SYMPOSIUM*
WATER

POTABLE WATER

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Sources

Potable water is obtained from a multiplicity of sources, but the ultimate source is always the sea, usually *via* evaporation, cloud formation and rainfall. The rainfall may either run off the surfaces on which it falls, giving rise to various surface water sources or may percolate into the soil, giving rise to various underground sources. Although this is the usual cycle of water supply, in some places of extreme water shortage or in emergency, potable water is obtained directly from the sea, either by distillation or by demineralisation with ion exchange compounds.

Collection of Rain

It is frequently imagined that the direct collection of rain will provide a very pure water, but this is very rarely the case. Rainfall will always collect dissolved gases and dust during its fall, particularly in industrial areas and must always be collected from a solid surface such as a roof, which even in remote country places may be a roosting place for birds and consequently be fouled by them. Such water will be very soft and probably plumbo-solvent so that lead piping, lead flashing and lead-lined tanks should be avoided. Collecting tanks should be watertight and fitted with close-fitting covers. Depending on the method of collection it may be necessary to filter and to disinfect such water.

Lakes

Lakes which may be natural or impounded in remote upland areas will vary according to the nature of the rock formation in the catchment area. Limestone areas will give rise to hard, alkaline waters, but insoluble rock formations especially if peat bogs are also present, will give rise to soft, acid waters which may be discoloured yellow or brown from the organic matter derived from the peat and are liable to be plumbo-solvent. In the absence of grazing animals, farms or villages with cess-pits or sewage systems draining into the catchment area, such sources should be bacteriologically pure. Growth of algæ is not likely to be troublesome owing to the absence of mineral salts in the water. Treatment of such waters may involve the addition of alkalis such as lime to neutralise excess acid, coagulation with alum to remove colour and treatment with chlorine primarily to remove bacteria. Elaborate filtration processes are not usually necessary as such waters are usually clear.

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Rivers and Streams

To obtain an adequate constant supply from rivers and streams it is usually necessary to abstract the water well below the source, by which time it has usually become polluted not only by natural surface drainage from the land through which the river flows, but from sewage effluents and industrial effluents. Twenty-five per cent. of the population of England and Wales now obtain water from rivers polluted in this way. Turbidity is liable to fluctuate widely with rainfall and such waters are usually hard.

This type of water cannot be used without careful treatment including storage, clarification and disinfection. Storage may serve three purposes; partial clarification due to settlement; bacterial improvement due to death of some of the polluting bacteria; and storage against drought. These advantages may be partially offset under certain conditions by some disadvantages. Water from polluted lowland sources will usually contain sufficient nitrogen compounds and dissolved salts to support the growth of algæ under suitable climatic conditions. Such growths will give rise to increased turbidity and colour with consequent filtration difficulties and to objectionable tastes derived either from the living cells or from their decomposition products. Control is facilitated by field observations and sampling to determine the best management of the reservoirs, sometimes helped by treatment with copper sulphate or, in the case of small reservoirs, with chlorine. Over suppression of one type of growth may lead to the appearance of a more objectionable type.

Under some climatic conditions thermal stratification may occur in deep reservoirs followed by de-oxygenation of the lower layers. When climatic conditions change, the de-oxygenated layers may mix with the rest of the water making the whole reservoir unusable for a time.

Clarification of stored river waters may be accomplished by filtration or chemical coagulation or a combination of the two. Filtration is frequently carried out in two stages, a rapid coarse filter followed by a slow finer filter, the rapid filter serving the purpose of prolonging the intervals between cleaning of the secondary filters and enabling a greater rate of filtration to be adopted. The rapid primary filters may take the form of coarse sand beds which are cleaned mechanically at intervals or may be rotary screens of fine stainless steel wire mesh which are automatically continually washed. Sand filtration improves the chemical quality of the water, but micro-straining is purely a physical process.

Secondary filters may take the form of large gravity-fed sand beds, where much of the filtration effect is dependent on the accumulation of an organic layer on the sand surface which may be cleaned by skimming manually or mechanically, or more recently by mechanical washing *in situ*. Secondary filters may also take the form of enclosed pressure filters containing sand, usually combined with alum coagulation, the precipitated aluminium hydroxide forming a filtering layer on the surface of the sand which is periodically washed by a scouring action or by rotation and inversion of the whole bed and container.

Clarification by coagulation with alum may be carried out without the need of a supporting sand bed. During the coagulation process

accompanied by stirring, suspended matter including some of the bacteria and algæ is trapped in the floc which then settles and is drawn off as a sludge. The process is usually a continuous one, the sludge forming a blanket through which the raw water passes, clear water overflowing at the top and sludge being drawn off at the bottom.

Sterilisation of river-derived water is usually achieved by chlorination or sometimes by ozonisation or, on a small industrial scale, by ultra-violet light. Chlorination and ozonisation will also remove some colour and taste by oxidation of the organic compounds causing these properties. The presence of much ammonia in the water will delay the disinfecting action of chlorine due to the formation of chloramines instead of free chlorine. The use of contact tanks is now usually advocated to ensure sufficient time for action between chlorine and bacteria to effect their destruction before the water passes into the distribution system. By the end of such contact time all the chlorine should have been deviated by the organic matter present, or if not, the excess can be removed with sulphur dioxide. Various types of apparatus are now available for automatic control of chlorine residuals.

Wells and Springs

Wells may be either shallow, sunk into the top pervious strata and collecting water from the immediate vicinity, or deep, sunk below an impervious stratum and collecting water possibly from considerable distances.

Shallow wells are very liable to surface pollution which may be intermittent and considerable care should be taken in siting and protective measures. Many are unsafe to use without filtration and disinfection. Various domestic types of apparatus are available for small installations.

Deep wells are usually of good bacteriological quality as the water usually has to travel considerable distances underground, being filtered on the way. Fissures and swallow holes in some rock formations may however provide access for gross pollution. Such waters are usually hard. Filtration is very rarely necessary. Disinfection with chlorine or ozone is usually carried out as a safeguard against sudden unpredictable pollution. Contact tanks are advisable to ensure adequate disinfection before distribution, but in the absence of organic pollution there will be little deviation of chlorine so that excess must be removed with sulphur dioxide before distribution.

POTABILITY

All statutory water undertakings are required to provide a supply of pure and wholesome water, but purity and wholesomeness are not precisely definable in scientific terms. Wholesomeness implies non-injury to health and absence of pathogenic organisms and toxic substances. As a chemically or physically pure water cannot occur in nature, purity implies pleasing to the senses, that is, absence of visible particles, turbidity, colour, taste and odour, and freedom from excessive amounts of substances in solution not normally detectable by the unaided senses.

Potability therefore implies physical attractiveness as well as safety. Such standards, however, are not satisfactory for all pharmaceutical purposes. All the sources described above can provide such a pure and wholesome water, but their suitability and ease of treatment for pharmaceutical purposes will vary.

Bacteriological Qualities

The most important function of the bacteriological control of potable water is to ensure freedom from the causative bacteria of so-called water-borne diseases, such as typhoid fever, paratyphoid fever, gastro-enteritis, dysentery and cholera. Cysts of amœbic dysentery and ova of intestinal helminths can also be carried by water and also presumably, the virus of poliomyelitis. All these organisms are of fæcal origin and their absence may be presumed with confidence by demonstrating the absence of the normal intestinal flora. These are the coliform group, the most important of which is *Escherichia coli* (*Bacterium coli*), the fæcal streptococci, of which the most important is *Streptococcus fæcalis* and anaerobic spore-forming organisms, of which the most characteristic is *Clostridium welchii*.

The Coliform Group

For waterworks control the organism normally sought is *E. coli* but from unknown sources or where interpretation of results is doubtful, enumeration of *Strep. fæcalis* and *Cl. welchii* is of value. Various culture media are used throughout the world for the primary detection of *E. coli*, but the one used for the standard method in Great Britain is MacConkey broth. This medium contains bile salt, which inhibits the growth of many of the common bacteria found in water, and lactose which is fermented by *E. coli* and other members of the coliform group, with the production of acid which is detected by an indicator change, and gas which collects in an inverted tube. Coliform organisms other than *E. coli* will also produce a positive result by this method. *E. coli* is much the most frequent type of coliform organism present in the human and animal intestine and is rarely found in sites free from excretal pollution. Other coliform types such as *Citrobacter freundii* (Intermediate types) and *Klebsiella aerogenes* (*Bact. aerogenes*) also occur in the intestinal canal, but in much smaller numbers. Outside the body these other coliforms have much greater powers of survival and even appear to be capable of multiplication under special conditions. In addition to being widely distributed in obviously fæcally polluted sites and in agricultural soils where their presence cannot be dissociated from manurial pollution, they can also be found in soil apparently free from fæcal pollution and in which *E. coli* is absent. The presence of *E. coli* in water denotes recent fæcal pollution; the other coliforms may indicate more remote pollution, but in some circumstances are of no epidemiological significance. *E. coli* can normally be distinguished from other coliforms by its ability to ferment lactose at 44° C.

It is usual to examine the water at all stages of purification from the raw

water before any treatment, to the final water as it reaches the consumer's tap. The frequency of examination of each stage will depend on the nature of supply, its liability to pollution and the stage in the purification process. Water passing into supply should be examined at frequent intervals, preferably daily, whereas raw or stored water need only be examined at less frequent intervals. Water sources not normally liable to pollution can be examined less frequently than those liable to pollution.

Streptococcus fæcalis

If a supply repeatedly gives a high coliform count in the absence of *E. coli* and their origin is in doubt, interpretation is facilitated by a search for *Streptococcus fæcalis* which is another organism undeniably indicative of fæcal pollution either of human or animal origin.

Clostridium welchii

Clostridium welchii, an anaerobic sporing bacillus is also a normal inhabitant in the human and animal intestine and is sometimes used as an indicator of fæcal pollution, but it has a different significance from the other fæcal organisms discussed, as its spores can survive for much longer periods and they are much more resistant to disinfection by agents such as chlorine. Their detection in a raw water in the absence of *E. coli* or *Strep. fæcalis* would be indicative of more remote fæcal pollution. It is for this reason that these organisms may still be found in a treated water derived from a polluted source, particularly at certain times of the year. It is not unusual to find them in 10 ml. quantities of water in London's river-derived supplies in winter. Their presence does not render the water unsuitable for drinking, but it does mean that it is unsuitable and dangerous to use for washing out syringes to be used for parenteral injections in the belief that it is sterile, or for the solution of tablets for hypodermic injection.

For further details of standard methods for the bacteriological examination of water, reference should be made to Report 71 of the Ministry of Health[1].

Colony Counts

In addition to examining water supplies for evidence of fæcal pollution, it is usual to attempt to obtain some information on the numbers of other bacteria present. The results will depend very considerably on the methods adopted and will be affected by the culture medium used and the time and temperature of incubation. No one combination of these conditions will be capable of permitting the growth of all the bacteria liable to be encountered.

As the method of counting depends on the ability of a bacterial cell to multiply into a visible colony in a nutrient medium, and that the bacteria initially giving rise to colonies may have occurred, singly, in pairs, chains, groups or dense clumps, not every individual cell will develop into a separate colony. It is therefore necessary when quoting colony counts to state the conditions under which they were obtained, and to express results as the number of colonies per millilitre.

An incubation temperature of 37° C. was chosen by most early workers as it was thought that organisms growing at that temperature would be of greater significance. Forty-eight hours has become recognised as the standard incubation time, although with river-derived waters there is usually little difference between 24 and 48 hours, whereas with shallow wells the difference might be considerable. The medium used as standard in Great Britain is a peptone yeastrel agar. Incubation for 3 days at 22° C. will give much higher results on river water, the ratio of the 37° C. to 22° C. counts being about 1 to 10. The ratio is often much lower on shallow well samples. The ratio is also lower after chlorination because normally only spore-forming organisms survive. A very much higher count will be obtained using various dilute nutrient agar media incubated at 22° C. for 15 days. Nutrients in such media are kept dilute to prevent colonies growing too large and interfering with each other. Such counts may be 100 to 1000 times as great as corresponding 37° C. counts.

Different types of water, all equally satisfactory for potable purposes may all have quite different colony counts. Such counts are not therefore of any direct value in themselves in assessing potability, but they are of considerable value once a series of counts has been obtained from any particular supply. Any gross variation from the normal count would then be of significance and its cause should be investigated. Water is used for many purposes other than domestic supply and for some manufacturing, food-processing and pharmaceutical purposes, large fluctuations in colony counts are undesirable. Large numbers of chlorine resistant aerobic saprophytic sporing bacilli are liable to cause considerable nuisance in some food-processing industries although they may be of no significance for domestic purposes. For pharmaceutical purposes, the relationship between bacterial counts, extent of pollution of raw water and the presence of pyrogens needs further study.

Bacteriological Standards

Standards for the bacteriological quality of potable water have been given by the Ministry of Health[1] and the following recommendations have been obtained from the Ministry Report. Ideally all waters intended for drinking should show no coliform bacteria in 100 ml., and it should be the aim of every water undertaking to provide water of this quality. Many natural waters reach this standard and, since it is readily attainable by effective treatment, no chlorinated supply should fail to give this result. The appearance of coliform organisms in a chlorinated supply as it leaves the works should at once occasion misgivings as to the adequacy of the chlorination process.

The following classification is suggested by the Ministry of Health for non-chlorinated piped supplies.

		Coliform count per 100 ml.	*E. coli count per 100 ml.*
Class 1	Excellent	0	0
Class 2	Satisfactory	1–3	0
Class 3	Suspicious	4–10	0
Class 4	Unsatisfactory	>10	0 or more

Throughout the year, 50 per cent. of samples should fall into Class 1; 80 per cent. should not fall below Class 2; and the remainder should not fall below Class 3.

In view of the hazards to which the water is exposed in the distribution system, a different interpretation must be placed on the results of samples taken on consumers' premises. It is difficult to lay down standards for supplies of this type and opinions differ on the subject, but the present writers are of the opinion that the standard for non-chlorinated waters can reasonably be applied to any samples taken from the distribution system because in most cases, by the time the water reaches the consumers, there is no residual chlorine remaining in the water, or at best, it would be "stale" chloramine with very feeble bactericidal powers.

There is always a reason for the appearance of coliform organisms in a sample taken from a consumer's tap and sooner of later the source can usually be found. The degree of investigation and remedial measures considered advisable will depend on the number and type of organisms isolated, but the presence of *E. coli* should be considered as a sure indication of dangerous fæcal pollution calling for immediate action.

Deterioration in Distribution System

Defects

Although water may be sent out from a works in a satisfactory condition, changes may occur in the distribution system before it reaches the consumer's tap. Contamination may occur through defective mains, hydrants, air valves etc. The construction of "dead ends" in distribution systems so that the water cannot freely circulate sometimes causes trouble and should be avoided wherever possible.

Service Reservoirs

Distribution systems normally also contain service reservoirs, situated in the highest parts of the district. These serve to equalise pressures and to act as a reserve against peak demands. Bacterial growth may occur to some extent in these in a river-derived water under warm conditions but the biggest danger is usually from leaks, especially in the roof, as these service reservoirs are normally covered. Defective ventilators will permit the access of small animals and birds particularly in elevated tanks. Regular bacteriological examination of these reservoirs is necessary to control these possibilities.

In some parts of Great Britain and elsewhere, service reservoirs are open and often of such a size that covering would be impracticable. Open service reservoirs would be quite impossible for a polluted river-derived supply owing to the growth of algæ which would occur, but with most upland surface supplies, no such difficulty arises. Such reservoirs often situated in built-up areas, are obviously liable to pollution of many kinds over which the water supply undertaking has little or no control. They are a feature of water supply practice which should be avoided wherever possible.

Cross Connections

Precautions must be taken to prevent cross-connections with, or to prevent back-syphonage from, various types of apparatus on consumers' premises, sewer flushing tanks, ships' ballast tanks or raw water hydraulic mains. Waterworks history has included many such accounts of unauthorised cross-connections in contravention of bye-laws, sometimes with serious consequences and more often with local minor inconvenience. Hot water and steam from neighbouring laundry boilers has issued from cold water taps, also dirty soapy water and in one case paraffin oil from a connection with a paraffin tank. In some of these cases, back-syphonage has occurred in spite of the insertion of non-return valves. What may appear to be unnecessarily strict bye-laws exist to prevent such occurrences, but instances of ignorance or deliberate flouting of them are continually being discovered.

Jointing Materials

Growth of bacteria may also occur on some of the materials such as jute yarn, used for packing joints in mains and the glands of valves or on mastic jointing materials used in concrete tanks. Tap washers particularly if made of leather, can also be a source of bacterial growth. Bacterial deterioration of this type in the distribution system may be revealed by increased colony counts or growth of coliform bacteria. The appearance of *E. coli* in the distribution system would indicate access of fæcal pollution from an outside source.

Cisterns

Bacterial deterioration in distribution systems cannot be considered without some discussion on the role of cisterns. It is usual to fit a tap off the rising main for "drinking water" where the other taps in a building are off a cistern. This tends to imply that cistern water is not suitable for drinking. If this is so, then it also implies that it is permissible to distribute such impure and possibly unsafe water throughout a building provided that there is also somewhere in the building a tap off the rising main, whether readily accessible or not. From the public health point of view this is certainly not meant to be the case and it must be accepted that, if water is conveniently available from any tap, people will drink from it, even though there is a special tap for drinking water in another part of the building.

The agents likely to cause bacterial contamination of a cistern are human beings, rodents, birds and possibly flies. No precautions will exclude the human factor, and indeed any man has a perfect right to open and inspect his cistern if he so desires. The inadequacy of protection often originates from the practice of carrying expansion pipes above the cistern, necessitating the cutting of holes in the cover, which consequently usually has to be made on the site from wood. Such holes, even if the cover is otherwise well made, permit the access of animals and birds, and it is not unusual following complaints of bad tastes or repeated illnesses, to find the decomposing remains of dead rats or birds in cisterns.

Such defects in covers could be overcome by the use of standard specifications for the method of plumbing to cisterns, and to permit the provision of a close-fitting, overlapping metal lid. The only disadvantage in using water from a properly protected cistern is that its temperature is likely to be higher with the possibility of flat or even slightly musty tastes. For pharmaceutical purposes, if there is any doubt as to the adequacy of protection of cistern water, it would be advisable to use mains water.

Where cisterns are exposed to weak alcoholic atmospheres within premises such as spirit distillers and bottlers, scent manufacturers, paint and varnish manufacturers and even the spirit department of a museum, sufficient nutriment may be present for certain fungi to grow profusely, forming gelatinous masses in the cisterns and around ball valves. Numerous bacteria are also found in these zoogleal masses and protozoa and nematodes soon make their home there.

Earthy Tastes

Earthy, musty or mouldy tastes have been frequently reported from distributing systems in large buildings when no taste could be detected from hydrants in the street. These tastes have usually been associated with warm semi-stagnant conditions in the pipes in the building such as obtains in large centrally heated office blocks with cold and hot pipes carried in the same ducts and not used outside office hours or at weekends, or where cold pipes run through basement boiler rooms or bake-houses. Under these conditions strains of fungi and sometimes of actinomycetes have been isolated producing strong mouldy, musty or earthy odours. The ability of moulds and actinomycetes to grow under these conditions has been demonstrated by Silvey, Russell, Redden and McCormick[2], a minimum temperature of 17° C. being necessary before tastes and odours are noticed. Dissolved organic matter is necessary to initiate this growth as it occurs in organically polluted river-derived water, but not in deep well waters. Complaints of this nature in the London area are obtained only from premises supplied with river-derived water and not deep well water. Mackenzie[3] reported that fungi would only produce a taste in water when in actual contact with or growing in the water, whereas actinomycetes produce a volatile substance that would dissolve in the water without direct contact with the organisms. Growth of these on damp wooden cistern lids or materials used for lagging cisterns could therefore lead to the production of volatile products dissolving in the exposed water and producing earthy tastes. Both Windle Taylor[4] and Silvey and colleagues[2] have shown that these tastes cannot be destroyed by chlorination, unlike many of the other tastes of organic origin. In fact, the addition of chlorine often increases the taste, due probably to the production of stable chloro-derivatives.

Chemical and Physical Qualities

Turbidity and Colour

As explained earlier, a chemically or physically pure water cannot occur in nature, so that purity of a water supply implies, pleasing to the senses,

and lack of substances likely to give rise to trouble. A potable water should be clear and bright and colourless. Turbidity may originate from rain washings or the flow of water over or through the soil, or from insufficiently treated sewage or industrial effluents, or it may arise as the result of growth of algæ in reservoirs, lakes or filter beds. It may also arise from precipitation of metals in solution or from action of the water on metal pipes or from deposition of chemicals used in treatment. These metallic impurities will be discussed later.

Colour may be derived from the organic matter in solution or the material causing turbidity may itself have a colour such as clay or iron oxide or green algæ. There will be traces of dissolved organic matter in any water derived from a surface source, the colour being deepest in water from acid peaty sources. Colour due to dissolved organic matter is partly removed by oxidation by aeration by chlorination or more effectively with ozone. Traces of organic matter can be completely removed only by distillation. Colour is measured in a colorimeter by comparison with a standard colour. Turbidity is measured photoelectrically and allowance is made for the effect of the colour.

Taste and Odour

A potable water should also be tasteless and odourless. Natural mineral waters, especially chalybeate waters will have their own characteristic tastes. Tastes originating from decaying vegetation or from micro-organisms such as fungi and actinomycetes growing on the decaying vegetation or in association with living algæ giving rise to weedy, musty, mouldy or earthy tastes have been described by Silvey and others[2], Silvey and Roach[5], and Ferramola[6]. Tastes may also arise from the growth of algæ, some algæ in the living state having very marked characteristic tastes and odour, e.g., "cucumber" and "geranium", and in the decaying stage the tastes produced will be similar to other decaying vegetation. Certain effluents will produce objectionable oily, tarry or phenolic tastes in a water. Tastes arising in the distribution system have already been discussed. Treatment with chlorine will remove some tastes as a result of oxidation of the organic matter or it may accentuate others such as earthy tastes due to actinomycetes. Chlorine combined with phenolic substances will produce chlor-phenol tastes which are detectable in extreme dilution and are very objectionable in nearly all beverages. Such tastes are very difficult to deal with. Excess of residual chlorine will produce its own characteristic taste.

The acceptance of a chlorinous taste is dependent on the education or conditioning of the water-drinking public. In some parts of the world the absence of a chlorinous taste is viewed with some alarm, whereas in other places, even slight chlorinous tastes produce strong complaints.

Residual Chlorine

As a result of interaction with ammonia, chlorine may be present in a chlorinated water as free chlorine, monochloramine, dichloramine or nitrogen trichloride. The proportion of the chlorine dose which remains

as residual chlorine in any one of these forms depends on the relative proportions of chlorine and ammonia, the nature and quantity of other impurities, the contact time, the pH and the temperature. A pure well water containing no organic matter will deviate negligible quantities of chlorine and excess chlorine should therefore be removed with sulphur dioxide or a carbon filter before passing into supply. A polluted surface water will deviate considerable quantities of chlorine and if the dose is suitably adjusted and a sufficient contact time is allowed, satisfactory sterilisation will be achieved with a negligible chlorine residual passing into supply. A simple test is available for the quantitative estimation of residual chlorine. Addition of *ortho*-tolidine produces an immediate yellow colour if free residual chlorine is present. If the residual chlorine is due to chloramines the yellow colour takes several minutes to develop.

Ammonia

Ammonia arises in a water supply as a result of breakdown of polluting nitrogenous organic matter, particularly sewage. It may be free in solution or combined as ammonium salts. During filtration through sand beds, especially through rapid gravity sand filters, rapid bacterial nitrification occurs when *Nitrosomonas* spp. oxidise the ammonia to nitrites, and *Nitrobacter* spp. oxidise the nitrites to nitrates. These organisms build up a layer around the sand grains and so long as the water temperature remains above about 4° C. they remain active. Below this temperature they tend to die out and ammonia increases in the filtrate. When the temperature rises again, there is a time lag before the bacteria increase in sufficient numbers to oxidise all the ammonia.

Ammonia is undesirable from the waterworks point of view because it interferes with chlorination. The chloramines which are formed when chlorine reacts with ammonia are much less effective sterilising agents than free chlorine, and they persist for much longer periods giving rise to chlorinous tastes in the distribution system. This effect of ammonia on chlorination can be overcome by means of "breakpoint chlorination". In waters with a high organic demand, however, this process is found to be too slow to be practicable and some ammonia is inevitable in supply in extremely cold weather.

Organic matter

All surface-derived water supplies, no matter how efficiently treated, contain traces of organic matter in solution. This is usually measured by estimating the albuminoid ammonia content of a sample and the oxygen consumed at a given temperature in a given time from acid potassium permanganate solution. The figure for the former may be high in upland surface supplies due to the ingress of products of decayed vegetation and river-derived supplies may show high figures for both albuminoid ammonia and oxygen absorbed contents. The 1955 Addendum to the British Pharmacopœia indicates that demineralised water suitable for pharmaceutical purposes should have an albuminoid ammonia figure of less than about 0·4 mg. per litre and an oxygen absorbed figure of less than

0·8 mg. per litre when tested at the temperature of boiling water for ten minutes. Examples of corresponding figures for a potable river-derived domestic supply are 0·08 and 2·75 mg. per litre respectively. From an epidemiological point of view, a water free from undesirable bacteria is not unsafe because it contains organic matter, but it might not be entirely suitable for pharmaceutical purposes.

Free Carbon Dioxide

Well waters in some formations contain free carbon dioxide in solution which may contribute to corrosion of iron, copper and galvanised steel pipes causing solution of traces of copper, iron and zinc.

Dissolved Salts

Chlorides occur in practically all waters and may be derived either from the strata over and through which the water flows, or by infiltration of sea water or from sewage. An excess causes a brackish taste and there is considerable variation in the amount which may be tolerated. It is usually recommended that chlorides should not exceed 350 mg. per litre as Cl but some communities are drinking up to 550 mg. per litre without observed effect.

Sulphates may occur as calcium, magnesium, sodium or potassium sulphates. The calcium and magnesium salts are the cause of permanent hardness and excess of magnesium or sodium sulphates may cause intestinal disturbance. It is recommended that sulphates should not exceed 250 mg. per litre as SO_4. The base exchange system of water softening will replace calcium and magnesium sulphates with sodium sulphate.

Carbonates will not occur in more than minimal amounts in waters containing calcium salts, as calcium carbonate is relatively insoluble, but bicarbonates will be found, calcium and magnesium bicarbonates being the cause of temporary hardness. Sodium carbonate and bicarbonate may occur in some soft waters and are present in many table and medicinal waters. Sodium bicarbonate will be formed as a result of base-exchange softening of water containing calcium and magnesium bicarbonates.

Nitrates present in water are probably derived mainly from oxidation of organic matter chiefly of animal origin. In river-derived supplies sewage is the main source of nitrates. Under some circumstances the nitrates may be reduced to nitrites and ammonia. Excess nitrates in water given to a very young baby can cause infantile methæmoglobinæmia. The risk is greatest in artificially fed infants as all the feeds are made up with water. For this reason nitrates should not exceed 100 mg. per litre as NO_3.

The quantity of fluorides present in natural waters varies considerably and it is not our intention to enter here into the question as to whether fluorides should be purposely added to control dental caries. The subject should be regarded as *sub-judice* until results are available from the trial areas which have been set up in various parts of the United Kingdom, namely, Kilmarnock, Anglesey and possibly Watford and Amersham. It should suffice to say at this stage that it has been observed that fluorides

in excess of 1·5 mg. per litre as fluorine are liable to cause mottling of the dental enamel whereas in areas with supplies containing less than 1 mg. per litre the incidence of dental caries is higher than in areas with more than 1 mg. per litre.

Metals

Some well and spring waters naturally contain iron in solution in the ferrous state giving the water a chalybeate taste. On exposure to air in reservoirs or tanks the ferrous iron is oxidised and precipitated as ferric hydroxide which may cause opalescence and brown stain on sanitary ware. A similar ultimate effect may be produced by the action of aggressive waters on iron pipes. The iron can be removed by aeration and filtration or by an ion exchange process. Trouble of this nature is likely to occur only when the iron content exceeds 0·2 mg. per litre, but a water may be rendered turbid by as little as 0·1 mg./l.

Manganese often occurs in conjunction with iron, particularly in upland surface waters and is not so readily removed. Waterton[7] has investigated its liability to precipitate in mains as a black crystalline deposit of manganese dioxide which periodically gets flushed into supply leading to complaints of dirty water. Manganese deposits may set up bimetallic systems leading to corrosion of copper pipes. Manganese in solution should not exceed 0·05 mg. per litre.

Contamination with metallic salts is likely to occur through the solvent action of some aggressive waters on pipes. Low pH and high free carbon dioxide content are liable to contribute to solvency of lead, copper, iron and zinc. Solution of lead, due to its cumulative toxic action is liable to be dangerous and great care must be taken with such waters to avoid the use of lead pipes. Steps are taken on some waterworks to correct the aggressive action of such waters. Zinc is relatively harmless, but solution can be so extensive as to cause opalescence due to precipitation of zinc hydroxide. With some well waters the zinc accumulates as hard grains of zinc carbonate which occasionally get flushed through the taps leading to complaints of "sand" in the water. When this occurs deterioration of galvanised iron ware is so rapid as to make this material unsuitable for use with such waters. Zinc content of the water should not exceed 5 mg. per litre at any point in the distribution system.

Corrosion of copper also varies according to the nature of the water. Pitting corrosion may occur in either hard or soft waters. Its prevention by the presence of certain organic inhibitors in water derived from organically polluted sources such as rivers has been studied by Campbell[8]. Traces of copper derived in this way are not likely to be harmful, but if such copper-containing water subsequently passes through galvanised pipes or tanks, zinc will be dissolved causing corrosion. In the same way copper-containing waters boiled in aluminium kettles will lead to deposition of the copper with solution and corrosion of the aluminium. Bimetal systems of this nature should therefore be avoided with waters containing no organic inhibitors. Copper piping is contra-indicated

where the water would take up more than 3 mg. per litre after standing in new pipes for sixteen hours.

Copper derived from copper sulphate treatment of algal growths in reservoirs is likely to be infinitesimal as it is nearly all precipitated as basic copper carbonate in the reservoir and settles to the bottom fairly rapidly.

Some other instances of solution of metals are difficult to explain except as a result of electrolytic action from stray electric currents originating from the practice of earthing electrical circuits to water pipes.

Toxic Elements

Toxic elements should be absent at the source and if present in greater amounts than given below should condemn the supply. The figures are low but are based on long continued ingestion.

Lead	0·1 mg. per litre and 0·3 mg. per litre after 16 hours contact with lead pipe.
Arsenic	0·2 mg. per litre.
Selenium	0·05 mg. per litre.
Chromium (hexavalent) ..	0·05 mg. per litre.
Cyanide	0·01 mg. per litre.

General Conclusions

There is considerable variation in methods of assessing purity of a water supply throughout the world, both bacteriologically and chemically and different standards are adopted. It is gratifying, therefore, to record that an International Study Group, under the auspices of the European Office of the World Health Organisation, has been investigating the standardisation of methods of analysis and standards of quality. In view of the developments which continue to take place in analytical methods it is not yet possible to specify single standard methods for each characteristic but some general principles have been agreed which will be of considerable advantage to all. The report is completed and the figures quoted here are largely derived from this document.

A summary has been given of the possible and limiting concentrations of constituents of potable water which is the "raw material" of the pharmacist for dilution purposes. With ever increasing demands for water, more impure sources have to be exploited. An increasing degree of protection is being applied to the sources and the water is efficiently purified wherever it comes from, so that it is safe to drink and palatable, but some of the characteristics remaining may be embarrassing to the pharmacist unless further treatment is given.

There will be traces of dissolved organic matter in any water derived from a surface source. Occasionally algal cells, bacteria and pyrogens may also be present. Dissolved salts may be in excess and metals may be present in traces. Residual chlorine and ammonia complete the list. Their content may in a lesser or greater degree determine the type of treatment necessary to render the public water supply suitable for specific pharmaceutical purposes.

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WATER FOR PHARMACEUTICAL PURPOSES

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Dr. Windle-Taylor and Mr. Burman have described the problems involved in the supply of a pure and wholesome, potable water and have pointed out that this water can be defined only in terms of its suitability for human consumption. In consequence, potable water is a very variable commodity from the chemical point of view, the nature and concentration of the impurities remaining in it depending on the locality from which it is drawn. No national chemical standard has been imposed for potable water and indeed such a standard is unnecessary for the water to fulfil its primary function.

Without further purification, potable water is not suitable for many applications in pharmacy and in chemical work associated with pharmacy. The extent of the further treatment to which it is subjected depends on the technical requirements for the water and on the cost of the treatment.

The impurities in potable water which necessitate its further purification for pharmaceutical purposes are:

(1) Micro-organisms and their breakdown products. This form of contamination should be slight in water which is freshly drawn from a mains supply. The bacterial breakdown products are not necessarily removed when the bacteria themselves are eliminated and these substances can cause the pyrogen reaction when they are present in water used for making solutions for injection. Solutions of this kind are therefore prepared from freshly distilled water and potable water is used only for sterilising syringes by boiling. Small amounts of hard water remaining in a syringe after sterilisation in this way may yield sufficient alkali to react chemically with medicaments, for example, to cause the precipitation of insulin.

(2) Inorganic salts and dissolved gases. A comprehensive list of the anions and cations normally present in potable water has been given in the first paper and many problems arise from their presence. The divalent cations such as calcium are particularly troublesome since they may cause precipitation reactions in addition to yielding alkalinity.

Potable water is now sanctioned in the B.P.C. 1954 and in the National Formulary, for making oral and other preparations. Where incompatibilities do not occur or are unimportant, the use of potable water can be justified on the grounds of economy. However, owing to the variation in the composition and proportion of the dissolved salts in potable water in different parts of the country, differences in the properties of certain pharmaceutical preparations are found. For example, the concentration of calcium ions present in the water affects the sol viscosities and gel strengths of alginate and pectin dispersions. The use of potable water

can therefore produce problems in formulation, as will all chemically unstandardised substances.

A major use of water in pharmacy is for cleaning apparatus and containers. If potable water is used for the final rinse, a residue is left on the apparatus after drying which may be alkaline and give rise to trouble with unbuffered solutions. Water which has been softened by treatment with zeolites or other cation exchangers, is economical to use with certain types of detergents but it does not obviate the trouble from residue left on drying.

Methods of purifying water

In the light of recent developments, methods for purifying water can be divided into two classes.

(1) The traditional process of distillation in which the water is separated as vapour from the small amounts of non-volatile impurities. This is an extravagant process, the high specific heat and latent heat of evaporation causing heavy power costs.

(2) Removal of the impurities from the water at normal temperatures by means of solid reagents which are themselves insoluble in water.

The development of stable ion exchange resins which can be reactivated many hundreds of times, renders this second method of purification very much cheaper than distillation[1].

Distillation

A recent paper by Greppin[2] describes an extensive study of various methods for preparing distilled water for pharmaceutical purposes. An all-glass apparatus was found to give the best product.

It is of course essential that a still should be cleaned regularly in order to prevent the growth of bacteria and fungi in the condenser, where they would cause contamination of the distillate. Sterilisation of the condenser can be carried out daily by steaming out before turning on the condenser water. When high rates of distillation are used, there is a danger of contaminating the distillate with droplets of liquid carried over from the boiler in the form of a fine spray. These entrained droplets will pollute the distillate with the non-volatile impurities present in the feed water. Entrainment is a particular problem in preparing water for injection solutions.

Shotton and Habeeb[3] have shown that, providing liquid is not swept as a film along the walls of the stillhead, the contamination due to entrainment in a simple still, is about 1 in 10,000. At this dilution, pyrogenic effects are unlikely to be serious if fresh water is fed into the still (Todd private communication). Pyrogenic effects observed with distilled water are more likely to have arisen in a condenser which has not been cleaned and sterilised regularly, or from careless storage of the water in a non-sterile condition.

In order to reduce power costs and to improve rates of distillation, two new methods for the electrical heating of water in continuous stills have been developed. One of these is the use of a bare nichrome element immersed in the water. When a current is passed through the wire, smooth

boiling starts very quickly and a rapid rate of distillation can be achieved without formation of deposits on the wire. As a result there is none of the bumping which so often causes trouble when immersion heaters are used. After a time, the boiling water becomes coloured due to a slow dissolution of the nichrome; however, providing that there is an efficient baffle system to prevent entrainment, the distillate is quite free from traces of nickel or chromium. The nichrome element requires replacement at regular intervals.

The second new heating method uses carbon electrodes which are immersed in the water to be distilled. It may be necessary to add a salt to this water to give it sufficient conductivity to start the boiling. A heavy alternating current is passed between the electrodes and sufficient heat is produced to boil the water. Both these types of direct electrical heating can give rise to volatile electrolysis products, such as chlorine, in the distillate and tests should be made to ensure that the product is free from these impurities.

Freshly prepared distilled water from a clean, well designed still is substantially free from micro-organisms. On standing, it rapidly becomes contaminated with airborne organisms, unless precautions are taken to prevent this. The storage of distilled water in carboys over long periods may result in heavy contamination. It is odd that distilled water, which should be free from nutriments, provides a growth medium for a number of organisms.

The presence of viable bacteria in stored water can give rise to pyrogenic substances and can ultimately impart a hazy appearance which is difficult to remove, giving the water the appearance of a dilute vaccine. In addition, the organisms can cause decomposition of materials dissolved in the water; for example, the growth of micro-organisms in Injection of Mersalyl has been observed to cause a pronounced increase in the sodium sulphide reaction for mercuric salts. Thus it is essential not only to sterilise Water for Injection immediately after collection if it is to be stored, but also to minimise the time interval between exposure of the water to contamination and the sterilisation of the solution prepared with it.

Treatment of water with solid reagents

The earliest solid reagents used in the treatment of potable water were the zeolites. These are complex silicates which are insoluble in water and which exchange the cations such as calcium in the water with sodium ions contained in the zeolite. The softened water obtained after treatment with zeolites is free from divalent metal ions and is as a result, more economical to use with certain types of detergents than is untreated potable water. The total amount of non-volatile matter in the water is not however reduced by the softening process. The development of stable ion exchange resins containing strongly ionised acidic and basic groups has led to an important new method for purifying potable water, which has been recognised by both the U.S.P. XV and the B.P. Addendum, 1955, for preparing water suitable for pharmaceutical purposes other than for injection.

The resins now generally used for this method of water purification consist of hard insoluble beads of a styrene-divinylbenzene polymer. The cation exchanger has sulphonic acid groups distributed throughout its mass, while the anion exchanger contains quaternary ammonium hydroxide groups[4]. When water containing dissolved salts is passed over a mixture of these two resins, all the cations in the water are replaced by hydrogen ions from the sulphonic acid resin while the anions are replaced by hydroxyl groups from the anion exchanger. The final result is that the dissolved salts are removed completely, being replaced by hydrogen and hydroxyl ions and the resulting water has a very high specific resistance. When the resins are spent they can be regenerated by separating them by flotation (the anion exchanger is considerably less dense than the cation exchanger), the anion exchanger is regenerated with sodium hydroxide solution and the cation exchanger with hydrochloric acid. The resins are then washed and re-mixed. These operations are carried out without removing the resins from the column.

Water purified in this way is called de-mineralised or de-ionised water. The product obtained from a single column containing a mixture of the two resins has a much higher resistance and contains less non-volatile matter than does water purified by a two column process in which each exchanger is contained in a separate column[5]. This may be due to the fact that the traces of resin breakdown products imparted to the water are absorbed by the resin of opposite type in the mixed resin method. For example, the anion exchanger in the hydroxyl form tends to give traces of an amine due to the reaction,

$$(R.N^{+}X_3)OH^{-} \longrightarrow ROH + NX_3$$

where R represents the resin and X, an alkyl group. This reaction accounts for the unpleasant fishy smell of some resins of this type. In the mixed resin column, any traces of amine dissolving in the water are immediately taken up by the cation exchanger.

At ordinary temperatures the rate of decomposition of the resins is small and although they are expensive they have a long life and can be regenerated hundreds of times. As a result the ion exchange process is a very much cheaper method for purifying water than is distillation. Details of the purification of water by this method have been given by Saunders[6] and others[7,8] and the subject was reviewed comprehensively by Professor Büchi[9] at the conference of the Federation Internationale de Pharmacie in London last year. Recently, a small laboratory apparatus suitable for laboratories or pharmacies, has been made available and has formed the subject of an equipment test report in the journal *Laboratory Practice*[10]. This apparatus supplies about eight gallons of purified water per resin charge when London tap water is used as the feed, the resins are not regenerated but the cartridge containing them is returned to the manufacturer when exhausted and is replaced by a freshly activated one.

The ion exchange treatment of potable water is more effective than distillation in removing ionised substances from potable water, de-mineralised water may however contain other contaminants.

Colloidal matter. If the feed water is a pure and wholesome potable water, it should contain only very small amounts of colloidal matter. Providing that the resins are regularly regenerated, the concentration of albuminous matter in de-mineralised water is less than that in the feed water, though the resins do not remove this impurity completely. As a precaution the B.P. Addendum monograph on purified water has specified an albuminoid nitrogen test to avoid the possibility of accepting a product which contains undesirable amounts of albuminous colloids.

Non-ionic impurities. The concentration of non-ionic materials in potable water is normally very small. Appreciable amounts of them in the feed water would lead to rejection of the demineralised water prepared from it, as a result of the non-volatile matter test.

Micro-organisms. By filtering bacteria and suspended organic matter from the feed water, an undisturbed resin column may become a breeding ground for micro-organisms and may finally give a "purified water" which passes all the chemical tests but which is more heavily contaminated with micro-organisms than the feed water. This result can be avoided by operating the de-mineralising plant under carefully controlled conditions with frequent regeneration[11] and back-washing of the column. It has been suggested that the treatment of the column with formaldehyde will reduce the bacterial population[12].

Greppin[2] has stated that "bacteriological controls (of demineralised water) indicate a contamination proportional to the length of service of the apparatus". We suggest that the same result would be obtained with any water purification apparatus, unless it is carefully cleaned and sterilised at regular intervals. Greppin reports a detailed examination of a sample of de-mineralised water drawn from an industrial apparatus which had been in service for two years but he gives no details about the way in which it had been maintained. This water had a specific resistance of 0·43 megohm. cm., a poor value for demineralised water. He found that it passed all the tests of the Pharmacopœia except the non-volatile matter test in which it gave a residue of 12 mg./l., the permitted limit being 10 mg./l., and the residue was dark coloured. He also stated that the sample was "infested with germs and pyrogenic substances" and quite correctly pointed out that specific resistance must be used cautiously as a criterion for the purity of de-mineralised water as it is greatly affected by traces of inorganic substances but is hardly changed at all by colloidal substances and micro-organisms. These results of Greppin illustrate the point that it is not good enough for pharmaceutical purposes to take an industrial water de-mineralising plant and run it for year after year without regular cleaning. The heavy contamination of Greppin's sample by micro-organisms was almost certainly due to inadequate maintenance of the ion exchange equipment.

Providing the resin column is kept free from heavy bacterial contamination, de-mineralised water can be used in place of distilled water for most purposes except for preparing injection solutions. It should be suitable for making preparations for oral and external use and for preparing

stock solutions of chemical reagents. In addition, its cheapness and freedom from non-volatile matter make it advantageous for use as the final rinse liquid in washing bottles and other containers for liquids.

Pyrogen-free water by ion exchange

Present methods for de-mineralisation of water are not suitable for preparing Water for Injection though there seems to be a reasonable possibility of developing reliable techniques for this purpose. The first problem to be settled is the sterilisation of the ion exchange column. According to the manufacturers of the Amberlite resins, the strong cation exchanger can be sterilised by heating it for 15–30 minutes with a steam pressure of 15 lb./in.^{2}, the anion exchanger in the hydroxyl form is liable to suffer degradation by this treatment though in the chloride form it can be heated for 15–20 minutes at this steam pressure (Rohm and Hass, private communication). Cold treatment with formaldehyde would appear to be a simpler method and this can be done with both the resins in their activated forms.

Removal of gaseous impurities

De-mineralised water is completely free from carbon dioxide and free ammonia, but its oxygen content will be the same as that of the feed water. On standing in air it rapidly absorbs carbon dioxide and its specific resistance falls to an equilbrium value of about 1 megohm. cm.

In distillation, the feed water is usually heated to a temperature above 95° C. before it enters a continuous still thus eliminating most of the dissolved gases, some remain however and pass over into the condensate.

It is necessary to ensure that gaseous impurities are removed from the water used for some preparations. For example, carbon dioxide precipitates certain barbiturates from solution, and the presence of oxygen will cause the destruction of materials such as ergometrine. In instances like these the dissolved gases are removed from the water immediately before it is used. Two methods frequently used for this are based on Henry's law, (i) the water is boiled for some time, and the gaseous impurities are swept out by the bubbles of water vapour, (ii) the dissolved gases can be replaced by an inert gas of low solubility, such as nitrogen, by bubbling a stream of the inert gas through the solution. Subsequent manipulations of the solution are carried out in an inert atmosphere.

Storage of purified water

Some of the problems associated with the storage of distilled water have already been mentioned. The best rule is to store small amounts and to use it as rapidly as it is produced; this applies equally to distilled water and de-mineralised water.

In storage two types of contamination can occur.

(i) Growth of micro-organisms. This can be avoided only by sterilisation followed by storage under aseptic conditions. Alternatively a preservative such as the phenyl-mercury nitrate borate as used by Greppin[2] can be used to kill micro-organisms present in the water and to prevent

subsequent growth. However the presence of compounds of this type in the water will be undesirable in many applications.

(ii) The solvent properties of purified water will lead to attack on the container and closure materials during prolonged storage. These materials are usually rubber and glass and their effects on water stored in them have been described in the Symposium of the British Pharmaceutical Conference held in London in 1953[13–15]. A variety of impurities may be introduced into the water by this means and the content of non-volatile residue will increase and in time may exceed the permitted limit of 10 mg./l.

Materials other than glass and rubber which are frequently used for storing water are metals and plastics. Metal storage tanks should always be enclosed otherwise appreciable amounts of suspended matter may appear in the water. Galvanised iron tanks are particularly objectionable because after a time when the inevitable corrosion occurs the water may be discoloured by ferruginous substances. Copper is not used for the storage of purified water since, appreciable amounts of the metal may be dissolved in pure water stored in a copper tank. Some kinds of stainless steel are suitable though expensive materials for storage tanks but traces of heavy metals are likely to be found in the water after prolonged storage. Perhaps during the discussion some disadvantages of stainless steels as storage tank materials may come to light. Large storage tanks can be made by fitting a polythene liner into a metal tank.

Silver lined condensers have been used for collecting distilled water to be used for injections[16]. The effect of the silver was probably considered an advantage in retarding the growth of micro-organisms between the time of collection and use. Whether this is a real effect when the silver is coated with a film of oxide or sulphide is not known. Presumably the object is to lessen the risk of pyrogens being formed in the water by inhibiting the growth of organisms.

De-mineralised water is so completely free from metal ions that it seems a pity to pollute it by allowing it to come into contact with metal piping or tanks. Fortunately the development of polythene "plumbing" and containers means that no metal need be used.

We have found that prolonged storage of good de-mineralised water is a polythene container causes very little change in the amount of non-volatile matter in the water. After three months this was unchanged at 2 mg./l. We have always found that the residues resulting from the evaporation of demineralised water are white, contrary to Greppin's observation[2].

The development of plastics which can withstand temperatures up to 120° C.[17] means that in the future it will be possible to sterilise water and solutions in plastic containers by means of heat treatment.

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DISCUSSION

MR. W. P. HUTCHINSON (Oxford) mentioned that large-scale sterilisation of water was possible using the gamma radiation from the spent fuel rods of atomic piles in atomic power stations. Bacteria would be killed and the necessity for chlorination eliminated.

MR. T. D. WHITTET (London) said that at University College Hospital, deionised water was used for rinsing bottles and for the preparation of some culture media and biochemical reagents. He agreed that the plants at present available were not suitable for preparing water for injection. He had found that the two column process was better for removing pyrogens from water than the mixed bed process, but it gave water of lower chemical purity. Little work seemed to have been carried out in this country on the pyrogenicity of tap water. It had been reported that Cardiff tap water was pyrogenic and Manchester tap water was not; but no information had been given about whether the samples were examined before or after sterilisation. His own work suggested that London tap water was pyrogenic when unsterilised, but autoclaving removed the pyrogenicity.

MR. G. R. WILKINSON (London) asked if some information could be given about the possible radioactivity of potable water. Had the authors any information on the factors responsible for the corrosion of aluminium collapsible tubes? In the second paper the non-volatile residue after storage in polythene containers was mentioned. Was it not possible that volatile materials, included during fabrication, might be taken up from the plastic and give trouble? Considering the source of purified water, low pressure steam, partially condensed, was used in some factories, and he wondered whether the authors had examined such water.

DR. G. E. FOSTER (Dartford) emphasised the importance of the proper maintenance of storage tanks, and said that for pharmaceutical purposes tap water should be drawn directly from the mains. He had experienced difficulties in preparing a standard solution containing 1 p.p.m. of chlorine for checking *o*-tolidine tablets. The chlorine in a stronger solution was estimated, and then the solution diluted to give the required concentration, but some impurity in the water used for dilution reacted with the residual

chlorine. Could the authors suggest how the difficulty could be surmounted?

It had been stated that potable water could be used for some pharmaceutical purposes. He recalled rejecting some barium sulphate because it did not comply with the official test for the absence of soluble barium compounds. It was found that tap water had been used in the preparation of the barium sulphate and the reaction given was due to the presence of calcium salts.

Some time ago silver-lined tanks had been used for the crystallisation of sodium chloride required for intravenous purposes. Due to the presence of some free acid, traces of silver were present in the sodium chloride and affected the blood cells. Was any information available about the silver content of water stored in tanks lined with silver?

MR. J. W. HADGRAFT (London) referred to an example of the contamination of distilled water with traces of nickel. The water was used for the dilution of a strong solution of sodium hypochlorite and immediately produced a brown colour which did not occur when water from another still was used. It was found that traces of nickel in the water were coming from the Monel metal condenser tubes of the still. The nickel was present in the nickelous state and was not easily detected by the B.P. test for heavy metals. A haze developed with sodium sulphide solution after prolonged standing. The amount of nickel present was insufficient to produce a detectable colour with dimethylglyoxime but the reaction with sodium hypochlorite solution was immediately apparent and was capable of demonstrating the presence of nickel below concentrations of 0·5 p.p.m. A test was devised giving a quantitative reaction, the method was to add 5 ml. of strong sodium hypochlorite solution containing about 12 per cent. of available chlorine to 50 ml. of water in a Nessler cylinder. The test was far more sensitive than the B.P. method for the detection of heavy metals using sodium sulphide.

The ability of certain organisms to exist in water was of importance not only in relation to injections, but also in preparations for oral administration. He had recently seen a sample of barium meal smelling strongly of hydrogen sulphide, and chemical tests established that the preparation contained barium sulphide. On bacteriological examination, it was found that a sulphate-splitting organism was present which had attacked the barium sulphate and produce barium sulphide. This organism attacked the barium sulphate not only in a dilute solution of alcohol and saccharin, but in a simple suspension in distilled water.

MR. J. L. LIVINGSTONE (London) said that some rivers might contain water which had passed through power stations for cooling purposes and had been deoxygenated, and also water which might have been used for chemical processes and returned in anything but its original state. In areas relying on deep wells for part of the supply, the levels of the wells were dropping inches every year due to excessive demands. Was it likely that a serious shortage of water would occur, especially in lowland areas, in another 5 or 10 years time?

He had seen some examples of very bad pitting of aluminium vessels which had been used to heat water which had passed through copper pipes. Polythene tubing used for cold water supplies was becoming increasingly common, but that material was permeable to gas and there was the hazard that a leak of coal gas might cause the water to be contaminated.

DR. J. G. DARE (Leeds) referred to published work showing that comparatively small departures from neutrality could reduce the resistance of pyrogens to autoclaving. It would be interesting to know the pH of the London tap water which Mr. Whittet had used and what sort of buffering capacity it had. If the water were comparatively neutral, it might be that the pyrogens to which Mr. Whittet referred were relatively unstable.

MR. E. W. RICHARD (Upminster) said there were some areas in which the water, as it reached the consumer, was very hard. Did any of the water authorities consider that the installation of softening plants would be of advantage? If so, would it be an economic proposition from the point of view of the supplier as well as of the consumer? There was, he believed, a water softening plant at Leatherhead, but he did not know of any others.

PROFESSOR J. P. TODD (Glasgow) said that in his view pyrogens were being confused with pyrogenic reactions. Reactions similar to those resulting from the injection of pyrogenic solutions could be obtained in a number of ways, for example by the transfusing of incorrectly matched blood.

Dr. Shotton and Mr. Whittet were flirting with the idea of using ion exchange resins for the preparation of water for injection. Very much more work was required before it would be wise even to consider replacing distillation by such methods. Examination candidates frequently explained the presence of pyrogens by the mysterious process of entrainment. Either Dr. Shotton was wrong in his figure of 1 in 10,000 or he (Professor Todd) did not understand it. It had been shown that if distilled water was to be made pyrogenic by entrainment the water must contain 10,000 times the minimum dose when one started to distil it. The figures were probably right, and it would seem that over the years the word "entrainment" had been used when really what was meant was dirty condensers or containers.

It would be interesting to know whether Dr. Burman was at all worried about the fall-out of Strontium 90.

MR. J. H. OAKLEY (London) said that he had found ammonia contamination of distilled water sometimes occurred in cold weather, and he would be grateful to have Dr. Burman's comments.

The sterilisation of ion exchange resins by formaldehyde could cause complications, particularly if the demineralised water was used in an alcoholic preparation which was to be exported. For exporting alcoholic preparations one claimed a drawback on the residual spirit duty, and it was necessary to guarantee that the alcohol was free from methyl compounds. The test was based on the formaldehyde colour reaction, and if

any trace of formaldehyde was present in the deionised water the Government chemist would immediately interpret that as implying the use of industrial methylated spirit.

If the resins were in two separate towers, debris was removed almost completely from the potable water in the first tower—the Zeocarb cation exchanger—and it was a good practice to give it a daily back-wash to remove dirt from the surface.

MR. J. C. HANBURY (Ware) said that some years ago he had heard a radio talk on the serious position—thinking in terms of 50 or 100 years—of the water supply in South-East England where there was a maximum concentration of demand and the worst climatic conditions to meet it. One had always understood that afforestation had a profound long-term effect on water precipitation and storage and the subterranean availability of water. He understood that afforestation was a favourable factor in both attracting moisture and storing it. It would be interesting if Dr. Burman would enlarge on the question of afforestation and possibly on the effects on the water supply of deforestation and the large built-up areas in South-East England.

On a more specific point, the sewage effluent from the new town of Stevenage was now being added to the sewage deposited on the Rye Meads gravel bed, and water from deep wells nearby was being used by a water authority. Was any danger anticipated of long term bacterial contamination from the Rye Meads beds reaching the water in the nearby wells?

DR. F. WOKES (King's Langley) referred to the problem of trace elements in water. They were present in very small quantities—much less than 1 p.p.m.—and therefore the intake from drinking water might not be considered serious. But some elements were stored in the bones so that over the course of years they could accumulate in toxic amounts and on liberation might be responsible to some degree for the development of diseases. Toxic elements had been suggested as the possible causative factor of disseminated sclerosis. A paper was published by Campbell in 1950 in which he found that in areas where the disease occurred there was a much higher concentration of lead in the water. Further, the teeth of those who had the disease contained up to ten times as much lead as the teeth of healthy persons of the same age.

On use of water in pharmaceutical processes, he commented that very large quantities were used in the preparation of malt extract. Work had been published showing that the rate of extraction from the malt varied with the type of water used, factors responsible being the pH and the salts present.

DR. W. ANDERSON (Liverpool) suggested that pyrogens were likely to be present in deionised water until a system could be constructed which would remove all colloidal matter.

MR. M. B. BROPHY (Dublin) said that activated charcoal had been used for removing pyrogens. Had the authors any information on distilling water in the presence of depyrogenising charcoal for that purpose?

Would it be worth while combining ion exchange resins and depyrogenising charcoal?

PROFESSOR H. BRINDLE (Manchester) said that some eight years ago when he had required some natural pyrogens, he had experienced the greatest difficulty in obtaining any public water supply which was contaminated with pyrogens. He would be interested to learn whether the authors had any information about the presence or absence of pyrogens in London water at that time.

MR. R. L. STEPHENS (Brighton) asked whether the authors could say anything about the presence of synthetic detergents in potable water. The problem of detergents to sewage disposal authorities was well known, and it had been shown that a proportion was returned to the rivers. Were any steps taken to remove the detergents from the public supply? Synthetic detergents were incompatible with cationic antiseptics.

Tin was a traditional material for lining laboratory apparatus, and on one occasion he had found his supply of distilled water heavily contaminated with stannous salts. After cleaning out the apparatus the salts ceased to be present.

On the possibility of plasticisers being present in polythene he said there was only one plasticiser used with polythene and that was completely insoluble in water.

DR. F. HARTLEY (London) asked if appreciable amounts of sodium fluoride were contained in water, and that water were exposed to radioactive waste, would the hydrofluoric acid or fluorides present affect the behaviour of the ion exchange resins with which the water might subsequently be treated?

MR. EDWARDS (Harrow) said that Dr. Shotton had implied that electrode boilers were a new development. This was not so—they had been in use for many years. No mention had been made of the steam compression still. By compressing steam it was possible to obtain the equivalent of triple distillation with something like 2 per cent. of the heat required for single distillation. It was possible to obtain 50 gallons per hour of pure pyrogen-free water for the equivalent of about 10 kW of energy. Entrainment was prevented by the use of a cyclonic separator.

MR. W. P. HUTCHINSON (Oxford) stated that sterilisation by gamma radiation did not induce radioactivity.

DR. N. P. BURMAN, in reply, said that his only knowledge of the pyrogen content of London water came from Mr. Whittet. When the bacteriologist referred to the presence of bacteria in water he was referring solely to viable bacteria, and there might also be millions of dead ones present.

He had no knowledge of sterilisation by gamma rays, fission products, etc. He was not aware of any research going on within the water industry on those methods.

Contamination of water by radioactivity was a matter that was being closely watched in the waterworks world, both from the point of view of discharges as a result of the development of nuclear energy and from

radioactive fall-out. He could do no better than advise those interested in the effects of fall-out to read the appropriate section of the recent publication of the Medical Research Council on "The Hazards to Man of Nuclear and Allied Radiations".

The type of pitting corrosion of aluminium he had mentioned referred to ordinary grades of the metal. He had no experience of the highly purified grade used in collapsible tubes. Pitting corrosion was considered to be due to minute traces of metals setting up electrolytic cells. There was another type of corrosion which occurred frequently in aluminium kettles, due to traces of copper in the water. Precipitation of the copper and solution of the aluminium was the result.

Storage tanks with faulty covers presented a problem. The water authority had no jurisdiction over storage tanks, and it was the owner's concern if something went wrong with them. A far better standard could be devised for storage tanks than the very indifferent individually made wooden covers which were placed on them. Tanks could be designed which would eliminate all possibility of the access of small animals and birds. The same problem occurred on a large scale in the distribution system, such as service reservoirs, water towers or elevated water tanks. Very great care was taken to exclude animals and small birds from such places, and regular inspection of ventilators and covers was necessary.

On the subject of obtaining a standard colour with *o*-tolidine reagent he commented that if one started with a known quantity of available chlorine in a solution and diluted with water it was necessary to exercise care in selecting the water with which dilution was carried out. It must be high quality distilled water, free from organic matter, because organic matter would deviate the chlorine. The deviation of chlorine in a river water was quite considerable, and gradually increased with time. It was now the general practice of the Metropolitan Water Board in the case of their river-derived filtered waters, to insert contact tanks between the chlorination plant and the point at which the water was pumped into supply. Chlorine was added to the water in the contact tank, which was large enough to contain up to two hours' supply; there were baffle plates in the tank so that water could not flow directly from the inlet to the outlet, and during that two hours the chlorine was completely deviated. Up to 1 p.p.m. of chlorine may be added at the beginning while very much less than 0·1 p.p.m. would be found at the outlet. With deep chalk well water, if the same procedure were adopted, 1 p.p.m. would be found at the outlet as the quality of the water had a considerable effect on the deviation of chlorine. Excess chlorine was removed by sulphur dioxide.

There were certain filters on the market operating with what was known as a catadyn sand. There were also available types of kieselguhr filters impregnated with catadyn silver which was reputed to render the water completely sterile. If any silver found its way into the water, it was in such minute quantities that it was not detectable by normal means. They seemed to last almost indefinitely with no reduction in the amount of silver on the filter itself.

As to sulphate-reducing organisms in water, the Metropolitan Water

Board were more concerned with sulphate reducers in clay soils, which under the anaerobic conditions existing could reduce sulphate in the presence of iron from iron pipes and produce hydrogen sulphide, which would cause deterioration of the iron and the production of ferrous sulphide. That would produce severe and, in some circumstances, rapid corrosion of the pipe. In some soils this was a very serious problem.

The question of cooling water from power stations had been very carefully considered in connection with the new power station being built up on the Lea. The temperature of the river water was raised but not sufficiently to cause trouble; the temperature of the discharge from the power station was the subject of control. Chemical effluents could often be far more serious than bacterial pollution, and if there were both together, the chemicals left in the water might have a sterilising action, so that a false picture was presented. The pollution of rivers had, since the war, become the responsibility of the River Boards which now possessed much greater powers of control.

In London alone there were hundreds of private wells in addition to those possessed by the Board. In some cases the level and quality of the water had deteriorated so much that the wells had fallen into disuse, but an interesting experiment had been conducted in the past two years to re-charge the underground waters. In the winter, water was allowed to flow down certain wells instead of being pumped up. Filtered, chlorinated river water was used, and in the summer, if it were required, the water was pumped into the supply. However, the quality of the water might be quite different from that obtained from the wells previously.

Polythene tubing could absorb coal gas quite readily. One serious instance had been encountered where a local gas main leaked, and the gas was absorbed by the polythene tubing which was buried in the ground nearby. An objectionable odour and taste was produced in water which passed through the tubing and the gas absorbed was continuously given off for some time afterwards. Before approving the use of polythene tubing in the Board's area, a number of experiments were carried out on water after passage through the tubing, and the only difference found was that sometimes there was an increase in the bacteria capable of growing at room temperatures. That might have some effect on subsequent pyrogenicity but none on potability.

Potable water as supplied in some areas was very hard and he had been asked about the economics of water authority softening. The softening of the whole of London's water supply would be a costly procedure and it had been estimated that it would put up the cost of treatment by about 100 per cent. The number of people who would benefit by that increase in cost was limited, and the demand for soft water had considerably declined due to the increased use of detergents.

The question of the ammonia content of water in cold weather was very interesting. A polluted water did contain ammonia and ammonium salts, and in works where rapid sand filtration was used before slow sand filtration for clarification, *Nitrosomonas* (which convert ammonia to nitrite) and *Nitrobacter* (which convert nitrite to nitrate) develop around

the grains of sand. However, they only function at temperatures above about 4° C. and in cold weather they die. When the temperature rises there is a time lag before they function again properly.

On the long term effect of afforestation, it was rather surprising to learn of a report to the British Association opposing afforestation in upland catchment areas because of the greater amount of evaporation which occurred. There would be a greater amount of evaporation initially, but the advantages far outweighed that disadvantage.

Small quantities of crude sewage had been deposited on the gravel beds at Rye Meads for many years. It was hoped now that larger quantities of good quality sewage effluent could be disposed of in a similar manner with equal success. This plan will considerably reduce the degree of pollution of the River Lea and it is not anticipated that it will affect the wells in the vicinity because they already carry some degree of pollution and are pumped into the adjoining New River and treated as for a river water.

He was not aware of any routine observation of the content of trace elements in water, in the very low concentrations mentioned.

It was known that a trace of detergent residue was left in some domestic water supplies but this amount was considerably less than that consumed as a result of surface-active material from household detergents left on unrinsed china and cutlery after washing. Enquirers were referred to the "Report of the Committee on Synthetic Detergents" recently published by H.M.S.O. and a paper by C. Hammerton, Senior Chemist of the Metropolitan Water Board, on "Synthetic Detergents and Water Supplies", to be published in the Proceedings of the Society for Water Treatment and Examination.

DR. E. SHOTTON, in reply, suggested that low pressure steam referred to might more aptly be described as waste steam. He had not heard of it being used to any great extent in pharmaceutical preparations. Some waste steams could be very dirty indeed, and he would not normally recommend its use as a source of distilled water. He was not advocating the use of silver-lined plant, but it was worth reporting that it had been used presumably for the oligodynamic effect.

He had never found that tin caused troublesome contamination of water and he had no knowledge of nickel causing contamination of distilled water, but he had had experience of nickel contaminating acid solutions, and the better quality stainless steels were much more resistant than Monel metal. Stainless steel was probably the most suitable metal for a condenser if glass could not be used. He had experience of sulphate reducing organisms in an aluminium phosphate suspension. The source of the aluminium had been aluminium sulphate and traces of sulphate present had been reduced to sulphide.

He was not advocating the use of ion exchange resins to produce water for injection. That would be very hazardous at the present stage. It might be, however, that in the future a technique would be evolved whereby such water could be obtained. He agreed that if formaldehyde were

used as a sterilising agent for the resins, it must be completely removed before the water could be used.

With reference to the suggestion of charcoal for the removal of pyrogens, he would stress that columns could be very dangerous, whether containing charcoal or ion exchange resin, in that bacteria might grow in them. It was worth while emphasising that charcoals were very variable. Many charcoals contained high quantities of lead, arsenic, copper, zinc and other metals, and should be treated before being used for pharmaceutical purposes.

He was aware that immersion electrodes had been used, and he knew of some buildings in which the steam supply was maintained by the installation of those heaters; but it was new in that recently in the literature the still had been advocated for pharmaceutical purposes. The economics would depend upon the scale of production. For small scale production the cost would probably be higher than when ion exchange resins were employed.

He was not aware that steam compression stills were in use for the production of distilled water, although they were employed for evaporation.

DR. L. SAUNDERS, in reply, said that the problem of the sterilisation of ion exchange columns had not yet been solved. Formaldehyde seemed unsatisfactory and a more suitable material had to be found.

As to polythene containers and plasticisers, although it was stated that no plasticiser was used in polythene, some fabricators occasionally included a plasticiser.

The possibility of nickel being present in water raised the question of the B.P. test for heavy metals in water. At the moment there was one not very sensitive overall test for a number of metals. There were many sensitive reagents available for individual heavy metals, and some modification of the test might be considered. He suggested that cationic exchange columns might be useful in estimating the content of trace metals in water.

Although he had no personal experience of the behaviour of fluorides with ion exchange resins, he did not think that their presence would have any harmful effect on the resin.

BRITISH PHARMACEUTICAL CONFERENCE DUBLIN, 1956

SCIENCE PAPERS AND DISCUSSIONS

(*continued from page 804*)

ANALGESICS AND THEIR ANTAGONISTS: SOME STERIC AND CHEMICAL CONSIDERATIONS

PART I. THE DISSOCIATION CONSTANTS OF SOME TERTIARY AMINES AND SYNTHETIC ANALGESICS; THE CONFORMATIONS OF METHADONE-TYPE COMPOUNDS

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INTRODUCTION

THE features thought to confer analgesic activity[1] at least equal to that of pethidine may be summarised as follows:

1. A basic centre which is ionised, or partially ionised at physiological pH, so as to associate with an anionic site in the receptor surface. 2. A flat aromatic structure in the molecule to allow a strong collective van der Waals' force bonding to a flat portion of the receptor reinforcing the ionic bond mentioned in (1). 3. The basic group and the flat structure to be in almost the same plane; this to be accomplished by a completely rigid molecule or a slightly less rigid one held in the correct configuration by steric or other constraints. 4. A suitably orientated projecting hydrocarbon moiety to form, with the basic centre and flat aromatic structure, a three dimensional geometrical pattern (see XVIII).

The purpose of the present series of papers is to present a detailed study of the cationic portion of the molecules of analgesics (and analgesic antagonists) in so far as it influences the first and third features.

Small changes in the alkyl groups attached to the basic centre of an analgesic are known to lead to profound alterations in the degree and the character of the biological response. Compounds produced by the replacement of the *N*-Me group of morphine by *N*-Et, *N*-*n*-Pr and *N*-allyl groups exhibit a transition from active analgesics to less active compounds and then to anti-analgesics as the series is ascended[2,3]. In this paper the dissociation constants and the conformation of methadone type compounds are considered, in Part II the effect of the type of basic group upon the properties of the compounds and the degree of analgesic effects will be considered, and in Part III the effect of the type of basic group upon the character of the biological response will be discussed.

Dissociation Constants of Analgesics and Related Compounds

The pK′a values of several series of methadone-type compounds were determined in water at 25° C. and ionic strength of approximately 0·013 M (Table III).

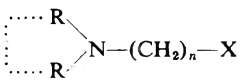
In Table I the reported analgesic activities and pK′a values of compounds of widely differing potencies are recorded, while in Table II the pK′a values of simpler amines are given.

Discussion

It is desirable to consider the observed △pKa values of the simpler amines of Table II before attempting to interpret those of the complex analgesics containing these basic groups. Since all the values quoted in the Table were not obtained using constant ionic strength of the medium, too much reliance cannot be placed upon minor differences. However, the consistent pattern upon changes of the various groups in the series indicates that the results may be used as the basis for generalisations.

The strength of an organic nitrogen base, as indicated by dissociation constants, is dependent upon the facility with which the lone pair electron cloud will attract and bind a solvated proton. Any effect which results in an increase in electron density in the lone pair orbital will increase the strength of the base. Any substituent exerting an electrical or steric effect to distort the orbital is considered to partially neutralise the electric charge around the nitrogen atom and be base weakening.

A substituent X may influence the basic strength of the tertiary amine (IV) by various mechanisms,



(IV)

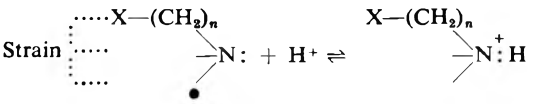
the observed effect being usually a complex combination of these.

(*a*) An inductive effect which is transmitted along the atoms of the chain may occur; it decreases rapidly with chain length.

(*b*) A direct electrostatic effect ("field effect") may operate through the solvent or free space between the substituent and the basic centre.

(*c*) The steric requirements of the substituent in proximity to the basic group may militate against the formation of the cation () which has larger steric requirements, due to solvation, than the corresponding unionised group ().

(*d*) The substituent will have a base weakening effect if there are greater steric interactions between the groups attached to the nitrogen in the 4-co-ordinated cation than in the 3-co-ordinated amine (B-strain)[20,21].



(strained) (more strained)

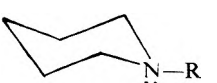
(*e*) The steric requirement of the substituent could result in greater difficulty of approach and increased facility of recession of a solvated proton. This is likely to be a relatively unimportant factor since it is known that the steric requirements of a proton are exceedingly small, e.g. the alkyl groups in 2:6-lutidine exert large steric effects sufficient to prevent combination with trimethyl borine and yet have no effect on the addition of a proton[20].

(*f*) The above effects may be further complicated by alteration of groups R and their inclusion as a ring structure, with or without atoms or groups possessing dipoles.

The observed differences in dissociation constants in the simple compounds in Table II may be explained in terms of the above effects. The values indicate that *N*-ethyl compounds are stronger bases than the corresponding *N*-methyl compounds by about 0·3 to 0·4 pKa units, e.g., piperidines 10 and 9 (△pKa 0·42), morpholines 13 and 12 (△pKa 0·29), pyrrolidines 24 and 23 (△pKa 0·38), trialkylamines 27 and 26 (△pKa 0·40). This is attributed to the ethyl group, which has a larger $+I$ effect than a methyl group, increasing the electron density on the nitrogen atom. Further increase in chain length produces no change, e.g., 2/3 and 4, and 10/11; the increase in inductive effect will be almost negligible and even this minor increase will be counter-balanced by the increased B-strain (with its base weakening effect) caused by increased chain length.

The fact that morpholino compounds are weaker bases than the corresponding piperidine analogues by about 2·65 pKa units is attributed to the inductive effect of the ring oxygen reducing the electron density on the nitrogen atom of the former compounds. The concordance of △pKa values of comparable compounds containing the two ring systems despite the wide variation of the *N*-alkyl groups supports this contention.

The dialkylamino-R compounds are slightly stronger bases than the corresponding piperidino compounds, e.g., 27/2 (△pKa 0·33), 28/3 (△pKa 0·17) (see later also). Six membered rings adopt a strain-free puckered conformation; it is difficult to account for the above difference in terms of strain in the ring favouring $\rangle N:$ at the expense of $\rangle \overset{+}{N}:H$ An increase in B strain in the ring compound may occur but it seems more reasonable to explain the difference as follows. An *N*-alkyl piperidine



(V)

will adopt the chair conformation (V), and by analogy with *cyclo*hexane, it would be predicted that the R group is equatorial since even a CH_3 group has been shown to have a greater steric requirement than an electron pair. The approach of a proton in cation formation is therefore sterically controlled (towards axial lone pair), since the movement of the lone pair electrons to other positions would cause ring conformation change involving an increase in non-bonded interactions between the

R group and the atoms of the ring. Such control will not operate in the analogous open chain compounds; cation formation will therefore be statistically more favoured in these as compared with the ring compounds.

The introduction of an α-CH_3 group into a piperidine ring has a base strengthening effect, e.g., 11/3 ($\triangle$pKa 0·24) 10/2 ($\triangle$pKa 0·27). The + I effect of the CH_3 group will increase the electron density on the nitrogen atom; its bulk in the vicinity of this atom will operate against cation formation. The inductive effect is presumed to play the major role since the CH_3-group will adopt an equatorial position and the rotation of the whole ring will result in the group keeping a fixed position from the *N*-atom lone pair electrons so that only a small steric effect will obtain.

The presence of a double bond $\beta\gamma$ to the N atom has a base weakening effect due to the reduced electron density on this atom.

The dissociation constants of analgesics

The pK′a values quoted in Table I are from the data of Farmilo and others[13], who used aqueous ethanol as solvent in many of the determinations. Values measured in water are likely to be slightly different, but those of Table I indicate that there is no simple relation between dissociation constants and analgesic activities.

TABLE I

DISSOCIATION CONSTANTS* AND ACTIVITIES† OF VARIOUS ANALGESICS

Analgesic	pK′a	Approx. analgesic activities (Morphine ≡ 100)
Morphine HCl	8·05	100
Diacetylmorphine	7·83	>100
Codeine phosphate	8·22	10
Dihydromorphinone HCl	8·15	450
Metopon HCl	8·08	1250
Levorphan tartrate	8·18	>200
Nalorphine HCl	7·83	—
Pethidine HCl	8·72	20
α-Prodine HCl	8·73	60–100
Methadone HCl	8·25	100
Isomethadone HCl	8·07	66
Phenadoxone	6·89	>100
6-Piperidino-4:4-diphenylheptan-3-one	6·8	100 or > 100

* Dissociation constants are taken from ref. 13. † Activities (in rats or mice) are taken from refs. 5 to12.

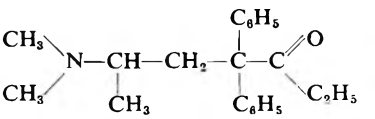
The values lie within the range of pK′a 7·8 to 8·9 (corresponding to about 86 to 98 per cent. ionisation as cations at physiological pH) with the exception of those of phenadoxone and its piperidino analogue. Titrations under aqueous conditions (see Part II) gave results for phenadoxone (pK′a 6·73) and a few of the analgesics in Table I in general agreement with the values obtained by Farmilo, but the piperidino analogue of phenadoxone was found to have a pK′a of 8·59 (Farmilo and others[13], 6·8). It seems probable that the basic group must be ionised at physiological pH, to allow association with the anionic site of the receptor, but only partly so. It is known that ions penetrate membranes less readily than the corresponding neutral molecules due to their charge and relatively greater size (due to hydration)[4,14]. Unionised molecules may be necessary in the case of analgesics to allow rapid penetration of

membranes; the molecules after penetration to the biophase at the receptor site will then partially ionise as cations to a degree dependent upon their pKa values and the pH of the medium.

The dissociation constants of methadone-type compounds

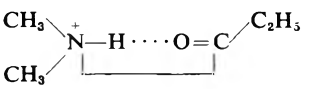
The pK'a values of these analgesics will be affected by interactions of the basic centre with other groups in the molecule.

In a previous paper[1], it was stated that the bulky groups attached to the quaternary carbon imparted a rigidity to the methadone molecule (VI).

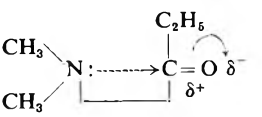


(VI)

The phenyl groups will occupy positions corresponding to the sides of a trihedral angle with the quaternary carbon at the apex. The nitrogen atom and carbonyl group will be held by electrostatic forces so that the conformation of the molecule is such that one phenyl ring and the nitrogen atom are capable of alignment with the flat surface and anionic site respectively of the proposed analgesic receptor surface (XVIII). It was suggested that hydrogen bonding of type VII might be the mechanism by which the nitrogen group (as a cation) and the carbonyl group are held in close proximity.

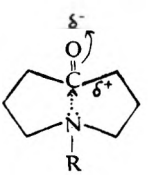


(VII)

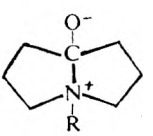


(VIII)

An alternative mechanism may be formulated (VIII), in which the lone pair orbital of the nitrogen atom interacts with the electropositive carbonyl carbon atom. Such a mechanism would be analogous to the transannular interaction[22] between amino nitrogen and carbonyl group in compounds of type IX. These have been shown to exist in certain conditions chiefly in the form X[22].

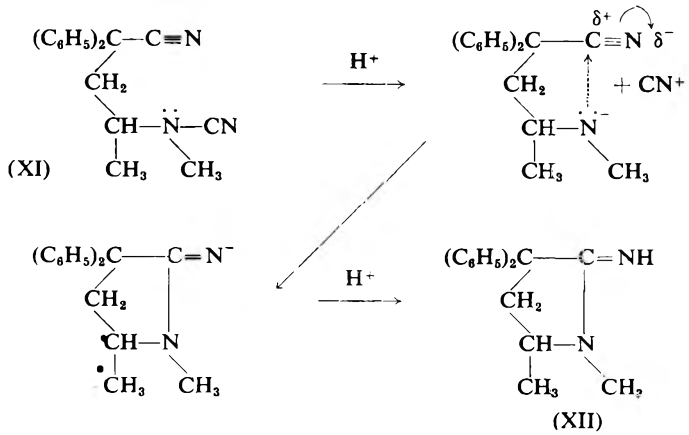


(IX)



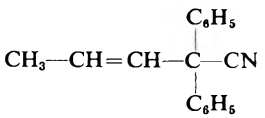
(X)

Certain chemical and physical data[23] indicated that this type of interaction rather than hydrogen bonding constituted the mechanism of the attractive forces. Acid hydrolysis of XI yielded the imino-pyrrolidine (XII); the mechanism shown seems probable (see also Wilson[24,25]).

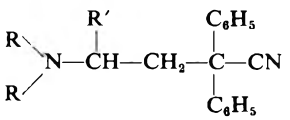


(XII)

The infra-red spectrum of XIII exhibited a characteristic nitrile absorption in the region of 2250 cm.$^{-1}$, whereas basic compounds of type

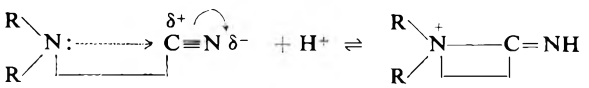


(XIII)



(XIV)

XIV showed only very weak nitrile absorption in this region, and in their salts the characteristic absorption peak was lacking. No evidence of C = NH absorption was found. It is presumed that nitrile-amino interactions occur but are not sufficiently powerful to result in the formation of XVI from XV upon salt formation.



(XV) (XVI)

The dissociation constants of several series of methadone-type compounds were determined (Table III) in an attempt to provide information on the above interactions under aqueous conditions.

The replacement of the H atom (R′ = H) by the cyano group (R′ = CN) in methadone type compounds (I) and (II) has a base weakening effect (△pKa values A, 0·89 and 1·07; D, 1·09 and 1·17; B, 1·14 and 0·8) (Table III). A smaller base weakening effect results upon the introduction of the ketonic group (R′ = COC_2H_5) (△pKa values A, 0·10 and 0·22; D, 0·19 and 0·49; B, 0·25 and 0·17). Since both the cyano and ketonic groups are electronegative in character, the observed differences may be attributed to the inductive effect along the chain separating these groups from the basic centre, or a field effect operating through space or solvent if the interacting groups are in close proximity. Until recently it has been impossible to assess the relative importance of the two modes of transmission in a particular compound. Grob and others[26] have now presented

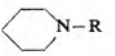
evidence, from considerations of the dissociation constants of certain betaine hydrochlorides, to show that the so-called general inductive effect appears as a function of the direct distance between electrostatically interacting groups and the dielectric constant of the intervening medium; this can be a chain of atoms, solvent or empty space according to the geometry of the system. The base weakening effect of a cyano or keto group in methadone type compounds would be expected to have little effect upon the basic centre if maximum group separation occurred. The results indicate that the molecules assume a conformation in which the cyano (or keto) group and basic centre are in close proximity.

The base weakening effects also indicate that the interactions are of the type shown in VIII rather than VII since the latter would be expected

TABLE II

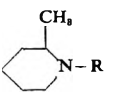
pK'a VALUES FOR VARIOUS TERTIARY AMINES
TEMPERATURE 25° C.; SOLVENT WATER

A



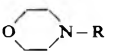
	R	pK'a
1	CH_3	10·15
2	C_2H_5 (*b*)	10·41
3	n-C_3H_7 (*c*)	10·48
4	n-C_4H_9 (*c*)	10·49
5	$-CH_2{\cdot}CH=CH_2$ (*d*)	9·68
6	$-(CH_2)_2N$ (*e*)	6·25 9·47
7	$-(CH_2)_3N$ (*e*)	7·9 9·95
8	$-CH_2{\cdot}CH_2OH{\cdot}CH_2{\cdot}N$ (*e*)	7·47 9·48

E



	R	pK'a
9	CH_3 (*c*)	10·26
10	C_2H_5 (*b*)	10·68
11	n-C_4H_9 (*b*)	10·72

B



	R	pK'a	ΔpKa A–B
12	CH_3 (*d*)	7·41	+ 2·74
13	C_2H_5 (*d*)	7·70	+ 2·71
14	$-CH_2-CH=CH_2$ (*d*)	7·05	+ 2·63
15	$-(CH_2)_2N\,O$ (*e*)	3·63 6·65	+ 2·62 + 2·82
16	$-(CH_2)_3N\,O$ (*e*)	5·25 7·25	+ 2·65 + 2·7
17	$-CH_2{\cdot}CH_2OH{\cdot}CH_2{\cdot}N\,O$ (*e*)	5·00 6·98	+ 2·47 + 2·50

C

	R	pK'a	ΔpKa A–C
18	CH_3 (*a*)	10·14	+ 0·01
19	*n*-C_4H_9	10·36	+ 0·13
20	$-(CH_2)_5N$ (*e*)	6·3 9·47	− 0·05 0·0
21	$-(CH_2)_6N$ (*e*)	8·03 10·04	− 0·13 − 0·09
22	$-CH_2{\cdot}CH_2OH{\cdot}CH_2N$ (*e*)	7·75 9·73	− 0·28 − 0·25

F

	R	pK'a	ΔpKa E–F
23	CH_3 (*c*)	10·26	0·0
24	C_2H_5 (*c*)	10·64	+ 0·04
25	*n*-C_4H_9 (*c*)	10·69	+ 0·03

D

$R_2'N-R$

	R'	R	pK'a	ΔpKa A–D
26	C_2H_5	CH_3 (*b*)	10·34	− 0·19
27	C_2H_5	C_2H_5 (*b*)	10·74	− 0·33
28	*n*-C_3H_7	*n*-C_3H_7 (*b*)	10·65	− 0·17
29	C_2H_5	$-(CH_2)_2N(C_2H_5)_2$ (*e*)	6·18 9·55	+ 0·07 − 0·08
30	C_2H_5	$-(CH_2)_3N(C_2H_5)_2$ (*e*)	8·2 10·18	− 0·30 − 0·23
31	C_2H_5	$-CH_2{\cdot}CH_2OH{\cdot}CH_2{\cdot}N(C_2H_5$ (*e*)	7·74 9·80	− 0·27 − 0·32

(*a*) See ref. 15. (*b*) See ref. 16. (*c*) See ref. 17. (*d*) See ref. 18. (*e*) see ref 19.

to have a base strengthening effect (e.g., the fact that basic groups with proximate rather than distal COO^- groups are the stronger bases of corresponding pairs, is attributed to hydrogen bonding $COO^-\cdots H-N^+$ in the former compounds[27]).

The △pKa values between the piperidino and corresponding morpholino compounds in these methadone type bases (Table III, △pKa A − B) are much lower than those for the simpler bases in Table II. This decreased base weakening effect of the morpholino oxygen in these methadone compounds is attributed to the competing electron attractive forces of the substituent in the *N*-alkyl chain which reduce the −I contribution of the oxygen atom.

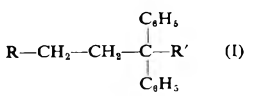
The presence of a CH_3 group on the α-carbon of the *N*-alkyl chain has a base weakening effect in these analgesically active ketones (the △pKa values 33/36, 46/49 and 40/43 are 0·28, 0·24 and 0·27 respectively). Consideration of molecular models in the conformation with carbonyl and

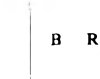
nitrogen function in close proximity indicates that the CH_3 group will impose a steric limitation in the vicinity of the N atom and so favour the neutral molecule rather than the larger cation. B strain will also be increased by the introduction of this group. These two base weakening effects will be more important in these compounds than the +I base strengthening contribution of the group. It is to be noted that the contribution of the methyl group is different from the base strengthening effect of such a group on the α-carbon atom of simple *N*-alkylated heterocyclic rings (e.g., *N*-alkyl-piperidines) (Table II). A CH_3 group on the β-carbon atom of the chain (see III) of *nor*methadone would be expected to have even a greater base weakening effect if the above interpretations are correct. Isomethadone nitrile (IIID) (pKa 7·9) and its piperidino analogue (pKa 7·54) are weaker bases than their respective *nor*-compounds by 0·41 and 0·53 pKa units respectively.

TABLE III

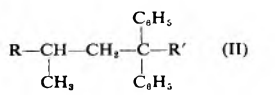
DISSOCIATION CONSTANTS (pK′a) OF METHADONE-TYPE ANALGESICS AND RELATED COMPOUNDS

TEMPERATURE 25° C.; SOLVENT WATER

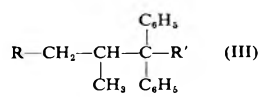


A R=  B R=  D R= 

Compound No.	R′	pKa′	Compound No.	R′	pK′a	ΔpKa A–B	Compound No.	R′	pK′a	ΔpKa A–D
32	H	8·96	39	H	7·25	+ 1·71	45	H	9·40	− 0·44
33	$-COC_2H_5$	8·86	40	$-COC_2H_5$	7·00	+ 1·86	46	$-COC_2H_5$	9·23	− 0·37
34	−CN	8·07	41	−CN	6·09	+ 1·98	47	−CN	8·31	− 0·24



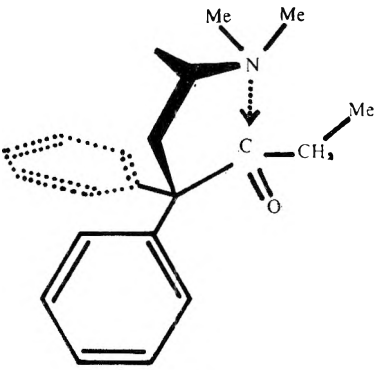
Compound No.	R′	pK′a	Compound No.	R′	pK′a	ΔpKa A–B	Compound No.	R′	pK′a	ΔpKa A–D
35	H	8·80	42	H	6·90	+ 1·90	48	H	9·48	− 0·68
36	$-COC_2H_5$	8·58	43	$-COC_2H_5$	6·73	+ 1·85	49	$-COC_2H_5$	8·99	− 0·41
37	−CN	7·73	44	−CN	6·10	+ 1·63	50	−CN	8·31	− 0·58



Compound No.	R′	pK′a	Compound No.	R′	pK′a	ΔpKa A–D
38	−CN	7·54	51	−CN	7·90	− 0·36

As in simpler compounds (Table II), the piperidino compounds of Table III are weaker bases than their dimethylamino analogues by about 0·4 units (see Table III, $\triangle$pKa A − D).

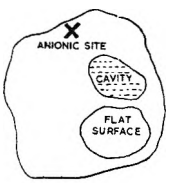
The infra-red and the dissociation constants data, as well as the chemical data briefly reported in the paper, all indicate a mutual interaction of basic and nitrile (or carbonyl) groups caused by their close proximity in the molecular conformation adopted, and the probability of the attractive forces being $N\text{–}C_{CN}$ and $N\text{–}C_{CO}$ interactions (see VIII).



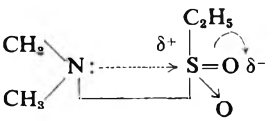
(XVII)

A preferred conformation of methadone in aqueous solution may be regarded as that portrayed in XVII; association with the proposed analgesic receptor site (XVIII) is therefore facilitated.

The morpholino and piperidino analogues of methadone and the corresponding "iso" (III; R = COC_2H_5) and "nor" (I; R = COC_2H_5) compounds may be regarded as adopting similar conformations. Replacement of the ethyl ketone group of methadone by the ethyl sulphone group gives an active analgesic[28]. It is reasonable to assume that this compound also has a similar conformation, the attractive forces between basic and sulphone groups being as shown in XIX.



(XVIII)



(XIX)

Experimental

All m.pts. are uncorrected.

Preparation (or source) of compounds

(The compound numbers correspond with those of Table III.)

The m.pts. of the bases or salts used in the measurement of dissociation constants are recorded in Table IV.

TABLE IV

THE MELTING POINTS OF THE BASES OR SALTS USED IN THE MEASUREMENT OF DISSOCIATION CONSTANTS

Compd. No.	Base or salt	M.pt. ° C.	Compd. No.	Base or salt	M.pt. ° C.	Compd. No.	Base or salt	M.pt. ° C.
32	HCl	211 to 212	39	HCl	207 to 208	45	HCl	144 to 145
33	HCl	175 to 176	40	base	115 to 116	46	HCl	173·5 to 175
34	base	70	41	base	81 to 82	47	HCl	200
35	HCl	214	42	HCl	198 to 199	48	HCl	151 to 153
36	HCl	117 to 120·5	43	(−)-base	60 to 61	49	HCl	233 to 234
37	HCl	203	44	base	106	50	(−)-base	100 to 101
38	HBr	249				51	HBr	223

3-Morpholino-1 : 1-diphenylpropyl cyanide (Compd. No. 41), m.pt. 81 to 82°C., 3-piperidino-1 : 1-diphenylpropyl cyanide (Compd. No. 34), m.pt. 70° C., and 3-dimethylamino-1 : 1-diphenylpropyl cyanide hydrochloride (Compd. No. 47), m.pt. 200° C. were prepared by the methods of Dupré and others[29] who quoted m.pts. of 82° C., 70 to 71° C. and 200 to 201° C. respectively for these compounds. Compounds No. 39 and 40 (see ref. 30). Compounds No. 33, 43, 44 and 46 (kindly supplied by Dr. J. Elks). Compounds No. 48 and 50 (see refs. 28 and 31). Compounds No. 36, 37 and 38 (kindly supplied by Dr. G. E. Foster). Compound No. 49 (Burroughs Wellcome Ltd.). Compounds 32, 35, 42 and 45 were prepared by the following general method. The appropriate 3-amino-1 : 1-diphenylpropyl cyanides or 3-amino-1 : 1-diphenylbutyl cyamides were refluxed with equal weights of sodamide in dry toluene for 16 hours. The excess of sodamide was filtered off, the solvent removed under reduced pressure and the resultant oil converted to the hydrochloride which was recrystallised from ethanol-ether: 3-morpholino-1 : 1-diphenylbutane hydrochloride (Compd. No. 42), m.pt. 198 to 199° C. (Bochmühl and Ehrhart[10] quote m.pt. 198 to 199° C.), 3-piperidino-1 : 1-diphenylbutane hydrochloride (Compd. No. 35,) m.pt. 214° C. (Bochmühl and Ehrhart[10] quote m.pt. 214 to 215° C.), 3-piperidino-1 : 1-diphenylpropane hydrochloride (Compd. No. 32), m.pt. 214·5 to 215° C. (Found: C, 75·95; H, 8·0 per cent., equiv. wt. 313. $C_{20}H_{26}N$ Cl requires C, 76·0; H, 8·3 per cent., equiv. wt. 315·5), 3-dimethylamino-1 : 1-diphenylpropane hydrochloride, m.pt. 144 to 145° C. (Found: C, 73·5; H, 8·0 per cent., equiv. wt. 278. $C_{17}H_{22}N$ Cl requires C, 74·0; H, 8·0 per cent., equiv. wt. 275·5.)

Compound 51. Prepared by recrystallisation of the hydrobromides of the mixed nitriles obtained in the methadone synthesis; 3-dimethylamino-2-methyl-1 : 1-diphenylpropyl cyanide hydrobromide crystallised from ethanol-ether as colourless prisms m.pt. 223° C. (Walton and Ofner[32] quote m.pt. 223 to 224° C.).

Measurement of dissociation constants

The dissociation constants recorded in Table III were measured in water at 25° C. by the method described in Part II of this series.

Summary

1. The dissociation constants of certain *N*-alkylated piperidines, morpholines, pyrrolidines and dialkylamines are discussed in terms of combined electrical and steric effects.

2. Interactions of the basic group with other groups in methadone-type molecules are briefly outlined.

3. Dissociation constants of several series of methadone-type compounds are recorded; consideration is given to the effect upon these values of the conformation of the molecules and combined electrical and steric effects.

4. Methadone type molecules are shown to adopt a conformation which permits their ready association with the "analgesic receptor site".

The author wishes to express his thanks to Dr. J. Elks (Glaxo Laboratories Ltd.) and Dr. G. E. Foster (Burroughs Wellcome and Co.) for supplying certain samples used in this investigation.

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ANALGESICS AND THEIR ANTAGONISTS: SOME STERIC AND CHEMICAL CONSIDERATIONS

PART II. THE INFLUENCE OF THE BASIC GROUP ON PHYSICO-CHEMICAL PROPERTIES AND THE ACTIVITY OF METHADONE AND THIAMBUTENE-TYPE COMPOUNDS

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No relation could be shown to exist between the analgesic activities of centrally acting analgesics and their dissociation constants[1]. However, minor modifications of the basic group in analgesics vary their activity[2–4] and it has been thought that association occurs between the basic group of the drugs and the anionic site of the analgesic receptor[5]. We are now concerned to measure the effect of changes in the basic group upon this association.

The factors contributing to the strength of the attractive forces between the basic group (as a cation) and the anionic site may be listed. They are, the strength of the base, the spatial arrangement of the basic centre in relation to the flat aromatic ring in the molecule, the size of the basic group, and the presence, in the groups attached to the cation, of groups or atoms with dipoles which introduce further attractive forces between the drug and the anionic site.

MEASUREMENTS

A measure of the bulk of the various cationic groups present in analgesics was made. Molecular models (Catalin) were constructed employing a quaternary nitrogen atom. The approximate width of the basic group was then measured at right angles to the plane joining the cationic head to the rest of the molecule. Piperidino and morpholino compounds were constructed in the chair conformation with equatorially orientated ring methyl groups in cases of the mono methyl substituted rings. The 2:6-dimethyl piperidino group was constructed with both groups equatorially orientated (the meso compound).

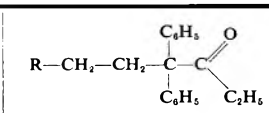
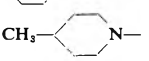
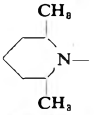
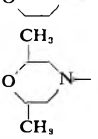
Some doubt exists about the correct measure of the effective "width" of the dialkylamino or aralkylamino groups because of the puckering and rotation of these alkyl chains. For the present purpose, the effective width is presumed to be the minimum width consistent with free rotation of the alkyl chains.

Methadone-type compounds

The effective widths, expressed in Ångström units. of normethadone compounds (II) are recorded in Table I along with the analgesic activities of the compounds in rats (activities compared against pethidine). It is well known that the relative activities of different analgesics vary in different animals and low activities are difficult to measure with accuracy.

TABLE I

ANALGESIC ACTIVITIES AND EFFECTIVE WIDTHS OF THE BASIC GROUP OF METHADONE-TYPE COMPOUNDS. (COMPOUNDS NOS. 5 AND 6 WERE PREPARED AND TESTED BY MORRISON AND RINDERKNECHT[12], THE REMAINDER BY DUPRÉ AND OTHERS[11].)

Compound No.	 R	Analgesic activity (in rats) (pethidine = 1)	Approx. width of basic group in Ångström units
1	$(CH_3)_2N$—	1	5·4
2	$(C_2H_5)_2N$—	⅔–1	6·7
3	$(n\text{-}C_3H_7)_2N$—	0–⅓	7·3
4	$(n\text{-}C_4H_9)_2N$—	0–⅓	8·3
5	$CH_2 = CHCH_2NCH_3$	½	6·3
6	$(CH_2 = CHCH_2)_2N$—	½	7·8
7	$(PhCH_2)_2N$—	—	
8	$(PhCH_2)N{\cdot}CH_3$	0	8·4
9		4	5·6
10		2–3	6·0
11		0	7·3
12		0–⅓	7·3
13		3	6·0
14		0–⅓	8·4
15		7	6·1
16		1½	7·5
17		1½	8·4

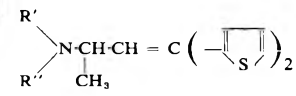
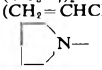
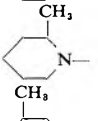
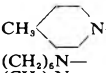
However, the activities quoted in Table I, and especially those used in Figure 1, most of which were obtained in one laboratory[11] may be used as a basis for broad generalisations about structure-activity relationships.

Thiambutene-type compounds

The analgesic activities of a series of these compounds and the effective width of their basic groups are recorded in Table II. The dissociation constants of certain of these compounds is given in Table III. The prep-

TABLE II

ANALGESIC ACTIVITIES AND EFFECTIVE WIDTHS OF THE BASIC GROUP OF THIAMBUTENE-TYPE COMPOUNDS

Compound No.	 R′R″N	Analgesic activities* morphine sulphate = 1	Approx. width of basic group in Ångström units
18	$(CH_3)_2N$—	1·07 (*b*)	5·4
19	$CH_3N{\cdot}C_2H_5$	1·7 (*b*)	5·8
20	$CH_3N{\cdot}n{\cdot}C_3H_7$	0·1 (*b*)	6·4
21	$CH_3N{\cdot}iso{\cdot}C_3H_7$	0·3 (*b*)	6·5
22	$CH_3N{\cdot}CH_2C_6H_5$	0 (*b*)	6·3
23	$(C_2H_5)_2N$—	1·0 (*b*)	6·7
24	$(n{\cdot}C_3H_7)_2N$—	0 (*b*)	7·3
25	$(CH_2{=}CHCH_2)_2N$—	0·3 (*b*)	7·8
26		0·7 (*b*)	5·6
27		1·1 (*b*)	6·0
28		0·5 to 0·6 (*a*)	7·3
29		0·6 to 0·9 (*a*)	7·3
30		0·6 to 0·9 (*a*)	6·0
31	$(CH_2)_5N$—	1·3 to 1·8 (*a*)	6·6
32	$(CH_2)_7N$—	0·05 to 0·10 (*a*)	6·9

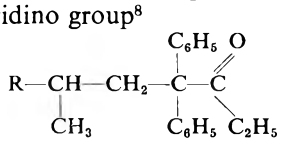
* Analgesic activities were determined in rats using heat and pressure methods; (*a*) present work, (*b*) abstracted from publication of Green[13].

aration of the compounds and the measurement of dissociation constants is described later in this paper.

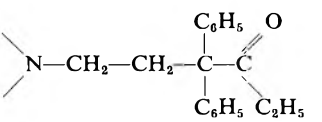
DISCUSSION

Methadone-type compounds

The replacement of the $-N(CH_3)_2$ group of methadone (I; R = $-N(CH_3)_2$) by $-N(C_2H_5)_2$ is attended by a considerable loss of activity[6]. If the 2 ethyl groups are joined to form a pyrrolidine ring, however, the analgesic activity is restored to the level of that of methadone[7]. The potency is retained upon replacing the basic group of methadone by a piperidino group[8]



(I)



(II)

and enhanced by a morpholino group[9]. Further increase in the ring size (I; R = $-N(CH_2)_6$ results in loss of activity[10]. Compounds of type II also exhibit similar variations in activity upon comparable changes in the basic group[11,12] (see Table I); a graphical representation of activities against width of the basic group is shown in Figure 1.

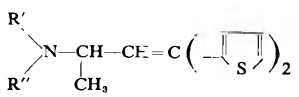
A simple relation between effective width of the basic group and analgesic activities is not apparent until the basic groups are divided into three different types, (*a*) those possessing alkyl or aralkyl substituents on the nitrogen atom, (*b*) those consisting of a heterocyclic ring other than morpholino, and (*c*) morpholino derivatives.

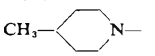
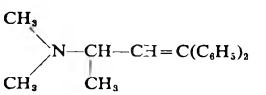
For types (*a*) and (*b*), two straight lines possessing different slopes represent the relationship between activities and width of basic group; activity is inversely proportional to width. Insufficient points are present to completely establish the relation involving morpholino compounds; it is reasonable to assume that once again a straight line relation exists as shown in Figure 1. The three lines intersect the "width" axis at approximately the same position corresponding to a width of basic group of 7·4 to 8·4 Å; analgesic activity in methadone type compounds possessing basic groups of larger effective width therefore seems unlikely.

TABLE III

DISSOCIATION CONSTANTS OF THIAMBUTENE-TYPE COMPOUNDS

SOLVENT, WATER; TEMP. 25° C.



No.		pK'a
18	$(CH_3)_2N-$	8·95
23	$(C_2H_5)_2N-$	8·90
27		8·46
28		7·46
29		8·28
30		8·20
31	$(CH_2)_6N-$	7·96
32	$(CH_2)_7N-$	7·52
33		9·26

The division of the basic groups into the three classes as shown appears logical on structural grounds if association of the cation with an anionic receptor site is an integral part of the total drug-receptor binding forces. If steric limitations near the anionic site are absent, van der Waals' forces between the receptor and the groups attached to the cationic head will reinforce the ionic bond. A complementary configuration of cationic head and anionic site will assist in the formation of this reinforced ionic bond. A piperidino and morpholino group may be regarded as equivalent in the steric sense; in the latter, the oxygen atom with its electron donating potentialities could well give rise to additional binding forces upon close contact with the receptor. The difference in dissociation constants of compounds containing the two types of group[1] is a further reason for the above separate classification of these basic groups in a consideration of analgesic activities. The classification of pyrrolidines (same dissociation constants as piperidines) and larger rings along with the piperidines is justified since although minor differences in the conformations of these rings exist, they would present roughly comparable surfaces to a receptor.

Increase in the effective width of the basic group is caused by the introduction of methyl groups into the 2 and 3 positions but not the 4 position of the 6-membered rings. The results (see Table I and Fig. 1) indicate that the incorporation of the cationic head into a ring structure is advantageous, presumably due to the increased van der Waals' force bonding between cationic head and receptor. However, steric requirements of the groups in the vicinity of the anionic site lead to reduction in activity with increasing width of the cationic head.

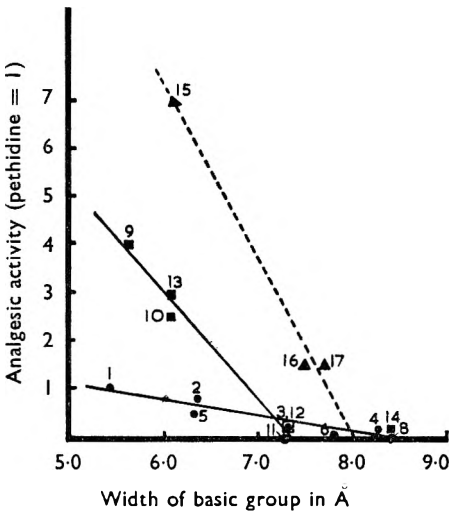


FIG. 1. Relationship between analgesic activity and width of basic group in methadone-type compounds. Compound numbers correspond to those in Table I.

- ● Alkyl or aralkyl group.
- ■ Heterocyclic group other than morpholino.
- ▲ Morpholino type group.

The dialkylamino groups are distinct from the above types, since separate rotation of the two alkyl groups is possible; the cationic head becomes less compact and less strong van der Waals' force bonding between the groups and the site would be expected. The reduced activity of these compounds, other than dimethyl, is attributed to this factor since the dissociation constants of dialkylamines, pyrrolidines and piperidines do not differ greatly. Increase in alkyl chain length results in a large increase in the effective width of the cationic head.

From these results it is possible to make the following observations: the ionic bond between cation and anionic receptor can be reinforced by van der Waals' bonding if the cationic head is suitably orientated in relation to the rest of the analgesic molecule; steric requirements in the vicinity of the anionic site reduce the bonding forces upon increase in effective width of the cation and completely prevent drug-receptor bonding if a width of approximately 8·0 Å is exceeded; increase in the dimensions of the groups attached to the *N*-atom along the axis between this atom and the rest of the molecule does not decrease the bonding forces (there is evidence to suggest that the bonding forces are increased—see Part III[14]).

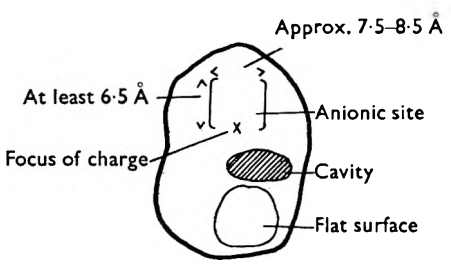
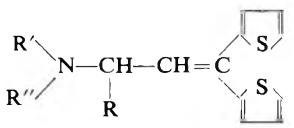


FIG. 2. Analgesic receptor site.

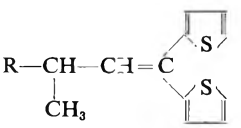
It may be concluded that the anionic receptor site has certain dimensions, and if the cationic head of an analgesic type molecule exceeds these, weaker association between the drug and the receptor results, with concomitant reduction in analgesic response. The analgesic receptor surface may now therefore be represented as in Figure 2.

Thiambutene-type compounds

Dithienylbutenylamines of type III exhibit powerful analgesic activity[14–16], and some are known to be antagonised by *N*-allylnormorphine[17,18]. It is reasonable to assume that they are adsorbed upon the same receptor surface as morphine, methadone and other analgesics, that they exist as semi-rigid structures, and that they adopt conformations similar to those of methadone-type compounds in solution (see Part I[1]).



(III)

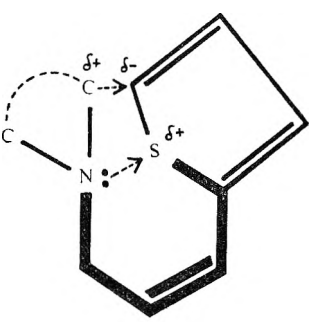


(IV)

If these conclusions are correct, the "effective width" of the basic group should influence the activity in a way comparable with that shown by the methadone-type compounds. Dissociation constants should provide information concerning conformations in solution although it is recognised that both inter- as well as intra-molecular interactions can affect the values. To test these conclusions, compounds of type IV, in which R consists of various amino groups, have been investigated.

Conformation of the molecules

The sulphur atom of a thiophen ring carries a partial positive charge due to resonance effects[19]. In agreement with Gero[20], it is considered that the two sulphur atoms in thiambutene-type compounds will tend towards positions of maximum separation due to steric and electrical repulsions. The double bond will contribute to the rigidity of the structure and the basic group will align itself in close proximity to one of the thiophen rings shown in V.



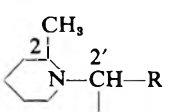
(V)

Gero[20] has emphasised that such a structure "imitates a piperidine ring", the ring being formed of N, S and the heavily outlined portion of (V). We believe the simulation of the piperidine ring to be unimportant in itself, but the above interactions stabilise the molecule in such a conformation that the basic centre, and the thiophen ring not involved in these interactions, are correctly orientated to allow ready association with the anionic site and flat portion respectively of the proposed "analgesic receptor site" (see Beckett and Casy[5] and Part I[1] of this series).

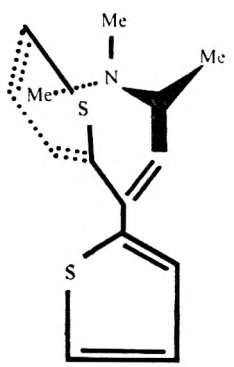
The adoption of such a conformation by this type of molecule will result in compounds, produced by varying the basic group, being more weakly basic than the corresponding simple *N*-alkylated compounds, due to the reduction of the electron density by the electrical effect and the steric limitations in the vicinity of the N atom[1]. The compounds will be weaker bases than predictable from considerations of inductive effect along the chain of atoms between the N atom and the thienyl groups.

Elsewhere[1] it was shown that compounds containing dimethylamino groups were stronger bases than the corresponding piperidino compounds by about 0·35 pKa units. Consideration of molecular models of dithienyl-butenylamines in the conformation shown in V indicated that basic groups constituting part of a ring will probably be sufficiently close to one of the thienyl rings to permit strong van der Waals' force bonding. This will increase the steric limitations near the N atom (base weakening effect) to a greater extent than in the corresponding methadone-type compounds; such steric effects will be further increased upon introducing methyl groups into the heterocyclic ring, especially in the α-position. Dialkylamino groups will be expected to have a reduced steric requirement since the group *as a whole* will be less firmly bound by the thienyl group and cation formation should consequently be less hindered by steric factors.

The dissociation constants of the series of dithienyl-butenylamines recorded in Table III substantiate the above predictions. Compound 33 (thienyl groups of thiambutene-type compound replaced by phenyl groups) is a stronger base (0·31 pKa units) than compound 18—the electrical effects shown in V being absent in compound 33. (The alternative explanation that the difference is due to the greater −I effect of two thienyl groups than two phenyl groups operating through a carbon chain of three atoms is inadequate.) Compound 18 is as strong a base as methadone (pK′a 8·99) but the piperidino compound 27, and especially the substituted piperidino compounds 28, 29 and 30, are weaker bases than the piperidino analogue of methadone (pK′a 8·58). Since it has been shown that the introduction of a CH_3-group into the ring of an *N*-alkyl piperidine has a base strengthening effect (+I effect of CH_3-group is a more important factor than its steric requirements near the N atom in these compounds—see Part I) the △pKa values between compounds 27 and 28 can be attributed to increased steric limitations about the N atom in this latter compound. Only if the N atom is near the S atom in III; (R = CH_3), and the basic group as a whole is in close proximity to one of the thienyl rings, will such steric limitations occur in these compounds. The alternative explanation that, in compounds of type VI, the interaction of the base strengthening 2–CH_3 group (△pKa 0·3) and the base weakening 2′–CH_3 group (△pKa 0·3) (see Part I[1]) might lead to a large base weakening steric effect is excluded since it



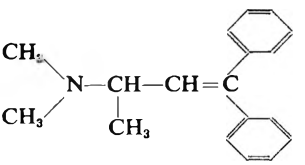
(VI)



(VII)

does not occur in simple compounds of this type[21]. The weakly basic character of compounds 31 and 32 is explicable in the same terms. It is therefore concluded that thiambutene-type molecules in solution adopt the conformation depicted in VII, so that a surface complementary to that of the analgesic receptor site (see Fig. 2) is present in this molecular conformation.

This conclusion is supported by the fact that compound VIII, in which the electrical forces responsible for holding the basic group in the correct orientation (as in VII) are absent, has negligible analgesic activity[22].



(VIII)

Effective width of the basic group and analgesic activities

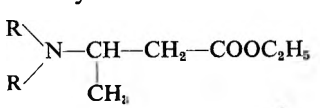
The analgesic activities of a series of thiambutene compounds and the effective width of their basic groups are recorded in Table II. The dissociation constants of certain of these analgesics indicate that no simple relation is possible between basic strength and analgesic activity. As in the case of the methadone-type compounds, the results indicate that there is some relation between the effective width of the cationic head and activity which is reduced upon increasing the width of the basic group. The pattern is not so consistent as observed for the methadone-type compounds (see Fig. 3).

Probably the greater $\triangle$pKa values between members of the thiambutene-type series (see Table III), in contrast to the methadone type, may be responsible for the reduced clarity of correlation, since the stability of the drug-receptor complex may be influenced by the strength of the base as well as the size.

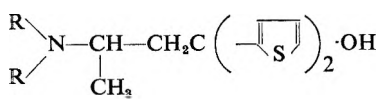
These results seem to provide further evidence for the adsorption of analgesics upon a common analgesic receptor incorporating an anionic site of circumscribed dimensions as indicated in Figure 2.

Preparation of the compounds

Compounds 18, 23 and 27 (Table III) have been described elsewhere[15]. The further compounds required were prepared by the condensation of the appropriate secondary bases with ethyl crotonate to yield the amino esters (IX) which, upon treatment with thienyl-lithium according to the method of Adamson[15], yielded the 3-amino-1 : 1-di(2′-thienyl)butan-1-ols (X). Dehydration of these amino-alcohols with dry hydrogen chloride



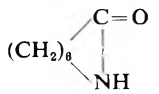
(IX)



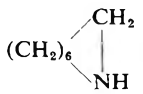
(X)

gave the corresponding 3-amino-1 : 1-di(2′-thienyl)-but-1-enes. (Table III, Compounds 28, 29, 30, 31, 32.)

The secondary bases used above, with the exception of heptamethyleneimine (XII), were commercially available. This base was prepared by the reduction of *cyclo*heptanone isoxime (XI) with lithium aluminium hydride



(XI)



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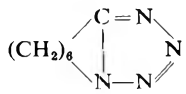

(XIII)

according to the method of Ruzicka and others[23]. These workers described two methods for the preparation of the isoxime; the rearrangement of *cyclo*heptanone oxime by means of concentrated sulphuric acid and the treatment of *cyclo*heptanone with hydrazoic acid. The latter method was recommended since the former was too vigorous and gave only low yields of the isoxime. In the hands of the present authors, the treatment of *cyclo*heptanone with hydrazoic acid gave largely 4 : 5-*cyclo*hexamethylene tetrazole (XIII) together with only a small yield (about 20 per cent.) of the desired isoxime. Rearrangement of *cyclo*heptanone oxime, however, according to the method of Wallach[24] gave the isoxime (XI) in 70 per cent. yield.

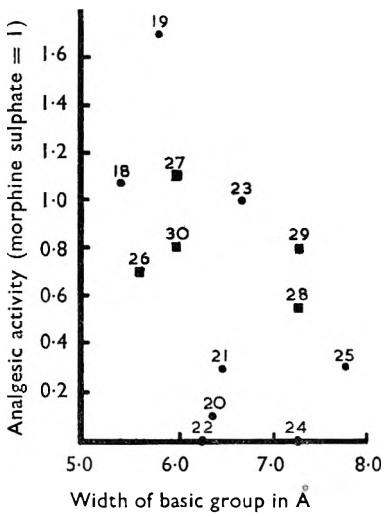

FIG. 3. Relationship between analgesic activity and width of basic group in thiambutene-type compounds. Compound numbers correspond to those in Tables II and III.

● Alkyl or aralkyl group.
■ Heterocyclic group other than morpholino.

Measurement of dissociation constants

In certain analgesic type compounds, e.g., methadone, the dissociation constant of the basic group is affected by solvent transmitted intramolecular electrical interactions between groups (see Part I)[1]. In other analgesics, e.g., morphine, the importance of such effects is reduced by the steric character of the molecule. Since the magnitude of these solvent transmitted effects is influenced by the dielectric constant of the medium, alteration of the composition of the solvent will not cause parallel effects upon the dissociation constants of both types of compound, e.g., Hall[25] found that the pK′a value in water of certain bases was a good index of base strength in organic solvents within a particular class, but compounds containing polar groups near the N atom belonged to a different class than those without such groups.

The low water solubility of many of these bases rendered impossible the titration of their salts to the point of half neutralisation. The use of

ethanol etc. to increase the solubility was inadmissible because of the above-mentioned factors; titrations at several concentrations and the extrapolation of the results to zero concentration of organic solvent was too time-consuming and wasteful in materials. However, it was possible to derive a relationship which permitted the calculation of pK'a values after titration to the point at which precipitation of the free base began.

Experimental

All m.pts. are uncorrected.

Microanalyses were by Mr. G. S. Crouch, School of Pharmacy, University of London.

Equivalent weights of the bases and picrates were determined by titration with 0·02 N perchloric acid in glacial acetic acid using Oracet Blue B[26] as indicator. Titration of the hydrohalide salts was carried out in non-aqueous media in the presence of mercuric acetate by the method described by Pifer and Wollish[27].

Cyclo*Heptanone isoxime*

(*a*) *From* cyclo*heptanone and hydrazoic acid.* A mixture of *cyclo*heptanone (25 g.) and hydrazoic acid (10·6 g.) in benzene (200 ml.) was added dropwise to a stirred, ice-cooled mixture of concentrated sulphuric acid (69 ml.) and benzene (100 ml.). The next morning, the mixture was made alkaline with strong aqueous sodium hydroxide and the precipitated oil extracted with chloroform. After drying (Na_2SO_4), the solvent was removed and the oil fractionally distilled to give the isoxime (5·3 g.) b.pt. 168° to 188° C./20 mm. and a residue of crude 4:5-*cyclo*hexamethylenetetrazole (7 g.), m.pt. 67° to 68° C. after recrystallisation from benzene (Ruzicka and others[28] give m.pt. 66° to 68° C.).

(*b*) *From* cyclo*heptanone oxime.* A mixture of *cyclo*heptanone oxime (5 g.), prepared by the method of Ruzicka and others[23], glacial acetic acid (5 ml.) and concentrated sulphuric acid (10 ml.) was gently heated until a lively reaction took place and the mixture boiled and turned black. Four more 5 g. batches of the oxime were treated in this way, the products bulked, made alkaline with ice-cold, strong aqueous sodium hydroxide, and the precipitated oil extracted with ether-chloroform. After drying (Na_2SO_4) the solvent was removed and the residue distilled to give the isoxime (18 g.) b.pt. 122° C./3 mm.

Heptamethylenimine

*cyclo*Heptanone isoxime (20 g.) in ether (20 ml.) was added dropwise, in the course of 50 minutes, to a stirred suspension of lithium aluminium hydride (8 g.) in ether (200 ml.), and the mixture refluxed for 3 hours. Excess of reagent was then decomposed with water and the mixture filtered. After drying (Na_2SO_4) the ether was removed and the residue distilled to give heptamethylenimine (12·6 g.) b.pt. 52° to 53° C./15 mm., n_D^{17} 1·4752, which gave a picrate, yellow needles, from ethanol-water, m.pt. 149° to 150° C. (Ruzicka and others[23] give n_D^{21} 1·4740 and m.pt. 147° C. for the picrate).

Ethyl 3-aminobutyrates (IX)

(*a*) (*IX* (*a*)). A mixture of hexamethylenimine (9·9 g.) and ethyl crotonate (11·4 g.) was refluxed for 3 hours and then fractionally distilled giving the amino-ester (IX (*a*)) (7·8 g.) b.pt. 142° to 144° C./15 mm., equiv. wt. 217 (calc. 213). It gave a *picrate*, yellow needles from ethanol, m.pt. 99° to 100° C. Found: C, 48·9; H, 5·9; N, 12·7. $C_{18}H_{26}O_9N_4$ requires C, 48·9; H, 5·9; N, 12·7 per cent.

(*b*) (*IX* (*b*)). A mixture of heptamethylenimine (11·3 g.) and ethyl crotonate (11·4 g.) was refluxed for 7 hours and then fractionally distilled giving the amino-ester (IX (*b*)) (13·4 g.) b.pt. 142° to 144° C./20 mm., equiv. wt. 225 (calc. 227). It gave a *picrate*, yellow needles from ethanol, m.pt. 63° to 64° C. Found: C, 49·4; H, 5·9; N, 12·4. $C_{19}H_{28}O_9N_4$ requires C, 50·0; H, 6·1; N, 12·3 per cent.

(*c*) (*IX* (*c*)). A mixture of 4-methylpiperidine (14·2 g.) and ethyl crotonate (16·4 g.) was refluxed for 7 hours and then fractionally distilled giving the amino-ester (IX (*c*)) (21 g.) b.pt. 122° to 126° C./20 mm. It gave a *picrate*, yellow needles from ethanol, m.pt. 111° to 112° C. Found: C, 49·1; H, 5·8; N, 12·9 per cent; equiv. wt. 439. $C_{18}H_{26}O_9N_4$ requires C, 48·9; H, 5·9; N, 12·7 per cent.; equiv. wt. 436.

(*d*) (*IX* (*d*)). A mixture of 3-methylpiperidine (18·5 g.) and ethyl crotonate (11·4 g.) was refluxed for 9 hours and then fractionally distilled to give the amino-ester (IX (*d*)) (17·8 g.) b.pt. 120° to 124° C./20 mm. It gave a *picrate*, yellow needles from ethanol, m.pt. 106° to 107° C. Found: C, 49·0; H, 5·7; N, 13·1 per cent; equiv. wt. 438. $C_{18}H_{26}O_9N_4$ requires C, 48·9; H, 5·9; N, 12·7 per cent.; equiv. wt. 436.

(*e*) (*IX* (*e*)). A mixture of 2-methylpiperidine (27·5 g.) and ethyl crotonate (15·8 g.) was refluxed for 17 hours and then fractionally distilled to give the amino-ester (IX (*e*)) (7·1 g.) b.pt. 128° to 130° C./20 mm. It gave a *picrate*, yellow needles from ethanol, m.pt. 109° to 110° C. Found: C, 48·2; H, 5·8; N, 13·3 per cent.; equiv. wt. 445. $C_{18}H_{26}O_9N_4$ requires C, 48·9; H, 5·9; N, 12·7 per cent.; equiv. wt. 436.

3-Amino-1 : 1-di(2′-thienyl)-butan-1-ols (X)

(*a*) (*X* (*a*)). Thiophen (5 g.) in ether (5 ml.) was added to a stirred solution of phenyl lithium in ether (50 ml.) prepared from lithium (0·85 g.) and bromobenzene (9·4 g.), and the mixture refluxed for 2 hours. The amino-ester (IX (*a*)) (4·3 g.) in ether (5 ml.) was added dropwise to the stirred product, cooled by an acetone-solid CO_2 bath. The mixture, after stirring for half-an-hour at room temperature, was poured on to crushed ice and acidified with glacial acetic acid (8 ml.). The solid which separated was washed with ether, the free base liberated with dilute aqueous ammonia and extracted with ether. After drying (Na_2SO_4) the solvent was removed and the residue distilled under reduced pressure to give an orange-coloured oil (3 g.) b.pt. 190° to 200° C./0·2 mm. which solidified on scratching in the presence of acetone. The solid was crystallised from ethanol to give colourless needles of the *amino-butanol* (X (*a*)) m.pt. 80·5° to 81·5° C. Found: C, 63·8; H, 7·2; N, 4·0 per cent.; equiv. wt. 341. $C_{18}H_{25}ONS_2$ requires C, 64·5; H, 7·5; N, 4·2 per cent.; equiv. wt. 335.

(*b*) (*X* (*b*)). Treatment of the amino-ester (IX (*b*)) (4·5 g.) with thienyllithium, as described above, gave a crude base which distilled at 216 to 230° C./0·1 mm. as a yellow oil (3·7 g.) that crystallised from methanol on standing in the refrigerator. Recrystallisation from the same solvent gave colourless plates of the *amino-butanol* (X (*b*)) m.pt. 43° to 44° C. Found: C, 65·4; H, 7·95; N, 4·2 per cent.; equiv. wt. 348. $C_{19}H_{27}ONS_2$ requires C, 65·3; H, 7·7; N, 4·0 per cent.; equiv. wt. 349.

(*c*) (*X* (*c*), (*d*) *and* (*e*)). Treatment of the amino-esters (IX (*c*), (*d*) and (*e*)) with thienyl lithium, as described above, gave the corresponding crude amino-butanols as solids which were crystallised from ethanol without prior distillation.

The *amino-butanol* (X (*c*)) was obtained as colourless plates m.pt. 89° to 90° C. Found: C, 63·9; H, 7·2; N, 4·0 per cent.; equiv. wt. 339. $C_{18}H_{25}ONS_2$ requires C, 64·5; H, 7·5; N, 4·2 per cent.; equiv. wt. 335.

The *amino-butanol* (X (*d*)) was obtained as colourless needles m.pt. 123° to 125° C. Found: C, 64·3; H, 7·4; N, 4·0 per cent.; equiv. wt. 339. $C_{18}H_{25}ONS_2$ requires C, 64·5; H, 7·5; N, 4·2 per cent.; equiv. wt. 335.

The *amino-butanol* (X (*e*)) was obtained as colourless needles m.pt. 101° to 102° C. Found: C, 63·9; H, 7·25; N, 4·4 per cent.; equiv. wt. 337. $C_{18}H_{25}ONS_2$ requires C, 64·5; H, 7·5; N, 4·2 per cent.; equiv. wt. 335.

3-*Amino*-1 : 1-*di*(2′-*thienyl*)-*but*-1-*enes* (*III*; $R = CH_3$)

Dry hydrogen chloride was passed for 10 minutes through a solution of the amino-alcohol (X (*a*)) (1 g.) in chloroform (5 ml.), the solvent removed under reduced pressure, and the residue in water stirred with charcoal for a few minutes at 60° C. The mixture was filtered, the base liberated with dilute aqueous ammonia and extracted with ether. After drying (Na_2SO_4), the solvent was removed to give 3-hexamethylenimino-1 : 1-di(2′-thienyl)-but-1-ene (0·8 g.) as a yellow oil. It gave a *hydrobromide*, grey-green plates from ether-ethanol, m.pt. 137° to 138° C. Found: C, 54·2; H, 6·0; N, 3·5 per cent.; equiv. wt. 406. $C_{18}H_{24}NS_2Br$ requires C, 54·3; H, 6·0; N, 3·5 per cent.; equiv. wt. 398.

Treatment of the amino-butanols (X (*b*), (*c*), (*d*) and (*e*)) with hydrogen chloride as described above gave the corresponding crude amino-butenes.

3-*Heptamethylenimino*-1 : 1-*di*(2′-*thienyl*)-*but*-1-*ene hydriodide* was obtained as grey-green plates from ether-ethanol, m.pt. 131° to 132° C. (*decomp*). Found: C, 49·4; H, 5·7 per cent.; equiv. wt. 462. $C_{19}H_{26}NS_2I$ requires C, 49·7; H, 5·7 per cent.; equiv. wt. 459.

3-(4-*Methylpiperidino*)-1 : 1-*di*(2′-*thienyl*)-*but*-1-*ene hydrobromide* was obtained as pale buff plates from acetone, m.pt. 148° to 148·5° C. (*decomp*). Found: C, 54·0; H, 5·9; N, 3·5 per cent.; equiv. wt. 402. $C_{18}H_{24}NS_2Br$ requires C, 54·3; H, 6·0; N, 3·5 per cent.; equiv. wt. 398.

3-(3-*Methylpiperidino*)-1 : 1-*di*(2′-*thienyl*)-*but*-1-*ene hydrobromide* was obtained as pale buff needles from ether-ethanol, m.pt. 180° to 181° C. (*decomp*). Found: C, 54·5; H, 5·8; N, 3·4 per cent.; equiv. wt. 399. $C_{18}H_{24}NS_2Br$ requires C, 54·3; H, 6·0; N, 3·5 per cent.; equiv. wt. 398.

3-(2-*Methylpiperidino*)-1 : 1-*di*(2′-*thienyl*)-*but*-1-*ene* was obtained as a

yellow oil. Found: C, 68·1; H, 7·3 per cent.; equiv. wt. 316. $C_{18}H_{23}NS_2$ requires C, 68·1; H, 7·25 per cent.; equiv. wt. 317.

Measurement of dissociation constants

Titrations were carried out in a glass micro-cell with a thick walled capillary tube sealed into the base. This permitted nitrogen to be bubbled up through the solution to stir it, remove other dissolved gases and maintain an inert atmosphere. The cell was immersed in a thermostat at 25° C. ± 0·1°, and the nitrogen freed from carbon dioxide by passage through 10 per cent. potassium hydroxide in a gas-washing bottle with a sintered glass disc and a trap, both immersed in the thermostat. Doran alkacid and micro saturated calomel electrodes were used in conjunction with a modified Cambridge pH meter, details of which will be published elsewhere. An Agla micrometer syringe was used as a burette.

Quantities of 10 mg. of the free bases or their salts were dissolved in sufficient 0·01N carbon dioxide-free hydrochloric acid to give a total concentration of approximately 0·013M in the region of the titration curve at which calculations were made, and the solutions diluted to 10 ml. with carbon dioxide-free distilled water. Aliquots of 5 ml. were titrated potentiometrically with 0·1N carbonate-free sodium hydroxide prepared by dilution from 18N solution followed by passage through a column of ion exchange resin IRA–400 as described by Davies and Nancollas[29]. Aliquot quantities were used since it was necessary to know the exact concentration of base for the purpose of the calculations.

pK′a values were calculated from the equation:—

$$pK'a = pH + \log \frac{Ca - Cb}{C - (Ca - Cb)}$$

where C, Ca and Cb are the total concentrations of organic base, strong acid and strong base respectively. Two points on each of at least two titration curves were used for the calculations. The reproducibility of the results was within ±0·05 pK units.

The dissociation constants for various thiambutene-type compounds are recorded in Table III. Compounds 28 to 32 were prepared in the present work. Compounds 18, 23 and 27 were kindly provided by Dr. H. T. Openshaw and Mr. A. F. Green. Compound 33 has been described previously[30].

Pharmacological testing

The analgesic activity of compounds 28–32 (see Table II) was tested in rats using heat and pressure methods. The piperidino compound (27) (see Green[13]) was used as a standard for comparison. The results are recorded in Table II (activities expressed against morphine to allow comparison with other results quoted in this Table).

Summary

1. The analgesic activity of several series of methadone type compounds is shown to decrease upon the increase in the "effective width" of the basic group.

2. The steric requirements about the anionic site of the analgesic receptor are discussed.

3. Dissociation constants and analgesic activities of a series of thiambutene-type compounds are presented. Evidence is given for their probable conformation in aqueous solution. Their activities are shown to decrease with increase in the "effective width" of their basic groups.

4. The preparation of certain thiambutene-type analgesics is described.

5. A method is described for the determination of dissociation constants in aqueous solution of sparingly water soluble bases.

The authors wish to express their thanks to Dr. H. T. Openshaw and Mr. A. F. Green (Wellcome Research Laboratories) for supplying Compounds 18, 23 and 27 and to Mr. Green for carrying out the pharmacological testing. They also thank Mr. C. Morton for advice in the measurement of dissociation constants.

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ANALGESICS AND THEIR ANTAGONISTS: SOME STERIC AND CHEMICAL CONSIDERATIONS

PART III. THE INFLUENCE OF THE BASIC GROUP ON THE BIOLOGICAL RESPONSE

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ELSEWHERE[1,2] the thesis was advanced that the basic group of the molecule influenced analgesic activity and evidence was adduced in support. In morphine-type compounds, a gradual transition from analgesic to anti-analgesic activity occurred as the group was changed from *N*-methyl to *N*-ethyl, *N*-*n*-propyl and *N*-allyl.[3–5]

A number of authors have attributed this anti-analgesic effect to drug-antagonist competition for unspecified "cell sites", or "susceptible enzyme systems" thought to be involved in the analgesic metabolic process[6–8]. Beckett and Casy[9] outlined the physical and chemical characters of the hypothetical "analgesic receptor site" and speculated on the mode of its physical interaction with the drug. It seems reasonable to assume that the mechanism of action of an analgesic antagonist involves competition with an analgesic for the "analgesic receptor site", but "fit" at the receptor surface does not of necessity mediate an analgesic response[9].

If the analgesic is represented by A and the receptor by S, the reaction between the drug and the receptor may be represented by (1) where k_1 and k_2 are the rate constants for the association of the components and the dissociation of the complex respectively.

$$A + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AS \qquad .. \quad .. \quad .. \quad (1)$$

The formation of this complex may be regarded as initiating a sequence of reactions which may be represented in the following way:

$$A + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AS \xrightarrow{k_3} XS + Y \rightarrow \underset{\uparrow \text{FURTHER REACTION?}}{\underset{\uparrow \text{ANALGESIC RESPONSE}}{X}} + S + Y \quad .. \quad (2)$$

(I) ADSORPTION (II) REACTION (III) DESORPTION

X represents the compound which causes analgesic action, or an essential intermediate in a further sequence of reactions which produces the biological effect. The desorption of X from the receptor S regenerates the latter for further combination with the drug. Assuming all receptors must be filled to obtund "pain", if A is present in less than an effective concentration in the biophase in contact with the receptor site, there will be incomplete saturation of the receptors which will result in a decreased

concentration, or rate of formation, of X leading to a reduced analgesic response. Complete saturation of the receptor sites by a particular analgesic will result in the full analgesic effect for the drug, (within its own analgesic "potency", which is itself limited by other considerations) and further increase in concentration may prolong, but will not increase, the level of the action.

Mechanism of antagonism

An analgesic antagonist B may be considered to exert its effect by one of the following mechanisms.

(1) The antagonist B may react with the analgesic A to form a stable complex AB which thus removes A from possible combination with the receptor S. However, since such analgesic antagonists as nalorphine and (−)-3-hydroxy-*N*-allylmorphinan differ from their parent analgesics only in the replacement of the *N*-methyl group by an *N*-allyl group, and have the same configuration, this mechanism may be ignored.

(2) The antagonist B may combine with the analgesic receptor and the formation of this complex may be followed by, (*a*) failure to undergo reaction II (see equation 2), with the result that the essential intermediate X is not produced; or (*b*) reaction II may proceed only with great difficulty so that X is liberated so slowly that only very low levels of analgesia are produced; or (*c*) a reaction sequence differing from that caused by an analgesic is initiated, and one of the intermediates in this sequence fails to react with one of the enzyme systems or receptor surfaces implicated in the analgesic metabolic sequence.

The following observations indicate that analgesics and their antagonists are adsorbed upon the same receptor sites.

(i) Morphine and the antagonist, *N*-allylnormorphine have the same configuration.

(ii) (−)-Dromoran, an active analgesic, becomes an analgesic antagonist upon replacing the *N*-methyl by an *N*-allyl group, whereas the corresponding change in the analgesically inactive (+)-dromoran fails to yield an antagonist[10].

(iii) When substitution of the hydroxyl groups of morphine-type compounds leads to a reduction in the analgesic activity, similar substitution in the corresponding *N*-allylnormorphine compounds leads to a reduction in anti-analgesic activity[3,4].

(iv) Antagonists based on the morphine or morphinan type structures, antagonise not only their parent molecules[10–15] but also many other active compounds[10,13–18].

This spectrum of antagonism is further demonstrated by the precipitation of the withdrawal phenomena by *N*-allylnormorphine in man[19] and monkey[20] addicted to any one of a range of analgesics. That antagonists seem to be adsorbed on the same receptors as analgesics and undergo comparable reactions less readily after adsorption, is indicated by the following:—

(*a*) *N*-allylnormorphine—an antagonist, possesses slight analgesic activity at high doses[13,21,22]; (*b*) alteration of the *N*-alkyl group of certain

analgesics can lead to compounds in which there is a gradual transition from analgesic to anti-analgesic activity as the groups are changed in the order, methyl, ethyl, *n*-propyl, allyl[3,4].

Scharenburg (unpublished, cited by Seevers and Woods[23]) claims to have shown that there is competition between nalorphine and synthetic analgesics of the pethidine, methadone and methorphinan type for cell sites in certain myelinated neurons. A similar phenomenon has been described for morphine and heroin[24]. Seevers and Woods[23] concluded from this and other evidence that "these compounds (morphine and synthetic analgesics), occupy receptors on certain myelinated neurons, and exert a pharmacologic or pathologic effect after occupation, and nalorphine competes successfully for the receptors ordinarily occupied by these agents, or displaces these agents after occupation".

Another possible way by which an antagonist may exert its effect is by directly blocking the reaction sequence initiated when a drug is adsorbed upon the receptor site subsequent to the formation of X (see later).

Nalorphine, in addition to antagonising the analgesic action of morphine, antagonises many of the other effects, e.g., respiratory and vasomotor depression. We are deliberately restricting our considerations in the present paper. The rapid reversal of morphine analgesia by nalorphine may be attributed to a direct central nervous stimulation by the latter rather than drug-receptor antagonism. The foregoing considerations and facts seem to favour the latter explanation, but the possibility of a dual mechanism of antagonism cannot be excluded (see also Miller and others[25]).

The reaction between an analgesic antagonist B and the analgesic receptor S may be shown thus:

$$\underset{\text{(I) Adsorption}}{B + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} BS} \xrightarrow[\text{(II) Reaction}]{k_6} \underset{\text{Negligible Analgesic Response}}{XS + Z} \rightarrow \underset{\substack{\text{(IV) Further Reaction?} \\ \text{(III) Desorption}}}{X + S + Z} \quad \ldots \quad (3)$$

chemical reactivity, and steric factors may affect the distribution of chemicals within the body, the similarity in structure and dissociation constants of analgesics and their antagonists, derived by alteration of the *N*-alkyl group only should ensure similar distributions in the body.

The relative stability of the analgesic and anti-analgesic-receptor complex

The stability of these complexes will probably be influenced by the following factors.

(*a*) The shape of the molecule, which may affect the closeness of the fit of the complementary surfaces and consequently the strength of the ionic and van der Waals' forces bonding the drug to the receptor; (*b*) the dissociation constant of the basic group which will affect the ionic interactions of the drug and the anionic site; and (*c*) the presence or absence in the drug molecule of other groups in addition to the basic groups and the flat aromatic ring. These additional groups may increase the attractive or repulsive forces when in proximity to the receptor surface, and may cause steric effects which will alter the fit of the drug to the receptor.

The influence of the factor (*c*) may be thought to be reduced to a minimum by considering the competitive antagonism of an analgesic and its antagonist derived by alteration of the *N*-alkyl group only. Winter and others[13] reported that nalorphine antagonised many times its molecular equivalent of analgesic drugs, although the drug-antagonist ratio seemed to vary with different drugs. Huggins (unpublished, cited by Siebert and Huggins[7]), in what appears to be a study of respiratory depression, comments on a blocking ratio of one molecule of nalorphine to 67 molecules of morphine. It may be postulated that, using equal doses, nalorphine may penetrate to the central nervous system in slightly higher concentration than morphine since the former is less ionised at physiological pH. Nevertheless, the slight concentration difference would probably be nullified by the lower basic strength of nalorphine leading to a less strong ionic binding at the receptor than occurs with morphine.

The increase in drug-receptor attraction upon replacing the *N*-methyl by an *N*-allyl group may be attributed to the increase in non-bonded attractive forces between drug and receptor effected thereby.

If the free energy change upon the combination of the antagonist (B) with the receptor (S) is $\triangle F_1$, and the corresponding change for the combination of the analgesic (A) with the receptor is $\triangle F_2$, then

$$K_{BS} = \frac{[BS]}{[B]\,[S]} = \exp(-\triangle F_1/RT)$$

$$K_{AS} = \frac{[AS]}{[A]\,[S]} = \exp(-\triangle F_2/RT)$$

In concentrations in the vicinity of the receptor at which the analgesic and the antagonist compete on equal terms for the site,

$$[BS] = [AS].$$

Then $\dfrac{[A]}{[B]} = \exp([\triangle F_2 - \triangle F_1]/RT)$

If [A] = 40[B] and T = 310° A. (body temp. = 37° C.)

$$40 = \exp([\triangle F_2 - \triangle F_1]/RT) = \exp([\triangle F_2 - \triangle F_1]/[1{\cdot}986 \times 310])$$

$$\therefore \triangle F_2 - \triangle F_1 = 1{\cdot}986 \times 300 \times \frac{\log 40}{\log e} \text{ cal.}$$

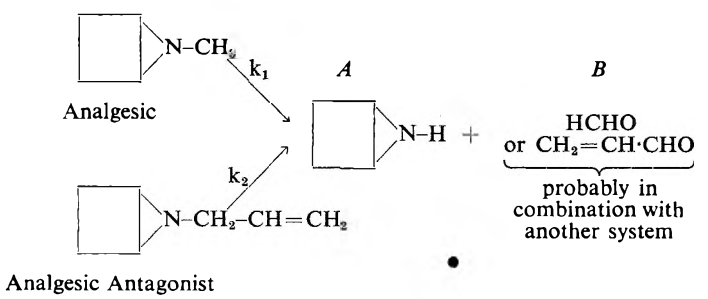
$$= 2270 \text{ cal.} = 2{\cdot}27 \text{ k. cal.}$$

Consequently, if the analgesic is present in the biophase in the vicinity of the receptor in 40 times the concentration of the antagonist, and the free energy change of BS formation is 2·27 k. cal. greater than that of AS formation, the two complexes would be formed in equal amounts. The difference of 2·27 k. cal. would be that expected for bonding involving an allyl as distinct from a methyl group since the former has a double bond in addition to 2 extra carbon atoms, and 5 carbon-carbon van der Waals' bonding forces would represent a free energy change of about 2·5 k. cal.[27].

If, as it seems possible, competition between the antagonist and the drug for the supposed analgesic receptor site is the major mechanism involved in reversal of analgesic action by an antagonist, the rapid character of the reversal requires consideration. It is possible that few analgesic receptor sites are available and that the reaction of AS to XS (2) and the utilisation of X is rapid; a relatively large excess of drug molecules possibly is necessary in the biophase to give continued receptor saturation. The antagonist molecules upon combination with the receptor could then be supposed to lead to an immediate antagonism of the analgesic response.

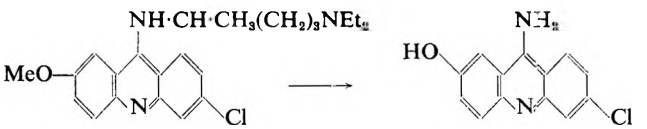
Consideration of the possible structure of X—the primary product of the reaction of an analgesic (and anti-analgesic) at the receptor site

If we assume that an analgesic and its antagonist analogue are adsorbed at the same receptor site, and our own observations support this concept, it may be useful to consider the possible nature of the first reactions involved. Since an anti-analgesic will antagonise a great variety of analgesics, it seems reasonable to assume that a common primary reaction step is involved for both. Because change in potency and change from an analgesic to an antagonist can occur by merely altering the *N*-alkyl group, it is now postulated that this group is involved in the primary reaction. This may be *N*-dealkylation by an oxidative mechanism resulting in small groups being removed more readily than larger ones. The reaction of an analgesic and its antagonist may be represented as follows, k_1 being much greater than k_2.



Either the dealkylated residue *A* or the alkyl fragment will be the essential intermediate involved in the sequence which produces analgesic activity. The latter possibility is precluded since some *nor*-compounds derived from active analgesics are not devoid of analgesic activity[28,29]. However, the low activity of the *nor*-compounds of morphine, pethidine and codeine, as compared with their parent molecule, does not invalidate the hypothesis that the *nor*-compounds are the essential intermediates. The assumption that the release of the *nor*-compounds at the receptor site in sufficient concentrations leads to an analgesic response does not imply that administration of these compounds by normal routes will give the same (or increased) effects. Differences will occur in the chemical reactivity, lipoid solubility and probably the membrane penetrating properties between the *nor*-compound and its parent molecule, e.g. *nor*-pethidine is extracted from a benzene solution of pethidine and *nor*-pethidine by a phosphate buffer solution[30].

Evidence is lacking concerning oxidative dealkylation by brain or central nervous system tissue but enzyme systems capable of effecting such reactions are known to be present in the body. Demethylation of a diverse range of compounds by animals and animal tissues has been demonstrated[31], e.g., demethylation of choline, monomethyl- and dimethyl aminoethanols (by dogs)[32]; monomethyl- and dimethylanilines (by rabbits)[33]; *N*-methyl and *NN*-dimethylsulphonamides (by man and mice)[34]; aminopyrine[35], ephedrine[36], methylamphetamine[37]. Evidence that dealkylations other than demethylations can take place is also available, e.g., the metabolism of phenacetin to *p*-aminophenol via phenetidine[38,39] and the de-ethylation of mepacrine[40]. The metabolism of mepacrine in man also demonstrates that large groups can be removed by dealkylation[41], the following change having been shown to occur:



Conversion of the removed alkyl groups to the corresponding aldehyde (methyl giving formaldehyde and ethyl, acetaldehyde) has been demonstrated in certain cases, e.g. ephedrine[42], amidopyrine[43] and monoethylaniline[44]. The dealkylation enzyme system, requiring both oxygen and reduced triphosphopyridine nucleotide, has been located in the microsomes of liver cells.

Bert and others[43] found that monomethyl-4-aminoantipyrine is more rapidly demethylated than the dimethyl analogue (amidopyrine) and showed that the size of the basic group has an influence on the ease of dealkylation (see Table I).

In analgesics, it has been shown using *N*-methyl-^{14}C labelled morphine, codeine, and pethidine, that *N*-demethylation occurs in rats and man[30,45–48]. The *nor*-compounds have not always been isolated although evidence confirming their presence has been reported. Burns and others[30] have isolated the demethylation products of pethidine, while

Plotnikoff and others[48], also using pethidine, established the presence of $^{14}CO_2$ in the expired air and identified *nor*-pethidine in the urine of rat and in man by counter current distribution. Brossi and others[49] recently reported that (+)-3-methoxyl-*N*-methylmorphinan was excreted as such, and in the form of three demethylated derivatives. On the other hand Shore and others[50] reported that neither of the isomers of 3-hydroxy-*N*-methylmorphinan are demethylated by intact animals or *in vitro* by a

TABLE I

DEALKYLATION OF VARIOUS AMINES

Substrate	4-Aminoantipyrine formed (μ moles)	Substrate dealkylated (per cent.)
Monomethyl-4-aminoantipyrine ..	1·93	36
Monoethyl-4-aminoantipyrine	0·60	11
Monobutyl-4-aminoantipyrine	0·56	9
Dimethyl-4-aminoantipyrine	0·62	11
Diethyl-4-aminoantipyrine	0·29	6
Dibutyl-4-aminoantipyrine	0·10	1

(5 μ moles of each alkylamine were incubated with liver homogenate. From Bert and others[43].)

demethylating enzyme present in liver. This last observation does not invalidate the present hypothesis because the very low concentration of the *nor*-compound, even after complete demethylation of the drug localised in the central nervous system, would pass undetected by the techniques adopted. The evidence provided by the work of Miller and Elliott[26] is apparently far more damaging to the present hypothesis. Using morphine-*N*-methyl-^{14}C, codeine-*N*-methyl-^{14}C and 2-^{14}C(±)-methadone, they determined the distribution of these analgesics in the central nervous system of the rat; peak levels correlated with pharmacological activity as measured by the pain reaction time method. Countercurrent distribution studies indicated that unaltered codeine and methadone (at least 90 per cent. unchanged) were present in the central nervous system 30 minutes after drug administration. The present hypothesis is therefore only tenable if relatively few of the analgesic molecules penetrating to the central nervous system are responsible for the biological response.

Recent investigations[51] have shown that the reduced, triphosphopyridine nucleotide dependent, microsomal enzyme system, which can demethylate morphine and other phenanthrene analogues—methadone and pethidine, is inhibited by certain *N*-substituted *nor*-morphines (the *N*-allyl and *N-iso*butyl derivatives exert the greatest action). The *N*-allyl compound has no effect on the enzymatic *N*-demethylation of cocaine, the side chain oxidation of hexobarbitone or the de-esterification of pethidine. It is of interest that *N*-allylnormorphine can itself be deallylated, but no evidence is available concerning the rate of deallylation in comparison with the rate of conversion of other *N*-alkyl*nor*morphines to *nor*-compounds.

The above hypothesis, that dealkylation is the primary step subsequent to adsorption of the drug upon the receptor site implies that, if the *nor*-compounds could be introduced directly into the biophase about the receptor, analgesic activity at least equal to that of the parent analgesics

would result. Investigations using morphine and *nor*-morphine were carried out in attempts to provide information on this point.

Pharmacological testing and results

Subcutaneous injections of morphine sulphate (2 mg./kg.) into rats gave much greater analgesic effects than those obtained using 50 mg./kg. doses of *nor*-morphine[52].

Solutions of the two compounds were injected intracisternally into mice and the degree of analgesia determined using a modification of the Singh Grewal method[53]. The results demonstrated that *nor*-morphine was rather more active than morphine at equal dose levels. Intravenous injection (mice) gave results indicating that *nor*-morphine had about 10 per cent. of the analgesic activity of morphine by this route; the onset of analgesic action using *nor*-morphine was preceded by short-lived convulsions in the animals.

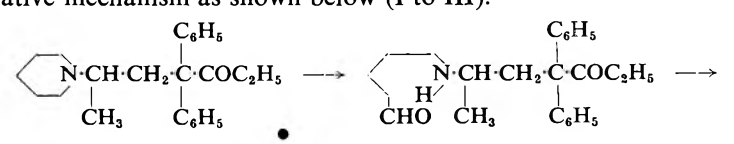
N-Allylnormorphine antagonised the analgesic action of intracisternally injected *nor*-morphine in doses comparable to those required to antagonise the action of morphine.

(The authors are grateful to Dr. M. F. Lockett and Mr. M. J. Davis for carrying out the above pharmacological testing which will be published elsewhere.)

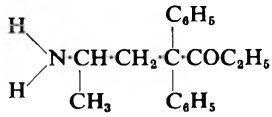
Mr. A. F. Green tells us that preliminary tests using the peristaltic reflex of isolated guinea-pig ileum show that *nor*-morphine has an inhibitory activity of about 10 per cent. that of morphine.

The greater activity of *nor*-morphine than morphine upon presentation of the drugs close to the analgesic receptors (intracisternally), in contrast to the negligible activity of *nor*-morphine upon subcutaneous injection, is consistent with the above hypothesis. The antagonism by *N*-allylnormorphine of the *nor*-morphine, as well as the morphine response, may be attributed to the former blocking the analgesic receptor so that although the essential metabolite X (*nor*-morphine) is available, it fails to be incorporated into the reaction sequence resulting in analgesia unless present upon the analgesic receptors.

The above discussion of oxidative dealkylation of analgesics has involved the consideration of compounds possessing one relatively small alkyl group attached to the *N*-atom (e.g., morphine and pethidine type compounds). The application of this hypothesis to methadone and thiambutene-type compounds possessing dialkylamino groups is self evident. (Methadone is demethylated by a reduced triphosphopyridine nucleotide dependent enzyme system[54].) Analogous compounds possessing piperidino, morpholino and pyrrolidino groups have high analgesic activity. It is presumed that ring opening and dealkylation occurs by an oxidative mechanism as shown below (I to III).



(I) (II)



(III)

Although it is suggested that increasing the size of the alkyl group attached to the nitrogen atom in morphine and pethidine-type compounds leads to anti-analgesic activity due to the greater difficulty of dealkylation, it is necessary to stress that the presence of electrical dipoles in the alkyl chain may affect the rate of dealkylation, e.g., the high activity of *N*-β-phenylethyl*nor*pethidine[55], despite its large alkyl group, may be attributed to this factor.

Summary

1. The mode of action of analgesic antagonists is considered in terms of competition with analgesics for the analgesic receptor surface.

2. The hypothesis is advanced that analgesics and their antagonists undergo a similar chemical reaction subsequent to adsorption, the rate constant for the former being very much greater than that for the latter.

3. Oxidative dealkylation to produce *nor*-compounds is presumed to be the first step in the reaction sequence leading to analgesia.

4. *Nor*-morphine has been shown to have a greater analgesic activity than morphine upon intracisternal injection into mice.

The authors wish to express their thanks to Mr. A. F. Green for supplying the *nor*-morphine and to Dr. M. F. Lockett and Mr. M. J. Davis for carrying out the pharmacological testing.

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DISCUSSION

The papers were presented by DR. A. H. BECKETT.

DR. A. MCCOUBREY (London) said that pKa is of undoubted importance in all basic drugs, but he felt that attempts to relate pKa to analgesic activity would be unlikely to succeed, especially should the activity be mediated by a degradation product. Correlation could be expected only of the active species in either the ionised or unionised molecule provided factors such as excretion and detoxication were controllable or negligible. Attempts to extrapolate findings with nonspecific enzymes in liver to the more specific functions of nervous tissue may be misleading. He was glad that another worker was considering metabolic activation in the analgesic group. He could not, however, see much fundamental difference between morphine and normorphine, though one remembered the curious dissociation of properties in the sympathins. Incidentally adrenaline has been stated to be more effective as an analgesic than noradrenaline by the intracisternal route. He felt doubtful of the validity of analgesic assay figures derived from animals that had recently suffered convulsive seizures. He realised, of course, that Dr. Beckett had not been actively concerned here. The drug SKF–525A has been stated to prevent the demethylation process or any other detoxication process in liver, at the same time increasing the action of various drugs, though he was not sure whether figures have been quoted for pethidine or morphine.

DR. J. B. STENLAKE (Glasgow) said he was critical of the use of infra-red measurements to supplement the conclusions drawn from dissociation constant measurements. The authors were concerned finally with the conformation of the structure in aqueous solution. While infra-red measurements would be made in non-aqueous media, he was therefore critical of their value as supporting evidence, although he agreed with the postulated transannular effects. He suggested that the author should take some pK measurements in a mixed solution, because it had been shown that there was a shift of pK values as a concentration of non-aqueous solvent was brought down to water, and the direction of shift of pK was a measure or an indication of whether ionisation of the proton was away from nitrogen or oxygen. Measurements of that type would, he suggested, provide much more satisfactory confirmatory evidence than infra-red for annular structure in methadone and related compounds. He had looked in the papers for the dissociation constant of normorphine but had been unable to find it. His own guess was that normorphine being a secondary base would in fact be a stronger base than morphine, and that meant that in all probability there would be much lower penetration to the surface receptor site. That would explain the difference in results which had been obtained by intracisternal and subcutaneous methods of testing normorphine.

DR. A. H. BECKETT, in reply, said while there was a danger in extrapolating results using liver when considering central nervous tissue, nevertheless, the enzyme system which was involved in demethylation of *N*-methyl compounds was also present in central nervous tissue. The important fact had to be considered that when the morphine was converted to form *N*-ethyl, *N*-propyl and *N*-allyl-normorphine, there was a change from active analgesic into an analgesic antagonist, yet the analgesic antagonist had some analgesic activity in itself. Therefore, it seemed reasonable to postulate that some reaction involving the alkyl group was implicated in the action. It was significant that since the paper was presented, work had appeared by Brodie and his colleagues in which dealkylation of analgesics had been carried out and this had been antagonised by *N*-allylnormorphine. He agreed that dissociation constants in various mixed solvents were required and that for the conformation of the molecules infra-red measurements cannot be used as a complete argument because these cannot be made in aqueous conditions. The authors were submitting that it was reasonable to believe that that dissociation constant measurements indicated the conformations existing in aqueous conditions and that the infra-red measurements showed that such conformations existed. He had no figures for the dissociation constant of normorphine. He agreed that penetration would be an important factor, but he also suggested that conjugation involving the free hydrogen on the nitrogen atom would also be important.

THE DETERMINATION OF RIBOFLAVINE IN PHARMACEUTICAL PRODUCTS

By L. Brealey and D. A. Elvidge

From the Physical Assay Division, Standards Department, Boots Pure Drug Co. Ltd.

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A variety of methods for the determination of riboflavine in pharmaceutical preparations are available to the analyst. Apart from the microbiological method, which suffers from the disadvantage of being time consuming, a number of procedures involving physical properties of the vitamin are well known. Probably the most widely used of these is the measurement of the fluorescence of riboflavine solutions at known pH values and this forms the basis of the official U.S.P. method. In this country, however, the technique has fallen into some disrepute, largely we believe because of the lack of sensitivity of commercially-available fluorimeters and consequently the microbiological method is favoured in the B.P.C. for all but the simplest preparations.

One of the intrinsic difficulties of the fluorimetric method is that high results may be obtained unless the riboflavine is in a pure state. To overcome this difficulty Conner and Straub[1] adsorb the riboflavine on a natural earth and elute it with a mixture of pyridine and acetic acid. Any remaining interference is then destroyed by treatment with potassium permanganate which does not attack riboflavine. The procedure is tedious and other workers[2,3] have introduced a dithionite treatment to reduce the riboflavine to a non-fluorescent form. Any residual fluorescence is caused by interfering substances which are stable and is subtracted from the total fluorescence. Polarographic methods have been described for the determination of riboflavine in simple solutions and tablets[4,5], but lack the flexibility necessary in a modern pharmaceutical laboratory where highly complex mixtures are often submitted for assay. This limitation also applies to the direct spectrophotometric determination[5,6], which otherwise has the advantage of speed and accuracy.

There exists, however, a general spectrophotometric technique which sometimes makes it possible to determine a single component in a complex mixture. If it is possible to perform a chemical reaction on a mixture containing more than one absorbing substance such that the absorption characteristics of one component only is altered, then it is a simple matter to determine that component by its difference spectrum. It seemed possible that the method could be applied to the determination of riboflavine because of the ease with which it undergoes reduction.

Reduction of riboflavine with dithionite has been widely used in fluorimetry and is included in the U.S.P. method. This reduction converts the riboflavine to its leuco-form which is non-fluorescent in the visible region; the absorption band at 445 mμ which gives rise to the fluorescence is also affected.

During preliminary investigations into the possibility of using this

reduction as the basis of a difference spectrophotometric method for the determination of riboflavine in complex mixtures, it soon became clear that the reduction is sufficiently vigorous to affect other materials often present. This applied particularly to dyestuffs. Hodson and Norris[2] in their fluorimetric method were able to make use of the oxidation reaction from the leuco-form back to riboflavine and it was found that a very mild oxidation was specific for riboflavine in all the pharmaceutical products examined.

Experimental

Materials and apparatus. Riboflavine—as supplied by the Medical Research Council.

Dithionite (sodium hydrosulphite) of reagent grade.

Buffer solutions of pH 2·0, prepared from 0·2 M KCl and 0·2 N HCl; of pH 4·0, 6·0 and 8·0 prepared from 0·2 M KH_2PO_4 and suitable quantities of H_3PO_4 or NaOH.

All spectrophotometric measurements were made on either the Unicam SP500 spectrophotometer or the Unicam SP600. When fluorimetric methods were used for comparison purposes the instrument was one built in this laboratory. This fluorimeter is extremely sensitive and will be described elsewhere.

It is well known that the absorption of riboflavine varies with hydrogen ion concentration[7] and it was considered necessary to investigate the reactions at different pH values. For these determinations a standard solution containing 100 μg./ml. riboflavine was diluted with the appropriate buffer to give final solutions containing 10 μg./ml.

The variations in spectral characteristics over the range pH 2·0 to pH 8·0 were found to be negligible, the data obtained being given in Table I. This agrees substantially with the reports of others.

TABLE I

Variation of riboflavine spectra with pH

pH	$\lambda_{max.}$	E (1 per cent. 1 cm.)	$\lambda_{max.}$	E (1 per cent. 1 cm.)	$\lambda_{max.}$	E (1 per cent. 1 cm.)	$\lambda_{max.}$	E (1 per cent. 1 cm.)
2·0	223	802	267	829	374	265	445	306
4·0	223	807	267	830	374	268	445	308
6·0	223	797	267	833	374	270	445	307
8·0	223	788	267	838	374	268	445	308

Reduction of riboflavine. From a spectrophotometric point of view, therefore, there is a wide pH range in which the absorption or riboflavine may be measured. The effect of dithionite on the spectrum of riboflavine was measured at all four pH values. Since dithionite decomposes in aqueous solution with products which absorb below 400 mμ, the spectrum of the leuco-riboflavine between 220 mμ and 400 mμ is not easily measured accurately and the spectrum shown in Figure 1 is typical. However, since the final method adopted makes use of the maximum extinction at 445 mμ where the dithionite decomposition products do not absorb, the spectrum below 400 mμ is of academic interest only. The leuco-riboflavine was formed by the addition of 0·1 ml. of the freshly prepared

TABLE II

REOXIDATION OF LEUCO-RIBOFLAVINE AT VARIOUS pH VALUES

	223 mμ			267 mμ			374 mμ			445 mμ		
pH	E_i	E_f	R	E_i	E_f	R	E_i	E_f	R	E_i	E_f	R
2·0	802	880	110	829	892	107	265	265	100	306	300	98
4·0	807	795	99	830	820	99	268	257	96	308	304	99
6·0	797	852	107	833	825	99	270	260	96	307	300	98
8·0	788	900	114	838	820	98	268	264	98	308	300	98

R = per cent. recovery.

TABLE III

EFFECT OF TIME OF AERATION ON RIBOFLAVINE SPECTRA

Time of aeration (min.)	$\lambda_{max.}$	E (1 per cent. 1 cm.)	$\lambda_{max.}$	E (1 per cent. 1 cm.)	$\lambda_{max.}$	E (1 per cent. 1 cm.)	$\lambda_{max.}$	E (1 per cent. 1 cm.)	Per cent. recovery at 445 mμ
0	223	797	267	833	374	270	445	307	—
1	223	825	267	830	374	274	445	303	98·7
5	223	810	267	830	374	269	445	302	98·4
10	223	805	267	828	374	270	445	303	98·7
20	223	820	267	830	374	269	445	300	97·8
30	223	828	267	842	374	272	445	307	100·0

dithionite solution (1 per cent. in water) to 5 ml. of the riboflavine solution used in the first experiment. The spectra were measured immediately using a blank of the appropriate buffer solution (5 ml.) to which 0·1 ml. of 1 per cent. dithionite had been added.

The general shape of the absorption spectra of the leuco-riboflavine does not vary much with pH. The maximum at about 250 mμ occurs at all pH values from 2·0 to 8·0 and the two absorption bands at 374 mμ and 445 mμ present in riboflavine are eliminated by dithionite treatment.

Reoxidation of leuco-riboflavine. On aeration of a solution of leuco-riboflavine the yellow colour re-appears and is shown to have the same spectrum as the original riboflavine.

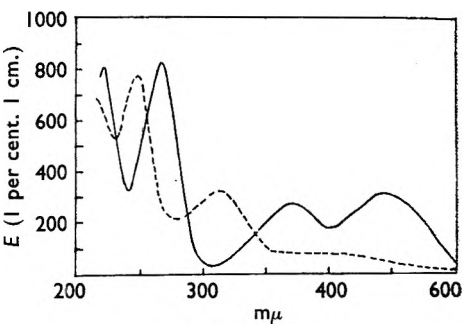

FIG. 1. Reduction of riboflavine with dithionite.

—— Riboflavine.
– – – Leuco-riboflavine.

Riboflavine solutions at pH 2·0, 4·0, 6·0 and 8·0 containing 10 μg./ml. were reduced with dithionite and then reoxidised by aerating for 1 minute. The aeration was carried out in a small tube into which a sintered glass filter stick was inverted, and after aeration the solutions were examined spectrophotometrically. Maxima were obtained at 223, 267, 374 and 445 mμ and the E (1 per cent. 1 cm.) values are shown in Table II.

The "recovery" of riboflavine in Table II is expressed as $E_f/E_i \times 100$, where E_f is the E (1 per cent. 1 cm.) value of the aerated solution and

E_i is the E (1 per cent. 1 cm.) value of the original solution. It will be seen that excellent results are obtained at 267, 374 and 445 mμ, in particular at the last wavelength. The variable results at 223 mμ may be attributed to either incomplete removal of dithionite decomposition products or absorption of oxygen.

Effect of time of aeration on the spectra of riboflavine. Aeration of reduced riboflavine solutions might have a possible detrimental effect if too prolonged, and the effect of aeration time was studied on a solution containing 10 μg./ml. Periods of aeration between 1 and 30 minutes gave the results shown in Table III. Reoxidation is complete after 1 minute and no decomposition occurs by aerating for periods up to 30 minutes. The aeration was carried out in diffuse daylight at room temperature.

Application to Pharmaceutical Materials

The spectrophotometric properties of riboflavine enable its determination to be carried out in two main types of preparation. In a first group, where there is no interference from other coloured materials a simple measurement of the maximum at 445 mμ in a suitable buffer solution suffices. In a second group, where other materials are present, including dyes and pigments, the change in spectrophotometric characteristics on addition of dithionite and on reoxidation can be measured and compared with the change under similar conditions of a pure riboflavine solution. However, each pharmaceutical preparation must be treated on its own merit and sufficient work must be carried out to ensure that only the riboflavine is being estimated. We have found that in many cases where artificial colouring matters are added these are reduced by dithionite but are not reoxidised by aeration. A number of preparations have been examined by the spectrophotometric and by either microbiological or fluorimetric methods. The details are given below.

(*a*) *Direct spectrophotometric method.* Sufficient sample was dissolved or diluted in buffer solution of pH 4·0 to give a concentration of between 10 and 20 μg./ml. riboflavine. The extinction at 445 mμ was measured in a 1 cm. cell on the Unicam SP500 or SP600. The concentration of riboflavine was calculated using an E (1 per cent. 1 cm.) value at 445 mμ of 308 for pure riboflavine.

(*b*) *Direct fluorimetric method.* Sufficient sample was dissolved or diluted in buffer solution of pH 4·0 to give a concentration of between 0·05 and 0μ1 μg./ml. riboflavine. The fluorescence was measured using an OX1 primary filter and an OY13 and OG3 as secondary filters. A solution of 0·1 μg./ml. pure riboflavine in buffer pH 4·0 was used as a standard. This method was used only for the simplest types of sample.

(*c*) *Dithionite spectrophotometric method.* Solutions were prepared as for the direct spectrophotometric method. The extinctions at 445 mμ were measured after addition of 0·1 ml. of 5 per cent. dithionite to 20 ml. of solution and allowing to stand for 1 minute, and also after aeration of the dithionite treated solution for 1 minute. The difference between

the last two readings was compared with that obtained for pure riboflavine under the same conditions, and the riboflavine content calculated using a value of 264 for the difference in E (1 per cent. 1 cm.) at 445 mμ between riboflavine and leuco-riboflavine.

(*d*) *Microbiological method.* The method employed *Lactobacillus casei* as the micro-organism.

Results

Preparations containing no interfering materials. A number of tablets and solids were examined by spectrophotometric method (*a*), and the fluorimetric method (*b*), the dithionite treatment being omitted. The results are given in Table IV.

TABLE IV

RIBOFLAVINE CONTENT OF SIMPLE TABLETS AND SOLIDS

Sample No.	Theory	Found	
		Method (*a*)	Method (*b*)
T1	1·0 mg./tab.	0·880 0·876	0·88
T2	1·0 ,,	1·01 1·01	1·03
T3	3·0 ,,	2·93 2·97	2·96
T4	1·0 ,,	0·985 0·991	1·01
T5	1·0 ,,	1·07 1·07	1·01
V1	35·6 μg./g.	35·5 35·0	35·0

TABLE V

RIBOFLAVINE CONTENT OF TABLETS WITH ADDED DYES

Sample No.	Theory mg./tab.	Found		
		Method (*c*) mg./tab.	Method (*b*) mg./tab.	Method (*d*) mg./tab.
P1*	0·50–0·69	0·530 0·536	0·54	0·54
P2	..	0·630 0·633	0·55	0·63
P3*	..	0·570 0·578	0·52	0·53
P4	..	0·610 0·608	0·55	0·63
P5	0·50	0·664 0·672	0·58	0·50
P6	..	0·558 0·570	0·56	0·51

Preparations containing added dyes. These included tablets, capsules and liquid vitamin preparations. As all the samples contained added dyes method (*c*) was used.

Tablets. The tablets were of two types, one containing riboflavine, vitamins A, B_1, C and D, the other riboflavine, vitamin B_1 and strychnine. In each case the coating material contained a dye and it was possible to determine the riboflavine before and after coating. The tablets were shaken with buffer solution until complete disintegration occurred, made up to a suitable volume and filtered. A typical aqueous extract is shown in Figure 2. It will be seen that the reoxidised solution shows an almost identical curve with the original (Fig. 2 (*a*)) for the uncoated tablets but there is a considerable difference for the coated tablets (Fig. 2 (*b*)). The tablets were also assayed by the microbiological method. The results are shown in Table V. Samples with an asterisk were uncoated.

Multivitamin capsules. Refluxing with buffer solution at pH 4·0 to ensure complete solution of riboflavine resulted in the gelatin coating also being dissolved. As this incorporated a dye method (*c*) was employed. A typical set of spectra are shown in Figure 3 (*a*). Here again the extraneous absorption is seen to be considerable. The difference between the reoxidised and reduced solutions is shown in Figure 3 (*b*). This compares favourably with that for pure riboflavine. The results

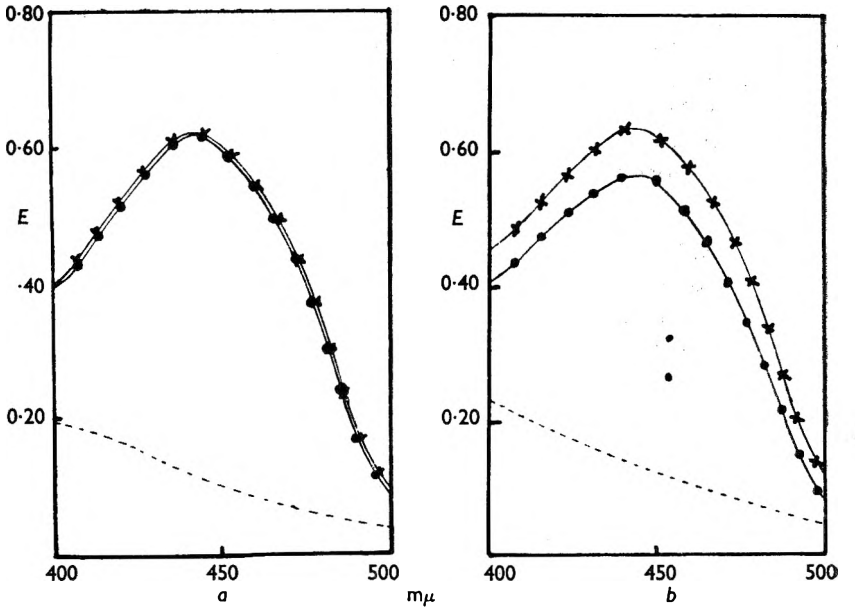

FIG. 2. (*a*) Extract of uncoated tablets. (*b*) Extract of coated tablets.
×——× Gross. - - - - After $Na_2S_2O_4$. ●——● After reoxidation.

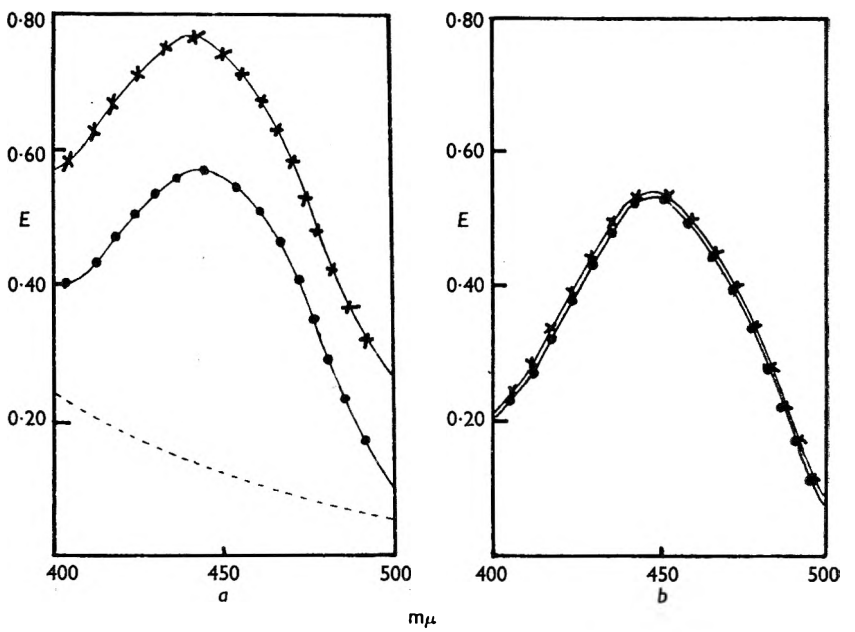

FIG. 3*a*. Extract of multivitamin capsules.

×——× Gross.
- - - - After $Na_2S_2O_4$.
●——● After reoxidation.

FIG. 3*b*. Difference of spectra for capsules and pure riboflavine.

×——× Reoxidised minus leuco for capsules.
●——● Reoxidised minus leuco for riboflavine.

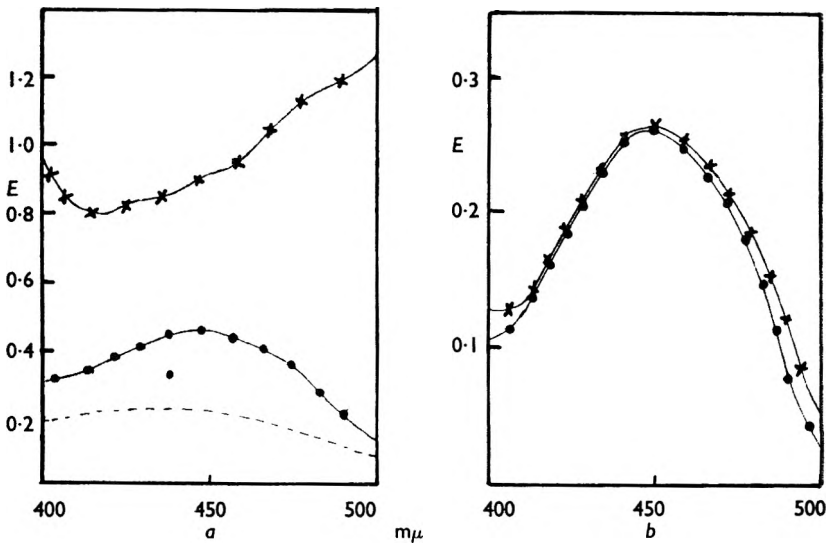

FIG. 4*a*. Liquid vitamin preparation.

×——× Gross.
– – – – After $Na_2S_2O_4$.
●——● After reoxidation.

FIG. 4*b*. Difference spectra for liquid preparation and pure riboflavine.

×——× Reoxidised minus leuco for mixture.
●——● Reoxidised minus leuco for riboflavine.

of three samples examined by spectrophotometric and microbiological methods are given in Table VI.

Liquid vitamin preparations. A typical sample is shown in Figure 4 (*a*). The extraneous absorption is considerable but after reduction and reoxidation a curve similar to that for pure riboflavine is obtained. Figure 4 (*b*) shows the difference curve for the sample and pure riboflavine. Results obtained by spectrophotometric and microbiological methods are given in Table VII.

TABLE VI

RIBOFLAVINE CONTENT OF MULTIVITAMIN CAPSULES

Sample No.	Theory mg./capsule	Found	
		Method (*c*) mg./capsule	Method (*d*) mg./capsule
CV1	0·425–0·575	0·57	0·55
CV2		0·55	0·54
CV3		0·52	0·50

TABLE VII

RIBOFLAVINE CONTENT OF LIQUID VITAMIN PREPARATION

Sample No.	Theory μg./ml.	Method (*c*) μg./ml.	Method (*d*) μg./ml.
E1	47	44·3 45·3	46·7
J1	375	268	288

Naturally occurring materials. Probably the most important natural source of riboflavine is yeast. Unlike synthetic vitamin preparations the chemical properties of the non-riboflavine pigments are unknown and it is virtually impossible to estimate the effect of reduction and reoxidation on these pigments.

Yeast was hydrolysed by refluxing for 1 hour with a mixture of acetone and 0·2 N H_2SO_4 (1 : 1). After adjusting to a suitable volume with

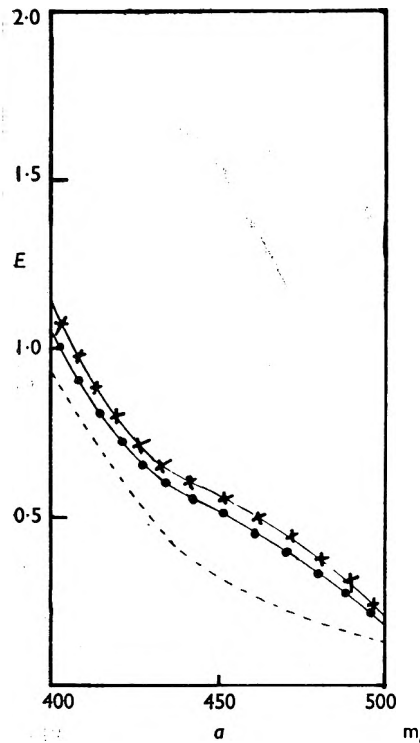

FIG. 5*a*. Extract of yeast.

×——× Gross.
- - - - After $Na_2S_2O_4$.
●——● After reoxidation.

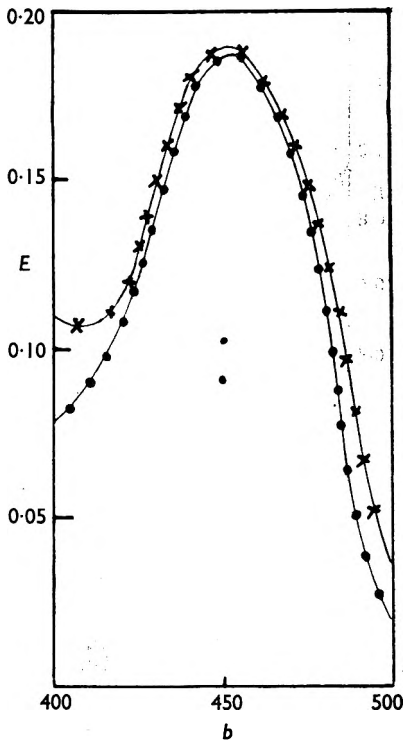

FIG. 5*b*. Difference spectra for yeast and pure riboflavine.

×——× Reoxidised minus leuco for yeast.
●——● Reoxidised minus leuco for riboflavine.

buffer solution at pH 4·0 the solution was centrifuged and filtered. A typical set of spectra are shown in Figure 5. Although only general absorption is obtained even after reoxidation (Fig. 5 (*a*)), the difference curve (Fig. 5 (*b*)) is similar to that for pure riboflavine. The results obtained on a number of samples by the spectrophotometric and microbiological methods are given in Table VIII.

TABLE VIII

RIBOFLAVINE CONTENT OF YEAST

Sample No.	Method (*c*) μg./g.	Method (*d*) μg. g.
Y1	54·1 55·3	53·0
Y2	64·4 65·0	53·5
Y3	55·5 55·3	54·3
Y4	64·8 65·2	56·3
Y5	57·4 57·2	53·0
Y6	65·7 61·4	55·0
Y7	59·0	57·5
Y8	60·8	66·8
Y9	58·8	58·5
Y10	51·5	57·0

SUMMARY

1. A spectrophotometric method has been described for the determination of riboflavine in pharmaceutical materials. It has a number of advantages over the present official methods.

2. It is much more rapid than the microbiological method.

3. By taking a series of spectrophotometric readings over the range 400 mμ to 500 mμ it can be shown that only riboflavine is determined because the difference curve will, in this case, be identical with that of pure riboflavine. In this respect it is more specific than most fluorimetric methods.

4. The method is more flexible than fluorimetric procedures in that the pH range over which it is applicable is very wide.

5. It is not necessary to prepare a standard riboflavine solution for each determination. Once the absorption values have been determined for the working conditions they can be applied with only very occasional checks because extinction readings are absolute and not comparative.

6. The results obtained on the samples of yeast are in general sufficiently close to those of the microbiological method to suggest that the spectrophotometric procedure could in due course replace the microbiological method in the B.P.C.

7. The principal shortcoming of the method lies in its poorer sensitivity than the fluorimetric technique, but this is not important in the assay of pharmaceutical products.

We are grateful to Miss F. N. Mulholland for the microbiological determinations.

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DISCUSSION

The paper was presented by Mr. L. Brealey.

Dr. G. E. Foster (Dartford) said he had tried the suggested method and found it satisfactory for multivitamin capsules. He asked if the authors had applied their method to the assay of malt extract.

Dr. F. Wokes (King's Langley) referred to the authors' statement that the fluorimetric technique had fallen into some disrepute because of the lack of sensitivity of commercially available fluorimeters. The riboflavine content of the various preparations examined was much higher than the figures he had published. He had made some calculations from the Tables giving the comparison of the microbiological and fluorimetric methods, and had found the latter to be on average 99·7 per cent. of the microbiological assay as compared with 108 per cent. for the spectrophotometric method. Therefore the data did suggest that the fluorimetric method was as accurate as the proposed spectrophotometric method.

Mr. S. G. E. Stevens (London) said that certain difficulties had arisen in the accurate determination of the riboflavine in animal feed substances.

It appeared that where certain grass meals were used, materials were being extracted which did not readily reduce, and they interfered with the fluorimetric determination of the re-oxidised riboflavine. On occasions it had been necessary to make use of the technique of adding known amounts of riboflavine to those extracts and to make a number of recovery experiments. He asked whether the authors had encountered this difficulty and had carried out recovery experiments.

MR. L. BREALEY, in reply, said that the method had not been used for malt extracts. The difficulty about using the method for materials low in riboflavine was in securing a solution of sufficient concentration to obtain good spectrophotometric readings. That was why the work had been limited to materials of relatively high vitamin content. The fluorimetric results were good because commercial instruments had not been used. They had built their own instrument and it was much more sensitive than the generally available commercial equipment. There were many arguments for and against the procedure of recovery experiments, but they had not made any such experiments.

STABILITY OF B VITAMINS IN PHARMACEUTICAL PRODUCTS

By F. Wokes and F. W. Norris

From the Ovaltine Research Laboratories, King's Langley, Herts, and the Department of Applied Biochemistry, University of Birmingham

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The standardisation of the vitamin B contents of pharmaceutical (including dietetic) products has been greatly facilitated by the development of physico-chemical and microbiological assay methods. These, as shown by comparison with the results of biological assays, can be relied upon to ascertain that the vitamin contents claimed are, in fact, present when the products leave the manufacturer. Much further information is, however, still needed by the pharmaceutical and medical profession about possible loss of vitamin potency during different storage conditions in the pharmacy, hospital or home. A certain amount of such information has already been given in publications dealing with vitamins A[1,2] and C[3–5]. In a recent paper[6] dealing with the content of A, B and C vitamins in samples of multivitamin products purchased in Canada, references are given to some results on the stability of B vitamins in pharmaceutical products obtained by several American and Canadian workers. Apart from the work of Parkington and Waterhouse[7] on the stability of different aneurine salts, very little appears to have been published about the stability of the B vitamins in pharmaceutical products under the storage conditions in general practice in this country.

Methods

Products Examined

Our findings are based on over 10 years' experience in the development of physico-chemical and microbiological assays and their application to a wide range of pharmaceutical products. To simplify the presentation of our data, we give here only our results on the following four classes of products.

(*a*) Vacuum dried dietetic specialities claiming vitamins in which the significant sources of B vitamins are usually malt and milk. One food concentrate which we have studied extensively also contains eggs. Some fortification with synthetic vitamins may be employed to allow for variations in the vitamin B contents of the raw materials.

(*b*) Bakery products in which B vitamins are obtained from cereals, with more extensive fortification than in (*a*).

(*c*) Vitamin concentrates prepared *in vacuo* in which the B vitamins are obtained from malt and in one product also from yeast. Fortification with B vitamins is employed.

(*d*) Multivitamin capsules* in which the B vitamins are supplied mostly in synthetic form in different media.

* After this paper went to press, H. E. F. Notton published (*Pharm. J.*, 1956, **177**, 69) further data on the stability of vitamins in capsules.

Our results were obtained on less than 20 different products out of more than a hundred on the market, and therefore cannot cover all the variations which occur in practice, though they may perhaps provide useful representative data.

Storage Conditions

The various products under investigation were stored, in the containers in which they are normally sold, in incubators at 27°, 37° or 43° C., in a refrigerator at about 3 to 4° C., and at room temperature. The relative humidity was below 70 per cent. except in certain experiments in which it was raised to 90 to 100 per cent. by means of suitable salt solutions. To test the effects of the fluctuating temperatures and humidities in the tropics, a number of field trials have been arranged in which samples are sent out to different tropical countries for periods ranging from 6 to 18 months for storage under the prevailing conditions, and then returned for examination.

Assay Methods

Vitamin B_1. Fluorimetric assays were by the S.P.A. method[8] with certain slight subsequent modifications. Microbiological assays were by the method of Fitzgerald and Hughes[9], with modifications proposed in the Report of the Thiamine (Microbiological) Panel of the S.P.A.[10], using *Lactobacillus fermenti* 36.

Riboflavine. Fluorimetric assays were by the method of Klatzkin, Norris and Wokes[11], and microbiological assays by the method proposed in the Report of the Vitamins Estimation (Microbiological) Panel of the S.P.A.[12], using *L. helveticus.*

Nicotinic Acid. Chemical assays were by the method of Klatzkin, Norris and Wokes[13]. Microbiological assays were by the method proposed in the Report above (for Riboflavine), using *L. arabinosus* 17/5.

Vitamin B_6 was assayed by the microbiological method using *Neurospora*[14], *Pantothenic acid* was assayed by the microbiological method using *Lactobacillus arabinosus*[15], Biotin was assayed by the microbiological method using *Lactobacillus arabinosus*[16], *Folic acid* was assayed by the microbiological method using *Streptococcus fæcalis*[17], and *Vitamin B_{12}* by the microbiological method using *Ochromonas malhamensis*[18].

At least two assays were carried out on each sample. If the results were not in sufficiently good agreement, more assays were made.

Results

Vitamin B_1

Dietetic specialities. Figure 1 shows that in the food concentrate stored in this country in tins with air-tight metal closures, there was no appreciable loss of vitamin B_1 even after more than 7 years' storage at room temperature, and the content always remained well above the claim. In other products without air-tight metal seals the rate of loss ranged

from 6 to 20 per cent. in 3 to 8 years at room temperature. At 37° C. the food concentrate in the metal sealed tins showed a very slow rate of loss, barely reaching 15 per cent. in 7 to 8 years. In the other products without metal-sealing, the rate of loss was increased, and after a year or two the vitamin content had fallen below the claim.

Bakery Products. In bakery products, vitamin B_1 is generally stable, both at room temperature and at 37° C., no appreciable loss being observed during over five years storage under reasonably dry conditions (relative humidity below 70 per cent.), whilst the content remained well above the claim (Fig. 2). The effect of baking on the vitamin does not seem to render it any less stable[19].

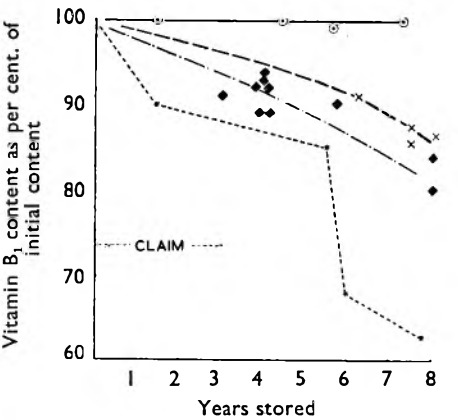

FIG. 1. Rate of loss of vitamin B_1 in vacuum-dried foods:

Stored at room temperature in hermetically-sealed tins .. .. ⊙——⊙
Stored at room temperature in containers not completely air-tight .. ◆-·-◆
Stored at 37° C. in containers not completely air-tight .. .. *----*
Stored at 37° C. in hermetically sealed tins .. .. ×---×

The initial vitamin B_1 content of the food concentrate averaged 14·2 μg./g. (as compared with a claim of 10·6 μg./g.) and of the other products ranged from 3 to 12 μg./g., with smaller "overages" than on the food concentrate.

Vitamin concentrates. The higher moisture content of vitamin concentrates seems to render the vitamin B_1 rather less stable (Fig. 3). Although the initial vitamin B_1 content may be 20 per cent. above the claim (this "overage" being provided to allow for the possible greater loss during storage), it is advisable to store the product in a cool place in order to ensure a shelf life of about 2 years. At 4 to 5° C., the rate of loss may be only about 5 per cent. in 2 years, provided that the product is stored in the original air-tight and completely filled glass containers. When these are opened to remove some of the contents, the rate of loss in the remainder is increased, but the vitamin B_1 concentration should not be significantly affected before the whole is consumed. In the original unopened jars stored at room temperature, the vitamin B_1 content of products with an overage of 20 per cent. should not fall below the claim until $2\frac{1}{2}$ to $3\frac{1}{2}$ years have elapsed.

Multivitamin capsules. The administration of vitamins in capsules raises a number of problems concerned with external factors such as storage temperature and exposure to light, moisture, etc., and also with internal factors such as the presence of minerals or other interfering substances in the capsules. The stability and assimilation of the vitamins

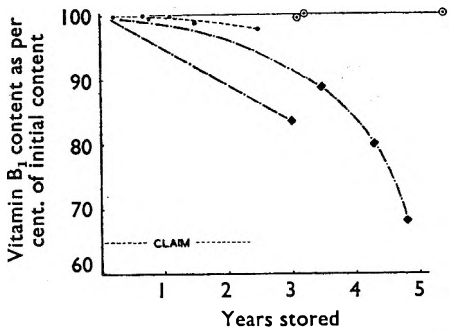

FIG. 2. Rate of loss of vitamin B_1 in bakery products:

Stored at room temperature in air-tight containers .. .. .. ○——○
Stored at room temperature in containers not completely air-tight .. ◆-·-◆
Stored at 37° C. in air-tight containers *----*

The initial vitamin B_1 content averaged 28 μg./g. (as compared with a claim of 17·6 μg./g.) in the main product investigated. In the other products, it ranged from 16 to 28 μg./g., with smaller "overages .

ing, most of the above disturbing factors have been eliminated. The possible deleterious effect of light on the contents is prevented by a pigment in the capsule shell. No minerals are present to interfere with stability or assimilation, and the B vitamins are rendered more stable by their incorporation in a special yeast medium. Our findings, will, therefore, not necessarily apply strictly to capsules in which such precautions have not been taken.

In this country, the capsules when stored at room temperature under the usual conditions retain their vitamin B_1 content remarkably well, no loss having been detected after several years (Fig. 4). In tropical climates, may be affected by minerals present. Vitamin C, for example, is less stable at a pH value above 6. The capsule shells may, under certain conditions, absorb some of the vitamins which, however, may still be available to the patient. But some vitamins may be lost by leakage if the shell becomes softened by atmospheric moisture as in humid tropical climates. On the other hand, intense dry tropical heat may make the shells brittle which could lead to leaks if the capsules were shaken too vigorously in their containers.

In the particular multivitamin capsules which we have been mainly investigat-

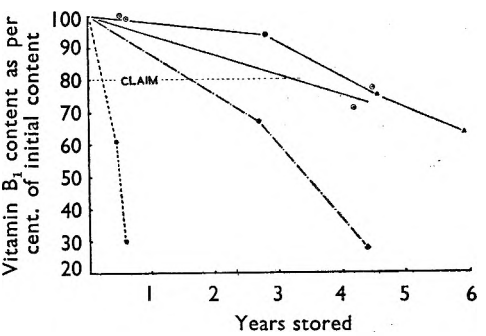

FIG. 3. Rate of loss of vitamin B_1 in vitamin concentrates:

Stored at 3–4° C. in completely filled jars .. .. ●——●
Stored at 3–4° C. in partly filled jars ▲——▲
Stored at room temperature in completely filled jars .. .. .. ⊙——⊙
Stored at room temperature in partly filled jars .. .. ◆-·-◆
Stored at 37° in partly filled jars .. *----*

The initial vitamin B_1 content averaged 15·4 μg./g. (as compared with a claim of 12·3 μg./g.) in the main product investigated. In the other products, it ranged from 7 to 15 μg./g., with smaller "overages".

the rate of loss varies widely according to the climatic conditions. If the capsules are kept in dry, sealed containers, temperatures up to 43° C. have had only slight effect on the vitamin B_1 content, so that the claim may still be met even after 2 years. If the capsules are not stored in absolutely air-tight containers, the rate of loss increases with the relative humidity, and may reach about 20 per cent. per annum. Significant losses of vitamin B_1 during storage in the tropics can be avoided only by stringent precautions.

When prolonged storage under unsuitable conditions has led to an appreciable loss of vitamin B_1 in foods and pharmaceutical products, substances may be formed which exert quenching effects and thus lower the per cent. recovery in fluorimetric assays. If the fluorimetric results are adjusted by means of this recovery (i.e., by multiplying by 100 and dividing by the per cent. recovery), the fully adjusted results are too high as can be shown by comparison with microbiological results (Table I), so that storage losses may be masked and escape attention when the usual fluorimetric procedures are adopted. This difficulty can be overcome by using a factor which has to be determined separately for each type of food by a method described elsewhere[20]. For food concentrates of the type we have been investigating, the factor has been found to be about 0·3. If the factor of 0·3 is multiplied by the increase in the result which would be obtained by making full adjustment for the per cent. recovery, a quantity is obtained which when added to the unadjusted result will give a value close to the true value.

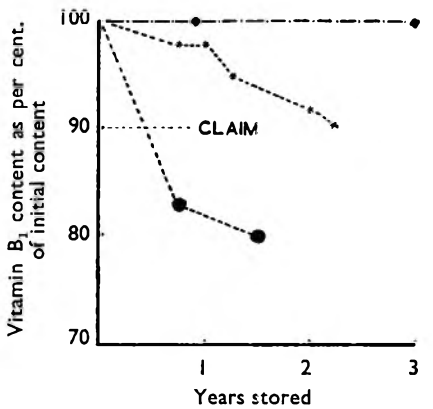

FIG. 4. Rate of loss of vitamin B_1 in multivitamin capsules:

Stored at room temperature in tins .. ◆–·–◆
Stored at 37° C. in screw-capped, sealed bottles .. .. .. *– – – –*
Stored at 37° C. in screw-capped sealed bottles at 90 to 100 per cent. relative humidity .. .. .. ●– – –●

The initial vitamin B_1 content averaged 2·75 mg. per capsule (as compared with a claim of 2·5 mg.) in the main product investigated. In the other products it ranged from 0·3 to 1 mg. per capsule, with similar "overages".

Riboflavine

Dietetic specialities. In samples of the food concentrate stored in the usual metal-sealed tins under normal conditions, the losses of riboflavine after 2 to 3 years have been found to lie between 5 and 10 per cent. In Figure 5, the riboflavine contents are given as per cent. of the initial content, and not in relation to any claimed content, since such claims are not yet being made for the food concentrate in this country. When the tins

have been opened to remove some of the contents, the rate of loss may be increased, so that after little more than a year's storage, the loss has become significant. Storage at tropical temperatures (37–43° C.) does not greatly increase the rate of loss in unopened tins. The metal-sealing, therefore, appears to be sufficient to prevent significant loss of riboflavine even during prolonged storage under tropical conditions, and the main precaution to be adopted is to avoid exposing the contents to air for too long a period after the tins have been opened. If such exposure does occur during storage in the home, it may be detected by the caking of the contents.

TABLE I

COMPARISON OF FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF VITAMIN B_1 IN A FOOD CONCENTRATE (ALL RESULTS AS μG./G.)

Age of sample years	Fluorimetric results		Microbiological results
	Adjusted for full recovery	Adjusted using factor	
4	14·0	14·0	14·0
2	13·9	13·4	13·7
3	14·2	14·0	14·6
2	16·6	14·0	13·7
4	18·4	14·4	13·7
Means	15·4	14·0	13·9

Bakery products. Under normal storage conditions, the rate of loss of riboflavine was found to be only 3 to 6 per cent. per annum, so that even after 3 years the content exceeded the claim (Fig. 6). Where there was exposure to a moist atmosphere, the riboflavine became less stable. In other storage experiments at 27 and 37° C., the rate of loss under satisfactory conditions was not much higher than at room temperature provided that there was no undue exposure to a moist atmosphere. In order to simplify the presentation of the data, these results have been omitted from Figure 6.

Vitamin concentrates. In the product we have mainly investigated, the stability of riboflavine was better than that of vitamin B_1 during storage in the original air-tight completely filled jars. Thus at 37° C., there might be only about 10 per cent. loss in 3 years, and at room temperature practically no loss after nearly 5 years' storage (Fig. 7). When the jars were opened and some of the contents removed, the riboflavine content still remained above the label claim throughout the time taken to consume the whole of the contents.

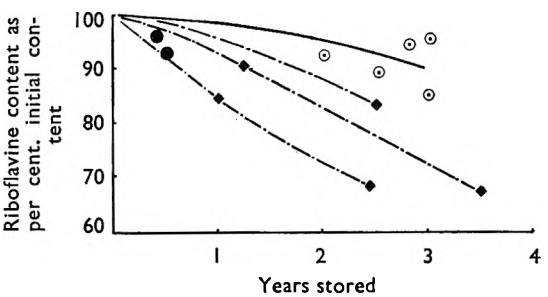

FIG. 5. Rate of loss of riboflavine in vacuum-dried foods:

Stored at room temperature in hermetically-sealed tins .. .. ⊙——⊙

Stored at room temperature in containers not completely air-tight .. .. .. ◆-·-◆

Stored at 37 to 43° C. in hermetically-sealed tins .. .. ●

The initial riboflavine content ranged from 3 to 20 μg./g. in the different products examined, with "overages" of 5 to 15 per cent.

As with vitamin B_1, prolonged storage under unsuitable conditions may lead to the development of substances which exert quenching effects in fluorimetric assays of riboflavine, thus lowering the recovery of added riboflavine. If this percentage recovery is used to adjust the fluorimetric results, the fully adjusted results may be too high so that the loss of vitamin during storage is obscured. When this difficulty is overcome by application of a factor, which has to be specially determined for each type of food, the partially adjusted results then become nearer to the true values, indicated by microbiological assays.

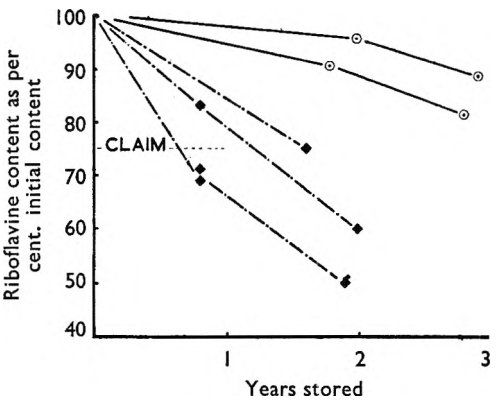

FIG. 6. Rate of loss of riboflavine in bakery products:
Stored at room temperature in air-tight containers .. .. ⊙——⊙
Stored at room temperature in containers not completely air-tight .. .. .. ◆-·-◆

The initial riboflavine content ranged from 5 to 30 μg./g. in the different products examined, with "overages" of 5 to 20 per cent.

Multivitamin capsules. Our findings emphasise the importance of avoiding undue exposure to atmospheric factors during the manufacture and storage of pharmaceutical products containing riboflavine. The problem is simplified when we turn to multivitamin capsules, in which the vitamins are protected from the action of air. The riboflavine in the multi-vitamin capsules we have been investigating did not undergo any significant loss during more than a year's storage under normal conditions at 37° C.

Nicotinic acid

This vitamin, in the form either of acid or of amide, has been found to be more stable than vitamin B_1 or than riboflavine. No significant losses have been detected either by chemical or by microbiological assays in any of the four classes of products we have been studying, when stored under normal conditions in this country for several years, even in opened containers. Thus, in one sample of the food concentrate stored at room temperature in opened tins for 4 years, a loss of about 7 per cent. was indicated by our assays, but this did not exceed the combined experimental errors of the assays. In another sample also stored in an opened tin at room temperature for 6 years, an increase of the same order was encountered, and it therefore seems safe to assume that the claim for nicotinic acid in this product will be met even when the storage period greatly exceeds the normal shelf life. Our experimental findings indicate that this applies also to bakery products

and vitamin concentrates. No losses of nicotinic acid have been detected in multivitamin capsules during several years' storage at room temperature. When the capsules were stored under tropical conditions at 37 to 43° C., losses of 6 to 16 per cent. were found in 1 to 3½ years' storage. However, the capsules had, under these conditions, ruptured the shells and released some of the contents, hence were obviously unsuitable for administration. This can be prevented by storage under suitable conditions.

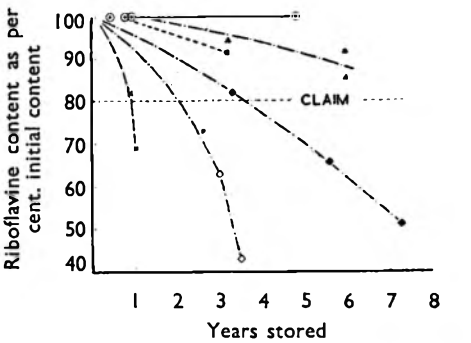

FIG. 7. Rate of loss of riboflavine in vitamin concentrates:

Stored at 3 to 4° C. in partly filled jars ▲-·-▲
Stored at room temperature in partly filled jars .. .. ◆-·-◆
Stored at room temperature in completely filled jars .. .. .. ○——○
Stored at 37° C. in completely filled jars .. .. *- - - -*
Stored at 37° C. in partly filled jars .. ×- - -×

The initial riboflavine content ranged from 5 to 12 μg./g. in the different products examined, with "overages" of 5 to 15 per cent.

Other B vitamins

The above mentioned vitamins (B_1, riboflavine and nicotinic acid) have for over 10 years been recognised by the Ministry of Food as being essential to health and liable to be deficient in human diets. Hence, they have been scheduled in the Labelling of Food Order (1946). During the last few years, claims have been made in increasing numbers for the presence in pharmaceutical products of other B vitamins which are not yet recognised by the Ministry of Food, although the Ministry of Health does not always frown on their being prescribed. Whilst our experience with these has been less extensive, their properties and stability are also important.

Vitamin B_6

Suggestions have recently been made[21] that deficiencies of this vitamin in low extraction flours may accentuate deficiencies of essential fatty acids, and its occurrence and stability in pharmaceutical products may, therefore, receive more attention in the future. In neutral or alkaline solution, vitamin B_6 is sensitive to ultra-violet radiation and suitable protection may, therefore, be needed for certain liquid or semi-liquid preparations, such as might be administered in multivitamin capsules. However, no losses of vitamin B_6 potency have been detected in the particular products we have been studying during storage for over a year at normal temperatures.

Pantothenic acid

Unequivocal pantothenic acid deficiency has not yet been reported in man, though it probably occurs as a complication in beri-beri and pellagra.

Results of microbiological assays show no significant losses of pantothenic acid during 18 to 20 months storage at room temperature in the products which we have studied.

Biotin

Biotin deficiency may occur in man. This B vitamin appears to be stable to heat, acids and alkalis, and no losses have been detected (microbiological assays) during 18 months storage at room temperature.

Folic Acid

Folic acid deficiency has been found in man, but on the other hand, administration of large doses of folic acid in vitamin B_{12} deficiency may precipitate neurological manifestations, hence the intake of both of these vitamins should be considered together. No losses of folic acid potency have been detected during 18 months storage at room temperature in those of the products we have been studying.

Vitamin B_{12}

The stability of this vitamin in the injections used for treatment of pernicious anæmia has been considered in a previous communication[22] from our laboratories giving results obtained both by spectrophotometric and by microbiological assays. For preparations given by mouth, the evidence rests entirely on microbiological assays, and is more scanty. However, in the products we have so far investigated, the vitamin B_{12} has been found to be stable for 18 months or more, provided that suitable precautions are taken. The occurrence and stability of vitamin B_{12} in dietetic products may receive more attention in the future, since human dietary deficiency of vitamin B_{12} has recently been reported[23] amongst persons (termed "vegans") living in this country on diets containing no animal food, not even milk or eggs.

Discussion

Our findings on the four different types of products indicate that the main precautions to be taken during their storage involve avoidance of exposure to atmospheric conditions, especially in hot humid climates. In certain circumstances (e.g., when riboflavine or other photo-labile vitamin is present) protection against the action of light is also desirable. Maximum stability is, of course, best achieved by storing these products in air-tight containers in a cool, dry, dark place. If the containers are opened and some of the contents removed, losses of B vitamins may set in after sufficient atmospheric moisture has been absorbed. The amount of air space above the contents can then become important, since it may diminish the stability of the riboflavine.

Campbell and McLeod[6] encountered serious losses of pantothenic acid in 2 out of 3 brands of multivitamin capsules stored for different periods up to 19 months under conditions not precisely stated. In the third brand they found very little loss, which is in agreement with our findings.

Our experiments on dry products detected no significant losses of pantothenic acid during 18 to 20 months storage at room temperature.

This is in agreement with the findings of Campbell and McLeod on one make of tablet. Their results on the other three makes of tablets which they were studying would have been interesting. They also agree with our findings that other B vitamins are reasonably stable in capsules, so that "overages" of 10 to 20 per cent. should be sufficient to ensure that the claims were met during a normal shelf life. Our experience on the stability of B vitamins in liquid multivitamin products has been rather limited. However, we agree with Campbell and McLeod in finding vitamin B_1 in these to be much less stable than in dry products.

This discussion would not be complete without some reference to the "overages" or excess vitamin contents initially provided by manufacturers to allow for losses during storage. Campbell and McLeod do not mention these in their paper, although their data show that appreciable "overages" must have been provided in most of the products they investigated. Examples of "overages" in actual use in this country are given in the present paper. Without a knowledge of the "overages" of the different vitamins initially supplied in any product, it is obviously not possible to make precise calculations about losses during storage, or to make any strict comparison of the relative stabilities of the vitamins in different products.

Campbell and McLeod have suggested that the manufacturer should be responsible for the maintenance of the potency of vitamin products over their normal shelf life. In our opinion, this responsibility can best be met by supplying the product in satisfactory containers, with advice to the pharmacist on the most suitable storage conditions, and by providing a sufficient "overage" of the different vitamins to ensure that the claims will still be met after a reasonable period of storage.

Summary

1. Storage experiments extending over 3 to 8 years have been carried out on representative samples of vacuum dried, dietetic specialities, bakery products, vitamin concentrates with a basis of malt extract and multivitamin capsules in which B vitamins have been claimed.

2. Changes in the contents of these vitamins have been followed by physico-chemical and microbiological assays.

3. The stability of the vitamins has been found to be affected by exposure to air as well as by temperature and relative humidity.

4. In vitamin concentrates with a malt extract basis, storage in a cool dry place is advocated to maintain the vitamin content during a reasonable shelf life. (1–2 years, depending on the "overages" provided.)

5. In multivitamin capsules, the stability of the vitamins may be affected by light as well as by exposure to high temperatures and relative humidity, and by interaction with other constituents. When precautions are taken to avoid these disturbing factors, the B vitamins in the capsules should not undergo significant loss during a reasonable shelf life.

We are indebted to a number of colleagues, including Miss Elaine Morphet, Miss Maureen Metcalfe and Mrs. Sheila Cowen, for assistance

in carrying out the considerable number of assays required for this investigation.

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DISCUSSION

The paper was presented by DR. F. WOKES.

DR. G. E. FOSTER (Dartford) referring to the assay of vitamin B_1, said it was well known that under some conditions, particularly in multivitamin capsules, there could be a decrease in the vitamin B_1 content on storage. It was possible that the decomposition products in the preparation under examination might produce some impurity which would quench the fluorescence of the thiochrome solution. It would be rather serious if the method used was unreliable. Could the authors give any information as to whether quenching interfered with the estimation?

DR. D. C. GARRATT (Nottingham) suggested that the paper might preferably be entitled "Stability of B vitamins in *Certain* Pharmaceutical Products". The preparations were obviously specialised, and he would hesitate to use the information in the paper as a basis for generalising on the stability of the B vitamins in pharmaceutical preparations. It was fairly well known that in spite of reasonable precautions in storage, these substances did not remain stable over the length of time set by the authors, and it would be interesting to hear of further experience with normal vitamin products.

MR. E. H. B. SELLWOOD (London) asked whether the authors had any information on the relative stability of aneurine chloride or nitrate, and riboflavine free or as phosphate.

MISS J. ASHWIN (Dorking) said that in the case of pantothenic acid it would be interesting to know whether any assays had been made after storage at higher temperatures, particularly of vitamin concentrates. She asked whether the authors could comment on the stability of vitamin B_{12} in liquid preparations containing vitamins B_1 and C.

DR. F. WOKES quoted extracts from a letter received from Dr. Campbell in Canada, in which he confirmed the findings of the authors and suggested the use of expiration data on container labels. Such data must be based on stability studies of each product.

DR. F. WOKES, in reply, said that the point raised by Dr. Foster had been dealt with to some extent in Table I, where it was shown that samples of different ages gave different recoveries, the lowest being in the older samples. In a previous paper a method had been described of allowing for recovery by using a factor which had to be determined separately for each type of product. In Table I of the paper under discussion it was shown that if such a factor were used, the average was close to the biological assays. The problem could alternatively be overcome by diluting the solutions, thereby diluting out the fluorescence. It had been shown that by sufficient dilution it was possible to raise the percentage recovery to nearly 100 per cent. and thus no correction was necessary. He accepted the criticism concerning the limitation of the work to *certain* pharmaceutical products, but pointed out that the paper stated clearly that only a limited number of products were covered which included not only specialities but some which were in the B.P. The authors had no data about the relative stability of aneurine in the form of different salts except for the hydrochloride. A good deal of the aneurine came from natural sources and would be in combined organic form. He had no information on the stability of pantothenic acid. The preparations tested for vitamin B_{12} were solids and he had no data on the stability of this vitamin in liquid preparations containing vitamins B_1 and C.

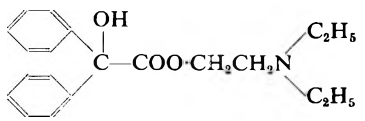
THE DETERMINATION OF BENACTYZINE

By J. P. Jefferies and J. I. Phillips

From Glaxo Laboratories Ltd., Greenford, Middx.

Received July 2, 1956

Benactyzine hydrochloride has been used in Denmark for the treatment of emotional and physical tension[1] and has been recently tested in this country[2–4]. It is usually administered orally, and tablets are marketed here under the trade name Suavitil. Benactyzine is 2-diethylaminoethyl benzilate, and has the structure



Protection of the drug from hydrolysis is of importance in the preparation of tablets and solutions for injection, as neither the benzilic acid nor the ester fragment of the molecule show any activity[5]. Analytical methods must therefore be capable of distinguishing benactyzine from the benzilic acid and 2-diethylaminoethanol produced on hydrolysis.

A. Solid Benactyzine Hydrochloride

Benactyzine hydrochloride shows typical benzenoid absorption in the ultra-violet region. Benzilic acid, as would be expected, has a similar absorption spectrum and the extinction coefficients of both compounds at their maxima are given in Table I.

The infra-red absorption curves (2000 to 700 cm.$^{-1}$) for both compounds as 0·5 per cent. w/v solutions in purified bromoform are given in Figure 1.

Benactyzine hydrochloride may be estimated by means of the strong carbonyl absorption peak at 1743 cm.$^{-1}$ and chloroform solutions containing 0·2 to 0·6 per cent. w/v with a cell path of 0·8 mm. give a rectilinear calibration. Benzilic acid shows strong absorption at the same wave number, so that for quantitative purposes absorption at 1743 cm.$^{-1}$ is not specific for benactyzine hydrochloride; any appreciable amounts of benzilic acid can, however, be detected from a qualitative examination of the spectrum in bromoform, particularly in the 1700 cm.$^{-1}$ region.

Conventional methods can be used to determine the ionisable chlorine and the total nitrogen content of benactyzine hydrochloride, but attempts to devise a gravimetric assay similar to that used for phenadoxone in the British Pharmacopœia proved unsuccessful. Picric acid failed to give a satisfactory precipitate from aqueous solution, and precipitation of the reineckate or picrolonate was not strictly quantitative.

The most useful procedure is based on complete hydrolysis of the benactyzine with strong sodium hydroxide, with subsequent steam distillation of the diethylaminoethanol produced and its titration with

TABLE I

SPECTROSCOPIC CHARACTERISTICS OF BENACTYZINE HYDROCHLORIDE AND BENZILIC ACID

Solvent	Wavelength of maximum mμ	Benactyzine hydrochloride		Benzilic acid	
		E (1 per cent. 1 cm.)	Molecular extinction	*E* (1 per cent. 1 cm.)	Molecular extinction
Ethanol	252½ 258½ 264½	10·9 12·4 9·8	397 451 357	16·8 19·7 15·3	383 450 349
0·1 N HCl in ethanol	252½ 258½ 264½	10·8 12·4 9·7	393 451 353	— — —	— — —
Water	251½ 258 263 (inflexion)	11·1 13·0 10·5	403 473 382	— — —	— — —

standard acid. The method is rapid and reasonably specific, and benactyzine can be adequately characterised by this assay and ultra-violet absorption measurements. The assay described in Section B for pharmaceutical preparations is specific, but due to the techniques involved it is insufficiently accurate for control of the pure material.

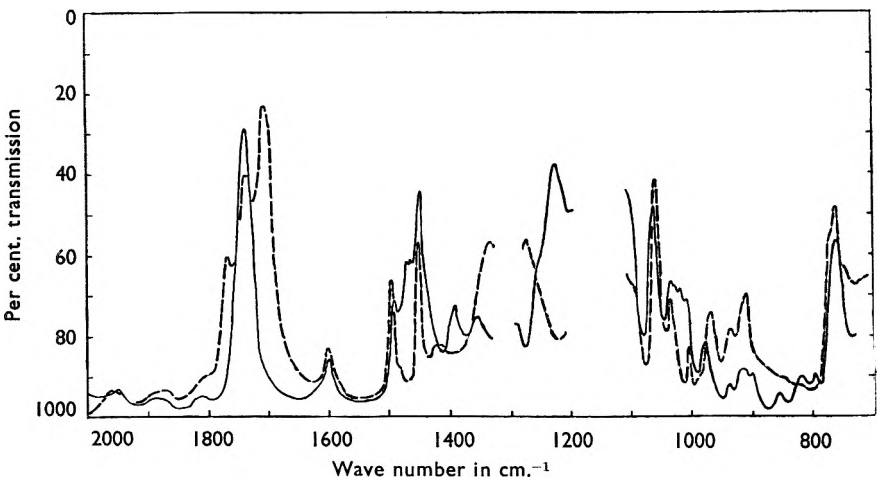


FIG. 1

FIG. 1. Infra-red absorption spectra of —— benactyzine hydrochloride (0·512 per cent. w/v in bromoform) and — — — benzilic acid (0·498 per cent. w/v in bromoform) both in 0·99 mm. cell using a sodium chloride prism.

EXPERIMENTAL

A sample of benactyzine hydrochloride was recrystallised twice from *iso*propanol and dried *in vacuo*. Analysis of the purified material gave C, 66·4; H, 7·1; N, 3·83; ionisable chlorine, 9·75 per cent.; ($C_{20}H_{25}O_3N.HCl$ requires C, 66·0; H, 7·2; N, 3·85; ionisable chlorine, 9·75 per cent.) m.pt. 182° C.

This material was used to establish the ultra-violet extinction coefficients given in Table I, the infra-red absorption spectrum shown in Figure 1

and for the recovery experiments in Section B. When tested by the recommended procedure the sample assayed 100·0 per cent. ± 0·2 per cent. Six specimens of commercial quality assayed between 99·0 and 100·0 per cent.

PROCEDURE

Reagents

1. *Boric acid solution.* Dissolve 20 g. boric acid A.R. in 1 litre of water.
2. *Indicator solution.* Dissolve 0·1 g. bromocresol green and 0·02 g. methyl red in 100 ml. 95 per cent. ethanol.

Transfer about 0·5 g., accurately weighed to a steam distillation apparatus. Add 20 ml. water and 20 ml. sodium hydroxide solution (20 per cent. w/v). Steam distil rapidly, collecting the distillate in 5 ml. of boric acid solution. When distillation of the volatile base is complete (about 100 ml. of distillate), titrate with 0·05 N hydrochloric acid, using 2–3 drops of the indicator solution. Make a blank titration and subtract the result from the sample titration.

One ml. 0·05N hydrochloric acid is equivalent to 0·01820 g. $C_{20}H_{25}O_3N.HCl$.

B. SOLUTIONS AND TABLETS

As a rule benactyzine is injected in solutions containing 5 mg. per ml. or taken in tablets containing 1 mg. For analysis of these products the methods outlined for the solid material are insufficiently sensitive and non-specific. Our attempts to adapt the sensitive sulphuric acid colour reaction for benzilic acid described by Snell and Snell[6] failed, since with benactyzine the intensity of the colour was not reproducible. Ultra-violet spectroscopy appeared to be the only promising method for the products envisaged, provided that it could be made specific by separating benactyzine from its hydrolysis products and other sources of irrelevant absorption.

Benzilic acid can be quantitatively extracted with ether from an acidified solution, leaving the benactyzine in the aqueous layer. After making alkaline with sodium bicarbonate, the benactyzine can be extracted with ether, the solvent removed and the residue dissolved in ethanolic hydrochloric acid for spectroscopic determination. Two solvent extractions completely remove the benzilic acid, but three extractions are necessary to recover all the benactyzine.

This procedure has been successfully applied to aqueous solutions and permits the determination of both benactyzine and any benzilic acid produced by hydrolysis. The official bacteriostatics used in solutions for injection do not interfere with the analysis of benactyzine, but their presence usually prevents the determination of any free benzilic acid.

Tablets are extracted with hydrochloric acid, and an aliquot of the filtered solution is assayed for benactyzine, as described for aqueous solutions. Any small amount of irrelevant absorption derived from the tablet excipients is removed by the acid ether extractions and does not interfere with the benactyzine assay. Determination of benzilic acid is

not a satisfactory measure of decomposition in tablets, and it is essential to assay the benactyzine. The chief source of error in the procedure is extraneous absorption from the apparatus.

Typical results obtained with solutions and tablets of known composition are shown in Table II.

TABLE II

ANALYSIS OF TABLETS AND SOLUTIONS OF BENACTYZINE HYDROCHLORIDE

Preparation	Benzilic acid added	Benactyzine hydrochloride added	Benactyzine hydrochloride found
	mg./ml.	mg./ml.	mg./ml.
Aqueous solution ..	0·48	0·0	0·0
	0·00	4·1	4·1
	0·24	4·1	4·1
	0·97	3·9	3·9
	0·00	*5·1	5·0
	mg./tablet	mg./tablet	mg./tablet
Tablets	—	0·00	0·02
	—	1·03	1·01
	0·10	0·92	0·88
	—	1·01	1·00
	—	1·00	1·02

* 0·2 per cent. of chlorocresol added.

PROCEDURE

Apparatus. All apparatus must be completely free from grease, and well washed with redistilled water.

Reagents

1. *Redistilled water.* Used throughout.
2. *N hydrochloric acid.* Dilute 45 ml. of hydrochloric acid A.R. to 500 ml. with water.
3. 0·5*N hydrochloric acid.* Dilute 22·5 ml. of hydrochloric acid A.R. to 500 ml. with water.
4. 0·1*N hydrochloric acid in ethanol.* Dilute 0·90 ml. of hydrochloric acid A.R. to 100 ml. with ethanol.
5. *Ether.* Anæsthetic ether redistilled in an all glass apparatus.

AQUEOUS SOLUTIONS. Transfer a suitable aliquot containing about 40 mg. of benactyzine hydrochloride to a separator, and dilute to 10 ml. with water. Add 10 ml. of N hydrochloric acid, and extract with 40 ml. and 25 ml. portions of ether. Wash the combined ether extracts with 2 × 5 ml. portions of 0·5N hydrochloric acid, and add the washings to the main aqueous solution. Reject the ether extracts*. To the solution add cautiously with swirling 1·4 g. of sodium bicarbonate A.R.; the solution should now be alkaline to litmus paper. Extract immediately with 50 ml., 30 ml. and 15 ml. portions of ether. Wash the combined ether extracts with 2 × 10 ml. of water containing a little sodium bicarbonate A.R. Filter the ethereal solution through a plug of cotton wool, and remove the solvent by distillation. Dissolve the residue in 100·0 ml. of cold 0·1N hydrochloric acid in ethanol and measure the

* In the absence of interfering substances these ether extracts can be evaporated, the residue being dissolved in ethanol and the benzilic acid estimated spectroscopically.

optical density of the solution in a 1 cm. cell at the maximum at 258½ mμ. Make a blank estimation omitting the benactyzine and correct the optical density of the sample solution*. Calculate the benactyzine hydrochloride content of the sample. The E (1 per cent. 1 cm.) of pure benactyzine hydrochloride at 258½ mμ is 12·4.

TABLETS. Weigh 30 tablets and powder finely. Transfer a weight containing about 25 mg. of benactyzine hydrochloride to a glass stoppered flask, and add 50·0 ml. of 0·5N hydrochloric acid. Mix by swirling, and

TABLE III

STABILITY OF BENACTYZINE HYDROCHLORIDE IN AQUEOUS SOLUTION (0·5 PER CENT. W/V AT 37° C.)

Solution	Time in days	Percentage of original concentration		
		Benactyzine hydrochloride	Benzilic acid calculated as benactyzine HCl	Total
Distilled water	9	89	7	96
	21	87	11	98
	53	85	17	102
	125	70	30	100
‡ Buffer pH 6·3	3	6	94	100
‡ Buffer pH 6·1	5	20	82	102
‡ Buffer pH 4·9	3	83	14	97
	7	70	29	99
	14	52	48	100
	27	30	68	98
‡ Buffer pH 3·5	3	99	2	101
	7	94	4	98
	14	94	8	102
	27	85	14	99
	38	82	20	102
	95	59	39	98
Citric acid pH 2·8 ..	10	94	1	95
	21	96	5	101
	43	92	9	101
	74	88	12	100
Citric acid pH 2·1 ..	10	98	2	100
	21	95	6	101
	43	92	9	101
	74	83	14	97

‡ Phosphate/citrate buffer solutions (Vogel, *Quantitative Inorganic Analysis*, 1945, 809).

set aside for five minutes. Stopper, shake mechanically for ten minutes and filter. Transfer 20·0 ml. of the filtrate to a separator. Continue as in Section A from "extract with 40 ml. and 25 ml. portions of ether . . .", dissolving the residue in 25·0 ml. 0·1N hydrochloric acid in ethanol.

Multiply the result by the appropriate correction factor to allow for the increase in volume of the 0·5N hydrochloric acid due to solution of the tablet excipients†.

C. STABILITY OF AQUEOUS SOLUTIONS AND TABLETS

Benactyzine is unstable in neutral or alkaline aqueous solution, but as the pH is lowered the stability progressively improves. Solutions with a pH below 3 show small losses (about 10 per cent.) when stored for three

* The blank should not exceed 5 per cent. of the sample absorption.
† For the tablets used in our experiments the correction factor is 1·04.

months at 37° C. Typical results for solutions of different pH are shown in Table III and are plotted graphically in Figure 2. The sums of the amounts of benactyzine and hydrolysed benactyzine (calculated from the free benzilic acid) show good agreement with the original benactyzine concentration, indicating that simple hydrolysis has occurred.

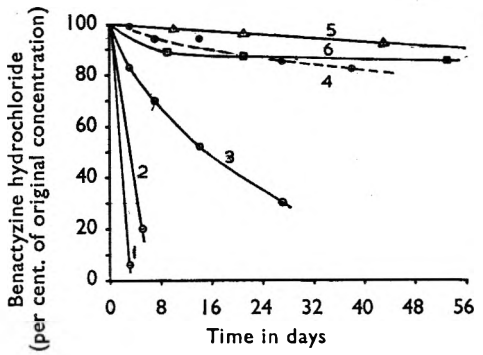


FIG. 2

FIG. 2. Decomposition of benactyzine hydrochloride in aqueous solution (0·5 per cent. w/v). 1. Buffer pH 6·3. 2. Buffer pH 6·1. 3. Buffer pH 4·9. 4. Buffer pH 3·5. 5. Citric acid pH 2·8 and 2·1. 6. Distilled water. Initial pH 5·5.

Trial batches of sugar coated tablets prepared with an excipient of starch and lactose underwent considerable hydrolysis on storage at 37° C., but when 1 per cent. of tartaric acid was added to the excipient the tablets showed good stability. Typical assay results obtained after storing stable and unstable tablets are recorded in Table IV.

TABLE IV

STABILITY OF BENACTYZINE HYDROCHLORIDE IN TABLETS

Storage time	Benactyzine hydrochloride mg./tablet			
	Unstabilised tablets		Stabilised tablets	
	Room temperature	37° C.	Room temperature	37° C.
Initial	0·98	—	0·98	0·98
2 weeks	0·91	0·88	1·00	0·97
1 month	0·93	0·83	0·97	0·99
2 months	0·88	0·70	0·95	0·98
3 months	0·87	0·72	0·97	0·98
6 months	0·92	0·74	0·98	0·96

SUMMARY

1. Infra-red and ultra-violet absorption spectra are presented for benactyzine hydrochloride.

2. An assay for benactyzine is proposed, based on hydrolysis and steam distillation of diethylaminoethanol.

3. Ultra-violet spectroscopic methods have been successfully applied to the determination of benactyzine hydrochloride in its pharmaceutical preparations.

4. Results are presented of stability tests on aqueous solutions and tablets of benactyzine hydrochloride.

The authors are indebted to Mr. W. H. C. Shaw for the infra-red examination, to Mr. J. A. Edwards for carrying out the microanalysis and to Mrs. M. F. Goodchild for technical assistance.

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DISCUSSION

The paper was presented by MR. J. L. PHILLIPS.

The CHAIRMAN said he could not follow why the method for procaine hydrochloride could not be applied to benactyzine. Was it because an insufficient quantity was present in the material under examination?

MR. L. BREALEY (Nottingham) said that difficulty had been encountered in using the described method with benactyzine tablets. The E (1 per cent. 1 cm.) of pure benactyzine was only about 20. That meant that any other material extracted interfered seriously with the assay and gave high results. It had been found impossible to eliminate the interference completely but it had been possible to make the method work satisfactorily if a three-point correction were used. It was an easy correction to apply because the benactyzine had three very sharp peaks, and a formula had been worked out using the values on the peaks and results had been greatly improved. Certainly all extraneous absorption had been found to be perfectly linear, but it was appreciable and should be corrected. What precautions did the authors take, as there must be a number of ways to eliminate the interference?

MISS A. E. ROBINSON (London) said that she also had encountered relatively high optical density of the blank determination using a variety of solvents. However, by using ether it had been possible to obtain a blank with an optical density of less than 0·01 in a 1 cm. cuvette at wavelengths ranging from 220 to 300 mμ. Unlike the authors, no extraneous absorption had been encountered.

DR. W. MITCHELL (London) asked what was the pH of a simple aqueous solution of the drug. He noted that the stability seemed to be greater between about 4 and 6·3.

DR. A. H. BECKETT (London) said he was worried about the accumulation of extraneous absorption from the apparatus. He suggested that in the determination of the solid materials, titration in non-aqueous media in the presence of mercuric acetate would prove to be convenient and rapid.

DR. G. E. FOSTER (Dartford) asked whether the authors had considered adding a certain amount of water and distilling rather than using steam distillation.

MR. C. A. JOHNSON (Nottingham) said it seemed a little unwise to use a method based on hydrolysis in the case of a material which was apparently so prone to it. Non-aqueous titration or spectrophotometric methods seemed preferable. The authors found a melting point of 182° C. but he had found that with repeated recrystallisation he could not achieve a

melting point higher than 180° C. It was possible that the soda glass tube was bringing about a certain amount of decomposition and lowering the melting point.

MR. J. L. PHILLIPS, in reply, said that the method suggested by the Chairman could not be carried out due to insufficient quantity of the drug being present. When the method was originally used there was a great deal of difficulty with high blanks. Redistilled ether was used, and it was also found that by using one set of perfectly clean separators, kept for this determination only, the difficulty of high absorption could be surmounted. It had not been found necessary to use a three-point correction. The pH of an aqueous solution of benactyzine hydrochloride was about 6. The difficulty about titration in non-aqueous solvents was that the dose was 1 mg. and titration would not be sufficiently sensitive for pharmaceutical preparations. The melting point was found to be 182° C. by the B.P. method and was sufficiently characteristic.

GLINUS OPPOSITIFOLIUS L. ROOT—A SUBSTITUTE FOR SENEGA

By R. M. Ridgway and J. M. Rowson

From the School of Pharmacy, Leicester College of Technology, and the Museum of the Pharmaceutical Society of Great Britain

Received June 26, 1956

In recent years, considerable quantities of materials named as "Senegas" derived from the Indian sub-continent have been offered on European markets, the extensive establishment of these materials probably having connection with the prevailing high price of *Polygala senega* L. The materials appear to be of varied origins and some have been investigated by a number of workers. Datta and Mukerjii[1] have described *Polygala chinensis* L. under the names of Chinensis, or Indian Senega, the material now being the subject of a monograph in the Indian Pharmaceutical Codex. Their description includes a somewhat sparsely illustrated account of the anatomy of the drug, which is said to have a wide geographical distribution throughout India, from the Punjab to Burma, S. India and Ceylon, but to be obtained in large quantities from the N.W. Provinces. Qazilbash[2] has ascertained that so-called Pakistan Senega, previously supposed to be derived from *P. chinensis*, is in fact derived from *Andrachne aspera* L. (Fam. Euphorbiaceæ) and is collected mainly from the Khattak hills, in the Peshawar district, N.W. Frontier Province. Dequeker[3] has reported the presence on the Belgian market of so-called Indian Polygala and has pointed out characters by which this material may be distinguished from *P. senega* and from Pakistan Senega. Dequeker has carried out a comparative evaluation of *P. senega* and two samples of Indian Polygala; the investigation was a preliminary one and the origin of the Indian Polygala was to be established at a later date. A further material, not of Indian origin, has been described by Paris and Lys[4] under the name of Syrian Polygala. This material, *Spergularia marginata* Kittel (Fam. Caryophyllaceæ) was investigated chemically and pharmacologically.

Samples of a material labelled Indian Senega Root have been offered on the English drug market, and also on the European continent, being claimed to possess the medicinal properties of *P. senega*. They are stated to be obtained from South Kanara, Malabar and the districts about Madura and Ramnad in the province of Madras. Samples of this material examined in the Museum of the Pharmaceutical Society did not appear to conform in structural characters with any of the other materials mentioned above, and it was thought worthwhile to investigate the macroscopical and microscopical characters of the material, in order that it might be adequately compared with the commercially related material. In order to determine the botanical origin, a specimen of an entire plant, including flowers, stem and root, was acquired; the root of this specimen possessed the macroscopical and microscopical

characters of the commercial Indian Senega Root. The material was identified in the Herbarium of the Royal Botanic Gardens, Kew, as *Glinus oppositifolius* L. A.DC. (= *Mollugo spergula* L. and *Mollugo oppositifolia* L.) Family Molluginaceæ (or Ficoidaceæ). Herbarium specimens seen in the Herbaria at Kew and at the National Science Museum, South Kensington, indicate that this plant is of a wide geographical distribution. References are made to its having grown in many different regions of India from the north to the south, it appearing to favour sandy soil and river banks. It grows also in China, Indo-China, the Malay Archipelago, the Phillipines, Australia, Africa (Sudan, Uganda, Nigeria, Tanganyika, Sierra Leone, Natal and the island of Madagascar) and South America.

Materials

The investigation was carried out on two samples of commercial material and a further sample sent from the University of Louvain through the kindness of Professor R. Dequeker. These samples, and the entire plant specimen identified at Kew, all showed the same morphological characters.

Macroscopical and Sensory Characters

The drug appears in commerce as pieces of root, largely unbroken, of a vertical direction of growth, and cylindrical in shape, with a slight taper from crown to tip (Fig. 1, *A*). The roots are unbranched, usually straight and only infrequently slightly contorted. The pieces vary in size from 6 to 16 cm. in length and from 2 to 6 mm. in diameter, and are of a pale brown colour. Rootlets are only occasionally attached as threadlike appendages up to 3 cm. in length and about 0·5 mm. in diameter, but the roots bear rather indistinct scars of detached rootlets, often as minute depressions in the surface. The root surface is longitudinally wrinkled, the wrinkles running spirally and being deeper in the more mature pieces. The crown of the root is knotty and bears the vestigial remains of from two to twelve aerial stems, the bases of which are surrounded by membranous scaly leaves which are generally green but sometimes pale brown in colour. The crown may occasionally bear buds as well as aerial stem remains. The stem portions, where not merely represented by scars, are the same colour as the roots; they are from 1 to 5 mm. in length and about 1 mm. in diameter, cylindrical, hollow, with a longitudinally striated surface and white interior parts. Examination of the transverse sections of the stems (Fig. 3, *A*) show a wide continuous ring of secondary xylem about a central parenchymatous pith, which latter commonly borders a wide lacuna. An anomalous structure is found due to a tertiary cambium having arisen in the phloem, producing an arc or ring of collateral bundles. These are separated, at least initially, by parenchymatous medullary rays which are wider towards the edge of the arc, where the youngest bundles are developing.

The smoothed transverse section of the root shows a number of rings of vascular tissue surrounding an eccentric vascular core, these appearing

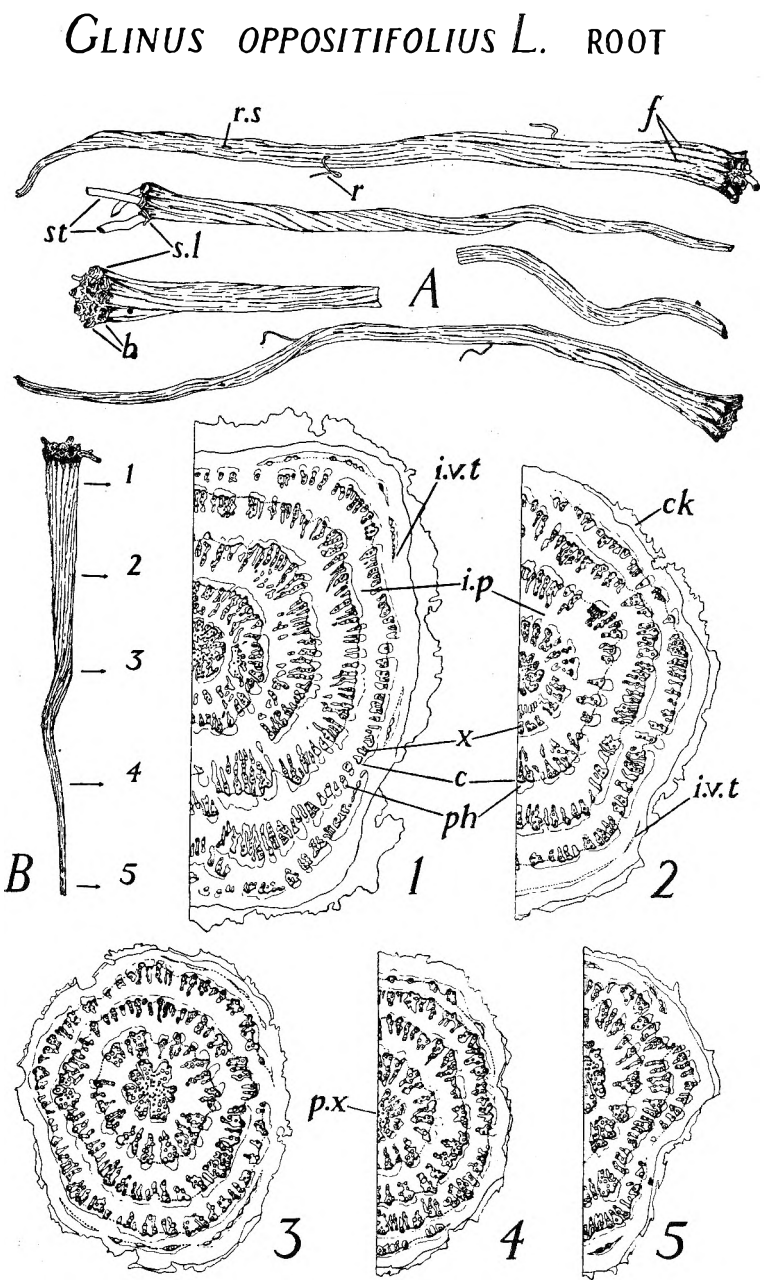

FIG. 1. Root of *Glinus oppositifolius* L. *A*, pieces of commercial root × 1. *B*, root (× 1) showing levels at which the corresponding smoothed transverse sections 1 to 5 were made. All sections × 20. *b*, stem buds; *c*, cambium; *ck*, cork; *f*, furrows; *i.p.*, intervascular parenchyma; *i.v.t.*, initiating vascular tissue; *ph*, phloem; *p.x.*, primary xylem; *r*, rootlet; *r.s.*, rootlet scar; *s.l.*, scale leaves; *st*, stems; *x*, xylem.

as white rings on a brown background (Fig. 1, *B*: 1 to 5). This appearance is due to the anomalous formation of successive rings or arcs of meristematic tissue. These successive meristems produce a number of annular zones or rings of growth which are more or less equidistant from one another and consist of xylem internally and phloem externally. The number of rings seen about the vascular core varies from one or more at the root tip to up to six at the crown. In the growth of these rings, an arc of vascular bundles appears intermittently on that side of the root having the greatest radius from the eccentral vascular core, developing in time to form a complete ring (Fig. 1, *B*; 2, *i.v.t.*). The outermost ring seen in a smoothed transverse section is consequently often discontinuous, the missing or least developed part of the ring usually occurring in that part of the section through which runs the shortest possible radius from the innermost xylem tissue to the cork. The rings of vascular tissue are separated from one another by intervening brown bands of about the same radial thickness as the rings. The central vascular core occupies from about one-tenth to a half of the total root diameter, the proportion decreasing with an increasing number of vascular rings in the section. Medullary rays between the individual vascular bundles of the rings may be made out with difficulty on viewing with a hand-lens magnifying 10 diameters; they appear as fine brown threadlines. The fracture is short and the roots have a springy resilience to bending. The odour is faint and the taste is not marked, being reminiscent of wheat grain. Chewing produces a very fibrous residue.

Anatomy of Root

In typical roots, the external cork tissue is of variable thickness, being made up of from 2 to 8 layers and frequently broken (Fig. 2, *A*). Individual cells are tabular and irregularly polygonal in anticlinal outline (Fig. 2, *E*); they measure about R = 7 to **15** to 26 μ, T = 12 to **33** to 53 μ and L = 17 to **48** to 91 μ*. The cells of the cork are suberised and slightly lignified. They stain brown with iodine and occasional cell contents stain black with ferric chloride. The phellogen is not distinct. Within the cork, the phelloderm is seen as a parenchymatous tissue up to about 8 cells in radial depth (Fig. 2, *F*), the individual cells being perceptibly larger than those of the parenchyma of the internal bands which separate the vascular rings and which are made up of from 10 to 18 layers of cells. In each of these bands of parenchyma, the cells are larger towards the outer part, and smaller towards the inner part, of the band. In the inner bands, the parenchyma cells tend to be tangentially elongated in a manner suggesting partial distortion by pressure. In roots showing five or more rings of vascular tissue, such elongated cells are commonly seen in the central and next outer band of conjunctive parenchyma tissue. The cells of the parenchyma measure about R = 10 to **26** to 51 μ, T = 20 to **43** to 89 μ and L = 24 to **46** to 71 μ.

* The letters R, T and L have the same connotations as devised by Moll and Janssonius[5]. That is to say, they symbolise the measurements of the cells in radial, transverse and longitudinal directions, respectively.

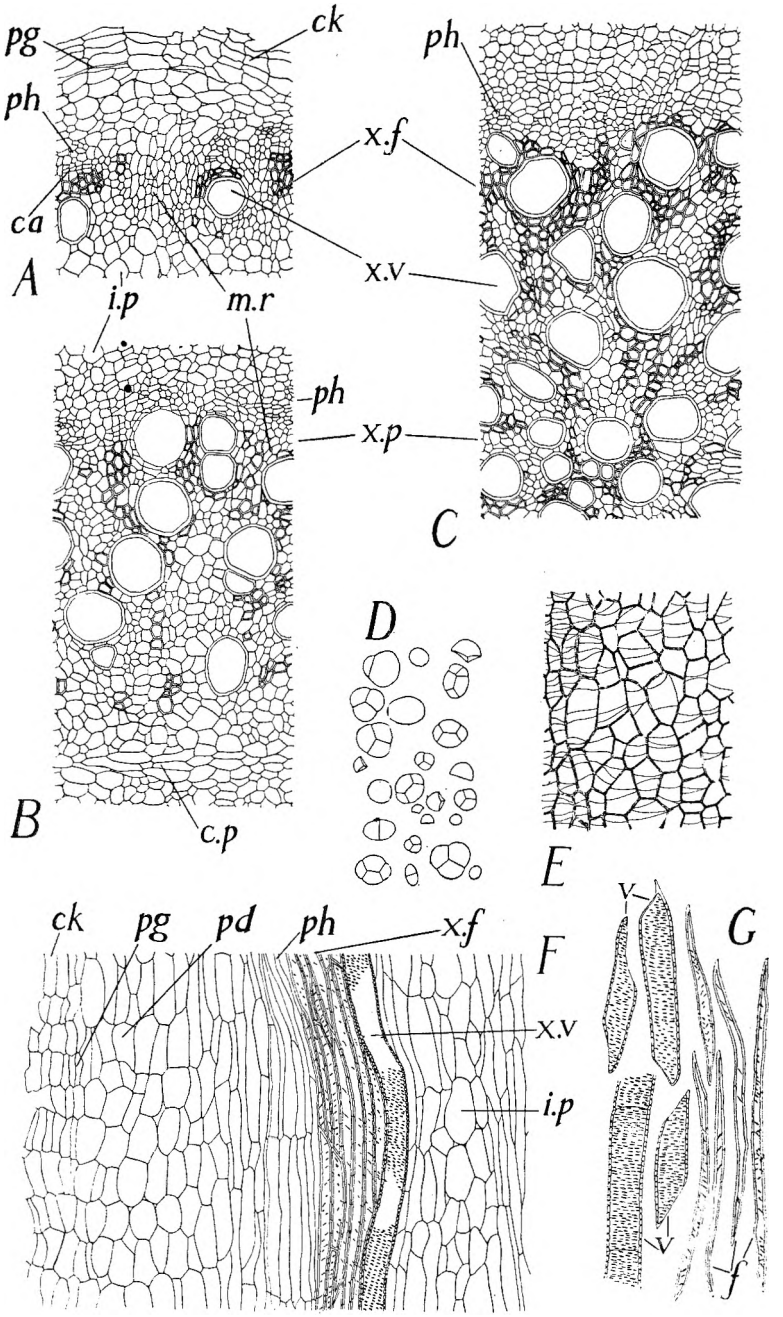

FIG. 2. Root of *Glinus oppositifolius* L. *A*, transverse section at level *B*1 (see Fig. 1), showing part of outermost ring of vascular tissue. *B*, transverse section at level *B*1, showing part of ring immediately outside the central vascular core. *C*, transverse section at level *B*1, showing part of central vascular core. *D*, starch grains. *E*, cork cells in surface view. *F*, radial longitudinal section of outermost ring of vascular tissue. *G*, isolated elements from the disintegrated xylem. *A–C*, *E–G*, × 140; *D* × 750. *ca*, cambium; *ck*, cork; *c.p.* collapsed parenchyma; *f*, fibres; *i.p.*, intervascular parenchyma; *m.r.*, medullary ray; *pd*, phelloderm; *pg*, phellogen; *ph*, phloem; *v*, vessels; *x.f.*, xylem fibres; *x.p.*, xylem parenchyma; *x.v.*, xylem vessels.

They contain numerous small starch grains, both simple and compound (Fig. 2, *D*); the latter are the commoner form, being made up of from 2 to 6 components and the most frequently occurring forms are those with 3 or 4 components. Hila were not made out, but the grains exhibit distinct crosses in polarised light. If compound grains be broken apart, as is commonly effected in powdering or scraping the root, the individual components are seen to have one larger curved side and 1 to 4 smaller flat surfaces depending on the number of components in the compound grain. Simple grains are subspherical or ovoid, two-component grains are ovoid and the more compound grains are spherical in outline. The complete grains vary in diameter from 1·5 to **4·5** to 13 μ.

The numerous collateral bundles which make up each ring are separated from one another by parenchymatous medullary rays of varying widths (Fig. 2, *A* and *B*); the cells tend to be smaller and more radially elongate than those of the parenchyma of the bands. The wider rays contain starch grains. There are more bundles in the rings as they occur nearer to the surface of the root; otherwise each ring of vascular tissue is of similar nature except for the peripheral incomplete ring. This peripheral ring shows immature bundles with a marked lack of vessels and, particularly at its edges, may be represented by undifferentiated cambium, elsewhere difficult to make out (Fig. 1, *B*:2, *i.v.t.*). The eccentral core contains both primary and secondary tissues and is of greater radial width than any of the rings.

Within the bands, the secondary phloem occurs in groups collateral with the xylem. These groups are up to 12 layers of cells in radial width and are made up of axially elongated parenchyma cells which are polygonal in transverse section (Fig. 2, *A*, *B* and *C*); the end walls may be tapering or, less frequently, at right angles. They measure about R and T = 3·5 to **7** to 15 μ and L = 17 to **100** to 160 μ. The sieve tubes are narrow and tend to be collapsed; the sieve areas are with difficulty distinguished. The cambia of the inner cylinders are not seen in a state of active division, but, where the outermost ring is initiating, an active meristem is found, consisting of from 2 to 6 layers of rectangular prisms measuring R = 2 to 6 μ, T = 3·5 to **8** to 15 μ and with longitudinally directed axes (Fig. 2, *A*, *ca*). Such adventitiously differentiated cambium arising in the external part of the phloem produces, firstly, new phloem externally; subsequently, xylem elements are produced internally. The xylem groups in the vascular rings, as seen in transverse section, are arranged in radially directed rectangular or wedge-shaped masses from 2 to 12 cells tangentially and up to about 30 cells radially (Fig. 2, *B*). The groups are made up of vessels, fibrous tracheids, sclerenchyma fibres and parenchyma, all but the latter having yellow lignified walls. The vessels appear singly or only infrequently paired. In transverse section, single vessels are more or less spherical in outline. They measure about R and T = 10 to **45** to 89 μ and the individual articulations measure L = 90 to **180** to 260 μ (Fig. 2, *F* and *G*). They are thick-walled with numerous small elongated bordered pits; the end-walls are either slightly tapering or at right angles to the longitudinal

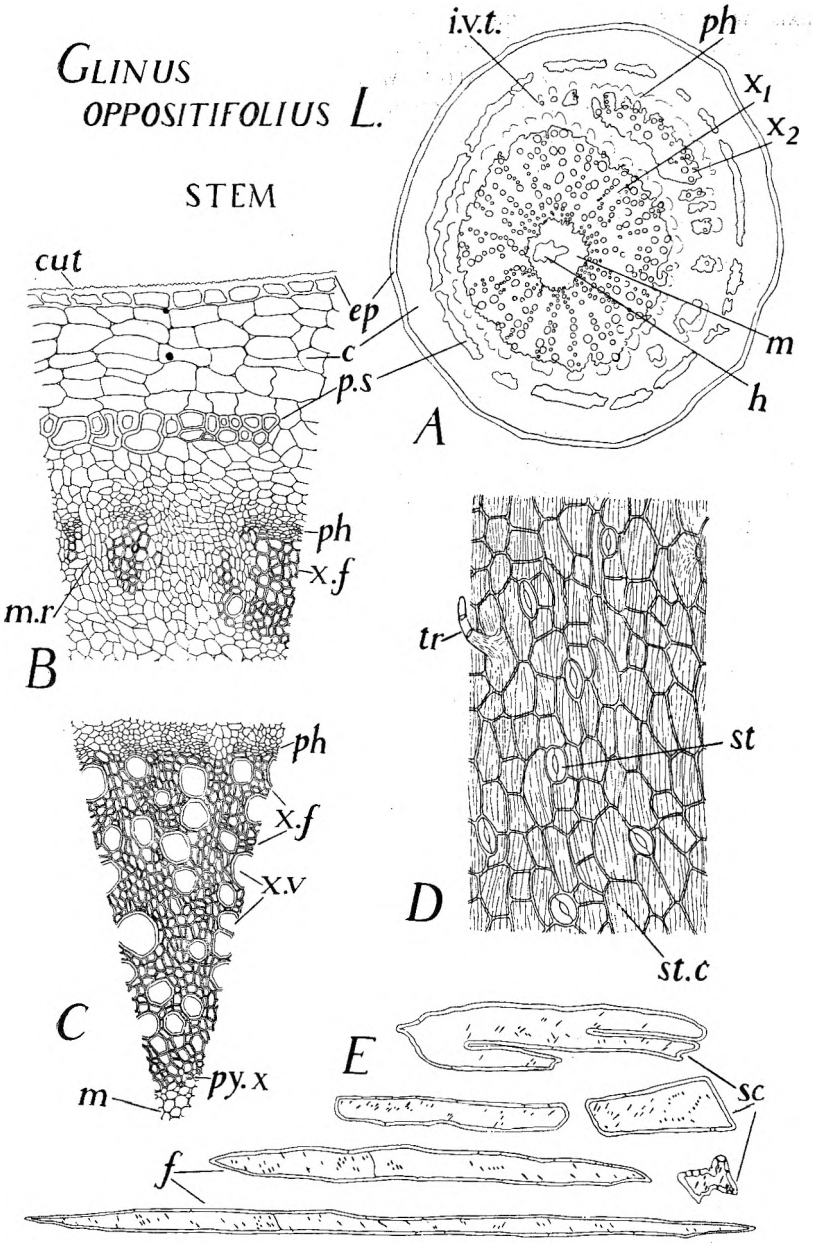

FIG. 3. Stem of *Glinus oppositifolius* L. *A*, transverse section of stem. *B*, transverse section of outer part of stem in region of initiating vascular ring. *C*, transverse section of inner part of stem. *D*, surface view of epidermis. *E*, isolated elements of the pericyclic sclerenchyma. *A* × 40; *B–D*, × 140; *E* × 160. *c*, cortex; *cut*, cuticle; *ep*, epidermis; *f*, fibres; *h*, lacuna; *i.v.t.*, initiating vascular tissue; *m*, medulla; *m.r.*, medullary ray; *ph*, phloem; *p.s.*, pericyclic sclerenchyma; *py.x.*, primary xylem; *sc*, sclereids; *st*, stoma; *st.c.*, striated cuticle; *tr*, trichome; x_1, secondary xylem; x_2, tertiary xylem; *x.f.*, xylem fibres; *x.v.*, xylem vessels.

axis, with, frequently, a small upper or lower tapering projection at the extremity. They have a large circular perforation. Sclerenchymatous xylem fibres are long and narrow with tapering ends, thickened walls and an irregularly penta- or hexagonal shape as seen in transverse section. They measure 7 to **12** to 17 μ in diameter, L = 130 to **285** to 450 μ and have a restricted slit-like pitting. The fibrous tracheids are comparatively broader, with extensive oval bordered pitting; they fall within the following measurements: R and T = 10 to **15** to 24 μ, L = 180 to **300** to 510 μ. Some fibres of intermediate characters also occur. Xylem parenchyma consists of unlignified axially elongated elements. The vascular tissue commonly does not run in any constant vertical plane, but tends to waver in its longitudinal direction.

Anatomy of Stem

The epidermal cells of the stem are tetra- to hexagonal prisms, measuring about R = 11 to **14** to 16 μ, T = 18 to **26** to 36 μ, L = 20 to **38** to 70 μ. The outer and inner walls are thickened, the outer walls having marked longitudinal cuticular striations (Fig. 3, *B* and *D*). There is infrequent beading of the anticlinal walls. Anomocytic stomata are common; they measure about 21 to 30 μ in length and have from two to four subsidiary cells. The axes of the stomata run more or less parallel to one another in the direction of the longitudinal axis of the stem. Trichome cicatrices are common. Trichomes are infrequent, uniseriate, up to 3 cells long. The basal part has a longitudinally striated cuticle, the cuticular striations of neighbouring cells of the epidermis converging upon this region. It has the shape of a truncated cone, and the remaining cells of the trichome are slightly warty, conical, often wider than the basal cell and the terminal cells is bluntly pointed. The cortex consists of parenchyma, about 5 or 6 cells in radial width (Fig. 3, *B*, *C*). The cells measure R = 8 to **21** to 23 μ, T = 30 to **52** to 83 μ and L = 20 to **52** to 95 μ. They show intercellular spaces and contain starch grains having the same characters as those of the root. The pericycle shows an interrupted ring of yellow lignified sclerenchymatous cells, one or two layers in radial width (Fig. 3, *B*, *p.s.*). The cells measure R = 8 to **23** to 46 μ, T = 9 to **22** to 44 μ and L = 150 to **310** to 710 μ; they comprise fibres and sclereids, the former being less frequent (Fig. 3, *F*). The fibres have thin walls and may be of variable width at different levels; they have a notched outline. Transverse partitions occur in some of the fibres. The sclereids are few and vary in shape from polygonal prisms with a longitudinally directed axis, or short cylinders, to very irregularly shaped cells, the lumen on occasion being U-shaped. They have thin walls and wide lumens. The pericyclic sclerenchyma cells exhibit simple pitting.

The conjunctive parenchyma lying within the pericycle consists of longitudinally directed thin-walled cells measuring about R = 5 to **11** to 26 μ, T = 6 to **18** to 38 μ and L = 33 to **70** to 85 μ, the R and T dimensions becoming progressively smaller from the pericycle inwards. The cells are densely filled with starch grains possessing the same characters as those of the root. Collateral with each ray of xylem, either secondary

or tertiary, is a cap of tangentially flattened phloem cells up to about six layers in radial width. The cells measure about R = 4 μ, T = 8 μ and L = 120 μ. The walls are thickened, especially in the angles of the cells and, in transverse section, the tissue appears somewhat whiter and more refractive than the cells of the conjunctive parenchyma (Fig. 3, *B* and *C*, *ph*).

The tertiary xylem resembles the secondary xylem except that there is an absence of vessels from its younger parts and that these are separated by parenchymatous medullary rays (Fig. 3, *A*, x_2 and *B*). The secondary xylem forms a continuous ring 35 to 40 cells in radial thickness and is made up of yellow lignified elements consisting of vessels in a ground tissue of fibrous tracheidal elements (Fig. 3, *A*, x_1 and *C*). Medullary rays, of which there are about 40, are up to five cells wide. The xylem vessels are more or less spherical in transverse section and occur singly. They measure about R and T = 10 to **24** to 48 μ and the individual articulations measure L = 100 to **186** to 290 μ; the end-walls are at right angles or slightly oblique, with a large circular perforation. They are covered with numerous small oval bordered pits. The tracheidal elements measure about R = 6 to **11** to 20 μ, T = 6 to **9** to 18 μ and L = 110 to **215** to 315 μ; they comprise slender fibrous cells with tapering ends and shorter elements with oblique or bluntly pointed ends. All have oval or elongated bordered pits. Primary xylem elements occur on the inside of the xylem rays as groups of lignified spiral and annular vessels. The pith consists of parenchyma cells measuring R and T = 10 to **21** to 34 μ, L = 66 to **110** to 150 μ. The cells are smaller towards the outside.

Differential Characters

Features by which *Glinus oppositifolius* root may be distinguished from that of senega are numerous and marked. In the entire condition, the roots of the former are longer and more slender; they are very much straighter and less branched. The colour is a uniform light brown, without any of the purple tints seen in senega; the crown is less bulbous and knotty, with fewer buds and aerial stem bases. The external surface shows uniform spiral furrowing and has neither the keel nor the transverse wrinkles of senega root. The transverse section of the root of *G. oppositifolius* shows up to about six characteristic concentric rings of anomalous xylem and phloem tissues arranged round an eccentric vascular core of secondary xylem and phloem. This differs markedly from the distribution of xylem and phloem tissues characteristic of senega. The taste lacks marked acridity and there is no distinct odour. When in the powdered condition, *G. oppositifolius* is a pale buff colour and has a dry starchy texture as against the grey colour and oily-damp texture of senega. Microscopically, the main distinguishing feature between the two powders lies in the contents of the cells of the parenchymatous tissues. In the case of *G. oppositifolius*, small starch grains, simple or 2 to 6 compound, are present in the cells and oily droplets are absent, whilst the converse condition is true of senega. There is no collenchymatous

parenchyma in *G. oppositifolius*. The xylem elements are rather similar in both roots, but the vessels in *G. oppositifolius* are up to about 90 μ in diameter and those of senega are up to 50 μ; the former has a few sclerenchymatous fibres. There are salient histological differences between the stems of the two materials. The epidermal cells of the stems of *G. oppositifolius*, described elsewhere in the paper, are readily distinguished from those of senega, which have sinuous anticlinal walls; the pericyclic sclerenchyma of *G. oppositifolius* is lignified whilst that of senega is unlignified. In powder, the very small amounts of stem present preclude their use as very significant diagnostic characters.

Evaluation by Haemolytic Indices

The root of *G. oppositifolius* is offered as a "Senega", and the powder, shaken with water, gives a copious persistent froth. Some preliminary investigations to assess the saponin content by determining the haemolytic index of the root have been made, using a moderately fine powder in an air-dry condition. After examining a number of methods for the determination of haemolytic index, that due to Runge[6], employing a geometric progression of dilutions, was adopted. The standard used was desoxycholic acid, maintained in a vacuum desiccator and prepared as a solution according to the method of Runge, immediately prior to its use. The use of a standard of purified white saponin (Saponinum purum album, Merck) was attempted, but rejected due to difficulties in obtaining consistent haemolytic responses with it under controlled conditions. Four extraction methods were employed, as follows:

1. 10 g. of the powdered drug was macerated for 24 hours with a little 60 per cent. ethanol and packed into a percolator. More of the solvent was added and the first 8 ml. of percolate collected and reserved. The drug was further percolated with solvent so as to yield 600 ml. of percolate, which was then evaporated in a stream of warm air, added to the reserved percolate and the whole made up to 10 ml. After 48 hours, the extract was centrifuged to obtain a clear liquid, of which one part was diluted for use with 49 parts of Runge's phosphate buffer pH 7·4 to give a 2 per cent. drug-to-solvent extractive.

2. 10 g. of the powdered drug was macerated as previously and percolated with 60 per cent. ethanol to yield 600 ml. of percolate. 60 ml. of this was evaporated to dryness in a steam of warm air and the residue dissolved in 60 ml. of methanol and filtered. The solvent was again evaporated and the residue taken up in 50 ml. of phosphate buffer pH 7·4, centrifuging if necessary to obtain a clear 2 per cent. drug-extractive.

3. One g. of the powdered drug was extracted with chloroform in a soxhlet apparatus for 6 hours and the solvent removed. The powder was then extracted for 10 hours with methanol in a soxhlet apparatus and the solvent removed and evaporated under reduced pressure. The residue was taken up in 50 ml. of phosphate buffer pH 7·4 to obtain a 2 per cent. drug-extractive.

4. A method due to Ruyssen (private communication) was employed, involving preliminary extractions with light petroleum and acetone and,

after drying, a form of controlled decoctive extraction of the powder with a phosphate buffer to yield a 1 per cent. drug-extractive.

Each of these extractions were applied at the same time to portions of a bulked sample of powdered senega root and the haemolytic indices determined.

The results were:—

	H.I. *P. senega* L.	H.I. *G. oppositifolius* L.
Method 1	2240	145
Method 2	1620	120
Method 3	1860	145
Method 4	1500	410

The first three of these methods give results which for *G. oppositifolius* are more or less consistent; for senega, there is a proportionately somewhat wider variation in values of haemolytic indices. Using these methods, the roots of *G. oppositifolius* show haemolytic indices which, by Runge's procedure, are from about 13 to 15 times lower than those of senega root. Method 4, which gives a lower H.I. value for senega, gives an appreciably higher H.I. value for *G. oppositifolius* than do the other three methods of extraction; in this case the H.I. value for *G. oppositifolius* is 3 to 4 times lower than that for senega. By this method of extraction, the total saponins were not taken up, as indicated by the capacity of the "exhausted" powders to yield a persistent froth on shaking with water. This may account for the lower H.I. value in the case of senega and indicates that the value for *G. oppositifolius*, though higher than by the other three methods, is none the less a minimal and provisional one. Work is being continued to elucidate the optimum extractive conditions for the material.

Summary and Conclusions

The diagnostic characters of *G. oppositifolius* are:

1. Macroscopical. The roots, pale brown in colour, are straight and unbranched, with small knotty crowns, longitudinally wrinkled surfaces and very few rootlets. The transverse section shows a series of concentric rings of vascular tissue arranged about an eccentric vascular core, the appearance being due to the formation of successive anomalous meristems external to a normal secondary cambium. Cylindrical, hollow stems, about 1 mm. in diameter, or their scars, or stem buds, occur on the crown.

2. Microscopical. Diagnostic characters include the thin-walled parenchymatous tissue containing small starch grains, single and compound, averaging about 4 or 5 μ and with up to six components. The xylem comprises pitted vessels up to about 90 μ in diameter, pitted fibrous tracheids and occasional sclerenchymatous fibres.

Preliminary determinations of the haemolytic index of *G. oppositifolius* show values ranging from about one-fifteenth to one-quarter of those of senega roots, depending on the methods of extracting the roots.

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DISCUSSION

The paper was presented by MR. R. M. RIDGWAY.

DR. J. W. FAIRBAIRN (London) said it was remarkable that a substitute for *Polygala senega* had such an increased haemolytic index with the fourth method used by the author, and yet the value for *Polygala senega* by that method had been reduced. The authors described the stomata as anomocytic. The term anomocytic indicated a group in which no definite arrangement was discernible: he had always hoped that this group would itself yield further categories. He noticed that the stomata tended to form a pattern of one large and three small subsidiary cells; it would be interesting to know whether the authors had examined a larger number than was shown in order to see whether the irregularity persists. He suggested that the word "sclerenchyma" used to describe xylem fibres was redundant.

DR. T. E. WALLIS (London) said that the description of the morphology and anatomy of root and stem provided a needed means of determining the origin of the material should it occur again. The authors did not state how many primary xylem groups were in the root.

MR. A. R. G. CHAMINGS (Horsham) commented on the use of the haemolytic index and enquired whether it had any relation to the toxicity of the drug.

MR. T. C. DENSTON (London) said that the new Indian Pharmacopœia of 1955 contained a monograph on Indian senega. That monograph was not very different from that which appeared in the precursor of the Indian Pharmacopœia. The paper referred to two samples of commercial material: were these available in commerce in Britain, or offered in India as complying with the Indian Pharmacopœial list?

MR. R. M. RIDGWAY, in reply, agreed it was remarkable that there was so great a difference in the haemolytic index of the two drugs by various methods of extraction. The particular pattern of stomata probably did not occur over a large number of stomata but was peculiar to the one drawing shown. He accepted Dr. Fairbairn's comment on the use of the word sclerenchyma. It had not been possible to determine the archy of *G. oppositifolius* as the material developed at too great a rate. The haemolytic index was not necessarily a measure of toxicity, and the pharmacological action of the drugs was not proportional to their haemolytic indices. The haemolytic index was merely a way of evaluating the drugs. The samples came from an English source.

THE EVALUATION OF BELLADONNA HERB*

PART I. THE QUANTITATIVE DETERMINATION OF SEED IN POWDERED HERB

BY R. G. ATKINSON AND C. MELVILLE

From the Department of Pharmacy, University of Manchester

Received June 29, 1956

BELLADONNA Herb B.P. consists of the leaves, or leaves and other aerial parts, of *Atropa belladonna* L., or of *Atropa acuminata* Royle ex Lindley, or of a mixture of both species, collected when the plants are in flower and dried. It contains not less than 0·30 per cent. of the alkaloids of Belladonna Herb, calculated as hyoscyamine.

Routine microscopical examination in this department of commercial samples of powdered belladonna herb revealed apparently excessive amounts of fragments of belladonna seed, which suggested that the drug was not being collected during the flowering period.

It has been shown by Kuhn and Schäfer[1] that, in all parts of the plant, the alkaloidal content increases rapidly in spring, reaching a maximum when the first flower-buds form. As flowering commences, the alkaloidal content falls rapidly, then rises again, and finally falls with the ripening of the fruit. Stems do not share in the second rise. With the flowering shoots and leaves, the second maximum is more marked than the first. The proportion of hyoscyamine in total alkaloid shows a similar curve with the two maxima.

Methods for ascertaining the amount of seed present in commercial material would be useful, and experiments were made, 1, to determine the number of testa cells per g. of belladonna seed, 2, to determine the number of testa cells present in a known weight of powdered belladonna herb, and hence the proportions by weight of each, and 3, to determine the maximum percentage of seed which might be expected in the flowering herb.

RESULTS

Mathematical Determination of Number of Testa Cells per g.

A mathematical method was devised to measure the surface area of the seed. Early in the 19th century Cauchy proved that the mean area of the projections of a convex body on to planes of all orientations is one-quarter of its surface area. The fraction is one-half for each plane element of surface, each projected area being double covered. Thus the surface area of a convex body can be accurately determined by projection on to the facets of a regular body with an infinite number of facets.

For convenience calculations were made to determine the error involved in reducing the number of facets to a small finite number. It was found that the cube, octahedron, dodecahedron, and icosahedron gave such

* This paper forms part of a Thesis to be submitted by one of us (R. G. A.) for the Degree of Master of Science of the University of Manchester.

good results that it was unnecessary to pursue the matter any further. The mean square error for these finite values was computed (Lighthill, personal communication) as in Table I.

Moran[2] calculated that, using the dodecahedron and icosahedron respectively, minimum values for the surface area would be 91·8 per cent. and 95·7 per cent. of the true area, and maximum values would be 107·9 per cent. and 104·8 per cent.

It will be noted, from Table I, that the mean square error for three mutually orthogonal directions is only 10 per cent., and that this can be reduced to 4 per cent. by the use of six planes. It was considered convenient and adequate to use the planes of the dodecahedron for practical application, and the following procedure was adopted. A projection of the seed was made in an arbitrary "initial direction", and then in five others all making an angle $\tan^{-1}(2)$, that is 63° 26′, with the initial direction, and equally spaced round it. Then the estimated area is four times the mean projected area, or two-thirds of the sum of the projected areas.

TABLE I

Number of planes	Projection on to faces of	Mean square error per cent.
3	Cube	10·16
4	Octahedron	7·52
6	Dodecahedron	3·96
10	Icosahedron	2·44

The practical details of the method are as follows. A microscope with low-power objective is set up in a dark room so that projections of the object can be made at a known magnification, calculated from the use of a micrometer slide.

A cork is cut in such a way that it has five equal lateral faces, each inclined at an angle of 26° 34′ to a fine pin which passes through the principal axis of the cork (Fig. 1, *a* and *b*).

The seed is placed on a glass slide on the microscope stage, and its outline projected and drawn. While still in this position, the seed is then impaled on the pin by pressing the pin in a direction normal to the glass slide.

The cork is turned on to the first face, and the pin pushed so that the seed is almost touching the slide. The outline is projected and drawn, and the procedure is repeated for the other four faces.

The areas of each of the six projections are determined by using a planimeter or by other means, and their sum, multiplied by two-thirds, and divided by the square of the linear magnification, gives the actual surface area of the seed. The result does not take into account any concavities in the seed, but these are not normally present in belladonna seed.

To test the method, larger regular objects of known surface areas ranging from 25·0 to 99·1 sq. cm. were projected at the given angles, using a point source of light and a screen. The per cent. errors on the known values for 17 objects were as follows, − 5·2; − 5·1; − 4·2; − 2·5; − 1·8; − 1·6; − 1·5; − 0·7; + 1·4; + 2·0; + 2·4; + 2·9; + 3·2; + 3·2; + 3·9; + 4·4; + 5·5.

Results for the surface areas of seeds, determined by the projection method, are shown in Table II.

TABLE II

SURFACE AREAS OF SEEDS BY PROJECTION

Sample	No. of seeds measured	Range (sq. mm.)	Mean	Standard deviation
1. Department stock	27	4·44–6·43	5·40	0·52
2. Harrow, 1952	20	5·14–7·47	6·19	0·59
3. "var. *lutea*", Chelsea, 1955	20	5·51–7·43	6·68	0·41
4. Chelsea, 1955	20	5·30–9·10	6·61	0·89
5. Jodrell Bank (from unripe fruit), 1954	20	5·10–7·67	6·68	0·63
6. Jodrell Bank (from ripe fruit), 1954 ..	60	6·75–9·20	7·91	0·55
7. Commercial sample	20	4·84–7·95	6·19	0·93
8. Commercial sample	20	4·72–6·48	5·48	0·53
9. Pharm. Soc. Exptl. Ground	20	5·49–7·69	6·70	0·61
All seeds	227	4·44–9·20	6·66	1·07

Determination of Number of Testa Celis in Unit Area

The testa of belladonna seed includes a single layer of cells which are characteristic in appearance. Counts were made of $\frac{1}{4}$ sq. mm. areas of the testa of the dry seed, using an Ehrlich eye-piece. One count was made on each seed, the flattest part of the seed being chosen. Portions

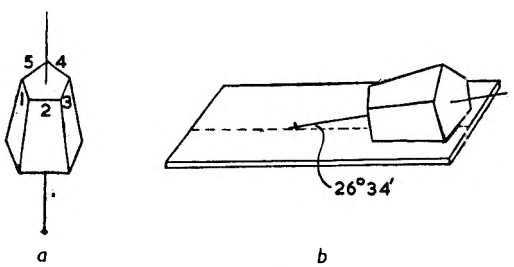

FIG. 1. *a*. Showing the cut cork. *b*. Cork on the slide.

of cells adjoining two adjacent sides of the field were included in the count, while portions of cells adjoining the other two sides were ignored (Table III). From these results were derived figures for the number of testa cells in unit weight (Table IV).

Determination of Number of Testa Cells per g. by Lycopodium Method

Wallis[3] has shown that the spores of the club-moss, *Lycopodium clavatum*, are uniform in size, and that 1 mg. of the powder contains approximately 94,000 spores. This figure was used as the basis of a second method of determining the number of testa cells per g. of belladonna seed.

Prepare a filter by folding a $2\frac{1}{2}$ in. diameter circle of closely-woven glass cloth into a cone, and support this in a glass funnel. Into the filter place about 0·2 g., accurately weighed, of belladonna seed, reduced to No. 60 powder.

TABLE III

NUMBER OF TESTA CELLS IN UNIT AREA ($\frac{1}{4}$ SQ. MM.)

Seed batch	No. of seeds	Range	Mean	Standard deviation
1	60	16–33	23·6	3·8
2	40	16–22	19·2	2·2
3	40	17–27	21·7	2·4
4	40	15–26	19·7	2·8
5	40	15–23	18·9	2·1
6	60	15–24	20·6	1·8
7	40	17–27	21·1	2·5
8	40	18–27	21·6	2·4
9	40	18–28	21·3	2·1
All seeds	400	15–33	21·0	2·9

Pour Schultze's Maceration Fluid, heated to near boiling point, slowly through the filter until the testa is bleached to a light brown and begins to disintegrate. At this point, pour about 10 ml. of hot solution of chloral hydrate (50 g. in 20 ml. water) through the filter, a few ml. at a time. Transfer the residue carefully to a small test-tube, using a small brush made from about 20 strands of stiff wire, diameter approximately 0·3 mm., fixed in a glass holder, and successive small volumes of solution of chloral

TABLE IV

NUMBER OF TESTA CELLS PER G.

Seed batch	Mean weight per 100 g.	Number of testa cells per g. (mean surface area × No. of testa cells per sq. mm. × No. of seeds per g.)
1	0·101	504,000
2	0·116	411,000
3	0·111	522,000
4	0·108	481,000
5	0·098	514,000
6	0·117	558,000
7	0·065*	802,000
8	0·066*	716,000
9	0·129	443,000
Mean	0·101	550,100
Standard deviation	0·022	127,900

* Most of the seeds of these two batches were unusual in being strongly flattened and occasionally biconcave in shape; endosperm was scanty. Both batches were taken from commercial herb received on two different occasions from the same supplier.

hydrate to a total volume of about 7 ml. Shake vigorously, and add 0·1 ml. of a suspension, in a suitable medium, containing about 0·05 g., accurately weighed, of lycopodium in 10 ml.

Place a small drop of the mixed suspension on a microscope slide, and cover with a $\frac{5}{8}$ in. cover slip. Count the total number of spores in the area bounded by the cover slip by scanning in strips equal in width to the field of view. Count the total number of testa cells by the same method, but with the assistance of polarised light. Include all testa cells of which the major portion is judged to be present; ignore those of which less than half is judged to be present.

Results obtained by this method on seed of Batch 1 are shown in Table V. To test the accuracy of this suspension method, further counts

were made using dry lycopodium powder, measured to an accuracy of 0·0001 g.; comparable results were obtained.

Change in Weight of Seeds on Powdering

Since the calculation of the number of testa cells per g. by the projection method was derived from the whole seed, it was necessary to determine whether any change in weight occurs on powdering.

TABLE V

NUMBER OF TESTA CELLS PER G. BY LYCOPODIUM METHOD

(Seeds—Batch 1; 10 mounts per macerate)

Macerate	Total weight of seed g.	Total of lycopodium	Spores	Testa cells	Testa cells/g.		
					Range	Mean	Standard deviation
1	0·314	0·1 ml. ≡ 53,600 spores	607	1807	356,600 to 696,000	521,940	91,300
2	0·247	do.	964	2218	375,500 to 648,800	496,010	82,600
3	0·205	do.	778	1197	358,400 to 537,400	410,740	54,700
4	0·226	do.	1688	3376	361,700 to 597,400	479,840	73,300
5	0·204	0·0026 g.	7485	2584	341,600 to 495,400	421,740	47,300
6	0·389	0·0069 g.	10,161	2513	321,000 to 589,600	424,110	89,000
7	0·233	0·0021 g.	7766	3382	259,000 to 489,900	385,470	71,600
8	0·205	0·0035 g.	5835	1436	300,900 to 469,500	403,100	52,400
Summation of macerates ..					259,000 to 648,800	446,390	85,400

Accurately weighed quantities of seed in No. 60 powder were exposed to the atmosphere, suitably shielded from dust, and re-weighed after three weeks.

Seventy separate weighings were made of seed from 15 different batches. 55 lost in weight under the conditions of the experiment; the maximum percentage loss was 4·22 and the mean 2·2 per cent. 11 samples increased in weight, but in only three of these was the increase more than 1 per cent. Gains and losses were evenly distributed within the individual batches. Hence it would appear that the powdering had no significant effect, and the value of the number of testa cells per g. as determined on the intact seed was equally applicable to the powder.

Determination of Number of Testa Cells per g. of Commercial Belladonna Herb

Seeds can be separated from commercial herb, or from powders coarser than No. 10, by sifting through a No. 10 sieve, and the percentage present may then be determined by weighing.

In powders finer than No. 10, it was found necessary to "concentrate" the testa cells by removal of as much extraneous material as possible, in order to obtain sufficient for each mount. The method finally adopted was maceration under carefully controlled conditions of time and temperature, such that the testa cells were not affected to any noticeable degree. The resulting cellulosic material was then dissolved out, again under carefully controlled conditions, and filtered rapidly through a sintered glass filter, on which the testa cells, isolated almost completely, were retained. The details of the method are as follows. Insert a clean No. 2 sintered glass filter, diameter $2\frac{1}{2}$ in., into a suitable flask in which the pressure can be reduced to $\frac{1}{4}$ atmosphere or less. Evenly distribute over the surface of the filter about 1·2 g., accurately weighed, of the sample, in powder not coarser than No. 60, and add one or two small crystals of potassium chlorate. Cover the powder with 25 ml. of a mixture of equal parts of nitric acid and water, heated to 70° C., and allow the reaction to proceed, without negative pressure, for about half a minute, or until bleaching just commences. At once reduce the pressure, wash the residue with two successive quantities of about 10 ml. of hot water, and partially dry by suction for a few minutes.

Transfer the residue to a 50 ml. beaker containing 25 ml. of a saturated solution of zinc chloride in hydrochloric acid, heated to 45° C. Maintain this temperature, with constant stirring, for about one minute, or until the solution begins to become clear. Without delay, transfer the contents of the beaker to the sintered glass funnel, and reduce the pressure fully. Immediately the liquid has passed through, wash the residue with two successive quantities, each of about 10 ml. of hot solution of choral hydrate.

Apply positive pressure momentarily to loosen the residue from the filter; transfer to a 50 ml. beaker, with the aid of the wire brush described previously and successive minimum quantities of solution of chloral hydrate, to a total of about 5 ml.

Add 0·1 ml. of the lycopodium suspension previously described, and stir thoroughly with a glass rod. Determine the relative numbers of spores and testa cells as before, and hence the number of testa cells per g. of the commercial sample.

To test the accuracy of the method, direct counts were made as follows:

A few mg. of the powdered herb, accurately weighed to 0·0001 g. on microscope slides, were evenly distributed in a drop or two of a hot, saturated solution of chloral hydrate in equal parts of hydrochloric acid and water. A $\frac{7}{8}$ in. cover slip was applied and the total number of testa cells counted with the assistance of polarised light. The acid in the mountant dissolves the calcium oxalate which tends to be troublesome under polarised light.

Table VI compares results obtained by both methods.

TABLE VI

DETERMINATION OF NUMBER OF TESTA CELLS PER G. IN COMMERCIAL POWDERED BELLADONNA HERB

By lycopodium method						By direct counting					
Batch	No. of macerates	Total No. of mounts	Range	Mean	S.D.	No. of mounts	Total testa cells	Total weight of powder (g.)	Range	Mean	S.D.
B	3	9	33,600 to 59,100	40,480	8980	11	1415	0·0262	33,880 to 86,700	56,870	18,000
H	3	30	2860 to 10,100	5130	2135	20	918	0·1570	3480 to 10,100	6040	1560
J	3	32	6050 to 28,330	13,060	4750	12	981	0·0662	8610 to 25,160	15,650	4,110
K	3	30	2200 to 14,800	5330	2670	20	1302	0·2131	4420 to 8450	6,170	1,180

Determination of Amount of Seed in whole Herb

Belladonna plants from various geographic sources were cut late in the season, and the aerial parts air-dried for one month. The seeds were separated from the fruits, air-dried, and weighed, and the remainder of the dried herb was also weighed (Table VII).

In an attempt to obtain upper limits for the percentage of seed, plants were included which had only one or two flowers remaining; in the second part of Table VII are shown results for some plants which had completely fruited.

DISCUSSION

The mathematical method described for the measurement of the surface area of belladonna seed has been shown to be accurate, and is suitable for the determination of the surface area of irregular convex objects of microscopic dimensions, or of larger objects. The method may conveniently be used in branches of science where it is necessary to estimate the number of discrete particles regularly distributed over the surface of such an object. The number of testa cells in unit area of belladonna seed was determined by direct counting, and the number per g. was estimated to be about half a million.

The accuracy of these estimates are supported by quantitative lycopodium determinations on the powdered seed. In initial experiments on the powdered seed, counting of the spores and cells was rendered difficult by refraction of light by the globules of fixed oil, which had proved resistant to de-fatting, except on prolonged exposure. The reduction of the seed to number 60 powder, and the use of hot solution of chloral hydrate, reduced this difficulty. The polyvinyl alcohol medium described by Hall and Melville[4] was used as a vehicle for the lycopodium suspension. Polarised light is very valuable in the counting of the testa cells; it should, however, be used with discretion, as non-refractive cells sometimes occur, particularly if the maceration has been allowed to proceed too far.

In the examination of commercial samples of powdered belladonna herb, it was found necessary to "concentrate" the testa cells in order to obtain sufficient for each mount. Removal of extraneous cellulosic material was accomplished by a method depending on the differential solubilities of each in a zinc chloride reagent. The dissolved cellulose produces a thick gel-like solution which is difficult to filter, and, as success depends upon leaving the testa cells in contact with this reagent for a minimum of time, it is essential to use relatively small amounts of powder and reagent, a clean filter with a large filtering area, and low pressures.

The above method may be adapted for the isolation from vegetable material of such resistant tissues as cork cells, or pollen grains.

The results obtained by this method for the sample B (Table VI) indicated that about 10 per cent. of seed was present in the powdered herb; this, at first, seemed a high proportion, but determinations on various samples of whole herb produced values as high as 16·6 per cent. (Table VII).

Information is insufficient at present for a "Limit of seed" to be suggested.

TABLE VII

PERCENTAGE OF SEED IN BELLADONNA HERB

Batch	Source and date	Per cent. of seed
1	University Experimental Grounds. Late August. Age of plant not known	16·6
2	Pharmaceutical Society's Experimental Grounds. Late August. Age not known	13·8
3	University Experimental Grounds. Late August. 1st year plant	4·0
4	Chelsea Physic Garden. Early September. Age not known	5·2
5	do.	11·9
6	Cheshire. Early October. 1st year plant	4·2
7	do.	5·9
8	Jodrell Bank, Mid-October. 1st year plant. No flowers	11·0
9	do.	14·3
10	do.	8·7
11	do.	14·8
12	do.	18·5
13	do.	13·7
14	do.	17·5

SUMMARY

1. A mathematical method is described for the determination of the surface area of objects of irregular shape, and applied to the measurement of the surface area of belladonna seeds.

2. From these results, and from direct counting of the testa cells in unit area, the number of testa cells per g. of belladonna seed is calculated. These figures are compared with those obtained by quantitative lycopodium methods.

3. A method is described for the separation of testa cells of belladonna seed from other cellulosic material in the powdered herb. This enables a quantitative estimate to be made of the amount of seed present in the powdered herb. The proportion of seed present in the dried entire herb, collected when in flower, was also determined.

For providing materials and facilities, the authors are indebted to Dr. J. M. Rowson, Curator, the Museum of the Pharmaceutical Society; Mr. W. G. MacKenzie, Curator, Chelsea Physic Garden; Dr. Adela Erith, University of Reading; the staff of the Manchester University Experimental Grounds. Thanks are also due to Professor M. J. Lighthill, F.R.S., Department of Applied Mathematics, Manchester University, for confirming and enlarging upon the mathematical approach.

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DISCUSSION

The paper was presented by MR. R. G. ATKINSON.

The CHAIRMAN said that the immediate object of the paper seemed to be to determine the amount of seed in the herb to ensure that it was not collected at a time when it did not contain enough atropine. It would seem that an alkaloidal assay of the material would have been a more direct method.

DR. T. E. WALLIS (London) said that belladonna seeds were rather flat, and he wondered whether the authors could have devised a simpler method of measuring the surface area. The method designed for determining the total surface area of convex bodies was checked by examining other regular objects in order to see whether the method worked. The authors did not describe the objects or give their shape, and it would be interesting to have more information about them. The methods described for estimating the amount of seed in the powder were difficult to follow, and it would have been valuable if complete details of at least one experiment could have been given. He would have thought the shape of the epidermal cells could have been identified for counting purposes without using polarised light. The specimens from Jodrell Bank were all first year plants and all contained a high proportion of seeds. Other first year plants mentioned in Table VII contained 4 to 6 per cent. of seeds as against a figure of up to 18 per cent. in the former group. It seemed remarkable that there should be such a large difference.

DR. J. W. FAIRBAIRN (London) observed that the standard deviation obtained with the whole seeds was 10 per cent. of the mean, which was good. When the lycopodium method was applied the standard deviation was about 22 per cent. of the mean, which was fairly wide; but when the method was applied in practice to a powdered herb the standard deviation was very wide indeed. In Table VI, sample K, the standard deviation

was about 50 per cent. of the mean, which meant that even if one did 25 determinations, which would take a very long time, there would still be an error of about ± 25 per cent. It was possibly due to the fact that the herb was bound to contain seeds at all stages of maturity; very slight variations in the oxidation process would lead to greater or less destruction of the cells of immature seeds. It was difficult to understand why the authors (in Table VII) collected plants so late in the season. One would assume that they desired to know what was the normal amount of seed present in B.P. belladonna, which should be collected when in flower. In London, belladonna flowered by early June. It would have been helpful to have a typical belladonna herb as a standard. The commercial samples HJK, in Table VI, contained from 1 to 3 per cent. of seeds, which was a fair amount, but those cited in Table VII were artificially high.

DR. J. M. ROWSON (London) agreed with the Chairman that determination of the total alkaloids and the proportion of hyoscyamine would have given an answer rather more readily than the lycopodium method. He pointed out that no reference to the original publication by Cauchy had been given. He asked the authors the number of counts made in Table V and whether they were based on 25 fields or more. He agreed with Dr. Wallis about the use of irregular bodies to test the projection method, and asked whether the authors would be able to state the accuracy of their value by that method compared with the first projection. The Jodrell Bank values for seed content seemed very high for one year plants. His own one year plants at Manchester did not produce many seeds. It would be interesting to know how the authors' method would behave for seeds of varying degrees of maturity.

MR. R. G. ATKINSON, in reply, said that he had hoped to find some relation between the amount of seed present at various stages and the alkaloidal content. However, it was rather more complex than was anticipated, depending on the age of the plant and the various conditions of growth. The maximum amount of seed, 16·6 per cent., which was found on material which was flowering, compared very closely with the 18·5 per cent. of Jodrell Bank with no flower, and it seemed doubtful that it would be possible to fix a limit on seed. It had been found that most of the seeds were convex. There were very few concavities. Regular bodies—cylinders of maximum surface area 90 sq. cm.—which could be measured relatively easily were chosen. The projection method in theory gave a mean of about 5 per cent. with a latitude of about 10 per cent. on either side. It had been found that polarised light was very useful as an adjunct to determine the number of cells. Maceration tended to reduce the refractibility to polarised light, and it was necessary to exercise care in its use. It was possible with practice to acquire the technique of passing the material through the filter without much loss of the testa material. The work of Cauchy was described to him by an expert in mathematics, but he was unable to find a reference in the literature. It would be seen from Table V that ten counts were taken for each of the macerates. The belladonna seeds where endosperm was found lacking were unusual.

THE PHARMACOGNOSY OF THE ASPIDOSPERMA BARKS OF BRITISH GUIANA*

PART IV. QUANTITATIVE NUMERICAL STUDIES OF THE LIGNIFIED ELEMENTS IN CASCARA AND IN *Aspidosperma* SPECIES

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BARKS derived from different species of the same genus often resemble one another very closely in the microscopical structure of their elements and cell contents. This has been shown in the previous communications for the barks derived from the genus *Aspidosperma*[1–3], where the detailed histology of *A. ulei*, *A. excelsum* and *A. album* has been described and illustrated. We have examined the detailed histology of the barks of *A. megalocarpon*, *A. oblongum* and *A. quebracho-blanco* and hope to describe them in subsequent communications. In these six co-generic species there are differences in tissue arrangements which disappear on powdering and other means were sought for distinguishing certain of these barks when in the powder form.

In recent years, numerical microscopical studies have been developed to ensure a more complete control of crude drugs, especially when in powder form. Numerical values determined microscopically can be subdivided into the two main classes of ratios, based upon two sets of numbers, and absolute values, based upon counts or upon measurements of area or of length. Compared with the numerous quantitative methods which have been applied to roots, stems, leaves, fruits and seeds in recent years, the number of investigations on barks is small.

Many barks contain sclerenchymatous tissue of stone cells and fibres, usually with heavily thickened walls which are frequently lignified; the striking appearance and the strong reaction for lignin of these cells rendering them easily identifiable. The possibility that these two types of cell-elements might occur in a fixed ratio one to the other in a given species was considered. Should this be proved to be so, it would be useful for identification purposes, and especially so if the ratio values differed between different species. The barks of *Aspidosperma* species contain both sclereids and fibres and were regarded as a suitable group in which to investigate this ratio.

In order to test these premises as stringently as possible within one species, some preliminary work was carried out upon the barks of *Rhammus purshiana*, since a wide range of samples was easily available differing greatly in age and size: also there were no intermediate forms between the sclereids and fibres in this species.

* The subject matter of this communication forms part of a thesis by one of us (J.D.K.) accepted by the University of Nottingham for the degree of Doctor of Philosophy in Pharmacy.

† Nottingham University Research Scholar.

Cascara Bark

To investigate the ratio between the sclereids and fibres present in cascara bark one may equate the number of each element per mg. of a powder; alternatively, the numbers of each element seen in a given transverse section of the bark may be counted and equated. Since each element has an approximately definite volume, it would also follow that the areas occupied by the sclereids and fibre groups as seen in transverse sections may also be equated. This last method is simple to apply and, if it yields a constant ratio for different samples of cascara bark, it should indicate that the ratio of numbers of sclereids and fibres per mg. is also a constant.

Transverse sections of cascara bark from different samples were cut, cleared by boiling in chloral hydrate solution, stained with phloroglucinol and hydrochloric acid and mounted in glycerine. The lignified areas were then traced on paper by means of an Abbé-type camera lucida. The areas of sclereids and of fibres were determined on the paper by cutting out the areas and weighing them separately. The results of three sets of experiments were tabulated in Tables I, II and III.

It is seen from these results that, although the per cent. area lignified varies between wide limits, the ratio of area of sclereids to area of fibres, as seen in transverse sections, is a constant in samples of cascara bark of different geographical origins, ages or thicknesses and between pieces of bark obtained from different aerial parts of the plant. Hence such a ratio may have diagnostic significance.

Aspidosperma Barks

Attempts were next made to apply this simple method to *Aspidosperma* barks, but the experimental difficulties made this impossible, largely because it was virtually impossible to get entire sections. It was therefore decided to find out if a constant ratio exists between the number of sclereids and the number of fibres present in the powdered barks of *Aspidosperma*.

The barks in No. 90 powder were used for all the quantitative work and microscopical examination showed that they contained sclereids both isolated and in groups. Only the powders of the barks of *A. album* and *A. oblongum* contain groups of fibres as well as the isolated fibres, the other four barks, namely *A. excelsum*, *A. megalocarpon*, *A. ulei* and *A. quebracho-blanco*, contain isolated fibres only. The methods available for counting the numbers of sclereids occurring in these masses were investigated.

Calculation of the number of cells in the groups of sclereids in powders

The individual groups of sclereids in No. 90 powder of the barks are usually of two kinds, one consisting of more or less isodiametric stone cells and the other of somewhat elongated stone cells of varying lengths; both types of cells were counted without distinction. By using the method of calculation devised by Wallis and Santra for pimento[4] and for olive stones[5], the number of stone cells in the masses was computed by counting the cells along two directions at right angles to one another and using

TABLE I

S/F RATIO (AREAS) IN CASCARA BARKS OF DIFFERENT THICKNESSES

Pieces	Thickness in mm.	Percentage area lignified	Percentage area of sclereids (S)	Percentage area of fibres (F)	Ratio S/F
1	1·81	0·93	0·52	0·41	1·25
2	1·67	1·33	0·73	0·60	1·21
3	1·95	1·87	1·04	0·84	1·25
4	2·09	1·85	1·04	0·81	1·27
5	2·34	4·78	2·77	2·01	1·31
6	2·27	3·91	2·14	1·76	1·24
7	1·39	7·85	4·35	3·49	1·24
8	1·95	6·82	3·78	3·04	1·24

Mean = 1·25

TABLE II

S/F RATIO (AREAS) IN CASCARA BARKS FROM DIFFERENT SAMPLES (MUSEUM OF THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN)

Sample	Thickness in mm.	Percentage area lignified	Percentage area of sclereids	Percentage area of fibres	Ratio S/F
1. North America, 1895	2·64 2·43	1·47 1·09	0·80 0·60	0·67 0·49	1·21 1·24
2. Wright, Layman and Umney, 1923	3·89 3·61	4·95 3·42	2·76 1·88	2·19 1·54	1·25 1·25
3. Imported U.S.A., 1930	3·48	1·75	1·02	0·72	1·27
4. H. O. Meek, 1938	1·81 1·95	0·70 1·26	0·40 0·69	0·30 0·57	1·25 1·25
5. H. O. Meek, 1937	5·56	4·03	0·26	1·77	1·29
6. H. O. Meek, 1933	6·39	2·38	1·26	1·13	1·17
7. Imported, 1930	3·41	4·58	2·53	2·04	1·24
8. Mossed, origin unknown	3·89	3·33	1·85	1·48	1·25
9. N. America, no date	2·50	4·06	2·25	1·80	1·25
10. Barts Hospital Museum, 1931 ..	2·92	5·86	3·29	2·57	1·28

Mean = 1·25

TABLE III

S/F RATIO (AREAS) IN CASCARA BARKS. SAMPLES OF DIFFERENT THICKNESSES AND FROM DIFFERENT POSITIONS ON THE SAME PLANT, COLLECTED FROM A CASCARA TREE GROWING AT BIRDSGROVE HOUSE, MAYFIELD, DERBYSHIRE

No.	Height from the ground	Thickness of stem mm.	Thickness of bark mm.	Percentage area lignified	Percentage area of sclereids (S)	Percentage area of fibres (F)	Ratio S/F	Mean S/F
1	7′	5	1·15	Not sufficiently lignified			—	—
2	6′	9	1·25	1·06 1·32	0·65 0·79	0·41 0·53	1·57 1·50	1·53
3	4·5′	16	2·78	1·97 1·98	1·10 1·09	0·87 0·89	1·26 1·22	1·24
4	4·5′	16	2·78	1·52 1·13	0·84 0·63	0·68 0·50	1·24 1·28	1·26
5	6″	31	3·40	3·70 3·93	2·07 2·19	1·63 1·75	1·27 1·25	1·26
6	2·5″	36	4·17	3·57 3·14	1·98 1·75	1·59 1·40	1·25 1·25	1·25

Samples 3 and 4 were taken at the same level on the same branch, No. 3 being from the outer side and No. 4 on the side nearest the trunk.

half their average as the radius of an imaginary equivalent sphere. Twenty-five typical masses were picked up on the point of a needle from a preparation stained with phloroglucinol and hydrochloric acid and each particle was placed on a slide with a drop of glycerine and a coverslip was applied. Counts were then made of cells along the two directions and the number of cells in each particle was calculated from the formula $4/3\pi r^3$. Then each of these particles was disintegrated by adding a drop of nitric acid 50 per cent. and the actual number of cells was counted after a brief maceration. The results are recorded in Table IV.

TABLE IV

NUMBER OF SCLEREIDS IN PARTICLES SELECTED FROM A NO. 90 POWDER OF THE BARK OF *A. album*

Number of cells along two directions		Total number of cells in each particle	
Longitudinally	Transversely	By calculation	By disintegration
4	3	22·5	24
5	3	33·5	32
5	5	65·5	68
3	3	14·0	16
5	3	33·5	34
6	6	113·0	95
4	4	35·5	30
7	5	113·0	125
4	4	35·5	48
5	4	48·0	31
4	3	22·5	24
6	4	65·5	59
5	4	48·0	44
4	3	22·5	24
5	4	48·0	51
4	4	35·5	33
5	4	48·0	40
5	3	33·5	44
8	3	87·0	78
4	3	22·5	21
4	4	33·5	37
4	3	22·5	19
5	3	33·5	36
5	4	48·0	39
4	4	33·5	33
Total number of cells in 25 particles		1112	1085

Close agreement was noticed in 16 of the experiments between the actual and calculated figures (522 and 526), and this indicated that if a sufficiently large number of particles was examined the errors cancelled out; totals for 25 particles being 1112 and 1085 for the calculation and disintegration methods respectively. These differences are within the error of experiments.

Determination of the number of stone cells and length of fibres per mg. of the powder

A weighed quantity of the powder and of lycopodium was gently triturated for three minutes in a glass mortar with sufficient nitric acid (50 per cent.) to make a thin paste. This trituration was just long enough to disintegrate all the fibres and most of the sclereid groups. Further reaction of the nitric acid was then stopped by the addition of 70 per cent. ethanol. The whole material was then transferred to

a centrifuge tube and the mortar was washed twice with small quantities of 70 per cent. ethanol and the washings transferred to the same centrifuge tube. After centrifuging for ten minutes, the supernatant liquid was carefully drained and 5 ml. of 1 per cent. solution of phloroglucinol in 90 per cent. ethanol was added to the material in the tube, it was shaken well, centrifuged and decanted. A few drops of strong hydrochloric acid were than added whereby the lignified tissues (sclereids and fibres) were stained red. The final volume of the suspension in the tube was made up to 6 ml. by addition of a suspending agent (2 vols. of glycerine, 2 vols. of water and 1 vol. of Mucilage of Tragacanth B.P.). The tube was then oscillated gently to ensure an even distribution. Four slides were prepared. The free sclereids were counted directly and the number of sclereids remaining in groups were calculated as described above. The total sclereids were thus determined in seven strips across the cover-glass as described by Wallis[6]. Further, the lengths of fibres, which are always broken, seen in the same seven strips across the cover-glass were marked on paper with the help of an Abbé-type camera lucida at a magnification of × 800 and thus the total length of fibres determined.

The methods of experiments and calculations of Wallis[6] were employed. Detailed counts and measurements obtained for three different samples of *A. album* bark are presented in Table V. Similar experiments were then carried on with the powders of various samples of all the other species of *Aspidosperma* and the summarised results are tabulated in Table VI.

The average mean length of fibre in the Aspidosperma *barks*

In order to calculate the number of fibres present in 1 mg. of powdered bark from the length of fibre per mg., which has already been determined, it is necessary to know the average mean length of fibre in each of these six species.

Ten small pieces of bark from each species were macerated in Schultzes maceration fluid for about half an hour. The pieces of bark were then teased out with the help of two dissecting needles to isolate the fibres. At least twenty of these complete fibres were then measured at random with the help of a calibrated eye-piece micrometer. Two samples of each species were used and thus about 400 to 500 microscopical measurements of fibres were obtained for each species. The statistical mean of the fibre lengths was found out by plotting a graph on the "arithmetical probability graph paper", of cumulative frequency as per cent. of total against the mean fibre length of each group. The results of these statistical calculations are tabulated in Table VII.

To determine the number of fibres per mg. of the powdered barks of Aspidosperma

By dividing the mean values of the lengths of fibres per mg. (Table VI) by the mean lengths of the fibres (Table VII), the mean number of fibres per mg. in the powdered barks have been derived. The results are tabulated in Table VIII.

TABLE V

COUNTS OF THE NUMBERS OF SCLEREIDS AND MEASUREMENTS OF TOTAL LENGTHS OF FIBRES (BROKEN PIECES) PER mg. POWDERED BARK OF *A. album*

Sample	Suspension	Number of sclereids per mg.	Fibre length per mg. in mm.
1949	I	8058, 8778, 8292 Average: 8376	118·25, 131·18, 125·16 Average: 124·86
	II	9148, 9109, 9175 Average: 9144	123·72, 125·20, 117·87 Average: 122·26
	III	8574, 8902, 9306 Average: 8927	120·46, 127·13, 124·53 Average: 124·04
1953	I	9246, 8492, 8921 Average: 8886	115·78, 121·44, 125·94 Average: 121·05
	II	8713, 8345, 9146 Average: 8734	116·48, 123·45, 128·16 Average: 122·36
1954	I	9140, 8986, 9456 Average: 9194	132·45, 129·28, 114·99 Average: 125·57
	II	8188, 8491, 9255 Average: 8645	130·04, 118·60, 114·78 Average: 121·14
Grand average		8844	123·04 mm.

TABLE VI

AVERAGE NUMBERS OF SCLEREIDS AND AVERAGE TOTAL LENGTHS OF FIBRES PER MG. IN THE POWDERS OF *Aspidosperma* BARKS

Species	Average number of sclereids per mg.	Average total length of fibres per mg. in mm.
A. album	8844	123·04
A. excelsum	7059	16·08
A. megalocarpon	11705	47·19
A. oblongum	11228	26·50
A. ulei	5749	65·59
A. quebracho-blanco	5052	40·80

TABLE VII

AVERAGE MEAN LENGTHS OF FIBRES IN THE *Aspidosperma* BARKS

Species	Sample	Mean lengths of the fibres in μ	Mean lengths in μ
A. album	1949 1954	1752 1798	1775
A. excelsum	1949 1954	2196 2208	2202
A. megalocarpon	1949 1954	1140 1222	1181
A. oblongum	1949 1954	1878 1930	1904
A. ulei	1949 1954	3684 3844	3764
A. quebracho-blanco	6B 6C	862 838	850

To determine the ratio of number of sclereids to number of fibres in the powdered barks of Aspidosperma

Finally, having determined for each species the number of sclereids per mg. and having calculated the number of fibres per mg., these two values were equated. The ratio was determined by dividing the values of number of sclereids per mg. (Table VI) by the number of fibres per mg. (Table VIII), and the results are tabulated in Table IX.

TABLE VIII

NUMBER OF FIBRES PER mg. OF THE POWDERED BARKS OF *Aspidosperma*

Species	Lengths of the fibres per mg. in mm.	Mean lengths of the fibres in mm.	Calculated number of fibres per mg.
A. album	123·04	1·7750	69
A. excelsum	16·08	2·2020	7
A. megalocarpon	47·19	1·1810	40
A. oblongum	26·50	1·9040	14
A. ulei	65·59	3·7640	18
A. quebracho-blanco	40·80	0·85	48

TABLE IX

S/F RATIO (NUMBER OF SCLEREIDS TO NUMBER OF FIBRES) IN THE POWDERS OF THE *Aspidosperma* BARKS

Species	Number of sclereids per mg. (S)	Number of fibres per mg. (F)	Ratio S/F
A. album	8844	69	128
A. excelsum	7059	7	1008
A. megalocarpon	11705	40	293
A. oblongum	11228	14	802
A. ulei	5749	18	319
A. quebracho-blanco	5052	48	105

DISCUSSION

Preliminary work on the bark of cascara sagrada has shown conclusively that in transverse sections the ratio of area of sclereids to area of fibres is a constant in samples of different geographical origins, ages or thicknesses and between pieces of bark obtained from different aerial parts of the plant. These results are tabulated in Tables I, II and III.

Similar ratios of areas could not be investigated because of section cutting difficulties in the barks of *Aspidosperma*, also a similar ratio in the entire bark would not have solved the problem of identification of these barks when in powdered form. In consequence, direct counts of the numbers of sclereids and a measure of the numbers of fibres present in each mg. of powdered barks were made.

A partial maceration of powdered *Aspidosperma* barks completely disintegrated the fibre masses but only incompletely disintegrated the

sclereid groups. Using the lycopodium method of Wallis[6], counting isolated sclereids and calculating the numbers in sclereidal masses by the method of Wallis and Santra[4,5], concordantly reproducible results were obtained for the number of sclereids per mg. in each species. The results in Table IV confirm the findings of Wallis and Santra[4,5] that the method of calculation gives satisfactory results for sclereid masses. It will be noted from Tables V and VI that the numbers of sclereids per mg. of powdered barks were constant, within experimental error, for each of the six species. Individual species could not be distinguished by this number alone, although three separate groups may be observed.

In order to determine the number of fibres present in 1 mg. of powdered bark an indirect method had to be employed since the fibres present in these fine powders were much broken. Hence total length of fibre per mg. was readily obtained by direct microscopical measurements; an independent measure of average fibre length in each species was made and, from these pairs of values, the numbers of fibre per mg. of powdered barks were calculated for each species.

From Tables V and VI, it will be seen that mean fibre length per mg. of powder is a constant, within experimental error, for barks of the six species examined. Moreover, four of the six species may be distinguished with some certainty, by this value alone, but values for *A. megalocarpon* and *A. quebracho-blanco* are similar as shown in Table VIII. In the same Table it will be seen that the mean fibre length, uniform within a species, differs considerably between species and ranging from 0·85 mm. in *A. quebracho-blanco* to 3·76 mm. in *A. ulei.* These values possess some diagnostic usefulness when the entire or broken barks are examined.

The number of fibres per mg. of barks as calculated and shown in Table VIII, is also a diagnostic character for each species but a clear distinction between the six species under examination is not possible on this character alone.

When the S/F ratio is calculated from these numbers of elements present per mg. of powdered barks, the values shown in Table IX were obtained. It will be seen that a clear distinction between each of the six barks can be made by means of this value alone. This separation is reinforced when the value of S (number of sclereids per mg.) and of F (number of fibres per mg.) are also considered.

Summary and Conclusions

1. The ratio of the areas of sclereids to fibres as seen in transverse sections of barks of *Rhamnus purshiana* has been shown to be a constant for samples differing considerably in sizes, ages or geographical origins.

2. The average mean fibre lengths in barks of six species of *Aspidosperma* have been determined.

3. The numbers of sclereids and numbers of fibres per mg. of powders of these six barks have been counted.

4. The ratio S/F (number of sclereids: number of fibres) is a constant for barks of each species and it serves to distinguish between them.

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DISCUSSION

The paper was presented by DR. J. D. KULKARNI.

DR. T. E. WALLIS (London) said that the authors had noted that the mean length of fibres per mg. offered a useful criterion for distinguishing barks, and suggested that they might have referred to the work of Dequeker, who was the first worker to suggest that that character could be used. It was a remarkable fact that the ratio of sclereids to fibres should be constant for a particular bark. Both types of sclerenchyma were separate in function. Sclereids hardened the tissue and fibres gave resilience and tensile strength. It would be of interest to know whether the authors could give any reason why the plant should produce those two tissues in constant proportions.

DR. J. D. KULKARNI, in reply, said that the authors had found the original method of Wallis and Santra for estimating the number of fibres and sclereids very useful.

DR. J. M. ROWSON, in reply, said that the authors could not say why the plant produced fibres and stone cells in constant proportions.

THE APPLICATION OF ENZYME INHIBITION TO THE ESTIMATION OF SMALL QUANTITIES OF DRUGS POSSESSING ANTICHOLINESTERASE ACTIVITY

The Assay of Injection of Neostigmine Methylsulphate

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When highly active substances are administered, it is often necessary to use tablets for oral administration or solutions for injection. These preparations contain accurately known small quantities or low concentrations of the drug and may present a considerable problem to the analyst. Neostigmine methylsulphate is officially assayed by determination of the quantity of elementary nitrogen in 0·5 g. of the sample. Since 0·5 g. of the substance corresponds to 1000 1-ml. ampoules of the official strength, determination of nitrogen as a method of assay for the injection is unsatisfactory. Further, while determination of nitrogen is a fairly satisfactory method of assay for a substance the purity of which can be established by a melting point determination, it constitutes a much less satisfactory method for assaying a preparation in which the drug may have undergone decomposition to a greater or lesser amount without loss of nitrogen.

Such difficulties may sometimes be overcome by the use of sensitive colorimetric or spectrophotometric methods. The drug may not, however, be coloured and may not contain a chemical grouping enabling conversion to a coloured derivative to be effected satisfactorily. Further, injections may contain, in a concentration large in comparison with the concentration of the drug, bactericides such as chlorocresol or phenylmercuric nitrate which interfere with the spectrophotometric assay. In some instances biological assay may be necessary. Biological assays are often time-consusming, costly and yield results with wider limits of error than are suitable for control or public analytical work. During the last few decades it has become established that a number of drugs exert their actions by inhibition of enzyme systems, in some, in very low concentrations. In 1942 Vincent and Morgin[1] used cholinesterase inhibition as the basis of a method for estimating physostigmine in calabar bean and extended the process to the estimation of other drugs possessing anticholinesterase activity such as morphine and codeine. The method was used in toxicological work by Vincent and Beaugar[2]. A similar process was used by Ellis Plachte and Strauss[3] for the estimation of physostigmine in 10^{-6} to 10^{-8} M concentration. Enzyme estimations are inexpensive and can be carried out in a chemical laboratory with as little trouble as, for example, the estimation of nitrogen. The limits of error usually lie between those of chemical and classical biological assays.

It seemed advisable, therefore, to investigate the extent to which

enzyme inhibition might be used for the assay of drugs in tablets and injections. As a result of the work of Stedman[4] relating chemical structure to anticholinesterase activity, Aeschlimann and Reinert[5] investigated a series of substituted esters of alkyl carbamic acids. Neostigmine proved to be the compound showing the most promising pharmacological properties. The pharmacological activity of neostigmine was shown to be closely related to its anticholinesterase activity; the kinetics of the enzyme inhibition have been extensively studied and, as mentioned above, it is inhibitory in such low concentrations as 10^{-7} M (see also below). The British Pharmacopœia lacks an assay of Injection of Neostigmine Methylsulphate. It seemed logical, therefore, in the first place to investigate the possibility of devising a satisfactory process based upon the inhibition of cholinesterase for the assay of this official preparation.

Experimental Methods

Materials. All the water used had been distilled in an all-glass still.

Cresol Red Solution. A stock solution, containing 0·8 g. of cresol red dissolved in 2·7 ml. 0·1 N NaOH and diluted with water to 100 ml., was diluted with three times its volume of water before use. Since 1 ml. of this product was used in each 50 ml. digest, this corresponded to a concentration of 0·004 per cent. cresol red.

Substrate. A salt of acetylcholine (ACh) was used as substrate for the pseudocholinesterase. In the earlier experiments the chloride was employed but later the non-hygroscopic perchlorate[6] was found to be more convenient. Glick[7] has shown that the rate of enzymic hydrolysis of ACh is independent of the nature of the associated anion. Both salts were of commercial quality.

Pseudocholinesterase (ψChE). Sterile horse serum[8,9] obtained commercially in 25 ml. vaccine bottles was used as the source of ψChE. It may be preserved with 0·2 per cent. chlorocresol (see below) or by saturation with chloroform[10]. When kept at 4° C. it retains sufficient activity for at least 6 months.

Determination of ψChE Activity. Cholinesterase activity is determined by the rate at which ACh (or some analogue) is hydrolysed.

Hestrin[11] used a colorimetric method to estimate the residual ACh after the period of hydrolysis. This method not only possesses the errors and difficulties of a colorimetric assay, but it is more suitable for experiments in which the bulk of the ACh is used up than for assays based on first order kinetics achieved by limiting the extent of hydrolysis of the substrate to one-third.

More usually, the acid liberated by the hydrolysis of the ACh is determined in one of several ways. (1) The acid may be caused to liberate CO_2 from $NaHCO_3$ solution and the volume of CO_2 determined either manometrically in the Warburg apparatus[12] or, in the case of microdetermination, by use of the Cartesian Diver technique[13]. Such methods, because of the specialised apparatus required, were considered to be unsuitable for an official assay method, especially since there is

no great increase in accuracy. (2) the acid liberated may be titrated with standard alkali either: (*a*) at the end of the hydrolysis, or (*b*) continuously during hydrolysis.

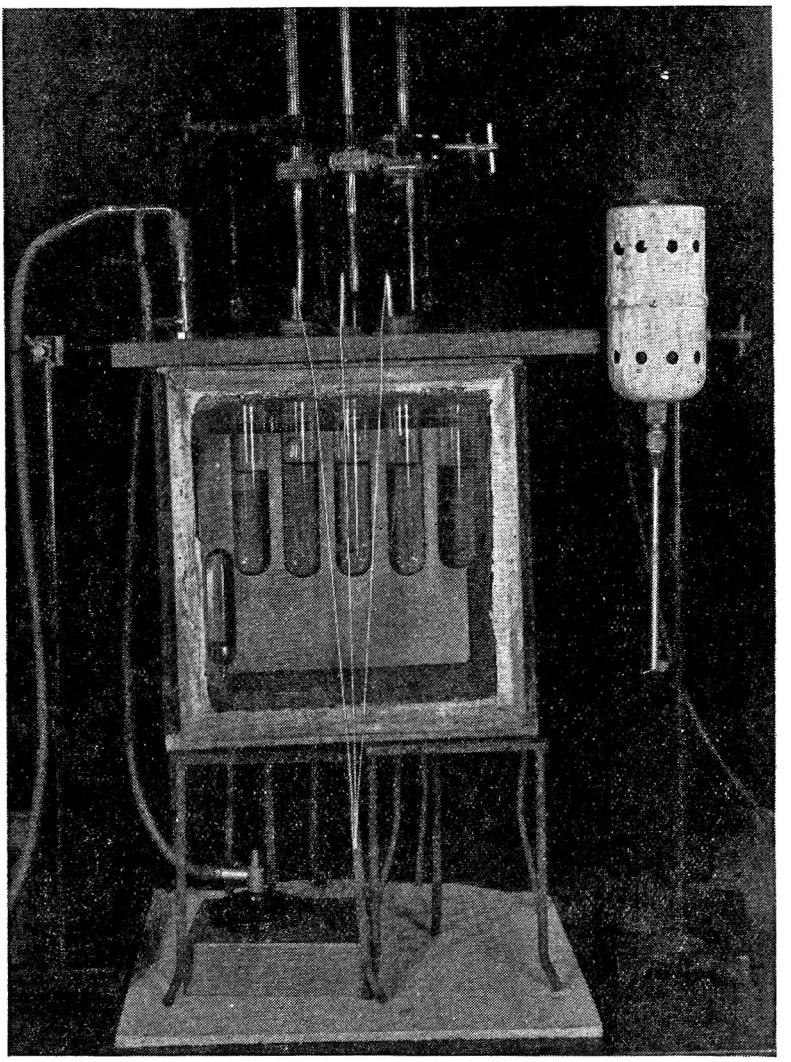

FIG. 1. Photograph of the apparatus used in the determination of ψChE activity.

Method (*a*) has the drawback that the pH and consequently the activity of the enzyme varies during the period of digestion unless a buffer is added, in which case determination of the end-point of the titration is difficult. In method (*b*) the pH is maintained constant during the digestion period. The maintenance of pH may be controlled by use of an indicator or electrometrically. Using an indicator 2 or 3

assays may be carried out simultaneously (Fig. 1) (e.g. with and without addition of inhibitor), whereas electrometrically this is difficult. It was found that variations were smaller between simultaneous replicates than between successive replicates. For these reasons a method based on (2) (*b*) using an indicator was adopted. The method, apparatus (Fig. 1) and technique are similar to those described previously for lipase[14].

One ml., accurately measured, of horse serum was mixed with 43 ml. of water containing 1 ml. of cresol red solution contained in a 3 × 20 cm. Pyrex boiling tube fitted with a stopper with two holes, one for the drawn out tip of a microburette containing 0·025 N NaOH and the other for a short glass tube through which passed a thread operating a glass-coil stirrer. The tubes were placed in a water bath at 40° C. and by addition of alkali the pH was rapidly adjusted to 7·9 (by comparison with the colour of two Pyrex tubes in the water bath containing respectively 1 ml. of cresol red solution and 49 ml. of B.P. buffer pH 7·8, and 1 ml. of cresol red solution and 49 ml. of B.P. buffer pH 8·0). After 15 minutes for temperature equilibration, 5 ml. of a 3 per cent. solution of ACh perchlorate was added (concentration in digest $1{\cdot}22 \times 10^{-2}$ M) the pH readjusted to pH 7·9 and the burette reading noted. The pH was then maintained between 7·8 and 8·0 by dropwise addition of alkali, the burette reading being noted after 5, 10 and 15 minutes. Maintenance between the two pH units is possible because the 1 ml. of serum has a buffering action such that addition of 1 drop of the alkali does not change the pH by more than 0·1 unit. All readings were corrected for non-enzymic hydrolysis ascertained by conducting an experiment with addition of 1 ml. of a dilute buffer in place of 1 ml. of serum. The correction was small, amounting to only 0·32 ml. for a 15 minute period. The corrected quantity of alkali added in each of the three 5-minute periods of the assay should be practically the same. If this were so, the alkali consumed in 15 minutes was recorded as a measure of the ψChE activity ("A_{15}" value).

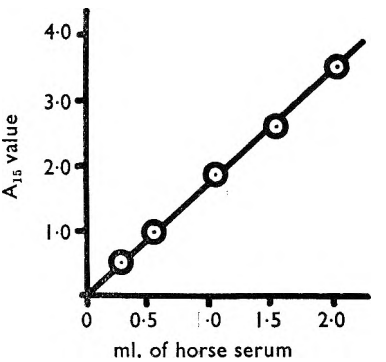

FIG. 2. Relation between the rate of hydrolysis and the quantity of horse serum used. Temperature 40° C. pH 7·9. ACh concentration $1{\cdot}22 \times 10^{-2}$ M.

The suitability of this process for the evaluation of inhibition is established by the straight line graph (Fig. 2) relating enzyme activity to the corresponding A_{15} values. A digest in which the enzyme is inhibited 50 per cent. will obviously require only half the alkali consumed in the absence of inhibition.

The sensitivity of the assay to various factors was determined as follows.

Substrate Concentration. Figure 3 shows that the A_{15} value of a given quantity of serum is practically independent of the substrate concentration.

Temperature. The results presented in Figure 4 show that 40° C. is in the neighbourhood of the optimum temperature under the conditions of the assay.

pH. Using the same sample of serum under identical conditions, A_{15} values of 2·46, 2·53 and 2·55 were obtained by maintaining the digests at pH values of 7·8, 7·9 and 8·0 respectively. The difference between A_{15} values determined at pH 7·8 and pH 8·0 thus amounts to about 1 per cent. This is the maximum percentage error which is likely to arise due to pH variation during the assay.

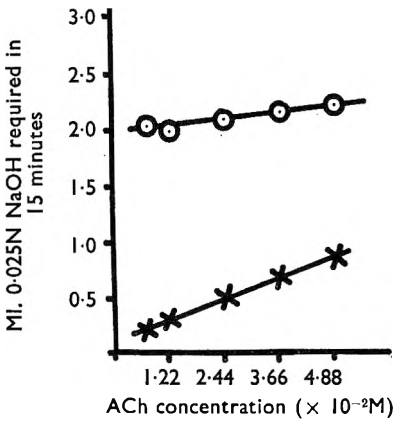

FIG. 3. Relation between the rate of hydrolysis and ACh concentration O—O corrected enzymic hydrolysis. X—X non-enzymic hydrolysis. 1 ml. of horse serum as source of enzyme. Temperature 40° C. pH 7·9.

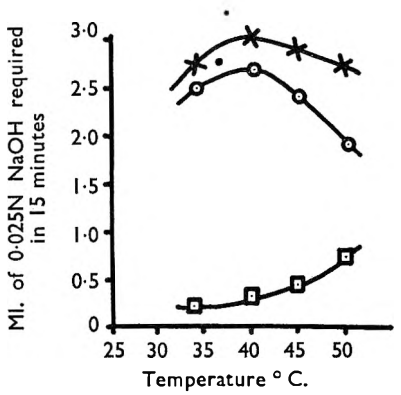

FIG. 4. Relation between the rate of hydrolysis of ACh and temperature ×—× Total hydrolysis. ○—○ Corrected enzymic hydrolysis. □—□ Non-enzymic hydrolysis. ACh concentration 1·22 × 10⁻²M. pH 7·9. 1 ml. of horse serum as the source of enzyme.

Antiseptics. Chlorocresol 0·2 per cent. or phenylmercuric nitrate 0·002 per cent. may be present as preservatives in Injection of Neostigmine Methylsulphate. Table I shows that such quantities of these preservatives as might thus find their way into the digests of the assay process would be without measurable effect on the ψChE activity.

TABLE I

NEGLIGIBLE ANTICHOLINESTERASE ACTIVITY OF BACTERICIDES 15 MINUTES PRIOR CONTACT OF ENZYME AND INHIBITOR AT 40° C.

Bactericide	Concentration in 50 ml. digest per cent.	A_{15} value	
		Bactericide present	Bactericide absent
Chlorocresol	0·002	2·75	2·76
	0·005	2·72	2·72
	0·01	2·73	2·76
	0·05	2·74	2·72
Phenylmercuric nitrate	0·0002	2·69	2·69
	0·0004	2·71	2·74
	0·001	2·65	2·62
	0·002	2·70	2·73

Accuracy of the Assay Process. Fourteen successive determinations using a single sample of horse serum gave an average A_{15} value of 3·24 ml., with a standard deviation of 0·06.

The ψChE Activity of Different Samples of Horse Serum. Six different samples of horse serum gave average A_{15} values of 3·24, 2·72, 3·04, 2·39, 2·58 and 2·78.

This variation from sample to sample of serum is important because it has been shown that the percentage inhibition caused by a given concentration of an inhibitor varies with the activity of the uninhibited enzyme preparation[15].

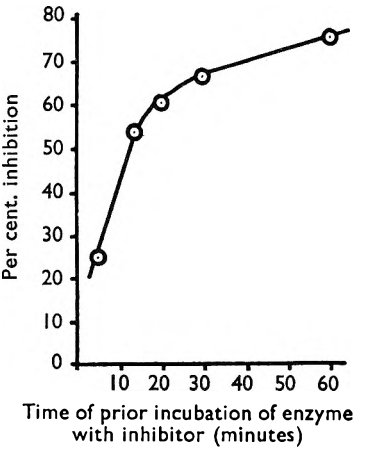

FIG. 5. Relation between the time of prior incubation of neostigmine with enzyme, and the degree of inhibition produced. Temperature 40° C. pH 7·9. ACh concentration $1·22 \times 10^{-2}$M. Neostigmine concentration $6·0 \times 10^{-8}$M. 1 ml. of horse serum as the source of enzyme.

Determination of the Percentage of ψChE Caused by Neostigmine

The percentage inhibition of ψChE caused by neostigmine varies with the time of "prior contact" between the enzyme and inhibitor before the addition of the substrate. Figure 5 shows that under the conditions used, the inhibition is not maximal even after 60 minutes prior contact. However, the curve begins to flatten after 15 minutes and this period of time was chosen for prior contact in the assay. To determine percentage inhibition, two ψChE determinations on the same sample of serum were carried out simultaneously, but whereas the control (A_{15} value = A) contained the usual 43 ml. of water, the test (A_{15} value = B) contained 38 ml. of water plus 5 ml. of a suitable concentration of neostigmine. The per cent. inhibition was $\frac{A-B}{A} \times 100$.

Figure 6 shows that the graph relating the negative logarithm of the molar concentration of neostigmine and the per cent. inhibition is a straight line in the region of 50 per cent. inhibition.

The Assay of Injection of Neostigmine Methylsulphate. According to Figure 6, 50 per cent. inhibition of ψChE is brought about by a $3·6 \times 10^{-8}$ M concentration of neostigmine. Therefore, to assay the Injection or indeed any solution of a salt of neostigmine, it is first quantitatively diluted until the concentration is approximately $3·6 + 10^{-8}$ M. From the experimentally determined per cent. inhibition caused by this dilution, the exact concentration of neostigmine in the digest can be read off from Figure 6 and the concentration in the injection calculated.

An injection was made up containing exactly 0·5 mg. neostigmine methylsulphate in 1 ml. A "first dilution" was prepared by diluting 1 ml.

accurately measured (for greater accuracy the injection may be weighed) to 250 ml. with water. Ten ml. of the first dilution diluted to 100 gave the "second dilution". Five ml. of the second dilution in a 50 ml. digest gave a concentration of $5{\cdot}98 \times 10^{-8}$ M neostigmine, hence from Figure 6 approximately 60 per cent. inhibition should result. Six separate assays on an injection made up to contain exactly 0·5 mg. of neostigmine methylsulphate per ml., gave per cent. inhibitions of 62·6, 62·9, 62·1, 62·3, 60·0 and 61·5: (average value = 61·9). A 2 per cent. variation in inhibition corresponds approximately to a 10 per cent. variation in concentration of inhibitor. Thus, by this method the injection can be assayed to within approximately 10 per cent. This conclusion is confirmed by the results recorded in Table II.

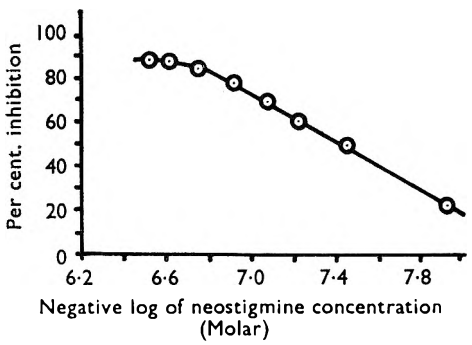

FIG. 6. Relation between concentration of neostigmine and the degree of inhibition produced. Temperature 40° C. pH 7·9. ACh concentration $1{\cdot}22 \times 10^{-2}$ M 1 ml. of horse serum as source of enzyme. Prior incubation of enzyme with neostigmine 15 minutes.

There are several objections to such an assay process. (1) The preparation of the calibration graph (Fig. 6) is time-consuming and since, as pointed out earlier, the per cent. inhibition caused by a given concentration of inhibitor varies with the ψChE activity of the serum used, a new calibration curve would be necessary at least for each sample of

TABLE II

VARIATION IN THE DEGREE OF INHIBITION OF ψChE WITH SMALL CHANGES IN INHIBITOR CONCENTRATION

15 MINUTES PRIOR CONTACT OF ENZYME WITH INHIBITOR AT 40° C.

Concentration of neostigmine in 50 ml. test digest $\times 10^{-8}$ M	A_{15} value		Inhibition per cent.
	Inhibitor absent	Inhibitor present	
5·38	2·29	1·16	49·3
5·68	2·36	1·16	50·1
5·98	2·20	1·06	51·8
6·28	2·28	1·02	55·2
6·58	2·30	1·01	56·1

serum. (2) The process is not very accurate (rather more than 10 per cent. error). It had, however, been noticed that simultaneous replicates gave closer results than successive replicates. Further, by comparing simultaneously a standard injection (of accurately known strength) with the injection to be assayed, it is possible to dispense with the preparation of a calibration curve. Based upon these considerations a process, perhaps best called a standardisation process rather than an assay, was

devised by means of which, in a single experiment, it can be proved that the strength of a sample of Injection of Neostigmine Methylsulphate does not deviate by more than 10 per cent. from the strength stated on the label.

The Standardisation of Injection of Neostigmine Methylsulphate

The Recommended Process

Prepare standard 1st and 2nd dilutions, as described above, from an accurately prepared aqueous 0·05 per cent. w/v solution (0·5 mg. per ml.) of neostigmine methylsulphate.

Prepare a test 2nd dilution from the injection to be examined, but in this case make the dilutions so that, assuming the strength of the neostigmine methylsulphate stated on the label to be correct, the test 2nd dilution will contain exactly the same concentration of neostigmine as the standard 2nd dilution.

In three tubes prepare three digests each containing 1 ml. cresol red solution, and 1 ml. horse serum, but in A 38·5 ml. of water + 4·5 ml. of standard 2nd dilution, and in B 38·0 ml. of water and 5·0 ml. of test 2nd dilution and C 37·5 ml. of water + 5·5 ml. of standard 2nd dilution. After mixing the contents of each tube, place the tubes in a water bath at 40° C. for 15 minutes before determining the remaining ψChE activity as described above. The injection under test is satisfactory if residual enzyme activity of digest B lies between those of digests A and C, i.e., if the A_{15} value of B lies between the A_{15} value of A and C.

Results

Table III shows the results of applying the proposed Method of Standardisation to a sample of Injection of Neostigmine Methylsulphate containing exactly 0·5 mg. per ml.

Two samples of serum were used, differing considerably in activity, but in each one of the 16 experiments the activity of the injection lay between the results corresponding to 10 per cent. excess and 10 per cent. deficiency.

Discussion

From the results reported inhibition of ψChE is a method which can be applied to the determination of anticholinesterase drugs like neostigmine in the dilute solutions used in pharmacy. 0·1 ml. would be more than sufficient for use in the assay of Injection Neostigmine Methylsulphate. Using a calibration curve relating enzymic inhibition of a particular sample of horse serum to the concentration of neostigmine present, this injection may be assayed with an error of not more than 10 to 15 per cent., depending upon the number of assays carried out. Using the comparison method described, the results recorded in Table III establish that a single experiment is capable of deciding whether a solution containing neostigmine (e.g., the injection) is of the strength stated on the label with less than a 10 per cent. error. It seems reasonable to

TABLE III

RESULTS OBTAINED BY TESTING A SAMPLE OF INJECTION OF NEOSTIGMINE METHYLSULPHATE BY THE PROPOSED METHOD. CONCENTRATION OF ACh = 0·0122 M. TEMPERATURE = 40° C. TIME OF PRIOR INCUBATION = 15 MINUTES

Serum	A_{15} digest containing 4·5 ml. of standard second dilution A	Difference A–B	A_{15} digest containing 5 ml. of test second dilution B	Difference B–C	A_{15} digest containing 5·5 ml. of standard second dilution C
A46750	1·48	0·08	1·40		
			1·35	0·13	1·22
	1·47	0·10	1·37		
			1·31	0·06	1·25
	1·54	0·09	1·45		
			1·40	0·03	1·37
E57060	2·56	0·02	2·54	0·04	2·50
	2·32	0·15	2·17	0·04	2·13
	2·50	0·12	2·38	0·06	2·32
	2·35	0·12	2·23	0·11	2·12
	2·27	0·02	2·25	0·14	2·11
	2·35	0·27	2·08	0·14	1·94
	2·35	0·36	1·99	0·06	1·93
	2·40	0·14	2·26	0·13	2·13
	2·32	0·19	2·13	0·13	2·00
	2·32	0·00	2·32	0·22	2·10

suggest that in the future, enzyme inhibition will become a recognised method for the determination of small quantities of certain drugs in such pharmaceutical preparations as tablets and injections.

SUMMARY

1. The suitability of cholinesterase inhibition as a method of assaying pharmaceutical preparations containing small quantities or low concentrations of certain drugs has been discussed and investigated with solutions containing neostigmine.

2. A method of assay for neostigmine accurate to within 10 to 15 per cent. has been elaborated.

3. A standardisation process has been described capable of ensuring, without the necessity of averaging replicate results, that a sample of Injection of Neostigmine Methylsulphate has a strength not more than 10 per cent. in excess or deficiency of the strength stated on the label.

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DISCUSSION

The paper was presented by the CHAIRMAN.

DR. D. C. GARRATT (Nottingham) said that the assay was really a challenge to the analyst in that the drug was simple in structure and should be quite capable of assay by conventional chemical means or, in small quantities, by physical methods. It would seem that the bacteriostatic prevented normal methods being used.

DR. G. E. FOSTER (Dartford) said he had repeated the authors' work, but he had been concerned about the very small titrations obtained. In his view it would be better to dilute the caustic soda to half strength. He considered the results presented in the paper should have been accorded statistical treatment.

MR. K. L. SMITH (Nottingham) said he was concerned that the authors had omitted to use the tools provided by the statistician. It was usual to apply statistical methods to those assays in which the result could not be established by theory and was subject to some variance. The assay under discussion fell into that category. Having had an opportunity of using the method with mustine hydrochloride, his impression was that the slope of the dosage response curve was much flatter than for neostigmine.

MR. A. R. ROGERS (Brighton) expressed the view that 15 minutes of prior incubation represented a short time in regard to the changing slope of the graph (Fig. 5) and that 25 minutes might not perhaps make the assay unduly long. It would give a better chance of obtaining good reproducibility.

The CHAIRMAN, in reply, said the Statistics Department of the University had been consulted, and the error had been assessed as being less than 10 per cent. If it were desired to carry out an estimation of how much nearer than 10 per cent. one could get, statistics would be required. It would be necessary to do a whole series of triplicates or 20 series of separate determinations. Dr. Foster's suggestion about using a more dilute caustic soda solution was a good one. There was no reason why 25 minutes for the prior incubation period should not be used. Accuracy to within 10 per cent. was obtained by using 15 minutes prior incubation.

HIGH FREQUENCY TITRATIONS IN PHARMACEUTICAL ANALYSIS

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From The British Drug Houses Ltd., London and Poole

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During the last ten years, considerable attention has been directed, particularly in the United States of America, to the detection of the end-point of a titration by the use of high-frequency methods. In ordinary conductometric analysis, two platinum electrodes are immersed in the solution and the change in electrical conductivity during the titration measured by means of a bridge circuit energised by an alternating current with a frequency of about 2000 cycles per second. This method has several disadvantages; the electrodes are costly and require careful washing and storing if their sensitivity is to be maintained, while in some instances the presence of the electrodes may have undesirable effects on the titration. In high frequency methods the electrodes are placed outside the titration vessel and by virtue of the high frequency of the energy applied to the electrodes, coupling between them and the solution takes place through the walls of the cell.

Measurements on systems of this kind are a composite function of both the dielectric constant and the conductance of the sample solution, as well as the cells walls and of any air space between these and the electrodes, and any system which displays significant changes in either or both of these properties with varying concentrations or composition will give a satisfactory high frequency end-point.

Instruments

The types of instrument used in high frequency titrations can be classified into several groups according to the electrical property actually measured. If the sample container is introduced into the tank coil of an oscillator, energy is absorbed from the circuit to an extent largely dependent upon the conductivity of the sample. This loss of energy can be followed by measuring the change in various circuit factors of the oscillator such as anode, grid or cathode voltages or currents as the conductivity of the sample changes during the titration. Instruments of this type have been described by a number of authors including Dowdall, Sinkinson and Stretch[1] with whose instrument we have had considerable experience.

The second group of instruments comprises those which indicate a change in capacitance of the cell by a change in frequency of the oscillator, which is measured either by mixing the output with that of a standard oscillator and measuring the change in frequency of the beat note produced or by some form of frequency discriminator. In addition to these two broad groups, a number of instruments have been devised which respond to both the conductance changes and the capacitance changes[2,3], while Hall and Gibson[4] have described the use of a high frequency

impedance bridge by which one can measure both the capacitance and conductance changes of a suitable cell independently.

Cell Design

The design of a cell suitable for high frequency titrations depends to some extent upon the type of instrument used for detecting the change in electrical properties of the sample during the titration. Blake[5] suggested the use of a glass tube with two metal bands round it to form the electrodes (Fig. 1) and he used this type of cell in his "Rectified Radio-frequency Impedance" method whereby energy from a signal generator was applied between one electrode and earth and the change in high frequency current

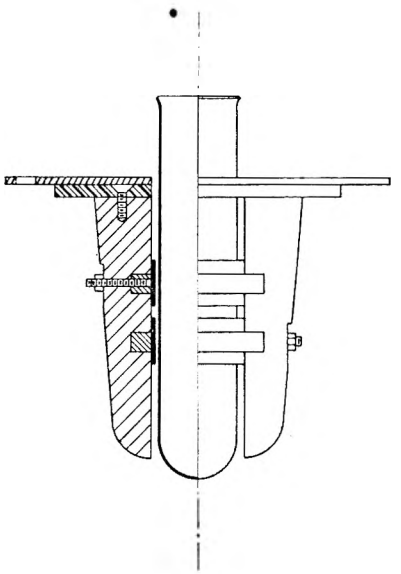

FIG. 1. Band type cell (after Blake).

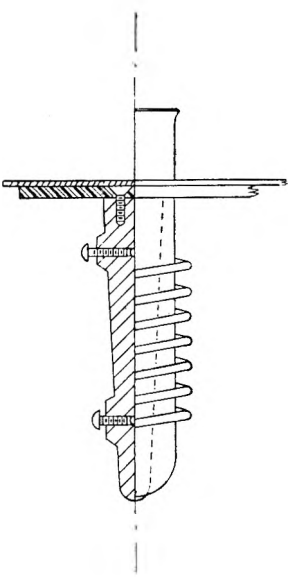

FIG. 2. Coil type cell.

measured, after rectification, between the other electrode and earth. We have used this method and we find that it is relatively insensitive to small conductance changes in solution; we also experienced some difficulty caused by the formation of resonant peaks in the response curve due to the band electrodes and the self-capacitance of the crystal rectifier.

This type of cell can be used with instruments measuring capacitative changes and Reilley and McCurdy[6], and Clayton, Hazel, McNabb and Schnable[7] have investigated the effect of cell geometry on the type of response obtained, the former using an impedance bridge and the latter a frequency discriminator type of instrument.

The coil type of cell (Fig. 2) is used in many published designs and, although it is somewhat more difficult to analyse the behaviour of such cells theoretically than is the case with the capacitor type cell, experience suggests that the choice between the two types must mainly depend upon the design of the instrument employed. In certain titrations, however,

we have found that while a satisfactory response is to be obtained with a coil type cell, no change of slope is obtained with the capacitor-type cell (Figs. 1 and 3) when using an instrument adapted for use with either type.

EXPERIMENTAL

In this work, we have used three methods of measuring the electrical changes at the end-point of a titration.

1. The tuned-anode-tuned-grid oscillator of Dowdall, Sinkinson and Stretch[1], was built with the modification suggested by Lane[8]. The layout of the circuit was varied from that suggested by the originators of the design so that it could be incorporated into a small steel instrument case. This entailed external connections to the batteries, but this has not in practice been found to have any disadvantages. As in the original, the titration cell was accommodated through a hole in the top of the case and the anode coil was rigidly mounted on a polystyrene frame secured to the inside of the case so that the cell could easily be removed for cleaning without disturbing the coil. The change in anode current during the titration was indicated by a Scalamp taut-suspension mirror galvanometer. The nominal frequency of oscillation of this instrument is approximately 16 megacycles per second.

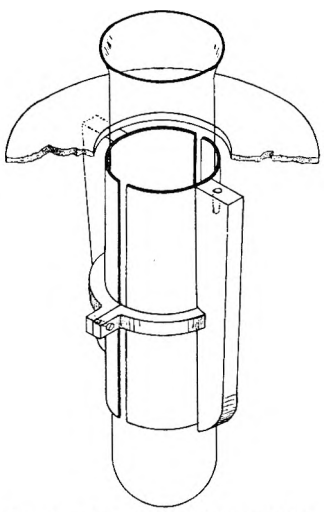

FIG. 3. One form of capacitative type cell.

2. Blake's "Rectified Radio-frequency Impedance" method[9,10], the circuit of which is shown in Figure 4 has been applied to a number of titrations. Blake described an oscillator giving energy at a frequency of 1175 kilocycles per second, but in an attempt to extend the range of concentrations to which this apparatus could be applied, we used an

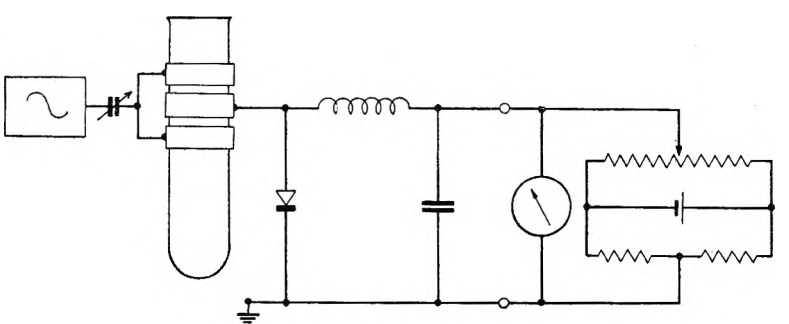

FIG. 4. Circuit of Blake's rectified radio-frequency impedence apparatus.

Advance Type E2 signal generator, taking the unmodulated ouput from the "full R.F." socket (1 volt into 1000 ohms). The frequency range of this instrument extends to 100 megacycles per second but reasonable responses could only be obtained at the resonance peaks, which with the particular cell and detector circuit used, occurred at 45 megacycles per second with the first harmonic slightly less pronounced at 90 megacycles per second. At other frequencies, the changes of current indicated by the galvanometer during the titration were too small to be of use. The occurrence of these resonant peaks is undoubtedly a function both of cell design and of rectifier design and modification of these portions of the apparatus may well enable satisfactory response curves to be obtained over a range of frequencies.

3. Using a coil type cell, any change in the electrical properties of the contents will affect the "Q-factor" of the coil. This can be defined as the ratio of the energy stored in the coil to the energy dissipated and it can be measured by means of a circuit magnification meter. In this instrument the coil is made part of a resonant circuit which is fed with a small voltage of known frequency from a generator of negligible impedance and the voltage drop across the coil measured at resonance by a valve voltmeter of negligible admittance.

Blake[5], uses the term "Q-metric" to describe the method whereby he measures the damping effect of a solution in a band-type cell on the Q-factor of a capacitor in the tank circuit of an oscillator when the cell is connected in parallel with the capacitor. The actual measurements made are of the anode current of the oscillator; thus the principle of the method is similar to that described by Dowdall, Sinkinson and Stretch[1]. Several types of circuit magnification meters are available commercially and we have used this method with both the Marconi circuit magnification meter type TF329G and the Advance "Q" meter.

In our experience, the most generally applicable instrument is the circuit magnification meter. The tuned-anode-tuned grid oscillator has been applied to a great many titrations satisfactorily, but it is somewhat more sensitive to the concentration of the solution in the cell and, although the formation of a precipitate during the titration adversely affects both instruments, this is not so marked with the Q-meter. We have not found Blake's rectified radio-frequency impedance method so sensitive as either of the above instruments; with non-aqueous titrations in fact, very little change in impedance appears to take place at the end-point. The results presented below, have been obtained using either the Advance Q-meter or the tune-anode-tuned-grid oscillator; all the titrations have been carried out with both instruments and, unless otherwise stated, either is equally suitable.

Results

The precise method of making a titration depends upon the particular instrument used. The cells were designed to take 1 in. diameter Pyrex glass boiling tubes and the following details are generally applicable.

Transfer a suitable quantity of the sample solution to the cell, dilute to

such a volume that the electrodes (bands or coil, etc.) are below the level of the liquid and titrate by adding the titrant in increments, stirring and reading the instrument after each addition. In our experience the best method of stirring is by means of a stream of nitrogen gas bubbles, the gas flow being stopped while a reading is taken; a satisfactory degree of

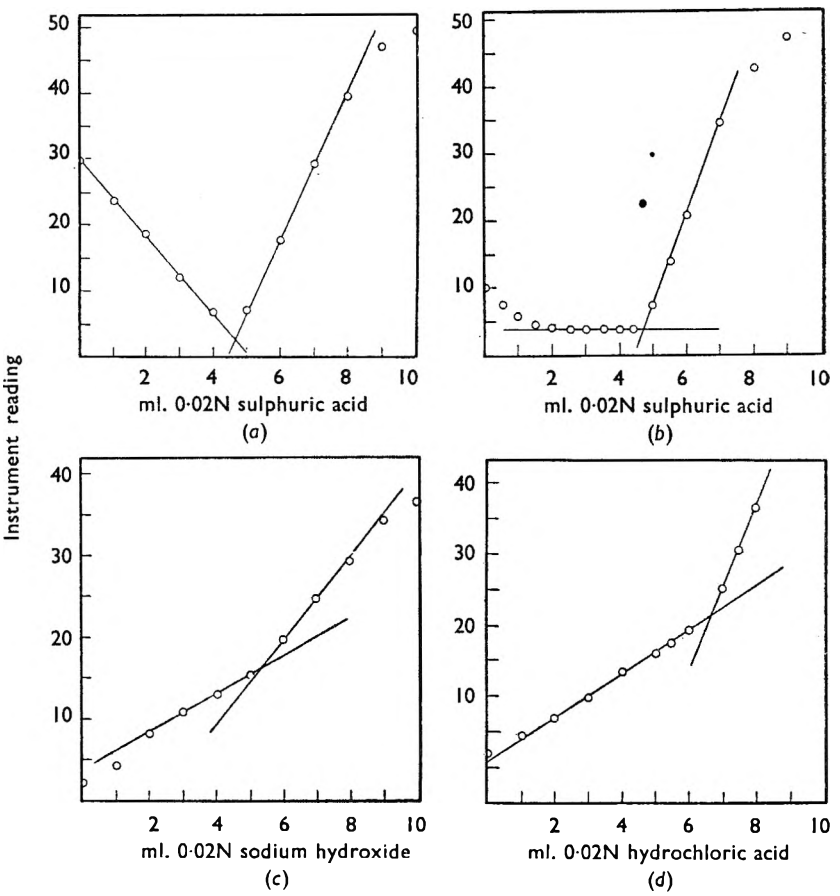

FIG. 5. Acid-base titration curves. (*a*) Sulphuric acid–sodium hydroxide. (*b*) Sulphuric acid sodium carbonate. (*c*) Sodium hydroxide–acetic acid. (*d*) Hydrochloric acid–pyridine.

mixing is obtained by turning on the gas-cylinder supply for about two seconds after each addition of titrant. In the case of the Q-meter, it is advisable to adjust the circuit capacitance after each addition to give the maximum reading; this is easily done by means of the trimmer provided.

Acid-base Titrations

No difficulty has been experienced with simple acid-base titrations using any of the three methods described. Using the tuned-anode-tuned-grid oscillator, and plotting change in anode current against volume of

titrant added, the findings of Dowdall Sinkinson and Stretch[1], have been confirmed and a representative selection of curves is shown in Figure 5. The optimum concentration for this apparatus is about 0·02N and we obtained the best results by diluting the sample under examination to about this concentration in base in the titration vessel and titrating it with 0·1N or 0·05N acid from a micro-burette. By this means undue dilution of the sample during the titration, with consequent production of curved plots, is minimised. Using Blake's method and applying energy at a frequency of 45 megacycles per second, good results were obtained at a concentration of 0·1N.

A sample of sodium phosphate B.P. has been assayed by titration with 0·02N hydrochloric acid using the simple tuned-anode-tuned-grid oscillator. The curve produced is shown in Figure 6. From this the purity of the sample is 99·78 per cent. By the official method 99·74 per cent. was indicated. A similar result was obtained using the Q-meter.

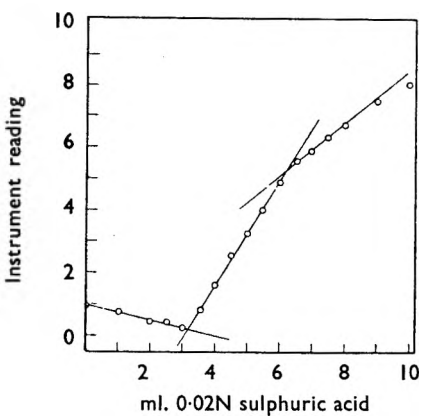

FIG. 6. Curve for the titration of sodium phosphate B.P. with sulphuric acid.

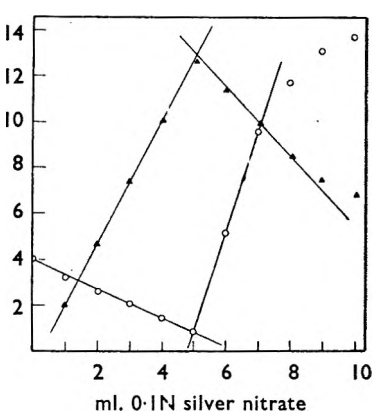

FIG. 7. Titration curves with silver nitrate. ○——○ Sodium chloride. ▲——▲ Hydrochloric acid.

Precipitation Reactions

(*a*) *Silver titrations.* No difficulties have been experienced in the titration of halides with silver nitrate at a concentration of about 0·02N so long as the presence of an excess of acid is avoided. Thus, sodium chloride and hydrochloric acid give normal response curves with a well-defined break at the end-point but the addition of hydrogen ion beyond that equivalent to the halide present increases the overall conductance of solution to an extent that masks any change at the end-point. Standardised solutions were used to obtain the curves reproduced in Figure 7.

Ammonium chloride, which is assayed in the B.P. by a modified Volhardt procedure, can be titrated directly. The curve is given in Figure 8 and the indicated purity of the sample used is 99·76 per cent. The corresponding figure by the official method is 99·81 per cent.

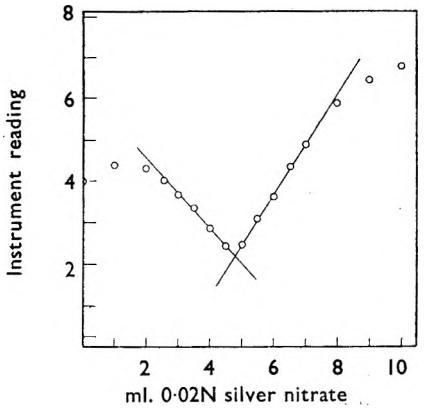

FIG. 8. Titration curve of ammonium chloride with silver nitrate.

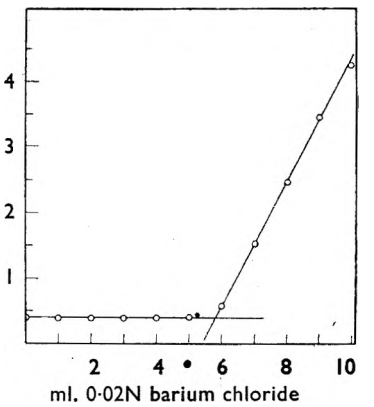

FIG. 9. Titration curve of sodium sulphate with barium chloride.

(*b*) *Titration of sulphate.* Milner[11], has reported that if the titration of sulphate with barium chloride solution is conducted in the presence of ethanol and is seeded with a trace of barium sulphate, a satisfactory end-point is given using an oscillator virtually identical with that of Dowdall, Sinkinson and Stretch, so long as the excess electrolyte calculated as sodium chloride is kept below a concentration of about 0·03M. Reasonable results have been obtainable only in the absence of excess of electrolyte and at a concentration of 0·02 to 0·05N. Within these limitations, a direct determination of sulphate is possible and the curve obtained by assaying sodium sulphate B.P. by titration with barium chloride under conditions recommended by Milner is given in Figure 9. The indicated end-point gives an assay figure of 99·6 per cent., while the official procedure carried out on the same sample gives 99·45 per cent., both calculated as the dry material. This swamping effect of excessive quantities of electrolyte, more particularly of hydrogen ion, is a major disadvantage of the apparatus used in this work. However, it is probable that the use of frequencies of the order of 200 megacycles per second and above may eliminate this. Work on devices employing these very high frequencies is in hand.

Non-aqueous Titrations

The most immediately useful application of the high frequency method of end-point detection is in non-aqueous titrations. Visual indicators are rarely entirely satisfactory and are, in fact, empirical, being based upon potentiometric results.

Using the simple oscillator described above, Lane[8] has shown that accurate and precise indications of end-point can be obtained with a variety of titrations and we have confirmed his work. In our hands, the method of Blake has proved insufficiently sensitive where non-aqueous media are concerned, whereas the circuit magnification meter with a coil type cell can give a satisfactory indication of the end-point in many cases. We have found that titrations using acetous perchloric acid give the best

results with this instrument and curves obtained in the standardisation of acetous perchloric acid with potassium hydrogen phthalate are given in Figure 10. The tuned-anode-tuned-grid oscillator cannot be used satisfactorily with this particular titration.

Beckett, Camp and Martin[12], have shown that the alkali metal salts of aliphatic and aromatic acids can be determined by titration with perchloric acid in glacial acetic acid solution, but they point out that the indicator colour changes at the end-point are affected by the type of cation present.

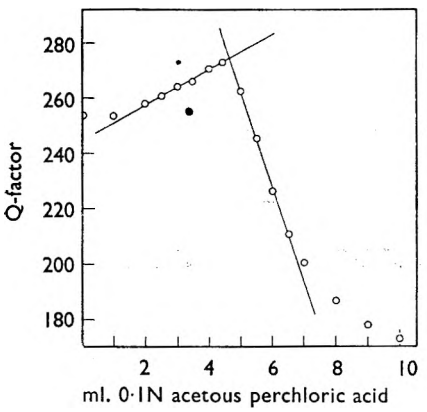

FIG. 10. Curve for the standardisation of 0·1N acetous perchloric acid with potassium hydrogen phthalate.

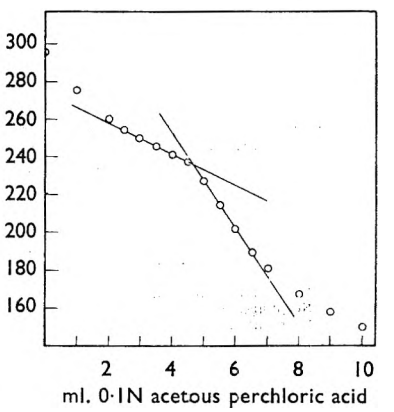

FIG. 11. Titration curve for sulphanilamide with acetous perchloric acid.

Using the Q-meter, good indication of end-point has been observed with a representative selection of this type of compound as shown in Table I.

Sulphonamides can be titrated satisfactorily with perchloric acid in glacial acetic acid[13]. Figure 11 shows the high-frequency titration curve of sulphanilamide; the results of the assay of a number of sulphonamide tablets are given in Table II together with the results by the official method for comparison. These have been obtained by crushing several tablets to a fine powder and warming a weighed portion (such that the solution in the cell was approximately 0·02N) with glacial acetic acid containing a trace of acetic anhydride. The sulphonamide dissolves but much of the excipient remains insoluble; this can be ignored and the mixture titrated with 0·1N acetous perchloric acid as described above. It is preferable to use the Q-meter for this particular titration.

The Q-meter is to be preferred for these titrations, but both this and the tuned-anode-tuned-grid oscillator can be used.

Potassium methoxide in benzene and methanol has not proved very satisfactory when the potassium salt of the acid being titrated is insoluble in the solvent system used. Precipitation appears to cause random deflections as the end-point is approached and it is difficult to select the exact breakpoint of the curve with certainty. This instability in the presence of a precipitate was reported by Lane[8], who found that in the reactions he studied, satisfactory indication of end-point could be obtained

TABLE I

COMPARISON OF OFFICAL ASSAY METHOD WITH THE HIGH FREQUENCY METHOD USING ACETOUS PERCHLORIC ACID

Chemical	Purity per cent.	
	Official method	High frequency method
Sodium citrate	99·70	99·70
Sodium benzoate	99·91	99·87
Sodium salicylate	99·90	99·86
Potassium citrate	99·90	99·90
Potassium benzoate	99·83	99·85
Potassium tartrate	99·98	99·89

TABLE II

COMPARISON OF THE OFFICIAL ASSAY METHOD FOR SOME SULPHONAMIDE TABLETS WITH THE HIGH FREQUENCY METHOD USING ACETOUS PERCHLORIC ACID

Tablet	Assay	
	Official method g. per tablet	High frequency titration g. per tablet
Sulphaguanidine (Sample I) ..	0·503	0·505
Sulphaguanidine (Sample II) ..	0·509	0·508
Sulphadiazine	0·505	0·501
Sulphathiazole	0·517	0·509

by taking the instrument reading at the same interval of time after adding each increment of the titrant. We have not found that this control of the time intervals during the titration effected any marked improvement in the particular reactions we studied. For the titration of very weak acids in non-aqueous media, Harlow, Nobel and Wyld[14], describe the preparation and use of tetrabutylammonium hydroxide and the possibility of detecting the end-point by high frequency means when this substance is used as titrant is being examined.

SUMMARY

1. A description is given of simple instruments which provide a satisfactory indication of the end-point of a titration using high-frequency energy.

2. The application of high-frequency titrations to a number of pharmaceutical analyses has been described.

3. The method is shown to give results comparable with those of the official assays.

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DISCUSSION

The paper was presented by MR. J. ALLEN.

DR. F. HARTLEY (London) said it was clear that the method had considerable potentialities. To be able to place electrodes outside the vessel in which the material was being titrated offered obvious advantages, and one would suppose that provided the excipients themselves were non-electrolytes there was no reason why the method should not be applied to some otherwise quite intractable mixtures.

MR. C. A. JOHNSON (Nottingham) said unfortunately the paper did not show that the method had any advantage over other available methods. In an American abstract of a paper read by Dr. Stuckey in May on the use of complexones in analytical work, he was reported as having said that end-point determinations in E.D.T.A. titrations often presented difficulties, and high frequency titration could be used with advantage. It was a pity that this was not brought out in the paper.

DR. A. H. BECKETT (London) said the authors were critical of Blake's method. They had changed the frequency in an attempt to extend that method's range and scope, whereas he had found that the electrode size and spacing was far more important than the frequency. For instance, at 2·5 megacycles, 1·0 cm. electrodes 1 mm. apart were suitable for about 0·1 to 0·2N, whereas 2 cm. electrodes similarly spaced were useful for about 0·1 to 0·01N. It was the easiest method to manipulate and had the advantage of being free from error due to thermal effect in the solution. There were no end capacity effects and crystal control could be easily used. In the non-aqueous titration field, Blake's method was at a disadvantage, because it was dielectric constant rather than conductance changes that were taking place in the medium. The authors seemed to be using a large volume of solution, and it would be interesting to apply the method to small amounts for measuring rates of hydrolysis.

MR. E. H. B. SELLWOOD (London) asked if the authors had any experience of the use of the simple oscillator of Alexander, consisting of a magic eye tuning indicator which functions as a crystal controlled oscillator and gives an indication of end-point at the same time. It was designed primarily for determination of dielectric constant and was based on the capacitance effect.

MR. J. ALLEN, in reply, emphasised that the paper was in the nature of an introduction. The instruments used were "low definition instruments". There were other high definition instruments with which one could obtain almost unparalleled sensitivity. It had been suggested that higher frequencies than those used might overcome the difficulty caused by electrolytes. That was in fact the case. At 250 megacycles one could

titrate satisfactorily in the presence of 10 per cent. potassium chloride. It was true that in the paper no application had been suggested which was better than standard methods. However, the method outlined was quicker than the normal potentiometric procedure, because it was not essential to plot many points around the end-point. As to the end-point of the compleximetric titrations, there were a number of anomalies which they were unable to explain at present. The Blake method had been tried, but it was insufficiently precise for any reasonable degree of analytical accuracy. The volume of solution depended upon the precision of the measurement of capacitance change. If one started with large capacitance one could measure small changes; but it was very difficult to measure smaller changes if the total capacitance were small. The only instrument which might satisfactorily do that was the very costly Twin "T" impedance bridge. The Alexander type of oscillator was applied to analytical work by Hall in the United States about 1952 and although in theory the use of the crystal controlled oscillator, which could be tuned in and out of oscillation by altering the capacitance in the anode circuit of the valve, and the use of the magic eye to indicate the sudden change in voltage as the valve came into and out of oscillation, was attractive, in fact the apparatus was not satisfactory because it "pulled". This means that different values of capacitance are obtained whether one begins with the instrument oscillating and tunes until it just stops or vice versa. Further, since this "pulling" is non-symmetrical about the critical point, depending on external conditions, it is not practicable to take a mean.

A METHOD OF DETERMINING BINARY MIXTURES BY DISTRIBUTION MEASUREMENTS, AND ITS APPLICATION TO THE ASSAY OF STRYCHNINE IN THE PRESENCE OF QUININE

By C. Morton and E. H. Tinley

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The combined determination of strychnine and quinine in various pharmaceutical preparations, and the estimation of strychnine in the presence of brucine, are typical examples of the analysis of binary mixtures. In the assay of alkaloidal mixtures, it is customary to rely upon repeated extractions with immiscible solvents, followed by further treatment of the residue obtained by extraction, as a means of separating the components of the mixture. The labour and cumulative losses incurred in the separation are often considerable, and it has been suggested that extraction processes should be replaced by instrumental methods of analysis. Infra-red and ultra-violet spectroscopy have been used in the estimation of mixtures of strychnine and brucine[1–3], and an ultra-violet spectrophotometric assay process for the combined determination of strychnine and quinine has been described[4]. In general, however, spectrophotometric determinations of binary mixtures suffer from the drawback that tedious calculations involving either the solution of simultaneous equations or the use of successive approximations are necessary in order to evaluate the results of the absorption experiments.

The distribution coefficient of a substance between suitably chosen immiscible solvents resembles its extinction coefficient at a given wavelength in being a specific physical property, characteristic of the substance. It is readily deduced from the Distribution Law that, when a mixture of two substances is dissolved in a suitable solvent and shaken with an immiscible solvent, distribution takes place in such a manner that the total weight of mixed solutes in each phase at equilibrium is a linear function of the composition of the original mixture. Thus if the weight of solutes in either phase is determined, the relative proportions of the components in the original mixture may be read on a linear graph connecting the weight of residue with the per cent. composition. For example, a rapid and accurate combined determination of strychnine and quinine may be carried out merely by dissolving a known weight of the mixed alkaloids in N hydrochloric acid and determining the weight of solutes obtained by one extraction with chloroform.

General procedure

It is shown in the theoretical section of this communication that the weight W of residue obtained by extracting a solution of 1 g. of a mixture of two substances A and B with an immiscible solvent is related to the percentage A_0 of the component A in the mixture by the expression

$$A_0 = K_1 W - K_2 \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where K_1 and K_2 are constants. If the distribution coefficients of the substances A and B between the selected solvents are known, K_1 and K_2 may be calculated, and a linear graph connecting the weight W of the residue with the composition of the mixture may be constructed with the aid of equation (1). A more reliable method is to construct an empirical graph from data obtained by using mixtures of known composition approximating to that of the samples for analysis; by this means, errors due to such sources as deviations from the Distribution Law, mutual solubility of the solvents, or the presence of traces of impurities in the solvents, may be eliminated.

Success in the attempt to apply this method to a given determination depends upon the practicability of selecting solvents with which a favourable ratio of the distribution coefficients of the two substances is obtained. The conditions which must be satisfied in order that high accuracy may be attained are discussed in the following sections. The method is capable of extension to mixtures of three or more components, but the requirements are then more exacting; in order to evaluate completely a mixture of m components, it is necessary to make use of (m − 1) pairs of solvents.

Theory

The theoretical basis of the method is as follows. Let x g. of a mixture of the substances A and B be dissolved in V_1 ml. of a solvent S_1, and let the solution be extracted with n successive volumes, each of V_2 ml. of an immiscible solvent S_2. Let the amounts of A and B remaining in the solvent S_1 after the first, second and nth extraction be a_1, a_2, a_n and b_1, b_2, b_n respectively, and let the partition coefficients of A and B between solvent S_1 and solvent S_2 be k_a and k_b respectively. If $V_2/V_1 = r$ and the weights of A and B initially present in solvent S_1 are a and b respectively, then after the first extraction we have $k_a = a_1 r/(a - a_1)$ and $k_b = b_1 r/(b - b_1)$ whence

$$a_1 = aK_a \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

and

$$b_1 = bK_b \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

where

$$K_a = k_a/(k_a + r) \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (4)$$

and

$$K_b = k_b/(k_b + r) \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (5)$$

Similarly, after the second extraction $k_a = a_2 r/(a_1 - a_2)$ and $K_b = b_2 r/(b_1 - b_2)$ or $a_2 = a_1 K_a$ and $b_2 = b_1 K_b$. It follows from equations (2) and (3) that $a_2 = aK^2_a$ and $b_2 = bK^2_b$. In general terms, the amounts of the substances A and B remaining in solvent S_1 after n extractions are $a_n = aK^n_a$ and $b_n = bK^n_b$ respectively, and the total weight of mixed solutes recoverable from the solvent S_2 after n extractions is

$$w = (1 - K^n_a)a + (1 - K^n_b)\, b \quad .. \quad .. \quad .. \quad .. \quad (6)$$

Substituting $b = x - a$ in this equation we obtain $100a = wK_1 - K_2x$

where $K_1 = 100/(K^n_b - K^n_a)$ .. .. (7)

and $K_2 = K_1(1 - K^n_b)$ .. .. (8).

The percentage of the substance A in the original mixture, viz.,

$A_0 = 100a/x$, is thus given by $A_0 = K_1W - K_2$ .. .. (1)

where $W = w/x$ is the weight of mixed solutes recovered from 1 g. of the original mixture.

When values of A_0 are plotted as abscissae against values of W as ordinates, a linear graph is obtained which, when produced, cuts the A_0 axis. The intercept on this axis gives K_2, and the slope of the graph is equal to $1/K_1$. When the sample for analysis consists merely of the component B, i.e., when $A_0 = O$, the weight of residue obtained from 1 g. of the sample is $1 - K^n_b$; similarly, it is readily shown that if only the component A is present ($a = x$) a residue $1 - K^n_a$ is obtained. As n and *r* are included in the terms K_1 and K_2, it follows that, throughout a given determination, the total number of extractions, and the ratio *r* of the volumes of the two phases, must be constant. In the following analysis of the conditions which must be satisfied in order that high accuracy may be attained, a ratio *r* of unity has been assumed for convenience; under these conditions, equations (4) and (5) simplify to $K_a = k_a/(k_a + 1)$ and $K_b = k_b/(k_b + 1)$ respectively.

Accuracy

Evidently, the highest accuracy is attained when the increment in the weight of residue due to a change of 1 per cent. in the proportion of the substance A is large; in other words, the solvents should be so chosen that the slope of the W/A_0 graph, viz.,

$$dW/dA_0 = 1/K_1 = (K^n_b - K^n_a)/100 \quad .. \quad .. \quad .. \quad (9)$$

is as steep as possible. When $k_a = k_b$, the slope dW/dA_0 is zero, and the weight of residue obtained is independent of the composition of the mixture. On the other hand, if k_a is very small and k_b is large, K^n_a and K^n_b approach zero and unity respectively, and the graph attains a maximal slope $dW/dA_0 = 1/100$, i.e., an increment in the weight of residue of 10 mg. per g. of mixture is obtained for a change in composition of 1 per cent. Assuming that the weight of residue obtained from 1 g. of the mixture is determined with an accuracy of $\pm$ y mg., the error in the determination of the substance A is $y/10(K^n_b - K^n_a)$; for example, when $y = \pm 1$ mg., $k = 10{\cdot}0$, and $k_a = 0{\cdot}5$, the error amounts to $\pm 0{\cdot}17$ per cent., if the determination is based upon a single extraction ($n = 1$) or $\pm 0{\cdot}14$ per cent. for $n = 2$. Thus whilst the highest accuracy is obtained when the solvents are so chosen that the ideal condition $k_b \gg k_a$ is satisfied, the requirements in this respect are by no means exacting. It is also clear that, in general, no substantial gain in accuracy is obtained by increasing the number of extractions. Indeed, calculation shows

that when $k_b < 5{\cdot}0$, the highest accuracy is attained by basing the determination upon a single extraction with the immiscible solvent.

In the preceding discussion, it has been assumed that the solutions behave ideally; complications due to such phenomena as association in the ethereal phase, dissociation in the aqueous solvent, or the influence of the solutes on the miscibility of the two layers have not been considered. In practice, errors due to deviations from the Distribution Law may be avoided by constructing the graph connecting the weight of residue with the composition of the mixture empirically from data obtained by using mixtures of known composition approximating to that of the samples for analysis.

The Assay of Strychnine in the presence of Quinine

Strychnine and quinine can be separated by extraction with chloroform from a strongly acid aqueous solution of the alkaloids. Evers[5] stated that strychnine can be removed completely from a solution in 2N hydrochloric acid by five extractions with chloroform, whereas quinine is only extracted to a very slight extent under these conditions. Haddock and Evers[6] used 0·5N hydrochloric acid semi-saturated with sodium chloride as the aqueous solvent, and subjected the residue obtained by five extractions with chloroform to further treatment in order to remove traces of quinine. Herd[7] separated the strychnine from a solution of the alkaloids in 7N sulphuric acid by five extractions with a 1 per cent. solution of dichloroacetic acid in chloroform. In the present work it was found that satisfactory results could be obtained by a single extraction with chloroform from a solution of the alkaloids in N hydrochloric acid.

Experimental

Preliminary experiments showed that when a solution of strychnine in N hydrochloric acid is shaken with chloroform, the weight of residue recovered by evaporating the chloroformic layer decreases by about 1 per cent. for 1° C. rise in the temperature of the solvents. In the experiments described below, solutions of strychnine and quinine were shaken with chloroform in a round bottomed flask immersed in a thermostat at 25° C. Equilibrium was attained after stirring the contents of the flask mechanically for one hour, a rubber sealed stirrer being used to prevent evaporation. The liquid was then allowed to separate into two layers. The chloroform was removed from the aqueous phase as quickly as possible, filtered through dry filter paper, evaporated to dryness, and the residue dried to constant weight at 105° C. All measurements of volume were made at 25° C.

Solutions of strychnine and quinine (0·2 per cent. w/v) in N hydrochloric acid were mixed in suitable proportions to produce a series of solutions in which the percentage of strychnine present in the total alkaloids varied between the limits of 0·0 and 100. A volume of 50 ml. of each solution was shaken with 100 ml. of chloroform as described above, and the weight of residue obtained by evaporating 75 ml. of the chloroformic solution was determined. A linear graph was constructed by plotting the weight W

of solutes obtained from 1 g. of the mixed alkaloids against the corresponding percentage A_0 of strychnine in the mixture, and from this the constants $K_1 = \delta A_0/\delta W$ and $K_2 = K_1 W - A_0$ of equation (1) were evaluated. The weight a (in g.) of strychnine in each mixture was calculated from the weight w of residue obtained from x g. of total alkaloids by means of the relationship $a = k_1 w - k_2 x$ where $k_1 = 0{\cdot}01\ K_1$ and $k_2 = 0{\cdot}01\ K_2$. The results are recorded in Table I.

TABLE I

$K_1 = 180{\cdot}0$ $K_2 = 2{\cdot}0$ $k_1 = 1{\cdot}80$ $k_2 = 0{\cdot}02$

Volume of 0·2 per cent. w/v quinine solution ml.	Volume of 0·2 per cent. w/v strychnine solution ml.	x = weight of total alkaloids taken g.	w = weight of residue from x g. of mixture g.	W = w/x = weight of residue from 1 g. of mixture g.	A_0 = per cent. of strychnine in the mixture	Weight of strychnine taken g.	Weight of strychnine found = a $= k_1 w - k_2 x$ g.
50	0	0·0917	0·0008	0·0087	0	0	−0·0004
45	5	0·0925	0·0066	0·0713	10·81	0·0100	0·0100
40	10	0·0934	0·0121	0·1296	21·42	0·0200	0·0198
30	20	0·0950	0·0234	0·2463	42·09	0·0400	0·0400
25	25	0·0959	0·0294	0·3067	52·16	0·0500	0·0508
20	30	0·0967	0·0347	0·3589	62·06	0·0600	0·0601
10	40	0·0983	0·0447	0·4546	81·35	0·0800	0·0780
5	45	0·0992	0·0503	0·5072	90·75	0·0900	0·0880
0	50	0·1000	0·0565	0·5650	100·0	0·1000	0·0990

In other experiments, mixtures approximating in composition to the residue obtained by complete extraction of the total alkaloids from 100 ml. of Easton's Syrup were prepared by adding amounts of strychnine varying from 0·0 to 0·05 g. to roughly equal weights (about 1·2 g.) of quinine. Each mixture was dissolved in 20 ml. of N hydrochloric acid and extracted with 50 ml. of chloroform at 25° C. The weight of residue obtained by evaporating 40 ml. of the chloroform layer was determined, and the values of k_1 and k_2 were calculated as described above. The results are recorded in Table II.

The samples of quinine and strychnine used in this work were assayed by means of moisture determinations and by titration with 0·5 N acetous perchloric acid, Oracet Blue B being used as indicator.

TABLE II

$K_1 = 215$ $K_2 = 1{\cdot}0$ $k_1 = 2{\cdot}15$ $k_2 = 0{\cdot}01$

Weight of anhydrous quinine taken g.	x = weight of total alkaloids taken g.	w = weight of residue from x g. of mixture g.	W = w/x = weight of residue from 1 g. of mixture g.	A_0 = per cent. of strychnine in the mixture	Weight of strychnine taken g.	Weight of strychnine found = a $= k_1 w - k_2 x$ g.
1·1130	1·1130	0·0051	0·0046	0	0	−0·0003
1·1856	1·1986	0·0116	0·0098	1·097	0·0130	0·0130
1·1421	1·1618	0·0145	0·0125	1·695	0·0197	0·0196
1·0667	1·0939	0·0194	0·0177	2·487	0·0272	0·0308
1·0815	1·1113	0·0191	0·0172	2·682	0·0298	0·0299
1·1904	1·2302	0·0246	0·0200	3·235	0·0398	0·0406
1·1573	1·2029	0·0269	0·0224	3·791	0·0456	0·0458

Conclusion

The weight a (in g.) of strychnine present in the weight x (in g.) of the mixture of strychnine and quinine obtained by complete extraction of the

total alkaloids from 100 ml. of Easton's Syrup may be determined by the following method:—

Dissolve the mixture of strychnine and quinine in 20 ml. of N hydrochloric acid, and shake the solution for one hour with 50 ml. of chloroform at 25° C. Separate the chloroformic layer, filter through dry filter paper, and evaporate 40 ml. of the filtrate to dryness. Determine the weight w (in g.) of the residue after drying to constant weight at 105° C. All volumes should be accurately measured.

The weight in g. of strychnine in the mixture of strychnine and quinine is given by $a = 2{\cdot}15w - 0{\cdot}01x$. Alternatively, the percentage $A_0 = 100a/x$ of strychnine in the mixture may be read on a linear graph constructed with the aid of the equation $A_0 = 215W - 1{\cdot}0$, where $W = w/x$ is the weight of residue obtained from 1 g. of the mixture.

Summary

1. When a mixture of two substances is dissolved in a suitable solvent and shaken with an immiscible solvent, the weight of mixed solutes present in each phase at equilibrium is a linear function of the per cent. composition of the original mixture, provided that the Distribution Law is obeyed; thus, if, after separation of the solvents, the weight of solutes in either phase is determined, the relative proportions of the components in the mixture may be read on a linear graph connecting the weight of residue with the percentage composition.

2. Errors due to deviations from the Distribution Law may be avoided by constructing the graph empirically from data obtained by using mixtures of known composition approximating to that of the samples for analysis.

3. A mixture of quinine and strychnine may be rapidly and accurately assayed by dissolving a known weight of the mixed alkaloids in N hydrochloric acid and determining the weight of residue obtained by one extraction with chloroform.

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DISCUSSION

The paper was presented by PROFESSOR W. H. LINNELL on behalf of the authors.

DR. F. HARTLEY (London) said if there were an appreciable quantity of alcohol present in the chloroform it would seem necessary to derive distribution figures for that particular sample, a procedure which destroyed some of the elegance and rapidity claimed for the method. To what extent was the method sensitive when up to 2 per cent. of alcohol was present in the chloroform?

DR. G. E. FOSTER (Dartford) said that the extraction method was based very largely upon work published by Dr. Evers some years ago. In that method the chloroform extract was shaken with ammonia in order to convert the alkaloids to bases. It would seem the authors evaporated the chloroform extract immediately. Was it strychnine hydrochloride or base which was weighed? He wondered why the chloroform extract was not tested with ammonia before evaporation. He also considered some of the chloroform additive compound was likely to be present.

MR. A. R. ROGERS (Brighton) said he could not agree that the fairly simple arithmetical calculations involved in spectrophotometric determinations were tedious, as was stated in the paper.

MR. R. L. STEPHENS (Brighton) asked whether the authors had considered the change of partition coefficient between the two substances with changes of temperature. The determination was carried out at 25° C. but the influence which $\pm 1°$ might have upon the precision of the results was not stated.

DR. D. C. GARRATT (Nottingham) said he felt that the method outlined was one which merited closer investigation. It would be interesting to know why it was necessary to take one hour to obtain equilibrium—one would have thought that equilibrium would be established in a short time and thereby the length of time of assay could be shortened.

PROFESSOR W. H. LINNELL, in reply, said he would refer the questions to the author.

MR. C. MORTON, in a written reply, states that the chloroform used was of Pharmacopœial quality, and the alcohol was not removed from it before use. When there are numerous samples for analysis, the most convenient procedure is to "calibrate" suitable solvents by means of standard mixtures, and reserve the solvents for use in the subsequent determinations. The linear relationship between composition and weight of residue is rigidly maintained, even when the proportion of strychnine in the mixture varies between the extreme limits of zero and 100 per cent. (Table I), and it is sufficient to use two standard mixtures in preparing the calibration graph. This method eliminates possible error due to varying alcohol content. In the absence of reliable information on the dissociation constants of strychnine, the degree of ionisation of its hydrochloride in aqueous solution, and the true distribution coefficients of strychnine and its hydrochloride between water and chloroform, it would be unwise to be dogmatic, but there can be little doubt that, under the conditions of the assay, the strychnine is present in both phases entirely as hydrochloride. It is also possible that the chloroform compound referred to by Dr. Foster is present in small amount. The observed distribution coefficient may accordingly be regarded as that of the hydrochloride rather than the free base, but is more correctly described as the apparent distribution coefficient of strychnine, i.e., the ratio of the concentration of the total alkaloid (in whatever forms it may be present) in the aqueous phase to that of the total alkaloid in the ethereal phase.

Provided that the concentration of acid in the aqueous phase is constant, the apparent distribution coefficient is also constant. Whilst it is true that, as Dr. Foster points out, the alkaloid is weighed initially as free base and finally as hydrochloride, the effect is merely to multiply the apparent distribution coefficient by a constant which, during the preparation of the calibration graph, is automatically included in the constants K_1 and K_2. Regenerating the free base after extraction would complicate the assay and necessitate redetermination of the constants K_1 and K_2 without improving the accuracy of the process. The proposed method, in which the relative proportions of the components in the mixture are read on a linear graph connecting composition with weight of residue, would appear to be less tedious than a spectrophotometric assay entailing the solution of simultaneous equations. It must also be borne in mind that the costly instruments required for infra-red and ultra-violet spectroscopy are not universally available in analytical laboratories, and that the suggested method may be applicable to determinations for which no spectrophotometric assay process can be devised. As pointed out in the paper, the weight of residue obtained by extracting a solution of strychnine in N hydrochloric acid decreases by about 1 per cent. for 1° C. rise in the temperature of the solvents. Thermostatic temperature control is accordingly necessary, and any departure from the recommended temperature of 25° C. would entail a redetermination of the constants K_1 and K_2. The length of time required to establish equilibrium obviously depends upon the efficiency of agitation, which cannot readily be standardised. The period can be considerably shortened if care is taken to ensure efficient shaking.

SUSTAINED RELEASE OF DRUGS FROM ION EXCHANGE RESINS

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The development of preparations to give sustained release of drugs in the body when administered by mouth, has been recently reviewed[1]. Most of these preparations are based on physical methods for retarding the release of drug as, for example, by making tablets which disintegrate slowly or by coating pellets with slowly soluble films[2,3]. A more continuous and uniform release over a long period is likely to result if the drug is chemically bound to a solid carrier, from which it is slowly released by the action of the digestive fluids.

The slowness of the uptake and release of alkaloids from ion exchange resins has been noted by Saunders and Srivastava[4,5] and it was thought that these resins might provide suitable chemical carriers for drugs in sustained release preparations. Ion exchange resins are extremely insoluble in aqueous liquids and have no toxic effects unless they are given in large enough quantities to disturb the calcium content of the body fluids. When they are administered by mouth, they are likely to spend about two hours in the stomach in contact with an acid fluid of concentration of about 0·1N hydrochloric acid. They will then be moved to the intestine where they will be in contact with a fluid of approximately neutral pH and ionic strength of about that of 0·1N sodium chloride, for several hours.

An outline of the properties and structures of ion exchange resins has been given in a review by Saunders[6]. The common cation exchangers contain either carboxyl or sulphonic acid groups distributed throughout the resin particles. Both types in the hydrogen form (the "form" of a resin is named after the exchangeable ion contained in it) absorb alkaloids from solution, forming resin salts, in which the alkaloid is chemically combined with the exchanger anion.

$$R\mathrm{SO_3^-H^+} + \mathrm{Alk} = R\mathrm{SO_3^-AlkH^+}$$

($R\mathrm{SO_3^-}$ represents the resin anion; Alk and $\mathrm{AlkH^+}$, the alkaloid and the alkaloidal cation, respectively.)

We have found that the ephedrine form of the carboxylic acid type of resin releases its ephedrine very rapidly in acid solution and is therefore not likely to be of much use for oral, sustained release preparations, since nearly all of the alkaloid would be eluted in the stomach. On the other hand, the sulphonic acid resins give a more moderate release in acid solution and release occurs at almost the same rate in neutral salt solutions; for example, in sodium chloride solution the alkaloid ion on the resin exchanges with the sodium ion in solution. The release of dexamphetamine was also studied.

Experimental Methods

Sulphonated, cross-linked polystyrene resins of varying divinyl-benzene content, were cycled twice between the sodium and hydrogen forms, and the final hydrogen forms were thoroughly washed with distilled water and surface dried at 40° C. until their moisture content was about 25 per cent. They were then sieved to give a series of fractions whose mean swollen particle sizes were measured by miscoscopic examination, of about 250 particles from each fraction. Those used are described in Table I.

TABLE I

Resin fractions

Resin	Nominal per cent. divenylbenzene	Fraction B.S.S.	Mean diameter	Moisture Per cent.	Mg. equiv. (110° C. dried form) capacity
			mm.		
a	4·5	30–36	0·70	28	5·25
b	9*	20–30	0·71	26·4	5·08
c	9	40–60	0·45	26·2	5·08

* This is the usual degree of cross-linking in the commercial cation exchangers of this type.

To prepare the ephedrine form, the resin in hydrogen form, was rotated in a closed tube with an aqueous ephedrine solution containing the required amount of alkaloid until absorption was complete. To prepare the dexamphetamine form, 0·1N dexamphetamine sulphate solution was passed through a weighed amount of the resin, in hydrogen form, until the pH of the effluent was exactly that of the solution entering the column.

Release of Ephedrine from the Resin

Rates of release of alkaloid from the resins were studied by three different techniques.

Closed tube method. A series of equal quantities of the hydrogen form of the resin were weighed and converted to the alkaloid form. Each sample was transferred to a stoppered glass tube and 50 ml. of eluting solution was added. The tubes were clamped to arms attached to a shaft which caused them to rotate in a water thermostat bath, at 25° C. for most of the experiments, so that the resin granules fell through the solutions 24 times in a minute. After each interval, a tube was removed and a sample of the liquid in it was analysed for ephedrine by measuring its extinction at 257 mμ.

Replacement closed tube method. One tube was prepared for each resin-alkaloid system studied. At appropriate time intervals the contents of the tube were removed through a filter stick and replaced by fresh eluant. After removal from the tube, the solution was analysed.

Infinite bath method. A continuous stream of eluant was passed over a bed of the alkaloid form of the resin, one particle thick, placed on a sintered glass disc in a closed cell, surrounded by a jacket through which water from a thermostat was passed (see Fig. 1). The eluant flowed

through a spray bulb on to the resin at a controlled rate of 74–76 ml./min. A stop-clock was started when the flow of eluant from a large flask immersed in the thermostat, was commenced. The cell was half filled with liquid before the effluent was released from the bottom of the cell; throughout the elution the cell remained half full. Ten seconds before

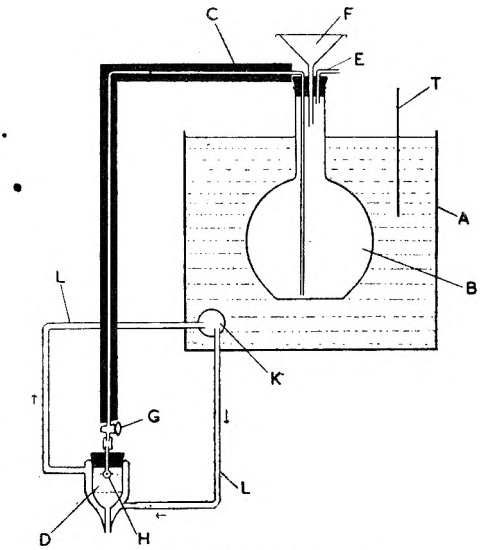

FIG. 1. Infinite bath apparatus.

A. Thermostat
B. Five litre flask
C. Glass siphon tube, insulated with asbestos tape
D. Elution cell with coarse sintered glass disc
E. Glass tube
F. Funnel
G. Stopcock
H. Spray bulb
K. Circulating pump
L. Thick walled rubber tubes
T. Thermometer

the scheduled end of elution the flow was stopped and the liquid in the cell drained. The resin was then washed free of eluant and analysed for alkaloidal content by one of the methods described.

With hydrochloric acid as eluant, the resin was transferred to a small column and a normal solution of sodium chloride was passed slowly through it for a period of several hours, until the pH of the effluent was exactly that of the original solution. The amount of acid in the combined effluent was determined by potentiometric titration with carbonate free 0·05N sodium hydroxide and this was equivalent to the amount of alkaloid released in the infinite bath experiment.

Infinite bath .. $H^+Cl^- + alkH^+RSO_3^- = alkH^+Cl^- + H^+RSO_3^-$

In column .. $Na^-Cl^- + H^+RSO_3^- = Na^+RSO_3^- + H^+Cl^-$

With sodium chloride or sodium bicarbonate as eluant in the infinite bath experiment the above method could not be employed since the

alkaloid released was replaced by sodium. The amount of sodium form of the resin present in the material taken from the elution cell was equivalent to the amount of alkaloid released and was determined by ignition of the resin and weighing as sodium sulphate[7].

Results

Figure 2 shows the results of elution experiments with 1 mg. equiv. of the ephedrine form of the resin (sample b, Table I) with 0·1N hydrochloric as eluant. When x, the percentage release of alkaloid is plotted against time of contact with eluant in hours, it is seen that there is a considerable difference between the closed tube method and the other methods, in which fresh eluant is brought into contact with the resin. This difference is believed to be due to the reversible nature of the elution reaction; in the closed tube method the released ephedrine remains in the eluting solution and re-absorption on to the resin occurs. According to the closed tube results, the rate of release of alkaloid falls off rapidly after two hours, whereas according to the other methods there is a sustained release for a period of more than six hours.

The differences between the results of the different experimental methods are accentuated when a mixture of ephedrine and hydrogen forms of the

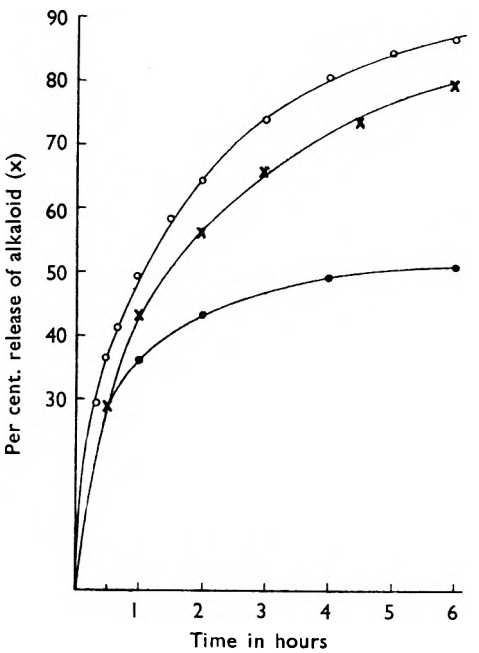

FIG. 2. Release of ephedrine using different techniques. 1 mg. equiv. of the ephedrine form of resin *b*; eluant 0·1N HCl.

● Closed tube.
× Replacement closed tube.
○ Infinite bath.

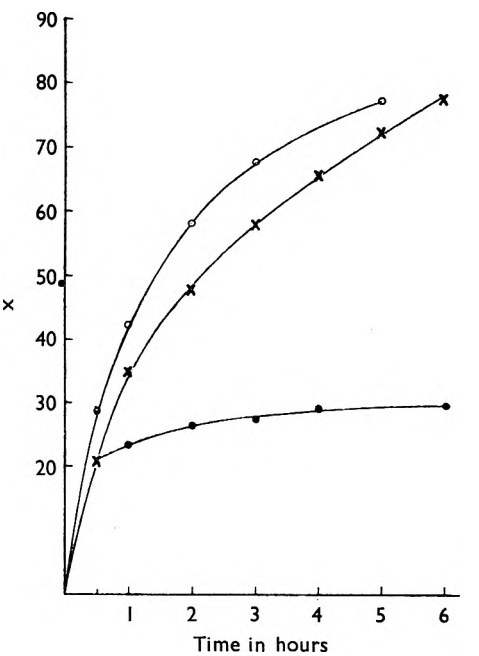

FIG. 3. Release of ephedrine from resin mixtures using different techniques.

● Closed tube.
× Replacement closed tube.
○ Infinite bath.

resin is eluted. In Figure 3, the results by the three methods for the elution of a mixture of 1 mg. equiv. of ephedrine form and 1 mg. equiv. of hydrogen form with 0·1N hydrochloric acid, are shown (both are resin fractions *b*, Table I). The presence of the hydrogen form greatly increases the reversibility effect in the case of the closed tube method but the replacement closed tube method gives completely different results. In the latter, the presence of the hydrogen form reduces the amount of ephedrine released in the early stages of elution (compare with Fig. 2) and in consequence, increases the amounts liberated in the later stages. Addition of the hydrogen form therefore produces a straightening of the release curve. A similar result is obtained with the infinite bath method.

In view of these results a detailed study of elution with the closed tube method was not made. The effects of various factors on the rates of release of alkaloid were studied by each of the other methods.

Infinite Bath Method

The infinite bath elution measurements were interpreted by the theory of Boyd, Adamson and Myers[8] to give rate constants. The results obtained are summarised in the Appendix. Some values for the percentage elution of ephedrine and dexamphetamine from 1 mg. equiv. of resin *b* are shown in Table II.

TABLE II

PERCENTAGE RELEASE OF BASES BY THE INFINITE BATH METHOD

Time (hours)	Ephedrine		Dexamphetamine	
	0·1N HCl	0·1N NaCl	0·1N HCl	0·1N NaCl
0·5	37·0	31·2	31·3	30·5
1·0	50·1	45·7	43·9	42·1
1·5	59·1	56·0	52·5	50·6
2·0	64·9	62·9	59·4	57·2
4·0	81·0	77·9	74·1	71·2
6·0	86·8	85·8	83·0	81·0

Replacement Closed Tube Method

The infinite bath method is tedious and troublesome. To determine a single curve, about 75 litres of eluant are required and the experiment takes one week. For practical purposes, the replacement closed tube method is more useful. When a spectrophotometric method of drug determination is used, it is necessary to condition the resin thoroughly by cycling and copious washing, in order to ensure that no traces of impurity are released from the resin which might affect the extinction of the eluting solution. Throughout the spectrophotometric work, a continuous check on this possible source of error was maintained.

Using this method, some further studies of ephedrine release rates were made.

(*i*) *Incompletely converted resin.* The rate of elution from 1 mg. equiv. of resin *b* completely converted to the ephedrine form was compared with that obtained with 2 mg. equiv. of *b* (hydrogen form) only half converted to the ephedrine form. The half converted resin gave a slower rate of release in the early stages than the completely converted form and the

release curve was almost identical with that obtained with a mixture of 1 mg. equiv. of completely converted ephedrine form (*b*) plus 1 mg. equiv. of the hydrogen form (*b*). With 4 mg. equiv. of resin, one quarter converted to the ephedrine form, a further straightening of the release curve was obtained, but the total elution after six hours was reduced (see Fig. 4).

(*ii*) *Variations in the hydrogen form of the resin used* (Table III). In these measurements 1 mg./equiv. of ephedrine form of the resin (*b*) was mixed with different types of hydrogen form. The results are summarised in Table III. In each case the added hydrogen form is shown at the top of each column. The resin fractions *a* and *c* in the hydrogen form (Table I) are both more effective in reducing the initial release of alkaloid and hence in straightening the release curve, than the hydrogen form of *b*. This is to be expected, since both *a* and *c* absorb ephedrine at a considerably higher rate than *b*. An exceptionally uniform rate of release is achieved from the mixture of 1 mg. equiv. of ephedrine form of *b* plus 3 mg. equiv. of hydrogen form of *a* (see Fig. 4).

TABLE III

PERCENTAGE RELEASE (X) OF EPHEDRINE FROM RESIN MIXTURES

Time, hours	No. H form	1 mg. equiv. of H form of *b*	1 mg. equiv. of H form of *a*	1 mg. equiv. of H form of *c*	3 mg. equiv. of H form of *a*
½	29·1	21·1	15·5	16·4	8·0
1	43·9	34·9	29·3	29·9	15·6
2	57·1	47·6	42·9	43·1	24·2
3	66·4	57·7	54·4	54·1	32·5
4	72·6	65·8	63·8	62·9	40·1
5	77·1	72·3	71·4	70·0	46·9
6	79·4	77·6	77·5	75·9	53·1

DISCUSSION

A sustained release of ephedrine and dexamphetamine over a period of at least six hours can be achieved when the drug form of a sulphonic acid resin is put in contact with 0·1N acid and salt solutions. The most convenient method for studying the elution of drug from the resin is the replacement closed tube method.

If the drug form of the resin is given by mouth, the released base will probably be absorbed quite rapidly by the mucous membrane of the gastro-intestinal tract and so there is not likely to be any major effect due to the reversibility of the base release reaction. It is therefore to be expected that the infinite bath and the replacement closed tube methods may more nearly reflect the drug release *in vivo* than the closed tube method. In the body, the resin is unlikely to meet more drastic eluting conditions than those of the infinite bath method. The fact that a steady, continuous release of ephedrine is maintained in the infinite bath elution shows that the slow displacement of ephedrine from the interior of the resin particles is only slightly altered by the conditions of elution, when the eluted alkaloid is continuously removed. This conclusion provides an important safety factor for the possible use of these resins in medicine.

The release curves for ephedrine are very much the same with 0·1N hydrochloric acid, sodium chloride and sodium bicarbonate solutions as eluants (see Appendix). The curves can be appreciably straightened by using resins which are only partially converted to the ephedrine form or by using mixtures of the alkaloid and hydrogen forms of the resin. A mixture of equivalent proportions of these two forms gives the same total release after six hours as the ephedrine form alone, but the initial release rate is reduced. Higher proportions of hydrogen form cause a reduction in the alkaloid release over a six-hour period.

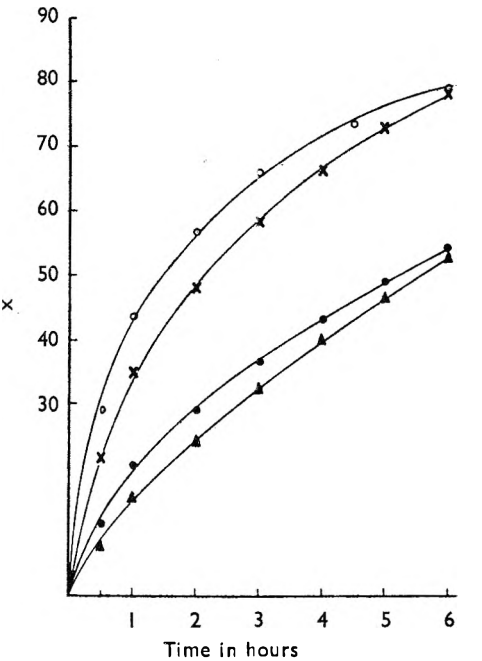

FIG. 4. Effect of hydrogen form on the release of ephedrine. Replacement closed tube method; 0·1N HCl as eluant.

- ○ 1 mg. equiv. of the ephedrine form of resin *b*.
- × 2 mg. equiv. of resin *b* (originally hydrogen form) half converted to the ephedrine form.
- ● 4 mg. equiv. of resin *b* (originally hydrogen form) one-quarter converted to the ephedrine form.
- ▲ Mixture of 1 mg. equiv. of the ephedrine form of resin *b* with 3 mg. equiv. of the hydrogen form of resin *a*.

APPENDIX

Infinite Bath Elution Results

The infinite bath elution results have been interpreted by the kinetic theory of Boyd, Adamson and Myers[8] and also by using the extended Table of functions given by Reichenberg[9]. The equation of this theory is shown below

$$F = 1 - \frac{6}{\pi^2} \sum_{n=1}^{n=\infty} \frac{\exp(-n^2 Bt)}{n^2}$$

Where n is an integer, $F = x/100$ i.e., F. is the fraction of alkaloid eluted from the resin in time t; B, the rate constant is defined as $B = \pi^2 D/r^2$ where D is the effective diffusion coefficient of the ion exchange process and *r* is the mean particle radius of the resin. F is dimensionless and B has the dimensions of reciprocal time, Bt is therefore dimensionless. Theoretical values of Bt for different numerical values of F can be computed from the above equation and numerical Tables of these quantities have been drawn up by Reichenberg. To calculate the rate constants B from the experiments, values of F are calculated from the measurements and the corresponding theoretical values of Bt are found from the Tables. These Bt values are plotted

against the experimental values of t and if the theory is correct a straight line passing through the origin, of slope B, should result. The value of B in sec.$^{-1}$ is found by measuring this slope. Deviations from the ideal Bt, t plot may arise if the rate of exchange is not controlled by the rate of diffusion within the resin particles; also, difficulty in deciding the exact time at which elution commences in a given experiment may give rise to a plot which although linear, does not pass exactly through the origin.

TABLE IV

ELUTION OF EPHEDRINE WITH VARIOUS ACID STRENGTHS

Normality of HCl	B in sec.$^{-1}$ $\times 10^5$	Time (min.) for half elution (F = 0·5)
0·025	—	112
0·05	—	73
0·075	—	62
0·1	8·7	60
0·25	13·3	38
0·5	19·4	26
0·75	24·8	20
1·0	28·6	18
1·5	32·5	15

TABLE V

PARTICLE SIZE EFFECT

Resin sample	Observed swollen diameter, mm.	B for 0·1N HCl Sec.$^{-1}$ $\times 10^5$	B for 1N HCl
b	0·71	8·7	28·6
c	0·45	27·7	75·9

The effects of various factors on the elution rate constant B, are summarised below.

(i) Concentration of hydrochloric acid in the eluant (Table IV). 1 mg. equiv. of resin sample *b* in the ephedrine form was used. The Bt, t plots were all linear but those for the lower acid concentrations below 0·075N did not pass exactly through the origin, the probable reason for this has already been mentioned. The uncertainty about the exact start of the experiment is likely to be greater when the rate of elution is slower as is the case with the more dilute acid solutions.

(ii) Resin particle size (Table V). 1 mg. equiv. of the ephedrine forms of resin samples *b* and *c*, were each eluted with 0·1 and 1·0N hydrochloric acid.

The value of B should be inversely proportional to the square of the particle radius, therefore the square root of the ratio of the values of B for a given acid concentration should equal the inverse ratio of the mean particle diameters. This inverse ratio of diameters is 1·6; for 0·1N acid the square root of the ratio of B values is 1·8 and for N acid it is 1·65. This represents a reasonable agreement with theory, considering the uncertainty of the mean particle diameter estimates.

(iii) Cross linking of the resin. The degree of cross linking of the resin is roughly measured by the proportion of divinyl benzene in the mixture which is polymerised to make the resin. By comparing the results for 1 mg. equiv. of the ephedrine forms of resins *a* and *b* with 0·1N hydrochloric acid as eluant, it was found that the more lightly cross linked resin gave a rate constant 5·9 times that of resin *b*.

(iv) Temperature. The effect of temperature on B is quite small; increasing the temperature from 25° to 35° C. increased B by a factor of 1·2, using 1 mg. equiv. of resin *b* and eluting with 0·1N hydrochloric acid.

(v) Quantity of resin. The value of B obtained using 2 mg. equiv. of ephedrine form of resin *b* was identical with that found with 1 mg. equiv.

(*vi*) *Flow rate.* The standard flow rate used was 75 ml./min. throughout these experiments. Increase of flow rate to 120 ml./min. did have some effect on B, increasing its value by a factor of 1·4.

(*vi*) *Eluant.* 0·1N sodium chloride solution and 0·1N sodium bicarbonate solution gave almost identical values of B when used to elute 1 mg. equiv. of the ephedrine form of resin *b*, showing that the anion in the eluant has no appreciable effect on the release rate. Both the sodium salts gave a B value which was slightly lower than that for 0·1N hydrochloric acid, the ratio being 0·86.

(*vii*) *Dexamphetamine.* The dexamphetamine form of resin *b*, when eluted with 0·1N hydrochloric acid gave a B ratio dexamphetamine/ephedrine of 0·72. With the more lightly cross-linked resin, *a*, the ratio was 0·81.

SUMMARY

1. The rates of release of ephedrine from sulphonic acid cation exchange resins in contact with 0·1N hydrochloric acid, sodium chloride and sodium bicarbonate solutions have been examined. Sustained release occurred over a period of more than six hours when a method involving removal of eluted alkaloid was used, even under the drastic conditions of the infinite bath method where a high continuous flow of eluant was employed. This provides an important safety factor for the possible use of these resin preparations in medicine. The release of dexamphetamine was also studied.

2. When the resin is only partly converted to the ephedrine form or when a mixture of the ephedrine and hydrogen forms of the resin is used, the initial release rate is reduced and the later rate is increased, producing a straightening of the release curve.

Our thanks are due to Professor W. H. Linnell for his interest in the work and to Clinical Products Ltd., for a grant.

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DISCUSSION

The paper was presented by MR. N. C. CHAUDHRY.

The CHAIRMAN asked whether any of the resin-bound drugs described had been tested clinically, and what were the classes of drugs which the authors had in mind for this type of preparation.

PROFESSOR W. H. LINNELL (London) said that in an American paper it was reported that in about 70 per cent. of some 300 or 400 cases the potency of resin-bound amphetamine was appreciably higher than amphetamine taken in the normal way. He had also seen thirty or forty clinical reports on an English preparation and they showed that there was an unexpected potency of the amphetamine-bound resin which might possibly be due to the slow release of the drug. The resins could be used with acidic or basic substances, but not for neutral compounds.

MR. D. JACK (London) said he was puzzled by the selection of 25° C. as the testing temperature. It was said that diffusion coefficient B increased by a factor of 1·2 for 35° C. as against 25° C. From the practical point of view one was really concerned with variations of F. It would be interesting to know what effect there was on the release in 2 hours when changing the temperature from 25 to 35° C. In Figure 4 the curve was straightened by adding the hydrogen form of the resin. A considerable straightening of the curve had been shown in the presence of hydrochloric acid. In the *in vivo* conditions in the stomach and intestine one would not expect such great changes in the shape of the curve with large quantities of cations and hydrogen ion, especially when the hydrogen form was introduced on a separate granule. That was important, because ideal sustained release forms should have a release curve which was linear with time. It would be interesting to have information on *in vivo* tests.

MR. A. AXON (Dartford) asked whether the authors would comment on (i) the difficulties of storage of a resin containing 25 per cent. moisture, (ii) the choice of preservative to prevent mould and fungal growth, and (iii) the release of drug from a dry resin.

DR. G. E. FOSTER (Dartford) asked how much of the resin had to be administered to give a dose of $\frac{1}{2}$ grain of ephedrine and whether the quantity would be small enough in bulk to be prepared as a tablet.

MR. N. J. VAN ABBÉ (Loughborough) asked whether the authors felt that they had adequately characterised the resins. Some clarification would also be helpful of the statement on page 976 "At appropriate time intervals". As far as could be seen, the curves for the method under discussion in Figures 2 and 3 could be anywhere between the other two curves, according to the appropriate time intervals chosen.

DR. K. R. CAPPER (London) said that the interest shown suggested the possibility that this type of sustained release medicament might become a standard form of presentation and, if so, it would have to comply with performance tests. The manufacturer of such a drug was, to some extent, in the hands of the manufacturer of the ion exchange resin, and the importance of the degree of cross linking was shown in the paper. It would be very useful to know whether the authors had found any difference in performance between batches of the resin supplied as identical by the manufacturer.

MR. T. D. WHITTET (London) said that a quinine ion exchange resin compound was used for the diagnosis of achlorhydria. He had tried to

use ion exchange resins as a method of concentrating the pyrogens in tap water, but he had not been able to elute them. It was interesting therefore to note the release over quite a long period.

DR. J. C. PARKINSON (Brighton) asked whether the release of the alkaloid from the resin was dependent on the resin remaining in the stomach for the whole period. Did release cease when the resin passed out of the stomach into the less acid conditions further down the gastro-intestinal tract?

MR. S. G. E. STEVENS (London) said he was rather puzzled by the use of the words "sustained release". Examination of the graphs in Figures 2 and 3 showed, in his interpretation, that sustained release was not being obtained, but a gradually decreasing release. He also asked whether the authors proposed to follow their work with a study of less soluble drugs bound to ion exchange resins, because he would expect the pattern of release to be somewhat different, and the conditions of testing might have to be very severely modified to provide useful criteria. On what evidence was the rotating tube at 12 r.p.m. selected? The authors were careful initially to select material of a narrow range of particle size, and he wondered whether any sort of attrition effects came into play during the test. He was doubtful whether the simple *in vitro* test was sufficiently accurately reflecting the *in vivo* picture.

MR. N. C. CHAUDHRY, in reply, said they had not carried out clinical tests or studies of the release of drugs *in vivo*. The rate of release would depend, among other factors, upon the molecular size and dissociation constant of the individual drug. The temperature coefficient was small and the percentage release of ephedrine with 0·1N HCl after two hours (Table II) was 69 at 35° C. instead of 64·9 at 25° C. (F = 0·69 at 35° C. instead of 0·649 at 25° C.). The straightening of the release curve is due to the presence of the hydrogen form of the resin which exerts a reversible reaction of absorption of the ephedrine being released. As long as the resin granules remain in contact with gastric medium where hydrochloric acid predominates, the effect of reversibility will probably continue. However, on entering the intestine, this effect should cease and the rate of release would increase for the subsequent time intervals, i.e., third hour onwards; the net result should be a further straightening of the release curve. As is seen in Table III, column 2, percentage release after the first two hours is 57·1, and the purpose of the study of the mixtures for producing straightening effects was to decrease this initial high figure. Further experiments have been made on the release of ephedrine from a mixture of equal portions of resin (*b*) in the ephedrine form and resin (*a*) in the hydrogen form, by eluting in the first two hours with 0·1N HCl, followed by elution with 0·1N $NaHCO_3$ or 0·1N NaCl up to six hours. It was found that the release curve was further straightened and the percentage total release after six hours was 82 instead of 77·5 (Table III). The resins were air dried to about 25 per cent. moisture content (Table I). They were allowed to come to equilibrium with atmospheric humidity so that they were stable for quantitative work. These resins have been

stored for more than two years and no fungal growth has been observed. The release of drugs from an air dried resin had not been studied, but the release may be slightly slower in the first half hour. The release of drugs from anhydrous sulphonic acid resins could not be recommended because of the possibility of fracture of the resin granules due to rapid swelling. One grain of air dried resin would absorb $\frac{1}{2}$ grain of dexamphetamine and 0·65 grain of ephedrine. The choice of time intervals was arbitrary and was made for experimental convenience. Two batches of the resin "Zeo-Karb 225" obtained in different years from the same manufacturer have shown identical performance, but variation between batches from different manufacturers is to be expected. A resin can be sufficiently characterized by determining its capacity and its volume expansion for a given exchange or change of solvent. This expansion measures the degree of cross-linking of the resin. The release of drugs from the sulphonic acid cation exchangers has been found to be almost independent of pH, and depends upon the cationic strength of the eluting solutions. Sustained release does not necessarily imply a uniform release. The rotation of tubes at 12 r.p.m. was selected because at this speed the rate of release was independent of the rate of rotation. Attrition in the tube did not seem to occur to any extent.

Dr. L. Saunders (London), in reply, said that the paper was of a preliminary nature. The temperature of 25° C. was chosen because the temperature coefficient was not very great. If one considered the volume of solution used in the infinite bath method it would be appreciated that considerable difficulty was experienced holding the temperature constant when working far from room temperature.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Adrenaline, Activity Ratio of, to Noradrenaline, in various Colour Reactions. T. Ozaki. (*Tohoku J. exp. Med.*, 1956, **63**, 225.) The activity ratio of adrenaline to noradrenaline, using such chemical methods as the permanganate, the corrosive sublimate, the iodine, the phosphotungstic and the arsenomolybdic estimations, was determined. The mean value of adrenaline to noradrenaline was 1·16 : 1 in the permanganate method, 1·31 : 1 in the corrosive sublimate method, 2·4 : 1 in the iodine method, 2·17 : 1 in the phosphotungstic method and 7·6 : 1 in the arsenomolybdic method. This variability in the ratio may be due to variations in the temperature and pH of the solutions. M. M.

Cardenolides, Dinitrobenzoic Acid Reaction of. A. L. O. M. Smithuis. (*Pharm. Weekbl.*, 1956, **91**, 253.) The colour reaction of 3:5-dinitrobenzoic acid with cardenolides does not give uniform results. Thus there is an increase in the extinction on boiling the glycosides and aglycones of strophanthus and digitalis with alcoholic acid, although g-strophanthin and 17-isocymarin do not show this phenomenon. On carrying out the reaction with some simpler butenolides, a difference in molecular extinctions was observed, showing that the structure of the β-compound linked to the butenolide group influences the stability and intensity of the colour. Examination of the ultra-violet spectra showed that boiling with alcoholic acid caused an increase in the absorption at 220 mμ due to destruction of the sugar part of the molecule. so that the aglycones show no increase or shift of wavelength. On boiling with alcoholic hydrochloric acid gitoxigenin shows an additional maximum at 338 mμ and the curve is identical with that of the compound $\triangle^{14,16}$-dianhydrogitoxigenin. It is to be supposed that digitoxin and digoxin will show a similar behaviour. The increase in the extinction after boiling the original glycosides or aglycones with alcoholic acid may be attributed to the formation of di-anhydro or mono-anhydro compounds, and the molar extinctions from the various cardenolides should have similar values. In the case of 17-isocymarin the butenolide group is in the transposition with respect to the C(14)hydroxyl, and the effect on the colour is eliminated. With g-strophanthin, this compound does not readily hydrolyse, nor does it easily form a 14-anhydro compound. The maximum colour intensity can only be obtained by using 4 milli-equivalents of sodium hydroxide. G. M.

Cardiac Glycosides containing Desoxy Sugars, Assay for. R. Dequeker. (*Bull. Soc. Pharm. Bordeaux*, 1955, **94**, 24.) The following method has been applied to preparations containing digitoxin, glycosides of *Digitalis purpurea*, digilanids A, B and C, ouabain and cymarin, with satisfactory results. Dissolve a quantity equivalent to 10–20 μg. of the sugar in 1 ml. of dry acetone in a 1·2 × 8-cm. test tube, add phosphoric acid to 5 ml., mix and heat at 35° C. for 15 minutes. Cool to 20° C. and measure the light absorption at 474 mμ, using as a blank a mixture of acetone and phosphoric acid treated in the same way. The quantity of the sugar is calculated from the light absorption, which follows the Lambert-Beer law with concentrations between 2 and 4 μg. of digitoxose per ml. The reaction is about 7 times more sensitive than the Keller-Kiliani test, the maximum intensity of colour is reached more rapidly and the colour is more stable and less dependent on the purity of the reagents. G. B.

Local Anaesthetics, Characterisation of. E. Hannig and W. Karau. (*Pharm. Zentralh.*, 1956, **95**, 187.) Local anaesthetics may be characterised by the formation of disulphimide derivatives which are prepared by simple mixing of equimolecular quantities of the substances in warm aqueous solutions. Melting points of the products are given in the Table below:

Disulphimide derivative	Falicaine	Procaine	Pantocaine	Xylocaine	Cocaine
4:4′-diaminodiphenyl disulphimide ..	163·5° C.	170·0° C.	142·0° C.	181·0° C.	—
4:4′-dichlorodiphenyl disulphimide ..	149·0° C.	141·0° C.	135·0° C.	151·0° C.	—
3:4:3′:4′-tetrachlorodiphenylsulphimide ..	131·0° C.	114·0° C.	132·0° C.	162·0° C.	151·0° C.

G. M.

Morphine in Poppy Capsules, Estimation of. S. Pfeifer and W. Keller. (*Pharm. Zentralh.*, 1956, **95**, 189.) The polarographic determination of morphine cannot be applied directly to poppy capsules, but a modification described by the authors gives satisfactory results: 0·3 g. of the finely powdered capsules (dried at 75° C.) is rubbed down with 1 ml. of water and, after 15 minutes, mixed with "acid" alumina in portions until a dry powder is obtained (about 7–8 g.). The mixture is filled into a tube of 2 cm. diameter above a layer of 4 g. of alumina, and eluted with water. About 20 ml. of eluate is collected, and it is rinsed through with 3·5 ml. of hydrochloric acid (25 per cent.). The solution is made up to 25 ml. To 5 ml. of this solution is added 2 ml. of N sodium nitrite solution and, after exactly 5 minutes, 3 ml. of 20 per cent. potassium hydroxide and 7 drops of gelatine solution. Nitrogen is bubbled through, and the morphine is determined polarographically. G. M.

Pholcodine, Paper Chromatography of. F. Sabon and R. Monnet. (*Bull. Soc. Pharm. Bordeaux*, 1955, **94**, 41.) Preparations of pholcodine were submitted to descending paper chromatography using paper soaked in 0·5M potassium chloride and dried. Butanol containing 2 per cent. v/v of hydrochloric acid, saturated with water was used as the developing solvent. Acetic or tartaric iodobismuthate reagent was used to mark the position of the alkaloidal spots. The R_F value for pholcodine was 0·17. Using paper soaked in 0·2M potassium dihydrogen phosphate, with butanol saturated with water as solvent, the R_F value was 0·04. In either case pholcodine was clearly separated from morphine, codeine and ethylmorphine which have much higher R_F values. The method is applicable to the identification of commercial preparations of pholcodine phenylacetate but a preliminary extraction of the base is required.

G. B.

Quaternary Ammonium Compounds, Determination of. P. A. Lincoln and C. C. T. Chinnick. (*Analyst*, 1956, **81**, 100.) Surface-active quaternary ammonium compounds are shown to be quantitatively precipitated by phosphotungstic acid; by weighing the quaternary phosphotungstate after drying and again after ignition, it is possible to calculate the amount of quaternary cation in an unknown sample and also the ionic weight of the quaternary salt. The main disadvantage of the method is that ammonia and amines form insoluble phosphotungstates and must therefore either be removed or other allowance made; volatile amines may be removed by making alkaline and boiling. In a preferred method a solution of quaternary ammonium compound in *iso*amyl alcohol (0·1 to 0·5N) is freed from amine by washing with N hydrochloric acid.

R. E. S.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

***Digitalis purpurea*, Chromatographic Examination of.** C. Gunzel and F. Weiss. (*Pharmazie*, 1955, **10**, 725.) The composition of digitalis extracts was examined by means of inverse phase chromatography, using paper treated with silicone. Comparison of a chloroform extract with one made with chloroform-*iso*propanol showed that the larger proportion of the primary glycosides which determine the value of the drug are in the chloroform-*iso*propanol extract. Extracts made by the method of the D.A.B. VI, and also cold water extracts, contained the purpurea glycosides A and B, digitoxin, gitoxin, gitoxigenin, diginin and a saponin. The cold water extract contained, however, a larger proportion of secondary glycosides and correspondingly a smaller one of primary glycosides. This is due to the partial breakdown of the genuine glycosides to the digitoxin stage as the result of the activity of naturally occurring enzymes which are partially destroyed by hot water. An infusion is therefore to be preferred to a cold water extract. Comparison of fresh infusions with one 4 months old showed a greater proportion of digitoxin glycosides and a smaller one of primary ones in the older preparation. It is uncertain whether this is due to enzyme action or to hydrolysis under the influence of pH. G. M.

***Digitalis purpurea*, Detection of New Components in the Glycoside Complex of.** K. B. Jensen. (*Acta pharm. tox., Kbh.*, 1956, **12**, 11.) A paper chromatographic method for separation of digitalis glycosides has been developed, and used to detect the presence of the glycosides gitorin, strospeside and digitalinum verum in leaf extracts of *Digitalis purpurea*. *iso*Propanol has been shown to be a more suitable solvent than ethanol or methanol for the preparation of these extracts, the glycoside showing greater stability in *iso*propanol. Several unknown substances were detected by the use of a chloroform-acetone-formamide solvent system, and examination of the spots in trichloroacetic acid-fluorescence reactions. They are distinguished as A or B type substances according to whether they give digitoxigenin or gitoxigenin on hydrolysis. Substances A_1, A_2 and B_3 gave a green colour with the trichloroacetic acid reagent, whereas B_1 and B_2 gave no reaction. With *m*-dinitrobenzene-R, sodium nitroprusside-R or picric acid-R all five substances gave blue, red or orange colours respectively like the known cardiac glycosides. In all, five new A substances and nine new B substances were detected by the chromatographic technique. J. B. S.

***Digitalis purpurea*, Paper Chromatographic and Fluorimetric Method for Cardiac Glycosides and Aglycones of.** K. B. Jensen. (*Acta pharm. tox., Kbh.*, 1956, **12**, 27.) A method is described for determining the cardiac glycosides in digitalis leaves, based on previously published chromatographic and fluorimetric methods. The method includes purpurea glycosides A and B, digitoxin, gitoxin, digitalinum verum, gitorin, strospeside, digitoxigenin, gitoxigenin, and various unknown glycosides, transformable into known purpurea glycosides by hydrolysis with digipurpidase or by sodium bicarbonate in aqueous methanol. Experiments show that lead purification of glycoside extracts cause transformation similar to those observed with sodium bicarbonate. These transformations were even more marked with the use of lead subacetate. Stability tests with tinctures showed that within one year no other changes occurred other than a limited transformation of unknown to known glycosides. Glycoside extracts prepared by purification with lead subacetate were stable for at least four weeks with *iso*propanol as solvent, but not with methanol or ethanol. J. B. S.

ORGANIC CHEMISTRY

Cephalosporin C, Isolation of. G. G. F. Newton and E. P. Abraham. (*Biochem. J.*, 1956, **62**, 651.) Cephalosporin C, a Penicillin-like antibiotic containing D-α-aminoadipic acid, was separated from cephalosporin N penillic acid, formed by treating crude cephalosporin N at pH 3, by chromatography on Amberlite IR–4B in ammonium acetate buffer (pH 5·0). Cephalosporin N penillic acid was eluted first, cephalosporin C being isolated from the later fractions as its crystalline sodium salt. Separations effected on Amberlite IR–4B using pyridine acetate buffer were more satisfactory because of the greater ease with which the buffer could be removed. Countercurrent distribution with the solvent system, phenol-carbon tetrachloride-aqueous acetic acid was also used to effect the separation. Cephalosporin C has also been separated directly from cephalosporin N by chromatography on Amberlite IR–4B, using pyridine sulphate as buffer. Desalting was effected by addition of barium hydroxide, which precipitated sulphate leaving the barium salt of the antibiotic in aqueous pyridine, the latter being removed *in vacuo* without loss of antibacterial activity. Separation of cephalosporins by this technique was incomplete, and more satisfactory results were obtained by chromatography on buffered paper at pH 5·5 to 6·0. Cephalosporin C has been assigned a provisional formula, $C_{16}H_{21}O_8N_3S$. Electrometric titration shows two acidic groups (pK 3·1 and $< 2{\cdot}6$) and one basic group (pK 9·8) indicating a monoaminodicarboxylic acid structure. The ultra-violet absorption spectrum shows a maximum at 260 mμ ($\epsilon_{max.}$ 9000), whilst the infra-red shows bands at 2·94; 3·06; 5·61 (c.f. carbonyl of β-lactam-thiazolidine ring system); 5·77 (ester or lactone); 6·05 and 6·57 (monosubstituted amide carbonyl); 6·29 (carboxylate ion), and 7·17 and 7·36 μ (*iso*propyl group). Cephalosporin C is relatively stable to acid, but rapidly inactivated in alkaline solution above pH 11. Cephalosporin C showed activity equivalent to that of cephalosporin N against *E. coli*, but only one tenth of that of cephalosporin N against *Salm. typhi* and *Staph. aureus*. J. B. S.

Choline Esters of Monobasic Carbonic Acids, Syntheses of. L. E. Tammelin. (*Acta chem. scand.*, 1956, **10**, 145.) The synthesis of acetylcholine, propionylcholine and butyrylcholine in more than 90 per cent. of the theoretical yields is described by the action of the appropriate acid anhydride on β-dimethylaminoethanol, and treatment of the condensation product with methyl iodide. J. B. S.

Dihydrostreptomycin, Structure of a Naturally Occurring Antagonist of. J. W. Cornforth and A. T. James. (*Biochem. J.*, 1956, **63**, 124.) The examination of a dihydrostreptomycin antagonist, previously isolated from *Pseudomonas pyocyanea*, is described. Chemical investigation has shown the antagonist to be a mixture of closely related 2-alkyl-4-hydroxyquinoline-*N*-oxides. The principal constituents were separated by partition chromatography of the product obtained on reduction with zinc in acetic acid, using an ethylene glycol-benzene-*cyclo*hexane solvent system. They have been identified as 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide and 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide in the approximate proportion 2:1. A small concentration of 2-*n*-undecyl-4-hydroxyquinoline-*N*-oxide is also present, and possibly also 2-*n*-octyl-4-hydroxyquinoline-*N*-oxide. 2-*n*-Heptyl, 2-*n*-nonyl- and 2-*n*-undecyl-4-hydroxyquinoline-*N*-oxides have been synthesised and the *n*-nonyl compound shown to have the highest activity against dihydrostreptomycin. A plausible mode of biosynthesis is suggested. J. B. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Human Serum Albumin from Placental Extracts, Procedure for the Preparation of. H. L. Taylor, F. C. Bloom, K. B. McCall and L. A. Hyndman. (*J. Amer. chem. Soc.*, 1956, **78**, 1353.) An improved procedure is described for the separation and purification of albumin from human placental extracts (see Gordon and others, *J. Amer. chem. Soc.*, 1953, **75**, 5859 for earlier procedure). Many of the improvements described have made the original method more adaptable to the large-scale production of albumin. The albumin is separated from haemoglobin and other plasma proteins under controlled conditions of pH, ionic strength, ethanol and zinc concentration and temperature. A. H. B.

Hyperglycaemic Factor in Urine, Identification of. F. Moya, J. C. Szerb and M. MacIntosh. (*Canada J. Biochem. Physiol.*, 1956, **34**, 563.) The precipitate obtained by the addition of two volumes of ethanol to acidified human urine has been found to be hypotensive and hyperglycaemic when injected into rabbits. The activity appears to be due to kallikrein. In dogs 12 μg. per kg. caused a marked fall in blood pressure, which was not antagonised by atropine or antihistamine compounds. The material did not contract the guinea-pig ileum itself, but when incubated with serum for one minute the mixture did cause a contraction. This contractile action was not blocked by atropine or promethazine. Incubation with soya bean trypsin showed that the material was not fibrolysin. There was a loss of activity when it was boiled with water for one hour, and like kallikrein it was inactivated following prolonged incubation with human serum. The hyperglycaemic action in rabbits also appeared to be due to kallikrein. G. F. S.

BIOCHEMICAL ANALYSIS

Adrenaline and Noradrenaline in Plasma, Fluorimetric Determination of. J. A. Richardson, A. K. Richardson and O. J. Brodie. (*J. Lab. clin. Med.*, 1956, **47**, 832.) This method is a modification of that used by Weil-Malherbe and Bone; the main alteration being the adapting of the Beckman model DU spectrophotometer for use as a fluorimeter. By such a method it was found that when known amounts of adrenaline or noradrenaline were added to dog plasma, the mean recovery of adrenaline was 93·4 per cent. $\pm$ 11·5 and of noradrenaline was 90·3 per cent. $\pm$ 9·9. Determination of the adrenaline and noradrenaline content of arterial blood of conscious dogs subjected to thoracic surgery several days previously, showed that the plasma level of adrenaline was 0·65 μg. $\pm$ 0·43/litre and of noradrenaline was 1·30 μg. $\pm$ 0·76/litre. Using a 15 ml. blood sample, plasma levels as low as 0·25 μg. of adrenaline and 0·5 μg. of noradrenaline per litre of plasma may be determined with accuracy. M. M.

Adrenaline, Noradrenaline and Hydroxytyramine in Urine, Fluorimetric Estimation of. H. Weil-Malherbe. (*Biochem. J.*, 1956, **63**, 4P.) The method of Weil-Malherbe and Bone for the fluorimetric estimation of adrenaline and noradrenaline gives high results with acid-hydrolysed urine, due to the presence of hydroxytyramine, 3:4-dihydroxyphenylacetic acid and possibly other acidic catechols, which form fluorescent derivatives with ethylenediamine. Acid and basic catechols were separated by ion exchangers, after separation of the catechol

fraction from the urine by chromatography on alumina. The purified basic catechols were examined by paper chromatography and shown to be the only constituents giving rise to yellow-green fluorescence with ethylenediamine, and can be estimated in this way. Adrenaline and noradrenaline are estimated separately by the method of Lund, hydroxytyramine being obtained by difference. J. B. S.

Phenobarbitone and Diphenylhydantoin in Blood, Simultaneous Determination of. G. L. Plaa and C. H. Hine. (*J. Lab. clin. Med.*, 1956, **47**, 649.) A method is described for the simultaneous determination of the two drugs in the same blood specimen. To extract diphenylhydantoin, place 5 ml. of oxalated whole blood in a 50 ml. glass stoppered centrifuge tube containing 0·5 g. of sodium bicarbonate and 0·2 ml. of 10 per cent. sodium hydroxide. Add 20 ml. of *cyclo*hexane and 1 ml. of *n*-butanol. Shake for 5 minutes and centrifuge. Transfer the *cyclo*hexane phase to another tube containing 10 ml. of N hydrochloric acid (retain the buffered blood residue), shake for 5 minutes and centrifuge. Transfer 15 ml. of the *cyclo*hexane phase to another tube containing 5 ml. of carbonate buffer (0·1M sodium bicarbonate and 0·09M sodium hydroxide, pH 11), shake and centrifuge. Remove the *cyclo*hexane layer and retain the aqueous phase for estimation. To extract the phenobarbitone, acidify the buffered blood residue retained above with 2 ml. of 25 per cent. acetic acid, after removing any *cyclo*hexane remaining in the tube. When no more carbon dioxide is evolved add 35 ml. of chloroform, shake and centrifuge. Remove the aqueous phase from the clotted blood and transfer the chloroform through filter paper to a 75 ml. centrifuge tube. Add 30 ml. of 10N sulphuric acid, shake and centrifuge. Remove the acid layer. Add 25 ml. of the chloroform layer to 5 ml. of 0·3N sodium hydroxide, shake and centrifuge and save the aqueous layer for estimation. A reagent blank is prepared by running 5 ml. of distilled water through the above procedures. For estimation of the barbiturate, pipette 3 ml. 0·3N sodium hydroxide into a silica cell and read against reagent blank at 260, 250 and 240 mμ on a D.U. spectrophotometer. Add 0·5 ml. saturated potassium bicarbonate solution to each cell and read again. Multiply the second readings by 1·17 to correct for dilution and subtract from the first readings. A barbiturate is present if a maximum positive optical density (O.D.) difference occurs at 260 mμ, approaching a minimal difference at 250 and a maximum negative difference at 240 mμ. Determine the amount from the standard curve for phenobarbitone in 0·3N sodium hydroxide. (10 mcg./ml. = 0·205 optical density units.) For diphenylhydantoin, pipette 3 ml. of the carbonate buffer into a silica cell and read against reagent blank at 260, 250, 240 and 235 mμ. Add 0·5 ml. of 10 per cent. sodium hydroxide to each cell and read again. Multiply the second readings by 1·17 to correct for dilution and subtract from them the first readings. Calculate the absorption due to the phenobarbitone in carbonate buffer at 260 and 235 mμ as follows:

O.D. phenobarbitone at 260 = 0·668 O.D. difference at 260.
O.D. phenobarbitone at 235 = 2·12 O.D. difference at 260.

Subtract these calculated optical densities from those obtained in the first readings to give the true hydantoin readings at wavelengths 260 and 235. This difference bears a direct linear relationship to the amount of hydantoin present. Determine the amount present from the standard curve for diphenylhydantoin in carbonate buffer. (10 mcg./ml. = 0·196 optical density units.) The method is sensitive enough for clinical and toxicological determinations with a high degree of specificity. G. F. S.

CHEMOTHERAPY

Spiramycin, *In Vitro* Study of. S. Pinnert-Sindico and J. Pellerat. (*Thérapie*, 1956, **11**, 308.) In tests by the dilution method against a variety of organisms, spiramycin was shown to have an antibacterial spectrum closely resembling that of erythromycin and carbomycin. Inhibiting concentrations were generally higher for spiramycin *in vitro*, but of the same order as those of erythromycin and carbomycin *in vivo*. The activity of the substance was not affected by the presence of serum. A strain of *Staphylococcus* resistant to spiramycin was susceptible to other antibiotics, but showed slight resistance to erythromycin and carbomycin. Strains resistant to other antibiotics remained sensitive to spiramycin. Spiramycin was especially active against Gram-positive organisms. Its activity was influenced by the reaction of the medium, being a maximum in alkaline solutions. Different strains of *Staphylococcus* varied considerably in sensitivity to this antibiotic. G. B.

Streptonivicin and Cathomycin, Antibacterial Activity of. W. F. Jones, R. L. Nichols and M. Finland. (*J. Lab. clin. Med.*, 1956, **47**, 783.) Streptonivicin and cathomycin are new antibiotics produced by *Streptomyces niveus* and *Streptomyces spheroides* respectively. Data concerns the *in vitro* activity of the crystalline monosodium salts of these two substances against various species of aerobic bacteria. It was found that these two substances exhibited essentially the same antibacterial activity in all of the tests to which they were subjected. Many strains of a variety of bacterial species showed wide differences in their susceptibility to these two agents, but both were equally active against each strain. The addition of serum or blood to agar, or of various concentrations of serum in broth used in the sensitivity tests, resulted in a decrease of susceptibility of a strain of *M. aureus*, the extent of the decrease being directly related to the concentration of serum, and the effect being identical with both antibiotics. The minimum inhibiting concentrations of both streptomycin and cathomycin for *M. aureus* and proteus, when tested in broth, were decreased progressively and to the same degree by progressive decreases in the pH of the medium. Increases in the size of the inoculum, in tests employing broth, resulted in decreases in the *in vitro* activity to the same extent with both antibiotics against staphylococcus and proteus. The rate of development of resistance in strains of *M. aureus* was the same for both antibiotics. It is thus concluded that streptonivicin and cathomycin are either the same or very closely related substances. M. M.

PHARMACY

NOTES AND FORMULAE

Association Colloid Solutions, The Effect of Decanol-1 on the Viscosities of Some. K. Passinen and P. Ekwall. (*Acta chem. scand.*, 1956, **10**, 215.) The effect of the relatively long paraffin chain alcohol, decanol-1, on the viscosity of association colloid solutions, has been studied using solutions of sodium oleate, sodium lauryl sulphate and sodium myristyl sulphate. At low oleate concentrations (below 0·15M) the viscosity increases slowly as the amount of decanol is raised, and no marked change occurs at the turbidity point where a new phase composed of decanol, sodium oleate and water begins to separate. With oleate concentrations above 0·2M, viscosity rises rapidly as long as the decanol is solubilised in the micelles, but as soon as the system becomes heterogeneous, viscosity decreases with increasing decanol concentrations, passing

through a minimum and finally rapidly increases. The minimum in the viscosity curve appears at a decanol concentration of approximately 0·3 to 0·4 mole of decanol per mole of oleate and is in the vicinity of the turbidity maximum in the same system. The effect of *p*-xylene on the viscosity of sodium oleate solutions has also been studied. Increased viscosity at low oleate concentrations is explained on the assumption of an increase in micelle volume, and at higher concentrations large changes in the form and structure of micelles must be assumed. J. B. S.

Cyclobarbitone, Stability of. S. Åhlander. (*Svensk farm. Tidskr.*, 1956, **60**, 249.) On storage, cyclobarbitone undergoes decomposition with formation of peroxides, probably at the methylene groups which are in the α position to the double bonds. This decomposition is not revealed by direct titration with alkali, but the bromometric assay shows it clearly. A number of old samples were examined. All titrated 99 to 100 per cent. by alkali, whereas the actual contents ranged from 79 to 99 per cent. Values below 80 per cent. were shown by samples respectively 8, 6 and 4 years old. The loss in strength was in all cases proportional to the peroxide value. Cyclobarbitone-calcium and hexobarbitone do not decompose on storage. Tablets of cyclobarbitone showed a considerable loss in strength after storage for periods from 1 to 4 years.

G. M.

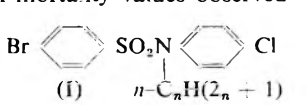
Soap Concentration where Interaction with Decanol-1 Begins and its Dependence on the Chain Length of the Soap. P. Ekwall, O. Söderberg and I. Danielsson. (*Acta chem. scand.*, 1956, **10**, 227.) Turbidity measurements show that the point at which interaction between decanol-1 and fatty acid soaps begins is dependent on the chain length of the soap. The soap concentration at which interaction begins decreases as the length of the hydrocarbon chain of the soap increases. This concentration lies very close to the lowest concentration at which hydrolysis in the soap solution leads to the formation of acid soap, or at which the soap forms acid soap when fatty acid is added. The variation of the upper limit of the first turbidity range with temperature is almost linear. The limiting association concentration lies considerably lower than the critical micelle concentration of the pure soap and below the concentration where micelle formation begins to increase rapidly in soap solutions that contain added alcohols. J. B. S.

Sodium Caprate Concentration where Interaction with Long-chain Alcohols Begins and its Dependence on the Chain Length of the Alcohol. P. Ekwall and C. F. Aminoff. (*Acta chem. scand.*, 1956, **10**, 237.) Turbidity measurements show that the interactions of hexanol-1, octanol-1, decanol-1 and dodecanol-1 with sodium caprate all begin at the same soap concentration and are independent of the chain length of the alcohol. The effect of temperature on the interaction between alcohol and soap is also independent of the nature of the alcohol. Higher molecular weight alcohols, tetradecanol-1, hexadecanol-1 and octadecanol-1 also show interaction at temperatures above their melting points, though interaction commences at slightly higher soap concentrations than with the shorter chain alcohols. It is shown that the association above the limiting concentration follows the law of mass action. It appears that the same forces that affect the association in pure soap solutions must take an active part also in the formation of soluble aggregates between soap and alcohols or fatty acids. Ion dipole interactions and hydrogen bond formation between carboxylate soap ions and alcoholic hydroxyl groups also contribute to the complex formation. J. B. S.

PHARMACOLOGY AND THERAPEUTICS

Benactyzine in Psychoneurosis. M. J. Raymond and C. J. Lucas. (*Brit. med. J.*, 1956, **1**, 952.) A pilot study carried out with benactyzine on 43 outpatients with various psychiatric disorders suggests that patients with a symptomatology in which anxiety and tension predominate respond favourably to the drug (of 18 cases, 8 were much improved and 4 improved), while those with depressive, hysterical and obsessive symptoms do not. About half the patients receiving 2 mg. three times a day experienced side-effects; these included a feeling of heaviness in the limbs, giddiness, ataxia and clumsiness, difficulty in reading small print, poor concentration, diarrhoea, increased anxiety, and drowsiness. On a dosage of 1 mg. three times a day side-effects did not occur and the higher dosage could be tolerated provided the increase was gradual. Most of the patients benefiting from the drug appeared first to notice an improvement towards the end of the first week of treatment. In 10 healthy volunteers given the drug subcutaneously subjective changes and EEG changes were noted. Changes in perception were predominant, and in half the subjects there was a marked diminution in the amount of alpha rhythm; this change is comparable to that following the administration of mescaline. S. L. W.

Dicophane, Substituted Benzenesulphonanilides as Synergists for. M. Neeman, A. Modiano, G. G. Mer and R. Cwilich. (*Nature, Lond.*, 1956, **177**, 800.) A series of 4-bromobenzenesulphon-4′chloroanilides (I) have been synthesised and examined for synergistic activity with dicophane (DDT) against dicophane-resistant houseflies. The compounds were tested together with dicophane by topical application of benzene solutions, and mortality observed after six hours. They were also tested in the form of residual deposits by the method of Mer and Davidovici. The proportional mortality values observed after topical application formed two statistically homogeneous groups of responses, significantly higher than the response to dicophane by itself, where $n = 1$ or 2, and where $n = 0$, 3, 4, 5, 6, 7 or 8 respectively. The proportional knock-down values observed after short duration contact with residual deposits formed three statistically homogeneous groups where $n = 6$, $n = 0$, 1, 2, 4, 5, 7, 8 and $n = 3$ respectively. A number of other synergists previously reported active were also examined. J. B. S.



(I)

Digitalis, Seasonal Variation in Response of the Pigeon to. R. A. Sachs, J. D. Highstrete and M. L. Pabst. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 248.) Examination of the data obtained over a 4-year period using the pigeon assay with the U.S.P. digitalis reference standard revealed a seasonal variation in the response of pigeons to digitalis. The animals were more resistant during the summer months, the maximum resistance occurring in August or September. On account of this seasonal variation it was found advisable to limit the interval between tests on the standard and the unknown to 30 days, when potency estimates were generally within the acceptable limits of error. Results outside the acceptable limits were more frequently encountered when the interval was increased to 60 days. G. B.

Meprobamate, Central Depressant and Anticonvulsant Activity of Compounds Isomeric with. F. M. Berger, C. D. Hendley, B. J. Ludwig and T. E. Lynes. (*J. Pharmacol.*, 1956, **116**, 337.) Meprobamate (Miltown, Equanil), an interneuronal blocking agent related to mephenesin, but with a longer duration of action, was compared for activity in mice with its seven isomers having the

basic 2-substituted trimethylene glycol dicarbamate structure, and with other structural isomers where alkyl substitution was made on one or more of the three carbons of the propanediol nucleus. All the compounds produced flaccid paralysis, either preceded or followed by excitement; meprobamate was the most potent of the series, being twice as effective as the next best compound. Against leptazol-induced convulsions and death, meprobamate gave most protection. The dicarbamates of 2-*n*-butyl-1:3-propanediol and of 2-*iso*butyl-1:3-propanediol afforded the same degree of protection against electroshock seizures as did meprobamate; taking the ratio between median paralysing dose and median seizure-modifying dose, meprobamate had the smallest ratio and 2-*n*-butyl-1:3-propanediol dicarbamate the greatest. The melting points of the compounds correlated well with paralysing potency and ability to protect against leptazol convulsions, but not with ability of the compounds to modify electroshock seizures. Clinically, meprobamate has been found to be of value in three types of condition: (1) anxiety states; (2) neurological and arthritic diseases involving muscle spasm; and (3) some forms of petit mal epilepsy. However, the closely related 2:2-diethyl-1:1:3-propanediol dicarbamate was of no value in anxiety states where beneficial results had previously been obtained with meprobamate. G. P.

Meratran, Clinical Trials with. W. G. A. Begg and A. A. Reid. (*Brit. med. J.*, 1956, **1**, 946.) This is a report on the use of a new stimulant drug meratran (α-(2-piperidyl) benzhydrol hydrochloride) in the treatment of over 200 psychiatric patients, the majority of whom were suffering from depression. In addition, 24 normal persons were given up to 3 mg. of the drug daily by mouth for periods of time up to a week. Two reported no change as a result of the drug, but the remainder reported an insidious elevation of mood (insufficient to be characterised as euphoria), with heightened confidence, greater ability to concentrate, and an increased work output. The effect of the drug lasted for 12 to 24 hours and there were no "hangover" effects. Some subjects reported interference with sleep. The drug was found to be most helpful in reactive depressions uncomplicated by anxiety, hysterical or obsessional traits. When obsessional features are present meratran tends to aggravate rather than relieve the condition; anxiety is also frequently increased by the drug. Of a group of 29 patients with the purer type of reactive depression 25 were helped by meratran, while of a group of 22 patients with reactive depression with hysterical features only 11 were improved. Dosage was started with a dose of 1 mg. three times daily and gradually increased to a maximum of 7·5 mg. a day in three doses; barbiturates were also given to counteract sleep disturbance. Patients with endogenous depressions were not so responsive to meratran as were the reactive depressives; of 65 patients suffering from severer depressions only 14 obtained any lasting benefit. Some of the patients in this group became worse during treatment with meratran and in some cases patients not previously showing obvious anxiety became acutely anxious, agitated, deeply depressed and even suicidal in the course of a day or two. The tendency of the drug to exacerbate pre-existing anxiety and produce unexpected aggravation of the mental state may often be successfully combated by combining each dose of meratran (2·5 mg.) with chlorpromazine hydrochloride 50 mg. or amylobarbitone sodium 100 mg. Meratran does not destroy appetite as the amphetamines do, but some patients may complain of nausea. No other serious side-effects were observed apart from the psychotic episodes mentioned (4 case reports are given). Unfortunately, owing to the lack of side-effects no warning of the imminence of

such episodes is given, and they can occur on minimal doses of the drug. Further clinical research is needed to ascertain more clearly the type of patient in whom these episodes are likely to occur. Promising results with meratran were obtained in 5 cases of spasmodic torticollis and in a number of patients suffering from post-leucotomy anergia. S. L. W.

Meratran in the Treatment of Schizophrenics. F. Houston. (*Brit. med. J.*, 1956, **1**, 949.) This is a study of group behaviour in chronic schizophrenics treated with the stimulant drug meratran. For the project a group of 20 male schizophrenics, the most deteriorated and withdrawn patients in a hospital of 900 patients, was selected. Their behaviour was studied under the headings of feeding, letter-writing, mutual aid, conversation, washing and dressing. After 6 weeks preliminary charting, 10 of the patients were given meratran (2 tablets three times daily) and 10 were given placebo tablets, of similar appearance and flavour, for 4 weeks. The nursing staff did not know which were the placebo tablets. After a 2-weeks interval the meratran and placebo order was reversed and administered for a further 4 weeks. The results in the fourth weeks of the two courses of treatment with meratran and placebo were compared and were found not to differ significantly. The results in the second week of the project were compared with the results in the final week, and a significant improvement in reading, washing and dressing was noted. This was attributable to increased nursing enthusiasm. Such a control experiment had not previously been carried out in the hospital, and it was the focus of considerable discussion, feeling and approval. The author concludes that caution and adequate control procedures are essential before any therapy or drug is accepted as beneficial in chronic psychotics, and that statistical analysis of results is essential, since in this particular group it showed that meratran was no more effective than placebo tablets yet an improvement in the behaviour of the patients occurred that would otherwise have been attributed to meratran. S. L. W.

Methitural, a New Intravenous Anaesthetic: Comparison with Thiopentone. S. Irwin, R. D. Stagg, E. Dunbar and W. M. Govier. (*J. Pharmacol.*, 1956, **116**, 317.) Methitural, sodium 5-(2′-methylthioethyl)-5-(1-methylbutyl)-2-thiobarbiturate, had about two-thirds of the anaesthetic potency of thiopentone in the cat, dog and monkey. With equivalent anaesthetic doses recovery from anaesthesia was more rapid with the methitural than with thiopentone. Cumulative action was also considerably less with methitural than with either thiopentone or thioamylal. The more rapid recovery from anaesthesia and low cumulative effects were due to a higher rate of destruction of the drug by the liver. Affinity of methitural for the fat depots of the body was the same as for thiopentone. In unanaesthetized dogs both thiopentone and methitural caused a transient hypotension, cardiac acceleration and reversible ectopic beats and bigeminy. Induction apnoea with thiopentone in dogs and cats was more often encountered than with methitural; also, intercostal respiration in the monkey and the dog was depressed more with thiopentone. Muscle relaxation was the same with both drugs. The incidence of side effects was slightly greater with methitural, more salivation and spontaneous coughing being noted in the cat. Atropine abolished salivation and reduced the incidence of coughing and cardiac arrhythmias; depth and duration of surgical anaesthesia also appeared to be prolonged. Morphine also prolonged, and leptazol partly reversed, anaesthesia with methitural. With continued administration of methitural or thiopentone to dogs, liver damage was considerably more frequent with the thiopentone. G. P.

3-(α-Naphthyl)-4-hydroxycoumarin, a New Synthetic Anticoagulant. J. Moraux. (*Thérapie*, 1956, **11**, 104.) Synthetic anticoagulants derived from coumarin were differentiated chemically into symmetrical and asymmetrical compounds: the former were twinned molecules, the single molecule being Vitamin K-like (e.g., 3-methyl-4-hydroxycoumarin) whilst the twinned molecule (e.g., dicoumarol) was anticoagulant. Symmetrical compounds were twinned at ring position 3, whilst the asymmetric were substituted at that position, giving such compounds as 3-(α-naphthyl)-4-hydroxycoumarin, which possessed marked antivitamin K properties. Pharmacologically, these anticoagulants have either short (ethyl biscoumacetate, phenylindanedione) or long (dicoumarol) duration of action. 3-(α-Naphthyl)-4-hydroxycoumarin is without many of the drawbacks of the other derivatives, being long-acting, of low toxicity, well tolerated, active at doses close to those of dicoumarol, and acting on at least three coagulation factors: prothrombin, factor VII (prothrombin conversion) and factor X (thromboplastinogenesis). It is without action on factor V (prothrombin activator) and is antagonized by the K vitamins. G. P.

3-[α-(4-Nitrophenyl)-β-acetylethyl]-4-hydroxycoumarin (Coumarin G. 23.350), Properties of. M. Leroux and B. Jamain. (*Thérapie*, 1956, **11**, 85.) Coumarin G.23.350 (Sintrom), an asymmetrical derivative of coumarin obtained synthetically by substituting a *p*-NO_2 into the phenyl group of the side-chain of Warfarin [3-(α-phenyl-β-acetylethyl)-4-hydroxycoumarin], was a potent anticoagulant. Sintrom contrasted with the closely related compounds Warfarin and Marcoumar in having a short duration of action closely similar to phenylindanedione and ethyl biscoumacetate. Sintrom differed from ethyl biscoumacetate in that maximum activity, when reached, remained steady for 12–24 hours, and then declined rapidly. Anticoagulant effectiveness in man, in order of diminishing potency was Sintrom (1); Marcoumar (2/3); Warfarin (2/5); phenylindanedione (1/5); dicoumarol (1/15); 3-(α-naphthyl)-4-hydroxycoumarin (1/20); ethyl biscoumacetate (1/45). Sintrom was an indirect anticoagulant, inactive *in vitro*, but active *in vivo*, inhibiting liver synthesis of proconvertin, prothrombin and factor X; vitamin K_1 was a rapid antidote. The toxicity of Sintrom in mice was less than with ethyl biscoumacetate, Marcoumar and phenylindanedione. Sintrom was tested clinically in 53 patients with no untoward effects; the initial dose was at most 20 mg. the first day and 16 mg. the second; maintenance doses (2 to 8 mg. a day) depended on the results of blood tests. The drug appeared therapeutically useful and presented some advantages over ethyl biscoumacetate. G. P.

Novobiocin: A Laboratory Investigation. G. Lubash, J. van der Meulen, C. Berntsen and R. Tompsett. (*Antibiotic Med.*, 1956, **2**, 233.) Novobiocin was found to be highly active *in vitro* on all strains of *Staph. aureus* tested, including strains highly resistant to all other commonly used antibiotics; it was also found active *in vitro* against other Gram-positive cocci, including certain strains of pneumococci and haemolytic streptococci. In general it was found relatively inactive against most Gram-negative bacilli with the exception of certain strains of *P. vulgaris*. The *in vitro* activity is markedly inhibited by human serum, probably because of its high degree of binding by serum albumin. It is well absorbed when administered orally and reaches high concentrations in the blood. It was not found, however, in cerebrospinal fluid or pleural fluid, but was demonstrated in bile. Novobiocin was administered to 30 patients in a dosage of 2 g./day in adults, given in 4 equal doses for periods up to

(ABSTRACTS *continued on p. 1000.*)

LETTER TO THE EDITOR

Bactericidal Activity and Soap Solution Structure

SIR,—A recent paper by Berry, Cook and Wills,[1] shows variation in the bactericidal activity with concentration of potassium laurate, in the range 0 to 100 millimoles/l. This took place in the presence of certain added phenols, and took the form of peaks or breaks in the bactericidal activity-concentration curve. Soap concentrations of the following limiting activity were reported:

(*a*) a first limit of about 30 millimoles/l., identified with the Critical Micelle Concentration. At this point maximal bactericidal activity was observed,

(*b*) a second limit of about 45 millimoles/l., at which point minimal bactericidal activity was observed, and

(*c*) a third limit above 80 millimoles/l., which corresponded to a point above which the concentration-bactericidal activity curve was "normal" and extinction time decreased with increased concentration.

The authors offered a number of suggestions to explain this anomalous variation. There is, however, a possible alternative explanation. Abrupt changes in the physical property-concentration curves of a number of colloidal electrolytes have been pointed out by Ekwall[2,3]. Measurements of equivalent conductance, solubilisation properties, hydroxyl-ion activity and partial molal volume when plotted against concentration of potassium laurate, gave curves which showed distinct breaks at 6, 20, 28 and 50 millimoles/l. Similar curves for other association colloids showed similar breaks at various concentrations, which Ekwall termed concentration limits. In corroboration of these results, Brudney and Saunders[4] found distinct breaks in the diffusion coefficient-concentration curve of potassium laurate at 28 and 50 millimoles/l. Breaks in the diffusion coefficient-concentration curves for sodium dodecyl sulphate,[5] and other association colloids[6] have also been reported and were in most cases in accord with Ekwall's findings.

With potassium laurate, there is an interesting similarity between the physical properties-concentration curves and the bactericidal activity curves of Berry and others.[1] Slight variations in the actual figures for concentration limits are inevitable with variations in temperature, conditions of purity, additive concentrations, or applied forces. Further, all the limits cannot necessarily be shown by any particular method. Thus, using the Gouy Diffusiometer, the limits at 6 and 20 millimoles/l. could not be shown, since these concentrations were outside the range of the apparatus. Similarly, these limits have not been shown by the bactericidal measurements. However, the fundamental pattern is the same, that is, anomalies occur in the property-concentration curves for potassium laurate between 0 and 100 millimoles/l., and these anomalies occur as distinct peaks or troughs in these curves.

Presumably these abrupt changes in physical and bactericidal properties could have a common explanation in terms of solution structure. Hartley's soap solution structure theory seems to provide a basis for the explanation of anomalous diffusion coefficient-concentration curves,[7] in the terms of a change in effect on the micelles, of the gegenions.

The obvious similarity of the physical property-concentration curves and bactericidal activity-concentration curves leads one to believe that bactericidal

activity is a function of solution structure. I suggest that on these lines an explanation of the mode of antibacterial action, with a stronger theoretical background, could be developed.

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(ABSTRACTS *continued from p. 998.*)

5 days. In 6 cases with uncomplicated pneumococcal pneumonia the clinical outcome was satisfactory; in a seventh patient the condition deteriorated on novobiocin and he was changed to penicillin therapy after 24 hours. Fourteen patients were treated for infections of the genito-urinary tract; cultures of urine prior to treatment all revealed *P. vulgaris*. Five of the patients were improved by novobiocin, though in 3 of these the cultures remained positive for *P. vulgaris*. Three patients derived no benefit, and in 6 the outcome was indeterminate. Proteus could be cultured from the urine of 8 of the 14 patients at the termination of therapy. The only side effects observed in this series of patients were mild skin eruptions which occurred in 2 cases; in one of these a second course of novobiocin did not cause a recurrence of the eruption. S. L. W.

Nystatin in Mycotic Infections. G. T. Stewart. (*Brit. med. J.*, 1956, **1**, 658.) Nystatin (Mycostatin) is an antibiotic prepared from *Streptomyces noursei*. It is an amphoteric crystalline polyene with the probable empirical formula $C_{46}H_{77}NO_{19}$, insoluble in water, but soluble in various alcohols and ethers. It is available as a pale-yellow lyophilised powder which is rapidly inactivated by heat, light and oxygen; tablets containing 500,000 units of the substance are prepared for clinical use. *In vitro* experiments show that nystatin inhibits cell-division and mycelial growth of candida and saccharomyces, including pathogenic strains isolated from a variety of human lesions. This effect is fairly complete against *C. albicans* at concentrations of 5–20 units/ml. Its mode of action is complex but highly specific. The presence of a chain of CH_2 groups, as in the alcohols, favours activity, while CHOH and CHO groups, as in various sugars, are antagonistic. Glycols, with combination of both, show intermediate properties. A series of 12 bronchitic patients with fungal hyphae demonstrable in direct examination of films made from sputum, and cultures positive for *C. albicans*, were treated with three to four daily oral doses of 500,000 units for 7 days. The moniliasis in these patients had developed as a sequel to antibiotic therapy. As a result of the treatment with nystatin 9 out of the 12 patients were rapidly and completely cleared of the mycotic infection. A similar result was obtained in 7 out of 8 cases of stomatitis. Apart from transient nausea no toxic effects were observed. Nystatin and antibacterial agents showed no mutual interference *in vitro* but when given prophylactically nystatin was not wholly successful in preventing mycotic superinfection in patients receiving antibacterial therapy. Resistance was not found to develop in strains after passage *in vitro* or on re-isolation during and after treatment. S. L. W.