REVIEW ARTICLE

THE APPLICATIONS OF ULTRA-VIOLET ABSORPTION SPECTROPHOTOMETRY IN PHARMACEUTICAL ANALYSIS

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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 $\pm$ 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 $\pm$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 $\pm$0·1 per cent.,[12] $\pm$0·22 per cent.,[13] $\pm$0·4 per cent.,[14] and $\pm$0·5 per cent.[15] It is fairly certain that a reproducibility of the order of $\pm$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

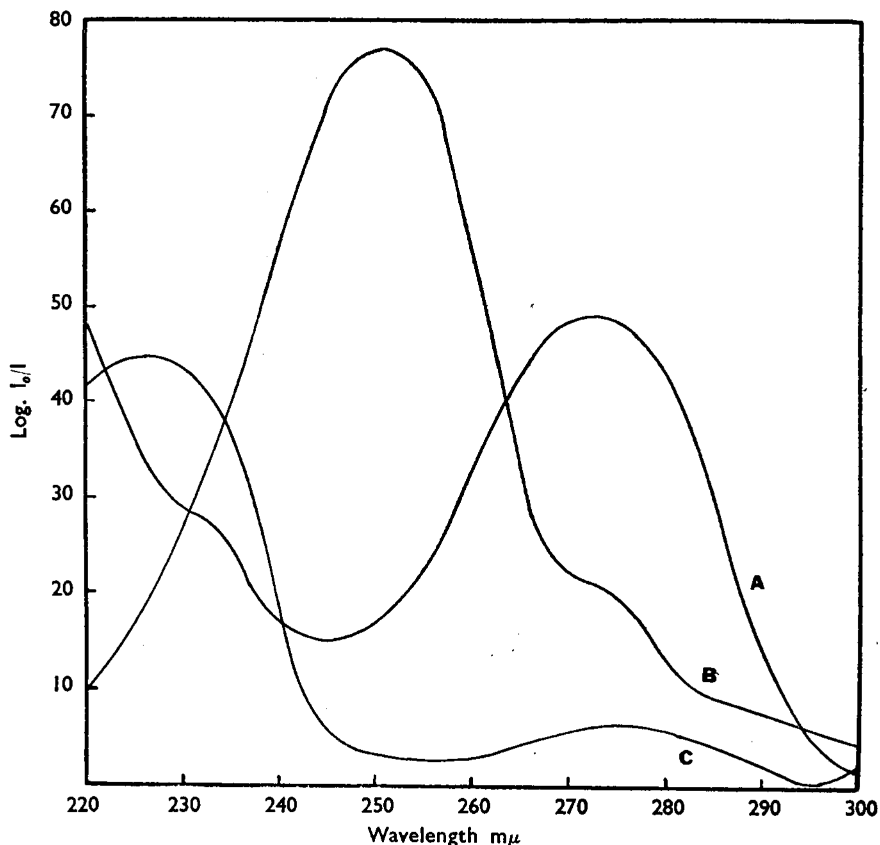


FIG. 1. Absorption spectra of aspirin, phenacetin and caffeine in ethanolic solution: A, Caffeine; B, phenacetin; C, aspirin.

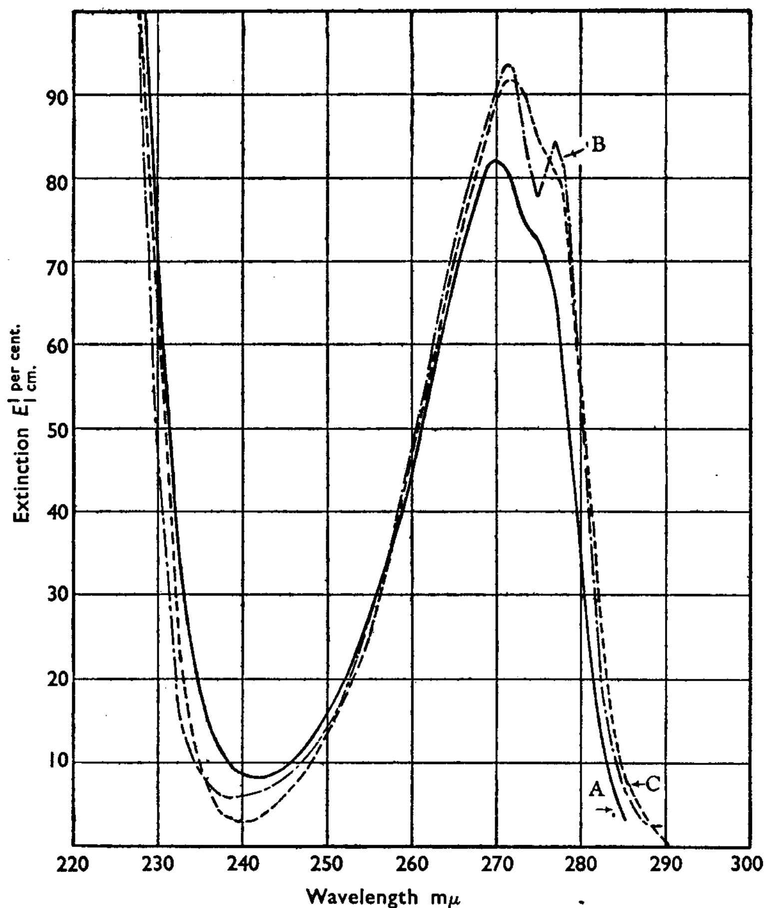


FIG. 2. Absorption spectra of mephenesin. A, in water; B, in *cyclo*hexane; C, in *iso*propanol.

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

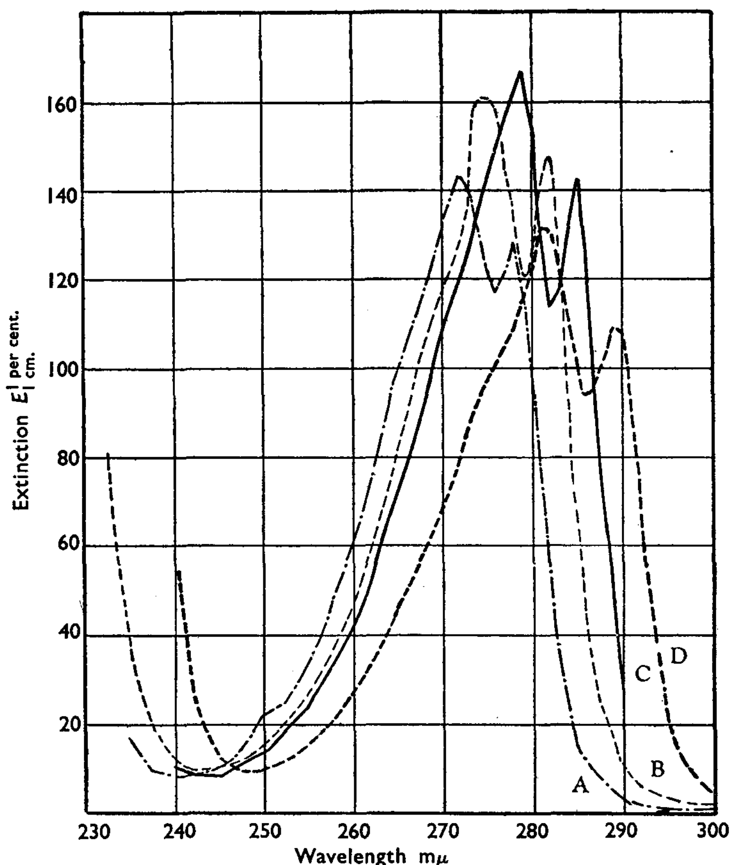


FIG. 3. Absorption spectra of phenyl ethers in *cyclo*hexane. A, *o*-cresyl ethyl ether; B, *o*-chloroanisole; C, *p*-cresyl methyl ether; D, *p*-chloroanisole.

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.

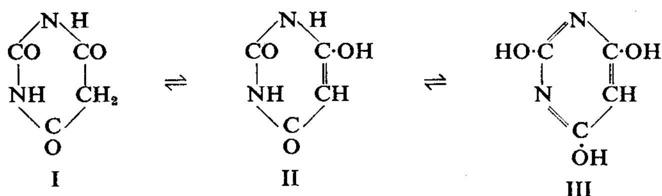


Figure 4 shows the variations in $E_{max.}$ with change in *p*H.

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

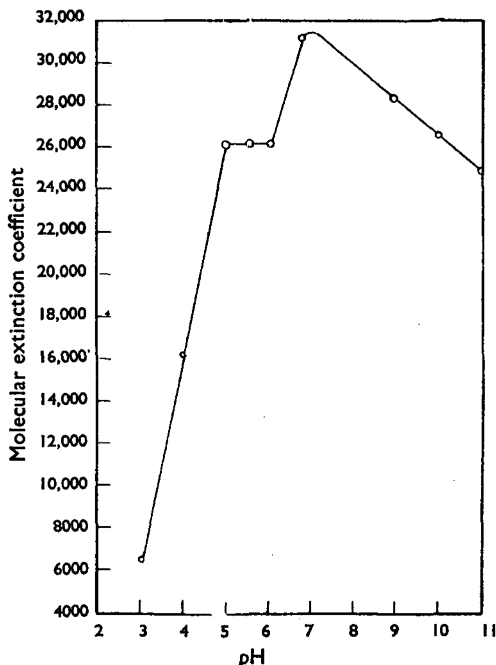


FIG. 4. Variation of absorption maximum of barbituric acid with change in *p*H. (Loofbourow and Stimpson).

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 $E^{1\ \text{per cent.}}_{1\ \text{cm.}} = 450$ 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 $E^{1\text{ per cent.}}_{1\text{ cm.}}$ about 570, $\lambda_{max.}$ 283 mμ $E^{1\text{ per cent.}}_{1\text{ cm.}}$ about 560 and $\lambda_{max.}$ 365 mμ $E^{1\text{ per cent.}}_{1\text{ cm.}}$ 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.

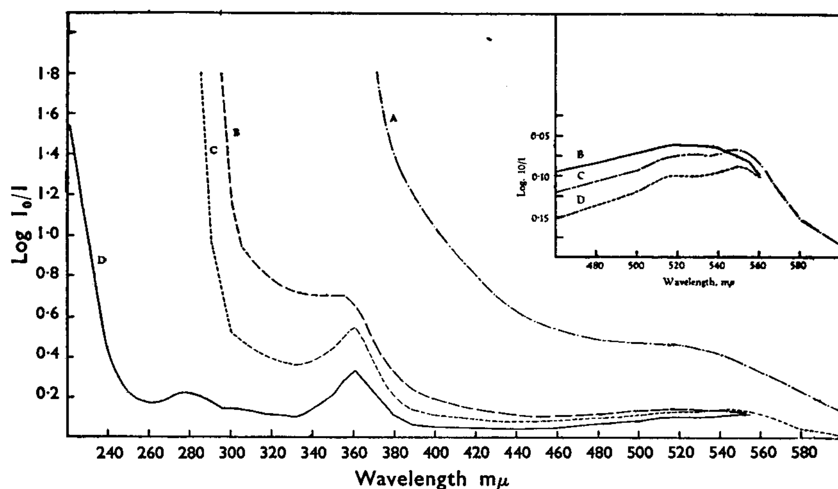


FIG. 5. Absorption spectra of vitamin B_{12} of varying purity. A, containing approximately 0·6 per cent.; B, containing approximately 2 per cent.; C, containing approximately 5 per cent.; D, pure vitamin B_{12}. The curves are adjusted so that they relate to the same total amount of vitamin B_{12} in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

$E_{1\,cm.}^{1\,per\,cent.}$ 361 mμ/$E_{1\,cm.}^{1\,per\,cent.}$ 278 mμ of 1·62 to 1·88, and for
$E_{1\,cm.}^{1\,per\,cent.}$ 361 mμ/$E_{1\,cm.}^{1\,per\,cent.}$ 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 $E_{1\,cm.}^{1\,per\,cent.}$ 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, $E_{1\,cm.}^{1\,per\,cent.}$ 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 $E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ 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 $E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ 240 mμ correcting for oils which interfered; deoxycortone acetate and testosterone propionate could be determined similarly. Ethinyl testosterone[51] showed $\lambda_{max.}$ 238 mμ $E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ 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

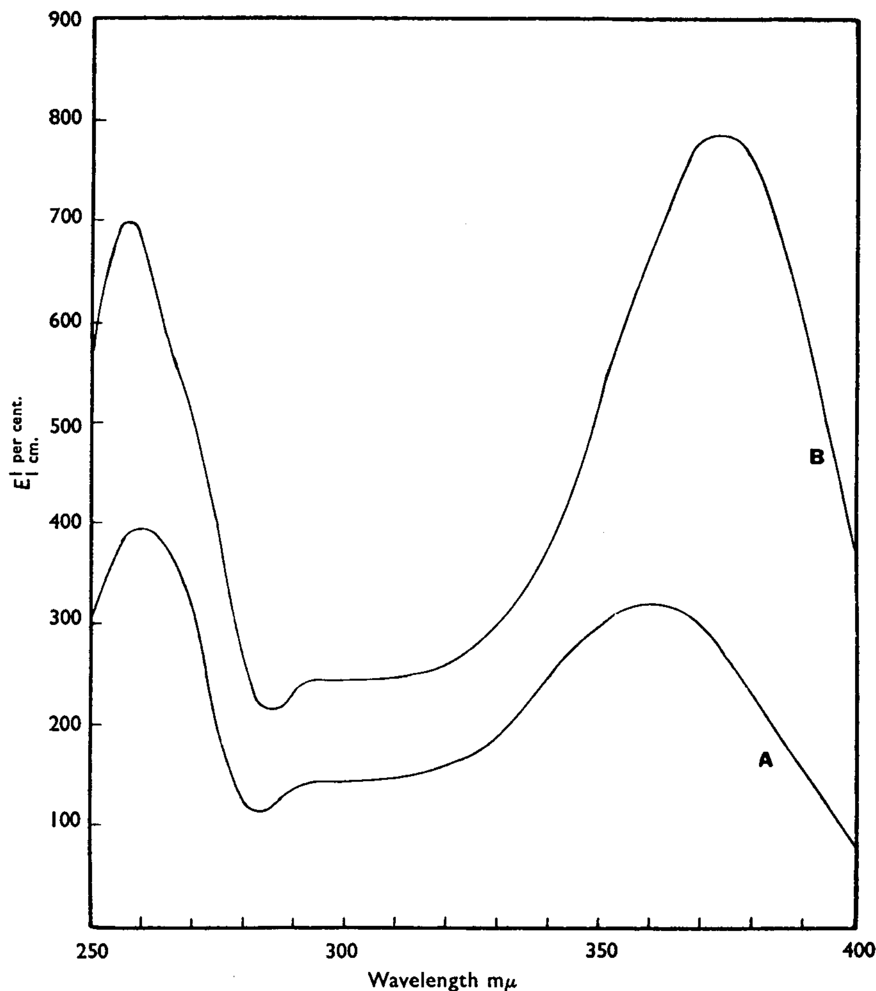


FIG. 6. Absorption spectra of rutin and quercetin in 95 per cent. ethanol containing 1 per cent. of 0·02 N acetic acid: A, rutin; B, quercetin.

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.

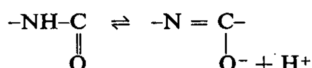
The ultra-violet absorption curves of aureomycin and terramycin[55] show multiple maxima and minima between 220 and 400 mμ. For analysis aureomycin preparations are hydrolysed by boiling with dilute sulphuric acid before measuring the absorption at 274 and 350 mμ; the aureomycin content is proportional 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.

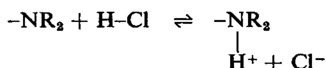
Urea Derivatives and Sulphonamides

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:



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.



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

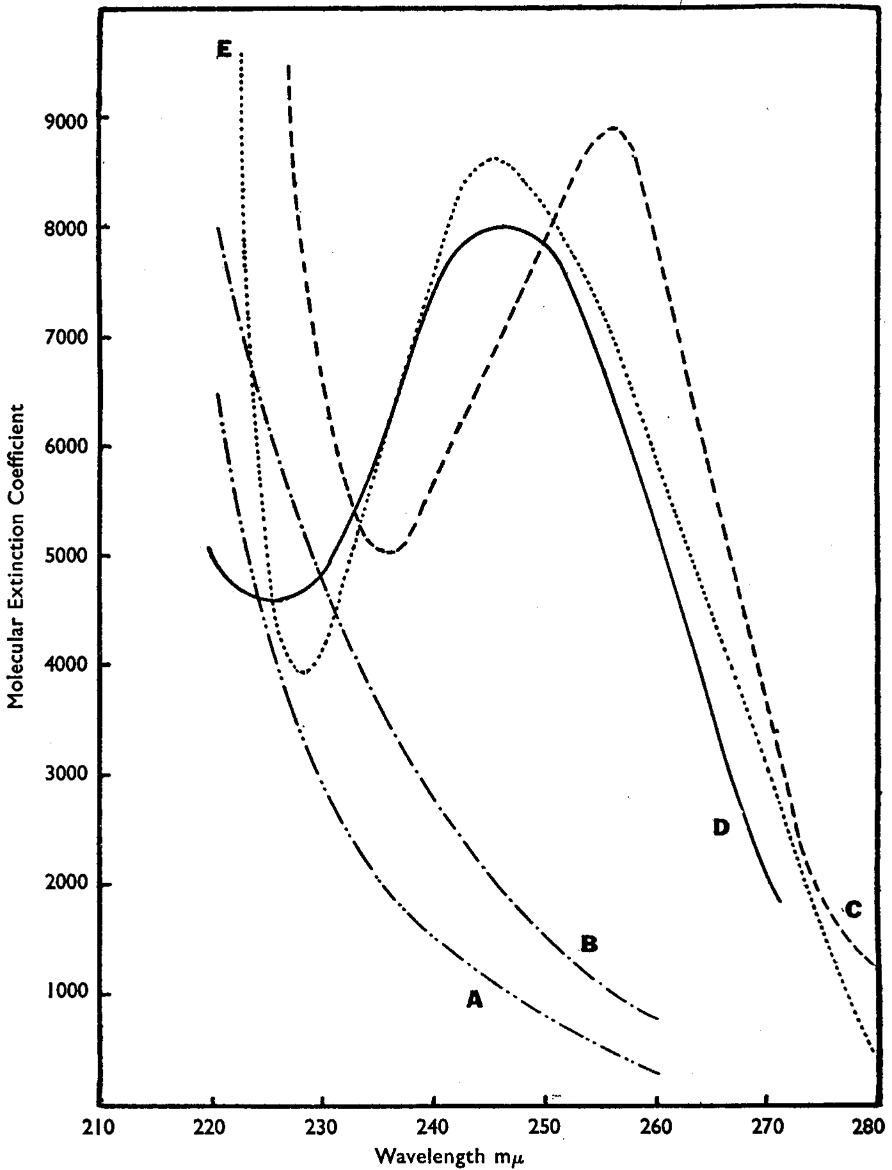


FIG. 7. Absorption spectra of barbituric acid derivatives: A, phenobarbitone in 0·1 N hydrochloric acid; B, phenobarbitone; 0·00025 M in water; C, phenobarbitone in 0·1 N sodium hydroxide; D, 5:5′-*iso*amylethyl barbituric acid in 0·1 N sodium hydroxide; E, barbitone in 0·1 N sodium hydroxide.

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 ($E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ *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 ($E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ 280 mμ *ca.* 50); morphine in alkaline solution shows a much higher absorption $E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ *ca.* 310 at 258 mμ, presumably due to the phenolic group. Apomorphine absorbs more strongly in acid solution ($E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ 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 $E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ 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 $E^{1\,\text{per cent.}}_{1\,\text{cm.}} = 183$. 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, Allchorne 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, Holiday 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**.