removed by treatment with magnesium oxide and zinc ferrocyanide plus, in some cases, permanganate oxidation; it is claimed that the method is both rapid and specific.

Alkaloids

Absorption spectra have been reported for most of the common alkaloids. The pure alkaloids and their salts usually absorb in the ultraviolet region although the intensity of absorption is not always sufficient to be of value for analysis; in few cases is the absorption so strong that it can be used without prior treatment for the determination of the alkaloid in a tincture or in a crude percolate. It is often useful, however, in the characterisation of the pure substance and in the analysis of simple aqueous solutions.

Analytical studies of cocaine and synthetic local anæsthetics have been made[65] and absorption maxima and minima have been recorded for orthocaine, benzamine hydrochloride, amydricaine hydrochloride, phenacaine hydrochloride, amylocaine hydrochloride and procaine hydrochloride. In examining the aqueous solutions of alkaloidal salts the effect of *p*H should be remembered; it is essential, in order to obtain reproducible results, that conditions prevail which will ensure complete ionisation.

For solutions of weak bases in aqueous media a strongly acid reaction is often necessary to "stabilise" the spectrum so that it is unaffected by small *p*H changes. The solanaceous alkaloids exhibit similar spectral properties with somewhat ill-defined absorption bands in the region 245 to 265 mμ; the intensity of absorption is low ($E^{1\%}_{1\,cm.}$ *ca.* 6) and the spectrum is thus of little value for analytical purposes. The ephedra alkaloids are also weakly absorbing[65] and although the spectra of the pure substances are of interest, again they are of relatively little assistance in pharmaceutical analysis.

Morphine, opium alkaloids generally, and other related alkaloids all show light absorption. The curves for morphine, codeine, and diamorphine as salts are very similar, the absorption being low in acidic solution ($E^{1\%}_{1\,cm.}$ 280 mμ *ca.* 50); morphine in alkaline solution shows a much higher absorption $E^{1\%}_{1\,cm.}$ *ca.* 310 at 258 mμ, presumably due to the phenolic group. Apomorphine absorbs more strongly in acid solution ($E^{1\%}_{1\,cm.}$ 373 mμ *ca.* 600) and this property can be used for its estimation in injection solutions and in tablets. The absorption spectra at varying *p*H values have been recorded for papaverine hydrochloride,[66] the peak value $E^{1\%}_{1\,cm.}$ 251 mμ of 1595 being very sensitive to *p*H changes; in alkaline solutions a higher value is shown, the minimum value occurring at *p*H 6·3.

Results obtained in a study of the curare alkaloids have been reported and Swann[67] has examined tubocurarine hydrochloride and dimethyltubocurarine iodide at varying *p*H values; the spectra were sufficiently different to permit the determination of the two compounds both together and in mixtures. The ergot alkaloids show well-defined spectra and ergometrine maleate can best be characterised in the pure state by its peak absorption at 312 mμ $E^{1\%}_{1\,cm.} = 183$. Emetine and its salts and strychnine and its salts[65] can also be determined spectrophotometrically. The absorption spectra of nicotine and related derivatives have been studied in some detail by Willits *et al*[68] and by Swain *et al*.[69] Vacher and Tounichon[70] identify and determine nicotine and similar compounds by treating with dilute cyanogen bromide solution followed by spectrophotometric estimation in the range 350 to 400 mμ. The alkaloids in cinchona bark were determined by Grant and Jones[71] by using the absorption at 316 mμ for quinine alkaloids and at 348 mμ for the cinchonine alkaloids, two component equations being used for calculating the final results.

Miscellaneous Pharmaceutical Applications

Absorption spectrophotometry is being increasingly applied in general pharmaceutical analysis and it is notable that in the standardisation and characterisation of new synthetic drugs, light absorption properties are now frequently specified. Thus spectrophotometric requirements have been included in the standards published by the American Medical Association for pyranisamine maleate,[72] antazoline hydrochloride,[73] benzpyrinium bromide,[74] and piperoxan hydrochloride[75]; in addition dimethyltubocurarine chloride and hydroxyamphetine hydrochloride are both assayed spectrophotometrically by measurements in the

ultra-violet. These standards reflect the growing use of direct measurements in the ultra-violet region on solutions of the substance itself, rather than the absorptiometric determination of a coloured derivative.

The examination of samples of *p*-aminosalicylic acid has presented some difficulty in that many chemical and physical methods of analysis do not differentiate between the various isomers; standards for this substance have recently been issued[76] which include requirements for the absorption spectra of a 0·0005 per cent. solution at 265 mμ ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$, 856) and 299 mμ, with minima at 244 and 285 mμ; the ratio of the optical densities, 265 mμ/299 mμ must fall between 1·50 and 1·56.

The presence of benzaldehyde as a contaminant in benzyl alcohol up to a concentration of 0·1 per cent. can be determined according to Rees and Anderson[77] by dissolving the sample in a water-methanol mixture and measuring the absorption at 283 mμ. Davidow and Woodard[78] have estimated benzene hexachloride by hydrolysis to 1:2:4-trichlorbenzene followed by the determination of the absorption at 286 mμ, using a base-line technique to avoid the interference of other substances. Dietz *et al*[79] show that the residual oil in petroleum wax can be determined from a knowledge of the ultra-violet absorption of the substances involved; in this field Lundren and Waller[80] have determined benzene and toluene in light petroleum by a similar process, the analysis of mixtures of phenols and cresols has been described by Robertson *et al.*[81] A paper dealing with medicinal liquid paraffin has appeared which makes a critical study of the BP. 1948 "acid test" by comparing it with results obtained from ultra-violet absorption measurements; it was concluded that the B.P. "acid test" does not afford a criterion of the true "quality" of liquid paraffin.[82]

The various methods available for the determination of khellin and visnagin are discussed at length by Ellenbogen *et al*,[83] who found the ultra-violet absorption method to be convenient and reproducible; solutions in *cyclo*hexane were used giving satisfactory results for mixtures of khellin and visnagin although crude extracts could not be analysed owing to interference at the relevant wavelengths. Shaw and Jefferies[84] have published the spectra of phenadoxone hydrochloride in water and ethanol and find that the extinction values, though relatively low, provide a basis for a satisfactory method of assay.

Sodium gentisate has been estimated in solution, powder, and tablets by Smith,[85] making use of the peak at 320 mμ in aqueous alkaline solutions; in this case the tablets were dissolved in water and examined directly, solvent extraction being unnecessary. The absorption of sodium heparin has been examined by Bell and Krantz,[86] who could not find any consistent relationship between light absorption properties and anti-coagulant activity.

In the field of oils, fats, and soaps the wide scope of the quantitative analytical methods available may be illustrated by the complex analysis of animal fats and soap due to Brice *et al*;[87,88] these workers use the natural absorption of the fatty acids to determine three components and then isomerise any unconjugated unsaturated acids to their conjugated ultra-violet absorbing form. The absorption is then studied in detail to

arrive at concentrations of three additional components, thus resulting in a six component analysis in steps. As an additional example, a complete analysis for saturated oleic, linoleic and linolenic acids can be carried out using absorption measurements before and after isomerisation with potassium hydroxide in ethylene glycol at 180° C., producing a band at 230 mμ characteristic of diene conjugation.[89,90,91] Linolenic acid, treated similarly, gives at 270 mμ a band due to triene absorption while saturated acids and oleic acids are unchanged; it is claimed that 0·2 g. of oil can be examined with an average percentage error of less than ±2 per cent.

Conclusion

The availability of photoelectric instruments has, during the past decade, greatly increased the use of ultra-violet absorption spectrophotometry in pharmaceutical analysis. The speed and accuracy with which absorption curves can be determined, together with the wide applicability of the method, suggests that light absorption properties will figure in many specifications and assay processes in the future; this will especially be true for many of the new organic compounds now being introduced into pharmacy.

References

1. Morton, *Practical Aspects of Absorption Spectrophotometry*, Royal Institute of Chemistry, 1938, p. 19 *et seq.*
2. Coggeshall, *Physical Chemistry of Hydrocarbons*, Academic Press, New York, 1950.
3. Morton, *The Application of Absorption Spectra to the Study of Vitamins, Hormones, and Coenzymes*, Adam Hilger, London, 1942, Chapter 2.
4. Braude, *Ann. Reports, chem. Soc.*, 1945, **42**, 105 *et seq.*
5. Lewis and Calvin, *Chem. Rev.*, 1939, **25**, 273.
6. Ferguson, *ibid.*, 1948, **43**, 385.
7. Jones, *ibid.*, 1943, **32**, 1.
8. Lothian, *Absorption Spectrophotometry*, Adam Hilger, London, 1949.
9. Brode, *Chemical Spectroscopy*, Wiley and Sons, New York, 1941.
10. Mellon, *Analytical Absorption Spectroscopy*, Wiley and Sons, New York, 1950, Chapter 6.
11. Cary and Beckman, *J. Opt. Soc. Am.*, 1941, **31**, 682.
12. Braude, *Ann. Reports chem. Soc.*, 1945, **42**, 108.
13. Rawlings and Wait, *Oil and Soap*, 1946, **23**, 83.
14. *Addendum* 1951 to the *British Pharmacopoeia* 1948, p. 93.
15. Bagnall and Stock, *J. Pharm. Pharmacol.*, 1952, **4**, 81.
16. *Photoelectric Spectrometry Group Bulletins* Nos. 1–4, April, 1949 to October, 1951.
17. Hermandez and Mattocks, *Bull. Nat. Form. Comm.*, 1951, **19**, 1.
18. Mattocks and Hermandez, *ibid.*, 1950, **18**, 113.
19. Morton and Stubbs, *Analyst*, 1946, **71**, 348.
20. Morton, *J. Pharm. Pharmacol.*, 1950, **2**, 129.
21. Cama, Collins, and Morton, *Biochem. J.*, 1951, **50**, 48.
22. Stross and Stuckey, *J. Pharm. Pharmacol.*, 1950, **2**, 549.
23. Stuckey, *Quart. J. Pharm. Pharmacol.*, 1940, **13**, 312.
24. Loofbourow and Stimson, *J. chem. Soc.*, 1940, 1275.
25. *Methods of Vitamin Assay*, Interscience Publishers, New York, 1947.
26. Morton, *The Application of Absorption Spectra to the Study of Vitamins, Hormones and Coenzymes.* Adam Hilger, London, 1942, p. 141.
27. *Third Addendum to the British Pharmacopoeia* 1932, p. 4.
28. Elvidge, *Quart. J. Pharm. Pharmacol.*, 1941, **14**, 138.
29. Kuhn, Gyorgyi, and Wagner-Jauregg, *Ber. dtsch. chem. Ges.*, 1933, **66**, 1034.
30. Warburg and Christian. *Biochem. Z.*, 1938, **298**, 164.
31. Booher, *The Vitamins*, Amer. Med. Ass., Chicago, 1939, 266.

32. Elvidge, *Quart. J. Pharm. Pharmacol.*, 1947, **20**, 263.
33. Daglish, Baxter and Wokes, *Quart. J. Pharm. Pharmacol.*, 1948, **21**, 344.
34. Hünecke, *Ber. dtsch. chem. Ges.*, 1927, **60**, 1451.
35. Hughes, Jellinek and Ambrose, *J. Phys. Colloid. Chem.*, 1949, **53**, 414.
36. Ellis, Petrow and Snook, *J. Pharm. Pharmacol.*, 1949, **1**, 60.
37. Brink *et al.*, *J. Amer. chem. Soc.*, 1949, **71**, 1854.
38. Hartley, Stross and Stuckey, *J. Pharm. Pharmacol.*, 1950, **2**, 648.
39. Johnson, *Biochem. J.*, 1936, **30**, 1430.
40. Bacharach, Alichorne and Glynn, *ibid.*, 1936, **30**, 2004.
41. Stern, Robeson, Weisler and Baxter, *J. Amer. chem. Soc.*, 1947,**69**, 869.
42. Elvidge, *Quart. J. Pharm. Pharmacol.*, 1941, **14**, 136–7.
43. Morton, *The Application of Absorption Spectra to the Study of Vitamins, Hormones, and Coenzymes*, Adam Hilger, London, 1942, p. 124.
44. Porter *et al.*, *U.S. Dept. Agr. Eastern Regional Research Lab.*, A.I.C. 159, 1947.
45. Sando and Lloyd, *J. biol. Chem.*, 1924, **58**, 737.
46. Tasaki, *Acta Phytochim (Japan)*, 1927, **3**, 259; *Chem. Zentr.*, 1927, II, 1951.
47. Tasaki, *ibid.*, 1927, **3**, 1–19.
48. Swann, *J. Pharm. Pharmacol.*, 1949, **1**, 323.
49. Hillmer and Hess, *Anal. Chem.*, 1949, **21**, 822.
50. Haskins, Sherman and Allen, *J. biol. Chem.*, 1950, **182**, 429.
51. Elvidge, *Quart. J. Pharm. Pharmacol.*, 1941, **14**, 135.
52. Elvidge, *ibid.*, 1939, **12**, 347.
53. *New and Nonofficial Remedies*, J. B. Lippincott, 1950, 577.
54. *The Chemistry of Penicillin*, Princeton University Press, 1949, p. 425.
55. Hiscox, *J. Amer. pharm. Ass., Sci. Ed.*, 1951, **40**, 251.
56. Stuckey, *J. chem. Soc.*, 1947, 331.
57. Stuckey, *Quart. J. Pharm. Pharmacol.*, 1941, **14**, 217.
58. Stuckey, *ibid.*, 1942, **15**, 377.
59. Elvidge, *ibid.*, 1941, **14**, 140.
60. Spinks, *Brit. J. Pharmacol.*, 1951, **6**, 35.
61. Hartley, *J. chem. Soc., Trans.*, 1905, **87**, 1796.
62. Gulland, Holicay and Macrae, *J. chem. Soc.*, 1934, 1639.
63. Loofbourow, Stimson and Hart, *J. Amer. chem. Soc.*, 1943, **65**,148.
64. Ishler, Finucane and Barker, *Anal. Chem.*, 1948, **20**, 1162.
65. Elvidge, *Quart. J. Pharm. Pharmacol.*, 1940, **13**, 229.
66. Foster and Macdonald, *J. Pharm. Pharmacol.*, 1951, **3**, 127.
67. Swann, *ibid.*, 1951, **3**, 843.
68. Willits, Swain, Connelly and Brice, *Anal. Chem.*, 1950, **22**, 430.
69. Swain *et al.*, *J. Amer. chem. Soc.*, 1949, **71**, 1341.
70. Vacher and Toanichon, *Bull. Soc. Chim. biol., Paris*, 1950, **31**, 1430.
71. Grant and Jones, *Anal. Chem.*, 1950, **22**, 679.
72. American Medical Association Council for Pharmacy and Chemistry, *J. Amer. med. Ass.*, 1950, **143**, 1156.
73. *ibid.*, 1950, **142**, 358.
74. *ibid.*, 1951, **145**, 487.
75. *ibid.*, 1951, **145**, 1135.
76. *ibid.*, 1950, **144**, 760.
77. Rees and Anderson, *Anal Chem.*, 1949, **21**, 989.
78. Davidow and Woodard, *J. Assoc. offic. agric. Chem. Wash.*, 1949, **32**, 751.
79. Dietz *et al.*, *Proc. Amer. Petroleum Inst.*, 1949, III, 29M, 60.
80. Lundgren and Waller, *Svensk. farm. Tidskr.*, 1950, **54**, 273.
81. Robertson *et. al.*, *Industr. Engng. Chem., Anal. Ed.*, 1946, **18**, 746.
82. Schnurmann, Martin and Maddams, *J. Pharm. Pharmacol.*, 1951, **3**, 298.
83. Ellenbogen *et. al.*, *J. Amer. pharm. Ass., Sci. Ed.*, 1951, **40**, 287.
84. Shaw and Jefferies, *J. Pharm. Pharmacol.*, 1951, **3**, 824.
85. Smith, *ibid.*, 1950, **2**, 439.
86. Bell and Krantz, *J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 95.
87. Brice and Swain, *J. Opt. Soc. Am.*, 1945, **35**, 532.
88. Brice *et al.*, *Oil and Soap*, 1945, **22**, 219.
89. Bradley and Richardson, *Industr. Engng. Chem.*, 1942, **34**, 237.
90. Mitchell *et al.*, *Industr. Engng. Chem. Anal. Ed.*, 1943, **15**, 1.
91. Beadle and Kraybill, *J. Amer. chem. Soc.*, 1944, **66**, 1232.

RESEARCH PAPERS

A STERILITY TEST FOR NEOARSPHENAMINE B.P. AND SULPHARSPHENAMINE B.P. FOR INJECTION

By G. Sykes, A. Royce and W. B. Hugo*

From the Microbiology Division, Boots Pure Drug Co., Ltd., Nottingham

with an Addendum on

THE SURVIVAL OF BACTERIAL SPORES IN ARSPHENAMINES

By C. E. Coulthard and B. H. Chantrill

From the Bacteriology Division, Boots Pure Drug Co., Ltd., Nottingham

Received March 17, 1952

THE British Pharmacopœia 1948 includes a large number of monographs on preparations for injection required to comply with tests for sterility, but this requirement has not so far been applied to neoarsphenamine or sulpharsphenamine used in making the respective injections. Neoarsphenamine and sulpharsphenamine are arsenobenzene derivatives which in most aqueous solutions are rapidly oxidised to the corresponding highly toxic arsenoxides, and it is probably on this account, and also on account of suggestions that the arsphenamines are self-sterilising, that sterility tests have been omitted. That these arsphenamines are not self-sterilising towards pathogenic bacteria is shown by Coulthard and Chantrill in the Addendum to this paper.

It is recognised that there are many difficulties in preparing batches of any compound used for injection which cannot be sterilised in its finished state. But, by applying appropriate aseptic methods during processing, bacterial infections can be adequately eliminated. Such methods are already used with a number of preparations, and they can be applied equally effectively to the arsphenamine derivatives. It is the purpose of this paper to present details of a method of testing for sterility which has proved satisfactory in detecting bacteria in various preparations of arsphenamine derivatives.

Any substance, toxic or potentially toxic to bacteria, can be tested for sterility provided it is adequately diluted in the test, or a suitable inactivating agent is included. The former method becomes impracticable, however, if dilutions of the preparation as great as 1 in 1000 or more have to be used. In the case of neoarsphenamine and sulpharsphenamine, the inhibitive concentrations to many of the commoner bacteria range between about 1 in 10,000 and 1 in 100,000 in nutrient broth, therefore, the alternative procedure of employing a suitable inactivating or neutralising agent is necessary.

Considerable work has been reported on the mode of action of arsenical drugs on living cells. As early as 1909, Ehrlich[1] theorised that these

* Present address: Department of Pharmacy, University of Nottingham.

compounds owed their toxicity to their affinity for thiol groups. Their mode of action against trypanosomes was studied by Voegtlin and colleagues in a series of papers published between 1920 and 1925. This work, summarised by Voegtlin,[2] showed that arsenobenzene derivatives were oxidised to the corresponding arsenoxides which then combined with essential thiol groups in biological systems, such compounds as glutathione (Rosenthal and Voegtlin[3]) or fixed thiol groups of proteins (Rosenthal[4]) being suggested. It also showed that arsenoxide derivatives were the biologically active ones and, furthermore, that their anti-trypanocidal activity could be removed by thiol compounds. The protective action of these compounds was confirmed by Eagle[5,6] and by Albert, Falk and Rubbo[7] in their respective studies on spirochætes and on trypanosomes and bacteria. This idea was again applied by Peters, Stocken and Thomson[8] when developing British anti-Lewisite (dimercaprol). Latterly, a large number of enzymes containing thiol groups have been described by Singer,[9] Gordon and Quastel[10] and Barron[11] the last of whom reviewed the importance of thiol compounds in biological processes generally.

It can thus be seen that arsenic interferes with some essential enzyme system containing a thiol group and that the action can be counteracted by adding to the system compounds themselves containing this group. Many such compounds have been used with greater or less success in reversing the toxic effects of arsenicals, typical examples being cysteine, thioglycollic acid, thiolactic acid, glutathione and dimercaptopropanol, and it was suggested by one of us (W. B. H.) that one of these compounds might be used in testing arsphenamines for sterility by incorporating it in the normal culture medium.

Experimental

As a result of work carried out many years ago on the sterility testing of arsphenamines considerable success was obtained in mitigating the toxicity developed, and so reducing inhibition of bacterial growth, by adding heat-coagulated muscle to the culture medium. This medium was, therefore, used in all subsequent routine batch testing. In such a meat medium thiol compounds occur naturally in variable amounts, and this could explain, at least in part, the results obtained. However, a more sensitive and reliable medium was required, and, taking into account other requisites for sterility test culture media, such as stability, ability to support growth of small inocula, availability and cost, a nutrient broth incorporating sodium thioglycollate appeared most promising. If used in sufficient concentration, this substance is inhibitory to bacterial growth, but experiments with a wide variety of organisms of the *Staphylococcus*, *Streptococcus*, *Bacillus*, *Bacterium* and *Clostridium* genera, showed that retardation of growth was apparent only in concentrations above 0·5 per cent.; hence 0·4 per cent. sodium thioglycollate was considered the maximum concentration which could be added.

In view of past experiences, two types of medium were examined: (*a*) a tryptic digest broth containing 0·4 per cent. of sodium thioglycollate,

and (*b*) the same medium with about a 1 cm. layer of heat-coagulated muscle added. They were filled in 50 ml. amounts into 4 oz. and 2 oz. screw-capped bottles, the former (with an air space above the medium) being designated for tests for ærobic bacteria and the latter for anærobic ones. The anærobic meat medium was always steamed for 30 minutes and cooled immediately before use. Although the oxidation-reduction potential of these media might appear to be held at too low a level for adequate ærobic growth, nevertheless, *Bacillus subtilis* grew very satisfactorily in both. Moreover, the anærobic organism, *Clostridium sporogenes*, grew luxuriantly in the meat medium (as might be expected), but sometimes failed to grow in the plain thioglycollate medium. The one disadvantage with this medium, namely, its susceptibility to oxidation if stored in plugged tubes, was readily overcome by preparing it in screw-capped bottles, a large air space being left in the ærobic test media. Under these conditions storage losses up to one month were negligible, but there was always a slight loss on sterilising in the autoclave usually representing about 0·05 per cent. in thioglycollic acid concentration. In all experiments reported here, freshly prepared media were used.

Sterile neoarsphenamine B.P. and sterile sulpharsphenamine B.P. (Boots Pure Drug Co., Ltd.) were used at final dilutions in the media of 1 in 100 or 1 in 200, these concentrations being found to be the most convenient in relation to the volumes of media employed and the size of dose in ampoules usually to be tested. The dilutions were made in each test in triplicate by first preparing a concentrated solution in distilled water and transferring appropriate amounts to bottles of medium as quickly as possible. These were then sown with 1 ml. of a highly attenuated dilution of a broth culture of the chosen test organism—in many cases less than 20 viable cells being inoculated per bottle, as shown by replicate plate counts. The test organisms chosen for detailed investigation were *Staphylococcus aureus* (F.D.A. strain), *Bacterium coli* (N.C.T.C. 86), *Bacterium prodigiosum*, a strain of *Bacillus subtilis*, and *Clostridium sporogenes* (N.C.T.C. 533), but similar responses were obtained with other unidentified species including a strictly ærobic spore-former and a strictly anærobic spore-former. All bottles, including controls without arsphenamines, were incubated at 37° C. for 10 days and plate counts were usually made daily; in the case of the anærobes, serial 10-fold dilutions were made in broth and the extinction point obtained.

Counts from typical experiments with neoarsphenamine at concentrations of 1 in 100 and 1 in 200 in thioglycollate broth and thioglycollate meat broth are given in Table I and illustrated in Figures 1 and 2. Results obtained with sulpharsphenamine (Table II) were not significantly different from those with neoarsphenamine. Control tubes without arsphenamine showed vigorous growths without exception within 48 hours, and at least 1×10^6 viable cells per ml. were present after 10 days incubation. It will be seen that, in general, growths from the very small inocula were initiated more readily and reached rather higher levels of viable cells in the weaker concentration of the arsphenamines. The

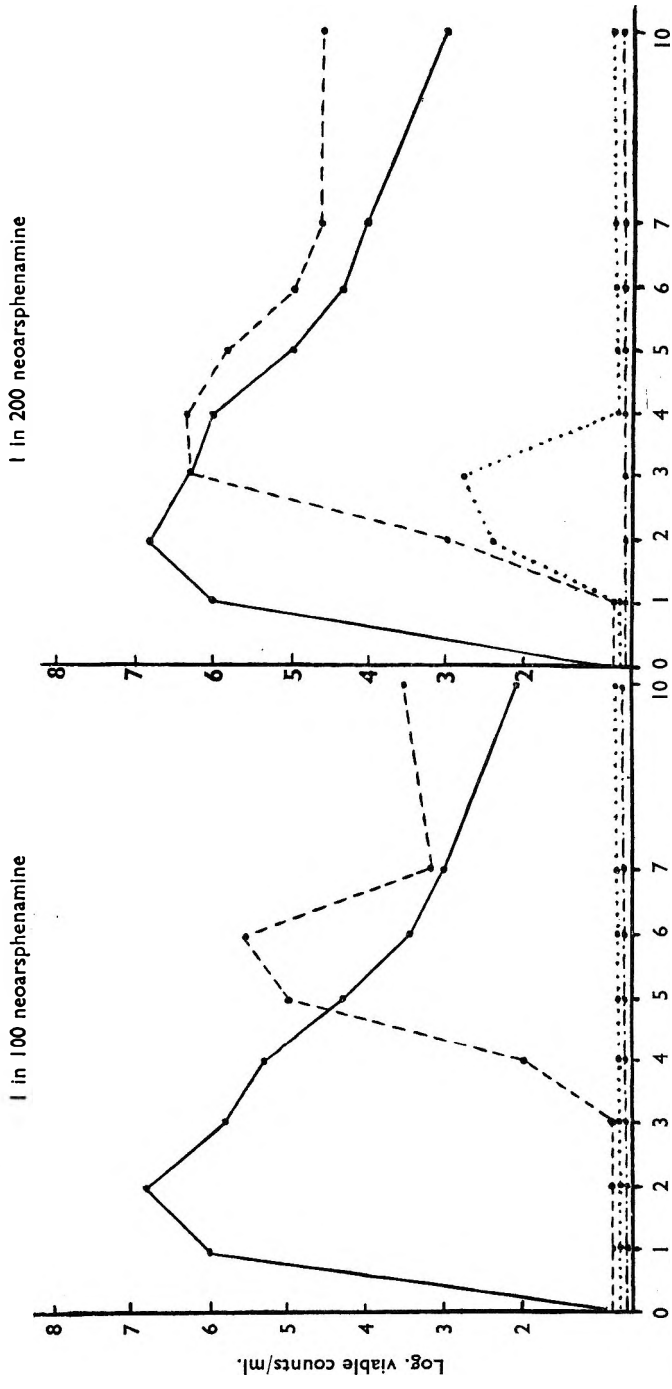


FIG. 1. Growth of bacteria with neoarsphenamine in 0·4 per cent. thioglycollate broth.

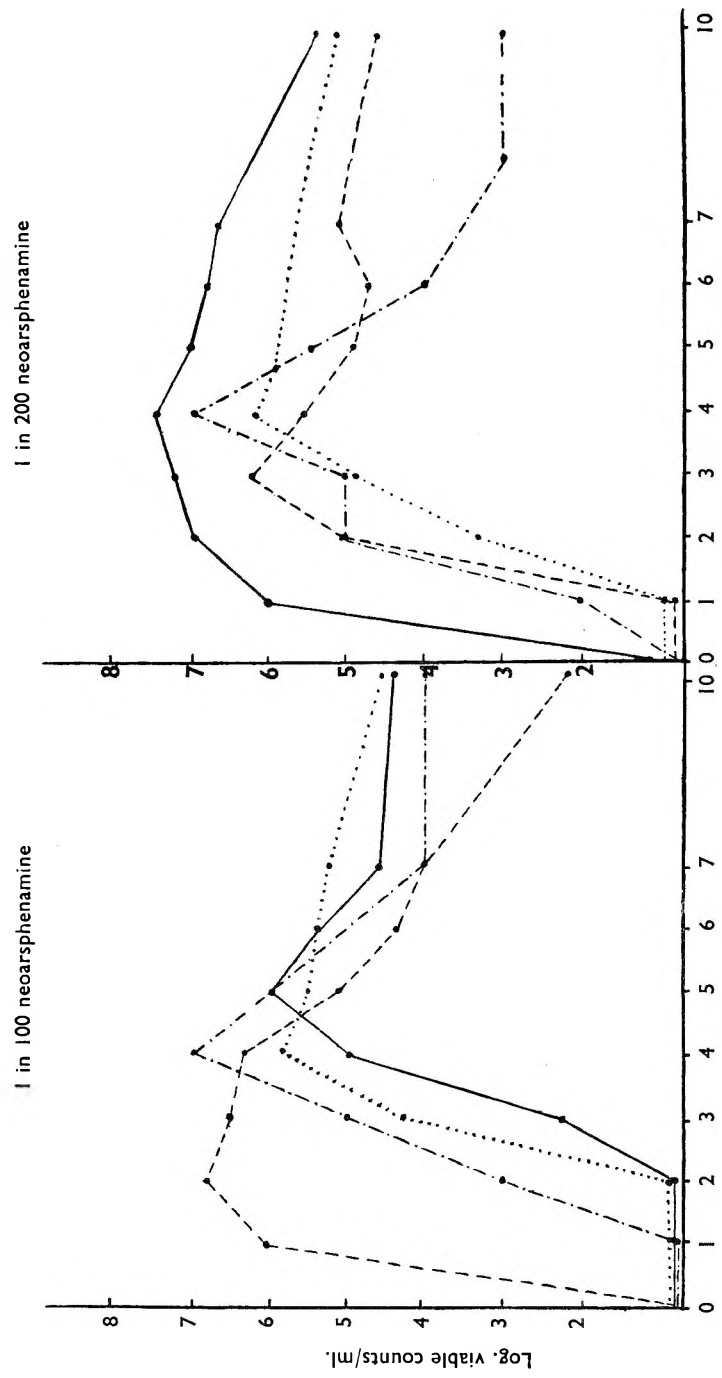


FIG. 2. Growth of bacteria with neoarsphenamine in 0·4 per cent. thioglycollate broth with meat.

TABLE I

GROWTHS OF BACTERIA IN STERILITY TEST MEDIA WITH NEOARSPHENAMINE B.P.

Concentration	Medium	Organism	Inoculum (organisms per 50-ml. bottle)	Logarithm of viable counts obtained per ml. after (days):— 1	2	3	4	5	7	10
1 in 100	Broth with 0·4 per cent. of sodium thioglycollate	*B. subtilis*	15	<2	<2	<2	2	5	3·2	3·6
		Staph. aureus	10	<2	<2	<2	<2	<2	<2	<2
		Bact. coli	8	6	6·7	6·2	5·6	4·1	2·6	2
		Bact. prodigiosum	10	6	7	6·3	5·8	4·5	2·5	<2
		Cl. sporogenes	26	<2	<2	<2	<2	<2	<2	<2
	Broth with 0·4 per cent. of sodium thioglycollate and heat-coagulated muscle	*B. subtilis*	15	<2	<2	2·5	5	6	4·3	4·3
		Staph. aureus	3	<2	<2	4·5	5·4	5·6	5·5	4·9
		Bact. coli	8	6	6·4	6·6	6·3	5·3	4·5	2·5
		Bact. prodigiosum	10	6	6·9	8	6·8	5·6	4·8	3·1
		Cl. sporogenes	20	<2	3	5	7	5	4	4
1 in 200	Broth with 0·4 per cent. of sodium thioglycollate	*B. subtilis*	15	<2	3·8	6·5	6·4	5·8	5·0	4·7
		Staph. aureus	10	<2	2·5	2·8	<2	<2	<2	<2
		Bact. coli	8	6	6·9	6·5	6·1	5·3	4·1	2·3
		Bact. prodigiosum	10	6	7	7·6	7·8	6·8	6	5·7
		Cl. sporogenes	26	<2	<2	<2	<2	<2	<2	<2
	Broth with 0·4 per cent. of sodium thioglycollate and heat-coagulated muscle	*B. subtilis*	15	<2	3·7	6·3	5·9	5·9	5·3	4·7
		Staph. aureus	3	<2	3	5	6·1	5·7	5·4	5·3
		Bact. coli	8	6	6·9	7·3	7·3	7·3	7·1	5
		Bact. prodigiosum	10	6	8	7·7	7·7	7	6	6
		Cl. sporogenes	20	2	5	5	7	4	3	<2

TABLE II

GROWTHS OF BACTERIA IN STERILITY TEST MEDIA WITH SULPHARSPHENAMINE B.P.

Concentration	Medium	Organism	Inoculum (organisms per 50-ml. bottle)	Logarithms of viable counts obtained per ml. after (days):— 1	2	3	4	5	7	10
1 in 100	Broth with 0·4 per cent. of sodium thioglycollate	*B. subtilis*	15	<2	4·6	6	5·4	5·4	5·1	2·6
		Staph. aureus	10	<2	<2	4	4·2	6·1	5·6	4·8
		Bact. coli	8	6	6·3	5	5	4	2	<2
		Bact. prodigiosum	13			5·7	7	5·6	2·3	<2
		Cl. sporogenes	26	<2	<2	<2	<2	<2	<2	<2
	Broth with 0·4 per cent. of sodium thioglycollate and heat-coagulated muscle	*B. subtilis*	15	<2	5·3	6	5·7	5·5	5·4	4·7
		Staph. aureus	3	2·3	5	6	5·7	5·3	<2	<2
		Bact. coli	8	6	6·3	5·8	5	4	<2	<2
		Bact. prodigiosum	13			6·7	7·5	7·7	6·6	5·2
		Cl. sporogenes	20	2	5	6	6	5	<2	<2
1 in 200	Broth with 0·4 per cent. of sodium thioglycollate	*B. subtilis*	15	<2	6·3	6	6·3	6	5·7	5
		Staph. aureus	10	<2	<2	5·3	7·4	6·2	5·8	4
		Bact. coli	8	6	6·8	6·8	5·5	3·5	<2	<2
		Bact. prodigiosum	13			6·7	6·9	7·6	6·9	5·2
		Cl. sporogenes	26	<2	<2	<2	<2	<2	<2	<2
	Broth with 0·4 per cent. of sodium thioglycollate and heat-coagulated muscle	*B. subtilis*	15	3·8	6·3	6	5·6	5·5	5	4·7
		Staph. aureus	3	2·3	4·3	6·7	6	5·5	4·9	5
		Bact. coli	8	6	6·9	7·3	7·3	6·5	4·7	<2
		Bact. prodigiosum	13			6·7	7·8	7·9	7·3	7·3
		Cl. sporogenes	20	2	7	7	7	5	4	4

Gram-negative organisms appeared to develop more easily than the Gram-positive ones. Maximum growths occurred between about the second and fourth days and beyond this period there was a decline in the number of viable cells. This decline was rather sharp with the higher concentration of arsphenamine. Nevertheless, easily detectable levels were present after 5 days incubation (the usual period for official tests for sterility), particularly when the meat medium was employed. Plain thioglycollate broth, whilst supporting growths of organisms in most experiments, failed to produce satisfactory growths of *Staph. aureus* in

the presence of either 1 in 100 or 1 in 200 neoarsphenamine, but was fairly good with sulpharsphenamine; it also yielded only delayed growths of *B. subtilis*. The foregoing results show that it is feasible to use a thioglycollate meat broth in testing for both ærobic and anærobic organisms. The tests could be carried out separately, the ærobic test in a container with a large volume of air above the medium, and the anærobic one in a filled bottle, steamed immediately before use; alternatively, they might be carried out simultaneously in the same container.

A Suggested Sterility Test Procedure

The following is an outline of a method for testing neoarsphenamine B.P. and sulpharsphenamine B.P. for sterility. It has been also applied to arsphenamine diglucoside B.P. and occasionally to other arsphenamine preparations.

The medium consists of a nutrient broth, made by the tryptic digest of heart muscle, with the addition of 0·4 per cent. of sodium thioglycollate (or the equivalent amount of the acid neutralised with sodium hydroxide) and a 1-cm. layer of heat-coagulated muscle. It is dispensed and sterilised in screw-capped containers, and should be relatively freshly prepared. The required amount of the test sample of the arsphenamine is dissolved in a sufficient quantity of this medium to give a final concentration not exceeding 1 in 200. The containers are incubated at 37° C. for 5 days. During incubation the medium will have become turbid, and subcultures are necessary to confirm presence or absence of growth. One loopful is inoculated into a fresh tube of the thioglycollate broth (ærobic test) or an agar-shake tube (anærobic test) and these are incubated for a further 24 to 48 hours at 37° C.

This medium has been applied successfully for many months in the detection of bacteria in routine tests for sterility of the arsphenamines.

The authors wish to express their thanks to Mr. D. V. Carter for his work on the stability of the thioglycollate media, and to Mr. C. Bowler for many hours of patient work at the bench.

Summary

1. A method is described for testing neoarsphenamine and sulpharsphenamine for sterility using a medium containing sodium thioglycollate and heat-coagulated muscle.

2. By this method very small inocula of a variety of bacteria can be detected.

References

1. Ehrlich, *Ber. dtsch. chem. Ges.*, 1909, **42**, 17.
2. Voegtlin, *Physiol. Rev.*, 1925, **5**, 63.
3. Rosenthal and Voegtlin, *J. Pharmacol.*, 1930, **39**, 347.
4. Rosenthal, *Pub. Hlth. Repts. Wash.*, 1932, **47**, 241.
5. Eagle, *J. Pharmacol.*, 1938, **64**, 364.
6. Eagle, *ibid.*, 1939, **66**, 436.
7. Albert, Falk and Rubbo, *Nature, Lond.*, 1944, **153**, 712.
8. Peters, Stocken and Thomson, *ibid.*, 1945, **156**, 616.
9. Singer, *Brewers Digest*, 1945, **20**, 85.
10. Gordon and Quastel, *Biochem. J.*, 1948, **42**, 337.
11. Barron, *Advances in Enzymology*, 1951, **11**, 201.

ADDENDUM

The Survival of Bacterial Spores in Arsphenamines

By C. E. Coulthard and B. H. Chantrill

Sterility tests carried out by us over many years have indicated that bacteria can be found in arsphenamines prepared without aseptic precautions and that they may remain viable for years in contact with the drug and under nitrogen. It seemed desirable to make some study of the virulence of such organisms and the following work was initiated for this purpose. It was obviously desirable to use an organism which would survive a drying process and which was pathogenic to small animals, and since members of the *Clostridium* group have frequently been found responsible for deaths after infection (see Coulthard and Sykes[1]) it was decided to use one of this group if possible. One difficulty with the clostridia is that some do not regularly produce spores, but after a prolonged search, we found that a *Clostridium septicum* isolated from a sheep by Mr. J. E. K. Lineham, at that time of our Veterinary Research Division, appeared to be suitable. It spored fairly readily on coagulated blood-serum and remained virulent on drying.

Experimental

The *Cl. septicum* was grown anærobically on coagulated blood-serum slopes at 37° C. for 24 hours. The growth, containing many spores, was suspended in Ringer solution and distributed in 0·2 ml. amounts into 5-ml. ampoules, which were immediately dried under vacuum at 50° C. Neoarsphenamine B.P. (Boots) 0·6 g. was then dispensed into each ampoule and these, after being filled with nitrogen, were sealed. These ampoules, half of which were stored at room temperature and half at 4° C., were tested for surviving bacteria at intervals by animal inoculation.

TABLE I

Survival of virulent bacterial spores in ampoules of neoarsphenamine under nitrogen

Test	Storage time of ampoules (weeks)	Dose of neoarsphenamine per mouse mg.	Method of sampling	Mice killed by injection from infected ampoule stored at:	
				Room temperature	4° C.
1	0	5·4	A	+	
2	0	5·0	B	+	
3	1	3·1	A	+	
4	1	4·0	A		+
5	2	5·0	B	+	
6	2	5·0	B		+
7	2	3·25	A	+	
8	2	2·26	A		+
9	14	5·0	B	+	
10	14	5·0	B		+
11	26	5·0	B	+	
12	26	5·0	B		+
13	48	?	A	+	+

A = Contents decanted and ampoule tested. B = Aliquot of entire contents tested.
Of the groups of 5 mice injected in each test, all died except in tests 1, 4, 9 and 10, where 4 out of 5 died. The presence of *Cl. septicum* was confirmed by autopsy and culture of at least one mouse from each test.

At first, to reduce the dose of neoarsphenamine, the major portion of the contents of the ampoule was decanted. The ampoule was then rinsed with 1·0 ml. of distilled water and 0·2 ml. of this injected intramuscularly into each of 5 mice. It was subsequently found possible to modify this procedure by dissolving the entire contents of the ampoule in 24 ml. of distilled water and injecting 0·2 ml. aliquots into each of 5 mice. To ensure that the deaths were not due to the toxicity of the drug a corresponding dose of sterile neoarsphenamine was injected into a control group of animals. When the first procedure was adopted this dose was calculated from the difference in the weight of the ampoule.

As a further control to make certain that the deaths were due to the multiplication of organisms and not to toxins carried over, autopsies were performed on representative mice and the presence of typical *Cl. septicum* confirmed by culture and animal inoculation.

Results

The results which are given in Table I show clearly that the dried *Cl. septicum* spores retained their virulence in contact with neoarsphenamine and under nitrogen for at least 48 weeks.

Summary

1. Spores of *Cl. septicum* in sealed ampoules of neoarsphenamine B.P. have been shown to survive and retain their virulence for at least 48 weeks.

Reference

1. Coulthard and Sykes, *Pharm. J.*, 1936, **137**, 39.

THE EFFECTS OF THIOURACIL, ALLOXAN AND NICOTINE ON THE SUPRARENAL GLANDS

By G. B. West

From the Department of Pharmacology and Therapeutics, University of St. Andrews Medical School, Dundee

Received March 17, 1952

The suprarenal medulla of most animals contains both adrenaline and noradrenaline. Although it is fairly certain that adrenaline is formed from noradrenaline by methylation, the exact way in which noradrenaline is produced in the body is still an open question. Recently, with the aid of paper chromatography, Goodall[1] showed that hydroxytyramine, in addition to adrenaline and noradrenaline, may be present in sheep suprarenal glands, and that dihydroxyphenylalanine may be found in the glands of thyroidectomised sheep. These two substances may therefore be precursors of noradrenaline. We have now examined suprarenal extracts of rabbits to which thiouracil has been given, to try to determine such precursors. We have also tested suprarenal extracts of rabbits made diabetic with alloxan, since Hökfelt[2] showed that the suprarenal glands of diabetic rats contained a very low content of adrenaline and noradrenaline. If suprarenal exhaustion occurs in this condition, it might be possible to detect precursors of adrenaline in the suprarenal gland.

Injections of nicotine over long periods (13 to 17 months) may produce chromaffin adenoma in the suprarenals of young rats and suprarenal hyperplasia in adults.[3] In rabbits, continued administration of nicotine (97 to 124 days) has been shown to decrease and then to increase the adrenaline content of the suprarenal glands.[4] Since this treatment may also be associated with changes in the concentrations of precursors of adrenaline, we have given daily doses of nicotine to rabbits, rats and guinea-pigs. After a suitable interval of time, we have examined extracts of their suprarenal glands. In some experiments, daily doses of methionine (a methyl donor) have also been given. It was expected that the methionine-treated animals would possess a more rapid rate of methylation in the suprarenals than that found in the other groups.

Methods

Rabbits, guinea-pigs and rats were killed by a blow on the head and their suprarenal glands were removed as soon as possible. After removal of the capsule, the glands were weighed and ground with sand and 1 to 5 ml. of 0·01 N hydrochloric acid/g. The extracts were centrifuged and the clear supernatant liquids were assayed for their adrenaline and noradrenaline contents by paper chromatography and biological assay (see Shepherd and West[5]). Careful examination of the paper chromatograms was carried out in every case to detect hydroxytyramine and dihydroxyphenylalanine.

Subcutaneous injections of thiouracil (in slightly alkaline solution)

were given daily (except Sundays) to 6 rabbits. Other rabbits received alloxan (100 mg./kg. intravenously) after food had been withdrawn for 48 hours. For the nicotine experiments, groups of 2 rabbits, rats or guinea-pigs were injected daily (except Sundays) with varying doses of nicotine acid tartrate. After a certain number of subcutaneous injections, they were killed either 6 hours or 7 days after the last injection. In other experiments, nicotine and methionine were given to groups of rabbits and rats. Solutions of *l*-adrenaline, *l*-noradrenaline bitartrate, hydroxytyramine hydrochloride, and *dl*-dihydroxyphenylalanine in 0·01 N hydrochloric acid were used as controls.

RESULTS

Effect of thiouracil in the rabbit. After 15 doses of thiouracil (50 mg./kg.), the mean value of extracts from 2 rabbits was 505 μg. of adrenaline/g., noradrenaline being absent. No major change has

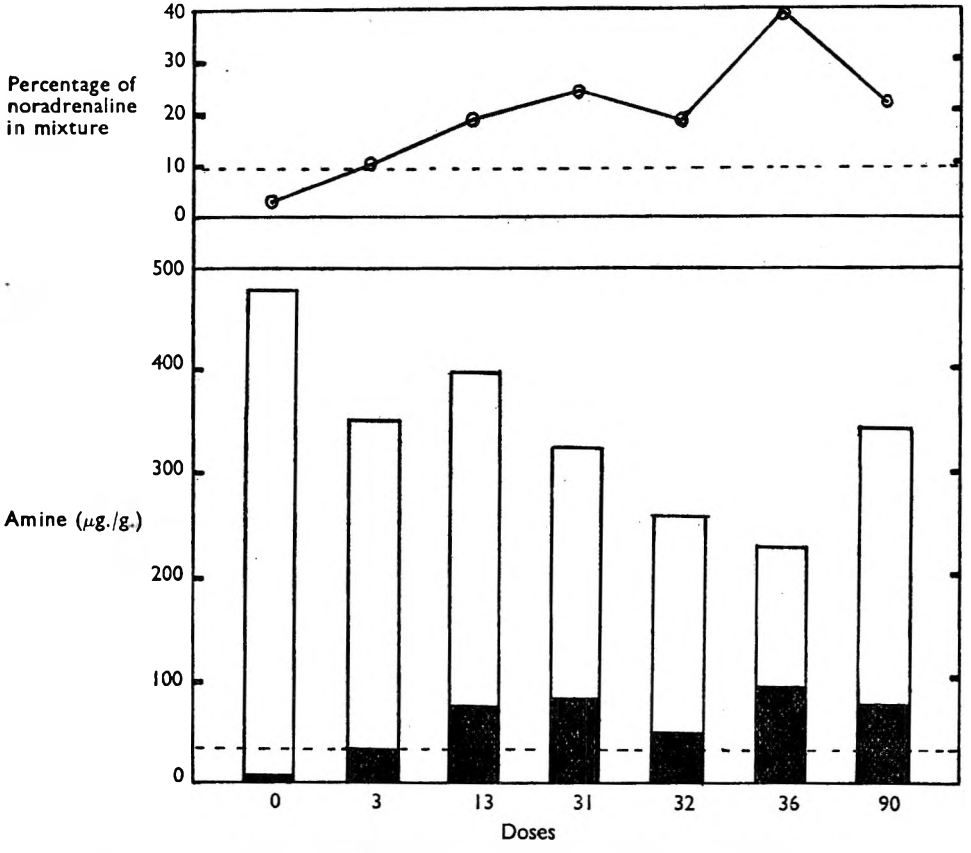


FIG. 1. The effect of daily doses of nicotine acid tartrate (16 mg./kg.) on the amine content (lower histogram) and the relative noradrenaline content (upper graph) of the suprarenal glands of rabbits. Plain areas, adrenaline; shaded areas, noradrenaline. The broken lines represent the maximal absolute and relative noradrenaline contents of the glands from 56 control rabbits.

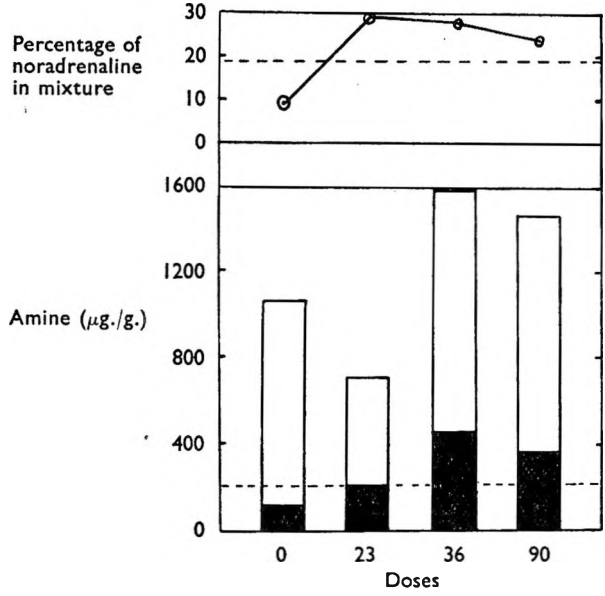


FIG. 2. The effect of daily doses of nicotine acid tartrate (2 mg./kg.) on the amine content (lower histogram) and the relative noradrenaline content (upper graph) of the suprarenal glands of rats. Plain areas, adrenaline; shaded areas, noradrenaline. The broken lines represent the maximal absolute and relative noradrenaline contents of the glands from 24 control rats.

occurred therefore in the amine content of their suprarenal glands, since the mean values of extracts from 56 adult control animals are 470 μg. of adrenaline and 10 μg. of noradrenaline/g. (Shepherd and West[5]).

2 other rabbits received 40 doses of thiouracil and lost weight (3·2 to 2·8 kg.). Their suprarenal glands appeared to be partly exhausted, values of 150 μg. of adrenaline and 2 μg. of noradrenaline/g. being recorded. Another 2 rabbits received 40 doses of thiouracil followed by insulin (4 I.U./kg. subcutaneously). 3 hours after the insulin, they were killed. Apart from further suprarenal exhaustion (mean value of 50 μg. of adrenaline/g.), no precursors of adrenaline were detected.

Effect of alloxan in the rabbit. When diabetes was confirmed in these animals, they were killed (about 10 days after the dose of alloxan). Suprarenal exhaustion had occurred (mean value of 61 μg. of adrenaline/g.) but again no precursors of adrenaline were detected.

Continued administration of nicotine. Rabbits received daily subcutaneous doses of 16 mg./kg. and the results are shown in Figure 1. Since the maximal noradrenaline content of the suprarenal glands from 56 control rabbits is 35 μg./g. (shown by the dotted line), it is clear that even after 13 doses of nicotine the noradrenaline values are raised, whilst total activity figures are somewhat decreased. Despite this increase in the relative noradrenaline content of the suprarenal gland, hydroxytyramine or dihydroxyphenylalanine were not identified. The rabbits

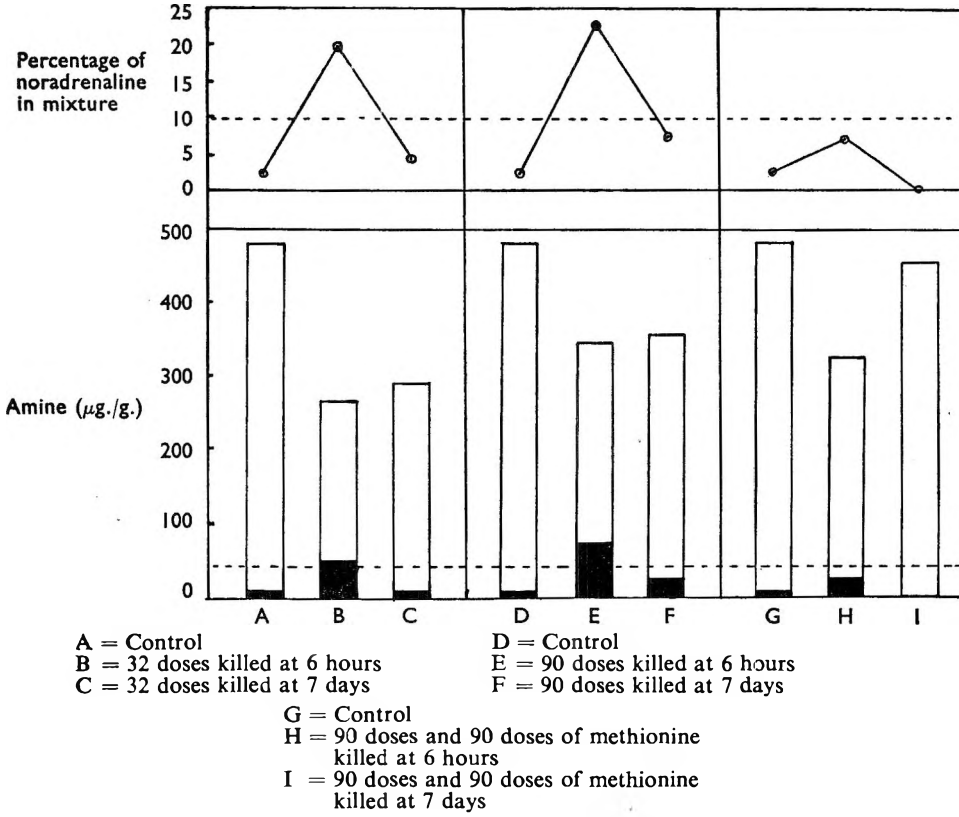


FIG. 3. The effect of daily doses of nicotine acid tartrate (16 mg./kg.) on the amine content (lower histogram) and the relative noradrenaline content (upper graph) of the suprarenal glands of rabbits. Plain areas, adrenaline; shaded areas, noradrenaline. The broken lines represent the maximal absolute and relative noradrenaline contents of the glands from 56 control rabbits. Also shown is the effect of 90 doses of nicotine and methionine (24 mg./kg.) on the amine content of the suprarenal glands of rabbits. Some animals killed 6 hours after the last injection, others left for 7 days. Note the protective action of methionine.

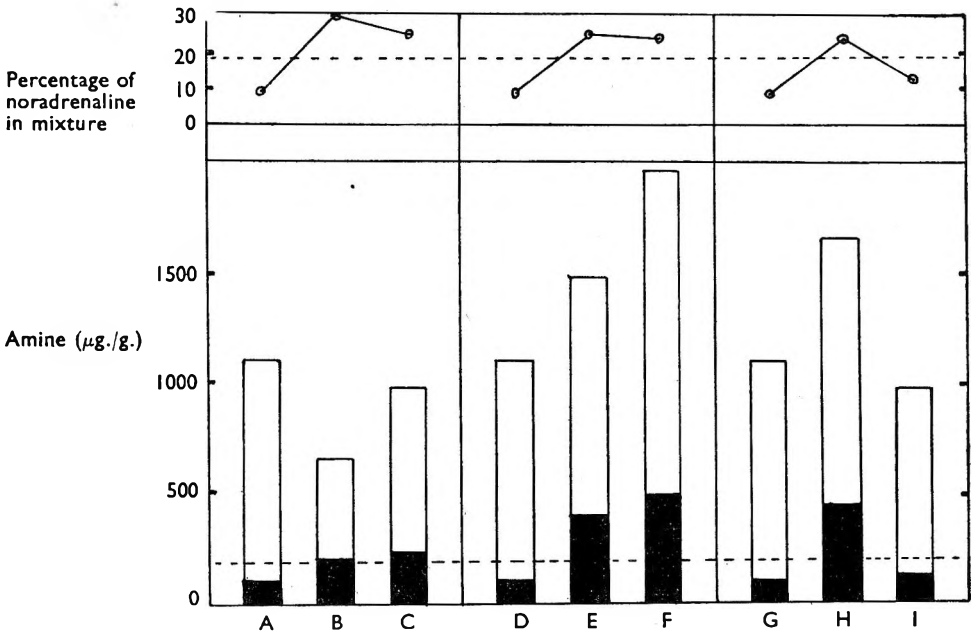
lost weight during the first 2 or 3 weeks of treatment, but then steadily gained. After each nicotine dose, respiration was stimulated and there was loss of power in the hind-limbs.

Rats received daily subcutaneous doses of 2 mg./kg. and the results are shown in Figure 2. With 23 doses there is a decrease in total activity, but after further doses there is an increase. As in the rabbit experiments, the increase in relative noradrenaline content was not accompanied by the appearance of detectable amounts of other precursors. Stimulation of the central nervous system (tail erection, circular movement sometimes backwards) was usually noted after each nicotine dose. Respiration was depressed, probably due to depression of the respiratory centre.

Guinea-pigs received 36 daily subcutaneous doses of 8 mg./kg. When killed, their suprarenal glands contained only adrenaline, and total activity values were low (mean of 50 μg./g.) compared with the mean

value of 60 control guinea-pigs (122·5 μg. adrenaline and 2·5 μg. noradrenaline/g. tissue). General depression was noted in these animals after each nicotine dose.

Continued administration of nicotine and methionine. In Figure 3 are shown the effects of 32 and 90 doses of nicotine (16 mg./kg.) and of 90 doses of nicotine and methionine (24 mg./kg.) on the amine content of the suprarenal glands of rabbits. The total activity was decreased in all three experiments, but whereas the relative noradrenaline content was increased following nicotine dosage it was not significantly increased when nicotine and methionine were given together. Therefore the depressant effect of nicotine on the rate of methylation in the rabbit may be reduced by the administration of excess of methionine (that is, by giving a methyl donor). In the rat, on the other hand (Fig. 4), it is clear that the rate of methylation is decreased even in the presence of excess of methionine (3 mg./kg.).



A = Control
B = 23 doses killed at 6 hours
C = 23 doses killed at 7 days
D = Control
E = 90 doses killed at 6 hours
F = 90 doses killed at 7 days
G = Control
H = 90 doses and 90 doses of methionine killed at 6 hours
I = 90 doses and 90 doses of methionine killed at 7 days

FIG. 4. The effect of daily doses of nicotine acid tartrate (2 mg./kg.) on the amine content (lower histogram) and the relative noradrenaline content (upper graph) of the suprarenal glands of rats. Plain areas, adrenaline; shaded areas, noradrenaline. The broken lines represent the maximal absolute and relative noradrenaline contents of the glands from 24 control rats. Also shown is the effect of 90 doses of nicotine and methionine (3 mg./kg) on the amine content of the suprarenal glands of rats. Some animals killed 6 hours after the last injection, others left for 7 days. Note the rapid return to normal levels in the presence of excess of methionine.

Also shown in Figures 3 and 4 are the results of leaving some treated animals for 7 days after the last injection before testing their suprarenal extracts. In rabbits receiving only nicotine, recovery of the rate of methylation occurred during this period although total activity remained low. In rabbits receiving both nicotine and methionine, recovery of both the rate of methylation and the total activity rapidly took place. In rats receiving 23 doses of nicotine, recovery was slow in this period, little change being recorded in the relative noradrenaline content of the glands. After 90 doses, however, there was a large increase in the total activity with little or no change in the raised relative noradrenaline content. In rats receiving both nicotine and methionine, recovery of both total activity and rate of methylation was almost complete in this period. Methionine therefore speeded up the recovery phase in the glands of both rabbits and rats.

Histological examination. Examination of frozen sections stained with hæmatoxylin and eosin, Heidenhain's iron hæmatoxylin, Mallory's stain, or acid fuschin and methyl green, indicated that only minor changes had occurred within the suprarenals. Reduction in the amount of stainable material in the medulla was particularly noticeable in the nicotine-treated animals. No neoplastic tissue was visible in the medulla.

Discussion

These experiments were designed to detect precursors of adrenaline by influencing the normal production of adrenaline in the suprarenal medulla. However, only noradrenaline has been found and then not in every extract. Since the limit for the detection of hydroxytyramine and dihydroxyphenylalanine by paper chromatography is 5 μg./g., less than 1 per cent. of the total catechols could be present as these substances.

In rabbits receiving thiouracil for 40 days, the total activity of the glands was reduced to one-third of the control value without affecting the relative noradrenaline content. Subsequent treatment with insulin produced further suprarenal exhaustion with little or no change in the relative amounts of the amines present. Therefore, the normal production of adrenaline in the medulla has not been altered by this thiouracil treatment. A similar finding was obtained with diabetic rabbits so that hypothyroidism and diabetes apparently do not influence the rate of methylation in the suprarenal medulla of rabbits.

The influence of nicotine is of considerable importance because of the great prevalence of smoking. The general response of the organism to nicotine is in part due to adrenaline, the output of which is increased soon after the injection and remains at a high level for several minutes. Since tumours of the suprarenal gland have been reported in the literature following nicotine administration over long periods, it was of interest to see the effects in the rabbit whose gland contains almost entirely adrenaline. Besides possible tumour formation, there seemed every possibility that more than one precursor of adrenaline would be identified. However, 90 doses of nicotine did not produce drastic changes. A steady decrease in total catechol activity was noted in rabbits, whereas the

decrease was followed by an increase in rats; the relative noradrenaline content increased in both rats and rabbits but no other precursor appeared. Methionine had little effect on the action of nicotine in rats but did allow of faster methylation in rabbits. When doses were stopped and the animals were left for 7 days, the methionine-treated animals recovered quicker than the controls, i.e. methionine accelerated the rate of methylation in animals whose suprarenals had been partly exhausted or stimulated. Previous workers have already shown that the methyl group of methionine given to rats may be found as the $N\text{-}CH_3$ groups of adrenaline isolated from the adrenal gland (Keller, Boissonas and du Vigneaud[6]).

Summary

1. If thiouracil or alloxan is given to rabbits, suprarenal exhaustion occurs but the relative noradrenaline content of the gland is not raised.

2. The administration of nicotine to rats and rabbits for periods up to 90 days results in a lowered rate of methylation of noradrenaline. Simultaneous dosage of methionine provides some protection. In the recovery phase following the injections, the methionine-treated animals possess a more rapid rate of methylation in the suprarenals than that of the control animals.

3. In no experiments were dihydroxyphenylalanine or hydroxytyramine detected in adrenal extracts by the chromatographic method.

I wish to record my thanks to Dr. D. M. Shepherd of this Department for most of the chromatographic studies, and to Dr. A. T. McQueen of the Physiology Department, University College, Dundee, for supplying the glands of alloxan-treated rabbits. Professor A. Haddow of the Chester Beatty Cancer Research Institute supplied the reference to work on experimental adrenal medullary tumours.

References

1. Goodall, *Acta Physiol. Scand.*, 1951, **24**, Suppl. 85.
2. Hökfelt, *ibid.*, 1951, **25**, Suppl. 92.
3. Staemmler, *Virchows Archiv.*, 1935, **295**, 365.
4. Kobayashi, *Fol. Pharmacol. Japon.*, 1937, **23**, 314.
5. Shepherd and West, *Brit. J. Pharmacol.*, 1951, **6**, 665.
6. Keller, Boissonas and du Vigneaud, *J. biol. Chem.*, 1950, **183**, 627.

THE PREPARATION AND TESTING OF THE PROVISIONAL BRITISH STANDARD FOR GLOBIN ZINC INSULIN

From The British Insulin Manufacturers' Biological Sub-Committee and The Department of Biological Standards, National Institute for Medical Research

Received March 25, 1952

The investigation described in this paper was planned and the experimental work carried out by Dr. C. W. Emmens, Dr. J. A. B. Gray, Dr. A. A. Miles and Dr. W. L. M. Perry representing the National Institute for Medical Research, and Mr. S. S. Adams, Mr. W. A. Broom, Miss J. M. Lesford, Mr. H. R. Rowlinson, Mr. K. L. Smith, Mr. G. A. Stewart, Dr. R. H. Thorp, Dr. S. W. F. Underhill, Dr. A. C. White, Mr. G. F. H. Whitney and Mr. P. A. Young representing the British Insulin Manufacturers (Allen and Hanburys Ltd., The British Drug Houses Ltd., Boots Pure Drug Co. Ltd., and Burroughs Wellcome and Co. (The Wellcome Foundation Ltd.))

INTRODUCTION

From the time of the introduction of globin zinc insulin in 1939, and up to the date of issue of the Provisional British Standard for Globin Zinc Insulin on January 1st, 1949, the potency of batches of globin zinc insulin was measured in terms of either soluble insulin or of protamine zinc insulin by their action on the blood sugar level in experimental animals. The measurement of potency of one kind of preparation of a biologically active substance in terms of another kind of preparation of the same substance, is in general undesirable in the light of the requirements for valid biological assay. It cannot be assumed that insulin bound in a complex with globin for the express purpose of altering its rate of action, will always have the same effect relative to other preparations of insulin in the test animal.

For this reason, the British Insulin Manufacturers' Biological Sub-Committee, in collaboration with the Department of Biological Standards of the National Institute for Medical Research, have established a standard preparation of globin zinc insulin for use in the assay of globin zinc insulin. This has been prepared by the British Insulin Manufacturers, distributed in ampoules in the freeze-dried state and presented to the Medical Research Council for custody in the Department of Biological Standards. The preparation has been designated The Provisional British Standard for Globin Zinc Insulin. In this connection it must be emphasised that the qualification "Provisional" does not imply any doubt about the suitability of the preparation as such. The standard was established provisionally solely to avoid unduly prejudicing any action that may in the future be taken to establish an International Standard for the substance.

Before a freeze-dried globin zinc insulin preparation could be adopted as a standard it was important to show that the freeze-drying and subsequent reconstitution had produced no changes in its properties.

It was felt that this point could be established by showing:—

1. That the potency was unchanged by freeze-drying and reconstitution and did not vary from ampoule to ampoule.
2. That the ratio of potencies as determined respectively by the rabbit and mouse assays was unchanged by these processes.
3. That the shape of the rabbit blood sugar curve before and after freeze-drying and reconstitution was unchanged.

Before the freeze-dried globin zinc insulin preparation could be used as a standard it was essential to show also that it was sterile and was sufficiently stable when stored under reasonable conditions.

I. Preparation and Properties of the Standard

Method of Preparation

The British standard for globin zinc insulin was prepared by freeze-drying a solution of globin zinc insulin made from a concentrated solution of crystalline insulin from ox pancreas. Globin and zinc chloride were added to bring the concentration of globin to 3·8 mg. and of zinc to 0·3 mg. for each 100 units of insulin. To this solution *o*-cresol was added to give a final concentration of 0·2 per cent. The *p*H of this solution before freeze-drying was 3·1.

The potency of the concentrated insulin solution used in the preparation of the original globin zinc insulin solution was not determined directly. The rest of this same concentrated solution was, however, by simple dilution converted into 9 commercial batches of insulin which were separately assayed by the mouse convulsion method. The results obtained in these assays are given in Table I. From them it can be calculated that the amount of soluble insulin converted to globin zinc insulin and present in each ml. of the original globin zinc insulin solution was 39·73 I.U. with limits of error (P = 0·95) from 38·45 to 41·05 I.U. This result did not differ significantly from the intended value of 40 I.U./ml. which was therefore accepted as the concentration of soluble insulin present and converted to globin zinc insulin.

The globin zinc insulin solution prepared as above was freeze-dried

TABLE I

Potency assays by the mouse convulsion method of commercial batches prepared from the same concentrated solution as the globin zinc insulin standard

Dilution No.	Labelled Potency I.U./ml.	Assayed Potency I.U./ml.	Log Potency	Weight = Reciprocal of Variance
1	40	41·1	1·6135	4075
2	40	38·7	1·5877	2469
3	40	41·6	1·6194	1802
4	20	20·9	1·3211	1239
5	80	78·8	1·8964	1295
6	20	19·0	1·2785	1419
7	20	19·7	1·2943	1570
8	20	19·5	1·2904	1827
9	20	19·2	1·2839	3257

and the solid material distributed in ampoules each containing approximately 40 mg.

A quantity of the globin zinc insulin solution before freeze-drying, termed hereafter the "original liquid," was set aside in order that the freeze-dried material, when reconstituted, could be assayed in terms of the former to ascertain whether a loss in potency had occurred during the freeze-drying process.

Calculated Potency from Weight Yield

Assuming no loss of potency on freeze-drying, the potency of the freeze-dried material could be calculated from the weight of freeze-dried globin zinc insulin obtained from a given volume of the original globin zinc insulin solution. This was determined in the following manner.

10 weighing bottles were packed with strips of absorbent paper and dried *in vacuo* over silica gel to constant weight. 8 of the bottles were then taken and 10 ml. of the original liquid was delivered into each. The 10 bottles were then redried *in vacuo* at 20° C. until the 8 bottles had attained constant weight and the 2 remaining bottles were the same weight as at the commencement of the experiment. This latter precaution was taken to ensure that a similar state of dryness was obtained at the end as at the beginning of the experiment.

The weight obtained from 10 ml. (400 units) of liquid globin zinc insulin was 44·02 mg. Therefore, the potency of the anhydrous material, assuming no potency loss was 400·0/44·02 = 9·09 U./mg.

To obtain the potency of the standard from this figure it was necessary to know its moisture content. This was determined by drying the contents of 4 ampoules of the standard preparation to constant weight in the desiccator used in the above experiment. Before drying the weight was 167·1 mg. and the loss in weight due to moisture 7·4 mg. The British standard for globin zinc insulin therefore has a moisture content of 4·43 per cent. Its potency calculated from that of the anhydrous material by correcting for the moisture content of the former was therefore

$$\frac{9{\cdot}09\ (100{\cdot}0 - 4{\cdot}43)}{100} = 8{\cdot}69\ \text{U./mg.}$$

II. Proof of the Identity of the Original Liquid and the Reconstituted Freeze-dried solid

As explained in the introduction a highly accurate determination of the identity of the original liquid and the reconstituted freeze-dried solid was absolutely necessary to the successful assignment of a potency to the British standard for globin zinc insulin. The present section describes the tests undertaken to prove this. The assays were carried out on both rabbits and mice.

Rabbit Assays

The rabbit assays were planned to a design which consisted of three triplet cross-over tests embodied in a 6×6 Latin Square. Similar designs have been discussed by Emmens[2] and Smith[3]. An example of one such square is shown in Table II.

TABLE II

6 × 6 LATIN SQUARE DESIGN CONSISTING OF 3 TRIPLET CROSS-OVER TESTS

	Day					
Group	1	2	3	4	5	6
1	T_3	S_1	S_3	T_2	T_1	S_2
2	S_1	T_2	T_3	S_2	S_3	T_1
3	T_1	S_2	S_1	T_3	S_2	T_2
4	S_3	T_1	T_2	S_1	T_2	S_2
5	T_2	S_3	T_1	S_3	T_3	S_1
6	S_2	T_3	S_3	T_1	S_1	T_3

S_1 = low dose—original liquid
S_2 = medium dose—original liquid
S_3 = high dose—original liquid
T_1 = low dose—freeze-dried reconstituted material
T_2 = medium dose—freeze-dried reconstituted material
T_3 = high dose—freeze-dried reconstituted material

The rows represent groups of rabbits, 3 or 4 animals being used per group, and the columns represent the different days of the test. The 6 treatments are provided by the 3 doses of original liquid and 3 doses of reconstituted material. In addition to the Latin Square, however, each pair of 2 days provides a complete triplet cross-over test in its own right. This design makes for flexibility in practice, since if anything occurs to invalidate the results on one particular day, the whole of the experiment is not rendered useless, as 2 complete cross-over tests can still be analysed; in addition, the analysis of the results can be carried out in both ways and the method having the smaller error used for estimating the potency. 6 such squares were worked through in 3 separate laboratories, providing 18 cross-over tests in all. Each square was randomised independently in each laboratory and the example given is one of those actually used. In addition all rabbits were allocated in the first place to the different groups at random, making use of a table of random numbers.

Methods. The methods used in the different laboratories were based on those in use for the routine assay of insulin on rabbits (Smith[3]). Initial blood sugar levels of fasting rabbits, determined from the mean of two separate bleedings, were recorded on each day of the test. The animals were then injected with the appropriate solution for each group and further bleedings were carried out at hourly intervals for 6 hours, single blood sugar determinations being carried out independently on each blood sample. It had been suggested that duplicate estimations of blood sugar should be done for each sample but Smith has shown (*loc. cit.*) that 12 rabbits with one determination of blood sugar at each time are equivalent to 10 rabbits with duplicate blood sugar determinations; it was therefore decided to carry out only single estimations. An interval of one week was allowed between each day of the experiment.

Results. As it would be impracticable to reproduce tables giving the detailed data for all the experiments in this collaborative assay, the data have been collected and bound, and are available for inspection in the library of the National Institute for Medical Research, Mill Hill, London, N.W.7, U.K.

Analysis. The functions of the data used for the estimation of the potency of the freeze-dried preparation in terms of its own original liquid were chosen so as to minimize the fiducial limits of error of that estimate. It became evident at an early stage of the analysis that nothing was to be gained by analysing the results of a complete Latin Square instead of by treating the individual cross-over tests separately. In fact, the fiducial limits of error obtained by analysing the whole square were wider than those obtained by analysing the cross-over tests separately. This is attributable to the fact that during a total period of 6 weeks the variation within animals is so large that the additional advantage gained by the use of a Latin Square design is largely offset. The analysis was, therefore, based solely on the individual cross-over tests. The various functions of the data used in the analysis were:

1. The mean percentage reduction of blood sugar over the hours 1 to 6 inclusive.
2. The mean percentage reduction of blood sugar over the hours 1 to 6 inclusive adjusted by covariance for the initial blood sugar.
3. The mean blood sugar level over the hours 1 to 6 inclusive.
4. The mean blood sugar level at selected hours only. The hours selected varied in different laboratories.

As explained earlier it was also necessary to ensure that the shape of the blood sugar curve was unaltered by the processes of freeze-drying and reconstitution. For this purpose graphs were drawn of all the individual curves. It was apparent that the biggest discrepancies occurred at different times in the different laboratories. The analysis mentioned in 4 above was, therefore, carried out at those hours at which the discrepancies were greatest. Such analysis would bring out any significant difference in the shape of the curves. It was apparent from the analyses that the best estimate of potency, that is the one giving the narrowest fiducial limits of error, was obtained from the analysis using as the variate the mean percentage reduction of blood sugar over the hours 1 to 6 inclusive, adjusted by covariance for the initial blood sugar. Table III shows a summary of the results, using this variate for analysis, for each laboratory separately; each estimate was weighted by the reciprocal of its own variance and all values for log. potency ratio (M) have been adjusted to refer to an assumed potency of 10 U./mg.

There was no heterogeneity of the estimates of potency calculated from the different cross-over tests either within any one laboratory or

TABLE III

ESTIMATE OF POTENCY BY THE RABBIT ASSAY

Laboratory	M	Weight	Potency U./mg.	Limits of Error P = 0·95
A	−0·07096	2191	8·49	7·71—9·36
B	−0·04600	2195	9·00	8·15—9·92
C	−0·08623	2206	8·22	7·44—9·04
Mean	−0·06776		8·56	8·09—9·04

between the different laboratories. The results of the tests which illustrate this are given in Table V.

Mouse Assays

Method. The mouse assays were carried out by the usual mouse-convulsion method and the data subjected to probit analysis. A (2 + 2) assay design was used throughout.

Results. The results of the individual mouse assays are not reproduced here, but may be inspected at the National Institute for Medical Research.

Analysis. Each assay was analysed by the method described by Bliss[4]; the combination of the results was carried out by the simplest method; namely, by obtaining the weighted mean potency, each estimate being weighted by the reciprocal of its own variance. This method tends to underestimate the limits of error (Perry[5]). Table IV shows the summarised results; all values for log. potency ratio (M) have been adjusted to refer to an assumed potency of 10 U./mg.

TABLE IV

ESTIMATE OF POTENCY BY THE MOUSE ASSAY

Laboratory	M	Weight	Potency U./mg.	Limits of Error P = 0·95
A	− 0·05651	2553	8·78	8·03—9·60
C	− 0·08323	2265	8·26	7·51—9·08
D	− 0·06208	3634	8·67	8·04—9·34
Mean	− 0·06607		8·59	8·18—9·02

There was no heterogeneity of the estimates of potency between the different individual assays within the laboratories nor between the different laboratories; the results of the χ^2 tests showing this are given in Table V.

TABLE V

CONSISTENCY OF ASSAY RESULTS

Test	Laboratory	χ^2	Degrees of freedom	P
Consistency with laboratories				
(*a*) Rabbit assays	A	2·7	5	0·7—0·8
	B	1·9	5	0·8—0·9
	C	1·7	5	0·8—0·9
(*b*) Mouse assays	A	12·09	12	0·3—0·5
	C	9·52	8	0·3—0·5
	D	21·85	16	0·1—0·2
Consistency between laboratories				
(*a*) Rabbit assays		1·81	2	0·3—0·5
(*b*) Mouse assays		0·96	2	0·5—0·7
Consistency between mouse and rabbit assays		2·78	1	0·05–0·1
Total ..		55·31	56	0·49

Assignment of Potency to the Standard.

From the 6 values for M and W (Tables III and IV) an overall estimate potency of the freeze-dried, reconstituted globin zinc insulin in terms of the original liquid was obtained by using the equation $\bar{M} = \frac{SWM}{SW}$;

this gave a value of 8·57 U./mg. with limits of error (P = 9·95) of 8·26 and 8·90 U./mg.

The consistency of the results was tested by making use of the approximate relationship $\chi^2 = SW (M - \overline{M})^2$ and Table V shows the results of all such comparisons. Since there is thus no evidence of heterogeneity of the estimates it is assumed that the combined potency of 8·57 U./mg. is the best available estimate of the true potency of the British standard for globin zinc insulin.

The criteria set out in the introduction for proof of the identity of the freeze-dried, reconstituted material with its original liquid have thus been satisfied. It was shown in Section I that, on the basis of the weight yield, the potency of the freeze-dried material should be 8·69 U./mg. assuming no loss on freeze-drying. The assay results are in full accord with this expectation, indicating that there is no significant loss of potency. The shapes of the blood sugar curves were compared in two ways; namely by graphical representation, which showed a close similarity in general appearance, and by analysis of the selected hours at which the most noticeable divergence in the shapes of the curves existed; the analysis showed no significant change in the potency ratio at these selected hours. Finally, the homogeneity of the potency ratios in different species indicated in the χ^2 tests, lends strong support to the conclusion that the original liquid and the freeze-dried, reconstituted preparation are identical in properties.

It was agreed, therefore, with the concurrence of the Medical Research Council, to establish this freeze-dried globin zinc insulin as the Provisional British Standard for Globin Zinc Insulin, and to define it as having a potency of 8·6 British units of globin zinc insulin per mg., one British unit of globin zinc insulin being the activity of one International unit of soluble insulin after conversion to the Provisional British Standard for Globin Zinc Insulin.

III. Stability and Bacteriological Tests

Stability

Samples of the standard were stored for 6 months both at room temperature and at 50° C. and thereafter assayed on mice against the same material stored in the cold. Assays were carried out by three laboratories. The results were as follows.

(a) Storage at room temperature for 6 months

Table VI gives the results. The combined weighted mean potency is 8·38 U./mg. with P = 0·95 limits of error 7·99 and 8·76 U./mg. A χ^2 test indicates that there is no heterogeneity between these three estimates. $\chi^2 = 1{\cdot}6261$ on 2 degrees of freedom. Assuming 8·57 U./mg. to be the potency at the beginning of the experiment, there has been a net loss of potency of 2·30 per cent. during storage at room temperature for a period of 6 months. This potency loss, however, is not significant: t = 0·772 P = 0·4 — 0·5.

TABLE VI

STORAGE AT ROOM TEMPERATURE FOR 6 MONTHS

Laboratory	Potency U/mg.	Log. potency	Weight
A	8·60	0·93450	3715
D	8·08	0·90741	3919
C	8·57	0·93278	1906
Mean	8·38	0·92303	

(*b*) *Storage at* 50° *C. for* 6 *months*

Table VII shows the results. The combined weighted mean potency is 6·74 U./mg. with P = 0·95 limits of error 6·47 — 7·02 U./mg. A χ^2 test indicates that there is no significant heterogeneity between the estimates at the P = 0·99 level, although there is significant heterogeneity at the P = 0·95 level. χ^2 = 7·9545 on 2 degrees of freedom. Assuming homogeneity there has been a net loss of potency of 21·41 per cent. during storage at 50° C. for 6 months.

TABLE VII

STORAGE AT 50° C. FOR SIX MONTHS

Laboratory	Potency U./mg.	Log. potency	Weight
A	7·09	0·85065	6822
D	6·36	0·80346	4403
C	6·07	0·78297	891
Mean	6·74	0·82852	

(*c*) *Estimated Stability of the Standard at* — 10° *C.*

The probable rate of deterioration of the standard at — 10° C. has been calculated from the above results. It has been assumed that "room temperature" may be taken as 18° C., and that the most probable values for the rates of deterioration at 50° C. and 18° C. may reasonably be derived from the estimated potencies after storage at these temperatures. Moreover, the comparisons were made against material stored at 0° C., and no allowance has been made for any deterioration at this temperature which would tend to lesson the apparent decay. The calculation of the probable loss of potency at — 10° C. is based upon the theoretical linear relationship between the reciprocal of the absolute temperature and the velocity of the reaction. On this basis at — 10° C. the standard would be expected to lose potency at the approximate rate of 0·5 per cent. per annum. The stock of standard held will not, at present rates of usage, last for more than 10 years; and in this period the total loss of potency should not exceed 5 per cent. Although the loss of potency has not been considered sufficiently serious to invalidate the use of the material as a standard, it is large enough to merit further investigation.

Bacteriological Tests

(*a*) *Sterility.* 6 ampoules of the standard preparation, reconstituted with distilled water, passed the sterility tests described in the British Pharmacopoeia 1948.

(*b*) *Bacteriostatic powers.* The contents of 5 ampoules of the standard preparation were mixed, reconstituted and distributed into tubes in 1·0 ml. lots. These were seeded with serial 10-fold dilutions in 0·85 per cent. saline solution of washed cultures of *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli*, and of *Bacillus subtilis* spores. The liquid, the *p*H of which was below 3·0, was bactericidal within 24 hours at 20° C. and 37° C. to the 3 vegetative bacteria added to make a final concentration of 10^4 living cells per millilitre. A small proportion of the *Bacillus subtilis* spores was killed, and the remainder failed to germinate in the globin zinc insulin solution even after 1 week at 37° C. The reconstituted material thus has bactericidal and bacteriostatic properties and it is unnecessary to add any antimicrobial substance to it.

IV. Conclusions and Recommendations for the use of the Standard

Suitability of the Freeze-dried Preparations for use as a Standard

From the tests already described it may be concluded that there was no evidence of a loss of potency during freeze-drying of the preparation; its potency did not appear to vary from ampoule to ampoule; and it was found to be bacteriologically sterile and sufficiently stable. That is, it satisfied the requirements for a stable biological standard.

Potency of the Standard

International unit potency for soluble insulin, which is the activity of 0·0455 mg. of the 2nd International Standard for Insulin, cannot be used directly to describe the activity of globin zinc insulin. Moreover, the potency of a preparation cannot be described in terms of the units of soluble insulin used in its manufacture without making unwarrantable assumptions about the molecular state of globin zinc insulin. An independent unit of globin zinc insulin potency is therefore required. However, globin zinc insulin defined in terms of the International unit of soluble insulin has already been in extensive clinical use, so that it was highly desirable to ensure that the units of soluble and of globin zinc insulin, although formally distinct, were related in such a way that the association between the two, already established clinically, was maintained. Consequently, unit potency of globin zinc insulin is defined as the activity of a weight of the Provisional British Standard for Globin Zinc Insulin and not in terms of the International Standard for Soluble Insulin. The unit of activity is the activity of 0·1163 mg. of the Provisional British Standard for Globin Zinc Insulin (i.e. the standard contains 8·6 British Units per milligram.); but this unit is related to the unit of soluble insulin in that one British Unit has the activity of one International Unit of soluble insulin after conversion to the British standard for globin zinc insulin.

Use of the Standard

The sealed standard preparation should be kept in the dark at temperatures of — 5·0° C. or below. Each ampoule of the preparation contains approximately 40 mg. of dried globin zinc insulin, and should be reconstituted for use by adding distilled water.

(*a*) *Potency Estimation.* The mouse convulsion or the rabbit cross-over method is recommended for the estimation of the potency of globin zinc insulin preparations in terms of the standard. The assay should be designed so that the fiducial limits of error of the estimated potency can be calculated from the data.

(*b*) *Estimates of Delayed Action.* No routine method of estimating the delayed action of globin zinc insulin can be recommended since a detailed investigation revealed that the rate of recovery of rabbit blood sugar following the decrease induced by globin zinc insulin is biometrically indistinguishable from that induced by soluble insulin unless an impracticably large number of animals is used in a cross-over test.

Summary

1. In the light of the requirements for valid biological assay it seemed desirable to establish a standard globin zinc insulin preparation. A suitable solution prepared by the British Insulin Manufacturers has been freeze-dried and tested in collaboration with the National Institute for Medical Research. The solid has been designated the Provisional British Standard for Globin Zinc Insulin.

2. Various tests, both chemical and biological, were carried out on the soluble insulin solution from which the globin zinc insulin solution was made and on the globin zinc insulin preparation before and after freeze-drying, in order to ensure the suitability of the freeze-dried material as a standard preparation and to determine its potency.

3. There was no evidence of a loss of potency during the freeze-drying of the standard; its potency did not appear to vary from ampoule to ampoule; and it was found to be bacteriologically sterile and sufficiently stable.

4. The unit potency of globin zinc insulin has been defined as the activity of 0·1163 mg. of the Provisional British Standard for Globin Zinc Insulin. That is, the solid contains 8·6 British Units per mg. This unit is related to the unit of soluble insulin in that one British Unit of activity is the activity of one International Unit of soluble insulin after conversion to the British standard for globin zinc insulin.

5. The use of the Provisional British Standard for Globin Zinc Insulin in assays of globin zinc insulin intended for therapeutic use has been discussed. Potency may be estimated by the mouse convulsion or the rabbit cross-over method. The assay should be designed so that the fiducial limits of error of the estimated potency can be calculated from the data. No method of estimating the delayed action of globin zinc insulin has been recommended owing to the fact that the rabbit blood sugar curve induced by globin zinc insulin is biometrically indistinguishable from that induced by soluble insulin unless an impracticably large number of animals is used.

References

1. Reiner, Searle and Lang, *J. Pharmacol.*, 1939, **67**, 330.
2. Emmens, *Principles of Biological Assay*, Chapman and Hall, Ltd., 1948.
3. Smith, *Hormone Assay*, Ed. Emmens, Academic Press Inc., 1950.
4. Bliss, *Ann. Appl. Biol.*, 1935, 22, 134, 307.
5. Perry, *M.R.C. Spec. Rep. Ser.*, No. 270, S.O. 1950.

THE EFFECTS OF INTRODUCING CERTAIN SUGGESTED SUBSTITUTES FOR TALC INTO THE PERITONEAL CAVITY AND INTO WOUNDS IN EXPERIMENTAL ANIMALS

By J. D. P. Graham and M. E. Jenkins

From the Department of Pharmacology, Welsh National School of Medicine, Cardiff

Received January 10, 1952

For many years talcum (a complex natural magnesium silicate) was the standard powder used for lubricating surgical gloves. A remarkably long time passed between the first use of talc for this purpose and the recognition by pathologists that it is a harmful substance. There is now a considerable literature on the subject,[1–19] of which Seelig, Verda and Kidd[7] give some 25 examples up to 1943. It has been established that the unabsorbable siliceous particles of talc cause a foreign body or giant cell reaction in tissues. The resulting granulomata may give rise to the most serious post-operative complications which entirely invalidate the benefits of surgery. Granulomata, pseudo-tuberculous reactions (leading to action based on a misdiagnosis), fistulæ, adhesions, sterility in females, and even acute abdominal crises causing death, have all been attributed with apparent justification to the evil effects of magnesium silicate. Experimental work with animals has shown that very small amounts of talc may cause severe damage in almost any cavity or tissue of the body, and that powder spilled in one part of the peritoneal cavity may be carried to other regions and there cause damage. Antopol[1] was perhaps the first to point out the dangers of talc, and Bethune[3] actually used it to produce fibrous obliteration of the pleural cavity, but its general use in surgery persisted. The attempt to substitute lycopodium for talc was abandoned when Erb[20] demonstrated that this substance was equally harmful. The undesirable effects of contamination of wounds with talc having been recognised, in recent years many attempts have been made to find a harmless and efficient substitute. The criteria for a desirable surgical glove powder have been outlined[21] and it is the aim of this paper to report the results of experimental trial of a number of such substitutes which are at present offered to the medical profession.

More serious than the accidental contamination of tissues with undesirable substances used as glove powders is the question of deliberate introduction of similar substances into wounds and cavities, frequently unwittingly on the part of the surgeon concerned, as a constituent part of chemotherapy applied in powder form—sulphonamides, antibiotics and mixtures of these. For this purpose wide use is made of a triturate of the sulphonamide with 5 per cent. magnesia mixed with penicillin, etc. (Dahl,[22] White[23]). Stearates have also been recommended for this purpose[24] because of their lubricity.

Finally, there is the question of introducing substances into tissues and cavities as a hæmostatic, or using them as a wound dressing. Such materials as Horsley's wax were long used to control hæmorrhage oozing

from bone, but granulomatous reactions and fistulæ as a result were not unknown. In America a whole group of "absorbable" hæmostatics has been developed and reported upon favourably. Fibrin foam with thrombin[25] has the advantage of being a biological product. Oxidised cellulose with thrombin[26] and gelatin with thrombin[27] have also been favourably received. In this country developments have occurred in the use of alginate (Blaine[28–31]). This substance, a phycocolloid, forms a continuous insoluble gel in the form of the calcium salt, whereas the sodium salt is water-soluble. These properties permit of its use in a great variety of ways, both medicinal and industrial. Varying proportions of the two salts result in substances which dissolve or are absorbed at differing rates depending upon the *p*H, vascularity and situation of the tissue or cavity where they are placed. Already a considerable literature is growing on the subject of the uses of alginates[32–36] in which it is suggested that this substance is harmless if used as a surgical glove powder, as a hæmostatic agent in many situations, or as an external dressing.

The substances investigated were (1) talc; (2), (3) and (4) three modified starches designated as starch (*a*), (*b*) and (*c*); (5) magnesia; (6) a mixture of magnesium carbonate and magnesium stearate; (7) magnesium stearate alone; (8) potassium bitartrate; (9) calcium and sodium alginate. All of these substances reach tissue wounds and cavities inadvertently or as a result of deliberate action during regularly executed therapeutic procedures.

Methods

The "commercial powders" were examined as received from the makers, other substances being of B.P. standard or equivalent standard obtained from reputable sources (e.g. talc, magnesia, bitartrate, stearate). The following tests were applied.

(1) An estimate of the mean particle size, before and after sterilising by the method recommended by the maker of the powder, or in an autoclave at 15 lb. pressure for 30 minutes, or with dry heat at 150° C. for 30 minutes.

(2) An estimate of lubricity by measuring in g. the weight necessary to be applied *via* a pulley to a block in order to overcome limiting friction. The block was enclosed in a portion of surgical glove attached with the inner surface of the glove facing outward, resting on a plane surface covered with a material which was selected from a number of fabrics, plastic, leather, rubber and other materials, treated and untreated skins, because it seemed in appearance, feel and other properties most similar to living skin. Dead human skin varies widely in its surface properties and can not easily be maintained in a constant state. Therefore it is not suitable for prolonged use which would require numerous fresh pieces of material of constant properties. The powders were applied in standard amount and by standard procedure between the opposed surfaces. Trials were made before and after autoclaving the powders.

(3) Samples of the powders selected from the depths of freshly opened packings as received were subjected to bacterial examination and again

after the recommended sterilising procedure. Incubation of samples proceeded in suitable media for 8 weeks, after which time any growth was subcultured and examined.

(4) A standard technique was applied to the examination of the effects of these substances upon living tissues. Rigorous aseptic technique was applied in the surgical work. Groups of 4 animals were used with each of the 9 powders. Kittens, rabbits, guinea-pigs, rats and mice were used. The larger animals were given a preliminary subcutaneous injection of atropine sulphate, all were anæsthetised with ether, and suitable areas of skin were shaved and washed with a solution of cetrimide. An attempt was made to produce (*a*) a standard 2-cm. incision through all layers of the skin of the back on the left side of the midline and (*b*) a standard laparotomy wound 2 cm. in length opening into the peritoneal cavity on the left side lateral to the recti and midway between the lower rib and the iliac crest, suturing being carried out carefully in layers with catgut for the peritoneum and muscle and fine silk for the skin. Into the skin wound was placed 10 mg. of powder, into the peritoneum 50 mg., in each case dry and tipped in loosely, and the wound closed. In the case of the mice the wounds were as small as could be conveniently made and less powder was used. The wound surface and the skin area, after suturing, were swabbed with the cetrimide solution and left undressed. Silk stitches were removed from skin after 10 days and the animals killed with coal gas after 6 to 9 weeks had elapsed. A careful post-mortem examination and report was made on each one, drawings of appearances were taken as necessary, and portions of tissue, etc. excised and examined by histological methods. The standard stain used was hæmatoxylin and eosin, but a polarising microscope was used on sections from all tissues in order to detect birefringent particles. An independent expert opinion was obtained on the appearance of most of the sections.

Results

The findings as to particle size and lubricity are summarised in Tables I and II respectively. Talc, as is well known, is a very fine powder, the mean particle size being the smallest of this series, but modified starch which approaches it in size is a much more even powder. Starch (*b*) is the most even of the three starches, the granules seeming to be largely unaffected by the process of hardening employed. The stearates have a

TABLE I

Particle size of powders in microns, after sterilisation

Substance	Mean	Range	Standard deviation	Mode
Talc	7·6	0·6 to 123·0	± 6·1	0·6
Starch (*a*)	10·1	1·2 to 19·5	± 1·4	8·5
(*b*)	11·1	2·4 to 19·5	± 1·4	11·0
(*c*)	11·3	2·4 to 27·0	± 1·4	11·0
Magnesia	16·1	2·4 to 134·0	± 6·3	6·1
Magnesium carbonate-stearate	30·3	2·4 to 244·0	±15·1	6·1
Potassium bitartrate	26·4	2·4 to 171·0	±11·0	6·1
Alginate	41·7	2·4 to 220·0	±16·0	30·5

TABLE II

LUBRICITY, EXPRESSED AS WEIGHT REQUIRED TO OVERCOME LIMITING FRICTION. APPEARANCE AND FEEL OF POWDERS

Substance	Before autoclaving	After autoclaving	Appearance and feel
	g.	g.	
Talc	107	125	same
Starch (*a*)	100	100	same
(*b*)	134	134	same
(*c*)	117	117	same
Magnesia	174	174	lumpy
Magnesium carbonate-stearate	93	93	lumpy
Magnesium stearate	125	—	solid
Potassium bitartrate	140	140	lumpy
Alginate	161	161	lumpy

greasy feel and melt with heat, which excludes them from use in a pure form where preliminary sterilisation is essential. This property probably accounts for an observed increase in particle size of the carbonate-stearate mixture after autoclaving. However, this powder retains its smooth feel and the "slipperiness" is not affected. No powder showed variation in the critical measure of limiting friction from sample to sample, and only talc was rendered less slippery by the process of sterilisation. No direct correlation exists between the particle size and the lubricity, other properties than size and shape of particles having an effect. Thus, neither magnesia nor potassium bitartrate is very slippery, both clump somewhat after autoclaving, and yet bitartrate is much more abrasive to the skin and to rubber gloves. Both alginate and the carbonate-stearate mixture form rather lumpy powders after autoclaving, but there is a marked difference in their lubricity. There is little difference in the particle size of the three modified starches tested, but some difference in lubricity.

TABLE III

STERILITY OF POWDERS BEFORE AND AFTER AUTOCLAVING (8 WEEKS GROWTH)

Substance	Before autoclaving	After autoclaving
Talc	sterile	sterile
Starch (*a*)	contaminated	sterile
(*b*)	sterile	sterile
(*c*)	contaminated	sterile
Magnesia	sterile	sterile
Magnesium carbonate and stearate	sterile	sterile
Magnesium stearate	contaminated	sterile
Potassium bitartrate	sterile	sterile
Alginate	contaminated	sterile

All these substances are capable of being sterilised by routine means: several of them are sterile in the raw state. Others are contaminated by streptothrix. From none of them were pathogenic organisms isolated. Nevertheless it is obvious that great care must be taken that no non-sterile substance reaches the tissues by the admixture of a non-sterile adjuvant to a chemotherapeutic powder.

The effect of the powders on the tissues of the skin and on the peritoneal cavity were assessed by applying the code shown in Table IV. The mean figure thus obtained from each group of 4 animals of one species is

TABLE IV

CODE APPLIED IN ASSESSMENT OF EFFECTS OF POWDERS ON SKIN WOUND AND PERITONEAL CAVITY OF ANIMALS

Skin	Peritoneum
0 = N.A.D.	0 = N.A.D.
1 = slight fibrosis	1 = omentum adherent to wound site only
2 = fibrosis or slight round-cell invasion	2 = as above plus fibrous tissue masses, "bloom" on spleen, etc.
3 = foreign body reaction—slight	3 = as above plus slight adhesions remote from site of wound, granulomata, etc.
4 = foreign body reaction—more severe, with crystals	4 = general and severe adhesions, marked foreign body reaction in organs, etc.

shown in Table V, together with the total score. Obviously the substance scoring the least total is the least harmful to living tissue in common laboratory animals, and therefore possibly, or even probably, least likely to be harmful in man. Examination with the polarising microscope revealed heavy contamination with doubly refractile particles in all tissues containing talc, in occasional tissues containing magnesia, and in tissues containing starch (*c*). Postelthwait *et al.*[18] describe and illustrate the granulomata which may be caused by this same preparation (starch *c*) and the curious doubly refractile particles which it contains. Alginate had a collagenous appearance and slightly affected the refractive powers. Sections from tissues contaminated with the other substances showed no evidence of refractile bodies being present.

TABLE V

MEAN EFFECTS OF SUBSTANCES ON SKIN WOUNDS AND PERITONEAL CAVITIES OF ANIMALS ASSESSED ACCORDING TO THE CODE SHOWN IN TABLE IV

Substance	Kitten		Rabbit		Guinea-pig		Rat		Mouse		Totals		Total (maximum 40)
	skin	peritoneum	skin	peritoneum	skin	peritoneum	skin	peritoneum	skin	peritoneum	skin	peritoneum	
Talc	4·0	4·0	2·0	4·0	4·0	4·0	4·0	4·0	2·0	4·0	16·0	20·0	36·0
Starch (*a*)	1·0	2·0	1·0	1·0	0·0	1·5	1·0	2·5	1·0	3·0	4·0	10·0	14·0
(*b*)	0·0	3·0	0·0	0·0	1·0	3·0	2·0	1·0	0·0	0·5	3·0	7·5	10·5
(*c*)	0·0	3·0	0·0	0·0	1·0	0·0	0·0	3·0	1·0	4·0	2·0	11·0	13·0
Magnesia	0·0	1·25	1·0	1·0	2·0	3·0	2·0	3·5	0·0	2·0	5·0	10·75	15·75
Magnesium stearate-carbonate	2·0	3·0	0·0	2·0	1·0	1·0	1·0	2·75	1·0	2·0	5 0	10·75	15·75
Magnesium stearate	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0 0	0·0	0·0
Potassium bitartrate	0·0	0·25	1·0	1·0	0·0	0·75	0·0	1·25	1·0	2·5	2 0	5·75	7·75
Alginate	1·0	3·0	4·0	2·0	1·0	3·25	1·0	3·0	1·0	2·0	8·0	13·25	21·25
Total	8·0	19·5	9·0	11·0	10·0	17·5	11·0	21·0	7·0	20·0	45·0	89·0	134·0

	Total	Skin	Peritoneum
Mean damage, per powder	14·88	5	9·88
Mean damage, per species	26·8	9	17·8

DISCUSSION

From this table it may be deduced that no substance is entirely harmless with the exception of stearate, which acts as a control for the other observations. This substance is precluded from being of practical importance by itself because it is frequently contaminated in the raw state and is melted by heat. It leaves a greasy appearance in the peritoneal cavity, but no evidence of inflammation other than a microscopical appearance of swelling of connective tissue cells. Furthermore, it appears

that the peritoneal cavity is a more sensitive indicator of irritation than is the skin, for each substance examined, except stearate which is harmless at both sites in all species. The skins of the 5 species exhibit a graded degree of sensitivity to the total insult offered them, the peritoneal cavities differ widely in response, but any one powdered substance may provoke a similar response in the skins of all species tested (talc is universally damaging, stearate is harmless) or a widely differing response in two species (alginate). Again, a similar response may be evoked in the peritoneal cavities of all species (talc and stearate) or a widely differing response in different species (starch *c*). Individual animals in any specific group varied little in their response to any given agent applied to either site. Small groups of animals are therefore a justifiable saving of expense in conducting such a screening test. In this test 180 animals were used.

Excluding talc as being universally damaging and stearate as unsuitable for sterilisation, it appears that bitartrate is the least harmful substance, which agrees with the findings of Seelig *et al.*[7] and Verda.[37] Unfortunately, as has been stated authoritatively, this substance tends to macerate the hands of the surgeon and to shorten the life of surgical gloves.[21] The latter consideration is not of prime importance, but the former may well be. In this test alginate powder proved damaging to a moderate extent in all species at both sites and accordingly may well be excluded from our choice. It must be stressed that no conclusion may be reached as to the use of alginate dressings and hæmostatic preparations. Magnesium oxide and carbonate-stearate mixture were likewise not free from adverse effects though a great improvement on talc. This leaves the modified starch powders which have been praised by Lee and Lehman,[12] Eberl, George, May and Henderson[38] and McQuiddy and Tollman.[39] There appears to be little to choose between them, but none of them is entirely harmless as frequently claimed. There is little information available as to the nature of starch (*a*), but starch (*b*) is a formaldehyde-treated selected starch, and starch (*c*) is described as a mixture of amylose and amylopectin which does not contain formaldehyde but does contain 1 per cent. of magnesia. In total damaging effect, starch (*b*) had slight advantages over the other starches and this improvement is evident in the findings in the peritoneal cavity, though in some species it was by no means harmless. It has been stated that the use of a formaldehyde-treated starch powder is completely safe in human beings.[39] Microphotographs of the appearances of tissues treated with talc and the starch powders, together with other findings, have been published. (Graham and Jenkins[40]).

Summary and Conclusions

1. Tests were made of the particle size, lubricity and sterility of a number of substances which have been advocated as lubricating powders for surgical gloves, or as adjuvants to chemotherapeutic powders used to increase their rate of flow, viz., talc, three separate modified starches, magnesia, magnesium carbonate with magnesium stearate, magnesium stearate alone, potassium bitartrate and alginate powder.

2. The effects of admitting these substances to the skin and peritoneum after sterilisation was determined in groups of animals of 5 species.

3. As a result of these tests and certain authoritative statements which have been published, it was concluded that modified starches appear to be the most practicable substitute for talc at present available, but that further effort to produce a substance which is entirely compatible with human tissue is needed.

Thanks are due to manufacturers for generous offers of information and samples of their products. Acknowledgments are also offered with gratitude to Prof. J. Gough, Department of Pathology, Welsh National School of Medicine, for his invaluable histo-pathology reports on specimens prepared in his Department.

References

1. Antopol, *Arch. Path.*, 1933, **16**, 326.
2. Faulds, *J. Path. Bact.*, 1935, **41**, 129.
3. Bethune, *J. thorac. Surg.*, 1935, **4**, 251.
4. Gardner, *Amer. J. Path.*, 1937, **13**, 13.
5. German, *Amer. J. clin. Path.*, 1940, **10**, 245.
6. German, *Surg. Gynec. Obstet.*, 1943, **76**, 501.
7. Seelig, Verda and Kidd, *J. Amer. Med. Ass.*, 1943, **123**, 950.
8. Lichtman, McDonald, Dixon and Mann, *Surg. Gynec. Obstet.*, 1946, **83**, 531.
9. Lichtman and McDonald, *Arch. Path.*, 1946, **42**, 69.
10. Roberts, *Brit. J. Surg.*, 1947, **34**, 417.
11. Eiseman, Seelig and Womak, *Ann. Surg.*, 1947, **126**, 820.
12. Lee and Lehman, *Surg. Gynec. Obstet.*, 1947, **84**, 689.
13. Mackey and Gibson, *Brit. med. J.*, 1948, **1**, 1077.
14. Walker, *ibid.*, 1948, **1**, 1079.
15. Smith, *ibid.*, 1948, **1**, 1078.
16. Swingle, *Ann. Surg.*, 1948, **128**, 144.
17. Ross and Lubitz, *ibid.*, 1949, **130**, 100.
18. Postelthwait, Howard, Schanker and Durham, *Surgery, St. Louis*, 1949, **25**, 22.
19. Gruenfeld, *Arch. Surg., Chicago*, 1949, **59**, 917.
20. Erb, *Surg. Gynec. Obstet.*, 1935, **60**, 40.
21. Editorial Annotation, *Brit. med. J.*, 1948, **1**, 1090.
22. Dahl, *J. Amer. pharm. Ass.*, 1945, **4**, 120.
23. White, *West. Drugg.*, 1946, **14**, 76.
24. Golden and Neumeier, *Amer. prof. Pharm.*, 1947, **13**, 153.
25. Bailey, Ingraham, Swenson, Lowrey and Bering, *Surgery, St. Louis*, 1945, **18**, 347.
26. Frantz, *Surg. Clin. N. Amer.*, 1945, **25**, 338.
27. Light and Prentice, *Arch. Surg.*, 1945, **51**, 69.
28. Blaine and Dollar, *Trans. ophthal. Soc. U.K.*, 1945, **64**, 187.
29. Blaine, *Lancet*, 1946, **251**, 525.
30. Blaine, *Ann. Surg.*, 1947, **125**, 102.
31. Blaine, *Chem. and Drugg.*, 1949, **151**, 214.
32. Passe and Blaine, *Lancet*, 1948, **255**, 651.
33. Fairbairn and Whittet, *Pharm. J.*, 1948, **160**, 149.
34. Mullard, *Thorax*, 1948, **5**, 233.
35. Rumble, *Brit. dental J.*, 1949, **86**, 203.
36. Oliver and Blaine, *Brit. J. Surg.*, 1950, **37**, 307.
37. Verda, *Hospitals*, 1944, **18**, 32.
38. Eberl, George, May and Henderson, *Amer. J. Surg.*, 1948, **75**, 493.
39. McQuiddy and Tollmann, *Surgery, St. Louis*, 1948, **23**, 786.
40. Graham and Jenkins, *Lancet*, 1952, **262**, 590.

THE APPLICATION OF TITRATION IN NON-AQUEOUS MEDIA TO PHARMACEUTICAL ANALYSIS

PART I. THE DETERMINATION OF ALKALI METAL SALTS OF ALIPHATIC AND AROMATIC ACIDS

By A. H. BECKETT, R. M. CAMP and H. W. MARTIN

From the Pharmaceutical Chemistry Laboratories of the School of Pharmacy, Chelsea Polytechnic, London, S.W.3

Received March 26, 1952

THE method of determination by titration in non-aqueous media of bases and acids which are too weak to be titrated in aqueous solution has been investigated and applied in analysis for many years.[1,2,3,4,5] It is only recently, however, that the method has received considerable attention, and it is now proving to be of wide applicability in the control of the purity of many diverse types of chemicals. Although many solvents have been used, glacial acetic acid has found the widest acceptance as a solvent for the titration of basic materials, and perchloric acid in glacial acetic acid has proved to be the most satisfactory titrant. This method allows not only of the titration of weak bases, but also of salts, because in many cases the anion is sufficiently basic under these conditions to give a good end-point.[6,7,8] It has recently been shown that hydrochlorides can also be titrated provided that mercuric acetate is added to the solution.[9] Titration in non-aqueous media does not require expensive equipment, and has been shown to be quick and accurate.[8,9,10] The completion of the titration can be determined either by the use of indicators or potentiometrically.

An examination of the B.P. and B.P.C. shows that many chemicals, at present being analysed by different procedures, some of which require a few hours to perform, could probably be analysed in non-aqueous media by a direct titration involving only a few minutes work. If this should prove to be possible, then the standardisation of the analytical procedures, and the simplicity, accuracy and speed of the method should be of great value in its application to pharmaceutical analysis, and this approach we have been investigating. Despite the fact that it has recently been demonstrated by other workers[8] that many of the compounds discussed in this communication can be titrated in non-aqueous media, we now present some of our results to show a comparison between the values obtained by this method and the values obtained by the procedure adopted in the B.P. and B.P.C., and to indicate the advantages and disadvantages associated with the method. We feel it necessary to place these results on record to emphasise the simplification of the B.P. and B.P.C. procedures which is possible. During the course of this work a number of publications have appeared showing the wide range of compounds, many being of pharmaceutical importance, which can be titrated in non-aqueous solvents.[9,11,12,13,14,15,16]

THEORY

By making use of the Brønsted-Lowry concept of acids and bases, the

titration of a base (B) in a solvent (SH) with an acid (HA) can be represented as follows:—

$$\begin{array}{rl} HA + SH \rightleftharpoons & SH_2^+ + A' \\ & \Updownarrow + B \\ & BH^+ + SH + A' \end{array}$$

If the reaction to BH^+ is to be complete (complete neutralisation) upon mixing one equivalent of base (B) and acid (HA), then B must be a stronger base than either SH or A′. For the titration of a very weak base in aqueous solution, the solvent ($SH = H_2O$) is too strong a proton acceptor, but it may be possible to titrate in glacial acetic acid (CH_3COOH) which has less tendency to accept a proton to become $CH_3COOH_2^+$. Thus titrations with perchloric acid of the base B in glacial acetic acid solution can be represented as follows:—

$$\begin{array}{rl} HClO_4 + CH_3COOH \rightleftharpoons & CH_3COOH_2^+ + ClO_4' \\ & \Updownarrow + B \\ & BH^+ + CH_3COOH + ClO_4' \end{array}$$

and so complete neutralisation of B to BH^+ becomes possible.

An aliphatic or aromatic carboxylic acid anion is also sufficiently basic to be titrated under these conditions because the equilibrium is considerably to the right in the following:—

$$RCOO' + CH_3COOH_2^+ \rightleftharpoons RCOOH + CH_3COOH$$

In the titration of hydrochlorides in glacial acetic acid,

$$BH^+ + Cl' + CH_3COOH_2^+ \rightleftharpoons BH^+ + HCl + CH_3COOH$$

the anion Cl′ is too weakly basic compared with CH_3COOH to result in a good end-point. However, by heating to remove HCl[17], or by adding mercuric acetate to remove the chloride anion as follows[9]:—

$$Hg(CH_3COO)_2 + 2Cl' \rightleftharpoons HgCl_2 + 2CH_3COO'$$

it is possible to obtain good results.

It is evident that the method therefore holds out considerable promise for the analysis of pharmaceutical substances because of the vast range of compounds which can be considered as falling within its scope.

Reagents, Solutions and Apparatus

Perchloric acid, Analar (72 per cent.).

Glacial acetic acid. Before use as a solvent it was treated with the theoretical amount of acetic anhydride to render it anhydrous.

Acetic anhydride. Redistilled before use.

Potassium hydrogen phthalate, Analar.

Crystal violet indicator solution (0.5 g. of crystal violet in 100 ml. of anhydrous acetic acid).

Standard solution of perchloric acid (0·1N). Perchloric acid (20·5 ml.) was added slowly, with stirring, to approximately 2000 ml. of anhydrous acetic acid in a large flask cooled in ice. Then acetic anhydride (50·5 ml.) was slowly added to react with the water in the perchloric acid, and the solution made up to approximately 2500 ml. with anhydrous acetic acid.

The solution was allowed to stand 24 hours before use, and then standardised against potassium hydrogen phthalate both potentiometrically and by using crystal violet indicator solution (2 drops).

Potentiometric titrations were carried out using a Morton D.C. Amplifier,[18] calibrated in *p*H and millivolt readings, and a glass electrode and a saturated calomel electrode (sleeve or fibre type). A mechanical stirrer was used.

Consideration of Various Factors Associated with the Titrations

In this paper we are dealing only with titrations with perchloric acid using glacial acetic acid as solvent.

Standardisation. Diphenylguanidine, sodium carbonate, and potassium hydrogen phthalate have been used by different workers as basic standards, and Seaman and Allen[10] studied the last standard in detail. We have found potassium hydrogen phthalate to be a reliable standard. The standardisation was performed both potentiometrically and using crystal violet as indicator, the indicator change at the end-point being blue→blue-green as shown in Figure 1.

Effect of Water-content upon the End-point. The results used in the preparation of the curves in Figure 2 were obtained with known percentages of water in solutions of 0·5 g. of potassium hydrogen phthalate in 50 ml. of acetic acid solution. The standard solution of perchloric acid was water-free. These results demonstrate that the presence of water must be avoided. A small percentage of water, even less than 1 per cent., leads to a slight flattening of the titration curve. The sharpness of the indicator end-point is impaired by 1 per cent. of water, and larger amounts of water ruin the end-point completely. The presence of excess of acetic anhydride has no effect upon the potentiometric titration or upon the indicator end-point. Consequently we have used an excess of 1 per cent. of acetic anhydride in all the acetic acid used in subsequent work to obviate the necessity of taking any precautions to exclude atmospheric moisture.

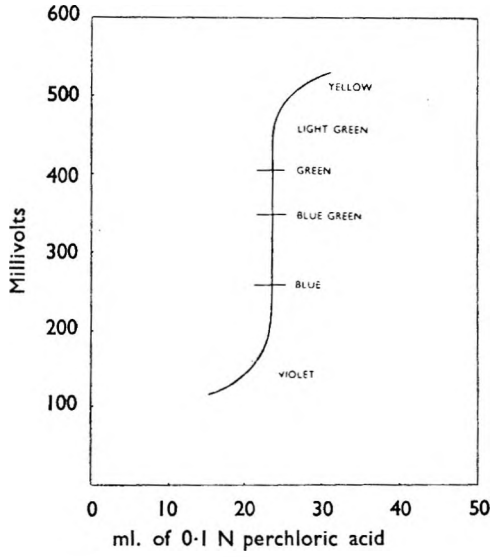


Fig. 1. The titration of potassium hydrogen phthalate using crystal violet as indicator.

Effect of Temperature. One of the disadvantages associated with titration in glacial acetic acid, and with non-aqueous solvents in general, is the rather large coefficient of expansion of these solvents. Seaman and

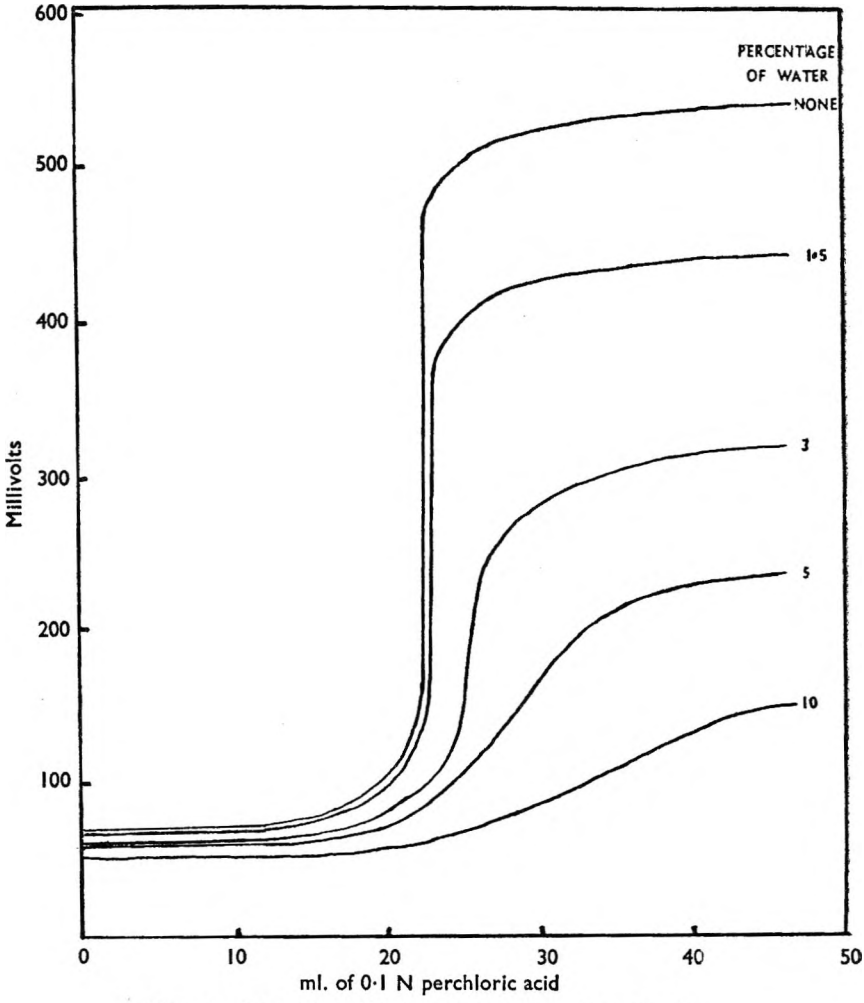


FIG. 2. The effect of water-content upon the end-point.

Allen[10] applied a factor of 0·11 per cent. per ° C. to the observed volumes of titrating solution, because the coefficient of expansion of glacial acetic acid is 0·0011 per ° C. We have determined the volumes of perchloric acid solution (0·1N) required at different room temperatures by 0·5 g. of potassium hydrogen phthalate dissolved in acetic acid (50 ml.), both the solution and the titrant being at room temperature before the commencement of the titration. The results are shown graphically in Figure 3 which indicates that a correction of 0·11 per cent. per ° C. is required. Thus, for accurate work, the perchloric acid must be standardised and used at the same constant temperature, or the burette readings must be multiplied by $1 - (n \times 0{\cdot}0011)$ if the temperature is above the standardisation temperature, and $1 - (n \times 0{\cdot}0011)$ if below, where n is the difference in ° C. from the standardisation temperature.

Potentiometric or Indicator End-point? The use of the Morton D.C. Amplifier[18] makes it possible to perform potentiometric titrations in a few minutes. However, because we consider it desirable to make the titration method applicable to general pharmaceutical analysis, without having to resort to instruments, we are attempting to find suitable indicators for the proposed analyses. It has been pointed out previously[10] that the slope of the curve at the end-point, and the colour change of crystal violet indicator at the end-point, are not only influenced by the strength of the base being titrated, but possibly by the concentration and nature of the ions in solution at this point.

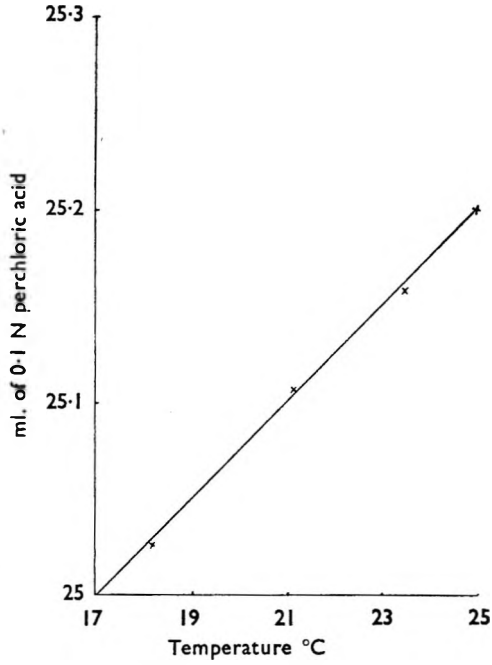


FIG. 3. The effect of temperature.

THE DETERMINATION OF ALKALI METAL SALTS OF ALIPHATIC AND AROMATIC CARBOXYLIC ACIDS OF THE B.P. AND B.P.C.

It has been shown[6,8] that salts of this type can be titrated in non-aqueous media. In the B.P. and B.P.C. are found three different procedures for the analysis of these substances, namely:—

(1) Ignition to alkali metal carbonate, addition of excess of 0·5N sulphuric acid, filtration and back titration with 0·5N sodium hydroxide, e.g., sodium potassium tartrate.
(2) Titration in almost boiling solution with 0·2N sodium hydroxide, e.g., potassium acid tartrate.
(3) Titration with 0·5N sulphuric acid, involving shaking with ether to remove most of the aromatic acid from the aqueous phase before a correct end-point can be obtained, e.g., sodium salicylate.

We suggest a simple standard analytical procedure for these substances as outlined later.

In Table I we present the mean results of analyses using the official procedures, and titration with perchloric acid in glacial acetic using the potentiometric and indicator methods.

The determinations were made on chemicals supplied as of B.P. or B.P.C. quality. The aqueous standard solutions, and the perchloric acid

TABLE I

Chemical	Determined purity percentage		
	Official method	Potentiometric method	Indicator method
Lithium citrate	99·40	99·56	99·52
Lithium salicylate	98·79	98·95	98·86
Potassium acetate	99·86	99·95	99·92
Potassium citrate	99·94	99·93	99·92
Potassium tartrate	100·16	99·97	99·95
Potassium acid tartrate	99·95	99·80	99·80
Sodium acetate	99·93	99·90	99·89
Sodium benzoate	99·83	99·92	99·90
Sodium citrate	99·64	99·75	99·68
Sodium acid citrate	100·04	99·93	99·90
Sodium potassium tartrate	100·3	100·2	100·2
Sodium salicylate	99·91	99·91	99·88

solutions in anhydrous acetic acid, were standardised against the same sample of potassium hydrogen phthalate. Many titrations were performed on the above salts in anhydrous acetic acid containing 1 per cent. of acetic anhydride, and the results indicated a reproducibility of ± 0·15 per cent. on a 25 ml. titration using either the potentiometric or indicator method.

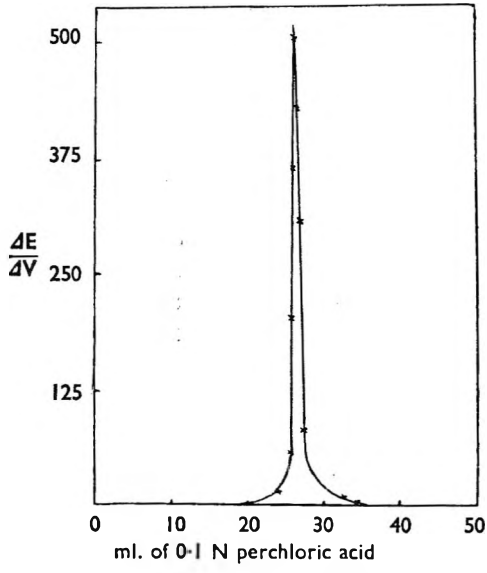


FIG. 4. A typical differential titration curve of alkali metal salts of aliphatic and aromatic carboxylic acids.

Potentiometric Titrations. Sufficient of the salt, to give a titration of approximately 25 ml. was dissolved in 50 ml. of anhydrous acetic acid. The change at the end-point was very sharp indeed in all cases, even when 0·01N perchloric acid solution was used as titrant. Figure 4 is a typical differential titration curve. In some titrations 2 drops of crystal violet indicator solution were added in order to observe the colour changes in the vicinity of the end-point. The millivolt readings recorded in the figures are meter readings (uncorrected).

Indicator Colour Changes. The colours near the end-point were affected by the type of cation present. For instance, the potassium salt produced a different colour from that of the corresponding sodium and lithium salt under comparable conditions, this fact being portrayed on the potentiometric curves in Figure 5. This may be connected with the fact that potassium perchlorate precipitates during the titration, whereas lithium and sodium perchlorates do not. When both sodium and potassium ions are present, the colour changes represent the mean of the changes produced

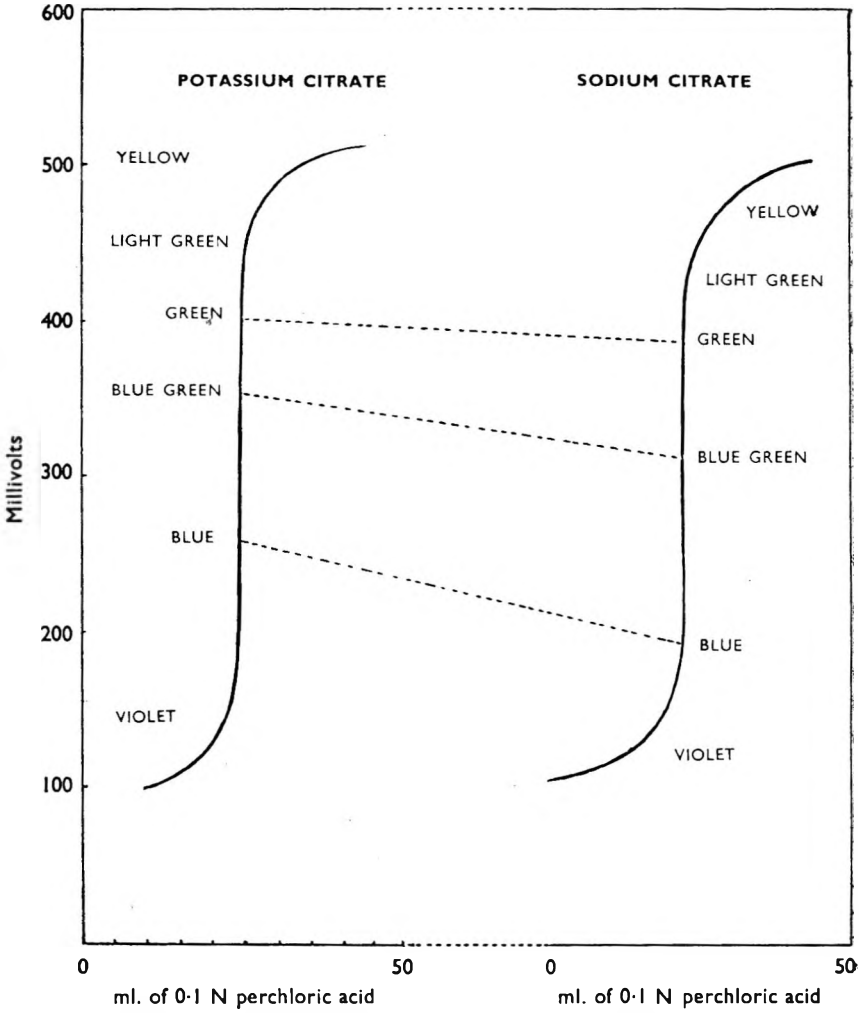


FIG. 5. Colour changes of the indicator (crystal violet) during the titration of sodium and potassium salts of organic carboxylic acids.

by these ions alone. However, in the determination of all the above salts, the end-point may be taken as the colour change from blue to blue-green, which is a sharp colour change. In fact, using sufficient salt dissolved in 50 ml. of anhydrous acetic acid to give a titration of 25 ml. of 0·1N perchloric acid solution a volume of 0·1 ml. of titrant is sufficient to change the colour from the last shade of blue to the full green.

Solution of the Salts. Sufficient of the salts could be dissolved in 50 ml. of anhydrous acetic acid to give a titration figure of 20 to 25 ml. of 0·1N perchloric acid, with the exception of the tartrates. Solution was affected in some cases by heating, with subsequent cooling before the titration. Too vigorous heating tended to cause charring in some cases. For the tartrates, more dilute solutions had to be employed, and 0·01N perchloric

acid was used as titrant. There is not sufficient water in the water of crystallisation of the salts to affect the end-point, and even if large percentages are present, the water may be removed prior to the titration by heating with glacial acetic acid containing excess acetic anhydride.

We consider the method of titration with perchloric acid in anhydrous acetic acid to offer considerable advantages over the official methods used for the determination of the salts mentioned in this communication, because of its speed, simplicity and standardisation of procedure without any loss of accuracy. Further communications will show its application to other groups of pharmaceutical substances. The major disadvantage is the effect of change of temperature upon the observed results, but a correction factor can readily be applied.

Recommended General Procedure

1. Use sufficient acetic anhydride to leave a 1 per cent. excess in the anhydrous acetic acid.

2. Record the room temperature at the time of standardisation of the perchloric acid solution and at the time of its use for determinations, and apply a correction factor.

3. For the salts (except tartrates) mentioned in this communication, weigh sufficient of the sample to give a titration of approximately 25 ml. of 0·1N perchloric acid, and dissolve in 50 ml. of anhydrous acetic acid, by the use of gentle heat if necessary. Cool to room temperature, add 2 drops of crystal violet indicator solution and titrate, taking the colour change from blue to blue-green as the end-point.

4. For tartrates, use 0·01N perchloric acid and proceed as above.

We have applied the general procedure to other alkali metal salts of organic acids such as sodium alginate, sodium glycerophosphate, sodium lauryl sulphate etc., with success. Although some of these substances are not pure material, and other analytical data will be required for the control of their purity, yet to specify a minimum figure for a determination in a non-aqueous medium such as outlined above, would certainly be of advantage.

Summary

1. Various factors concerned with titrations in non-aqueous media are examined, and the advantages and disadvantages of the method in respect to its application to the analysis of pharmaceutical substances are considered.

2. Comparative results are quoted for the determinations of alkali metal salts of aliphatic and aromatic carboxylic acids using the B.P. and B.P.C. methods, a potentiometric method, and an indicator method.

3. A general indicator method suitable for routine analysis is recommended.

References

1. Vorlander, *Ber. dtsch. chem. Ges.*, 1903, **36**, 1485.
2. Folin and Wentworth, *J. biol. Chem.*, 1909–10, **7**, 421.
3. Folin and Flanders, *J. Amer. chem. Soc.*, 1912, **34**, 774.
4. Hall and Conant, *ibid.*, 1927, **49**, 3047.

A NOTE ON THE ESTIMATION OF ISONICOTINIC ACID HYDRAZIDE

By Teodor Canbäck

From Apotekens kontrollaboratorium, Stockholm, Sweden

Received April 4, 1952

The current interest in isonicotinic acid hydrazide has prompted the need for a rapid assay method. It is well known that hydrazines in slightly alkaline solutions are oxidised by iodine according to the scheme:—

$$RNHNH_2 + 2I_2 = RI + 3HI + N_2.$$

Mono-acylhydrazides are said to behave in a different way[1]:—

$$2RCONHNH_2 + 2I_2 = RCONHNHCOR + N_2 + 4HI.$$

However, isonicotinic acid hydrazide consumes 4 equivalents of iodine when oxidised as described below. Thus the mechanism of the reaction seems to be analogous to that of hydrazines.

Isonicotinic acid hydrazide may be titrated in the following manner:— Dissolve 50 mg. of the substance in 50 ml. of water, add 1 g. of sodium bicarbonate and 25 ml. of 0·1N iodine. Allow to stand for 15 minutes. Cautiously add 10 ml. of 5N hydrochloric acid and titrate the excess of iodine with 0·1N sodium thiosulphate using starch solution as indicator. 1 ml. of 0·1N iodine corresponds to 0·003429 g. of $C_6H_7ON_3$.

Two samples of the hydrazide from different manufacturers, m.pt. 168° to 169° C. and 170° to 171° C. (Kofler), assayed 97·8, 97·8, 98·0 and 98·4, 98·6, 98·7, 98·2 per cent.

Commercial tablets containing 50 mg. of the hydrazide were analysed in a similar way, the only difference being that the suspension of the tablet powder was centrifuged before the addition of the sodium bicarbonate. The following results were obtained:—47·7, 48·1, 48·3 and 48·2 mg. per tablet.

Reference

1. *Chemistry of Carbon Compounds*, edited by E. H. Rodd, Elsevier, London, 1951, 1A, 601.

Titration in non-aqueous media. References continued.

5. Lavine and Toennies, *Amer. J. med. Sci.*, 1933, **185**, 302.
6. Blumrich and Bandel, *Angew. Chem.*, 1941, **54**, 374.
7. Tomicek, *Collection. Czechoslov. Chem. Communs.*, 1948, **13**, 116.
8. Markunas and Riddick, *Anal. Chem.*, 1951, **23**, 337.
9. Pifer and Wollish, *J. Amer. pharm. Ass., Sci. Ed.*, 1951, **40**, 609.
10. Seaman and Allen, *Anal. Chem.*, 1951, **23**, 592.
11. Moore, McCutchan and Young, *ibid.*, 1951, **23**, 1639.
12. Pifer and Woolish, *ibid.*, 1952, **24**, 300.
13. Fritz, *ibid.*, 1952, **24**, 306.
14. Fritz and Keen, *ibid.*, 1952, **24**, 308.
15. Markunas and Riddick, *ibid.*, 1952, **24**, 312.
16. Terry, Eilar and Moe, *ibid.*, 1952, **24**, 313.
17. Higuchi and Concha, *J. Amer. pharm. Ass., Sci. Ed.*, 1951, **40**, 173.
18. Morton, details of this instrument are to be published later.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

Ergot Alkaloids, Paper Chromatography of. A. M. Berg. (*Pharm. Weekbl.*, 1951, **86**, 900.) The six most important alkaloids of ergot can be separated by using buffered filter paper (*p*H 5) and ether as solvent. The best results are obtained by circular chromatography, which gives narrower bands than are obtained with the usual strip method. These bands represent, in order from the centre, ergometrine, ergometrinine, ergotamine, ergotaminine, ergocristine and ergocristinine. They are located by their fluorescence in ultra-violet light.

G. M.

Gelsemicine, Structure of. M. M. Janot, R. Goutarel and W. Friedrich. (*Ann. pharm. franc.*, 1951, **9**, 305.) Gelsemicine is relatively rare and owing to difficulties similar to those met in the elucidation of the structure of gelsemine, the formula is not yet certain. That adopted is $C_{19}H_{24}O_3N_2$. The alkaloid has been obtained by benzoylation and a laborious chromatographic purification on alumina followed by recrystallisation. The product melted at 262° C. and is considered to be an oxynorhydrogelsemine. J. R. F.

ANALYTICAL

Alkaloids, Paper Chromatography of. R. Munier and M. Macheboeuf. (*Bull. Soc. Chim. biol.*, 1951, 33, 846.) In dealing with mixtures of alkaloids with unknown chromatographic characters, it is necessary first to determine the physicochemical conditions (*p*H) giving the most sharply defined spots, and then to find a solvent giving R_F values showing good separation. With a dissociation constant of the base not less than 10^{-2}, neutral or acid solutions should be used; at K = 10^{-3} to 10^{-7}, alkaline solutions; and at K = less than 10^{-12}, either acid, alkaline or neutral. Examples of the separation of groups of alkaloids are given; the figures given representing R_F values. *Atropine group:* *n*-butanol + 14 per cent. of glacial acetic acid: atropine, 0·71; hyoscyamine, 0·72; homatropine, 0·64; scopolamine, 0·60; tropine, 0·43. *Nicotine group:* *n*-butanol + 20 per cent. of hydrochloric acid (d = 1·19): nicotine hydrochloride, 0·27; pyrrolidine hydrochloride, 0·53; pyridine hydrochloride, 0·36. *Alkaloids of broom:* *n*-butanol + 20 per cent. of glacial acetic acid: genisteine, 0·84; sparteine, 0·73. *Pseudocinchona group:* *n*-butanol + 4 per cent. of hydrochloric acid (d = 1·19). Distinct spots are given by corynanthine, corynantheine cryst., corynanthidine, corynantheidine and yohimbine. For the development of alkaloidal spots two solutions are used: 0·85 g. of bismuth subnitrate with 40 ml. of water and 10 ml. of glacial acetic acid, and 8 g. of potassium iodide with 20 ml. of water. Equal volumes of these two solutions are mixed, and 10 ml. of the mixture is diluted with 20 ml. of glacial acetic acid and 100 ml. of water. The paper is dipped in this reagent for a few seconds, and dried between filter paper. The spots show up red on a pale orange ground.

G. M.

Ephedrine, Colorimetric and Chromatographic Determination of. A. Capone. (*Boll. chim.-farm.*, 1951, **90**, 465.) Ephedrine, 1 to 15 mg., is placed in a 25-ml. flask, with 4 ml. of 16 per cent. sodium chloride solution, 0·45 ml. of 0·1N sodium hydroxide, 6 drops of 33 per cent. hydrogen peroxide, and water to make 25 ml., and the flask is kept in a boiling water-bath for 5 minutes. A reddish-violet colour is produced and the reading is taken in a Hellige colorimeter. The amount of ephedrine present is read from a curve previously obtained by treating known quantities in the same way. Many drugs likely to be mixed with ephedrine, such as diethylbarbituric acid, calcium chloride, codeine, magnesium chloride, terpene hydrate cause only slight inaccuracies but many, such as lactic acid, calcium gluconate, chloral hydrate and potassium bromide and iodide hinder the reaction. The author therefore devised a chromatographic method. 0·01 ml. of the solution to be examined is placed on a sheet of Schleicher and Schull filter paper No. 597 36 cm. high, placing the drops 25 mm. apart for aqueous solutions and 30 mm. apart for oils and ointments. The solvent is allowed to ascend for 30 cm. in a suitable closed glass cylinder at room temperature. To develop the chromatogram the paper is kept immersed in an atmosphere saturated with iodine vapour for 12 hours. For aqueous solutions the solvent is 100 parts of water-saturated butanol with 25 parts of ethanol, for oils and ointments it is equal parts of butanol, ethanol and water. The quantity of ephedrine should be about 50 μg. H. D.

Hyoscyamine and Atropine, Decomposition of. W. Schneider. (*Arch. Pharm., Berl.*, 1951, **284**, 306.) The cause of the ease of racemisation of hyoscyamine lies in the character of the tropic acid fraction of the molecule, which has a enolisable hydrogen atom attached to the asymmetric carbon atom. The racemisation is favoured by the same factors as the enolisation. This also applies to hyoscine and homatropine. Hyoscyamine and atropine behave similarly with respect to hydrolytic decomposition. The proportion of atropine and tropine in a partially hydrolysed solution may be determined by shaking out the bases and determining their equivalent by titration against standard acid. Comparing the effect of sodium carbonate, ammonia and sodium bicarbonate on atropine, the greatest amount of hydrolysis is produced by sodium carbonate, less by ammonia, and only a small amount by sodium bicarbonate. In assaying drugs, using the ammonia method, high values are therefore due not only to traces of residual ammonia, but partially to tropine. G. M.

Hyoscyamine and Hyoscine, Separation of. G. Schill and A. Ägren. (*Svensk farm. Tidskr.*, 1952, **56**, 55.) A chromatographic method has been worked out for the separation of hyoscyamine and scopolamine. For the pure substances a solution in chloroform is added to a column of kieselguhr acidified with hydrochloric acid, and eluted with chloroform. The eluate contains the hyoscyamine as hydrochloride which is converted to base by passing the eluate through a kieselguhr column containing sodium carbonate; the alkaline column is washed with chloroform to remove the hyoscyamine. The scopolamine is eluted from the first column with chloroform saturated with ammonia; recovery experiments with known amounts of the alkaloids were satisfactory. The same principle is used for separation of belladonna alkaloids in pharmaceutical preparations; an amount of the preparation equivalent to about 0·15 g. of alkaloids is made alkaline with M sodium carbonate and extracted with 200 ml. of chloroform by percolation. The eluate in turn passes three columns; the first containing 4 ml. of M hydrochloric acid and 15 g. of kieselguhr retains the scopolamine, the second containing 4 ml. of M sodium carbonate and 15 g. of

kieselguhr will convert the hyoscyamine hydrochloride to base, while the third containing 4 ml. of 0·5M phosphoric acid and 15 g. of kieselguhr retains the hyoscyamine; the inert substances pass through the columns. The first column is then eluted with 250 ml. of chloroform and the eluate may pass the second and third columns; the scopolamine is eluted from the first column with 250 ml. of chloroform saturated with ammonia, and the solution may pass 10 g. of aluminium oxide. The second column is washed with 50 ml. of chloroform and the eluate is added to the third column. The hyoscyamine is eluted from the third column with 250 ml. of chloroform saturated with ammonia, and the eluate may pass 10 g. of aluminium oxide. The second column is necessary in order not to get low values since if it is omitted part of the hyoscyamine hydrochloride will pass the third column. Recovery experiments were satisfactory. R. E. S.

Nicotinic Acid in Pharmaceutical Products, Determination of. A. Mueller and S. H. Fox. (*J. Amer. pharm. Ass., Sci. Ed.*, 1951, **40**, 513.) When nicotinic acid reacts with cyanogen bromide in the presence of ammonia, a bright yellow colour is produced. The intensity of the colour is proportional to concentration when absorption at the maximum wavelength is plotted against concentration. The maximum wavelength is 408 mμ and the colour reaches its maximum intensity after two to two-and-a-half minutes. The maximum wavelength for nicotinamide is at 389 mμ, reaching its maximum intensity after 6 minutes. Since the rate of reaction with nicotinamide is slower than with nicotinic acid, it is better to hydrolyse the amide; either acid or alkaline hydrolysis may be used. The method of determination is to heat a suitable number of capsules, tablets, etc., with water on a steam-bath until the sample is dispersed; if a fatty basis is present, 3 to 5 ml. of ethylene dichloride is added to dissolve the fat. After cooling, the solution is made up to volume and filtered. If hydrolysis is necessary an aliquot part of the filtrate is evaporated to low bulk with hydrochloric acid, diluted with water and the *p*H adjusted to 2 to 12 with potassium hydroxide; alternatively the filtrate may be heated with 0·5 N sodium hydroxide. The test solution is then treated with a buffered ammonia solution and a 10 per cent. solution of cyanogen bromide in water, and the absorption measured. The experiments are repeated using a standard solution of nicotinic acid, and the result calculated from a given formula. With coloured solutions, a blank determination is also carried out and the absorption of the blank subtracted from the total. Data given showing results obtained with multivitamin capsules with or without ferrous sulphate, ferrous gluconate, liver concentrate, whole dried liver, yeast, and mixtures of these indicate that the method is precise in the application to pharmaceutical preparations.

G. R. K.

Phenol, Bromination of, in Assays. R. Reimschneider. (*Chim. et Ind.*, 1951, **66**, 806.) A number of assay processes depend upon the treatment of phenol with bromine in acid solution, tribromophenol bromide being formed. Methods which measure only the amount of free bromine absorbed in the reaction give results in good agreement with theory. Such methods involve treating phenol with an excess of bromine and reducing the excess with standard arsenious acid or thiosulphate. Methods which depend upon a determination of the quantity of bromide ions remaining in solution after the reaction of the phenol with bromine, estimated by titration after removal of the precipitate and excess of bromine, yield high results, whereas those which depend on weighing the tribromophenol bromide or estimation of bromide in the precipitate, yield low

results. The errors appear to be due to the solubility of the bromo-derivative in acid solutions and possibly also to secondary reactions. G. B.

Sulphonamides, Identification of, on Paper Chromatograms. A. E. Steel. (*Nature, Lond.*, 1951, **168**, 877.) A solution of *n*-butanol, acetic acid and water in the proportions of 50:15:35 by volume has been found a satisfactory solvent for developing sulphonamides on paper chromatograms. R_F values found range from 0·55 (sulphaguanidine) to 0·85 (sulphamerazine). Detection of the spots, carried out in alcoholic solution to minimise spreading, is by spraying the dried paper with nitrous acid solution, a mixture of 0·1 g. of sodium nitrite in 1 ml. of water shaken first after adding 10 ml. of *n*-butanol, and again after the addition of 0·3 ml. of concentrated hydrochloric acid. This is followed after 1 minute, by a 1 per cent. solution of dimethyl-α-naphthylamine or a similar coupling reagent, red or pink indicating a positive result. 1 μg. is detectable by this method, which has also been used for quantitative measurements. J. R. F.

Zinc, Identification and Volumetric Determination of. H. Wachsmuth and J. Cornelis. (*J. Pharm. Belg.*, 1951, **6**, 389.) The addition of a few drops of 0·5 per cent. solution of potassium ferricyanide to a solution containing a small amount of zinc and adrenaline gives a pink colour (sensitivity 1 in 10,000), with diphenylamine a violet colour (sensitivity 1 in 10,000), with benzidine a bluish-violet (blanks should be tried in this case) (sensitivity 1 in 50,000), and with potassium iodide and starch a blue colour (sensitivity 1 in 1,000,000). Nickel, cobalt, copper and manganese interfere giving different colours. For volumetric determination a standard solution containing 32·935 g./l. of potassium ferricyanide and another containing 16·615 g./l. of potassium iodide are used. To 5 ml. of ferricyanide solution add 5 ml. of potassium iodide and an exactly measured volume of the solution to be tested. Titrate the liberated iodine with 0·01N sodium thiosulphate. 2 atoms of iodine are liberated for 3 atoms of zinc. The solution should be neutral or slightly acid with hydrochloric acid, it will have a yellow colour at the end of the titration owing to the excess of ferricyanide. Bismuth, mercury, barium, magnesium, chromium, tin and boric acid have no influence on the reaction, but nickel and cobalt hinder it, as well as copper, but it is easy to determine the latter and then titrate the zinc in the same sample. Add some crystals of potassium iodide and a little starch, wait a quarter of an hour and titrate the copper with sodium thiosulphate and then titrate the zinc as described. Iron must be removed as follows. If necessary, neutralise with 10 per cent. solution of sodium carbonate and then add 10 per cent. acetic acid until the precipitate disappears. Heat to 70° to 80° C. and saturate with hydrogen sulphide. Allow to stand for 10 minutes and centrifuge; wash the precipitate with 10 per cent. acetic acid. If there is much iron repeat the operation. Dissolve the precipitate in 1 ml. of concentrated hydrochloric acid, dilute to 40 ml. and boil off the hydrogen sulphide. Cool and determine the zinc as above. H. D.

ESSENTIAL OILS

***Lippia carviodora* from Somaliland, Oil from.** H. T. Islip and W. S. A. Matthews (*Colonial Plant and Animal Prod.*, 1951, **2**, 96.) A sample of dried leaves from the Somaliland Protectorate yielded on steam distillation 3·15 per cent. of a pale yellow oil having an odour reminiscent of caraway. The characters and composition of the oil were compared with oils distilled in Kenya from the same species in 1944 and 1945 respectively. Sample A was distilled

from a mixture of 36-lb. flower heads and 1-lb. each of leaves and twigs which yielded only 0·99 per cent. The composition of the three oils is summarised in the table. Although the oils from the two localities were similar the yield from the Somaliland leaves was much greater than that from flower heads or leaves from Kenya. It is considered that the oil should find a market in the United Kingdom especially as a source of carvone.

	Sample from Somaliland	Sample A from Kenya	Sample B from Kenya
	per cent. w/w	per cent. w/w	per cent. w/w
Ketones, mainly *d*-carvone	67·3	67·9	60·2
Free alcohols as linalool	2·9	2·4	3·5
Acids, as acetic	Negligible	0·1	0·1
Esters as linalyl acetate	0·7	1·3	4·1
Terpenes (mainly *d*-limonene) sesquiterpenes and undetermined	29·1	28·3	32·1

G. R. A. S.

FIXED OILS, FATS AND WAXES

Niger Seed Oil from Tanganyika. R. W. Pearman, W. D. Raymond and J. A. Squires (*Colonial Plant and Animal Prod.*, 1951, 2, 101.) Three samples of seed of *Guizotia abyssinica* Cass., grown in Tanganyika yielded to extraction with light petroleum from 35 to 38 per cent. of fixed oil. The characters of these oils are tabulated and compared with published figures. Niger seed oil being a drying oil finds application in paints and can also be used for the manufacture of soap. As an edible oil it is satisfactory when fresh, but has the disadvantage that it deteriorates rather rapidly. G. R. A. S.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

Sapote Gum, Composition of. E. Anderson and H. D. Ledbetter. (*J. Amer. pharm. Ass. Sci. Ed.*, 1951, **40**, 623.) Crude Peruvian sapote gum is the exudate which flows from the wounds of trees which have been tapped for the latex from which chicle is prepared. It contains 6 to 10 per cent. of resin which may be separated by heating with water and filtering. The water-soluble gum may be purified by decolorising with bromine and precipitating with ethanol. It contains calcium and magnesium salts of a methoxyuronic acid containing about 2 uronic acid and 7 anhydropentose groups to each methoxyl. The pentosan material (64·7 per cent. of the crude gum) is derived from D-xylose and L-arabinose in the ratio 8·5 :1. Most of the L-arabinose is liberated by hydrolysis in dilute acid at 80° C. for 2 hours, and heating for 4 hours removes most of the D-xylose, 2 sugar units remaining attached to each uronic acid group. Drastic hydrolysis (autoclaving) fails to remove all the D-xylose from combination with the uronic acid. G. B.

GUMS AND RESINS

***Podophyllum emodi* Wall, New Compounds from.** M. V. Nadkarni, P. B. Maury and J. L. Hartwell. (*J. Amer. chem. Soc.*, 1952, **74**, 280.) In the search for tumour-necrotising components of the resin from different species of *Podophyllum*, the resin from *P. emodi* Wall was investigated using chromatography with activated alumina. Three colourless crystalline compounds were isolated, their order of decreasing strength of absorption being podophyllotoxin (36 to 39 per cent. yield), demethylpodophyllotoxin (I) (1·7 per cent.) and

1-O-(β-D-glucopyranosyl)-picropodophyllin (II) (1·8 per cent.). Compounds I and II are new. Compound I crystallised in colourless transparent prisms, m.pt. 250·0° to 251·6° C., from ethanol, and methylation of this substance with diazomethane produced podophyllotoxin. Compound II crystallised in long, thin, colourless needles from 75 per cent. methanol or water, m.pt. 237·0° to 238·2° C. Hydrolysis of II with dilute hydrochloric acid gave D-glucose and picropodophyllin. The glucoside II was shown to be probably a β-glucopyranoside. Compound I is active in producing hæmorrhage and necrosis in Sarcoma 37 in mice, while II is inactive even in high doses. A. H. B.

TOXICOLOGY

Arsenic in Hair, A Method of Localisation for Use in Toxicology. H. Griffon and J. Barbaud. (*Ann. pharm. franç.*, 1951, **9**, 545.) Hairs are cut off at the scalp, fastened together keeping the cut ends level, washed in acetone, ethanol, water and again in acetone and dried, before being submitted to neutron bombardment to induce radioactivity. By means of a Geiger-Muller counter and a lead slit, the activity is determined over each 2 mm. portion along the length of the bundle of hairs. Radioactivity induced in the natural constituents of the hair decreases in a regular manner from the cut end to the tip, on account of the decrease in thickness of the hair and of the decay in radioactivity during measurement. For normal hair the graph relating radioactivity to length is a straight line, and undulations indicate the presence of adventitious arsenic which can be confirmed by study of its radioactive period (26 hours 8 minutes). From the rate of hair growth and position of the arsenic deposits, the time of arsenic intoxication can be calculated, and this generally accords more exactly with the facts than do the results of chemical methods which do not localise the arsenic along the length of the hair with such great accuracy. G. B.

β-*p*-Hydroxyphenylpropionic Acid in Viscera, Identification of. G. Roche Lynch. (*Analyst*, 1951, **76**, 610.) The crude isolate is purified by grinding with light petroleum (40° to 50° C.) and recrystallised from benzene. The crystals are soluble in water and benzene but not in light petroleum. The mol. wt. is 166 and the empirical formula $C_9H_{10}O_3$. The crystals, a pure sample of the acid and a mixture of the two, melt at 126·5° to 127·5° C., thus confirming the nature of the isolate. The acid when treated with bromine in acetic acid yields a crystalline bromo derivative m.pt. 112·5° to 113·5° C. ex water. Also it gives a very strong brownish red colouration on coupling with diazotised sulphanilic acid in alkaline solution. J. R. F.

β-*p*-Hydroxyphenylpropionic Acid in Viscera, Note on the Occurrence of. L. C. Nickolls. (*Analyst*, 1951, **76**, 609.) An extraction of the viscera from an exhumed body, subjected to anærobic conditions, with the purpose of isolating a suspected barbiturate fraction, yielded yellow crystals the m.pt. of which was raised by purification to 128° C. This crystalline compound gave results similar to barbiturates with a series of chemical reactions, but sodium fusion and microanalysis showed a negative result for nitrogen, and mixed m.pts. with the common barbiturates were much lower. A pure sample of β-*p*-hydroxyphenylpropionic acid gave all the reactions for the unknown material and had m.pt. and a mixed m.pt. with the unknown of 128° C., thus confirming identity. It is suggested that the mixed m.pt. should always be employed in the identification of suspected barbiturates in viscera. J. R. F.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Adrenaline, Inhibition of Oxidation by Borate. E. M. Trautner and M. Messer. (*Nature, Lond.*, 1952, **169**, 31.) When dilute solutions of adrenaline, M/500 to M/2000 were allowed to oxidise in phosphate and borate buffers, it was found that the oxygen uptake in phosphate-buffer increased rapidly as the *p*H was increased above 7·0, but with borate buffer remained very low and almost unchanged up to a *p*H of just above 9·0. Above *p*H 9·5 the oxidation-rates increased markedly, though they were still lower than in other buffers at the same *p*H. Adrenaline was also allowed to oxidise in phosphate buffer in the presence of varying concentrations of borate. About 50 per cent. inhibition occurred when adrenaline and borate were present in equimolar proportions, at *p*H 7·8, 37·5° C., the concentrations of each being M/500. It is assumed that the borate forms complexes with the adrenaline, blocking the hydroxyl groups and hindering the onset of oxidation. Apparently borate also forms complexes with the oxidation products of adrenaline. The complexes are apparently unstable above *p*H 9 and on dilution. When adrenaline-borate mixtures were injected intravenously into an anæsthetised cat, they showed neither inhibition nor intensification, nor prolongation of the adrenaline effect on the blood pressure and the nictitating membrane. A. H. B.

Antibiotics, Microscopic Identification of. O. Landgren. (*Farm. Revy.*, 1951, **50**, 781.) The crystal characters of antibiotics may be determined by the following scheme. For the test a series of liquid mixtures of *iso*-amyl *iso*-valerate, α-bromonaphthalene, liquid paraffin and methylene iodide are prepared, showing a range of refractive index at 0·01 intervals. The observations are made as follows:—The solid substance is observed microscopically in a liquid having about the expected refractive index. After focusing, the tube is raised, when a bright line is observed following the contour of each crystal. On raising the tube this line wanders towards the medium (crystal or liquid) which has the highest refractive index. By a series of trials with different liquids the refractive index of the crystal is determined. The preparation is observed in polarised light, without analyser: if all crystals are equally bright, the material is monochroic; if there are varying colours, it is pleochroic; and if some are light and

	Crystal character	Refractive index
Chloramphenicol	monochroic anisotropic	1·52 to 1·53
Dihydrostreptomycin sulphate	isotropic, glassy	1·54 to 1·55
do.	dichroic, anisotropic, microcrystalline	1·55 to 1·56
Streptomycin sulphate	isotropic, glassy	1·54 to 1·55–1·56
Streptomycin-calcium chloride	isotropic, glassy	1·55 to 1·56–1·57
Penicillin-potassium	pleochroic, anisotropic	1·57 to 1·58
Procaine-penicillin	monochroic, anisotropic	1·57 to 1·58
Penicillin-sodium	anisotropic laminae	1·59 to 1·61
Aureomycin base	pleochroic, anisotropic, tabular	1·70 to 1·74
Aureomycin hydrochloride	monochroic, isotropic, glassy	1·66 to 1·67
Penicillin-calcium	isotropic, glassy	1·57 to 1·58
Bacitracin	isotropic, amorphous	1·54 to 1·55
Polymyxin B sulphate	isotropic, amorphous	1·53
Terramycin hydrochloride	monochroic, anisotropic	1·55 to 1·56
Tersavin (ephedrine-penicillin)	dichroic, anisotropic, laminae	1·56 to 1·57
Usnic acid	dichroic, anisotropic, columns	1·61 to 1·62

others dark, it is dichroic. Between crossed Nicols, if all crystals are dark they are isotropic; if they are light or coloured in certain positions, they are anisotropic. With the latter there is more than one refractive index, and if the form of the crystals is such that they lie in different positions these can be determined. Observed characters of antibiotics are as shown in the table (where 3 values are given for the refractive index, the last figure represents a higher value which was noted after several hours). G. M.

Fungicides, Organic, A New Class of. A. R. Kittleson. (*Science*, 1952 **115**, 84.) *N*-trichloromethylthio derivatives of imides, hydantoins, 2:4-oxazolidinediones and sulphonamides were prepared as follows. Disperse 1 mole of the sodium derivative in 1000 ml. of benzene and add during 2 to 3 hours, by dropping funnel, 1 mole of perchloromethyl mercaptan, stirring and heating gradually and continuing to boil under a reflux condenser for 4 to 6 hours. Filter to remove sodium chloride, concentrate, cool and collect the precipitated *N*-trichloromethylthio derivative on a filter. *N*-trichloromethyl-thiomorpholine was prepared similarly from perchloromethyl mercaptan and an excess of morpholine. Derivatives were stable compounds, obtained in good yield and had LD50 less than 10 parts per million against *Alternaria solani* and *Sclerotinia fructiola* by the slide germination technique. G. B.

Riboflavinyl Glucoside. L. G. Whitby. (*Biochem. J.*, 1952, **50**, 433.) A new derivative of riboflavine is described, prepared by incubation of riboflavine with an enzyme obtained from rat liver. An aqueous solution is stable at 100° C., but N sodium hydroxide at 100° C. rapidly destroys the flavine by attacking the *iso*alloxazine nucleus; the compound is hydrolysed by strong acids, with the production of riboflavine, the reaction being completed in 2·5 hours in N hydrochloric acid at 100° C. The molecular absorption coefficient at 450 mμ and the positions of the maxima and minima are the same as reported for riboflavine by Singer and Kearney, although in the region 440 to 310 mμ the absorption of the new flavine is consistently 2 to 3 per cent. less intense. The elementary composition of the substance was found $C_{23}H_{30}O_{11}N_4$; the structure of the compound is discussed and is identified as 5′-D-riboflavine-D-glucopyranoside (riboflavinyl glucoside). It is suggested that the glucosidic linkage has the α-configuration. Preliminary investigations of the enzymic reaction indicate that the enzyme catalyses a transglycosidation of D-glucose from maltose or glycogen to riboflavine. R. E. S.

BIOCHEMICAL ANALYSIS

Aneurine, Microbiological Assay of. S. C. Fang and J. S. Butts. (*Proc. Soc. exp. Biol., N.Y.*, 1951, **78**, 463.) The test organism used is *Lactobacillus fermenti* 36 and the extent of growth is determined by measurement of the turbidity by means of a Klett-Summerson photo-colorimeter using a 54 filter after 16 hours at 37° C. The medium contains takadiastase-digested starch which has a stimulant effect on the growth of the test organism; it also contains filbert nut meal extract which appreciably increases the rate of growth. In the assay of foodstuffs the sample is digested with a mixture of takadiastase and papain, and it is likely that any starch will break down into dextrin and maltose which stimulate the growth of the organism. The authors believe that enzyme-digested starch is one of the many stimulants which may be responsible for erratic results in the assay of the B vitamins of some natural products, and recommend its addition to the assay medium. H. T. B.

Aneurine Salts, Titration of, with Perchloric Acid. C. W. Pifer and E. G. Wollish. (*J. Amer. pharm. Ass. Sci. Ed.*, 1951, **40**, 609.) Aneurine in glacial acetic acid solution may be titrated with perchloric acid. Halogen ions must be removed by the addition of mercuric acetate before titration. The following method is suggested for the assay of aneurine hydrochloride. Dissolve about 0·6 g. by warming with 80 ml. of glacial acetic acid, cool, add 10 ml. of 6 per cent. mercuric acetate reagent and titrate with 0·1N perchloric acid. The end-point is detected potentiometrically or by using crystal violet as indicator; each molecule of aneurine neutralises 2 equivalents of acid. Mercuric acetate need not be added when aneurine mononitrate is being assayed. The reaction is not specific and a number of compensating or limit tests for specific impurities is suggested. For example, 2-methyl-5-ethoxymethyl-6-aminopyrimidine, 4-methyl-5-β-(hydroxyethyl)thiazole and 2-methyl-5-bromoethyl-6-aminopyrimidine can be extracted with ether from aneurine in alkaline solution, dissolved in glacial acetic acid and titrated with perchloric acid; 6-amino-5-aminoethyl-2-methylpyrimidine may be estimated by the red colour (absorption maximum, 560 mμ) which it gives with ninhydrin. G. B.

Calcium in Biological Fluids, Determination of. I. J. Greenblatt and S. Hartman. (*Anal. Chem.*, 1951, **23**, 1708.) The determination depends on the fact that calcium in an ionic state, in the presence of ammonium purpurate produces a pink colour and when titrated with a solution of disodium dihydrogen ethylenediamine tetra-acetate dihydrate, the calcium is firmly bonded in an ionised soluble complex which turns orchid-purple. The biological fluid is pipetted into a suitable vessel, the *p*H adjusted with sodium hydroxide and ammonium purpurate is added to produce a salmon-pink colour. The mixture is then titrated with disodium dihydrogen ethylenediamine tetra-acetate dihydrate, until a stable orchid-purple colour is obtained, which, upon the addition of another drop of disodium dihydrogen ethylenediamine tetra-acetate dihydrate, will not alter; a standard calcium solution is treated similarly. Excellent agreement was obtained in comparison with potassium permanganate methods in which the calcium was precipitated as an oxalate. In hæmolytic serum with a marked increase of ionic iron, and in jaundice serum with a marked increase in bile pigments, a blurring of the sharp, distinct end point ordinarily obtained in titrating clear serums was experienced. R. E. S.

Phenylpyruvic Acid in Urine, Estimation of. J. P. Berry and L. I. Woolf. (*Nature, Lond.*, 1952, **169**, 202.) Urine from phenylketonurics contains an ether-soluble acid, phenolic in nature which gave false high results in Penrose and Quastel's method (*Biochem. J.*, 1937, **31**, 266) for phenylpyruvic acid, and the following method was therefore worked out. The urine is acidified (*p*H 1) and extracted with ether; the ether is evaporated in a stream of nitrogen at room temperature and the residue dissolved in a glycine-sodium chloride-hydrochloric acid buffer at *p*H 2·2. Ferric chloride is added and the resulting colour read at its maximum (2 to 3½ minutes after mixing) in a photoelectric colorimeter using Ilford 607 filter ($\lambda_{max.}$ = 600 mμ); fading was rather slow. A straight line graph is given, obtained with pure phenylpyruvic acid; recovery of phenylpyruvic acid added to normal urine varied from 98 to 100 per cent. The results are quoted for several urines in which phenylpyruvic acid was estimated (*a*) by this method and (*b*) by the dinitrophenylhydrazone method (modified for photoelectric colorimetry); in all cases method (*a*) gave results considerably lower than those obtained by method (*b*). *p*-Hydroxyphenylpyruvic acid and homogentisic acid

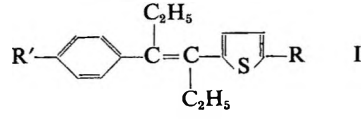
do not interfere in the proposed method, since the blue-green colour they give fades completely within 1 minute. By the dinitrophenylhydrazone method, *p*-hydroxyphenylpyruvic acid gives, mole for mole, 1·3 times the colour intensity given by phenylpyruvic acid. The nature of the interfering substance is unknown. R. E. S.

Propylthiouracil in Urine, Determination with 2:6-Dichloroquinone-chloroimide. R. A. McAllister. (*J. clin. Path.*, 1951, **4**, 432.) Take 100 ml. of urine and adjust the *p*H to 6·0. Transfer 50 ml. or an aliquot containing up to 5 mg. of propylthiouracil, to a small separating funnel. Extract 3 times with 100 ml. amounts of peroxide-free ether. Pool the extracts and wash once with 100 ml. of water. Evaporate the pooled extracts to dryness and dissolve the residue in 2 ml. of aldehyde-free absolute ethanol. Wash the solution into a flask and make up to 100 ml. Take, in 2 tubes, aliquots of 2 and 1 ml. and adjust the volume of each to 5 ml. with water, add 5 ml. of borate buffer *p*H 8 and 0·1 ml. of 0·4 per cent. chloroimide reagent. Mix the contents in each tube and allow to stand during 45 minutes. Add 10 ml. of chloroform to each, and shake until the yellow colour is extracted. Allow to settle and remove the aqueous supernatant liquid by suction. Filter through a small No. 42 Whatman paper and read in a Spekker absorptiometer against chloroform using the violet filter. Prepare a standard reference graph by applying the colour reaction to 10, 20, 30, 40, 50 and 100 μg. of propylthiouracil, each in 5 ml. of water, and extract the colours with 10 ml. of chloroform. J. R. F.

CHEMOTHERAPY

Barbituric Acid, Thiocyanate and *iso*Thiourea Derivatives of. G. S. Skinner and W. H. Waitz. (*J. Amer. chem. Soc.*, 1952, **74**, 498.) A series of thiocyanate and *iso*thiourea derivatives of barbituric acid of type $RR'C(CONH)_2CO$ where R = C_2H_5–; *n*-C_3H_7–; *n*-C_4H_9–; *i*-C_5H_{11}; *n*-C_5H_{11}; and R′ = $NCSCH_2CH_2$–; $(H_2N{=}C(NH_2)SCH_2CH_2–)^+Br^–$; $HN{=}C(NH_2)SCH_2CH_2$–, were prepared and subjected to pharmacological testing. Both the thiocyanates and the *iso*thiouronium salts were easily prepared in good yields by the action of potassium thiocyanate and thiourea, respectively, on the β-bromoethylbarbituric acid derivates in ethanol. The *iso*thiouronium salts were converted to the *iso*thiourea derivatives by treatment of their warm aqueous solutions with a slight excess of ammonia. The thiocyanates resisted hydrolysis without cleavage of the ring but the *iso*thiourea derivatives were smoothly hydrolysed to the mercaptobarbituric acids. None of the compounds produced hypnosis or anæsthesia, but the *iso*thiouronium bromides exhibited some anticonvulsant activity. A. H. B.

Diethylstilbœstrol, Some Thiophene Analogues of. W. R. Biggerstaff and O. L. Stafford. (*J. Amer. chem. Soc.*, 1952, **74**, 419.) Although compound Ia (R′ = H, R = H) had previously been shown to possess only a low order of œstrogenic activity, compounds Ib (R′ = OH, R = H) and Ic (R′ = OH, R = Br) were prepared because the introduction of a 4-hydroxyl group into the α:α'-diethylstilbene nucleus greatly enhanced the œstrogenic activity.



I

Compound Ib was much more active than Ia and produced 50 per cent. œstrus (rats) in 100-μg. doses and 100 per cent. at 250 μg. It also appears to be much more active than its benzene analogue, 4-hydroxy-α:α'-diethylstilbene. The introduction of a bromine atom in the thiophene ring (compound Ic) results in a decided lowering of the activity. The synthesis of the above compounds is described. A. H. B.

***N*-Substituted Diallylbarbiturates, Antipyretic and Analgesic Activity of.** F. Sandberg. (*Svensk farm. Tidskr.*, 1951, **55**, 698.) A number of derivatives were tested for antipyretic and analgesic action. These were 1-(*N*-phenylcarbamylmethyl)-5:5-diallylbarbituric acid; 1-(*N*-*p*-ethoxyphenylcarbamylmethyl)-5:5-diallylbarbituric acid; 1-(*N*-*p*-allyloxyphenylcarbamylmethyl)-5:5-diallylbarbituric acid; 1:3-di (*N*-phenylcarbamylmethyl)-5:5-diallylbarbituric acid; 1:3-di(*N*-*p*-ethoxyphenylcarbamylmethyl)-5:5-diallylbarbituric acid and 1:3-di(*N*-*p*-allyloxyphenylcarbamylmethyl)-5:5diallylbarbituric acid. Their action was very slight. This result was unexpected, since it would be expected that the metabolism for these compounds would be similar to that of other aniline derivatives such as acetanilide or phenacetin. It was also found that a molar mixture of diallylbarbituric acid with acetanilide, phenacetin or *p*-allyloxyacetanilide produced a greater antipyretic and analgesic effect than the chemical compounds of these components, owing to an additive synergism. There was, however, one exception—there was no synergism between the analgesic effect of diallylbarbituric acid and *p*-allyloxyacetanilide. It was also noted that the latter compound, unlike acetanilide and phenacetin, appreciably shortened the duration of the anæsthetic action of diallylbarbituric acid. G. M.

PHARMACY

DISPENSING

Calcium Gluconate Solutions, Stabilisation of. S. Balasundaram and V. Subrahmanyan. (*Indian J. Pharm.*, 1951, **13**, 179.) Solutions containing 10 per cent. of calcium gluconate were stabilised by the addition of 0·6 per cent. of boric acid; 15 per cent. solutions required 0·9 per cent. of boric acid. Adjustment of the *p*H to 6·8 to 7·0 resulted in a slight decrease in the amount of boric acid required. The following concentrations of other stabilisers were necessary to prevent crystallisation in 10 per cent. solutions of calcium gluconate: calcium saccharate, 0·8 per cent.; calcium galactonate, 0·8 per cent.; and calcium lactobionate, 0·6 per cent. 1 per cent. of calcium lactate stabilised a 9·6 per cent. solution of calcium gluconate. The following substances were unsatisfactory as stabilisers: lactic acid, glucose, fructose, sucrose, sodium chloride, sodium bromide and sodium iodide. G. R. K.

NOTES AND FORMULÆ

Antiseptic Power of Some Ointments, Influence of Excipients on. A. Mirimanoff and F. Ducommun. (*Pharm. Acta Helvet.*, 1951, **26**, 387.) Bacteriostatic power against *Staphylococcus aureus* was determined for a number of substances. The most powerful bacteriostatics tested were phenylmercuric borate (merfen), domiphen (bradosol) and désogène, a mixture of methosulphates of quaternary trimethylammonium bases. The bacteriostatic power of certain substances was increased in the presence of cationic detergents, for example, formaldehyde, mercurochrome and boric acid with bradosol and phenol with désogène. This exaltation could not be confirmed with anionic

detergents. No exaltation or bacteriostatic power was observed with the non-ionic detergents crillex 11, carbowax 1500 and tween 20. Ointments were tested against *S. aureus* by a cylinder-plate method. Zones of inhibition were measured after 36 hours' incubation with ointments prepared from the following bases: (1) soft paraffin/lanolin (water in oil), (2) glyceryl monostearate and paraffins (oil in water) and (3) bentonite. Formaldehyde, boric acid, mercuric oxycyanide, silver proteinate, merfen, mercurochrome and penicillin were incorporated. In general the antiseptics diffused most readily from the bentonite base and least from the water in oil base. It was not possible to give a general rule, but it was frequently observed that anionic detergents increased the diffusion of the antiseptic while non-ionic detergents decreased it. The method of testing was not suitable for cationic detergents. G. B.

Chlorcyclizine Hydrochloride (Di-Paralene Hydrochloride). (*New and Non-official Remedies, J. Amer. med. Ass.*, 1952, **148**, 286.) Chlorcyclizine hydrochloride is 1-*p*-chlorobenzhydryl-4-methylpiperazine hydrochloride and occurs as a white, odourless, bitter, crystalline solid, m.pt. 222° to 227° C., soluble in water (1 in 1·6), ethanol (1 in 10·4) and chloroform (1 in 3·6) and almost insoluble in benzene and ether; a 1 per cent. solution has *p*H 5·0 to 5·5. The picrate obtained by treating a 1 per cent. solution with picric acid melts at 215° to 219° C., with decomposition. Chlorcyclizine hydrochloride loses not more than 2 per cent. of its weight when dried for 3 hours at 120° C., and yields not more than 0·20 per cent. of residue on ignition. A 0·001 per cent. w/v solution in ethanol exhibits an ultra-violet absorption maximum at 2300 Å. ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$, 443 ± 10) and a minimum at 2180 Å. It is used as a histamine antagonist in a dose of 50 mg. 2 or 3 times daily. G. R. K.

PHARMACOGNOSY

Agar-yielding Seaweeds from the Philippines. M. Cantoria, G. T. Velasquez and P. Valenzuela. (*J. Philipp. pharm. Ass.*, 1951, **38**, 295). A survey of the red seaweeds growing near the Philippines and likely to yield agar has been made. The identification and characters of the three most promising plants *Hypnea musciformis* var. *hipporoides* (Kuetz.) Web. v. B., *Gracilaria canaliculata* (Kuetz.) Sond. and *Gracilaria lichenoides* (L.) Gmel. are discussed. The appearance of transverse sections of the thallus, drawings and photographs of which are given, together with the staining effects of ruthenium red solution B.P. were found useful in identifying the plants. J. W. F.

***Hyoscyamus muticus* L. Morphology and Histology of the Flowering Tops.** A. H. Saber and S. I. Balbaa. (*Reports Pharm. Soc., Egypt*, 1951, **33**, 29.) A detailed description of the inflorescence and of the macroscopical and microscopical characters of the flower are given, as well as a table comparing the characters with those of the flower of *Hyoscyamus niger* L. J. W. F.

***Myroxylon pereirae* Klotzch, a Source of Nerolidol.** R. Cortesi. (*Bull. Soc. Pharm., Bordeaux.*, 1951, **89**, 141.) Portions of the trunks of healthy and of wounded trees of *Myroxylon pereiræ* from San Salvador were examined anatomically and chemically. The heart wood is distinguished chiefly from the sap wood by the presence of a reddish-brown secretion; this secretion appears to originate in the medullary rays and passes into the neighbouring fibres and finally into the vessels in which it resinifies and blocks up the lumen. The amount of volatile fraction of the secretion in the wounded trunk was

30 per cent. higher than in the healthy one, thus confirming the general rule that pathological conditions increase the flow of secretion. The most important constituent of this volatile fraction is nerolidol which is a valuable source material for the synthesis of α-tocopherol. Nerolidol is obtained from balsam of Peru (the commercially prepared oleoresin from *Myroxylon pereiræ*) but is present only in traces, whereas the volatile fraction secreted in the heart wood of this tree contains up to 70 per cent. Probably, during the stages of burning, boiling in water, etc., involved in the preparation of balsam of Peru, the bulk of the volatile nerolidol is lost. The author suggests that by taking these facts into account the tree could be used as a valuable source of natural nerolidol.

J. W. F.

PHARMACOLOGY AND THERAPEUTICS

Aconitine and Lappaconitine, Toxicity of. F. Dybing, O. Dybing and K. B. Jensen. (*Acta Pharmacol. Toxicol.*, 1951, **7**, 337.) Lappaconitine is the chief alkaloid in *Aconitum septentrionale*, a plant common in parts of Scandinavia, which occasionally causes poisoning in cattle; the dried root contains up to 5·8 per cent. of total alkaloids of which about 80 per cent. is lappaconitine. Experiments on mice showed lappaconitine to have 1/20 to 1/40 the toxicity of aconitine, according to the method of administration. Both aconitine and lappaconitine are relatively stable to heating in aqueous solutions at *p*H 3, whereas they are easily hydrolysed in neutral solutions. Lappaconitine and its hydrolytic products—picrolappaconitine and lappaconine—can be identified by their R_F values in paper chromatography. Picrolappaconitine shows blue fluorescence in ultra-violet light and can be diazotised and coupled with β-naphthol to produce a red colour. Lappaconitine and picrolappaconitine can be isolated from and chromatographically identified in urine from rats given sub-lethal doses of lappaconitine. A method is described for the isolation of aconitine from organic material, and its biological demonstration by a vomiting-like reflex produced in mice by subcutaneous injection. S. L. W.

Adrenaline and Noradrenaline, Concentrations in Adrenal Glands at Different Ages. G. B. West, D. M. Shepherd and R. B. Hunter. (*Lancet*, 1951, **261**, 966.) The amine content of the adrenal glands of adults and babies has been examined. The total pressor activity in exhausted adrenal glands of 36 cases was 0·303 mg./g. About 14 per cent. of this was noradrenaline. In 9 cases of hypertension the relative amount of noradrenaline in the gland was not significantly different from that found in other conditions. Lack of methylation was not considered the cause of hypertension in these patients. The activities of the medullary and cortical components of the glands were compared in 9 cases. 14 per cent. of the total activity in the medulla but only 3 per cent. in the cortex was found to be due to noradrenaline. This suggests that noradrenaline is formed in the medulla. Extracts from glands of children under 70 days old showed incomplete methylation, about 90 per cent. of noradrenaline being present. Large quantities were also found in the organs of Zuckerkandl. In one case of Addison's disease 50 per cent. of the total catechol amines was noradrenaline.

J. R. F.

Adrenaline and Noradrenaline; Elimination of. A. Lund. (*Acta Pharmacol. Toxicol.*, 1951, **7**, 297.) The destruction of adrenaline and noradrenaline in the rabbit has been studied on intact animals and isolated organs; both substances behaved in a similar manner. In the intact organism either substance disappeared from the blood circulation at a rate of about 10 μg./kg./minute. In

the perfused liver there was a maximum rate of destruction of 10 mg./kg./minute. In the perfused hind limb the maximum rate of destruction was about 3 μg./kg./minute. The prompt elimination of adrenaline and noradrenaline would appear to be due to a combination of the following processes: (1) complete destruction, consisting chiefly in oxidative deamination of adrenaline and noradrenaline in the blood passing through the liver; (2) prompt diffusion from the blood circulation into the muscular tissue; followed by (3) a slower oxidation through the adrenochrome stage, effected by the cytochromoxidase; and possibly (4) excretion of adrenaline and noradrenaline, partially transformed (esterified), through the kidneys. S. L. W.

Adrenocorticotrophic Hormone in Slow Release Medium. H. M. Bruce and A. S. Parkes. (*Lancet*, 1952, **262**, 71.) The clinical use of adrenocorticotrophic hormone is probably highly inefficient due to over-rapid absorption after the parenteral injection of a rapidly absorbed preparation. It remains in the circulation for less than 30 minutes and adrenal stimulation may be ended within an hour of its absorption. In practice this short duration of the action is counteracted by the frequent injection of small doses. With the object of eliminating the need for frequent injection experiments were carried out on nestling rats to compare the effectiveness of different media in prolonging the activity of the hormone, assays being based on the decrease in weight of the thymus glands of the experimental animals. Suspension in a medium consisting of 5 per cent. of beeswax in arachis oil increased the effectiveness of daily injections by at least 10 times as compared with solution in saline solution or suspension in oil alone. Large doses given as a single injection in the oil-wax medium produced an effect for several days. A preparation containing aluminium stearate was less effective. No work has yet been done on the effect if any of the particle size of the suspended hormone. H. T. B.

Alkyl Sugar Derivatives, Absorption and Excretion of. F. R. Skelton, H. M. McConkey, J. K. Souch and G. A. Grant. (*J. Amer. pharm. Ass. Sci. Ed.*, 1951, **40**, 626.) In rats, the 3-ethyl, 3-propyl and 3-butyl derivatives of glucose, and the 3-methyl and 1-methyl derivatives of fructose are almost quantitatively excreted after they have been administered intraperitoneally. Under the same conditions, about 70 per cent. of 3-methylglucose is excreted. Varying amounts are excreted after a single oral administration and similar results are obtained when alkyl sugars are added in a proportion of 20 per cent. to a synthetic diet. 3-methylglucose appears to be completely absorbed from the gut. Diuresis is most pronounced following administration of 3-methylglucose and 3-ethylglucose. No toxicity or metabolic adaptation is observed when 20 per cent. of 3-methylglucose is included in the diet for 1 year. In dogs, diuresis and loss of sodium are most marked for 3-methylglucose and 3-ethylglucose. In repeated administration the former is the more effective diuretic.

Compound	Percentage excreted in the urine following oral administration	
	Rats	Dogs
3-methylglucose ..	85	85
3-ethylglucose ..	25	68
3-methylfructose ..	20	17
1-methylfructose ..	60	75
3-propylfructose ..	27	
3-butylfructose ..	35	

G. B.

p-Aminosalicylic Acid and m-Aminophenol, Antithyroid Effect of. K. Kjerulf-Jensen and G. Wolffbrandt. (*Acta Pharmacol. Toxicol.*, 1951, **8**, 376.) *p*-Aminosalicylic acid given to rats in a daily dose of 10 mg. for 10 days caused a moderate cellular thyroid hyperplasia which was prevented by the simultaneous administration orally of dried thyroid tissue, but not of sodium iodide. Feeding with *p*-aminosalicylic acid also caused thyroid hyperplasia in mice and rabbits, but not in guinea pigs. *m*-Aminophenol, one of the degradation products of *p*-aminosalicylic acid in the body, also caused thyroid hyperplasia in rats. The thyroid hyperplasia induced by either of these substances is qualitatively comparable with that produced by thiouracil; quantitatively, however, the effect of *m*-aminophenol in rats as a maximal thyroid blocking agent was found to be only about 1 per cent. of that of 6-methyl-2-thiouracil. The degree of antithyroid activity may be compared with that of *p*-aminobenzoic acid. These experiments, together with clinical observations, indicate that the development of artificial myxœdema and goitre may, although reversible and rare, be a possible side-effect of *p*-aminosalicylic acid therapy. S. L. W.

Benzilic Acid Derivatives, Pharmacology of. O. C. Forbes and P. B. Marshall. (*Brit. J. Pharmacol.*, 1951, **6**, 634.) 11 benzilic acid derivatives structurally related to diphenhydramine and trasentin were found to possess varying degrees of general spasmolytic, antihistamine and local anæsthetic properties. Their antihistamine properties were low compared with diphenhydramine and mepyramine, but anti-acetylcholine activity was higher in several of the compounds than that of trasentin. Two compounds, namely diethylaminoethyl diphenyl-(β-morpholinoethoxy)-acetate (3–0257) and β-diethylamino*iso*propyl diphenyl-(β-dimethylaminoethoxy)-acetate (3–0281)), are more potent local anæsthetics than procaine, being more than twice as active. All the compounds lower arterial blood pressure in the cat, and this effect is not abolished by atropine. The presence of a morpholino group in the molecule was found to diminish all types of pharmacological action. S. L. W.

Boric Acid, Phagocyticidal and Antibacterial Action of. M. Novak and W. I. Taylor. (*J. Amer. pharm. Ass., Sci. Ed.*, 1951, **40**, 428.) Using blood drawn aseptically from healthy adult male donors, phagocytosis was found to be inhibited by concentrations of boric acid higher than 2 per cent. at temperatures of 37° or 40° C. It was partially inhibited by a 2 per cent. concentration, and totally inhibited by higher concentrations, at 34° C. Using a suspension of *Micrococcus pyogenes* var. *albus* a 4 per cent. boric acid concentration was not toxic to 100 per cent. of the cocci but was toxic to 100 per cent. of the phagocytes. Boric acid is soluble at room temperature to the extent of about 5 per cent. and this concentration is often used as a so-called mild antiseptic. The solubility of boric acid at 0° C. is 1·95 per cent. and it is recommended that refrigerated boric acid solutions only should be employed to ensure non-phagocyticidal concentrations. S. L. W.

Chloramphenicol Palmitate; Use in Pædiatrics. V. de P. Larkin. (*Proc. Soc. exp. Biol., N.Y.*, 1951, **78**, 191.) A suspension of chloramphenicol palmitate containing 125 mg. of chloramphenicol in 4 ml. was used in the treatment of 17 patients, whose ages ranged from 11 days to 9 years, with a variety of diseases. The dosage was 50 mg./kg. of body weight in 24 hours, divided in 4 or 6 equal doses and continued until the patient was clinically cured, from 3 to 7 days. All the patients responded promptly, the suspension being taken without difficulty. No instances of nausea, vomiting, diarrhœa, drug fever or drug eruption were observed in this series of cases. S. L. W.

LETTER TO THE EDITOR

The Determination of Santonin in Artemisia—Solubility Correction

SIR,—Many methods of assay for santonin in artemisia depend, in their final stages, upon the weight of santonin crystallised from ethanol (15 per cent. w/w). In a method recently described by Qazilbash[1] a solubility correction of 0·0064 g./90 ml. is applied, whereas published solubilities[2,3] for santonin in this solvent vary from 0·04 to 0·06 g./100 ml. When his method was applied to a santonin-free sample of herb to which a known quantity of santonin had been added low results were obtained, whereas a recovery of 99 per cent. was obtained using a correction based on a solubility of 0·044 g./100 ml. of solvent determined in this laboratory. Furthermore, in the assay the ultra-violet absorption spectrum of the mother liquor left after removing the crystals agrees with such a correction.

On the basis of the solubility determined in these laboratories, a correction of 0·04 g. would be valid and, therefore, any artemisia containing less than 0·4 per cent. of santonin would yield no crystals and the assay figures would be reported as nil. On the other hand, if Qazilbash's correction is valid, it should be possible to obtain crystals from artemisia containing as little as 0·07 per cent. of santonin. Reference to his Table II shows that the 24 samples examined fall into two groups, the smaller group containing more than 0·5 per cent. of santonin and the larger group reported as nil, but which on the basis of our suggested correction may have contained up to 0·4 per cent. of santonin. It would, therefore, be of interest to workers in this field if the data on which the 0·0064 g. correction is based, were brought forward.

Analytical Control Division,
May and Baker, Limited,
Dagenham, Essex.

J. ISAACS.

April 16, 1952.

REFERENCES

1. Qazilbash, *J. Pharm. Pharmacol.*, 1951, **3**, 105.
2. British Pharmaceutical Codex, 1934, 926.
3. Coutts, *Quart. J. Pharm. Pharmacol.*, 1932, **5**, 369.

ABSTRACTS (*Continued from page* 422)

Dihydrostreptomycin Sulphate, Toxicity for Auditory Nerve. C. Don and J. Gregory. (*Lancet*, 1952, **262**, 72.) An extensive review of published reports of the toxicity of dihydrostreptomycin for the auditory nerve suggests that the hydrochloride is more toxic than the sulphate, possibly because its chemical purity is lower. A follow-up investigation was carried out on 26 patients who had been treated for more than 6 weeks with 1 g. daily of dihydrostreptomycin sulphate of a minimum purity of 88 per cent. No cases of miliary or meningeal tuberculosis were included since auditory effects are known to occur in these patients without any form of chemotherapy. 4 cases showed auditory impairment: 1 with audiometric loss only, 1 with slight deafness, 1 with moderate deafness and 1 with severe deafness. The results obtained suggest that the purest form of dihydrostreptomycin sulphate at present available is toxic to the auditory nerve. H. T. B.

BOOK REVIEWS

PHYSICAL BIOCHEMISTRY, by Henry B. Bull. Second Edition. Pp. viii + 344 and Index. Chapman and Hall, London. 1951. 46s.

This new and revised edition of Professor Bull's book is a valuable contribution to the teaching of the sciences in which physical and physico-chemical methods are applied to biological problems; the copious references to the literature are a useful key to the large number of recently published research papers in these fields. The book is written in an interesting and straightforward manner with a minimum of complicated mathematical treatments and the text is fully illustrated with graphs and diagrams of apparatus. Apart perhaps from the last few chapters, the subject-matter should present no great difficulty to readers whose mathematical knowledge is limited. Professor Bull's use of the word "biopolymers" in place of "colloids," has much to recommend it. His frank admission (p. 106) concerning electrode sign conventions is refreshing and his statement that these signs can usually be guessed from a knowledge of chemistry is essentially that of a practical electrochemist who cannot be bothered with elaborate sets of rules.

Some criticisms of the book are: Chapter 2 dealing with energetics (i.e. thermodynamics) is much too short to give any real grasp of the subject; similarly the section dealing with dielectric constants and dipole moments (pp. 15–19) is too brief; p. 56, expressions for "probable errors" are given—these quantities have been long discarded by most experimental workers; p. 194, the imperfections of the Wilhelmy balance as an absolute method of measuring surface tensions should have been stated; pp. 198–9, the deduction of the Gibbs adsorption isotherm given here is not very satisfactory, since it does not clearly reveal the limitations of this approximate form of the equation; p. 268, in the deduction of the Donnan membrane equilibrium equation, the large amount of electrical work required to transfer one mole of chlorine or sodium ions from one compartment to another, is ignored. L. SAUNDERS.

PRACTICAL PHARMACOLOGY, by J. H. Burn. Pp. viii + 72. Blackwell Publications, Oxford. 1952. 12s. 6d.

In pharmacology there has long been a need for a book of simple practical exercises. This book is therefore especially welcome. In compiling it the author has borne in mind the restrictions imposed on the student by the Cruelty to Animals Act. The book contains 21 experiments illustrating the actions of drugs on isolated organ preparations, on the perfused heart and blood vessels, and on the cardiovascular system of the spinal and decerebrate cat; as well as a number of quantitative determinations and an experiment on man. Throughout, the experimental techniques are very clearly described with fully illustrated diagrams and some really excellent demonstration tracings. It seems a pity that the opportunity was not taken to include a description of the phrenic nerve diaphragm preparation, Dr. Bulbring's original description often not being easy of access. The book tends to be too thorough, for it leaves little to the student's personal observation. It is exceedingly well printed on surprisingly good paper and there are few errors, apart from an unfortunate series of misprints in the legend to Figure 30. There is a discrepancy between the text and Figure 18 where the coronary flow is increased by adrenaline in the rabbit; the activity of the International Standard Digitalis Powder, quoted on page 61, has not been brought into line with the 3rd International Standard. This excellent little book has much to recommend it and should become extremely popular in the teaching of practical pharmacology. G. F. SOMERS.