REVIEW ARTICLE

THE PHYSICAL CHEMISTRY OF THE LECITHINS

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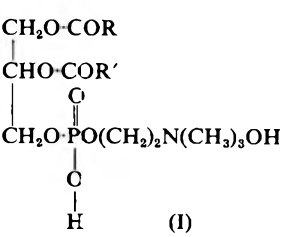
The lecithins belong to the group of natural fats or lipides known as phosphatides, which occur in nearly all living cells. It has been shown that when animals are subjected to nutritional deficiencies, the amounts of phosphatides present in the various organs remain constant[1,2], indicating that these substances are structural components.

The universal distribution of phosphatides in animal cells and the facility with which fatty films are formed by them, suggest that they constitute the central structure of the cell wall (assuming that this structure is of the form proposed by Danielli and Davson[3] and Danielli and Harvey[4]). As is described later in this review, lecithin sols in water possess the property of forming stable films at a boundary made between the sol and water. These films consisting of membranes of fatty material and separating two aqueous liquids, have some of the properties of simple cell walls. Further studies of them and of the physical chemistry of lecithin sols would seem to offer a promising approach to the elucidation of the structure of cell membranes. Such work could also lead to the development of model membranes which might be helpful in the quest for further knowledge concerning the physico-chemical mechanisms of drug action.

In this review, we summarise the present state of research into the physical chemistry of the lecithins.

Chemical Structure of the Lecithins

As a result of their hydrolysis experiments on lecithin prepared from egg yolk, Diakanov[5–7], and Strecher[8] in 1868 proposed the structure (I) for the compound where R and R′ are hydrocarbon chains, which in the natural lecithins, may contain one or more double bonds.



(I)

The basis of this structure is the glycerol nucleus; this is joined by ester links to two long chain fatty acid radicals and to an orthophosphate group, which in turn is linked to the strongly basic choline group by means of another ester bond. The resulting molecule has a soap-like structure with water-insoluble long chain fatty acid groups joined to the rather complex water soluble head group (the part of the molecule other than the hydrocarbon chains R and R′).

The structure (I) has the phosphate radical attached to the α-carbon atom of the glycerol; optical isomers are possible as the β- or central carbon atom of the glycerol is asymmetrical. The alternative structure in which the phosphate is attached to the β-carbon and R and R′ are identical, would not give optical isomers. The natural lecithins are always optically active. Hydrolecithins, which are prepared by reducing the fatty acid radicals of natural lecithin and have also been isolated directly from natural sources have $[\alpha]_D^{20}$ varying between $+6{\cdot}25°$ and $+7{\cdot}10°$[9,10].

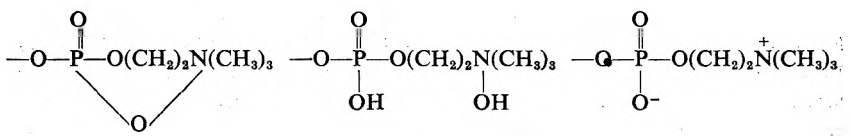
Pure (dipalmitoyl) lecithin prepared from natural sources was found to be dextrorotatory; it was shown to have the same configuration as synthetic L-α-(dipalmitoyl) lecithin. This configuration also applied to egg and brain lecithins, since the hydrolecithins obtained by reduction with hydrogen, were dextrorotatory. In addition, Long and Maguire[11] have shown that after hydrolysis of natural lecithin amounts of α-, L-α- and D-α-, glycerophosphoric acid were recovered, which were identical with those obtained by hydrolysis of synthetic L-α-lecithin, and quite different from those obtained by the hydrolysis of synthetic β- or DL-α-lecithins. It was therefore proved that natural lecithins have the L-α conformation.

Fatty Acids. A great deal of work has been done on the identification of the fatty acids obtained by hydrolysis of lecithin. Lecithin prepared from natural sources normally yields a fairly complex mixture of acids. Palmitic and stearic were the only saturated acids present[12–15] in egg lecithin, they comprised about 35 per cent. of the total fatty acid mixture. A wide range of unsaturated acids have been found[14–19]. Levene and Rolf[16] identified oleic (C_{18} with one double bond), linoleic (C_{18} with two double bonds) and arachidonic (C_{20} with four double bonds) acids in egg lecithin hydrolyzates. Subsequent work[14,15] has shown the presence of a group of highly unsaturated, long chain acids described collectively as "clupadonic acid". A typical analysis of the fatty acid mixture resulting from the hydrolysis of egg lecithin is,

Stearic acid	4·1 per cent.
Palmitic acid	31·8 per cent.
Oleic acid	42·6 per cent.
Linoleic acid	8·2 per cent.
"Clupadonic acid"	13·3 per cent.

The unsaturated fatty acid composition of egg lecithin can vary according to the diet of the hens[20].

Zwitterionic Structure. Grun and Limpacher[21] suggested that the phosphate-choline part of the lecithin molecule could have one of two



(II) (III) (IV)

structures, the anhydro-form II or the hydrated form III. Jukes[22], however, proposed a zwitterionic structure IV and this seems most likely to be correct in view of the fact that both choline and phosphoric acid (first stage of dissociation), are strong electrolytes. This zwitterionic structure was supported by potentiometric titration evidence[22,23], and by the observations that lecithin was dielectrically active in both water[24] and ethanol[25].

Hydrolecithin. This name is given to lecithins containing only saturated fatty acid radicals[26]. They can be prepared by the hydrogenation of natural lecithins, by isolation in the pure state from natural sources[9,10], and by synthesis[34].

Lysolecithin. Lysolecithins have the same structure as the lecithins except that only one fatty acid radical is present. They occur in association with the natural lecithins, and it is only quite recently that chromatographic methods have been developed which ensure the complete separation of lysolecithin from natural lecithin.

Lysolecithin is formed by partial hydrolysis of lecithin and a highly specific catalyst for this reaction is the lecithinase enzyme; this occurs in a very active form in snake venoms[27,28]. An important development in the study of lysolecithin is the discovery by Hanahan and others[29] that the venom dissolved in water will act on an ethereal solution of lecithin. Lysolecithin is insoluble in ether and so is precipitated as it is formed. It can therefore be removed from the reaction mixture by centrifugation and treated to give a high yield of pure lysolecithin.

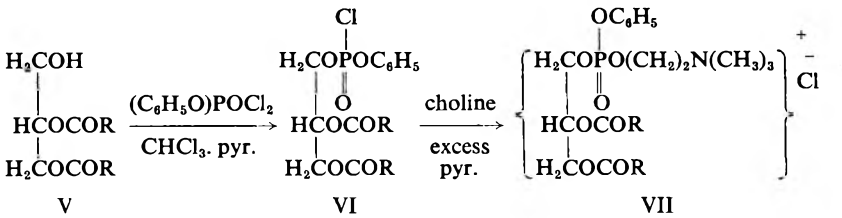
It has been found by various oxidative procedures[30,31] that the fatty acid in lysolecithin is attached to the β-carbon atom of the glycerol group. When it is prepared from natural lecithin containing unsaturated acids the lysolecithin possesses only saturated fatty acids; so it seems that in lecithin, unsaturated acids are attached to the α-carbon atom[32,33]. Hanahan and others[29] have found that the snake venom reaction occurs with pure (dipalmitoyl) and (dipalmitoleyl) lecithins giving the corresponding pure lyso-compounds.

Lysolecithin has a strong lysing action on erythrocytes, this is presumably caused by dissolving out the lecithin film which forms the membranes of the cells. The lyso-compound also probably plays an important part in the dispersion of lecithin in aqueous fluids.

Synthesis of Lecithin

The synthesis of lecithin is a difficult problem. Many of the earlier synthetic materials were in fact mixtures. A recent synthesis of hydrolecithins by Baer and Kates[34], used a D-α,β-diglyceride (V) as the starting material (prepared by the method of Sowden and Fischer[35]). This was treated with monophenyl phosphoryl dichloride in the presence of pyridine and the whole reaction mixture was treated with choline chloride in the presence of a large excess of pyridine. The resulting material was isolated as a reineckate, converted to a sulphate, catalytically hydrogenated to remove the phenyl group and then the sulphate was removed by means of barium carbonate. In this way (dimyristoyl-), (distearoyl-)

and (dipalmitoyl-) lecithins have been prepared in an optically active form. The stages in the synthesis are shown below.



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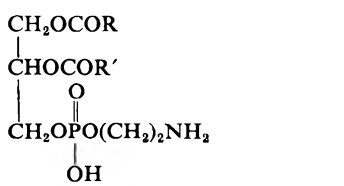
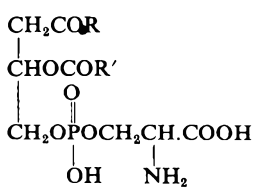
This year, Baer, Buchnea and Newcombe[36] have reported the synthesis of the unsaturated L-α-(di-oleyl) lecithin. D-Acetone glycerol was phosphorylated with phenyl phosphoryl dichloride and the product was esterified with ethylene chlorhydrin. After removal of the phenyl and acetone groups, the L-α-glyceryl-phenyl-phosphoryl-ethylene chlorhydrin was isolated as its barium salt and treated with oleyl chloride. The resulting compound was heated with trimethylamine, giving a mixture of L-α-(di-oleyl) lecithin and the corresponding lysolecithin. The pure lecithin was isolated by chromatography.

Preparation of Lecithin from Natural Sources

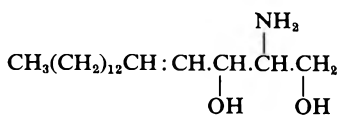
In order to prepare lecithin, the starting material is first extracted with ethanol. This breaks up the lipo-protein complexes[37] and dissolves the phosphatides together with other fats. After removal of ethanol the residue is extracted with acetone; the phosphatides are insoluble in acetone whereas the other lipides such as cholesterol, dissolve. Alternatively, the starting material can be extracted first with acetone and then with ethanol. The major components of this extract other than lecithin are:



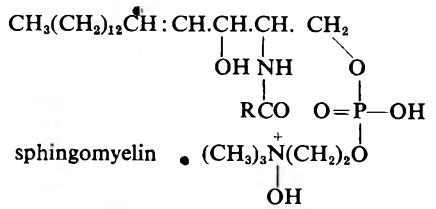
cephalin



phosphatidyl serine



sphingosine



sphingomyelin

Fractionation of this mixture to give lecithin was until recently carried out by a method devised by Bergell[38] in 1900 and developed by MacLean[39],

Levene and Rolf[13,40] and by Pangborn[41–44]. In this a complex between cadmium chloride and lecithin is formed which is only sparingly soluble in ethanol. It is precipitated by adding a saturated cadmium chloride solution to an ethanolic solution of the mixed phosphatides. The complex is further purified by extraction or by partition between immiscible solvents. Lecithin is liberated from the complex by adding methanolic ammonia to a chloroform solution[13,40], which precipitates the cadmium as hydroxide. Alternatively the chloroform solution of the complex is extracted with 30 per cent. ethanol in water[41–44], or it is broken up by using ion exchange resins[45].

Reasonably good samples of lecithin, free from amino nitrogen and with the correct N/P ratio, have been obtained. However, the phosphatides are prone to associate with one another in solution and fractionations based on precipitation are not clear cut. The ideal procedure for separating such materials is one in which the molecules are dealt with almost individually, for example, by adsorption on to the surface of a solid followed by elution. Several chromatographic procedures based on this principle have been successfully developed for fractionating phosphatide mixtures. A disadvantage of the cadmium chloride purification method is that it is almost impossible to remove all traces of cadmium from the lecithin, and traces of cadmium have a considerable effect on some of the properties of lecithin sols[46]; the chromatographic method avoids this contamination.

Hanahan[47,48] and his colleagues have used alumina for the chromatography of ethanolic solutions of phosphatide mixtures. They found that all the amino-containing substances were removed by the column and the effluent gave a solid product with a reasonable N/P ratio. Faure also used this method[49].

Lea and Rhodes[50,51] have found that the product from alumina chromatography has a low fatty acid/P ratio and they attributed this to the presence of lysolecithin. Using a mixed silicic acid and Celite column, with 20 per cent. methanol in chloroform as solvent, they obtained three chromatographic fractions from egg yolk phosphatides. The first fraction had an R_F value of 0·9 and consisted of the amino-containing compounds, mainly cephalin, the second fraction ($R_F = 0{\cdot}6$) consisted of the lecithins and had the correct N/P and fatty acid/P ratios. The third fraction ($R_F = 0{\cdot}1$) was eluted from the column with methanol and consisted of lysolecithin.

This chromatographic method of Lea and Rhodes is the only process so far developed by which lecithin can be completely separated from lysolecithin.

Direct paper chromatography of phosphatide mixtures does not seem to have been very successful[52,53]. Lea, Rhodes and Stoll[54] have, however, developed separation procedures using silica impregnated paper. An attempt has been made to separate lecithin from an egg extract by counter current partition chromatography on paper[55]; some success has been claimed for two dimensional paper chromatography using acetone-methanol and phenol[56].

Light magnesium oxide has been used as a chromatographic material; all the phosphatides were adsorbed from a solution in petroleum ether but only the lecithins were eluted by methanol[57]. Chromatographic methods are valuable for preparing lecithin as they do not involve any harsh chemical treatment. Lecithins are easily decomposed by heating or by the action of acids or alkalies.

No separation of lecithins according to the degree of unsaturation of their fatty acids has so far been achieved by chromatography. Escher[58] in 1925 reported such a separation by fractional crystallization from ether at low temperatures, but his results have not been confirmed; his separation may have been one of lysolecithin from the lecithin.

Physical Properties of Lecithin

The purified lecithin prepared from natural sources such as egg yolk, contains a variety of fatty acids, but as it is not been resolved into separate components by techniques so far developed, it can be regarded as a chemical entity.

It is a white, somewhat waxy solid and is very hygroscopic. On exposure to air, oxidation of the unsaturated acids occurs; the hydrolecithins are more stable. All the natural lecithins and lysolecithins are optically active, the former are dextrorotatory while the latter are reported to be laevorotatory[28]. No definite melting points have been reported but synthetic (dipalmitoyl) lecithin sintered at 40–42° C. and formed a meniscus at 235° C.

With water, synthetic hydrolecithins form sols which settle out quite rapidly[59] in contrast to the natural lecithins whose sols are stable over long periods. These natural lecithin sols are turbid. The lysolecithins give optically clear sols in water.

The hydrolecithins are soluble in warm ethanol and are insoluble in ether, acetone and cold ethanol[34]. The natural lecithins are insoluble in acetone but freely soluble in ethanol, ether, and chloroform. Lysolecithin is insoluble in ether and acetone but freely soluble in warm chloroform, water, and ethanol.

X-ray diffraction patterns of crystalline, synthetic hydrolecithins showed variations with the length of fatty chain in the compound[34]. From low angle X-ray diffraction studies[60], layer spacings were deduced, and on plotting these against fatty acid chain length a straight line was obtained. The slope of this line was characteristic of the arrangements of the hydrocarbon chains while extrapolation to zero chain length gave the contribution of the end group to the layer spacing (23 Å).

The natural lecithins can be prepared in crystalline form by cooling a saturated solution in a mixture of acetone and methyl ethyl ketone. The crystals show a parallel extinction under the polarising microscope[61].

When water is put on to solid lecithin, twisting cylindrical forms (myelin forms) grow out from the surface of the solid, these often develop into intricate patterns, and ultimately the solid disperses completely. Leathes[62] has studied the effects of various additives on these; he found that their appearance was changed when various acids and alcohols were

put in contact with the lecithin. Lime water prevented their growth but baryta water did not. Leathes considered that the outgrowths were due to the hydrophilic nature of the lecithin molecule. Hydrolecithins give few myelin forms, and so the unsaturated acids in natural lecithin must play some part in the process of growth. It has been suggested that the myelin forms are liquid crystals[63].

Physical Chemistry of Lecithin Sols

General

With water, natural lecithin forms sols which are easily precipitated by small concentrations of divalent metal salts but are stabilised by higher concentrations. Large concentrations (10 per cent.) of lecithin can be dispersed in water, the surface tension of the sol being lower than that of water. The turbidity and stability of the sol to electrolytes are both dependent on the proportion of lysolecithin in the dispersed material. It is only since Lea and Rhodes'[50] work in 1954 that natural lecithin free from lysolecithin has been made, and so it must be recognised that all earlier work has been carried out on preparations containing varying amounts of lysolecithin.

The sols, which are turbid white liquids, are made by shaking or triturating solid lecithin with water[64], or by adding an ethereal solution of lecithin to water with vigorous stirring and removing the ether by bubbling nitrogen through the liquid[65]. In the absence of inorganic salts, they are quite stable over a period of days. De Jong and his colleages[66] claim to have prepared clear sols by adding a hot ethanolic solution of lecithin to hot water and stirring, however, this procedure undoubtedly results in some decomposition of the lecithin.

The mean molecular weight of the monomer of egg lecithin can be calculated as 773[67]. Boiling point measurements with solutions in ethanol gave a value of 797, whereas in benzene a value of 3388 was found[68]. Dialysis experiments have confirmed that in polar non-aqueous solvents such as ethanol the lecithin is present as the monomer whereas in non-polar solvents, it is in a micellar form[69]. In benzene and heptane the micelles are believed to have the paraffin chains of the molecules directed outwards. In water, lecithin exists in the form of very large micelles in which the polar parts of the molecule are on the outside. X-ray diffraction studies[70,71] of concentrated sols indicate that bimolecular leaflets are present. The long spacing, representing the thickness of the bimolecular leaflet plus the distance between the leaflets, was found to be 69 Å for a 33 per cent. sol. Both potassium and calcium chlorides reduced this figure, the latter salt having the greater effect.

Surface and Interfacial Tension Studies

Neuschlosz[72,73] reported that lecithin did not lower the surface tension of water. Subsequently, more accurate work[68] has shown that the surface tensions of the sols varied as a function of pH, being a maximum at the isoelectric point; sodium and calcium chlorides shifted the maximum o lower pH values. These effects were attributed to changes of charge

on the micelles. Filtration experiments showed that the largest particles in the sol possessed the surface activity, the filtrate having a surface tension nearly as high as that of water[75]. Addition of cholesterol modified the pH/surface tension curves.

At the water/benzene interface, small amounts of lecithin dissolved in the benzene phase caused a lowering of interfacial tension; the same amount of lecithin dissolved in the aqueous phase had a much smaller effect[76].

Hirt and Berchtold[77] studied the effect of a large number of substances on the lowering of interfacial tension at the chloroform/water interface by lecithin. Adrenaline-like substances, which possessed an acidic or phenolic *ortho* dihydroxy group, had the greatest effect in reducing the lowering of interfacial tension.

Effect of Electrolytes on Lecithin Sols

Most of the work on this subject suffers from the use of impure materials. The authors have shown that small amounts of impurities, particularly divalent metals or soap-like substances, have a considerable effect on the stability of lecithin sols in the presence of electrolytes.

Early studies showed that the higher the valency of the ions in the added salt the more effective was the coagulating effect on lecithin sols[78–82]. In accordance with the usual theory of colloid precipitation, it has been found that the concentrations of salts which produced coagulation were those required to neutralise the charge on the sol particles[82]. On addition of acid, the sols flocculated at a pH value of about 2[65,81,83,84]. The rate of coagulation was increased by increase of temperature[85].

Some workers[65,86] have reported that divalent metal chlorides gave two precipitation zones with a region of peptisation between them; we have confirmed this observation with calcium chloride[46]. In an extensive study of the coagulating effects of potassium and calcium chlorides on sols of lecithin which had been prepared by various methods, Elworthy and Saunders have found that chromatographic lecithin, which was free from lysolecithin, gives sols which are much less stable to salts than did lecithin prepared by the cadmium chloride method. The sols were stabilised by the presence of small traces of soaps, for example a 0·5 per cent. sol of chromatographic lecithin was precipitated by 2×10^{-3} M potassium chloride; the addition of sodium dodecyl sulphate to a concentration of 5×10^{-5} M rendered the sol stable to 0·1 M potassium chloride. Lysolecithin exerted a similar protecting effect and it is probably the presence of this compound which stabilises lecithin dispersed in biological fluids.

Coacervation studies by de Jong and his colleagues[87–96] have been made with clarified lecithin sols, but the method of clarification almost certainly caused decomposition of the material. Horvath[97] suggested that complex coacervation was a possible form of protein-phosphatide association in living organisms, but this view has been criticised by Bull[98].

Viscosity

The addition of ethanol to lecithin sols increased their viscosity while the addition of electrolytes caused a decrease[99,100]. Old sols had a lower

viscosity than fresh ones. Divalent cations had a greater effect than monovalent ones[101]. However, when viscosity was studied by observing the time taken for a rotating sol to come to rest, it was found that calcium chloride increased the viscosity at concentrations where it reversed the charge on the sol particles[89–91]. Sodium salicylate had a similar effect.

Conductivity

It has been reported that as the concentration of a lecithin sol was increased the specific electrical conductivity passed through a minimum[103]. It was also found that further purification of lecithin decreased the specific conductivity; ageing of the sol increased conductivity[104]. We have found[61] that when a lecithin sol was passed through a column containing mixed ion exchangers, the conductivity was reduced almost to that of pure water though the lecithin content of the sol was almost unchanged. This result indicates that the conductivities previously reported were due to the presence of electrolytes as impurities in the lecithin. Lecithin itself has a very small conductivity in an aqueous sol, as is to be expected if only very large particles are present.

The mean size of the micelles in lecithin sols has yet to be determined. Some early measurements of osmotic pressure indicated a molecular weight of the order of 350,000 but the material used was of doubtful purity[9]. Ultrafiltration studies showed that the lecithin micelles were bigger than haemoglobin molecules[105]. Saunders[106] has found that the diffusion coefficient of lecithin sols clarified with small amounts of soap were of the order of 10^{-8} cm.^{2} sec.$^{-1}$, indicating a molecular weight for the micelles of several millions.

Electrophoretic Studies and Isoelectric Point

Lecithin sols showed a low electrophoretic mobility[107,108] at neutral pH values; the point of charge reversal was reported as pH 2·7 in the earlier work. Addition of barium chloride decreased the mobility and shifted the isoelectric point to a higher pH. Price[109] found that cholesterol had little effect on the mobility of lecithin, he reported the isoelectric point of lecithin as pH 2·7. Other workers reported values between 1·73 and 2·8[65,83,84].

Theoretically the isoelectric point of lecithin should be about pH 7 since both the acidic and basic groups in the molecule have the character of strong electrolytes[110]. Careful mobility studies of purified lecithin gave a value of 6·7[111], the addition of small amounts of cephalin lowered this slightly[112]. Aged lecithin sols were found to have much lower isoelectric points[113], and it has been suggested that the very low values originally obtained were due to free fatty acids present in the sol which gave the micelles an adsorption charge; the amount of free acid present would increase with the age of the sol, due to hydrolysis of the lecithin.

Dialysis

Dialysis has been used for the purification of sols since lecithin only dialyses slowly[114]. When lecithin-sodium oleate mixtures were dialysed

the lecithin did not affect the rate of dialysis of the soap; with lecithin-sodium glycocholate mixtures the rate of passage of soap through the membrane was increased by a factor of 4.

Turbidity

Levy[115] has studied the turbidity of lecithin sols. De Jong[92] and his colleagues have used turbidity measurements to study the coacervation of the sols.

Hydrotropism

The addition of soaps, particularly bile salts, clarified lecithin sols; double bonds in the fatty acid of a soap made it a better hydrotropic agent[116,117]. Sols have been clarified by polyethylene oxide stearates[106], sodium dodecyl sulphate[118], and sodium cholate[119]; lysolecithins can be used as clearing agent for lecithin sols.

Lecithin as an Emulsifying Agent

Lecithin has been used to emulsify various fish oils and animal fats in water[120]. Emulsions of both types can be formed, for example with sunflower oil and water the type of emulsion formed in the presence of lecithin, depended only on the relative proportions of the two liquids[121]. Studies have been made of the effect of cholesterol on the types of emulsions obtained with lecithin[122,123] and on the behaviour of lecithin as an emulsifying agent with different substances[124,125].

Bull[126] has suggested that phosphatide complexes with proteins or carbohydrates are better emulsifying agents than pure phosphatide , and this has been confirmed[127]. However, it has been reported[128] that pure soya bean lecithin is a better emulsifier in some cases than the crude material containing protein.

Oil in water emulsions prepared with lecithin resembled soap emulsions in that the oil tended to separate on standing. The effects of electrolytes on these emulsions have been studied[129].

Lecithin as a Protective Colloid

Lecithin acted as a protective colloid for dispersions of carbon and calcium carbonate in xylene[130,131], the lecithin was thought to be adsorbed at the surface of the solid particles giving them a charge which was generally negative. Red gold sols were protected from precipitation by potassium chloride[132] as were titanium oxide sols[133]. Lecithin has been used as a protective colloid in the manufacture of foodstuffs and vitamin A preparations[134].

The Effect of Lecithin on Gels

Yumikura[135] studied the rates of diffusion of some drugs in gelatin gels. He found that the presence of lecithin increased the rate of diffusion of cocaine and various surface active substances; the opposite effect was found with alypine and eucaine. Diffusion of various acids and alkalies in gels was said to be inhibited by lecithin but promoted by cholesterol[36,137].

The effects of lecithin and cholesterol on the swelling of gelatin gels at different pH values have been studied[138–141]. The results of different workers are conflicting probably due to the impurity of the lecithin samples used.

THE SURFACE CHEMISTRY OF LECITHIN

Air/Water Interface

Leathes[142,143] found that when lecithin was spread on the surface of water, an expanded film was formed. At a surface pressure of 1·4 dynes per cm. the area per fatty acid chain in the lecithin molecule was 55 Å^2 for one sample and 58 Å^2 for another. There was no sign of condensation of the film when the pressure was increased by a factor of twelve. Hydrolecithin gave a condensed film with an area per fatty acid chain of 28 Å^2 at 1·4 dynes per cm. When the pressure was increased considerably the area diminished but did not become as small as that for a palmitic acid chain in a condensed film (about 21 Å^2 in ethyl palmitate[144] and 26·3 Å^2 in α-monopalmitin[145]). This suggests that the polar part of the lecithin molecule prevents close packing of the fatty acid chains; the unsaturated acids present in natural lecithin caused the high areas per acid chain found with this material. The area occupied by a lecithin molecule in the film was found to be reduced by the addition of cholesterol to the film, due to interaction between the two lipides. It has been reported that at high compressions, the area per molecule of hydrolecithin could be reduced to 41·4 Å^2 [148].

Surface potential studies of films of this type by Hughes[146] indicated an area per fatty acid chain of 58 Å^2 for lecithin; lysolecithin gave a more distended film with an area per molecule of 108 Å^2. Values for the vertical component of the electric moment were 8·8 and 4·3 $\times$ 10^{-19} e.s.u. respectively. On altering the pH of the water on which the film was spread it was found that a rise in surface potential occurred for lecithin between pH 6 and pH 3. Lysolecithin only showed this effect between pH 2 and pH 1. Lecithin films were easily oxidised while lysolecithin films were only slowly affected. The surface potential studies confirmed the observation that the area per lecithin molecule was smaller in a mixed lecithin-cholesterol film than in a pure lecithin film. Injection of snake venom below a lecithin film on water gave a rapid decrease in surface potential due to the conversion of the lecithin to lysolecithin by the enzyme lecithinase present in the venom[147].

The sulphonamides were found to have no action on lecithin monolayers on water, and so it was concluded that the biological activity of these compounds did not depend on their influence on the permeability of the cell membrane[149]. The logic of this conclusion is rather dubious, since it seems unlikely that a lecithin film at the air/water interface bears much resemblance to a cell membrane. Plant growth hormones were found to accumulate in lecithin monolayers and increased the area per molecule in the film, but there was no relation between growth promoting activity and their effect on the monolayer[150].

Saponins caused a rise in surface pressure when injected under mixed lecithin-cholesterol films and this effect was more pronounced in the presence of calcium chloride. This result fitted in with the observation that the haemolysis of red blood cells by saponins was more rapid when calcium chloride was added[151].

Injection of sodium penicillin beneath compressed lecithin monolayers gave a rise in surface pressure and a change in surface potential; the penicillin remained in the surface layer after repeated expansion and compression of the film[152].

Oil/Air Interface

Lecithin formed a condensed film at the glyceride/air interface in which the polar parts of the molecule were thought to be protruding upwards with the fatty acid chains in the liquid phase. An estimate of the thickness of the film at this interface gave a value of 0·55 microns, very much bigger than the maximum dimension of the lecithin molecule[153].

Oil/Water Interface

At the benzene/water[154] interface both lecithin and lysolecithin films were found to be of the vapour expanded type; at high pressures, the areas approximated to those found at the air/water interface which suggested that there was the same type of packing at the two surfaces. The results were interpreted by the equation of Langmuir's theory of expanded films

$$(F—F_o)\ (A—A_o) = \text{const.}$$

where F is the force on the film, F_o is the negative spreading force due to the hydrocarbon chains, A is the area per molecule in the film and A_o is a constant to allow for the area occupied by the head groups. Up to F = 15 dynes per cm. the results fitted this equation. As was expected, the value of F_o which was quite considerable at the air/water interface, was very small at the benzene/water surface. A_o had almost the same value at both interfaces.

Further work on lecithin films at the benzene/water interface showed that injection of salicylic acid above the lecithin film caused a large increase in interfacial tension. Other substances such as benzoic acid, phenol and *ortho*-cresol caused only small increases in tension. The effect of salicylic acid was more pronounced when the acid was undissociated, and is thought to be due to the formation of a complex with the lecithin at the interface[155].

At the xylene/water interface a changing contact angle was found when the ring or plate method was used to study lecithin films; this difficulty was overcome by coating the plate with a deposit of carbon from a benzene flame. The compressibility of a lecithin film at this interface was greater than that at the air/water surface[156].

The Lecithin Sol/Water Interface

Many attempts have been made to correlate the action of substances on films at various interfaces with their biological activity. Most of these

attempts have failed because the interface studied bore only a very remote resemblance to the boundary which separates a living cell from a liquid environment. The simple cell membrane is a film built on a fatty structure which contains lecithin and cholesterol and which separates two *aqueous* fluids. Air/water and oil/water interfaces having a non-aqueous fluid on one side of the boundary, cannot be expected to give a very close representation of a cell membrane, since many substances cannot pass across the boundary from one fluid phase to the other.

Langmuir and Waugh[157] tried to overcome this difficulty by transferring pre-formed protein films to make boundaries between two aqueous phases, believing that such boundaries would have some of the properties of cell surfaces. Their efforts were not successful as the protein films broke up on transfer; addition of lecithin tended to strengthen them.

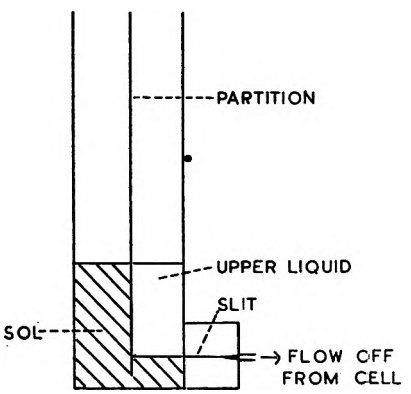


FIG. 1. Original cell for boundary formation.

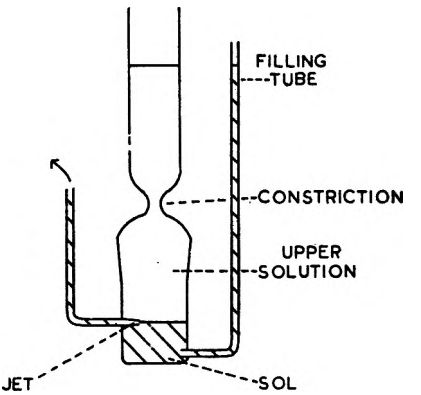


FIG. 2. Infra-red cell for surface force measurements.

Saunders[158] studied this problem of forming stable films between two aqueous liquids by examining the sharp boundaries formed when two such liquids were caused to flow through a common orifice. The apparatus used is shown in Figure 1. The cell was filled with the denser liquid on both sides of the central partition to the depth of the slit, the denser liquid was then added to the left hand side of the cell at the same rate as the lighter liquid was added to the right-hand side and both liquids were drawn off together through the slit. With care, a sharp boundary between the liquids could be made and maintained while flow through the slit continued. After flow was stopped, diffusion normally occurred and the progress of this diffusion could be followed by an optical technique (the Gouy interference method). With a lecithin sol as the lower liquid and water as the upper one, it was found that a boundary which was stable for a period of several days was formed. The presence of a coherent film at this boundary was demonstrated by the fact that when a platinum ring was drawn up through the interface, a definite pull of the order of 0·2 dynes per cm. was required to cause the ring to break through the boundary.

Elworthy and Saunders[61] have studied these surface forces at the lecithin sol/water interface in detail and have examined the effects of various substances on them. A different cell was used for most of this work and this is shown in Figure 2. This cell was filled to the constriction with the lecithin sol and then water or an aqueous solution was carefully put into the upper part of the cell, in this way a sharp boundary was produced in the constriction. Sol was allowed to flow out through the side arm causing the boundary to move down to the level of the jet, inside the cell. The boundary was finally sharpened at this position by continued flow through the jet; during these operations a platinum ring suspended from the arm of a torsion micro-balance was immersed in the lower liquid. After the boundary had been sharpened the force required to pull this ring through the sol/water interface was measured (the surface force). No appreciable force was required to raise the ring through the liquid until it reached the boundary, then an increasing force had to be applied until the ring suddenly broke through.

Using egg lecithin purified through the cadmium chloride complex we found that there was no surface force if all small ions were removed from the sol by passing it through mixed ion exchange resins, the force originally obtained with lecithin alone was almost certainly due to traces of cadmium chloride remaining in the lecithin. Addition of 0·01 M potassium chloride gave a small force of 0·01 dynes per cm. In the presence of small concentrations (0·0001 M) of divalent metal chlorides, surface forces of 0·1–0·25 dynes per cm. were obtained. The order of activity in producing these forces for the metal chlorides was Cd>Mg>Ca>Ba.

Sols of lecithin prepared by the chromatographic method of Lea and Rhodes[50,51] were unstable to electrolytes and they were rapidly flocculated by 0·01 M potassium chloride. They also gave appreciable surface forces in the presence of this salt, differing in both these respects from the sols made with cadmium complex prepared lecithin. The sols of chromatographic lecithin could be stabilised by adding small traces of soaps and these additions caused the disappearance of the surface force. This suggested that there was a relation between the occurrence of a surface force at the sol/water boundary and the stability of the sol. Further work has confirmed this view. By studying sol stability and surface force in the ternary system of lecithin-soap-salt, the authors have found that for a given soap concentration in the lecithin sol, the lowest concentration of a salt which causes an appreciable surface force when placed in the upper solution in the cell, is very much the same as the concentration of salt which causes precipitation of the sol. The surface force therefore appears to be due to a film of insoluble lecithin-salt complex formed at the boundary between the sol and the upper liquid[46].

It should be noted that this surface force differs from a surface or interfacial tension; there are no unbalanced molecular attractive forces at the sol/water interface, as there are at the benzene/water interface. Surface force is more akin to the work of extension of a membrane than to the work of extension of a liquid/liquid interface.

It is not difficult to imagine that cell membranes are formed in some such

way as this. The intracellular fluid is rich in phosphatides but contains very little calcium, and the lecithin in it is presumably stabilised to the monovalent metal salts present by soap-like substances, probably lysolecithins. When it meets a fluid of higher calcium content, an insoluble lecithin-calcium complex film is formed around the cell and this is fixed and rendered more insoluble by adsorption of proteins and insoluble lipides such as cholesterol. We hope that studies of the lecithin-lysolecithin-divalent metal salt systems will give a more definite picture of cell membrane formation and hence a better understanding of the structure and properties of these membranes.

Correlation of Some Properties of the Sol/Water Interface with Those of Some Biological Membranes

Both these two boundaries form surfaces of separation between two aqueous fluids. The magnitudes of the surface forces at the boundaries are very similar; the surface forces of the lecithin sols can be varied between 0·01 and 0·25 dynes per cm. while those of some simple cells are, *Arbacia punctulata* egg 0·03–0·2 dynes per cm., *Busycon caniculatum* egg 0·5, *Triturus viridescens* egg 0·1, erythrocytes 0·25, *Amoeba dubia* 1·0[159–162].

Ionic effects can be compared. Immersion of various Amoebae in potassium chloride solutions caused dispersion of the plasmalemma and this effect could be prevented by adding calcium chloride[102,163]. We have found that at certain calcium chloride concentrations, addition of potassium chloride reduces the surface force but at higher calcium chloride concentrations this effect does not occur. Another similarity is in the toughening effect of calcium salts on the membranes of some Amoebae species and Fundulus eggs[74], and on the lecithin films.

In the present state of our knowledge it is inadvisable to carry this comparison too far but it seems reasonable to forecast that the lecithin sol/water interface will serve as a useful model for the study of mechanisms of drug action.

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BRITISH PHARMACEUTICAL CONFERENCE DUBLIN, 1956

SCIENCE PAPERS AND DISCUSSIONS

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THE STERILISATION, STABILITY AND TOXICITY OF CONGO RED INJECTIONS

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CONGO red consists mainly of the disodium salt of 4:4'-bis-(1-amino-4 sulpho-2-naphthaleneazo)diphenyl and it usually contains traces of sodium chloride. It is chiefly used in an 0·5 to 1·5 per cent. solution for the detection of amyloidosis.

There have been conflicting reports on the toxicity of the dye, on methods of preparing the injection and on its stability in solution[1–7]. Congo red is recognised as being a rather variable product and the British Pharmaceutical Codex 1954 includes biological tests for freedom from undue toxicity and for the absence of pyrogens. There is, however, no statement about limits of pH value. The United States Pharmacopeia XV gives the pH of a solution of Congo red (no strength stated) as 8 to 9·5 and states that solutions decompose on exposure to acid fumes. Injection of Congo Red U.S.P. is required to have a pH between 7·0 and 9·0.

The United States Foods and Drugs administration[8] has stated that "in the preparation of solutions of Congo red it is important that no free colour acid should be present. The presence of free colour acid may be due to exposure of the solid or of solutions to acid".

While large numbers of injections of Congo red have been made without toxic symptoms[9–18], there have been reports of untoward reactions such as abdominal pain, palpitation and rigors[19,20], and a few fatalities have occurred[21]. Some of these have been attributed to anaphylactic reactions in previously sensitised people.

For the preparation of Congo red solutions for injection the British Pharmaceutial Codex 1949 suggests that the dye should be dissolved in Water for Injection immediately before use. The Extra Pharmacopœia (Vol. I, 1941) states that such solutions should be used as soon as possible since the dye slowly hydrolyses in solution. However, the availability of commercially prepared solutions and the results of Richardson and Dillon[2] suggest that the dye is more stable in solution than is

generally supposed. Richardson and Dillon[2] prepared solutions containing 1 per cent. of Congo red with 5 per cent. of dextrose and found that they were stable almost indefinitely at 4° C. Solutions in physiological saline were only stable for 24 hours due to a salting out effect.

Wallace[5], who first described the use of the Congo red test in this country, prepared the injection by dissolving the dye in hot water, filtering through fine filter paper and boiling. He stressed the need for using specially purified dye and stated that solutions should be used within 12 hours or rigors may occur. He considered reactions were probably due to the use of concentrations over 1 per cent.[22]. Others have injected 1·5 per cent. solutions without ill effects[6].

In view of these conflicting statements we have investigated the effects of different methods of sterilisation, of pH changes and storage conditions on the toxicity of Congo red solutions. A preliminary note of our results was read at the General Assembly of the International Pharmaceutical Federation in 1953, leading to the adoption by the British Pharmaceutical Codex 1954 of autoclaving or filtration as methods for sterilising injection solutions[23].

Experimental and Results

Materials

The same sample of Congo red powder was used throughout the investigations except where otherwise stated, and was a commercial product intended for injection. The dye had been biologically tested by the manufacturers. Commercial solutions were obtained on the open market.

Methods

Preliminary experiments indicated that a 1 per cent. solution was suitable for the toxicity tests. Solutions were prepared by dissolving the dye in Water for Injection with the aid of heat and filtering through a No. 3 sintered glass funnel. The solutions were then submitted to different sterilisation and storage procedures. The samples were tested for acute toxicity by the intravenous route in mice. Male albino mice, weighing between 16 and 24 g., were randomised into groups of 10 mice and respective groups were injected at ascending dose levels. Mortalities were observed over 48 hours from which the LD50 doses and their limits of error were calculated[24].

Tests for the absence of pyrogens were carried out in rabbits by the method described in the British Pharmaceutical Codex 1954.

Effect of autoclaving, steaming and filtration. Part of a solution of Congo red was transferred to 10 ml. ampoules, half of which were autoclaved at 115° C. for 30 minutes and the rest steamed at 90 to 100° C. for 30 minutes. The remainder of the solution was filtered through a 5/3 bacteria-proof sintered glass funnel and the filtrate distributed aseptically into 10 ml. ampoules. These solutions were tested for toxicity: the results obtained are shown in Table I. There was no significant difference in the toxicities of the solutions subjected to the different processes.

Effect of Storage. A fresh solution of Congo red was prepared, distributed into three groups of ampoules and treated by the above methods. Half the number of ampoules in each group was stored at 4° C. and the remainder at room temperature, over a period of three months. The physical appearance of these solutions and their toxicities after storage were compared with a freshly prepared solution. The results are shown in Table II.

TABLE I

INTRAVENOUS TOXICITY IN MICE OF 1 PER CENT. CONGO RED SOLUTIONS

Solution	LD50 ml./20 g.	Limits of error (P = 0·95) per cent.
1. Autoclaved	0·75	76 to 132
2. Steamed	0·60	90 to 113
3. Filtered	0·63	94 to 106

The samples stored at 4° C. all showed a slight deposit, this being greatest in the samples sterilised by filtration. The deposit redissolved on warming and did not reappear on cooling to room temperature. The toxicity results show that the autoclaved and steamed solutions had not increased in toxicity during storage. The bacteriologically filtered solutions were much more toxic than the others, an observation which we found difficult to explain. We repeated

TABLE II

INTRAVENOUS TOXICITY IN MICE OF 1 PER CENT. CONGO RED SOLUTIONS STORED FOR THREE MONTHS

Solution	Appearance	LD50 ml./20 g.	Limits of error (P = 0·95) per cent.
1. Control; freshly prepared solution	Bright and clear	0·69	97 to 103
2. Autoclaved and stored at room temperature	Bright and clear. Slight deposit on inverting ampoule in bright light	0·69	97 to 103
3. Autoclaved and stored at 4° C.	Bright and clear, but considerable deposit on inverting ampoule in bright light	0·75	97 to 103
4. Steamed and stored at room temperature	Bright and clear. Slight deposit on inverting ampoule in bright light	0·66	94 to 106
5. Steamed and stored at 4° C.	Bright and clear, but considerable deposit on inverting ampoule in bright light	0·64	94 to 106
6. Filtered and stored at room temperature	Bright and clear, no deposit	0·48	95 to 105
7. Filtered and stored at 4° C.	Copious deposit	0·47	97 to 103

The deposits were readily soluble on warming and shaking the ampoules; the resulting solutions appeared and remained bright and clear.

the filtration method with a new solution and this time no increase in toxicity occurred during storage.

Ampoules remaining from the batch subjected to filtration and used in the earlier tests (solution 3 in Table I), when tested for toxicity after seven months' storage at room temperature, had an LD50 of 0·62 ml./20 g. mouse. Thus no increase in toxicity had occurred. The single occurrence of a solution with a high toxicity after filtration could not be ignored

and we investigated the possible causes further. In the preparation of the toxic solution filtration was prolonged. The solution took four hours to pass through the filter and the sterile solution was left overnight in a sterile sealed flask. The following morning some deposit was observed in the filtrate but this readily disappeared on shaking before the solution was transferred aseptically to sterile ampoules. Possible causes of the increased toxicity may have been oxidation, the effect of carbon dioxide during prolonged exposure to air, or contamination with acid from an imperfectly cleaned filter. These were investigated in turn.

Effect of prolonged exposure to air. A fresh solution was prepared, filtered through a 5/3 sintered glass funnel and the sterile filtrate kept in the flask overnight as before.

The LD50 of this solution was 0·61 ml./20 g. mouse with limits of error from 95 to 105 per cent. No increase in toxicity had therefore occurred. In another experiment the filtered solution was kept in a partly filled sterile 500 ml. infusion bottle for seven days with occasional thorough shaking. This solution also did not increase in toxicity. After storage at 37° C. for three months, in partly filled 10 ml. ampoules, the toxicity remained the same. Exposure to air could not have been the cause of the increased toxicity in our stored solutions after filtration, so we turned our attention to pH. Unfortunately the pH of our original toxic solution had not been taken and no more was available, so a fresh solution had to be prepared.

Effect of pH. The pH of a freshly prepared 1 per cent. solution of our sample of Congo red, measured electrometrically, was 10·1 and did not change during autoclaving. This was higher than the upper limit stated in the U.S.P. XV. The pH of a commercially prepared injection solution was 9·8 and of a 1 per cent. solution of another commercial powder intended for injection was 9·4. None of these samples, therefore, complied with the U.S.P. requirements.

To study the effects of increased acidity, graded amounts of 0·1N hydrochloric acid were added from a microburette to 10 ml. quantities of a freshly prepared Congo red solution. The pH values and the appearance of each solution were recorded and the toxicity determined. The results are shown in Table III.

Immediately a drop of acid was added to the dye solution a bluish-black precipitate appeared, but this readily dissolved on shaking to leave the solution bright red and clear down to pH 7. Any further increase in acidity caused a darkening in colour and the development of an opacity. Below a pH of 6·5 the solution changed to a purple colour and solid material was precipitated. These changes affected the toxicity of the solutions. Below pH 6·8 there was an increase in toxicity and at pH 6·5 solutions which contained solid material were immediately lethal to mice. The change in pH is therefore a probable explanation of the higher toxicity of the solution sterilised by filtration.

Toxicity of a solution of Congo red from another hospital. We also had the opportunity of examining some ampoules of a 1 per cent. solution of Congo red prepared in another hospital. The dye, from a different

TABLE III

EFFECT OF ADDING ACID TO CONGO RED SOLUTION

Ml. of 0·1 N HCl added to 10 ml. of 1 per cent. solution	Appearance of solution	pH	Mortality of mice
None	Blood red colour. Bright and clear. No precipitate	9·7	9/20
0·01	Blood red colour. Bright and clear. No precipitate	9·7	4/10
0·20	Blood red colour. Bright and clear. No precipitate	7·2	1/10
0·22	Blood red colour. Bright and clear. No precipitate	6·8	5/10
0·24	Blood red colour. Bright and clear. No precipitate	6·8	5/10
0·30	Dark red. Opaque. Trace of precipitate appeared on standing	6·5	3/3 immediately
0·40	Dark brownish red colour with trace of precipitate, becoming reddish violet with marked precipitate on standing	6·5	5/5 immediately
0·50	Brown colour with marked precipitate becoming violet with a thick deposit on standing	6·4	2/2 immediately

The mice were all given a dose of 0·64 ml. of 1 per cent. solution each, by intravenous injection. This represents the approximate LD50. Except where otherwise stated the mortalities were observed over 24 hours.

source than ours, was reported to give a dense precipitate when the solution was autoclaved.

The LD50 of this solution was 0·38 ml./20 g. mouse with limits of error from 90 to 110 per cent. The solution was much more toxic than usual. A fresh solution of the original solid material had an LD50 of 0·52 ml./20 g. mouse with limits of error from 95 to 105 per cent. We conclude that this was a bad batch of Congo red.

TABLE IV

PYROGEN TEST ON 1 PER CENT. CONGO RED SOLUTIONS

Solution	Mean maximum rise in body temperature in 3 rabbits
1. Autoclaved sample of our Congo red (sample 2, Table II)	0·11° C.
2. Commercial solution	0·34° C.
3. Solution from another hospital	0·42° C.
All solutions comply with the B.P.C. test.	

Effect of incompletely dissolved dye. Solutions of Congo red may be prepared extemporaneously by the addition of Water for Injection to an ampoule containing a weighed amount of Congo red. Without filtration there must be a danger of injection of solid material due to incomplete solution which is not easy to detect. With one sample of Congo red it took several minutes of vigorous shaking before the solution became bright and clear. Before solution occurred the injections were highly toxic to mice.

Effect of sodium chloride. A solution of Congo red in physiological saline was found to be much more toxic than an aqueous solution, confirming the findings of Richardson and Dillon[2].

Toxicity of a commercially prepared solution. We have examined the toxicity of a prepared solution of 1 per cent. Congo red for intravenous

use, made by the same firm who supplied our solid material. This sample had been stored at room temperature for at least six months. The pH of this solution was 9·8 and its LD50 was 0·74 ml./20 g. mouse, so its toxicity was the same as that of our own preparations.

Test for pyrogenicity. Examination of the three samples recorded in Table IV showed them to be within the limits for pyrogenicity prescribed by the British Pharmaceutical Codex 1954.

Conclusions

While solutions of Congo red can be prepared extemporaneously by the addition of water for injection to the solid powder, there is a danger of incomplete solution of the dye which is difficult to detect.

Intravenous injection of the solid dye may cause immediate death, so a filtered and sterilised solution of Congo red is to be preferred. We have described how such a solution may be prepared and we have shown that solutions of Congo red may be safely autoclaved, steamed or subjected to bacterial filtration.

Such solutions are stable for at least seven months at room temperature. Storage in a refrigerator is not recommended because the dye may precipitate and become difficult to redissolve. In the preparation of the solution there is no danger in prolonged exposure to the air, provided evaporation does not take place, but care must be taken to avoid contamination with acid. A real source of danger is incomplete washing of sintered glass filters.

Solutions with a pH less than 6·8 are opaque and dark red in colour. They are also highly toxic. We recommend that solutions for injection should be bright red and clear and have a lower limit of pH 7·0 as stipulated by the U.S.P.

The inclusion of sodium chloride in Congo red solutions is contra-indicated for the toxicity is increased and the solutions are unstable. Solutions made with dextrose if autoclaved might also become dangerous as a result of a fall in the pH.

Summary

1. A method is described for the preparation of solutions of Congo red for injection.

2. Solutions may be autoclaved, steamed or filtered through a bacteria-proof filter.

3. Solutions are stable at room temperature for at least seven months and storage in a refrigerator is not recommended.

4. Prolonged exposure to air does not increase the toxicity provided evaporation does not take place.

5. The pH of the solution is very important. Acid solutions are dangerously toxic and we recommend the adoption of a lower limit of pH 7·0, as directed by the United States Pharmacopeia.

6. The addition of sodium chloride increases the toxicity of the solutions which may be unstable.

7. No solution of Congo red should be injected which is not bright red and clear.

8. The pyrogenicity of three different samples of Congo red was within the limits set by the British Pharmaceutical Codex, 1954.

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DISCUSSION

The paper was presented by MR. T. D. WHITTET.

DR. F. HARTLEY (London) said that different batches of Congo red might vary considerably in toxicity. It would be interesting to know whether the so-called "pyrogenicity" of different batches was a proper use of that term, as it seemed doubtful that bacterial pyrogenic contamination was the cause. One was a little puzzled as to how the authors reached their conclusion that in a particular batch of ampoules which were alleged to be toxic, this was due to the dye.

MR. K. L. SMITH (Nottingham) asked whether the suitability of Congo red could be established by physical characteristics. Would the sample used have passed the B.P.C. test for toxicity?

MISS M. H. NEAL (Kuala Lumpur) asked whether, in view of the somewhat short period for which the authors recommended the solution should be kept, it might not be advisable to make some labelling recommendations. It would seem that no storage tests were conducted at tropical temperatures. She pleaded with workers to put on storage tests at 34 to 37° C. and to record those results for the benefit of those who worked overseas.

MR. S. G. E. STEVENS (London) asked at what point the authors would anticipate toxic reactions if evaporation were conducted under aseptic conditions.

MR. T. D. WHITTET, in reply, agreed with Dr. Hartley that the toxicity was not the result of contamination with pyrogens. The reason for the B.P.C. test for pyrogens was probably due to the fact there had been complaints of unpleasant reactions, including rigors. Only one sample was used in the main work, but samples from commerce were tested and found to be of similar toxicity. If any solid material were present in the solution it was likely to be very dangerous. Some Congo red dye issued as an indicator was tested and found to have no greater toxicity and a pH nearer to the U.S.P. range than one issued for injection. The indicator dye passed the B.P.C. test for pyrogenicity with ease. Seven months at least were laid down for storage time. Solutions which had been kept since 1953 had not increased in toxicity.

DR. G. F. SOMERS, in reply, said that the material used by the authors had passed the B.P.C. test. On evaporation if a concentration of 1·5 per cent. were exceeded then the Congo red would readily precipitate. If stored in a refrigerator there was a danger of crystallisation of the dye; the crystals tended to grow and were difficult to redissolve.

PERCUTANEOUS ABSORPTION USING DIIODOFLUORESCEIN ^{131}I

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Many different methods have been described for studying the absorption of medicaments from ointment bases applied to the intact skin. Some have been based on the application of pharmacologically active substances such as alkaloids[1] and anticholinesterase drugs. We have previously described a method using the anticholinesterase drug eserine in the rat[2]. Other methods have been based on the inunction of an easily recognised substance such as potassium iodide or a salicylate[3–6] which can be estimated chemically in the blood, urine or faeces.

The introduction of radioactive tracer substances, which can be readily detected in biological materials, even in minute quantities, offers a new approach with the advantage of greater sensitivity[7–9] We have made a preliminary study of the percutaneous absorption of diiodofluorescein ^{131}I in the rat from a number of typical ointment bases with two main objects in view: (*a*) to estimate the value of radioactive tracer techniques in assessing percutaneous absorption in laboratory animals and (*b*) to obtain information on the absorption of medicaments from white soft paraffin, lard, cetomacrogol, hydrous ointment and hydrous emulsifying ointment.

Experimental

Materials

Diiodofluorescein ^{131}I was chosen because it was readily available and possessed solubility characters similar to those of a number of substances commonly applied to the skin. It is only sparingly soluble in water and slightly soluble in chloroform, ether and fixed oils. But it is readily soluble in ethanol and aqueous solutions of propylene glycol. Its hydrophilic and lipophilic properties are therefore fairly evenly balanced. ^{131}I has the advantage of a short half life, eight days, which is a long enough time for the experiments to be performed but does not require a long storage for decontamination of apparatus and animals.

Methods

The preparation of ointments containing radioactive diiodofluorescein raised some interesting pharmaceutical problems.

Preparation of Ointments

White Soft Paraffin and Lard. Non-emulsified ointments, in which the diiodofluorescein was insoluble, were prepared by the following technique:

Diiodofluorescein ^{131}I, approximately	20 mg.*
Base	5 g.

The base was melted in an evaporating dish over a water bath and the diiodofluorescein transferred to a warm glass mortar. The medicament was triturated with the melted base and the ointment transferred to a modified "Plim" syringe, described below. In carrying out these operations, the hands were protected by rubber gloves to prevent skin contamination and a lead screen (thickness $1\frac{1}{2}$ inch) was interposed between the operator and the radioactive material to minimise exposure to radiation.

Cetomacrogol Base. Non-emulsified ointments in which the diiodofluorescein was completely soluble were prepared as follows:

Diiodofluorescein ^{131}I, approximately	20 mg.*
Propylene Glycol	3 g.
Cetomacrogol	2 g.

The cetomacrogol was dissolved in the propylene glycol by heating over a water bath. The diiodofluorescein was added and the ointment heated until solution was complete. It was then transferred to the modified "Plim" syringe.

Hydrous Ointment. Emulsified ointments were prepared as follows:

Diiodofluorescein ^{131}I, approximately	20 mg.*
Propylene Glycol	1·25 g.
Purified Water	1·25 g.
Ointment of Wool Alcohols	2·5 g.

The ointment of wool alcohols was melted in an evaporating dish over a water bath. The diiodofluorescein was dissolved in a mixture of the propylene glycol and purified water and the solution, after heating over a water bath, was transferred to the melted base. The mixture was stirred until cool and transferred to the modified "Plim" syringe.

Hydrous Emulsifying Ointment.

Diiodofluorescein ^{131}I, approximately	20 mg.*
Propylene Glycol	1·25 g.
Purified Water	1·25 g.
Emulsifying Ointment	2·5 g.

This ointment was prepared by a similar technique.

Measurement of Dose. In measuring the amounts of ointment to be applied to the skin, a method was devised to avoid excessive manipulation of the ointment. The ointment was placed in a special syringe so that it could be subdivided into doses without weighing. This syringe is illustrated in Figure 1. It consists of a "Plim" syringe in which the piston has been replaced by one actuated by a screw-thread. The nozzle of the syringe can be removed for filling the barrel with ointment. When

* The actual amount of diiodofluorescein varied with different batches of material. The quantity used was estimated to give a total activity of approximately 2 mc. in 5 g. of ointment.

the screw-thread is turned, the ointment is extruded in an amount determined by the number of turns of the thread. The activity of each dose was determined as described under physical methods.

Animal Experiments. Twelve rats of the same sex, weighing about 180 g., were used in each experiment. On the day before the experiment, the hair was removed from the skin of the back with electric clippers and any rats which showed cutaneous lesions were rejected. To prevent oral contamination and to facilitate handling, the rats were anæsthetised with an intraperitoneal injection of urethane (0·4 ml. of a 25 per cent. solution per 100 g. body weight). In addition, a cardboard collar was fixed round the chest as an added safeguard. Each rat was injected subcutaneously with heparin (200 units per 100 g. body weight) to prevent clotting during collection of the blood. The measured amount of ointment was applied with a metal spatula to an area of 4 cm. square marked on the back, and rubbed in with the spatula for exactly one minute. The rats were then placed into individual tins lined with cartridge paper. In each experiment, three rats were killed after one, two, three and four hours, and blood was collected from the heart by the following procedure to avoid contamination. The rat was deeply anæsthetised with chloroform and then placed on its back on a piece of cotton wool in a lined enamelled tray. The skin was removed from the thorax and abdomen, using instruments reserved for the purpose, since the fur usually became contaminated with radioactive material. The thorax was then opened with new instruments, by cutting through the sternum, and blood was removed from the heart with a teat pipette and measured in a 10 ml. measuring cylinder containing heparin. The volume of each blood sample was recorded and it was then transferred to a labelled test-tube for subsequent counting.

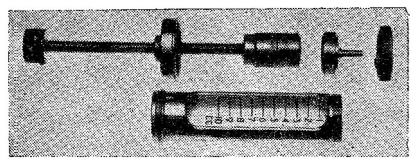

FIG. 1. Modified "Plim" syringe.

Physical Methods. The ointment was divided into 12 approximately equal amounts and the relative activities accurately compared by measuring each at 25 cm. from a Geiger Muller tube (G10 Pb cathode). The amount of blood obtained from the rats varied but, for measuring purposes, it was diluted to 10 ml. and counted using an Ekco scintillation counter. From previous work with ^{131}I, the ratio of the counting rate on an Ekco scintillation counter, from a source diluted to 1 litre, to the counting rate due to the same source at 25 cm. from the G.M. was known to be 43·4. It was therefore possible to relate the radioactivity of the blood to the amount of ointment applied and to determine the proportion absorbed. Since the blood volumes of the rats were unknown, the results have been expressed as percentage of diiodofluorescein absorbed per 10 ml. of blood.

If D = counting rate of ointment at 25 cm. from G.M. tube
A = „ „ „ 10 ml. of blood

Then, ointment diluted to 1000 ml. would give a counting rate of $43{\cdot}4 \times D$ in scintillation counter, and the percentage absorbed into 10 ml. of blood $= \frac{A}{43{\cdot}4D}$

RESULTS

The absorption obtained in different animals showed considerable variation. This is illustrated in Table I, which gives the results obtained with the cetomacrogol/propylene glycol base. The results obtained with

TABLE I

ABSORPTION OF DIIODOFLUORESCEIN FROM CETOMACROGOL BASE

Time (min.)	Dose*	Blood activity†	Absorbed ‡ per cent.	*Average
60	2667	31	0·0003	
60	2544	12	0·0001	0·0002
60	2591	15	0·0001	
120	2684	14	0·0001	
120	2740	96	0·0008	0·0005
120	2676	74	0·0006	
180	2579	68	0·0006	
180	2542	—	0·0045	0·0019
180	2597	62	0·0005	
240	2792	79	0·0006	
240	2696	948	0·0081	0·0035
240	2628	210	0·0018	

* Counts per minute determined at 25 cm. distance from Geiger Muller tube (G10 Pb cathode) = D.
† Counts per minute of 10 ml. blood determined in Ekco scintillation counter = A.
‡ Per cent. absorbed per 10 ml. of blood = $\frac{A}{43{\cdot}4D.}$

the five different bases are shown in Table II and illustrated in Figure 2. They show that diiodofluorescein was absorbed from all the ointments we tested and that the amount absorbed increased with increasing time, with the possible exception of white soft paraffin. Compared with the initial doses, the amount absorbed was extremely small. Because of the

TABLE II

ABSORPTION OF DIIODOFLUORESCEIN FROM DIFFERENT OINTMENT BASES

Time (min.)	Per cent. of applied dose absorbed per 10 ml. blood = A/43·4D				
	Soft paraffin	Lard	Cetomacrogol	Hydrous ointment	Hydrous emuls. ointment
60	0·0004	0·0004	0·0002	0·0003	0·0003
120	0·0008	0·0006	0·0005	0·0009	0·0004
180	0·0010	0·0007	0·0019	0·0008	0·0007
240	0·0005	0·0023	0·0035	0·0030	0·0008

wide animal variations, we have not attempted to evaluate our results statistically. To obtain results suitable for statistical analysis, large numbers of rats would have to be used, raising difficulties of expense, radiation hazards and disposal of contaminated animals. Our results were most variable with the bases, lard and white soft paraffin, in which the diiodofluorescein was insoluble. Absorption was best from cetomacrogol and hydrous ointment, in both of which the diiodofluorescein

was dissolved by adding propylene glycol. With the cetomacrogol base, the tracer was dissolved in a homogeneous mixture, whereas in the hydrous ointment, it was present in the dispersed phase of a water-in-oil emulsion. Absorption was poor from hydrous emulsifying ointment in which the diiodofluorescein was again dissolved in propylene glycol, but here it was present in the continuous phase of an oil-in-water emulsion.

A possible explanation of these findings can be made on the theory that the skin surface is repellent to aqueous solutions, and, in the case of oil-in-water bases, the external aqueous phase has a retarding effect on absorption. When the external phase is oily, as in hydrous ointment, absorption is facilitated by miscibility with the sebum, which allows the medicament to come into contact with the absorbing cells at the base of the follicles. Cetomacrogol, possessing both lipophilic and hydrophilic properties, may therefore assist penetration and absorption by a similar mechanism.

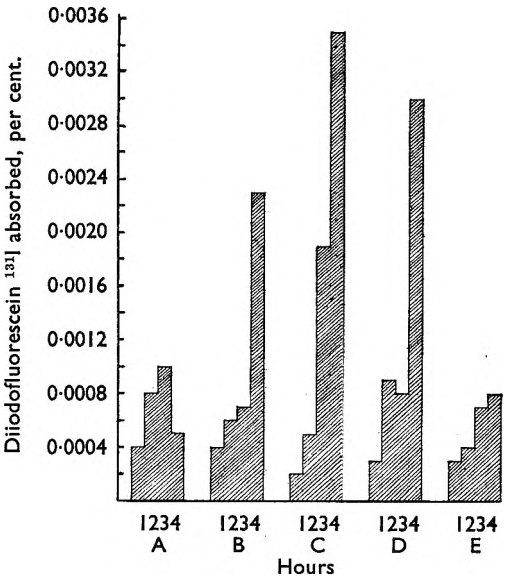

FIG. 2. Absorption of diiodofluorescein ^{131}I from five different ointment bases. A. Soft paraffin. B. Lard. C. Cetomacrogol. D. Hydrous ointment. E. Hydrous emulsifying ointment.

SUMMARY

1. The percutaneous absorption of diiodofluorescein ^{131}I from five different ointment bases in rats was tested.

2. The amount absorbed was extremely small and there was a wide variation between individuals. The numbers needed to obtain an accurate answer would considerably increase the radiation hazards.

3. Absorption was better from hydrous ointment and cetomacrogol than from lard, white soft paraffin and hydrous emulsifying ointment.

The authors wish to thank Ann Coram, Marion Hodges and Pamela Middleton for their assistance in carrying out the experimental work. They also wish to express their thanks to Mr. J. Shephard for supplying the photograph.

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DISCUSSION

The paper was presented by MR. J. W. HADGRAFT.

MR. J. H. OAKLEY (London) said it was disappointing that the newer hydrous emulsifying ointment base had given such poor results. It would be interesting to see the result of using emulsifying ointment as a base instead of hydrous emulsifying ointment, because the lyophobic nature of the base allowed penetration of the skin; the ointment could be easily emulsified and removed from the skin when required.

MR. S. G. E. STEVENS (London) said that a recent paper in the *Journal of the American Pharmaceutical Association* had drawn attention to the increase in penetration of ointment bases incorporating hyaluronidase. The advantage of hyaluronidase seemed to be that non-aqueous ointment bases could be used with very high absorption.

MR. R. L. STEPHENS (Brighton) asked for an assurance that the figures in Table II were the averages for three animals.

DR. K. R. CAPPER (London) suggested that it was possible, even with a small number of animals, to assess statistically the significance of the results, and he doubted whether there was any significant difference between lard, cetomacrogol and hydrous ointment.

DR. B. A. MULLEY (London) asked if the authors could suggest the reason for the fact that in Figure 2, experiments B and D, there was a sudden increase in the absorption of diiodofluorescein between hours 3 and 4. It appeared from the results that the rate of absorption was still increasing in certain experiments. Had the authors made any measurements over longer periods of time?

MR. N. J. VAN ABBÉ (Loughborough) asked the authors to comment on the validity of drawing any conclusions about human percutaneous absorption from experiments with rats. The conclusions in relation to oil-in-water emulsions would be affected by the time of inunction during the experiment, and by the thickness of the film applied. In many oil-in-water emulsions there would be loss of continuous phase, and there might even be reversal of the emulsion after four hours. Percutaneous absorption would depend largely on the nature of the emulsifying agent.

MR. K. L. SMITH (Nottingham) said that one ought to be careful of the conclusions drawn unless they were shown to be statistically significant.

MR. D. H. O. GEMMELL (Glasgow) asked if any attempt was made to estimate the iodine present in the urine of the rats. Was not the concentration of the ointment more important than the amount applied.

DR. G. BROWNLEE (London) said it was intriguing why more diiodofluorescein had not passed from the skin into the animal. By measuring

the concentration of radioactivity in the blood, the assumption was made that there was a relation between blood radioactivity and the diiodofluorescein which had passed through the skin. The authors might comment on whether there was any dye in the urine or staining fatty tissue.

MR. J. B. LLOYD (Manchester) asked if the authors had considered the possible effect of characteristics such as molecular size, basicity and acidity on absorption.

MR. W. P. HUTCHINSON (Oxford) said that by tracer technique in the case of iodine the minimum weight which could be detected using a Geiger counter was 10^{-12} g. It was necessary, therefore, to mix the isotopes carefully into the ointment base. The accuracy of the counter was of the order of about 10 per cent. and it was necessary to do 12 counts and then take the mean.

MR. T. D. WHITTET asked whether the authors had considered measuring the rate of decay of radioactivity at the site of application as a measure of the absorption.

MR. J. W. HADGRAFT, in reply, said that it did not necessarily indicate disappointing results if the amount of systemic absorption from an ointment base was poor. In general one used ointments for localised action, and in many cases systemic absorption was not wanted when an ointment was applied. The authors were concerned not only with determining the systemic absorption from ointment bases, but they hoped at a later stage to examine the levels of penetration of ointments. The incorporation of hyaluronidase had not been considered. The results given in Table II represented an average of a minimum of three animals, and in the case of hydrous emulsifying ointment, soft paraffin and lard, averages of more than three animals. The urine of the animals had not been examined for iodine, but in a number of experiments radioactive iodine was detected in the thyroids. He agreed that diiodofluorescein was not an ideal substance for the study of percutaneous absorption, but the difficulty was to obtain substances in a radioactive state, and diiodoflorescein appeared to be the most satisfactory of those available from the point of view of solubility and radioactivity. The mixing of the tracer in the base was checked in each experiment. If the medicament had not been evenly dispersed through the base it would have shown up in the variation in the dose measured by means of the syringe which was used. The determination of the decay of radioactivity at the site of application had not been considered.

DR. G. F. SOMERS, in reply, said that the conclusions had, perhaps, been drawn a little too strongly, and that it might be more accurate to say that the results "suggested". Referring to the question of increase in rate of absorption, the sudden increase was a reflection of absorption and excretion, both of which must be going on at the same time. The tissues had not been examined, but a great deal of radioactive material was left on the skin. Over 24 hours something different might happen, but it was difficult to maintain the rat under anaesthesia for that period.

THE ACTION OF ION EXCHANGE RESINS ON PYROGENS

PART I. EFFECT ON THE PYROGENICITY OF TAP WATER

BY T. D. WHITTET

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WATER purified by ion exchange materials is now included in the British and United States Pharmacopœias under the title of "Purified Water". The monographs in both these publications state that purified water is unsuitable for the preparation of injections, although several workers have reported that treatment with ion exchange resins can reduce the pyrogenicity of solutions.

Harrison, Myers and Herr,[1] in one of the earliest papers on demineralised water for pharmaceutical purposes, carried out the U.S.P. test for pyrogens on samples of New Brunswick City water before and after treatment with ion exchange resins.

The untreated water gave a mean rise in temperature in five rabbits of 0·86° F. (0·48° C.), whereas the treated sample gave practically no rise in temperature, 0·12° F. (0·07° C.), in five rabbits. The authors stated that it could not be presumed that water once contaminated with pyrogenic material would be purified by treatment with ion exchange resins, but that the treatment did not result in water acquiring pyrogenicity. They did not, at that time, recommend the use of demineralised water for preparing parenteral solutions but considered it possible that future investigation might show it to be a practical application of the process.

Smith and Pennell[2] used an ion exchange agent "Decalso" for reducing the pyrogenicity of concentrated protein solutions. They showed that it had appreciable effect by itself and was very effective in conjunction with Seitz filter pads. "Decalso" is a synthetic sodium aluminium silicate, in the form of 60–90 mesh granules, and acts as a cation exchange material. It is not so stable as the resins and can be used only in neutral solution.

Reid and Jones[3] used ion exchange resins in the production of human blood plasma protein fractions. They reported that a pyrogenic solution (*Aerobacter cloacae* pyrogen), which gave a strong reaction when diluted 100 times and a weak reaction when diluted 10,000 times, after being passed through a 107 cm. column of mixed anion and cation exchange resins and then through an 84 cm. column of cation exchange resin gave no detectable response. These workers did not state the types of resins used. Dr. R. Heiz, in a personal communication, has informed me that the ion exchange resins Amberlite IR120 (cation exchanger) and Amberlite IR410 (anion exchanger) used as mixed columns will remove from solutions quantities of a lipopolysaccharide "Pyrexal" (Wander), a pyrogen from *Salmonella abortus equi.*

Fischer[4] described two plants intended for the production of bacteria-free and pyrogen-free water using the same Amberlite resins together with Seitz filtration. He tested three samples of water after they had been passed through a Seitz filter, sealed into ampoules and sterilised. All three samples were apyrogenic.

Unfortunately he did not record whether he tested samples before Seitz filtration, so it is impossible to tell whether pyrogenicity was removed by the resins or by the filter pads. Co Tui[5] has shown that these pads can absorb appreciable quantities of pyrogens. Wilke[6] has described a similar ion exchange-filtration apparatus.

Hatta and others[7], working with pyrogens from several organisms, including moulds, stated that the pyrogenic substances were completely eliminated by passing solutions through beds of ion exchange resins. The results given in their paper, however, do not show complete removal of pyrogenicity in every case.

The papers quoted above give a considerable amount of evidence that the pyrogenicity of solutions can be markedly reduced or even abolished by means of ion exchange resins. On the other hand, Suzuki[8] reported that treatment with both cation and anion exchange resins was practically ineffective for depyrogenising a glucose injection contaminated with a pyrogen of fungal origin.

In view of these conflicting reports, experiments were carried out to determine whether demineralised water is apyrogenic and, if so, to ascertain which resin is responsible for removal of the pyrogen.

Experimental

The resins used were Zeocarb 225 (cation exchanger) and Deacidite FF (anion exchanger). They were placed in glass tubes resembling percolators with stopcocks at the lower end. Glass wool was used to prevent the resins from entering the stopcock.

The size of the Zeocarb 225 bed was about 30 cm. long by 7 cm. diameter and that of the anion bed about 60 cm. long by 7 cm. diameter, these being the proportions of the resins necessary for demineralisation. These sizes were chosen to give columns of about the same size as in some of the commercially available plants.

The source of pyrogen was London tap water which has always been found to be highly pyrogenic[9]. Before use the columns were thoroughly washed with several bed volumes of freshly distilled water from a still known to give pyrogen-free water.

The rabbits used were New Zealand whites of 2·0 to 3·0 kg. They were known from previous experiments to give a marked response to injections of pyrogens and had not shown signs of tolerance.

All the water samples were adjusted to isotonicity with sterile pyrogen-free concentrated salt solution. All the injections were made into the ear veins and the dose given was in every case 10 ml./kg. body weight.

During the experiments the rabbits were kept in the usual type of restraining box and their rectal temperatures were measured by means

of thermistor electrical thermometers. Their temperatures were recorded for at least one hour before and three hours after injection.

Effect of Uncharged Resins

To determine whether the resins were capable of removing pyrogens by simple absorption or adsorption, tap water was passed through uncharged columns.

The effluents were injected immediately after collection into four groups of three rabbits.

The maximum rise in temperature for each animal is shown in Table I and the mean temperatures for all the animals before and after injection are plotted in Figure 1.

TABLE I

EFFECT OF TREATING PYROGENIC TAP WATER WITH ION EXCHANGE RESINS

Effect of untreated tap water (control)		Uncharged Zeocarb treated water		Uncharged Deacidite treated water		Zeocarb–Deacidite treated water	
Rabbit	Response °C.	Rabbit	Response °C.	Rabbit	Response °C.	Rabbit	Response °C.
468	1·30	139	1·30	140	0·55	148	−0·05
573	1·40	141	1·52	131	0·78	400	0·13
466	1·00	143	1·60	133	0·83	436	0·10
573	1·63	140	1·65	123	1·75	122	0·03
444	1·50	139	1·73	124	1·75	143	0·18
466	0·95	141	1·90	131	2·00	426	0·18
483	1·25	148	1·68	573	0·40	467	0·05
150	0·80	400	0·33	837	0·60	482	0·00
145	1·63	430	1·53	978	0·28	444	−0·02
145	1·68	466	0·30	426	0·65	837	0·60
143	1·83	439	2·20	148	0·93	141	−0·25
147	1·43	436	1·56	444	1·00	145	0·13
Total ..	15·52	Total ..	17·30	Total ..	11·52	Total ..	1·08
Mean ..	1·29	Mean ..	1·44	Mean ..	0·96	Mean ..	0·09

Dose in all cases 10 ml./kg. body weight, injected intravenously.

The maximum total rise in temperature in twelve rabbits must not exceed 6·6° C. for water to pass the B.P. test for absence of pyrogens.

Effect of Charged Resins

The columns were then charged and tap water was passed through the cation and anion exchange columns. The resulting deionised water was injected, immediately after collection, into four groups of three rabbits. The rate of flow through the columns was standardised at about the optimum for columns of this size as suggested by the manufacturers of the resins.

The four groups of three tests were carried out on different days and the resins were not regenerated between tests. The columns were kept closed between tests and, before use on each occasion, were washed with several bed volumes of freshly distilled water. At least two bed volumes of the water to be tested were passed through the columns before taking the test samples.

As controls, samples of tap water from the same source were injected in the same dose into an equal number of rabbits.

The results of all these tests are shown in Table I and Figure 1.

Effect of Individual Resin Columns

As tap water deionised by passage through these two ion exchange columns appeared to be completely freed from pyrogens, experiments were carried out to ascertain which of the resins is responsible for this effect.

Tap water was passed separately through the same cation and anion exchange columns used in the previous experiments and the effluent from each was injected into different groups of three rabbits.

The effluent from the cation exchange column had a pH of about 2·5 and was highly pyrogenic, whilst that from the anion exchange column had a pH of about 10·5 and was apyrogenic. To make sure that the physical effect of the larger bed of the anion exchange column was not responsible for the difference in action, a column of the cation exchanger of the same size as the anion exchange column was tested and was still found to be ineffective in reducing pyrogenicity. In later work anion exchange columns as small as 15 cm. long and 3 cm. in diameter were found to be capable of removing large quantities of pyrogens.

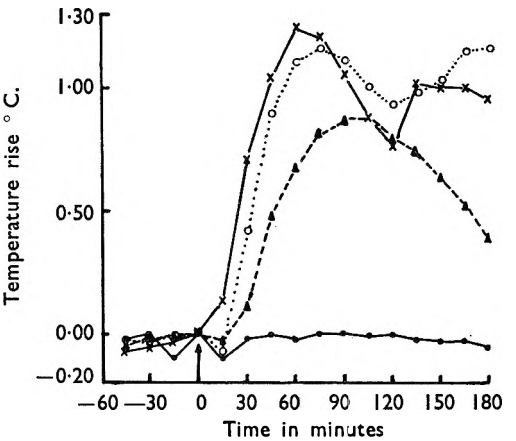

FIG. 1. Effect of ion exchange on the pyrogenicity of water.

●——● Tap water passed through charged columns.
×——× Untreated tap water.
○....○ Tap water passed through uncharged Zeocarb (cationic) column.
▲- - ▲ Tap water passed through uncharged Deacidite (anionic) column.
All points are the mean of 12 tests.

To confirm that acidity of the cation exchange effluent was not the cause of the rise in temperature, physiological saline, known to be pyrogen-free, was adjusted with autoclaved 0·1 N hydrochloric acid to pH 2·5 and was injected into an equal number of rabbits as a control. The results of all these tests are shown in Table II and Figure 2.

To show that the alkalinity of the water from the anion exchange column was not causing destruction of the pyrogenicity, tap water was adjusted to pH 10·5 with autoclaved 0·1 N sodium hydroxide solution and was injected into an equal number of rabbits.

To ensure that the temperature rise caused by the intravenous injection of tap water is not due to living or dead organisms, one sample was steamed for 30 minutes to destroy living vegetative organisms and another was sterilised by passing through a bacteria-proof sintered glass funnel. Both of these samples were found to be highly pyrogenic on injection into rabbits. The sterility of the filtered sample was confirmed by the usual tests.

TABLE II

EFFECT OF INDIVIDUAL CHARGED ION EXCHANGE RESINS ON PYROGENIC TAP WATER

Zeocarb treated water		Deacidite treated water		Acidified apyrogenic saline pH 2·5 (negative control)		Alkalised tap water pH 10·5 (positive control)	
Rabbit	Response °C.	Rabbit	Response °C.	Rabbit	Response °C.	Rabbit	Response °C.
409	1·18	439	0·08	439	0·05	400	0·60
426	1·65	467	0·10	575	0·18	468	1·28
482	1·53	495	0·80	978	0·15	837	1·43
400	0·68	148	0·20	468	0·00	426	1·55
436	1·60	467	0·18	483	1·03	466	0·93
495	1·03	483	0·23	837	0·48	444	1·10
148	1·13	409	0·35	145	0·38	439	1·40
436	1·43	482	0·23	445	0·28	573	1·08
467	1·43	468	0·43	466	0·13	978	1·08
122	1·13	483	0·75	148	0·08	409	1·73
139	1·40	978	0·20	400	0·20	443	0·45
469	1·65	141	−0·15	436	0·10	448	0·98
Total ..	15·84	Total ..	3·40	Total ..	3·06	Total ..	13·61
Mean ..	1·32	Mean ..	0·28	Mean ..	0·25	Mean ..	1·14

Dose in all cases 10 ml./kg. body weight, injected intravenously.

The maximum total rise in temperature in twelve rabbits must not exceed 6·6° C. for water to pass the B.P. test for absence of pyrogens.

A column of 15 cm. length and 3 cm. diameter was made from a sample of Deacidite FF which had been in continuous use for two-and-a-half years. This was also found to give complete removal of pyrogenicity.

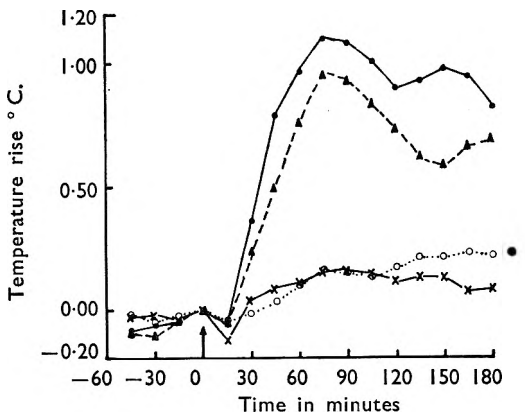

FIG. 2. Effect of individual ion exchange resins on the pyrogenicity of tap water.

●——● Tap water passed through charged Zeocarb (cationic) column.

×——× Tap water passed through charged Deacidite (anionic) column.

○....○ Apyrogenic water adjusted to pH 2·5 (Negative control).

▲– – –▲ Tap water adjusted to pH 10·5.

RESULTS

The results in Tables I and II and Figures 1 and 2 show that untreated tap water and tap water adjusted to about pH 10·5 are highly pyrogenic. Treatment with the uncharged ion exchange columns does not remove this pyrogenicity, showing that pyrogens cannot be removed by adsorption on the resins.

On the other hand, water deionised by passing through both the columns or treated by the anion exchange resin easily passes the B.P. test for absence of pyrogens. This is a severe test, as untreated tap water gives a mean rise of about 1·3° C. in the dose used for these tests and a response of about 0·6° C. in a dose as low as 0·4 ml./kg. body weight. Acidification of pyrogen-free saline does not render it pyrogenic. This confirms the observations of Seibert[10]. A sample of the

anion exchange resin which had been in continuous use for two-and-a-half years is still capable of completely depyrogenising tap water.

Discussion

Several groups of workers have shown that water deionised by ion exchange resins can give water which will comply with the B.P.[11], D.A.B.6[5], Pharm. Helv.[5,12] and U.S.P.[1] tests.

Saunders[11] showed that, provided a correct technique is used and only water of specific resistance greater than 1 megohm per cm. is collected, the purity of demineralised water prepared from London tap water is at least equal to that of distilled water B.P.

The results given above and some experiments carried out with a commercially available ion exchange plant containing the same resins in single columns indicate that deionised water of this chemical purity may also be apyrogenic.

The effect of operating technique on the bacteriological content of water from deionising plants has been studied by Cruikshank and Braithwaite[13] and by Eisman, Kull and Mayer[10].

The results in this paper also show that the removal of pyrogens from London tap water must be a function of the charged anion exchange resins, since uncharged resins of both types and the charged cation exchange resin are without effect. This suggests that some pyrogens, at least, must be negatively charged molecules. It is interesting to note that Caillaud and Vincent[15] were able to depyrogenise water completely by means of an electro-osmotic cell.

Work is proceeding to test the effect of several different ion exchange resins on as many different pyrogens as possible. All pyrogens so far tested have been removed by anion and unaffected by cation exchange resins. Some evidence has been obtained that the anion exchange resins are more effective against some pyrogens than others.

It seems probable that deionised water of sufficient chemical purity to comply with the pharmacopœial requirements for purified water may also be apyrogenic, but whether the demineralising plants at present available will be suitable for the production of water for injection remains to be determined. It should be possible to construct an apparatus to give deionised pyrogen-free water.

Summary

1. The effects of two ion exchange resins (Zeocarb 225 and Deacidite FF) on the pyrogenicity of London tap water have been examined.

2. It is possible to produce large quantities of pyrogen-free deionised water from London tap water by passing it successively through columns of the charged cation and anion exchange resins.

3. The uncharged resins and charged cation exchange resins have practically no effect on the pyrogenicity.

4. The anion exchange resin is responsible for removing the pyrogenicity from the water.

I wish to thank the Permutit Company for generous supplies of resins and Mrs. C. Friedenthal for translating several papers from German.

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DISCUSSION

The paper was presented by THE AUTHOR.

DR. L. SAUNDERS (London) asked if the author had any information on the effect of the ion exchange resins on pyrogenic distilled water. Was the removal of pyrogens connected with the removal of salts from tap water? The author suggested that some pyrogens must be negatively charged molecules, but there was another possibility. If the pyrogens were polysaccharides, catalytic hydrolysis might destroy the pyrogenic properties.

MR. J. W. HADGRAFT (London) pointed out that the author did not state whether the water was drawn directly from the mains supply or from a tank supply. That might be of some importance since his own experience with London mains water did not support the author's statement. One sample taken from his hospital's mains supply and tested for pyrogens was described as being the least pyrogenic preparation tested for some considerable time.

DR. J. C. PARKINSON (Brighton) said he was puzzled about the origin of pyrogens in tap water. London tap water was often surprisingly free from bacteria, and he wondered whether it was possible that chlorination, for example, could leave matter in the water which might give a "pyrogenic" response.

DR. J. G. DARE (Leeds) hoped that the author would continue his work in a more quantitative way and establish whether the resins were effective with heavily contaminated water.

MR. T. D. WHITTET, in reply, said he had not yet tried the effect of the resins on pyrogenic distilled water, but he hoped to do so. He agreed that removal of pyrogens might be connected with catalytic hydrolysis of polysaccharides. It was a point of interest that resins in the borate form would absorb large quantities of polysaccharides. His experience

was that London tap water was always pyrogenic provided it was not sterilised, but he had found in the course of his work that the thermal stability of pyrogens in London tap water was very low. It was difficult to think of a reason why any chemical in water should affect pyrogenicity. Dr. Windle Taylor, whom he had consulted, was of the opinion that there was not enough residue from chlorination to have any effect on the temperature of rabbits. He had compared the water from the mains taps with that from other taps in the department and had found no difference in any of them before sterilisation.

PAPER CHROMATOGRAPHIC DETECTION OF NEW CONSTITUENTS OF *DIGITALIS LANATA*

By Barbara J. Aldrich, Margaret L. Frith and S. E. Wright

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Since digoxin was first isolated from *Digitalis lanata*[1], a number of new cardiac glycosides derived from this plant have been described. These substances fall into two main groups, the so-called primary glycosides, lanatosides A, B, and C, first isolated by Stoll and Kreis[2], and the secondary glycosides, digitoxin, gitoxin, and digoxin, which are derived from the lanatosides by loss of glucose and an acetyl group. The primary glycosides are more polar and more water-soluble than the secondary glycosides and it is possible to separate the two groups by several methods of paper chromatography. In our experience, the clearest separation has been obtained by using the method of Silberman and Thorp[3], in which the developing solvent is a mixture of ethyl acetate, benzene, and water. If 1 per cent. of ethanol is added to the developing phase, the secondary glycosides travel with the solvent front, but the primary glycosides separate clearly.

When this system was used to study the lanatoside content of *Digitalis lanata*[3,4], it was observed that the chromatograms always showed several distinct spots closer to the starting line than the lanatosides. At first it was thought that one of these apparently highly polar substances was lanatoside C, but this was later shown to be incorrect[5]. We have now examined these substances more fully and have obtained some information about their properties. In addition we have shown that these apparently new constituents are present in very young seedlings before any trace of the lanatosides can be detected, and we have followed the development of the glycosides during the early growing season of a crop of *Digitalis lanata*.

Experimental

Solvents. Ethyl acetate first washed with potassium carbonate and then dried and distilled. Benzene, thiophene free, redistilled. Formamide, Light and Company. Chloroform, redistilled and 1 per cent. ethanol added. Butanol, redistilled (b.pt. 116° C.).

Chromatography. System 1[3]. Ethyl acetate—benzene—water, 84 : 16 : 50 (by volume). The solvents were shaken and allowed to separate clearly. The lower (aqueous) phase was placed in the bottom of the tank (11 × 11 × 17 inches high) and the atmosphere kept saturated by filter paper sheets dipping into the stationary phase. To the upper (organic) phase, 1 per cent. of ethanol was added just before chromatography. This organic phase was then placed in a glass trough resting on the bottom of the tank and used to develop the chromatograms by the upward method for four to five hours after the paper had been equilibrated in the tank

overnight. The temperatures found most suitable for development were between 22–24° C.

A small volume of the extract, usually 0·01 ml., was spotted on to the paper using a micropipette. Whatman No. 1 paper was used placing 7 spots on an 8 inch wide paper. On chromatograms where the developed spots were to be eluted, Whatman No. 3 paper was used, and up to 0·1ml. of the extract spotted on to the paper.

System 2. Strips of Whatman No. 4 paper (11 inches × ½ inch) were dipped into formamide—methanol, 1 : 1, and then exposed to the atmosphere for a short time. The plant extracts were streaked on to the strips 2 inches from the end, and development was carried out in horizontal trays at 32° C. for sixteen to twenty hours. The developing solvent was chloroform—benzene—butanol, 78 : 12 : 5 (by volume), saturated with formamide.

The chromatograms from System 1 were dried at room temperature and sprayed with freshly prepared 33 per cent. trichloroacetic acid in chloroform to which 1 drop of 100 volume hydrogen peroxide per 10 ml. of reagent had been added[7]. The chromatograms were then heated at 110° C. for ten minutes. With this reagent, as with the chloramine-trichloroacetic acid reagent of Jensen[8], glycosides belonging to the A series (digitoxin derivatives) fluoresced yellowish-brown in ultra-violet light. Those of the B series (gitoxin derivatives) fluoresced bright blue, and those of the C series (digoxin derivatives) fluoresced pale blue.

The glycosides were detected on the chromatograms of System 2, by dipping the strips in 5 per cent. *m*-dinitrobenzene in benzene, followed by 20 per cent. aqueous sodium hydroxide. Characteristic blue bands which faded quickly were obtained.

Extraction of plant material[3]

Fresh material. Fresh seedlings 12 g., collected in the early morning, were mashed with 6 g. of ammonium sulphate and shaken for six hours with 60 ml. of ethyl acetate. The mash was then shaken for one hour with 30 ml. of ethyl acetate, decanted and shaken with a further 30 ml. for one hour. The extracts were filtered on a Büchner funnel, and the filtrate was evaporated under reduced pressure to approximately 1 ml. The contents of the flask were absorbed on diatomaceous earth and allowed to stand overnight in an evaporating dish. The diatomaceous earth was then extracted with light petroleum in a Soxhlet extractor for about eight hours and then the constituents were washed out of the diatomaceous earth with chloroform-methanol, 1 : 1. The chloroform-methanol extract was evaporated under reduced pressure, almost to dryness, at a temperature not exceeding 30° C. The extract was then made up to 4 ml. in 80 per cent. ethanol. This extract was used for chromatography.

Dried material. Approximately 2·0 g. of each sample of dried leaf, previously broken up in a mortar, were shaken for three hours with 30 ml. of chloroform-methanol solvent 1 : 1, followed by filtration on a Büchner funnel. This was repeated twice, shaking the residue with 10 ml. of

solvent for one hour. The combined filtrates were evaporated to small bulk under reduced pressure, at a temperature not exceeding 30° C., and then absorbed on diatomaceous earth. Chlorophyll and other impurities were next removed by Soxhlet extraction of the powder with low boiling point light petroleum. The glycosides were redissolved from the diatomaceous earth with chloroform-methanol, 1 : 1, followed by filtration and evaporation to small bulk under reduced pressure. The extract was finally adjusted to 10 ml. in 80 per cent. ethanol.

Elution of glycosides from chromatograms

Whatman No. 3 paper was used as larger volumes of extract could be spotted on to this paper. The substances were localised by spraying a strip of the chromatogram, and the areas were cut up finely and shaken with 30 ml. of chloroform-methanol, 1 : 1, for one hour, decanted and shaken with a further 30 ml. for half an hour. The solution was filtered and a small portion of the filtrate evaporated to dryness and taken up in 0·1 ml. of methanol. This was then used for spotting on System 1 in order to check the purity of the substance. The substance was then run on System 2 as a further check of purity.

Acid hydrolysis of eluted glycosides

The glycoside solution eluted from the paper was evaporated to dryness and 3 ml. of ethanol and 3 ml. of 1 per cent. hydrochloric acid added. The solution was then boiled under reflux in a water bath for thirty minutes. The solution was neutralised with a 1 per cent. sodium hydroxide solution, and extracted with chloroform for one and a half to two hours. This was then evaporated to small volume and used for spotting.

Results

A typical paper chromatogram obtained after running extracts of mature dried leaves on System 1, is shown in Figure 1*e*. Four distinct spots were observed travelling behind lanatoside C. These have been named L1, L2, L5, and L6. Of these, L5 showed a yellowish-brown fluorescence in ultra-violet light after trichloroacetic acid treatment, indicating that it probably belongs to the A series of glycosides. The other three spots fluoresced blue under the same conditions. L5 was eluted from the paper and rechromatographed with deacetyl lanatoside A on System 1. Distinct separation was obtained. Similarly it was found that deacetyl lanatosides B and C did not correspond with L1, L2, or L6, when they were eluted and rechromatographed on both Systems 1 and 2.

The position of these substances on the chromatogram indicated that they might be tannoid complexes and the following test was carried out on the plant extracts[9]: To 4 ml. of the plant extract, 0·6 ml. of a suspension of basic lead acetate in 80 per cent. ethanol was added. The solution was then filtered and centrifuged. To the filtrate 1 ml. of a saturated solution of disodium hydrogen phosphate in water was added and the solution again centrifuged. The decanted liquid was adjusted to 8 ml. with ethanol and then spotted on to paper chromatograms. The position of

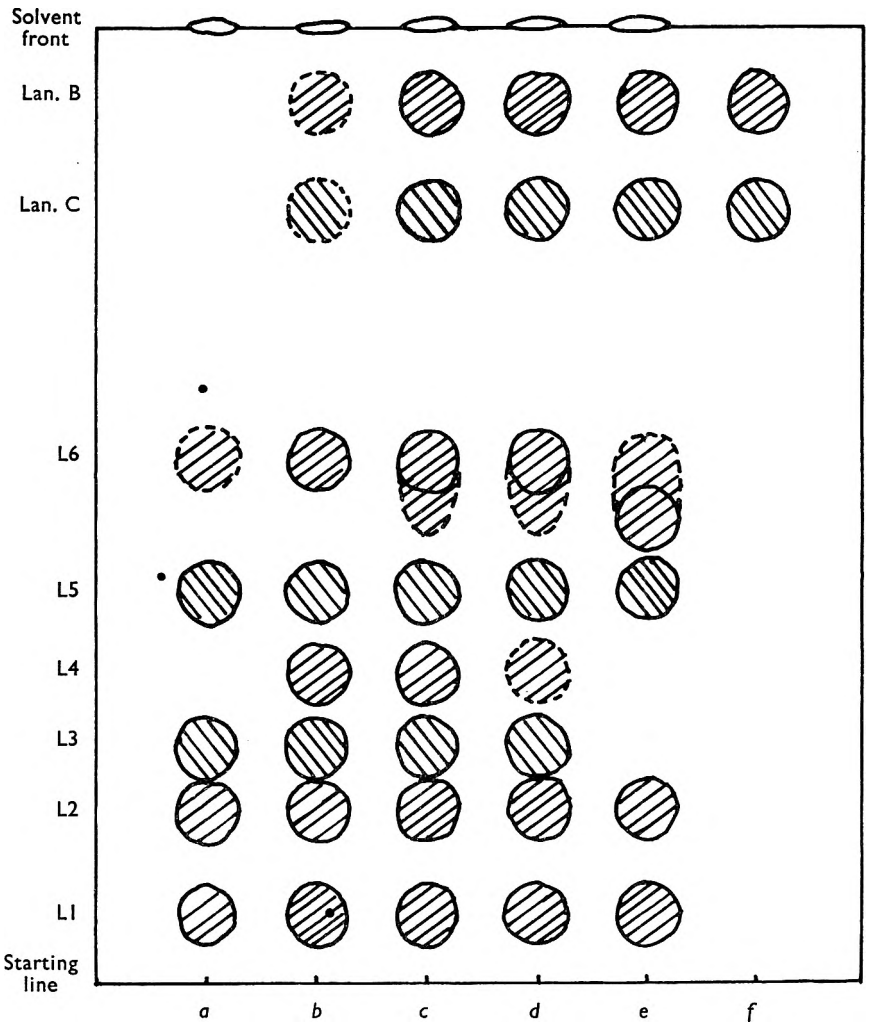

FIG. 1. Chromatography on System 1.

a. Extract of plant 40 days after sowing.
b. ,, 60 ,,
c. ,, 80 ,,
d. ,, 100 ,,
e. ,, approx. 8 months after sowing. (Dried material).
f. Lanatoside B and Lanatoside C.

the spots on the chromatograms after treatment with basic lead acetate was the same as that obtained from the original extract, indicating that the unknown substances are not tannoid complexes.

Investigation of L2 and L6

As chromatograms of many plant extracts showed that L2 and L6 fluoresced more strongly than L1 and L5, only the former two have so far

been investigated. Eluates of L2 and L6 checked for purity by re-chromatography on both systems were evaporated to dryness, redissolved in Ringer solution and tested for toxicity on embryonic chick hearts according to the method of Lehman and Paff[10]. Typical heart blocks were obtained at two dilutions. Both L2 and L6 gave intense blue colours with alkaline *m*-dinitrobenzene and when eluates of L2 and L6 were tested with xanthydrol reagent, pink colours were obtained and persisted for twenty-four hours. A weak Keller-Kiliani test was also obtained with L2.

Hydrolysis of L2

A quantity of L2 obtained from approximately 0·25 g. of air-dried plant by elution from several chromatograms was hydrolysed with dilute acid. The hydrolysed material was chromatographed on System 2 and shown to contain 3 constituents (Fig. 2). One of the bands (B, Fig. 2) appeared to

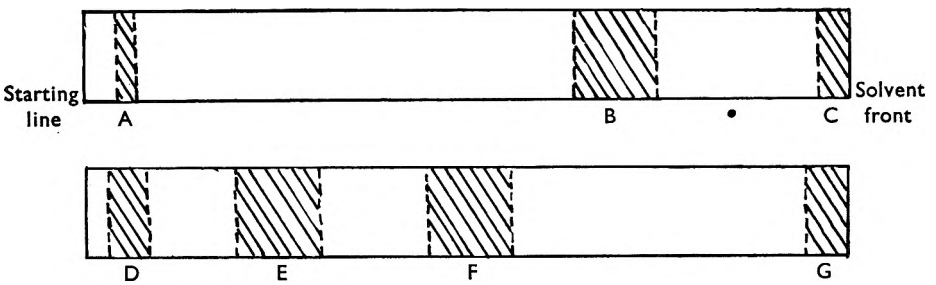

FIG. 2. Chromatograms on System 2.
(1) Hydrolysis of L2 A and C—unknown, B—digoxigenin.
(2) Hydrolysis of L6 D, E, F, G—unknown.

run similarly to digoxigenin and when eluted and re-run with digoxigenin, no separation was obtained on System 2. It did however, separate from gitoxigenin on this sytem. These results were confirmed using the reversed phase system of Tschesche, Grimmer, and Seehofer[11]. The other bands on the chromatogram were not identified, but band A(Fig.2) separated distinctly from the original material on System 1.

Hydrolysis of L6

A quantity of L6 eluted from the same chromatogram as L2, was hydrolysed with dilute acid. In this case, 4 bands of material were detected on chromatograms of the hydrolysate using System 2 (Fig. 2). Of these, band E was the most intense, and was shown to separate from gitoxigenin and digoxigenin. Band F was also shown to separate from gitoxigenin and digoxigenin.

Chromatography of plant extracts obtained from young seedlings

Extracts of freshly picked leaves of *Digitalis lanata* seedlings were taken 40, 60, 80, and 100 days after planting the seeds. Approximately the same quantity of each extract was run on the chromatogram for comparison

as shown in Figure 1. The extract of the youngest seedlings (40 days) showed three spots which on elution and rechromatography could not be separated from L1, L2, and L5 obtained from chromatograms of mature plants. Traces of L6 could also be detected and a new yellow spot appeared travelling just above L2. This has been named L3. There appeared to be no visible trace of the lanatosides. At 60 days, L1, L2, L3, L5 and L6, were visible as well as a further blue spot which travelled between L3 and L5 and has been named L4. A faint trace of the lanatosides B and C could also be detected at this stage. At 80 days the picture was the same with an apparent increase in intensity of L4 and L6 and the lanatosides were beginning to show more prominently. At 100 days after planting, a distinct spot indicated the presence of lanatoside B. Lanatoside C was also visible and L4 fluoresced only faintly. In the two later extracts, L6 which had been increasing in intensity with the age of the plant, appeared to be splitting into two separate constituents.

Discussion

Paper chromatograms of extracts of dried mature plants of *Digitalis lanata* showed clearly the presence of 4 unknown constituents which may be cardiac glycosides. These substances were apparently more water soluble than the lanatosides (digilanids) and separated from them quite clearly. One of these substances (L5) could belong to the A series (digitoxigenin derivatives) but the only evidence for this was the yellow-brown fluorescence in ultra-violet light after treatment with trichloro-acetic acid. Two of the constituents (L2 and L6) appeared to be present in reasonably large amounts. Both were cardiotoxic, gave the characteristic colour reactions of cardiac glycosides, and possibly contained digitoxose or related sugars. Paper chromatographic evidence indicated that one of them (L2) yielded digoxigenin on hydrolysis. The other substances (L6), although showing a blue fluorescence in ultra-violet light after trichloroacetic acid treatment was apparently not a derivative of either digoxigenin or gitoxigenin.

These substances in addition to several others of similar polarity or solubility, were also present in very young seedlings of *Digitalis lanata* before the lanatosides or other glycosides could be detected. It is possible that some of these substances may be precursors of the lanatosides containing for example, more sugar residues, but the present study gives little indication of their chemical nature. It does, however, indicate the complexity of the problem of the constituents of *Digitalis lanata.*

Summary

1. Extracts of mature and young plants of *Digitalis lanata* have been examined by paper chromatography and shown to contain a number of new substances which are apparently cardiac glycosides and more polar in nature than the lanatosides.

2. The eluates of two of these substances obtained from the paper chromatograms have been examined in more detail. Both substances are cardiotoxic and one appears to contain digoxigenin as its aglycone.

3. These new substances are present in very young plants before the lantosides themselves appear and most of them persist throughout the life of the plant.

The authors wish to thank Professor R. H. Thorp and Dr. H. Silberman of the Pharmacology Department, University of Sydney, for their helpful advice, and Mrs. D. A. Thorp for the supply of plant materials used in this work.

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DISCUSSION

The paper was presented by DR. S. E. WRIGHT.

The CHAIRMAN asked why the seedlings were collected "early in the morning".

DR. J. M. ROWSON (London) enquired if seedlings collected at other times of the day had been examined to determine whether the content of the new compounds varied with the time of collection. He had harvested at three hourly intervals up to five days and had found some variations. What was the ratio of the content of compounds L1, L2, L5 and L6 to the total glycosides present? Had the authors any information about the cardiac toxicity of the compounds in relation to the better known glycosides, and their possible therapeutic action? How quantitative was the elution of the glycosides from the chromatograms?

PROFESSOR H. BRINDLE (Manchester) said the authors used a very drastic system of extraction. Were they satisfied the new substances were not decomposition products? The substances were very labile, and he asked if simpler extracts of *Digitalis lanata* had been examined, and if so were the same substances obtained?

MR. G. J. RIGBY (Manchester, in a written contribution read by Professor Brindle) said that he had carried out similar work using *Digitalis purpurea* and had found several unidentified substances. Had the developed chromatograms been examined under ultra-violet light before treatment of the paper with trichloroacetic acid? Had the authors determined whether the compounds L1 and L5 reacted with xanthydrol and alkaline reagents used to demonstrate the presence of characteristic lactone rings?

DR. G. E. FOSTER (Dartford) suggested that the use of a large number of solvent systems in chromatographic examination of digitalis was

increasing the complexity of the problem. He asked whether the authors could give any information about the way in which their compounds behaved with solvent systems other than the one described in the paper.

DR. J. W. FAIRBAIRN (London) said that while the authors did not hesitate to label lanatosides B and C on their chromatogram, was it possible that L5 should be labelled lanatoside A?

DR. S. E. WRIGHT, in reply, said the plants were always collected in the morning because it was felt that they should be picked at a constant time. The possibility of variation during the day had not been investigated. It was impossible to say anything about the quantities of L2 and L6 present. They fluoresced brilliantly but that could not be taken as a measure of their concentration. As regards the relevant cardiac toxicity, judging by the colour reaction it fell somewhere on the dose response curve which would be expected for an aglycone. He had no information about the therapeutic effect. In the elution process not less than 90 per cent. recovery had been obtained. The extraction was based on the original method used by Stoll for lanatosides. The temperature did not rise above 40° C., and if decomposition occurred he would expect less polar compounds. They had tried formamide systems, but the substances did not travel very far. L6 did fluoresce before spraying with trichloroacetic acid. It also gave typical cardiac glycoside reactions. They had not tried the reaction of L1 and L5 because the quantities present were very small. Lanatoside A always travelled ahead of lanatoside B.

STUDIES IN THE GENUS DIGITALIS

PART V. FERMENTATIVE DEGRADATION OF *D. purpurea* LEAF

BY J. M. ROWSON AND S. SIMIC

From the Museum of The Pharmaceutical Society of Great Britain

Received June 26, 1956

THE deterioration in therapeutic potency of the leaves of *Digitalis purpurea* when incorrectly dried or stored is well known. This is generally attributed to enzymic degradation of the glycosides in the presence of moisture but the nature of these changes is not fully understood. It is probable that the work of Cloetta[1] is responsible for the widely held view that digitoxin and gitoxin readily hydrolyse in the moist drug to liberate the much less potent aglycones digitoxigenin and gitoxigenin together with free digitoxose. The presence of free aglycones in digitalis leaf is supported by Kedde[2] who, using adsorption methods, found that up to 50 per cent. of the glycosidal mixture was of free aglycone. Svedsen and Jansen[3], using paper chromatographic methods, found that stabilised digitalis leaf contained much primary glycoside with smaller amounts of secondary glycosides and aglycones, whereas the dried leaves which had not been stabilised contained less primary glycosides but more secondary glycosides and aglycones.

Tattje and Van Os[4–6] found small amounts of aglycones present in the majority of the leaf samples examined. An average of figures which they record for 15 samples shows the glycosidal complex to contain 79 per cent. primary glycosides, 5 per cent. secondary glycosides and 16 per cent. aglycones in stabilised leaves whereas for non-stabilised leaves the corresponding figures are 32 per cent., 58 per cent. and 10 per cent. respectively. They used the picrate and modified Keller–Kiliani methods of estimation upon the chloroform extracts from cold water macerates of the powdered leaf prepared by either $1\frac{1}{4}$ hours shaking, or 3 days maceration at 30° C. The glycosidal complex in the $1\frac{1}{4}$ hour maceration was of primary and secondary glycosides also of aglycones. During the more prolonged maceration at 30° C. the primary glycosides were all converted to secondary glycosides, but these authors maintained that no degradation of secondary glycosides to aglycones occurred; thus the difference between the picrate and the Keller-Kiliani estimations for this extract was a measure of the aglycones present. The estimation of the primary glycosides depended on the difference in the Keller-Kiliani values for the two extracts, since in the former only two of the three digitoxose molecules present in these glycosides reacted, whereas all three molecules reacted after removal of the glucose by fermentation[7]. These authors have also pointed out that the recently isolated strospeside would be estimated as aglycone by their method since it contains gitoxigenin and digitalose[8].

Neuwald and colleagues[9–12] found good agreement between modified picrate-genin and modified Keller-Kiliani estimations of total glycosides and concluded that no free aglycones were present in the three leaf

samples examined. Wolfgramm and Weiss[13] used the picrate and Keller-Kiliani reagents to examine the chloroformic extracts from cold water macerates prepared by varying times of contact between water and drug from 1¼ hours to 48 hours. There was good agreement between the two assay values at each period of maceration, with only occasional exceptions, and the authors concluded that free aglycones were absent. Since the assay value increased with periods of maceration up to 24 hours, they concluded that secondary glycosides only were separated by chloroform, leaving the primary glycosides in the aqueous phase; on fermentation these were broken down to secondary glycosides and were then extracted; hence the increases in values as a result of prolonged soaking gave a measure of primary glycosides present. For this reason these authors preferred their methods of extraction to that used by Langejan and van Pinxteren[14] in which a mixture of chloroform and ethanol was employed to remove primary as well as secondary glycosides.

Wegner[15,16] used cold water extracts of digitalis leaf powder, macerating for varying periods up to 24 hours, also digitalis enzyme preparations were added to extracts prepared by hot infusion. Picrate and Keller-Kiliani assay values increased with increasing periods of fermentation, the increase being more marked for the sugar estimation until parity of values for the two assay processes was reached after 24 hours of maceration. Wegner thus concluded that there was no strong evidence for the presence of free aglycones; that part only of the primary glycosides were extracted from the aqueous phase with chloroform, of which only two digitoxose molecules reacted with the Keller-Kiliani reagent; fermentation converted all primary into secondary glycosides. He also found that, after such fermentation, 30 per cent. of the glycosides originally present in the aqueous phase were chloroform-insoluble. Fish and Todd[17] examined the stability of digitalis tinctures and calculated that the initial fall in activity was due to the conversion of primary to secondary glycosides, after which the tinctures were quite stable with no further degradation to aglycones.

There is thus some real conflict in the published evidence on the occurrence or formation of free aglycones in dried digitalis leaf, the more recent work tending to suggest that little is found. All the foregoing comparative work had been based upon stabilised or normally processed drugs and we have no information about faulty processing, other than the behaviour of the leaf in the assay by 1–3 day fermentation itself, in which the secondary glycosides are said to be stable. A real conflict of evidence as to the extraction of the primary or secondary glycosides with chloroform also exists. To investigate these problems the following tests were made.

Extraction and Assay Methods

Cold water extracts were prepared by mixing powdered digitalis leaf 0·5 g. with water 50 ml. and shaking gently for 1¼ hours; decolourisation was effected by adding 5 ml. of 15 per cent. aqueous solution of lead acetate, shaking, allowing to stand, decanting and filtering. 37 ml. of filtrate (equivalent to ⅓ g. leaf) was shaken out with 4 × 20 ml. quantities

of either chloroform or a mixture of equal volumes of chloroform and ethanol 95 per cent. The mixed chloroformic extracts were dried over anhydrous sodium sulphate, filtered, the filter washed with chloroform, the filtrate adjusted to a suitable volume and divided into two halves each of which was evaporated to dryness. Using one dry chloroformic residue the glycosidal content was determined by the Keller-Kiliani reagent at

TABLE I

AQUEOUS MACERATES. DINITROBENZOIC ACID AND KELLER-KILIANI ASSAYS. EQUIVALENT DIGITOXIN CONTENTS (PER CENT.)

Leaf sample	A		B		C	
	Chloroform	Chloroform and ethanol	Chloroform	Chloroform and ethanol	Chloroform	Chloroform and ethanol
d_1	0·25	0·57	0·19	0·45	0·31	0·51
K_1	0·25	0·35	0·23	0·32	0·27	0·37
d_3	0·39	0·56	0·32	0·45	0·35	0·50
K_3	0·35	0·41	0·33	0·35	0·36	0·38

590 mμ as previously described[18], results being expressed as equivalent digitoxin (K_1). The second dry chloroformic residue was dissolved in 4 ml. of ethanol 17·5 per cent. and the glycosidal content determined by the dinitrobenzoic acid reagent at 535 mμ[18,19], results being expressed as equivalent digitoxin (d_1). The entire process was then repeated but macerating the leaf in water at 30° C. for 3 days: values K_3 and d_3 being determined.

TABLE II

DINITROBENZOIC REAGENT; ETHANOLIC AND COLD WATER EXTRACTS OF LEAVES. EQUIVALENT DIGITOXIN CONTENTS (PER CENT.)

Leaf sample	70 per cent. ethanolic extract of leaf	Aqueous maceration of leaf. Chloroform and ethanol extracts
A	0·57	0·56
B	0·50	0·45
C	0·53	0·50

Using three leaf samples A, B and C, the assay process was carried out in duplicate, employing chloroform and chloroform with ethanol in parallel experiments. The results are given in Table I. The assay values obtained by extraction of the leaf with ethanol 70 per cent., decolourisation and direct estimation with dinitrobenzoic acid[18,19] are compared with the 3-day maceration results in Table II.

It will be seen from this second Table that the results for each leaf by the two extraction methods are in good agreement and hence it is concluded that both methods are quantitative in extracting total glycosides which react with dinitrobenzoic acid reagent. The comparison of values in Table I shows that, from 3-day macerates, chloroform with ethanol extracts about 30 per cent. more material reacting with dinitrobenzoic acid than does chloroform alone; but this material appears to contain little or no digitoxose, since the K_3 values show almost insignificant increases. Hence this material is behaving as an aglycone in the estimation, it is not gitoxigenin or digitoxigenin which are chloroform-soluble and it is more probably digitalinum verum or strospeside which are free from digitoxose and are insoluble in chloroform.

Disparity of results is the more marked between the different solvent extractions from 1¼ hours aqueous macerates: the digitoxose-free glycoside such as digitalinum verum accounts for a portion of the difference in d_1 values, the extraction of primary glycosides by chloroform and ethanol accounting for the remainder. The good agreement between d_1 and d_3 values when based on chloroform with ethanol extracts should be noted. The K_3 values are also a maximum for these extracts due to the conversion of all primary to secondary glycosides in the 3-day fermentation with consequent availability of all three sugar molecules to react. Hence the 3-day aqueous maceration process followed by chloroform with ethanol extraction give results most nearly agreeing with those obtained by ethanolic extraction of this leaf. The guinea-pig biological assay of chloroform with ethanol and of chloroform extracts from decolourised tinctues of leaf sample E, as prepared for dinitrobenzoic acid assay[18,19], are reported in Table IV. It will be noted that about 30 per cent. of the glycosidal complex, as shown by potency, is insoluble in chloroform but soluble in chloroform with ethanol and this agrees well with the d_3 values recorded in Table I.

The presence of small amounts of chloroform-soluble free aglycones in leaf sample A is indicated by the difference $d_3 - K_3$ for the chloroform extract (Table I), this is approximately 10 per cent. of total glycoside present. Chloroform-soluble free aglycones are absent from leaf samples B and C.

Fermentation Studies

A preliminary test to investigate the behaviour of the glycoside content as shown by dinitrobenzoic acid assay in leaves subjected to different periods of humidification was set up. Weighed quantities of three leaf powders were each moistened with water, placed in covered petri dishes and incubated at 30° C. for periods of from 2 days to 6 weeks. Each

TABLE III

Fermented leaf. Dinitrobenzoic acid assay of 70 per cent. ethanol extracts

Leaf sample	D		E		F	
Period of fermentation	Loss in weight per cent.	Equivalent potency I.U./g.	Loss in weight per cent.	Equivalent potency I.U./g.	Loss in weight per cent.	Equivalent potency I.U./g.
0 days	—	11·9	—	11·8	—	12·5
3	26	9·7	—	—	—	—
7	29	7·2	35	7·1	—	—
14	—	—	41	2·4	—	—
21	—	—	44	2·1	—	—
28	—	—	—	—	49	3·2
35	—	—	—	—	47	3·4
42	—	—	—	—	46	3·0

sample was stirred daily and kept moist by adding water when necessary. When humidified for the requisite period of time, the dish lids were removed, the contents dried at 55° C. and re-weighed: losses in weight due to fermentation were calculated and weights of the materials necessary to make tinctures equivalent to 1 in 10 of the original leaf samples were taken.

Assays of these tinctures were made by the dinitrobenzoic acid process[19] and the equivalent potencies are given in Table III. Biological assays of sample E after 7 days and after 21 days' fermentation were also carried out and these are recorded in Table IV.

TABLE IV

BIOLOGICAL ASSAYS (GUINEA-PIG METHOD). LEAF SAMPLE E

Treatment of leaf	Equivalent leaf Potency I.U./g.
Decolourised 70 per cent. ethanolic tincture[18,19]:—	
extract with chloroform and ethanol	10·8
extract with chloroform	7·4
Leaf fermented 7 days	2·4
Leaf fermented 21 days	0·7

The fermentation process was repeated for periods of time up to 21 days, using two further leaf samples. Tinctures of the fermented materials were prepared and assayed as described above, the equivalent potencies being given in Table V. Further samples of the same fermented materials were also examined by the cold water extraction process for 1¼ hours or for 3 days at 30° C. as described above; allowance being made for the losses in weight due to fermentation. Glycosides were extracted from the 1¼ hour aqueous macerates with chloroform and ethanol; the 3 day aqueous

TABLE V

FERMENTED LEAF. DINITROBENZOIC ACID ASSAY OF 70 PER CENT. ETHANOL EXTRACTS

Leaf sample	G		H	
Period of fermentation	Loss in weight per cent.	Equivalent potency I.U./g.	Loss in weight per cent.	Equivalent potency I.U./g.
0 days	—	13·1	—	11·6
3	23	12·2	25	10·3
6	30	10·4	32	9·2
9	39	8·6	43	8·1
12	42	9·4	44	7·5
15	45	4·1	48	7·6
18	49	4·2	49	5·5
21	49	1·5	48	6·9

TABLE VI

FERMENTED LEAF SAMPLE G. AQUEOUS MACERATES. EQUIVALENT DIGITOXIN CONTENTS (PER CENT.)

Period of maceration	1¼ hours		3 days			
Extraction solvent	Chloroform and ethanol		Chloroform		Chloroform and ethanol after chloroform	
Period of fermentation	d_{1T}	K_{1T}	d_3	K_3	d_{3CA}	K_{3CA}
0 days	0·52	0·32	0·36	0·32	0·13	0·04
3	0·35	0·33	0·30	0·28	0·05	0·03
6	0·35	0·28	0·32	0·25	0·05	0·04
9	0·30	0·27	0·30	0·23	0·04	0·03
12	0·33	0·23	0·29	0·20	0·04	0·03
15	0·17	0·19	0·16	0·15	0·01	0·04
18	0·17	0·20	0·16	0·16	0·02	0·05
21	0·05	0·13	0·04	0·07	0·01	0·04

macerates were extracted first with chloroform and then with a mixture of chloroform and ethanol. Dinitrobenzoic acid and Keller-Kiliani assays were then applied to each fraction and these are recorded in Tables VI and VII.

TABLE VII

FERMENTED LEAF SAMPLE H. AQUEOUS MACERATES. EQUIVALENT DIGITOXIN CONTENTS (PER CENT.)

Period of maceration	1¼ hours		3 days			
Extraction solvent	Chloroform and ethanol		Chloroform		Chloroform and ethanol after chloroform	
Period of fermentation	d_{1T}	K_{1T}	d_3	K_3	d_{3CA}	K_{3CA}
0 days	0·50	0·32	0·34	0·31	0·12	0·04
3	0·36	0·26	0·31	0·26	0·05	0·03
6	0·31	0·25	0·28	0·22	0·04	0·03
9	0·29	0·24	0·26	0·21	0·03	0·03
12	0·26	0·23	0·27	0·20	0·02	0·03
15	0·18	0·20	0·13	0·14	0·01	0·04
18	0·18	0·19	0·16	0·14	0·03	0·04
21	0·03	0·08	0·03	0·03	0·02	0·04

TABLE VIII

FERMENTED LEAF SAMPLES G AND H. APPARENT CONTENTS OF PRIMARY AND SECONDARY GLYCOSIDES AND OF AGLYCONES (PER CENT.)

Leaf sample	G			H		
Period of fermentation	Primary glycosides	Secondary glycosides	Aglycones etc.	Primary glycosides	Secondary glycosides	Algycones etc.
0 day	0·15	0·25	0·08	0·11	0·26	0·07
3	Nil	0·33	0·02	0·11	0·20	0·03
6	"	0·28	0·03	Nil	0·25	0·03
9	"	0·27	0·02	"	0·24	0·02
12	"	0·23	0·05	"	0·23	0·01
15	"	0·19	Nil	"	0·20	Nil
18	"	0·20	"	"	0·19	"
21	"	0·13	"	"	0·08	"

DISCUSSION

The presence of about 30 per cent. of the total glycoside complex in the form of chloroform-insoluble but chloroform with ethanol-soluble material has already been noted in leaf samples A, B, C and E as recorded in Tables I and IV. Similar results were obtained for leaf samples G and H when controls were examined by means of the 3-day aqueous macerate method, the d_{3CA} value being about 26 per cent. of the total glycosidal material estimated, as shown in the first line of Tables VI and VII. The almost complete absence of digitoxose sugar in this chloroform-insoluble material was also confirmed by the very low assay figures with the Keller-Kiliani reagent (K_{3CA}). This material is neither digitoxigenin nor gitoxigenin, but may be a chloroform-insoluble glycoside free from digitoxose such as digitalinum verum.

Using the results for leaf sample A, Table I, as an example it is seen that 0·15 per cent. ($d_{3CA} - K_{3CA}$) of this chloroform-insoluble glycoside is present. Thus the total amount of other glycosides and aglycones originally present in the leaf is 0·41 per cent. ($d_{1CA} - 0·15$). It thus follows

that chloroform alone fails to extract 0·16 per cent. ($0·41 - d_1$) of this group, and this will be primary glycoside which is very feebly soluble in chloroform. Now in the chloroform with ethanol extract the difference between the two Keller-Kiliani values is one-third of the amount of primary glycoside present since only two digitoxose molecules react in the primary glycoside molecule (K_{1CA}) whereas all three digitoxose molecules react after degradation to the secondary glycoside (K_{3CA}). Hence the leaf contains 0·18 per cent. of total primary glycosides ($3 \times (K_{3CA} - K_{1CA})$) and so the 0·16 per cent. of primary glycoside, not extracted by chloroform alone after $1\frac{1}{4}$ hour aqueous maceration, comprises practically the whole of the primary glycosides present in the leaf. Similar calculations may be made for leaf samples B and C (Table I). Undue reliance should not be placed on such absolute calculations for it seems probable that the solubilities of the components in the glycosidal complex will differ from those determined on pure glycosides. Nevertheless, these results support the findings of Wolfgramm and Weiss[13] that the primary glycosides are not extracted by chloroform from aqueous leaf macerates, the results also support the work of Langejan and van Pinxteren[14] that primary glycosides are extracted from such aqueous macerates by means of chloroform and ethanol.

The good agreement between the dinitrobenzoic acid assay results for ethanolic extractions of leaves and for aqueous macerates followed by chloroform with ethanol extractions is of importance (Table II). Since aglycones appear to be almost completely absent, even in deliberately fermented samples, and since digitalinum verum is of high potency, it follows that the former method of extraction may be used with confidence, for it extracts maximum glycosidal material.

The fall in total glycosidal material, as estimated by dinitrobenzoic acid, in ethanol extracts of leaf samples which have been subjected to progressive humidification at 30° C., is marked as is seen in Tables III to V and very low values are reached after about 14 days. This decrease, associated with a fall in dry weight of the leaf tissues, is most readily explained by fermentative degradation of cell contents. It is reasonable to hypothesise that the aglycone portions of the glycoside molecules are progressively degraded by these fermentative changes, at least in so far as the butenolide group, which reacts with the dinitrobenzoic acid reagent, is concerned.

The most outstanding fact which arises from these investigations is that it has proved impossible to produce free aglycones in leaf samples submitted to extremely bad conditions of storage. These moist, warm conditions rapidly affected the samples adversely as judged by odour, colour and general appearance also by loss in dry weight of up to 49 per cent. The rapid fall in dinitrobenzoic acid assays of 70 per cent. ethanolic extracts, associated with these changes, has already been pointed out and similar decreases were found with this assay on aqueous macerates prepared from these fermented materials as shown in Table VI and VII. The decreases in Keller-Kiliani assay values with increased periods of leaf fermentation are of the same order, but not quite so great as the decreases in dinitrobenzoic acid values. Hence the difference $d_3 - K_3$, which is a measure

of aglycone, becomes smaller and eventually reaches a zero value. Thus in these degradation changes the aglycone portion of the glycoside molecule, or at least the butenolide linkage thereof, disappears, as rapidly or slightly more rapidly than the digitoxose sugar moiety. There is never any evidence that the digitoxose is split off from the aglycone, and, if this does occur, the subsequent degradative changes are more rapid, for free aglycones do not accumulate.

The amount of primary glycoside originally present in the leaf sample G, as indicated by the difference in Keller-Kiliani values on chloroform with ethanol extracts, had completely disappeared after 3 days of fermentation of the material; in leaf sample H it had disappeared after 6 days' fermentation. This was to be anticipated and confirms the findings of other workers which were discussed above. The chloroform-insoluble glycosides free from digitoxose, such as digitalinum verum, which are associated with the primary glycosides (d_{3CA}, Tables VI and VII) also disappear relatively rapidly as fermentation proceeds. Hence the relative stability of the secondary glycosides is demonstrated. The more rapid fall in biological assay values after 7 and 21 days' fermentation (Table IV) should however be noted.

If the methods of calculation, proposed by Tattje[5] for the estimation of primary and secondary glycosides and of aglycones, are applied to the chloroform and ethanol values in Tables VI and VII, the figures of Table VIII are obtained for leaf samples G and H.

Digitalinum verum and other non-digitoxose glycosides are here shown as aglycones. Because of differences between solubilities of individual glycosides and of their mixtures, these calculated values cannot be regarded as absolute but the results do support the general conclusions already made.

Summary and Conclusions

1. Leaf powders of *Digitalis purpurea*, when moistened and allowed to ferment at 30° C. for periods of time up to 42 days showed: (*a*) a rapid conversion of primary to secondary glycosides; (*b*) a progressive degradation of secondary glycosides, involving both the aglycone and sugar parts of the molecule; (*c*) free aglycones did not accumulate at any stage.

2. The glycoside complex of *Digitalis purpurea*, extracted with either water or 70 per cent. ethanol may contain about 50 per cent. of material insoluble in chloroform, but soluble in chloroform with ethanol and possessing high pharmacological activity; this comprises: (*a*) practically the whole of the primary glycosides, and (*b*) up to 30 per cent. of glycosidal material free from digitoxose.

3. The dinitrobenzoic assay process, applied to decolourised preparations of 70 per cent ethanol extracts of leaf powders, estimates the whole of this material.

Our thanks are due to the Laboratory Animals Bureau for the biological assays of Table IV.

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DISCUSSION

The paper was presented by DR. J. M. ROWSON.

The CHAIRMAN said that workers on digitalis frequently referred to fermentation. Was this fermentation hydrolysis caused by micro-organisms which might be a definite species associated with the drug or by enzymes? Had the authors any evidence that the surface of the leaves was sterile?

PROFESSOR H. BRINDLE (Manchester) said the evidence for the presence of aglycones in digitalis was conflicting. The authors used the extraction method but the value of certain parts of their work would have been enhanced if they had also used chromatography. For example, they speculated that one substance might be digitalinum verum, whereas had chromatography been applied they would have been sure. Speaking from memory, he had found in unfermented leaf a higher ratio of primary glycosides to secondary than the authors had. In Table VIII the primary glycosides present in Sample H had not changed after three days' fermentation—was this a misprint?

MR. G. J. RIGBY (in a written contribution read by Professor Brindle) said it was surprising the authors had not employed chromatography in their investigations. The authors had calculated that one leaf sample contained 0·18 per cent. of total primary glycosides, and said that 0·16 per cent. of primary glycosides not extracted by chloroform alone approximated closely to that figure. The paper would be less difficult to understand if the symbols used for this calculation had been clearly defined. The value 0·18 per cent. had been calculated from the expression $3(K_{3CA} - K_{1CA})$ which took no account of the different molecular weights of the primary and secondary glycosides. The authors had assumed that 2 mg. of a secondary glycoside would react with the Keller-Kiliani reagent to the same degree as 3 mg. of the corresponding primary glycoside. In fact,

one molecule of each were reported to react to the same degree. That required a correction in the paper. The percentage of total primary glycoside present should be calculated as 3·63 ($K_{3CA}-K_{1CA}$). The figure 0·18 per cent. would then become 0·22 per cent., showing that chloroform alone extracted about 30 per cent. of the total primary glycosides and not 11 per cent. as suggested by the authors. The term "equivalent digitoxin" was ambiguous. Did the authors mean "producing the same extinction as x mg. of digitoxin", or did they mean that two g. molecules of desacetyl-digilanid A was equivalent to two molecules of digitoxin?

DR. G. E. FOSTER (Dartford) said he understood the authors to state that the activity of moist digitalis leaf was completely destroyed on storage. That had not been his experience. He had found that it fell fairly rapidly for a time but then remained constant over a long period.

DR. S. E. WRIGHT (Sydney) said the authors had tended to reject the idea that genins were present in digitalis. That was difficult to establish as the genins were not easy to identify. It was interesting to note that Kaiser and co-workers had isolated some compounds recently which were formyl derivatives.

MR. E. H. B. SELLWOOD (London) referred to the relative amounts of the biological activity extracted in chloroform, with and without alcohol. He had extracted the chloroform-soluble glycosides from a number of samples of leaf and isolated them in solid form, obtaining a yield of approximately 1 g. assaying biologically at about 400 units/g. from a pound of leaf. It was obvious that a considerable amount of biological potency was unaccounted for because this was equivalent to about 1 unit per g. of leaf, whereas the leaf itself assayed at 10–12 units per g. The missing activity may be in the water soluble fraction.

DR. J. W. FAIRBAIRN (London) said that with anthraquinone drugs he had found that during storage or extraction the glycosides broke down, but one could not recover a corresponding amount of the aglycones. At the beginning of the paper the reader was offered two statements of fact. The first was that there were aglycones present, and the second that they were not present. At the end of the paper the authors stated aglycones did not accumulate. It would be interesting to hear how they explained the first statement.

DR. J. M. ROWSON, in reply, said that no attempt had been made to control or to follow the nature of the fermentation. The leaves were kept moist in dishes, and after several days' fermentation there was a good growth of mould on the surface. He had not examined the leaves for micro-organisms, but he felt sure that they would not be sterile. The naturally occurring enzymes were present since these are only destroyed by hot water extraction. He had resisted the use of chromatography up to the present time because he knew that others had undertaken chromatographic studies, and it was undesirable that the work should overlap too much. With regard to the high proportion of secondary to primary glycosides, his recoveries were in good agreement with those of Van Os and Tattje which were quoted. The presence of digitalinum verum in

Digitalis purpurea leaves of different geographical sources had been proved by other workers; he accepted their findings and made use of the new evidence. There was no misprint in Table VIII. The symbols d_{3CA} and K_{3CA} etc. were adequately defined in the paper. The calculations in the paper were correct, since all results were expressed as equivalent to digitoxin. Mr. Rigby has wrongly interpreted this fact. As to the activity of the leaf being destroyed on storage, the leaf he was examining was very moist and it fermented. It was not comparable with the slow drying or leaf storage. The formyl compounds of Kaiser were interesting. Early references to the presence or absence of aglycones in the dried leaf were not clear, and Cloetta in 1927 contributed much to the idea of their presence.

DETERMINATION OF THE DIGITOXIN CONTENT OF *DIGITALIS PURPUREA*

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There is no simple method of determining the digitoxin content of leaves of *Digitalis purpurea* and often it is not easy to decide the suitability of a crop for the preparation of this glycoside other than by extracting a substantial quantity of leaf. It is well known that both the amount and composition of the glycosidal mixture are subject to wide variation depending on botanical, geographical and ecological factors. Neither biological nor chemical assays of the leaf or crude extracts prepared from it give a reliable indication of the amount of digitoxin present. However by using paper chromatography the separation of the glycosidal mixture is considerably simplified and the individual glycosides may be estimated colorimetrically. A method along these lines for the estimation of digitoxin in small samples of leaf is described here and results obtained in applying the procedure to several samples are reported.

Propylene dichloride was used to isolate the glycosides because it is more selective than chloroform[1] and the system carbon tetrachloride-ethanol-water[2] was found suitable for the chromatographic separation of digitoxin from the propylene dichloride-soluble glycosides. This system consists of readily available solvents which require no further purification and are easily removed from the completed chromatograms. One drawback is that the capacity of the system is small compared with those of systems based on formamide, but this is not serious as digitoxin is the main constituent of the glycosidal mixture and it was not intended that trace constituents should be studied. The best known and most sensitive of the colorimetric assays for digitoxin is that using sodium picrate but this needs 100 to 150 μg. of digitoxin to give a useful optical density. This would necessitate running a considerable number of spots and the extraction of a correspondingly large area of paper. A sensitive assay method with xanthydrol was developed from the qualitative test for digitoxin described by Arreguine and Pasqualis[3]; the digitoxose side chain is the basis of the reaction and quantities of the order of 20 μg. only are required.

Arreguine and Pasqualis heated the reaction mixture in a water bath until the red colour developed. Pesez[4] reported that the colour is stable if the mixture is heated in a boiling water bath for exactly three minutes followed by cooling in ice for five minutes. These conditions have been used by Tschesche, Grimmer and Seehofer[5] and by Tuzson and Vastagh[6] respectively in their quantitative chromatographic methods. The modification of the Arreguine and Pasqualis procedure described here, in which the reaction takes place at room temperature, avoids any critical conditions of heating and there is no risk of errors arising from uptake of

moisture. In addition, when using paper chromatograms, extraction of the paper and colour formation proceed simultaneously.

Preliminary autolytic fermentation is employed to hydrolyse the initial complexes to the secondary glycosides. Purpurea glycosides A and B are apparently not extracted by propylene dichloride, for on chromatograms they would be expected to remain close to the solvent front whereas no more than a trace of unidentified material has been found in this position on chromatograms from unfermented leaf. Moreover, after the preliminary hydrolysis both the content of propylene dichloride-soluble glycosides and of digitoxin show substantial increases. The breakdown of the secondary glycosides into aglycones does not take place and according to Neuwald[7] there are no enzymes in the leaf capable of such hydrolysis. In the present work no more than a trace of digitoxigenin has been detected and this is in agreement with Neuwald's findings.

Experimental Methods

Propylene Dichloride-Soluble Glycosides

20 g. of dried powdered leaf was mixed with 20 ml. of distilled water, placed in a 250 ml. flask and incubated in a water bath at 37° C. for 65 hours. At the end of this time 40 ml. of distilled water and 140 ml. of industrial absolute ethanol were added. The flask was then shaken for an hour and the 70 per cent. ethanol extract was obtained by filtering through a fluted 531 paper. To 50 ml. of the extract was added 100 ml. of distilled water and the solution distilled under vacuum until free from ethanol, using a silicone antifoam to prevent frothing. The glycosides were then extracted with four quantities each of 25 ml. of propylene dichloride taking care to avoid emulsification. These extracts were mixed together and washed with two quantities each of 25 ml. of 1 per cent. sodium carbonate solution (2·7 per cent. of $Na_2CO_3 \cdot 10H_2O$) and finally two volumes each of 25 ml. of water. The propylene dichloride was then removed under vacuum and the residue taken up in 100 ml. of *iso*propanol. 5 ml. portions of this solutions were mixed with 5 ml. of sodium picrate reagent (1 per cent. aqueous solution of trinitrophenol 95 ml., 10 per cent. sodium hydroxide solution 5 ml.) and the maximum optical density at 495 mμ measured (filter 623 in the EEL photoelectric colorimeter). The results were calculated from a linear calibration curve relating optical density with biological potency, previously prepared using samples of digitoxin of known biological potency. As no solid fraction is isolated in this method results were expressed as units per g. of leaf.

Chromatography

Equal volumes of carbon tetrachloride B.P., industrial 95 per cent. ethanol and distilled water were shaken together and allowed to separate. The lower layer then formed the mobile phase and the upper layer was placed in the bottom of the tank. The solution to be chromatographed was obtained by distilling to dryness, *in vacuo*, 50 ml. of the above prepared *iso*propanol solution and taking up the residue in 5 ml. of dry methanol.

0·01 ml. quantities of this were applied to the paper using an Agla micrometer syringe. A strip of Whatman No. 1 paper 46 × 9 cm. was used and on this were pencilled (*a*) a starting line 19 cm. from one end and (*b*) two longitudinal lines running one along each side, 1·5 cm. from the edge. Six spots were placed on the starting line, one in the centre of each of the side divisions and four, one centimetre apart, in the middle of the strips, the outside spots of the latter group thus being 1·5 cm. from the parallel markings. The solvent system in the tank was allowed to come to equilibrium as usual but no preliminary saturation of the paper was used. The strip together with a blank marked in the same way was dipped into the trough and downward development was continued until the solvent front was near the bottom of the test strip (about seven hours) by which time the solvent front on the blank had already reached the bottom. The papers were then removed and allowed to dry in the air. By cutting along the two pencilled lines the chromatograms corresponding to the two outer spots were obtained and the position of the glycosides on these was revealed by spraying with trichloroacetic acid in chloroform, "aged" with hydrogen peroxide. By placing these strips alongside the unsprayed part of the original strip the positions of the zones in the latter were determined and these were then cut out. The corresponding areas of the blank paper were also cut out to serve as controls in the colorimetry. In preliminary experiments the glycosides were also located by using antimony trichloride[8], phosphoric aid[9] and xanthydrol, respectively.

Colorimetric Assays using Xanthydrol Reagent

The rectangular pieces of paper were then further cut up and placed in small beakers, one to each zone. To each was added 10 ml. of a 0·125 per cent. solution of xanthydrol in glacial acetic acid (A.R.) and 0·1 ml. of hydrochloric acid. The contents of each beaker were then stirred and the beakers placed under inverted amber glass jars. At the same time a reagent blank consisting of 10 ml. of xanthydrol solution mixed with 0·1 ml. of hydrochloric acid was placed in a corked colorimeter tube. At the end of two hours, during which time the contents of the beakers were stirred occasionally, the solutions were poured into colorimeter tubes and corked. Readings of optical density were taken, using filter 624, on the EEL Colorimeter, the instrument being balanced to zero with the reagent blank in position. Further measurements were made at intervals until the maximum density was reached (2–4 hours from the time of mixing) the tubes being kept away from strong light between observations. The optical densities recorded from the areas of blank paper were then subtracted from those for the corresponding areas of the chromatogram and the glycosidal contents calculated from a standard graph based on digitoxose.

RESULTS

Study of the Isolation Procedure

A number of estimations were made using one of the leaf samples (A24) in order to study the period of fermentation required to complete

enzymatic hydrolysis, the proportion of water needed for this process and the completeness of extraction into propylene dichloride. It was found that the content of propylene dichloride-soluble glycosides indicated by assay with sodium picrate increased rapidly during the first eight hours of fermentation, then increased slowly to reach a maximum in about three days (Table I). Variations in the amount of water used, with corresponding alterations in the quantity added with ethanol for extraction, showed that the ratio one part of water to one part of leaf is satisfactory (Table II). By assaying each 25 ml. volume of propylene dichloride in the extraction process, after washing with sodium carbonate solution and with water, it was found that the total yield could be increased by about five per cent. if a fifth volume of the solvent was used (Table III).

TABLE I

EFFECT OF THE DURATION OF FERMENTATION ON THE APPARENT CONTENT OF PROPYLENE DICHLORIDE-SOLUBLE GLYCOSIDES (ASSAYED WITH SODIUM PICRATE)

Length of fermentation at 37° C. in hours	Units per g. of leaf propylene dichloride-soluble fraction
0	1·36
8	2·52
18	2·60
24	2·56
48	2·60
72	2·75
115	2·75

TABLE II

EFFECT OF THE PROPORTION OF WATER USED DURING FERMENTATION FOR 65 HOURS AT 37° C. ON THE APPARENT CONTENT OF PROPYLENE DICHLORIDE-SOLUBLE GLYCOSIDES (ASSAYED WITH SODIUM PICRATE)

Weight of water/Weight of leaf	Units per g. of leaf propylene dichloride-soluble fraction
0	1·28
0·25	2·12
0·5	2·40
1·0	See Table I
3·0	2·45

TABLE III

EXTRACTION OF PROPYLENE DICHLORIDE-SOLUBLE GLYCOSIDES INTO SUCCESSIVE PORTIONS OF THE SOLVENT (ASSAYED WITH SODIUM PICRATE)

	Units per g. of leaf propylene dichloride-soluble fraction	
	Per portion	Progressive total
1	1·13	1·13
2	0·59	1·72
3	0·36	2·08
4	0·22	2·30
5	0·13	2·43

Chromatography of the Propylene Dichloride Soluble Glycosides

Chromatograms were prepared of the propylene dichloride-soluble glycosides obtained without preliminary fermentation and those obtained after 72 hours of such treatment. The two strips were similar in appearance but the zones from the latter were denser. Digitoxin formed elongated spots extending from R_F 0·26 to R_F 0·42 and assays with xanthydrol reagent showed the apparent digitoxin content of the unfermented leaf to be 0·073 per cent. and that of the fermented leaf, 0·157 per cent. Further runs of the "72 hours" sample were made on wider sheets of paper with digitoxin, gitoxin and the aglycones as controls. Such a run, in which the solvent has been allowed to drip from the bottom of the paper is shown in Figure 1A. By continuing development for 24 to 48 hours, further resolution of the slower running spots into additional components takes place but no attempt has been made to study them further. A typical chromatogram of the propylene dichloride-soluble glycosides is shown in Figure 1B. There is sometimes a trace of digitoxigenin running

faster than digitoxin and normally some gitoxigenin slightly slower than digitoxin. Somewhat slower still is the spot due to gitoxin and between this and the starting line is an unidentified member of the B series. There is usually a slight "ghost" on the starting line itself.

Assays with Xanthydrol Reagent

When a solution of digitoxin is mixed with xanthydrol reagent and warmed in a water-bath the initial yellow colour rapidly changes to an intense red, then becoming brown and finally green. At lower temperatures the reaction is much slower, the red colour reaching maximum intensity in two to four hours at room temperature. The maximum sensitivity is shown when using filter 624 (about 520 mμ), and Beer's Law is obeyed. Some fading takes place in sunlight and the presence of a trace of water greatly impairs colour formation, 1 per cent. producing a significant effect. Gitoxin on treatment gives a colour with an optical density similar to that of digitoxin while, as expected, the aglycones produce no colour at all. The reaction is about four and a half times as sensitive as that with sodium picrate, the value of E (1 per cent. 1 cm.) using digitoxin being about 900. Because of the difficulty in obtaining a pure sample of digitoxin, digitoxose was used as standard and a value of E (1 per cent. 1 cm,) = 1520 was obtained.

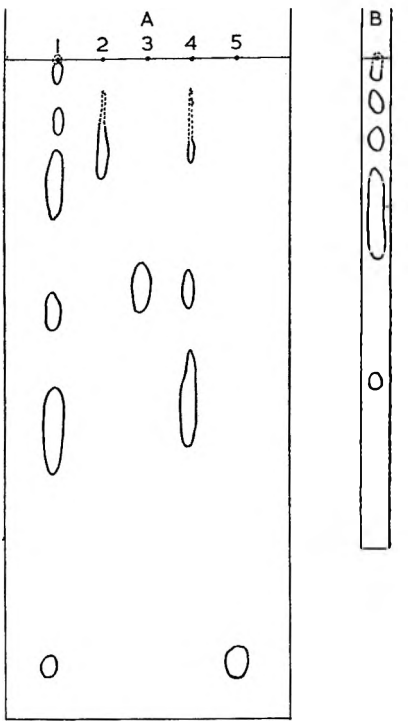

FIG. 1. Chromatograms of the propylene dichloride–soluble glycosides of digitalis. *A*. 15-hour run. *B*. 7-hour run.

1. 10 μg. Propylene dichloride–soluble glycosides. 2. 1 μg. Gitoxin. 3. 0·4 μg. Gitoxigenin. 4. 10 μg. Digitoxin. 5. 10 μg. Digitoxigenin.

Estimation of Digitoxin on Paper Strips

Eight samples of digitoxin all containing gitoxin were examined chromatographically, running duplicate chromatograms from 1 in 1000 solutions in methanol. The digitoxin and gitoxin zones were then assayed using xanthydrol reagent, and direct assays of the methanol solution were also made by placing 0·04 ml. quantities in colorimeter tubes, evaporating the solvent and adding the reagent to the residue. Recoveries from the strips were calculated by adding together the optical densities produced by the digitoxin and gitoxin zones and expressing the sum as a percentage

of that obtained by direct assay. The average recovery was found to be 95·1 per cent., the lowest being 90·5 per cent. and the highest 99 per cent. (Table IV).

On these chromatograms R_F values for digitoxin and gitoxin were somewhat lower than the R_F values for these substances when constituents of the propylene dichloride-soluble fraction, and the fringes of the two

TABLE IV

RECOVERY FROM CHROMATOGRAMS, 0·04 ml. PORTIONS OF APPROXIMATELY 1 IN 1000 SOLUTIONS OF DIGITOXIN ASSAYED WITH XANTHYDROL, DIRECTLY AND AFTER CHROMATOGRAPHY

Sample	Optical density × 100 direct assay (A)		Optical densities × 100 of chromatographic zones		$\frac{(d + g)}{A} \times 100$
			Digitoxin (d)	Gitoxin (g)	per cent.
1	28·0 27·0	27·5	21·5 24·5	4·3 2·0	94·0 96·2
2	31·0 31·0	31·0	20·6 22·0	8·0 6·3	92·3 91·2
3	33·5 33·5	33·5	30·0 29·6	2·6 2·0	97·4 94·5
4	32·5 32·5	32·5	26·6 24·9	5·0 4·6	97·2 91·0
5	27·5 27·5	27·5	23·8 22·5	3·4 3·7	99·0 95·2
6	32·0 31·0	31·5	27·0 29·0	3·8 1·8	97·7 97·7
7	34·5 35·0 35·5	35·0	24·6 26·2	7·0 6·5	90·5 93·5
8	36·0 35·5 36·5	36·0	27·1 28·5	8·3 6·0	98·5 96·0

zones tended to merge. Consequently with some of these samples although agreement between parallel runs was good in respect of total glycosides it was rather poorer for the two components due to the difficulty in deciding where to make the cut between them. Using this procedure to study the amount of gitoxin present in samples of digitoxin it was found better to develop the chromatograms for up to 15 hours, allowing the solvent to drip from the bottom of the paper, under which conditions good separation was obtained.

Examination of Leaf Samples

Samples of experimental crops of digitalis grown at Ware by Dr. Rowson were by his kind permission collected in September, 1954, dried at 55° C., powdered and stored in well closed tins. The propylene dichloride-soluble glycosides were estimated by the procedure outlined above, using two 20 g. samples of each batch. Duplicate chromatograms were run from each of the resulting extracts and the digitoxin contents were determined using xanthydrol reagent. The results are shown in Table V. The remaining glycosides in the chromatograms were estimated

as a single zone and direct assays of the methanol solution were also carried out. The percentage of the glycosides recovered from the chromatograms was somewhat lower than with samples of digitoxin, but averaged 91·5 per cent. For one strip it was only 83·5 per cent. but for the others it was above 86·5 per cent., while the highest was 99 per cent. (Table VI).

To find whether free digitoxose interferes in the determination of the digitoxin content of the leaf 25 mg. of digitoxose was dissolved in 100 ml. of distilled water and submitted to the isolation procedure. The propylene dichloride solution, after washing with sodium carbonate solution and with water, was distilled to dryness. No residue could be seen in the flask. However, glacial acetic acid was added and the solution assayed with xanthydrol reagent. No digitoxose was detected. Moreover when digitoxose was chromatographed it remained on the starting line and could not therefore be mistaken for digitoxin.

TABLE V

EXAMINATION OF SAMPLES OF DIGITALIS LEAF (i) PROPYLENE DICHLORIDE-SOLUBLE GLYCOSIDES (ii) DIGITOXIN CONTENT

Leaf	Propylene dichloride-soluble glycosides Units per g. of leaf	Percentage of digitoxin
A22	3·14 3·06	0·179 0·185
A23	2·64 2·54	0·115 0·113
A24	2·75 3·02	0·157 0·177
A25	2·84 2·76	0·164 0·166
A26	2·30 2·24	0·101 0·096
A27	2·50 2·72	0·139 0·150

It was thought desirable to test if digitoxin of the order indicated by these assays could be isolated from the leaf. The propylene dichloride-soluble glycosides were isolated from 1 kg. of leaf A22, yielding 5·17 g. of green solid assaying chemically at only 455 units per g. This solid was only partly soluble in 50 per cent. ethanol and the solution, after filtration, was treated with strong solution of lead subacetate, refiltered and the excess of lead removed as sulphate. The lead-free filtrate was distilled to remove the ethanol and the glycosides extracted with chloroform and isolated by precipitation of the concentrated dried chloroform solution in petroleum spirit. 2·08 g. of pale green solid was obtained assaying chemically (sodium picrate) at 905 units per g. and biologically at 1063 units per g. In the chromatographic assay the sample was found to contain 67 per cent. of digitoxin and 5·6 per cent. of gitoxin.

DISCUSSION

The results of the assays after various periods of fermentation, besides showing that most of the hydrolysis takes place in the first eight hours also serve to confirm the reproducibility of the method of extraction. The results in Table III indicate that it might be desirable to use a fifth volume of propylene dichloride in the extraction thereby increasing the total yield of propylene dichloride-soluble glycosides by about five per cent. Whether this is associated with a corresponding increase in the amount

of digitoxin detected on the paper chromatograms has not been studied and it is probable that further quantities of the solvent would continue to extract, in addition, small amounts of water-soluble glycosides for which the partition ratio into propylene dichloride is unfavourable. The sum of the glycosides extracted when using four volumes of propylene dichloride is somewhat lower than that indicated in Table I, but this is

TABLE VI

RECOVERY FROM CHROMATOGRAMS. SOLUTIONS OF PROPYLENE DICHLORIDE-SOLUBLE GLYCOSIDES ASSAYED WITH XANTHYDROL, DIRECTLY AND AFTER CHROMATOGRAPHY

Sample		Optical density × 100 direct assay (A)		Optical densities × 100 of chromatographic zones: Digitoxin (d)	Other glycosides (g)	$\frac{(d+g)}{A} \times 100$ per cent.
A22	1	43·5 44·0 44·5	44·0	30·7	6·0	83·5
				32·5	7·0	89·5
	2	40·5 41·5 40·0 41·0	40·7	32·4	4·0	89·5
				33·1	2·9	89·0
A23	1	30·5 30·5	30·5	20·7	7·7	93·0
				20·0	7·3	89·5
	2	29·5 28·5	29·0	20·2	6·8	93·0
				20·1	6·0	90·0
A24	1	39·0 39·0	39·0	28·5	7·0	91·0
				26·7	7·0	86·5
	2	40·5 40·5	40·5	30·4	6·3	90·6
				32·5	7·3	98·5
A25	1	39·0 39·0	39·0	27·9	6·8	89·0
				30·0	6·2	93·0
	2	36·0 35·5 35·5 36·5	35·9	29·8	5·7	99·0
				28·8	6·4	98·0
A26	1	28·5 29·5 28·0	28·7	17·6	9·2	93·5
				18·0	8·6	92·6
	2	26·5 26·5 25·5 26·5	26·2	16·9	8·2	96·0
				17·1	7·0	92·0
A27	1	39·0 39·0	39·0	24·6	9·5	87·5
				24·5	9·8	88·0
	2	37·5 38·0	37·7	26·3	8·2	91·5
				26·8	8·6	94·0

probably due to losses during the washing of each volume twice with alkali and twice with water.

Assays with sodium picrate were carried out to follow the progress of extraction for, although chemical determinations give for crude materials, results higher than biological assays[10] the optical densities in these assays appear to run parallel with the content of digitoxin. Thus both the digitoxin content (chromatographically) and the content of propylene dichloride-soluble glycosides (assayed with sodium picrate) are greater

when fermented leaf is examined, while in Table VI the leaf samples assaying highest by the sodium picrate method also have the greatest digitoxin content. However in a routine estimation of the digitoxin content of a sample of leaf, the picrate method of assay is unnecessary and may be omitted, the residue from the distillation of the propylene dichloride being therefore dissolved in methanol for application to the paper strips.

From Tables IV and V it is seen that elution from the chromatograms is practically complete, but to ensure full recovery it is necessary to treat a larger area of paper than that representing the limits of the fluorescent zones. Thus in estimating digitoxin a further 1–2 cm. below the lower limit of the spot should be included and at the other end of the zone a cut midway between the apparent edges of the digitoxin and gitoxin zones is suitable. The gitoxigenin present in this region is not determined in the assay.

The yield and potency of the solid isolated from leaf A22 are in agreement with the digitoxin content indicated by the assay. No attempt was made to purify the product further, for in one or more stages of recrystallisation considerable weight losses could be expected, but it is evident that the isolated solid is a potential source of digitoxin of high potency. Leaf samples A22–A27 are believed to represent specially selected strains and the digitoxin contents are considered distinctly higher than those of commercial samples of digitalis although no assays on such samples have so far been carried out.

Summary

1. A new method of estimating the digitoxin content of digitalis leaf has been described.

2. This consists in preliminary autolytic fermentation followed by extraction of the propylene dichloride-soluble glycosides from which digitoxin is separated chromatographically and estimated colorimetrically.

3. During the preliminary fermentation the residual plant enzymes release digitoxin from the initial complexes, but there is no evidence of any further breakdown into digitoxigenin.

4. Xanthydrol reagent is suitable for the colorimetric estimation of the digitalis glycosides eluted from chromatographic zones. The reaction is four and a half times as sensitive as that with sodium picrate.

5. The recoveries from chromatograms average 95·1 per cent. using samples of digitoxin and 91·5 per cent. for the propylene dichloride-soluble glycosides.

6. The method has been applied to the estimation of the digitoxin content of six samples of leaf; crude digitoxin of the order indicated by assay has been isolated from one of these.

The Author thanks Dr. J. M. Rowson for the samples of experimental crops of digitalis, Dr. H. O. J. Collier for the biological assay and Mr. G. L. Matchett for technical assistance in running the chromatograms.

Thanks are also expressed to Mr. C. J. Eastland, F.P.S., F.R.I.C., for his advice and criticism during the progress of the work.

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DISCUSSION

The paper was presented by MR. E. H. B. SELLWOOD.

DR. J. M. ROWSON said that the xanthydrol reaction was a useful tool, but the time of 2 to 4 hours required before peak colour development was a disadvantage. The best known colorimetric agent might be sodium picrate, but it was not the most sensitive. Had the author proved the efficiency of his extraction of the leaf with propylene dichloride and had he any idea of the absolute efficiency of this solvent to extract digitoxin? What else did it extract, e.g., how much gitoxin? Describing some unpublished work, he said he had tested six samples from the same clones as those examined by Mr. Sellwood and found the potency by chemical assay to be between 13 and 17 units/g. That was equivalent to 0·4 to 0·7 per cent. of total glycosides. He had found in those six samples that the content of the B series (gitoxin) comprised from 44 to 75 per cent. of the total. If it were assumed that the A series made up the remainder, then he estimated this to be from 25 to 56 per cent. of the total glycosidal complex. Therefore, the amount of digitoxin present in the leaves would be 0·14–0·25 per cent., which agreed with the author's results in Table V. On behalf of Mr. Rigby he asked what tests the author had applied to assess the purity of the digitoxose used as a standard.

DR. G. E. FOSTER (Dartford) said he was not sure whether the author was trying to estimate the amount of digitoxin in *Digitalis purpurea*, or whether he was suggesting that the method should be used for standardising *Digitalis purpurea*. In Table I he referred to units/g. of leaf and the highest figure was 2·75. Powdered digitalis was standardised to contain 10 units of activity per g.; was there any connection between the digitoxin content and the biological activity of the leaf examined?

DR. S. E. WRIGHT (Sydney) pointed out that in Table IV the author showed that 40 μg. of digitoxin were put on the paper, but on the chromatograms it was observed that the amounts were never more than 10 μg. It would seem that 40 μg. was an excessive loading; what was the separation between gitoxin and digitoxin when the paper was loaded to that extent? Perhaps the author would also comment on the temperature sensitivity of the xanthydrol reaction.

MR. E. H. B. SELLWOOD, in reply, agreed that the time of development of the colour of 2 to 4 hours was quite long, but in practice it was balanced by the fact that there was no critical condition of heating. He agreed that he should have said the sodium picrate method was the more sensitive of the older and better known methods. It was very difficult to decide the efficiency of the extraction with propylene dichloride without using some other extraction procedure. It was gratifying to learn that the percentages of digitoxin found were in fair agreement with those determined by Dr. Rowson. Whereas chemical methods in general tended to overestimate digitoxin content, he had obtained a somewhat low result. The digitoxose used was prepared by the standard method of hydrolysis and had a satisfactory melting point. The object of the work was to develop an assay procedure which would indicate whether a sample of leaf was suitable for extraction of digitoxin on a manufacturing scale. The figures for units/g. quoted were for the propylene dichloride soluble fraction and not for the total activity of the leaf. It was indicated in the paper that the spots were in all cases for 10 μg. digitoxin and the 40 μg. were made up of four separate spots of 10 μg., so there was no overloading. He could give no indication at all of the temperature sensitivity of the xanthydrol reaction: his work had been done as closely as possible to 20° C.

THE REACTIONS OF ANTIBACTERIAL SUBSTANCES WITH BACTERIA

PART I. METHODS USING NITROFURAZONE AND *AEROBACTER AEROGENES*

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THE interaction of antibacterial substances and bacteria may involve changes in the drug concentration and the production of degradation products. The precise measurements of these substances with nitrofurazone and *Aerobacter aerogenes* as models was attempted. They may be expected to add to our knowledge of the mechanism of action of antibacterial substances[1–4].

Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) has been used in the treatment of a variety of localised infections[5] and in veterinary medicine[6]. It has been shown to be metabolised in rats[7], and by tissue slices[8–10], isolated enzymes[11–14] and bacteria[15,16].

EXPERIMENTAL METHODS

Materials

Nitrofurazone. Commercial material was recrystallised from dimethylformamide-ethanol solution to a constant ultra-violet absorption; m.pt. 238° C. with decomposition (Raffauf[17] gave 238° C.). A solution in water gave log ϵ 4·12 at λ max. 260 mμ and log ϵ 4·20 at λ max. 375 mμ. (Raffauf[17] gave log ϵ 4·12 and 4·20 respectively at these wavelengths.)

The culture medium was prepared from Analar reagents and was of the following composition:—KH_2PO_4 0·36, $MgSO_4$ 0·004, $(NH_4)_2SO_4$ 0·10, D-glucose 2·0 per cent. w/v in distilled water. The salts were dissolved in water, the pH was adjusted to 7·0 with sodium hydroxide and the solution sterilised. The glucose was dissolved in water, sterilised and added aseptically immediately before inoculation.

Organism. Aerobacter aerogenes was obtained from Dr. A. M. James; it was originally supplied by the N.C.T.C. (see Lowick and James[18]).

Preparation of the Bacterial Suspensions

A. aerogenes was grown at 40° C. for 16 hours with positive pressure aeration in 250 ml. quantities of medium. The culture was centrifuged at 13,000 r.p.m. for 4 minutes, the cells washed twice and resuspended in the medium either with or without glucose. The final volume of the suspension was adjusted so that, upon twentyfold dilution before exposure to the drug, the optical density at 500 mμ was about 0·65; this dilution corresponded to Brown's opacity tubes No. 3 and approximated to 900 $\times$ 10^6 viable organisms per ml.

When bacteria were to be separated before quantitative examination of the solution, exposure to the drug was in capped nylon centrifuge tubes for small volumes (less than 40 ml.) and in glass stoppered flasks for larger volumes. The test substance dissolved in distilled water was introduced into the exposure container immersed in a water bath at 40·0° C. At timed intervals after addition of the bacteria, aliquot parts of the suspension were withdrawn, centrifuged and the supernatant solution (suitably diluted if necessary) used for ultra-violet absorption and polarographic measurements.

Spectrophotometric Measurements

A Hilger Uvispek spectrophotometer was used. The slit width was opened to double the band width of the incident light and maximum sensitivity was used for measurements involving drug solutions containing suspended bacteria.

Measurements in the presence of bacterial suspensions. 1. In certain experiments, portions of the drug-bacteria suspension were transferred to the cuvettes of the spectrophotometer at intervals; the reference cuvette being filled with a comparable bacterial suspension without drug.

2. In determinations of reaction rates, the drug solution and an equal volume of water, for the reference suspension, were warmed to 40·0° C.; to each was added the same volume of a bacterial suspension and after rapid and thorough mixing the suspensions were transferred to matched cuvettes maintained at 40·0° C. thermostatically. With the constant density of bacterial suspension, matched 1 cm. cuvettes were used for a drug concentration of 10 μg. per ml., 5 mm. cuvettes for 20 μg. per ml. and 1 mm. cuvettes for 50 μg. per ml.

Polarographic Measurements

A Tinsley polarograph in conjunction with twin dropping mercury electrodes and mercury pool anodes was used with the technique previously described[19].

Sufficient Analar potassium chloride was added to give a final concentration of 0·1M in all solutions examined polarographically.

Preliminary Results

Ultra-violet absorption measurements. The ultra-violet absorption curve for nitrofurazone in culture medium diluted twentyfold at pH 7·0 is shown in Figure 1, curve 1; maxima were obtained at 260 mμ and at 375 mμ. Beer's Law was obeyed at 260, 275 and 400 mμ by solutions of the drug containing up to 10 μg. per ml., equivalent to a density of 0·8 at 375 mμ in a 1 cm. cell in distilled water and in diluted culture medium.

Dilute solutions of nitrofurazone slowly decompose. Light absorption measurements show that after 2 days' exposure to light or after 5 days when protected from light there was little decomposition, and that no uptake occurred upon glass surfaces or upon the nylon centrifuge tubes used.

Some of the spectral curves obtained by the measurement of the optical densities of the solutions produced by centrifuging aliquot parts of a bacterial suspension in medium without glucose in contact with 10 μg. per ml. nitrofurazone are recorded in Figure 1. The nitrofurazone peak at 375 mμ disappeared rapidly during the bacteria-drug contact, to be replaced by a peak at 330–335 mμ; the latter peak showed a hypochromic shift on prolonged standing at room temperature. The 260 mμ peak suffered a hypochromic shift and a small bathochromic shift to 270 mμ as the reaction proceeded, but prolonged contact gave a shift back to 260 mμ and a hypochromic effect as the 330 mμ peak became reduced.

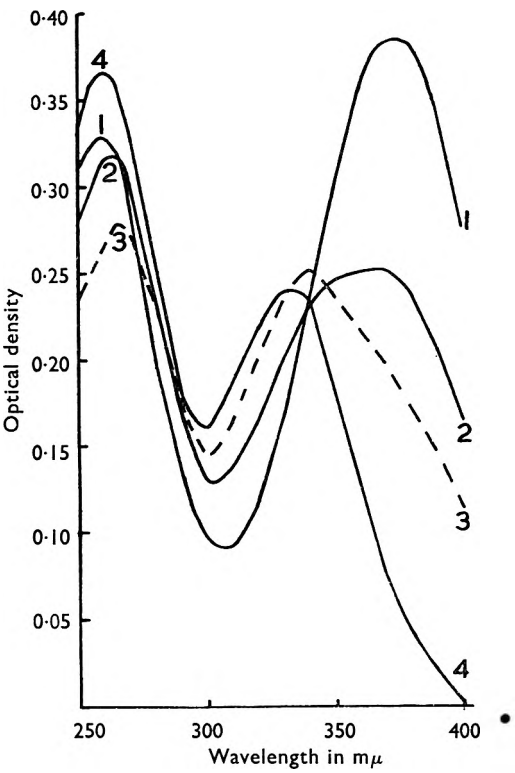


FIG. 1. Ultra-violet absorption curves of supernatant solutions obtained by contact of *A. aerogenes* with 10 μg. per ml. nitrofurazone in presence of culture medium (glucose omitted). The solutions were diluted 1 in 2 and the pH was 7·0 throughout. Curve 1, 5 μg. per ml. nitrofurazone (reference curve). Curves 2, 3 and 4 represent the solutions obtained after ½, 1 and 23½ hours respectively.

These results indicated that nitrofurazone gave a product with maxima at 330–335 mμ and 270 mμ which underwent further change to yield a product with a maximum at 260 mμ. Complete interpretation of these curves was complicated by the presence of unchanged drug as well as metabolic product, or products in some of the solutions and by the possibility of leakage from the cells of materials with absorption in the 260 mμ region[20–24]. Procedures involving separation of bacteria before examination of the solution were inconvenient for the determination of reaction rates, and for the preparation of solutions containing reaction products free from the last trace of nitrofurazone. A combination of ultra-violet and polarographic measurements was therefore adopted.

Polarography. Polarography has been used by Cramer[16] to measure decrease in nitrofurazone concentration upon metabolism. Sasaki[25] reported two reduction steps in aqueous solutions of nitrofurazone the first, reduction of the nitro group and the second, reduction of the

—CH=N⁻ bond in the side chain. Both half wave potentials varied with the pH of the solution, the former being within the range of an aromatic nitro group. Sasaki concluded that a 4 electron irreversible reduction to the hydroxylamino compound was effected. Polarography seemed to offer a suitable method to measure the metabolism if the nitro group only were involved, and also to check whether the semicarbazone chain had been affected.

Ultra-violet absorption measurements in the presence of bacteria. The problems involved in these measurements are discussed in the appendix.

Results

Polarography

Nitrofurazone in diluted medium without glucose at pH 7·0 showed two reduction steps (see Fig. 2). ($E_{\frac{1}{2}}$ — 0·35V. and — 1·35V. against mercury pool anode.) Plots of nitrofurazone concentration between 1–20 μg. against diffusion current gave a straight line calibration employing the first reduction step.

The buffering power of the medium in concentrations equivalent to those used in metabolism experiments was adequate in the absence of glucose.

The polarographic reduction of nitrofurazone was unaffected by the presence of eluate from *A. aerogenes*, or the metabolic product exhibiting an ultra-violet absorption peak at about 330 mμ.

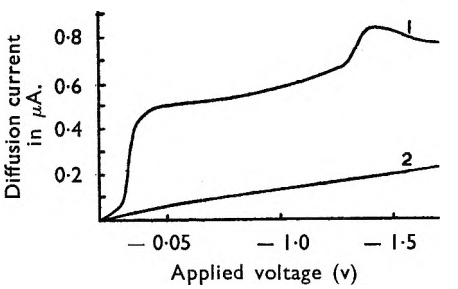


FIG. 2. 1. Polarogram of 10 μg. per ml. nitrofurazone in diluted culture medium (1 in 20) at pH 7·0 in presence of 0·1 M potassium chloride. 2. Residual current.

Using the twin capillaries with their separate calibration curves, and taking the precautions described in a previous paper[19], nitrofurazone could be determined at concentrations of 2–10 μg. per ml. in solutions equivalent to those obtained under biological conditions, with an accuracy of $\pm$ 3 per cent.

Combined Ultra-violet and Polarographic Determinations

The spectrum of the centrifuged solution measured after contact of *A. aerogenes* with 10 μg. per ml. nitrofurazone in presence of glucose-free medium is shown in Figure 3, curve 2. Simultaneously, the nitrofurazone content of a portion of the solution was determined polarographically. A solution of nitrofurazone, of concentration equal to that determined polarographically, was placed in the reference cuvette, and the ultra-violet absorption spectrum of the contact supernatant solution determined (Figure 3, curve 4). The curve of this nitrofurazone concentration is also shown (curve 3). The relatively low optical density at 375 mμ shown in curve 4, and the shape of this curve proves that curve 2, between 310–

400 mμ, represents the summation of the light absorbing properties of the unchanged nitrofurazone and its metabolic product exhibiting a peak (330–335 mμ) and reveals the light density additivities of these substances. The peak in the 260 mμ region in curve 2, probably represents the summation of light absorption of unchanged nitrofurazone, eluate from the cells, metabolic product exhibiting a peak at 330–335 mμ and the product (or products) of decomposition of the metabolite.

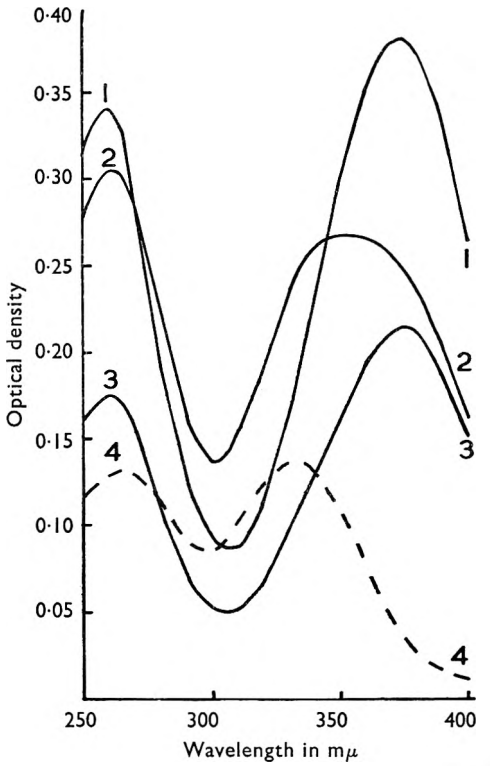


FIG. 3. Ultra-violet absorption curves from combined light absorption and polarographic measurements.

1. 5μg. per ml. nitrofurazone (reference curve).
2. Composite curve obtained after contact of *A. aerogenes* with 10 μg. per ml. nitrofurazone in presence of culture medium (glucose omitted) at pH 7·0 for 2 hours. The supernatant solution was diluted 1 in 2.
3. 2·7 μg. per ml. nitrofurazone, the concentration of drug found polarographically in the diluted (1 in 2) supernatant solution.
4. Absorption curve of the metabolic product obtained by placing 2·7 μg. per ml. nitrofurazone in the reference cuvette and measuring the absorption of the 2 hour supernatant solution described in 2.

The characteristics of curve 4 between 360–400 mμ, especially in the region of 375 mμ illustrate that the concentration of unchanged nitrofurazone has been correctly determined, and that the experimental techniques adopted are valid. Curve 4, between 300–400 mμ, exhibiting an absorption maximum at 333 mμ thus represents the true light absorption in aqueous solution at pH 7·0 of the metabolic product obtained from the interaction of nitrofurazone with *A. aerogenes*.

Ultra-violet Absorption Measurement in the Nitrofurazone—A. aerogenes *System*

When the drug solution was mixed with the bacterial suspension in glucose-free medium and optical densities immediately measured at 375 and 500 mμ against an identical bacterial suspension (see experimental), the readings at 375 mμ were those expected for the concentration of drug present, and those at 500 mμ were zero. The supernatant solution obtained upon centrifuging the drug containing suspension gave identical readings at 375 mμ (water as reference). Consequently, negligible

quantities of drug are absorbed by the bacteria, and the light scattering properties of the latter remain unaltered after a brief duration of contact. Readings at 500 mμ remained virtually zero during 6 hours indicating no change in size, of character of the surface, or in the number of organisms during this period. Suspensions containing drug concentrations of 10, 20 and 50 μg. per ml. behaved similarly. A number of the factors, enumerted in the Appendix, which complicate measurements are therefore absent in the present system.

In the experiments in which measurements of the rate of metabolism of nitrofurazone in presence of bacterial suspensions were involved, measurements at wavelengths of 375, 400 and 500 mμ were made for the following reasons. The wavelength of 375 mμ is the position of the light absorption maximum of nitrofurazone. At pH 7, the metabolic product has negligible absorption in this region (see Fig. 4, curve 2). Reduction of density at this wavelength is a measure of the loss of drug from the solution provided that the density reading at 500 mμ remains zero. Development of acidity in the solution causes a bathochromic shift in the metabolic product which then has significant absorption in this region (Fig. 4, curve 1) and so invalidates the use of reduction in density as a measure of the loss of the drug. The wavelength of 400 mμ, although corresponding to a rather steep portion of the light absorption curve of the drug, proved to be useful for measurement because the metabolic product has negligible absorption even in acid solutions. The wavelength of 500 mμ was chosen as the point at which to check the scattering power of the bacteria—neither drug nor metabolic product absorbs in this region. In all experiments both with and without glucose, readings at 500 mμ remained virtually zero throughout, indicating that neither change in the characteristics of the bacteria nor any possible changes in their number had interfered with the observed optical density readings at 375 and 400 mμ.

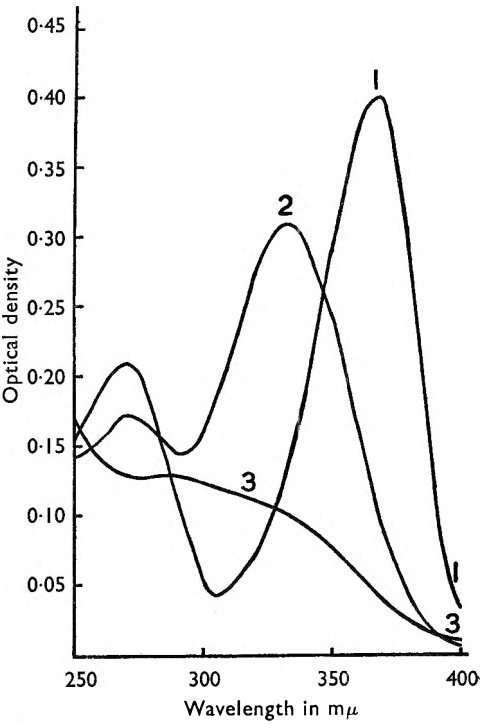


FIG. 4. Effect of pH change on the absorption curve of the metabolic product. 1. pH 3·0. 2. pH 7·0. 3. pH 10·8.

The validity of the method was checked occasionally by taking optical density measurements before and after centrifuging the above suspension, the readings from the supernatant solutions at 375 and 400 mμ (water as reference) were identical with those of the suspensions.

Ultra-violet absorption measurements in the presence of bacteria made possible the determination of the drug content in small samples (1 to 3 ml.) within 1 minute of withdrawal from a bulk suspension. The correct timing of centrifugation of the bulk to yield solutions at the desired stage of metabolism could be readily assessed. The studies of the rates of reaction of the drug and the bacteria under various conditions were facilitated by the use of thermostatically jacketed cuvettes. (Since these measurements can thus be accurately made within 45 seconds of mixing the drug with the suspension, and readings can be taken at 5 second intervals thereafter, a detailed study of the rapid reactions between drugs and bacteria becomes possible. Such studies will be reported in subsequent papers.)

Reactions of A. aerogenes *with Nitrofurazone*

Exposure of viable bacteria to the drug in concentrations of 10 μg. per ml. in glucose-free medium gave the results already described (see Figs. 1 and 3). The rate of metabolism of the drug varied greatly between experiments but in general it was relatively slow. In some, the nitrofurazone was incompletely metabolised even after a 20 hour contact period. With 50 μg. per ml., no trace of the metabolic product could be detected during 20 hour contact periods. A progressive hypochromic shift with increasing time between 300 and 400 mμ indicated that a slow process of adsorption (or absorption) was occurring. The uptake of drug was negligible in the first 10 minutes of contact (in contrast with the rate of adsorption of many drugs).

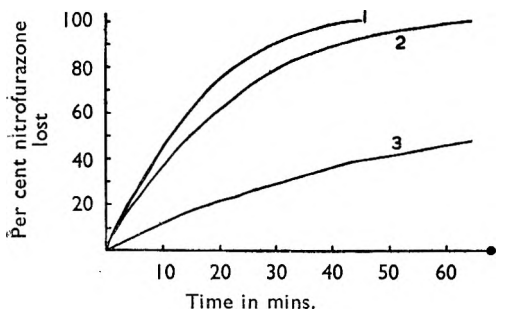


FIG. 5. Rate of loss of nitrofurazone in presence of *A. aerogenes* in culture medium at three drug concentrations:—1. 10 μg. per ml. 2. 20 μg. per ml. 3. 50μg. per ml.

In the presence of culture medium containing glucose, however, the rates of metabolism of nitrofurazone were rapid and much more reproducible, and higher concentrations could be metabolised. Figure 5 shows the rates of metabolism of solutions of nitrofurazone containing 10, 20 and 50 μg. per ml. The times required for the complete metabolism of 10 μg. under these conditions only varied between 50 and 90 minutes using suspensions prepared on different days from different cultures. The increased rate in the presence of glucose is in agreement with the work reported by Asnis and others[15] who showed that the rate of nitrofurazone

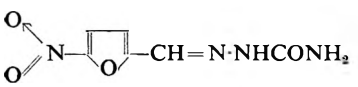
reduction by susceptible strains of *Escherichia coli* and *Staphylococcus aureus* was increased in presence of oxidisable substances.

Cells, after steaming for 20 minutes, produced little effect upon solutions containing 10 and 50 μg. of the drug during exposure periods of 2 hours. At the end of this period, the light absorption curves were identical in shape with those of nitrofurazone and the densities indicated that less than 3 per cent. of the drug had been adsorbed (or absorbed) by the bacteria.

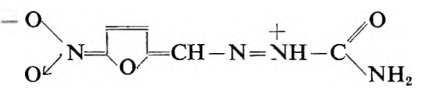
The above results are of a preliminary character to indicate the use of the physical techniques described in this paper. Further work is in progress on the effect of various conditions upon the uptake and metabolism of nitrofurazone. The question of the structure and reactions of the metabolic product, however, have been the subject of more detailed work described below.

The Structure and Reaction of the Metabolic Product

The absorption peak (λ max. 375 mμ log ϵ 4·20) of nitrofurazone (I) can be explained in terms of structure (II), in which there is the equivalent of 5 conjugated double bonds, being the chief contributing structure to the resonance hybrid (see Raffauf[17]).



(I)



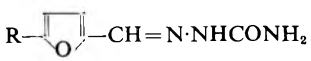
(II)

The ultra-violet absorption spectrum of the metabolic product in a solution in which the metabolism had just been completed (10 μg. per ml.; contact in absence of glucose) is shown in Figure 4, curve 2. It is reasonable to assume from the results of storage experiments of this metabolic product (Fig. 6), that a negligible quantity had decomposed during the time involved in its production. If its molecular weight is presumed to be approximately that of nitrofurazone, then log ϵ will be of the order of 4·1 at λ max. 333 mμ.

The intensity of the absorption, and the position of this absorption peak of the metabolic product, indicate that the furan ring and the semicarbazone side chain are intact in this compound, and that only the nitro group is implicated in the metabolism of nitrofurazone. This conclusion is supported by the results of the polarographic measurements which demonstrate that the typical reduction step of the nitro group disappears quantitatively in proportion to the disappearance of the 375 mμ absorption peak upon nitrofurazone metabolism, but that the reduction step attributable to reduction of the —CH=N— bond of the side chain is unaffected.

Biological reactions involving the nitro group could possibly lead to the removal of the group, the partial reduction and linking of two of the furan molecules *via* an azo group, or reduction to give a nitroso, hydroxylamino or amino group. That metabolism of nitrofurazone does not involve removal of the nitro group is demonstrated by the occurrence of the peak of furaldehyde semicarbazone at 293 mμ. Formulation of the

metabolic product as involving the azo-link is contra-indicated since such a compound would be expected to give an absorption maximum at wavelengths longer than the nitrofurazone peak and would be reducible polarographically. The formulation as a nitroso group is unacceptable on the polarographic evidence, since nitroso groups attached directly to aromatic systems have been shown to be reduced at lower potentials than corresponding nitro groups[26]. The formulation of the metabolic product as the hydroxylamino compound (III; R = $-NHOH$) or as the amino compound (III; R = $-NH_2$) is consistent with the ultra-violet and the polarographic data.



(III)

The metabolic product underwent a bathochromic and hyperchromic shift during some metabolic studies in the presence of glucose due to the medium becoming increasingly acid. The effect of changes of pH on the light absorption of solutions containing the metabolic product is shown in Figure 4. The spectral shifts affected by changes of pH between 8·0 and 3·2 were reversible, but the rate of reversibility was too rapid to measure.

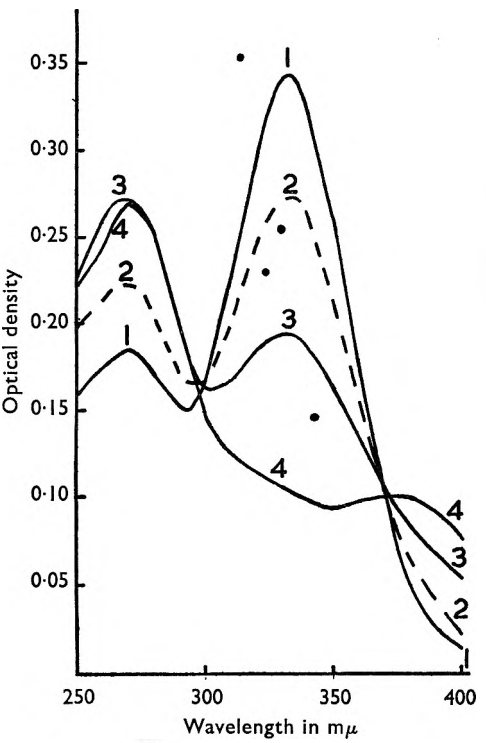
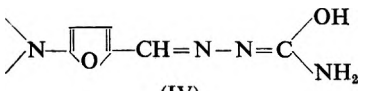


FIG. 6. Effect of storage at room temperature on the ultra-violet absorption curve of the supernatant solution obtained after contact of nitrofurazone with *A. aerogenes* in the presence of culture medium (glucose omitted) at pH 7·0. 1. Immediate readings. 2. 24 hours' storage. 3. 48 hours' storage. 4. 72 hours' storage.

The elimination of the absorption peak at more alkaline pH values (see Fig. 4, curve 3) was irreversible and required about 60 minutes at pH 10·8 at room temperature to complete the change shown. Curves obtained by plotting optical density at 335 and 370 mμ against changes of pH in the range where the effects were reversible, gave the value of approximately pH 4·5 (apparent dissociation constant pKa 4·5) as the mid-point of the change between the two molecular species responsible for the 333 and 370 mμ peaks.

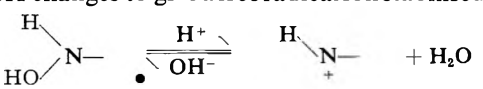
It seems reasonable to assume that the peak of 333 mμ may be attributed to the contribution of the molecular species IV (equivalent to 4 conjugated double bonds) to the resonance hybrid.



(IV)

The bathochromic shift to 370 mμ (presuming ionisation is involved because of the rapid reversibility) seems to indicate the improbability of the metabolic product having the amino structure (III; R = $-NH_2$), since the conversion of unionised amino group conjugated with an aromatic structure to the ionised form upon pH changes is known to give hypsochromic shifts. It is recognised, however, that the semicarbazone side chain attached to the furan ring might possess proton accepting properties although an increase in conjugation upon such a change seemed highly improbable. The absorption peaks of nitrofurazone and of furaldehyde semicarbazone in aqueous solution did not change in wavelength over the pH ranges of 2 to 10 and 2 to 6 respectively. The *p*-nitrobenzaldehyde semicarbazone peak (λ max. 325 mμ in water) was unaffected by pH changes in the range of 1·25 to 5·5 whereas the peak (λ max. 330 mμ in water) of *p*-dimethylaminobenzaldehyde semicarbazone underwent the expected hypsochromic shift of an aromatic amino compound to give λ max. 280 mμ at pH 2·1. If the amino structure (III; R = $-NH_2$) is accepted as the formula for the metabolic product, it must be presumed therefore that the furan nucleus differs from the benzene nucleus in the properties it confers to compounds of comparable structure.

It is equally difficult to explain the bathochromic shift exhibited by the metabolic product as the pH changes from neutral to acidic if the hydroxylamino compound (III; R = —NHOH) is accepted as the structure. The possibility was considered of the following reaction proceeding rapidly and reversibly with pH changes to give a free radical ion stabilised by conjugation



with the furan ring and side chain with consequent shift to longer wavelength at acid pH values. The peak (λ max. 230 mμ) of freshly prepared phenylhydroxylamine in water underwent a hypsochromic shift in solution at pH 3 so that its measurement at this pH using the present instrument was precluded. The proton acceptance by the N atom of this compound in acidic solution had reduced the conjugation as expected. Consequently the above formulated change for the hydroxylamine group in a furan compound seems improbable.

All attempts to prepare reference compounds by the chemical reduction of nitrofurazone have so far proved unsuccessful. Attempts to follow the ultra-violet absorption during contact of this compound with reducing agents under aqueous conditions have also failed to yield information of value in the solution of the problem. Until further evidence is available, the structure of the metabolic product remains in doubt, although certain reports favour the formulation of the product of metabolism of nitrofurazone by the xanthine oxidase-hypoxanthine system[11], or by certain bacteria, as the hydroxylamino compound (III; R = —NHOH)[14,15]

Stability of the Metabolic Product

The metabolic product in solution at pH 7 at room temperature gradually decomposed—reduction of the 333 mμ peak occurred with the simultaneous increase in the peak in the 260 mμ region (see Fig. 6). The rate of decomposition increased with rise of temperature. Acid solutions lead to a more rapid decomposition, while in alkaline solutions greater than pH 10, the decomposition occurred almost immediately.

The metabolic product is devoid of antibacterial activity (see also Asnis and others[15] and Cramer).

Appendix

Light Absorption Measurements in Presence of Bacterial Suspensions

Some of the problems involved in attempting to determine the light absorbing characteristics of organic molecules in solutions in which bacteria were suspended are as follows.

1. *The scattering spectrum of bacteria.* The scattering of the incident light by the bacteria will obviously contribute to the optical density. A typical curve obtained by plotting optical density against wavelength between 300 and 1000 mμ of a dilute washed suspension of *A. aerogenes* in distilled water is shown in Figure 7. The optical density of the bacterial suspension will be dependent partly upon the position of the cuvette in relation to the photometer, and partly on the aperture of the latter, since some of the forward scattered light will be included in the transmitted beam. At wavelengths from 220 to 300 mμ it has been shown[27] that the constituents of bacteria e.g., nucleic acids absorb incident light and that the observed densities are the sum of scattering by the cells and absorption by the cell constituents. A typical curve obtained for *A. aerogenes* is shown in Figure 8. The effect of variation of the age of the culture upon the characteristics of the scatter curve was not investigated because all experiments reported in this paper were carried out using a 16-hour culture.

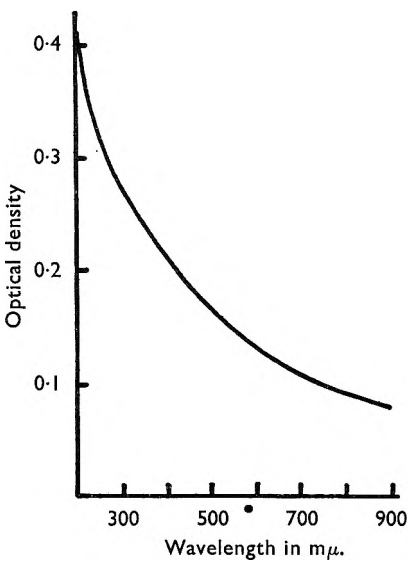


Fig. 7. Scattering spectrum of a 16 hour culture of *A. aerogenes* in distilled water.

The adsorption of, or chemical attack by, a drug upon a bacterial surface may alter the size of the organism or the characteristics of its surface considerably; such changes will be attended by significant

alteration of the scattering spectrum. We have found, for instance, that the adsorption of small amounts of certain phenols doubles the light scattering properties of the suspension immediately. On the other hand, adsorption of relatively large quantities of acridine-type compounds gives only a slight increase in the scattering power[28].

When compounds with negligible light absorption properties at wavelengths longer than 400 mμ are to be determined in bacterial suspensions, check measurements between 500 mμ and 1000 mμ will indicate (*a*) any change in the number of bacteria present in either the test or the reference suspension caused by the presence of either the complete culture medium or the drug or both, (*b*) any change in the light scattering properties caused by the drug. (If the light scattering properties are altered it is possible to extrapolate back from the scattering curves between 1000 mμ and 500 mμ to obtain a measure of scattering density in the region of light absorption by the drug).

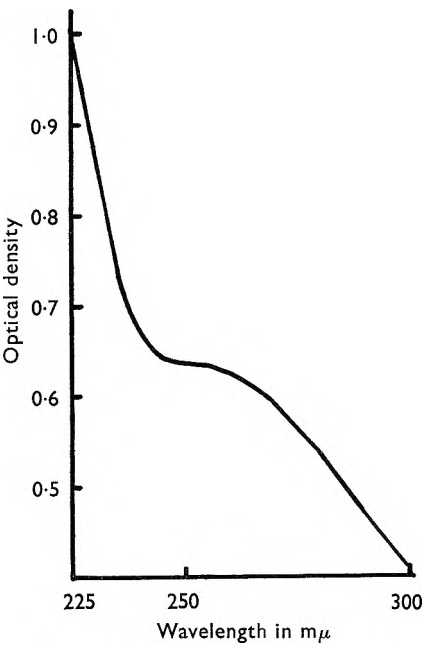


FIG. 8. Scattering and absorption spectrum of a 16 hour culture of *A. aerogenes* in distilled water.

Care must also be taken to ensure that sedimentation of the organisms does not occur during the course of an experiment.

2. *Adsorption of the drug.* If the drug is adsorbed upon the bacterial surface in significant amounts, shifts in the wavelengths of the absorption maxima or changes in the intensity of light absorption may occur. We have found, for instance, that there is a considerable reduction in the light absorbing properties of acridine molecules when they are "bound" upon bacterial surfaces.

3. *Measurements between 200 and 300 mμ.* The scattering of light by suspensions increases rapidly with decreasing wavelength, especially below 350 mμ. Consequently, only relatively dilute bacterial suspensions can be employed if measurements between 220 to 300 mμ are involved (see later, however). Secondly, if the uptake or metabolism of a drug is dependent upon the viability of the bacteria, it may be unwise to carry out repeated readings, since bacteria are known to be particularly sensitive to ultra-violet radiation at 260 to 270 mμ. Thirdly, it is known that many

agents in contact with bacteria cause a "leak" from the cells of substances exhibiting absorption maxima *ca.* 260 mμ[20-24].

4. *pH changes during experiments.* The absorption spectra of many organic molecules are affected by pH changes. Using live bacteria, pH changes may occur during an experiment with consequent complications in the composite light absorption pattern. Although buffered solutions are usually employed, metabolism in presence of growth media may yield acids in sufficient quantity to produce pH changes, e.g., in some experiments involving nitrofurazone and *A. aerogenes* in the presence of the growth media, the absorption peak of *ca.* 330 mμ underwent a gradual bathochromic shift with increasing time of drug-bacteria contact due to pH changes.

5. *Scattering density in relation to solution absorption density.* It has already been stated that only dilute bacterial suspensions may be used for readings at wavelengths lower than 350 mμ. Even under these conditions, the scattering spectrum may constitute such a high proportion of the observed light absorption under normal working conditions of the instrument, that accurate measurements of drug or metabolite concentrations become difficult. The use of an identical suspension of bacteria (omitting the drug) in the reference cuvette allows the use of higher drug concentrations and more accurate determination of their values. It was found, for instance that, in the 350 mμ region, densities equivalent to 1·5 against water could be used in the reference cell in this relative method when the slit width was opened to double the bandwidth of the incident light and maximum instrument sensitivity was employed.

It was found, under these conditions, using a heat killed suspension of *A. aerogenes* exhibiting a light density reading of 1·2 at 350 mμ (against water) in the reference cell, that Beer's Law was obeyed by nitrofurazone in concentrations equivalent to optical density readings of up to 0·8.

6. *Choice of wavelength for measurement of optical density in rate experiments.* Ideal wavelengths at which to take measurements would be (*a*) a few positions in the region in which incident light is scattered by the bacteria but not absorbed by the drug or metabolic product(s); (*b*) positions of light absorption maxima of the drug and of its metabolic product (or products); (*c*) the 260 mμ region in which light absorption by certain cell constituents released by the bacteria is exhibited. In practice, a compromise is usually necessary especially since there may be much overlapping of the spectra of the drug and its metabolic products. In rate experiments, the choice of wavelengths may also be influenced by the instrument working conditions, e.g., lamp and photocell changes must be avoided if the light absorbing systems are changing rapidly.

Summary

1. The application of spectrophotometric and polarographic methods of determination to the investigation of the reactions of nitrofurazone with *A. aerogenes* is described.

2. A method for the determination of organic molecules in bacterial suspensions is outlined, and some of the problems associated with such determinations are discussed.

3. The production of a nitrofurazone metabolic product by *A. aerogenes* is described and its structure investigated.

The authors wish to thank Messrs. Menley & James Ltd., for gifts of nitrofurazone 'Furacin' and other chemicals used in this investigation.

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DISCUSSION

The paper was presented by MISS A. E. ROBINSON.

MR. S. G. E. STEVENS (London) said that the authors had chosen a difficult chemical system, and it was not surprising that they had experienced trouble in isolating and identifying some of the reduction compounds. They quoted a shift of the peak from 375 to 335 mμ, and it might be of interest to record that by using dithionite or hydrazine the peak could be shifted to 305 mμ. There was no doubt that nitrofurazone was photosensitive and if a weak alcoholic solution of the compound was allowed to stand in the light, the 375 mμ peak was completely eliminated leaving the pattern shown in Figure 6. He found difficulty in accepting the statement that the absorption peaks of nitrofurazone and of furaldehyde semicarbazone in aqueous solution did not change over the pH ranges of 2 to 10 and 2 to 6 respectively, and suggested that there might have been a transposition of the values. Nitrofurazone developed an intense red colour in alkaline solution, and he felt that this was accompanied by a shift of the peak from 375 mμ. He wished to amplify the statement that nitrofurazone was stable in solution as there could be some misunderstanding. It was true that under the conditions of the authors'

test the solution could be considered stable, but one application of nitrofurazone in solution, namely, the treatment of coccidiosis in poultry where the method of administration was in the drinking water could, however, produce a different picture. Aqueous solutions were stable over the period during which the birds received their drink, provided that the drinking troughs were of good quality galvanised ware. If, however, old chipped and rusty tanks were used these would act as an electrolytic cell and there would be a very rapid breakdown of the nitrofurazone.

DR. J. C. PARKINSON (Brighton) said that on page 1073 the authors referred to comparable bacterial suspension made at time zero; but after reaction had taken place the comparable suspension would surely have grown, and if in the test suspension there were bacteriostasis, it would not be the same. The authors stated that the effect of variation of the age of culture upon the characteristics of the scatter curve was not investigated. Surely after a short lapse of time there would be a change, and then the test cuvette would not be the same as the reference cuvette? Would not that affect the measurements? It was stated that the metabolic product was devoid of antibacterial activity. It was rather a hypothetical substance and it would be interesting to know whether the authors meant that it was devoid of activity against *A. aerogenes* or whether it was tested against a large number of bacteria.

MR. K. A. LEES (London) asked the authors what motivated their choice of the culture medium used for growing the inoculum, and also the conditions, i.e., the high concentration of glucose used. It would also be of interest to have more details of the technique used for washing bacteria, and whether medium, saline or sterile water was used. The washing of bacteria could in general be regarded as a technique which left the bacteria in a state of shock, and that was possibly connected with the statement in the paper that the uptake of drug was negligible in the first 10 minutes of contact. How long were the washed bacteria allowed to remain before the addition of nitrofurazone, and was there any period of adaptation? Was the nitrofurazone reduced by non-sensitive bacteria?

MR. A. R. ROGERS (Brighton) referred to the structure of the metabolite and asked whether the authors considered that it might be a hydrazo compound. Chemical reduction methods had been tried, but the metabolite might be amenable to electrolytic reduction. It might be possible to isolate the metabolic product either by the cation exchange method, or by paper chromatography.

DR. K. R. CAPPER (London) said it would be interesting to know what was really happening to the organisms during the experiment. It was stated on page 1077 that the readings indicated no change in size, character or number of organisms; but did they remain viable, and was there any change which was not being detected? It would seem that with 50 μg./ml. no trace of metabolite could be detected, but one was dealing not with the antibacterial activity of nitrofurazone, but with the mechanisms of resistance. It would appear that there were susceptible strains and resistant strains. Did resistant strains show greater activity in metabolising

nitrofurazone than susceptible strains? Had the authors obtained exactly the same result when glucose was present and did their readings indicate that there was any change in the number of organisms during the six hour period?

Miss A. E. Robinson, in reply, said that it is known that chemical and bacterial reductions may proceed by different mechanisms, and care must be taken in the choice of an analogous chemical reduction, both routes being explicable in terms of accepted chemical theories. With regard to the effect of pH 10 on nitrofurazone solution, they used a phosphate buffer and no distinct red colour was observed. There was a slight colour change but no difference in the location of the peak of nitrofurazone. The readings were taken immediately, the solution not being allowed to stand. Spross, carrying out research on the breakdown of nitrofurazone in aqueous solution, found that it was affected by the presence of metals. With regard to the use of comparable bacterial suspension at time zero, the suspension would be similar in both cuvettes. The effect of incubation would possibly lead to growth, although the concentration of the culture medium was fairly weak; however, the fact that no difference in the ultra-violet absorption readings over a longer wavelength was observed seemed to indicate that there could have been no difference between the two systems after the various contact times. No growth could have occurred, nor could there have been any leakage of cell constituents. Tests had not been made on a range of bacteria, but earlier work had indicated that once the drug was reduced no further antibacterial activity was observed. The culture medium chosen was a simple one and was that used by Hinshelwood in his work. The concentration of glucose was considerably higher than one would perhaps expect, and there was a possibility of the organisms capsulating. The technique for washing bacteria was described in the paper. It was a centrifuging technique and the washing fluid was either the complete medium or the medium without glucose. No lapse of time occurred before the addition of the drug. With regard to the reduction by non-sensitive bacteria, reference should be made to the work of Asnis and others who used sensitive and resistant strains of bacteria and showed that a different rate of reduction occurred between organisms which were resistant and those which were susceptible. The possibility of the formation of hydrazo compound had not been considered. On looking at the possibility it was rather difficult to explain the effect of pH on the compound. If a proton were added to one of the two –NH– groups and two furan molecules were linked, one would expect the formation of two peaks beyond 300 mμ in the ultra-violet absorption spectra. In fact only one peak had been observed, and it seemed to contraindicate the possibility of that compound. Electrolytic reduction had been considered but had so far met with no success. With reference to Sasaki's work, the system was in fact irreversible, which prevented calculation of the electron change by normal means. They had obtained similar results to those of Sasaki. Isolation of the metabolite did not seem likely because of its instability, as was indicated in Figure 6. The rate of decomposition was

too rapid to permit the use of ion exchange and chromatographic methods at present. As to whether the bacteria were still viable, one might refer to Cramer's work, in which once the drug was reduced, growth did ensue in the normal way. The question on results obtained in the presence and absence of glucose could best be answered by the fact that in the presence of glucose the reaction was faster than if it were absent, and probably a reduction of the drug occurred before any growth was effected.

DR. A. H. BECKETT, in reply, emphasised that the authors were always checking the scattering spectrum at higher wavelengths between 500–1000 mμ. It had been shown that if constant checks were made throughout this range, the absence of development and of growth could be guaranteed. In every experiment quoted, such checks were made.

ANTITUBERCULOSIS ACTIVITY IN THE PHENAZINE SERIES. ISOMERIC PIGMENTS OBTAINED BY OXIDATION OF *O*-PHENYLENEDIAMINE DERIVATIVES

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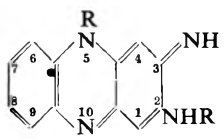
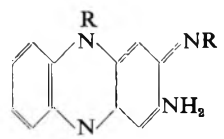
Introduction

The products obtained on oxidation of derivatives of *o*-phenylenediamine with ferric chloride or *p*-benzoquinone have been under investigation in these laboratories for some years. The main products formed are derivatives of anilino*apo*safranine (2-anilino-3 : 5-dihydro-3-imino-5-phenylphenazine), I (R = Ph), and its isomer, 2-amino-3 : 5-dihydro-3-phenylimino-5-phenylphenazine, II (R = Ph).



I



II

Anilino*apo*safranine is strongly inhibitory of the growth of *Mycobacterium tuberculosis* in serum enriched media and it has been shown to exert a suppressive effect in experimental tuberculosis in mice and guinea-pigs[1–4]. It is also claimed to show a curative effect in lepromatous leprosy[5]. The present paper describes the evaluation in mouse and guinea-pig tuberculosis of a number of new derivatives of structure II and a comparison of their activity with that of the corresponding isomers of structure I where these were available. The chemistry of these compounds has been described recently[6–8].

Experimental Methods

The experimental methods have been described previously[9]. In brief, groups of 10 albino mice (Schofield or Parkes) of average weight 25–30 g. were infected intravenously with 0·1 mg. (moist weight) of the bovine Ravenel Rv strain of *Myco. tuberculosis* grown for 9 days on a Dubos type liquid medium.

The drugs were administered in a ground biscuit diet for 14 days, beginning on the day of infection. The measured daily intake of drug, used in the Tables, is the mean of the daily intakes during the five days, second to the sixth inclusive.

Every mouse which died was examined to ascertain the cause of death. The criterion of antituberculosis activity was a significant increase (19/20 probability, or better) in the median survival time (MST) of the treated mice over the controls. The MST of untreated control mice varied in different experiments from 13 to 23 days.

For the investigations in guinea-pigs, animals of average weight 625 g. were used. Each animal was infected subcutaneously in the left flank with 0·01 mg. of the Ravenel Rv strain grown as above.

Drugs were incorporated in the ordinary diet of crushed oats. Liberal quantities of greenstuffs, mainly cabbage, were also provided. From the daily intake of the drug-oats mixture the mean daily intake of drug over the period of treatment was calculated.

In determining the Disease Index[9] extensive disease in the lungs, liver, spleen, site of inoculation and adjacent lymph nodes combined, is awarded 35, 30, 25 and 10 points, respectively; a total maximum of 100 points. Moderate disease is awarded, 25, 20, 15 and 10 points; slight disease 15, 10, 5 and 5 points.

Results and Discussion

The investigation has shown (Table I) that the isomer (II, R = Ph) of anilino*apo*safranine and certain derivatives of both isomers are more active in suppressing experimental tuberculosis in mice than anilino*apo*-safranine (283). Of the 11 derivatives of structure I, Nos. 283, 628, 616, 623, 658, 637 and 662 show significant activity and of these only No. 628, I, (R = $C_6H_4Cl(p)$) had outstanding activity. High activity in this group is associated with the presence of a methyl or higher alkoxy group in the *p*-position of the phenyl substituents on the 2:5-nitrogens. A similar pattern of behaviour is evident in the compounds of structure II. Here again the outstanding compound is the chloro-derivative, 629, (R = $C_6H_4Cl(p)$) which is isomeric with compound 628, although the *p*-ethoxyphenyl derivative 630, is little behind it in activity. Included in this group of compounds are a series of alkyl and *cyclo*alkyl derivatives, and one aralkyl derivative of type II, the corresponding I isomers of which could not be prepared. On the whole they showed low activity with the exception of the *cyclo*hexyl derivative, 430.

Apart from the type I compound, 628, which is the most active of all the compounds examined, it appears to be true that on the whole the type II compounds show higher activity than the group I isomers. This is clear from the comparison of the following 7 pairs:—283, 595; 616, 619; 422, 607; 658, 659; 637, 636; 656, 657; 662, 661. The last pair are exceptional in that both isomers show identical activity.

We have subsequently confirmed the high antituberculosis potency of compound 630 in a mouse infection which had been allowed to progress for 5 days before treatment was initiated. The increase in MST in this case was 56 days. (Dosage = 130 mg./kg. daily for 14 days.)

The chlorinated derivatives are of especial interest. No. 639, the 8-chloro-derivative of No. 283, showed a reduced MST. Despite this the disease in the lungs of the mice was small and their early death suggests a toxic effect. No. 640 the only other compound with a chlorine substituent in the phenazine structure proper, showed no activity. Apparently the substitution of this chlorine atom has destroyed the activity of the parent 595. The only pair of I, II, type isomers available for comparison in this chlorinated group was 628 and 629. In both of these compounds

TABLE I

ACTIVITY IN MOUSE TUBERCULOSIS

No.	Compound	Measured daily intake of drug (mg./kg.)	Change in MST (days)
293	II, R = methyl	161	−2
510	II, R = allyl	191	+0·5
550	II, R = *n*-propyl	111	+2·5
617	II, R = *iso*propyl	252	0
449	II, R = *n*-heptyl	110	+1·5
415	II, R = benzyl	119	+3·5 S*
653	II, R = *cyclo*pentyl	155	+2·5
430	II, R = *cyclo*hexyl	268 193 140 62	+27 S +24 S +19 S −1·5
283	I, R = phenyl	70 41	−7·5 S +1·5
595	II, R = phenyl	350 125 66 41	+14 S −12·5 S +10·5 S 0
628	I, R = *p*-chlorophenyl	120 92·5	−117 S +159 S
629	II, R = *p*-chlorophenyl	145 127	+108 S +57 S
622	I, R = *o*-tolyl	158 58	−1·5 +4 S
616	I, R = *p*-tolyl	144	+11 S
619	II, R = *p*-tolyl	213	+26 S
623	I, R = *o*-methoxyphenyl	172 90	+9 S +8·5 S
627	II, R = *o*-methoxyphenyl	156 82	0 +6 S
422	I, R = *p*-methoxyphenyl	200	+2·5
607	II, R = *p*-methoxyphenyl	295 200 111 71	+28 S +30 S +31 S +14 S
630	II, R = *p*-ethoxyphenyl	117 65	+91 S +30 S
658	I, R = *p*-*n*-propoxyphenyl	70	+18 S
659	II, R = *p*-*n*-propoxyphenyl	131	+28 S
637	I, R = *p*-*iso*propoxyphenyl	168	+12·5 S
636	II, R = *p*-*iso*propoxyphenyl	162	+44 S
656	I, R = *p*-*iso*butoxyphenyl	75	+3
657	II, R = *p*-*iso*butoxyphenyl	131	+20 S
662	I, R = *p*-*sec*.butoxyphenyl	115	+30 S
661	II, R = *p*-*sec*.butoxyphenyl	115	+30 S
640	II, R = phenyl (Also Cl in the 7-position)	135	−1
639	I, R = phenyl (Also Cl in the 8-position)	120	−4 S

*S = significant at 19/20 level or better.

the chlorine is situated on the periphery of the molecule and its introduction has had a remarkable enhancing effect on the antituberculosis activity (see Table I).

The activity in mice of compounds 628, 629 and 630 is of a higher order than we have obtained with PAS, the thiosemicarbazones or streptomycin, and when compared on a molecular basis is only equalled by certain isoniazid derivatives.

TABLE II

RESULTS IN GUINEA-PIGS INFECTED WITH *Myco. tuberculosis* RAVENEL RV (0·01 MG. S.C.) AND TREATED FOR 21 DAYS COMMENCING ON THE DAY OF INFECTION. THE ANIMALS WERE THEN KILLED

No. of drug all given 0·5 per cent. in diet	Disease Index maximum = 100	Lungs showing macroscopic evidence of tuberculosis	Lungs giving positive cultures for *Myco. tuberculosis* on Lowenstein-Jensen medium
628 (260 mg./kg.)	7	0/5	0/5
629 (230 mg./kg.)	11	0/5	0/5
671 (50 : 50 mixture of 628 and 629) (243 mg./kg.)	17	0/5	0/5
630 (197 mg./kg.)	29	0/5	4/5
Control	45	4/5	5/5

The above three compounds were further investigated in experimental tuberculosis of guinea-pigs and the results are set out in Tables II, III and IV. In the first experiment (Table II) the drugs were administered in the diet for 21 days from the day of infection and the animals were then killed. As was found in mice the protective effect of the drugs was impressive. No. 671 which was a 50 : 50 mixture of compounds, 628 and 629, showed no better results than those obtained with either constituent.

TABLE III

RESULTS IN GUINEA-PIGS INFECTED WITH *Myco. tuberculosis* RAVENEL RV (0·01 MG. S.C.) AND TREATED FOR 21 DAYS COMMENCING ON THE DAY OF INFECTION

Drug, all given 0·5 per cent. in diet	Mean survival time in days	Disease Index maximum = 100	Disease index in lungs maximum = 35
628 (260 mg./kg.)	127	77·0	15
629 (230 mg./kg.)	97	77·5	17·5
671* (243 mg./kg.)	118	80·0	20·0
630 (197 mg./kg.)	87	86·0	35·0
Control*	59	82·5	35·0

* One animal from each of these groups of 5 animals was lost through intercurrent infection and was not included in the analysis.

In the second guinea-pig experiment (Table III) the animals received similar infection and treatment to that described for the first experiment. In this case, however, when the administration of the drug was discontinued after 21 days, the disease was allowed to run its course. Final assessment of the activity was based on mean survival time and on the extent of the

TABLE IV

RESULTS IN GUINEA-PIGS INFECTED WITH *Myco. tuberculosis*, RAVENEL RV (0·01 MG. S.C.). TREATMENT* COMMENCING ON THE 22ND DAY AFTER INFECTION AND CONTINUED UNTIL THE 83RD DAY

Drug	Median survival time time (days)	Disease Index in lungs maximum = 35
628* (241 mg./kg.)	70	9·4
629 (191 mg./kg.)	63	13·5
671 (238 mg./kg.)	76	15
630 (182 mg./kg.)	79	19
Pre-controls	—	12
Controls*	51	30·5

* One animal from each of these groups of 10 animals was lost through intercurrent infection and was not included in the analysis.

disease. For an experiment of this kind the extension of the mean survival time is impressive particularly with Nos. 628, 629 and 671.

Table IV shows the results obtained with an established infection in guinea-pigs. At start of treatment, i.e., 21 days after infection, 5 animals (precontrols) were killed. These had a disease index of 45/100. As far as increase in MST is concerned, the results embodied in this Table are not striking. It will be noticed, however, here as well as in Tables II and III, that the amount of infection in the lungs is markedly less than that in the control animals. It is characteristic of these coloured substances that they accumulate in many organs and persist there long after treatment has been discontinued. This phenomenon is less noticeable with No. 630. It may be that the compounds remain in lung tissue to a large extent without extensive decomposition.

The disparity between the activity exhibited by these pigments in mouse and guinea-pig tuberculosis is interesting and is reminiscent of the different effect obtained by streptomycin treatment in established disease in the same animal species, except that with this drug the disparity noted was in the opposite direction. An obvious explanation in our case would be that the guinea-pig metabolises the phenazine derivatives more rapidly than does the mouse. Alternatively, we have noted that guinea-pigs receiving these compounds in the diet, eat much less well than the control animals. The lessened food intake may be in itself enough to explain the species difference in results.

We have already pointed out[2] the formal resemblance in structure between these phenazines and riboflavine and suggested that their activity might be related to this fact. Since riboflavine does not antagonise their growth-inhibitory properties for *Myco. tuberculosis*, it may be that this was too simple an approach to the problem of their mode of action. All these compounds contain a *p*-quinonoid system which is readily reduced, and reoxidised in the air. It is not improbable that they become involved in the hydrogen transfer system of the bacillus, accepting hydrogen from reduced diphosphopyridine nucleotide and transferring it to oxygen with the formation of hydrogen peroxide in the cell.

Winder[10] in these laboratories has already postulated that the antimycobacterial action of isoniazid is due to the intracellular formation of peroxide. In support of our hypothesis we have shown that hydrogen peroxide is produced when these phenazine pigments are exposed to air after catalytic reduction.

If this hypothesis is correct, high activity in this series should be related to the ease of reduction and re-oxidation of the compound and also to its capability of undergoing this reversible transformation repeatedly without decomposition. Some evidence has already been obtained which shows that the most active compounds are those which form the most permanent redox systems, and investigation is continuing in this field.

Further support of this hypothesis is provided by the fact that compound 595 which exerts a moderate suppressive effect in mouse tuberculosis (increase in MST, 12·5 days at 125 mg./kg. dosage) has been found by us to be highly active against a mouse infection induced by an isoniazid-resistant variant of the same strain as was used in the other experiments. Thus in the latter experiment compound 595 at a dose of 153 mg./kg. caused an increase in MST of 43 days. It is well established that isoniazid-resistant strains of *Myco. tuberculosis* are much more susceptible than isoniazid-sensitive strains to the action of hydrogen peroxide[11].

Other factors which will determine activity in these series will be those physico-chemical properties of the molecules which govern absorption from the gastrointestinal tract and which allow access to the bacilli present in the tuberculous lesions and then to diffuse into and be held in a suitable orientation in the bacillary cell. Included in these will be lipoid/water solubility, the planar area of the molecule and the disposition of its active groupings.

Summary

1. A number of phenazine compounds obtained by oxidation of derivatives of *o*-phenylenediamine have been investigated for antituberculosis activity in mice and guinea-pigs. Considerable activity was found in mice in more than half of 30 derivatives examined.

2. The activity was outstanding, however, and of the order obtainable with isoniazid, with 2-*p*-chloroanilino-5-*p*-chlorophenyl-3 : 5-dihydro-3-iminophenazine (No. 628), its isomer, 2-amino-5-*p*-chlorophenyl-3-*p*-chlorophenylimino-3 : 5-dihydrophenazine (No. 629), and the analogue of the latter in which the chlorine is replaced by ethoxyl (No. 630).

3. The results in guinea-pigs were clear cut but less impressive.

4. The mode of action of these compounds may depend on their ability to produce hydrogen peroxide within the mycobacterial cell.

Grateful acknowledgement for financial assistance is made to J. R. Geigy, S.A., Basle, Switzerland. Thanks are also due to Messrs. D. Malone, L. Murphy and T. Murray for technical assistance.

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DISCUSSION

The paper was presented by DR. VINCENT C. BARRY.

DR. G. BROWNLEE (London) said he welcomed this painstaking structure-action study. Like Dr. Barry he found the peroxide hypothesis stimulating but not quite acceptable as a theory. The equal antibacterial actions against both susceptible and resistant isoniazid strains, and the observed non-inhibitory actions of serum were both stumbling-blocks to accepting, in its simplest form at least, the peroxide hypothesis. The discovery of a reversing agent, perhaps to be sought for among the ribose-nucleic acids, would help to test the theory.

MISS A. E. ROBINSON (London) said that it seemed on consideration of the shape, size and chemical characteristics of the phenazine molecules that by analogy with the acridine type of compounds, penetration of the bacterial cell was precluded. The authors, however, seemed to suggest that penetration of the bacterial cell was necessary for their activity, although a hydrogen transfer mechanism was postulated. Was it not possible that the site of metabolic interference was in fact at the cell surface? It would be interesting to know whether the authors had undertaken any bacterial uptake or metabolism studies using those compounds. The authors had come out strongly in favour of one hypothesis concerning the mode of action of isoniazid, namely, peroxide formation, and they suggested that the phenazine compounds acted by a similar mechanism. Could they give a little more information concerning that choice to the exclusion of other currently postulated mechanisms; for example, chelation of trace metals, formation of diphosphopyridine nucleotide analogue, and direct inhibition of various enzyme systems?

DR. R. F. TIMONEY (Dublin) asked whether the authors had considered the possibility of testing sulphur analogues of these compounds. A number of sulphur compounds had been shown to be effective in the treatment of leprosy.

DR. V. C. BARRY, in reply, said that sulphur derivatives had not been considered because he did not like mixing his ideas! He had expected a violent reaction to his hypothesis. The hydrogen peroxide hypothesis was not being put forward very strongly. It was being submitted because there was some evidence which seemed to support it. A number of isoniazid derivatives were quite active, but they could not chelate. Evidence had been produced that isoniazid decomposed in solution liberating hydrogen peroxide. It was known that isoniazid gets into the

cell, and it was not asking much to expect that under proper conditions it would decompose, in that way liberating peroxide. Isoniazid did not accumulate. The planar area of the molecule and the disposition of the polar groupings would depend on how they orientated. The stability of the molecule was also all important. Reduction and reoxidisation were carried out a number of times without decomposition. It was well known that isoniazid-resistant bacilli were more resistant to hydrogen peroxide than sensitive strains. It was possible that the hydrogen peroxide was formed within the bacillus cells as well as outside it, and that that gave the increased curative effectiveness. It might also be that the small amount of catalase present in the tubercle bacilli was near the outer membrane, and its function might be to remove peroxide from the environment.

THE CHEMISTRY OF SANTONIN

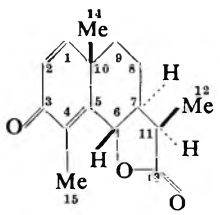
PART II[1]. PREPARATION OF SOME DERIVATIVES WITH POSSIBLE ANTHELMINTIC ACTIVITY

BY WESLEY COCKER AND T. B. H. MCMURRY

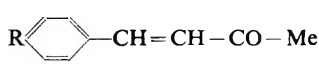
From The University Chemical Laboratory, Trinity College, Dublin

Received June 1, 1956

BALDWIN[2] in an excellent summary of his own and earlier work has suggested that three features of the santonin (I) molecule are essential for anthelmintic activity. They are (*a*) an intact butanolide ring, (*b*) unsaturation at C (3), and (*c*) an angular methyl group at C (10). However, inspection of the structure of many of the active non santonin-like compounds investigated by Baldwin reveals that the most active agents are those which form chelate complexes with metals. This is demonstrated by the greater activity of *o*-hydroxy- over *p*-hydroxyacetophenone, and the very great activity of 2:2′-dipyridyl and related compounds. The activity may be due to interference with an enzyme system in the nematode, by the removal of an essential metallic ion. Other promising series of compounds studied by Baldwin were the substituted benzylidene acetones (II), the phenol carbamates, and the aminothiazoles (III).



(I)



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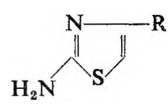


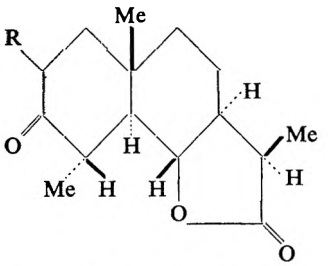
(III)

In our investigations we have applied the above principles to the santonin molecule; we have synthesised various derivatives which incorporate the three essentials mentioned by Baldwin[2] with one or more features which might be expected to increase biological activity.

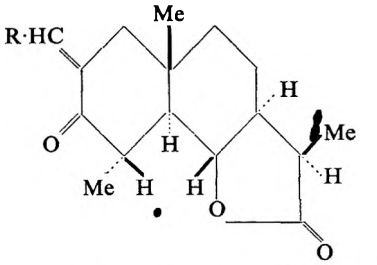
The so-called* α-tetrahydrosantonin (IV*a*) was used as starting material for our syntheses. On the basis of experiments not yet published we have tentatively assigned to it the *trans* A/B ring fusion shown[4], which is the reverse of the assignment of Yanagita and Tahara[5].

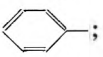
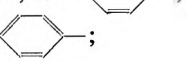
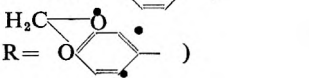
Condensation of α-tetrahydrosantonin with ethyl formate under basic conditions affords its 2-hydroxymethylene compound, which since it gives a purple ferric reaction must be (V*a*) and not (VI). The 2-benzylidene (V*b*), 2-anisylidene (V*c*), and 2-piperonylidene (V*d*) derivatives of α-tetrahydrosantonin were obtained by base catalysed condensation of this compound with the corresponding aldehyde.

* When the stereochemistry of this compound is established it will be named according to the suggestions of Cocker and Cahn[3] and Cocker and McMurry[1].

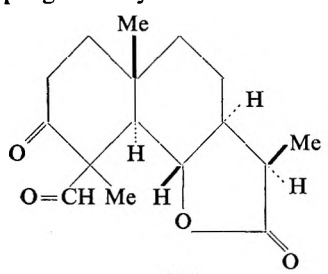


(IV; *a*, R=H; *b*, R=OH; *c*, R=OAc)

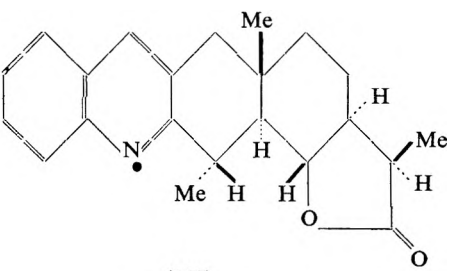


(V; *a*, R=OH; *b*, R=  ;
c, R= MeO  ;
d, R= )

It is interesting to compare the ultra-violet absorption spectra of these condensation products with those of the corresponding benzylidene acetones (II). Fieser and Fieser[6] have shown that in the α : β-unsaturated ketones substitution of an alkyl group for hydrogen in the α-position produces a batho-chromic shift of about 100 Å in the position of the low wavelength band. In the system under examination (ArCH = CR—CO—) it will be observed (Table I) that the effect of the alkyl substituent (R) diminishes as the aromatic nucleus is progressively substituted.

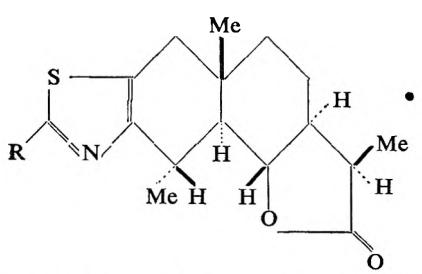


(VI)



(VII)

Condensation of α-tetrahydrosantonin with *o*-aminobenzaldehyde gave the quinoline (VII), whilst 2-bromo-α-tetrahydrosantonin[5], in which the bromine is equatorially substituted[4], when reacted with thioacetamide gave the methyl thiazole (VIII*a*).



(VIII; *a*, R=Me; *b*, R=NH_2; *c*, R=NHAc)

Substitution of thiourea for thioacetamide in the last reaction gave the aminothiazole (VIII*b*) from which its acetyl derivative (VIII*c*) was obtained. The latter compound should chelate with metal ions.

TABLE I

ULTRA-VIOLET ABSORPTION DATA OF DERIVATIVES

α-Tetrahydrosantonin derivative	$\lambda_{max.}$ Å (log ε)	Acetone derivative	$\lambda_{max.}$ Å (log ε)	$\Delta\lambda_{max.}$ Å
Benzylidene	2940 (4·29)	Benzylidene	2850 (4·35)[7]	90
Anisylidene	2320 (3·89) 3220 (4·34)	Anisylidene	2320 (4·08) 3180 (4·37)[8]	40
Piperonylidene	2500 (3·98) 3375 (4·29)	Piperonylidene	3360 (5·19)[8]	15

$\Delta\lambda$ = [$\lambda_{max.}$ (Santonin derivative) − $\lambda_{max.}$ (Acetone derivative)] is a measure of the influence of the alkyl substitutent introduced by the *cyclo*hexanone ring.

2-Acetoxy-α-tetrahydrosantonin (IV*c*) was prepared by the action of mercuric acetate in acetic acid[10] on α-tetrahydrosantonin. Hydrolysis gave 2-hydroxy-α-tetrahydrosantonin (IV*b*) which again should be capable of chelation. Yields of this compound were however too low to permit tests of its anthelmintic activity to be performed.

Preliminary screening tests carried out on the compounds described using *Ascaris lumbricoides* have so far failed to reveal any interesting degree of activity in these compounds.

EXPERIMENTAL

All ultra-violet spectra were measured by a Beckman DU spectrophotometer, using ethanol as solvent. Specific rotations were measured in chloroform. Melting points are uncorrected.

2-*Hydroxymethylene-α-tetrahydrosantonin* (V*a*). A mixture of α-tetrahydrosantonin (1·25 g.) anhydrous sodium methoxide (1·4 g.) and ethyl formate (1 ml.) in dry benzene (20 ml.) was shaken for 12 hours, and then poured on to ice and dilute sulphuric acid. The benzene layer was separated, dried and the solvent removed leaving a gum which separated from dilute ethanol as needles, thus affording 2-*hydroxymethylene-α-tetrahydrosantonin* (1·13 g.) m.pt. 143–144° C. $[\alpha]_D^{15}$ + 94·0° (c, 1·2). Light absorption: maximum 2800 Å (log ε 4·05) (in acidified ethanol). (Found: C, 68·7; H, 8·0. $C_{16}H_{22}O_4$ requires C, 59·1; H, 7·9 per cent.).

2-*Benzylidene-α-tetrahydrosantonin* (V*b*). A solution of α-tetrahydrosantonin (2·5 g.), redistilled benzaldehyde (1·06 g.) and solid potassium hydroxide (1·12 g.) in ethanol (10 ml.) was set aside for 64 hours at room temperature, during which time a deep red colour developed. The mixture was diluted with water (60 ml.), acidified with hydrochloric acid and steam distilled. The solid residue was collected and recrystallised from aqueous ethanol as pale yellow needles (2·86 g.), m.pt. 125–126° C. $[\alpha]_D^{15}$ − 131° (c, 2·64). Found: C, 77·8; H, 7·5. $C_{22}H_{26}O_3$ requires C, 78·1; H, 7·7 per cent.)

2-*Anisylidene-α-tetrahydrosantonin* (V*c*). This compound was obtained from α-tetrahydrosantonin (2·75 g.), anisaldehyde (1·25 ml.) and potassium hydroxide (1·25 g.) in ethanol (25 ml.) after standing for 48 hours. The product, 2-*anisylidene-α-tetrahydrosantonin* was obtained (3·44 g.) as

pale yellow plates (ethanol), m.pt. 195–196° C. (Found: C, 75·6; H, 7·5. $C_{23}H_{28}O_4$ requires C, 75·0; H, 7·6 per cent.)

2-*Piperonylidene-α-tetrahydrosantonin* (V*d*). α-Tetrahydrosantonin (2·5 g.), piperonal (1·5 g.) and potassium hydroxide (1·16 g.) in ethanol (15 ml.) after 48 hours gave 2-*piperonylidene-α-tetrahydrosantonin* (2·8 g.) as yellow rhombs (ethanol) m.pt. 148–149° C. $[\alpha]_D^{17}$ — 198·7° (c, 2·2). (Found: C, 71·6; H, 7·0. $C_{23}H_{26}O_5$ requires C, 72·3; H, 6·8 per cent.)

Condensation of o-*aminobenzaldehyde with α-tetrahydrosantonin.* A solution of α-tetrahydrosantonin (0·65 g.), o-aminobenzaldehyde (0·35 g.), and sodium hydroxide (1·35 g.), in ethanol (12 ml.) and water (8 ml.) was kept for 64 hours at room temperature. It was diluted with water, extracted with ether, and acidified with hydrochloric acid. The volume was reduced to 5 ml., and the mixture was carefully neutralised with sodium hydroxide. The quinoline derivative (VII) was deposited and it was crystallised from ethanol as needles (0·82 g.), m.pt. 263° C. Light absorption: maxima, 2325, 3075, 3210 Å (log ε 4·85, 3·73, 3·85 respectively); *cf.* 2-methylquinoline[11] which shows maxima at 2740, 3150 Å (log ε 3·54, 3·6 respectively). (Found: C, 78·2; H, 7·1. $C_{22}H_{25}O_2N$ requires C, 78·8; H, 7·5 per cent.)

The quinoline (0·2 g.) failed to yield a methiodide after refluxing with methyl iodide (2 ml.) for 30 minutes.

Methylthiazole (VIII*a*). A solution of 2-bromo-α-tetrahydrosantonin (0·65 g.) and thioacetamide (0·2 g.) in pyridine (2 ml.) was heated on the water bath for 45 minutes. The mixture was poured into water and the solid (0·45 g.) collected. After treatment with charcoal in boiling benzene the product was recrystallised from ethanol as rhombs, m.pt. 234° C. Light absorption: maximum 2530Å (log ε 3·64). *Cf.* Thiazole[12] which shows a single maximum at 2400 Å (log ε 3·60). (Found: C, 67·0; H, 7·5. $C_{17}H_{23}O_2NS$ requires C, 66·9; H, 7·5 per cent.)

Aminothiazole (VIII*b*). A mixture of 2-bromo-α-tetrahydrosantonin (0·65 g.), thiourea (0·20 g.) and pyridine (2 ml.) was heated on the water bath for 45 minutes. The mixture was poured into water, and the solid (0·51 g.) collected, decolourised with charcoal in benzene solution and recrystallised from ethyl acetate, from which the *aminothiazole* was obtained as rhombs, m.pt. 271–272° C. Light absorption: maximum 2630° Å (log ε 3·80); *cf.* 2-aminothiazole which gives a maximum at 2520 Å (log ε 3·8)[13]. (Found C, 62·1; H, 7·0. $C_{16}H_{22}O_2N_2S$ requires C, 62·7; H, 7·2 per cent.)

Acetamidothiazole (VIII*c*). A mixture of the aminothiazole (VIII*b*) (0·2 g.) and acetic anhydride (2 ml.) was heated for 1 hour at 100° C. The solution was poured into water and the acetic acid neutralised with sodium hydrogen carbonate. The solid (0·21. g.) was collected and recrystallised from ethanol as needles, m.pt. 308–309° C. Light absorption: maximum, 2790 Å (log ε 4·04). (Found: C, 62·0; H, 6·8. $C_{18}H_{24}O_3N_2S$ requires C, 62·1; H, 6·8 per cent.)

2-*Acetoxy-α-tetrahydrosantonin* (IV*c*). A mixture of α-tetrahydrosantonin (1·25 g.) and mercuric oxide (1·17 g.) in glacial acetic acid (15 ml.) was refluxed for 2·5 hours. A solid rapidly separated and then

slowly dissolved. At the end of the reaction the solution was decanted from mercury, diluted with water, neutralised with sodium hydrogen carbonate, and the resulting gum collected in chloroform. The gum was dissolved in ethanol, and allowed to stand overnight when a solid (0·4 g.) was deposited. It was decolourised by charcoal in benzene solution, and recrystallised from ethanol as needles, m.pt. 173–174° C. (Found: C, 65·8; H, 7·8. $C_{17}H_{24}O_5$ requires C, 66·2; H, 7·8 per cent.)

2-*Hydroxy-α-tetrahydrosantonin* (IV*b*). A solution of 2-acetoxy-α-tetrahydrosantonin (0·4 g.) and potassium hydroxide (0·6 g.) in methanol (30 ml.) was refluxed for 2 hours. The solvent was removed, the residue was acidified with hydrochloric acid, and the mixture was extracted with chloroform. The extract was shaken several times with sodium hydroxide solution, and the combined alkaline extracts were acidified and extracted with chloroform. The resulting gum gave a positive ferric reaction, and on extraction with ethanol gave the required compound (0·23 g.) as needles, m.pt. 157–158° C. (Found: C, 67·6; H, 7·6. $C_{15}H_{22}O_4$ requires C, 67·7; H, 8·3 per cent.)

α-*Tetrahydrosantonin thiosemicarbazone*. A mixture of α-tetrahydrosantonin (1·0 g.), thiosemicarbazide (1·0 g.) and fused sodium acetate (0·4 g.) in methanol (25 ml.) was refluxed for 6 hours. The solvent was removed, the solid residue was washed with water and crystallised from ethanol from which the *thiosemicarbazone* (1·05 g.) separated as needles, m.pt. 234° C. (Found: C, 59·1; H, 7·8. $C_{16}H_{25}O_2N_3S$ requires C, 59·4; H, 7·8 per cent.)

α-*Tetrahydrosantonin semicarbazone*. This compound had m.pt. 236–237° C. (rapid heating). Weinhaus and Oettingen[14] record m.pt. 256–258° C.

Summary

1. A number of derivatives of santonin incorporating the β-methyl group at C (10), the ketonic group or its equivalent at C (3), and the intact butanolide fused at C (6)–C (7) to the bicyclic system have been synthesised.

2. These derivatives were related to non santonin-like compounds which have anthelmintic activity.

3. The combination of features of the santonin molecule might reasonably have been expected to produce activity. Preliminary screening tests have unfortunately shown no promise of such activity.

The authors gratefully acknowledge the financial support given by Messrs. T. and H. Smith, Ltd. of Edinburgh and the Medical Research Council of Ireland.

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8. Wilds, Beck, Close, Djerassi, Johnson, Johnson and Shunk, *J. Amer. chem. Soc.*, 1947, **69**, 1985.
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10. Triebs and Bast, *Ann.*, 1948, **561**, 165.
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12. Braude, *Ann. Repts.*, 1945, **42**, 128; Ruehle, *J. Amer. chem. Soc.*, 1935, **57**, 1887.
13. Vandenbelt and Doub, *ibid.*, 1944, **66**, 1633.
14. Weinhaus and Oettingen, *Ann.*, 1913, **397**, 219.

DISCUSSION

The paper was presented by MR. T. B. H. MCMURRY.

DR. G. E. FOSTER (Dartford) said that santonin became discoloured very readily in light. He wondered whether any of the compounds which the authors had made possessed similar photochemical properties, and if so were they able to relate these properties to the structure.

DR. W. MITCHELL (London) said he was puzzled by the opening paragraph where reference was made to the fact that certain active compounds could chelate metals. Immediately afterwards reference was made to compounds II and III which could not, so far as he could see, chelate metals. It would be interesting to know whether compound Va could chelate metals.

DR. A. H. BECKETT (London) suggested that the chelation of trace metals was a vital factor. It was amazing to observe the avidity with which bacteria could compete with chelating agents.

MR. T. B. H. MCMURRY, in reply, said that form IVa did not discolour in light. This change was associated with double bonds and the ketone groups in santonin. With regard to chelation, the statement was based on the fact that of the compounds which were examined by Professor Baldwin only 2:2′-dipyridyl and related compounds had the same activity as santonin. The other compounds examined were not nearly as active. 2:2′-Dipyridyl was too soluble for use: it was quickly eliminated from the system. Compound Va will chelate, and one can obtain a very good reaction with ferric iron.

PROFESSOR W. COCKER added that the testing of the materials was entirely out of the authors' hands.

SOME STATISTICAL ASPECTS OF THE ANALYTICAL CONTROL AND STANDARDISATION OF TABLETS

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Introduction

The problem of the standardisation of tablets has been discussed by Bandelin[1], and by Denston[2], who described many of the tests which are available. Some of the tests proposed have statistical implications which form the subject of this paper.

It is well known that tests which are of considerable use to the manufacturer may be unsuitable for incorporation in a book of standards such as the British Pharmacopœia. They may also be unsuitable for public analysts, because they work on small and not necessarily representative samples. Furthermore, each test must be so designed that it is not too difficult for the manufacturer to produce batches of tablets complying with the requirements, while adequately protecting the interests of the consumer.

Weight Variation

The function of the test for weight variation is to maintain a suitable standard of elegance, and to ensure accurate doses since provided that the granules are uniform[3], the weight of active ingredient will be directly proportional to the weight of the tablet[4]. Two factors have to be considered: (i) Gross errors, not normally distributed, due to variation in the setting of the machine, mixing of batches, etc. (ii) Small errors, probably normally distributed, due to irregular filling of the die, separation of granules in the hopper, and slackness of the compressing machinery.

The desirable criteria are therefore that no tablets in a batch shall vary from the mean weight by more than (say) 10 or 20 per cent., and that the variation shall be limited within (say) $\pm$ 5 per cent.

(*a*) *Tests based on Standard Deviation*

The most obvious test would be a specification of the permitted standard deviation or coefficient of variation of weight. This is the method of the Swedish Pharmacopœia, which states that "the variation in the weight of tablets of the same production batch shall not be greater than that corresponding to a relative standard deviation of 4·5 for uncoated tablets and 6·5 for coated tablets" (*cf.* Denston[2]).

There is a limited amount of data in the literature[4–6] on the coefficients of variation of weights of normal production batches. A graph which relates coefficient of variation and mean weight shows a rapid increase in the coefficient of variation for tablets weighing less than about 150 mg. (see Fig. 1). That is, small tablets show a greater amount of variation in relation to their weight than large tablets.

An improvement on the Swedish specification would be to vary the permitted maximum coefficient of variation according to the mean weight of the tablets. A suitable expression would be that "the coefficient of variation of the weights of tablets shall not exceed that corresponding to a standard deviation of 2 mg. plus 2 per cent. of the mean weight, or 0·06, whichever is the less". A line representing this maximum is included in Figure 1.

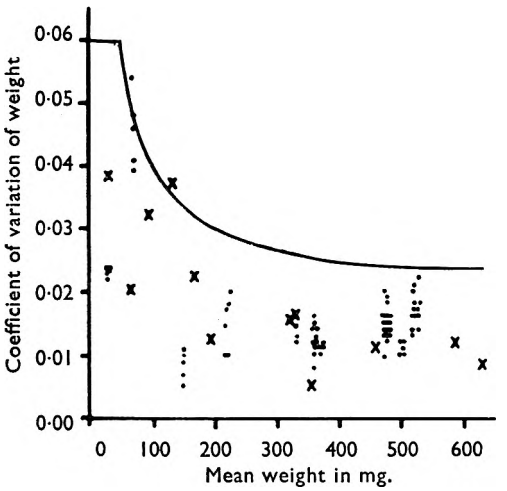

FIG. 1. Coefficients of variation of weights of production batches of tablets. × Data by Evers[4]. • Data by Dunnett and Crisafio[6]. — Suggested maximum.

It is of interest to calculate the effect on the coefficient of variation of (say) one punch out of twenty-five in a rotary compressing machine being wrongly adjusted. If σ' is the standard deviation of a mixture, in the proportion $p:(1-p)$, of two populations with means $\bar{x}_1$ and $\bar{x}_2$ respectively and the same standard deviation σ, then

$$(\sigma')^2 = \sigma^2 + p\,(1-p)\,(\bar{x}_1-\bar{x}_2)^2.$$

Consider a maladjusted machine producing 24 tablets with $\bar{x}_1 = 100$ for each tablet with $\bar{x}_2 = 105$, with $\sigma = 2$ for each population; then $\sigma' = 2{\cdot}22$. This appears to be a marked increase in the standard deviation, but allowance must be made for the errors associated with small samples. For a sample of 20 tablets, assuming normal distribution of weights, application of the t-test shows that an estimate of σ' as high as 2·22 might arise by chance 45 times in 100 even when the true value was 2·00. For $P = 0{\cdot}95$ of detecting the mixture of populations with a sample of 20 tablets, $\bar{x}_2$ would have to be at least 109 (or less than 91). That is, with a machine intended to produce tablets of mean weight 100, one punch out of twenty-five must be incorrectly adjusted to the extent of nearly 10 per cent. for there to be reasonable certainty of detecting the error by determination of the standard deviation of the weights of the tablets in a sample of twenty.

If a test based on standard deviation were introduced into the British Pharmacopœia, it would be desirable to insert a safeguard against gross variations which might not otherwise be detected. A suitable expression of this type would be that "no tablet shall vary from the mean weight by an amount greater than that corresponding to five times the permitted coefficient of variation".

The particular advantage of a test based on the estimation of the standard

deviation is the freedom it allows the analyst. The same test may be used for a sample of any size. If only a small sample (*e.g.*, five tablets) is available, then the estimate based on this small sample will be subject to wide limits of error, and the batch would be rejected only if the 0·95 (say) limits of confidence did not include the coefficient of variation permitted by the specification. The manufacturer, on the other hand, could obtain a more precise estimate of the coefficient of variation of the batch by taking a large sample (e.g., 50 or 100 tablets) or by some other more convenient method of quality control.

(*b*) *Tests based on Range*

Although the range is an inefficient estimate of the standard deviation with large samples, it is of considerable convenience for samples of not more than ten. Smith[7] has referred to its use in the control of variation of weight of tablets, and some data are given by Spengler and Schenker[8], and Beeler[9].

The ratio of the mean sample range to the standard deviation can be obtained from Tables (e.g., Table XX of Fisher and Yates[10]). Consider a batch of tablets of mean weight = 1 g. with standard deviation = 20 mg. The mean of the ranges of weights in samples of ten tablets will be about 60 mg., and a sample of ten tablets with a range of weights of 120 mg. would be so unusual as to justify rejection of the batch.

If a batch is permitted a standard deviation not exceeding σ, then a suitable upper limit to the range of weights permitted in a sample of ten tablets is 6σ, or 5σ for a sample of five tablets. Using the recommended limits of section (*a*) above, the test would read "weigh five tablets individually and calculate the mean weight. The difference in weight between the heaviest and the lightest tablets shall not exceed 10 mg. plus 10 per cent. of the mean weight, or 30 per cent. of the mean weight, whichever is the less".

This test has the advantage over (*a*) that no special skill is required in calculating the range of weights, whereas it may not be desirable to rely on the accuracy of junior staff in calculating coefficients of variation. It is particularly suited to the application of a simple form of quality control by a manufacturer during the run of a batch. Its main disadvantage is its inefficiency, and it would probably not be acceptable as the basis of an official specification, since in effect the batch is accepted or rejected according to the weights of only two tablets.

The test of the British Pharmacopœia may be regarded as including a limit to the range of weight variation. The maximum variation permitted in a sample of either ten or twenty tablets with mean weight exceeding 5 grains is 10 per cent. in one direction and 5 per cent. in the other, i.e., a range of 15 per cent. of the mean weight; for smaller tablets, the permitted range is 22·5 or 30 per cent. of the mean weight. Since the greatest variations will act in the same direction in half of the samples, the test is actually somewhat less severe than these figures suggest.

(*c*) *Tests of the B.P. Type*

The majority of pharmacopœias use a test of the form: "Of a sample of n tablets, not more than 10 per cent. shall deviate from the mean weight by more than x per cent., and no tablet shall deviate by more than $2x$ per cent." (*cf.* Dunnett and Crisafio[6]). Thus, the British Pharmacopœia takes $n = 20$ (or $n = 10$ if 20 tablets are not available) and $x = 5$, 7·5 or 10 per cent. according to the size of the tablets.

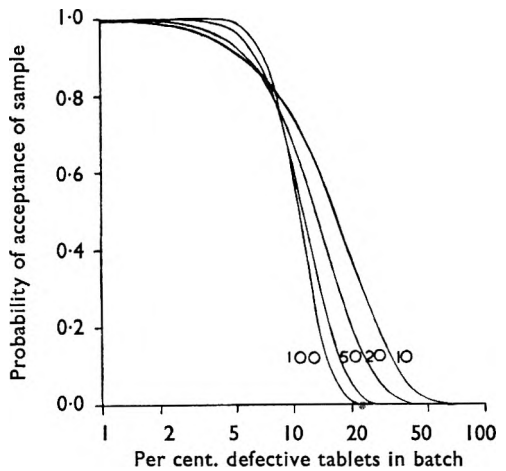

FIG. 2. Operating characteristic curves for samples of the stated size with not more than 10 per cent. of defective tablets in the sample.

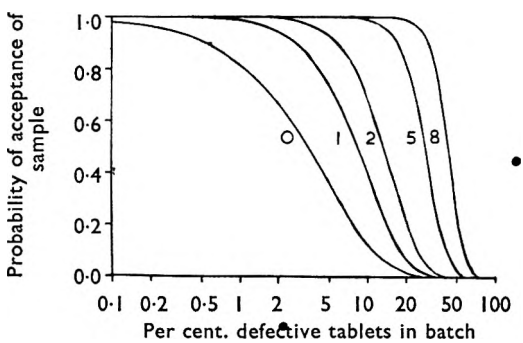

FIG. 3. Operating characteristic curves for samples of 20 with not more than the stated number of defective tablets in the sample.

The proportion of samples which will pass the test for a given percentage of faulty defective tablets in the batch can be calculated by expansion of the appropriate binomial, which for large batches is a satisfactory approximation to the hypergeometric variable which should be used. Dunnett and Crisafio[6] gave curves showing the relation where $n = 10$, 20, 50 and 100, although they used an approximate and complicated method of calculation. Evers[11] gave some related data in tabular form.

Curves of this type, known as "operating characteristic curves", are given in Figures 2 and 3. In calculating the data, the proportion of tablets deviating from the mean by more than $2x$ per cent. has been ignored. The effect of considering them would be to increase very slightly the probability of acceptance of batches by the official test (*cf.*, for example, Figure 3 of this paper with Table I of Smith's paper[7] in which the proportion of "double defectives" has been calculated by assuming normal distribution of weights).

A good test will show a high probability of accepting satisfactory batches and a low probability of accepting unsatisfactory batches. In terms of operating characteristic curves, the greater the sigmoid character

of the curve, the greater the ability of the test to discriminate between satisfactory and unsatisfactory batches.

Values of x given by various pharmacopœias have been tabulated by Smith[12] and Denston[2]. They are shown in a diagrammatic manner in Figure 4. The method of calculating x given by the Danish Pharmacopœia (namely, x is 4 mg. plus 5 per cent. of the mean weight, or 10 per cent., whichever is the less) provides a gradual narrowing of the limits with increase in mean weight, and is probably to be preferred. In any case, it would seem desirable for the British Pharmacopœia to specify mean weights in metric rather than in imperial units, when tabulating values of x.

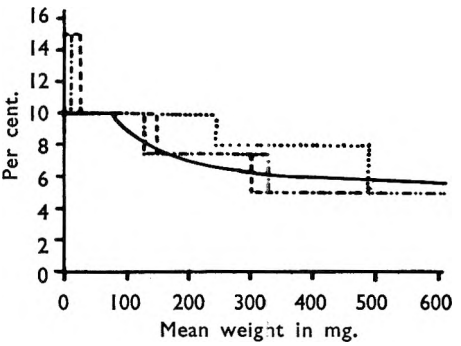

FIG. 4. Limits of deviation from the mean weight of not more than 10 per cent. of tablets in the sample permitted by various pharmacopœias. —— Denmark IX (1948). – – – Egypt 1953. –.–.– B.P. 1953; U.S.P. XV (1955). .... Finland VI (1937); Norway V (1939); Switzerland V (1933).

It is clear from Figure 2 that large sample sizes improve the ability of the test to discriminate between satisfactory and unsatisfactory batches. Reference to Figure 3 shows that the curve can also be made more markedly sigmoid by increasing the proportion of tablets in a sample of fixed size which are allowed to deviate from the mean weight by a stated percentage x. In the latter instance, the probability of acceptance of a batch increases whether the percentage of defective tablets in the batch is large or small. This may be corrected by a decrease in the value of x; thus a test approximately equivalent to that now official would be that not more than eight tablets out of a sample of twenty should deviate by more than $0{\cdot}67x$ per cent. of the mean weight, and that none should deviate by more than $2x$ per cent.

The particular advantage of this test is that it operates relatively near to the inflexions of the distribution curve, whereas the test of the British Pharmacopœia operates relatively near to the "tails". Provided that the distribution is normal, this is of little importance, but it is common for unsatisfactory batches to show deviations from normality. Batches showing platykurtosis, i.e., a preponderance of weights remote from the mean, such as might be caused by maladjusted punches in rotary compressing machines, would be likely to be rejected by the test, although the sample might pass the test of the British Pharmacopœia. Conversely, leptokurtic batches, with a preponderance of weights close to the mean (a desirable characteristic) would be likely to be accepted by this test.

The tests of the British Pharmaceutical Codex for uniformity of weight of pastilles and of lozenges may also be considered here, since the problems are similar. The pastille test appears to be incorrectly stated: "The average weight is determined by weighing 20 pastilles. When weighed

singly not more than one of the pastilles deviates from the average weight by more than 15 per cent., and none of the remainder deviates by more than 10 per cent.". This implies that not more than one pastille may deviate from the mean weight by more than 10 per cent., but that one pastille may deviate by an unspecified amount. It is presumably intended to require that not more than one pastille shall deviate by more than 10 per cent., and no pastille by more than 15 per cent. Reference to Figure 3 shows that this test gives a curve which is even less sigmoid than that of the British Pharmacopœia test applied to tablets, whereas a more markedly sigmoid curve would be desirable.

The lozenge test is similar to the test of the British Pharmacopœia for tablets weighing not more than 2 grains (i.e., $x = 10$ per cent.), except that no lozenge is permitted to vary by more than 15 per cent. (i.e., $1{\cdot}5x$ compared with $2x$ for tablets). This will not make the test appreciably more severe on satisfactory batches, but will reject a greater proportion of unsatisfactory batches, and so is to be preferred.

(d) Sequential Analysis Tests

Smith[7] recommends application of the methods of sequential analysis to the problem, on the grounds that it allows uniform batches to be accepted with fewer weighings than the test of the British Pharmacopœia and removes the rigid division between acceptance and rejection in borderline cases.

He selects as the important criteria the probabilities of accepting batches which contain $p_0 = 5$ per cent. and $p_1 = 25$ per cent. of defective tablets respectively. Using the data of Figures 2 or 3 (or of Table I of Smith's paper[7]) relating to the test of the British Pharmacopœia, the desired probability of rejection of the more acceptable quality p_0 is $\alpha = 0{\cdot}08$, and the desired probability of acceptance of the less acceptable quality p_1 is $\beta = 0{\cdot}08$, giving the acceptance and rejection numbers $0{\cdot}128\ m \pm 1{\cdot}323$ for a sample of size m, and this leads to the Table I (*cf.* Table II of Smith's paper[7]).

If the probabilities at the $p_0 = 5$ per cent. and $p_1 = 30$ per cent. levels are considered, then the acceptance and rejection numbers are $0{\cdot}146\ m - 1{\cdot}632$ and $0{\cdot}146\ m + 1{\cdot}190$ leading to Table II. Table III gives the data for $p_0 = 15$ per cent. and $p_1 = 20$ per cent. The criteria for acceptance and rejection of batches can thus be varied by using data from different parts of the operating characteristic curve based on the specification of the British Pharmacopœia.

Just as a test of the B.P. type can be improved by use of a greater number of tablets in the sample, so the sequential analysis test can be improved by altering the probabilities desired. Table IV gives the criteria for $\alpha = \beta = 0{\cdot}01$, with $p_0 = 5$ per cent. and $p_1 = 25$ per cent.; it is clear that a greater number of tablets is required before a decision can be made.

Smith also describes an "improved" procedure, involving counting the numbers of "half-defectives", and gives data based on the assumption of normal distribution of tablet weights. This procedure is equivalent to reducing x in tests of the B.P. type to $0{\cdot}5x$, and has certain advantages

TABLE I

CRITERIA FOR ACCEPTANCE AND REJECTION WHERE $p_0 = 5$ PER CENT., $p_1 = 25$ PER CENT., $\alpha = \beta = 0{\cdot}08$

Number of defectives observed	Accept if number of tablets weighed is not less than:	Reject if number of tablets weighed is not more than:
0	11	—
1	19	—
2	26	5
3	34	13
4	42	20
5	50	28
6	58	36

TABLE II

CRITERIA FOR ACCEPTANCE AND REJECTION WHERE $p_0 = 5$ PER CENT., $p_1 = 30$ PER CENT., $\alpha = 0{\cdot}08$, $\beta = 0{\cdot}03$

Number of defectives observed	Accept if number of tablets weighed is not less than:	Reject if number of tablets weighed is not more than:
0	12	—
1	19	—
2	25	5
3	32	12
4	39	19
5	46	26
6	53	32

which have been discussed above. The particular advantage here is the reduction in the number of weighings when there is evidence of uniformity.

As Smith himself has pointed out, "decisions with a small number of weighings would be possible only when the batch was uniform", and so the test is unsuited to inclusion in an official book of standards, since evidence of uniformity of a batch would not in general be available to a public analyst or other small-scale consumer. The method is well-suited for use by manufacturers, especially in well-controlled production units.

TABLE III

CRITERIA FOR ACCEPTANCE AND REJECTION WHERE $p_0 = 15$ PER CENT., $p_1 = 20$ PER CENT., $\alpha = 0{\cdot}61$, $\beta = 0{\cdot}19$

Number of defectives observed	Acept if number of tablets weighed is not less than:	Reject if number of tablets weighed is not more than:
0	12	—
1	18	—
2	24	6
3	30	12
4	35	18
5	41	24
6	47	29

TABLE IV

CRITERIA FOR ACCEPTANCE AND REJECTION WHERE $p_0 = 5$ PER CENT., $p_1 = 25$ PER CENT., $\alpha = \beta = 0{\cdot}01$

Number of defectives observed	Accept if number of tablets weighed is not less than:	Reject if number of tablets weighed is not more than:
0	14	—
1	23	—
2	32	4
3	41	13
4	50	22
5	59	31
6	68	40

Coated Tablets

The foregoing discussion relates to uncoated tablets. While it is true that the weights of pan-coated tablets are not directly proportional to the weights of active ingredient contained in them, there is a positive correlation, and a high standard deviation of the coated weights would indicate a poorly-made batch. In the case of tablets coated by compression, there should be no difficulty in securing uniformity of weight.

If it were considered desirable to extend the test for uniformity of weight to coated tablets, wider limits would need to be set, at any rate at first. Suitable values would be twice the deviations allowed for uncoated tablets. Thus for tests of type (*a*), a suitable limit would be that "the coefficient of variation of the weights of coated tablets shall not

exceed that corresponding to a standard deviation of 4 mg. plus 4 per cent. of the mean weight".

Disintegration

The function of the test for disintegration is to ensure adequate release of the active ingredient[13].

Apparatus and methods have been described by many workers[14–33], and reviews are given by Brown[16], Hoehn[34], Sperandio, Evanson and DeKay[24], Smith[12], Denston[2] and Münzel and Kägi[35]. Mention must also be made of the apparatus described by official bodies, including the American Pharmaceutical Manufacturers' Association, the Association of Official and Agricultural Chemists, the American Pharmaceutical Association, the Fédération Internationale Pharmaceutique, and pharmacopœia commissions of many countries. Other related papers have been published[36–39] suggesting suitable time limits for disintegration, and Bukey and Brew[40] have given information about *in vivo* disintegration times as measured by X-ray methods. The method of setting the specification has been discussed by Hoyle[21], Prance, Stephenson and Taylor[22], and Evers[11]. Enteric and hypodermic tablets lend themselves to the same statistical treatment.

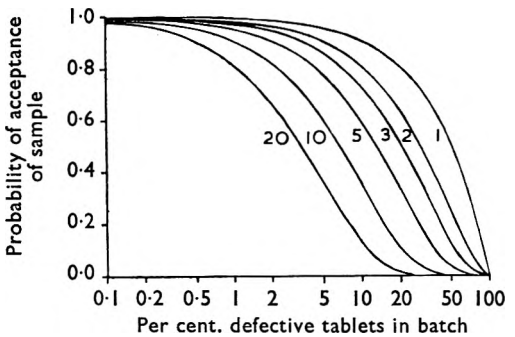

FIG. 5. Operating characteristic curves for samples of the stated size with no defective tablets in the sample.

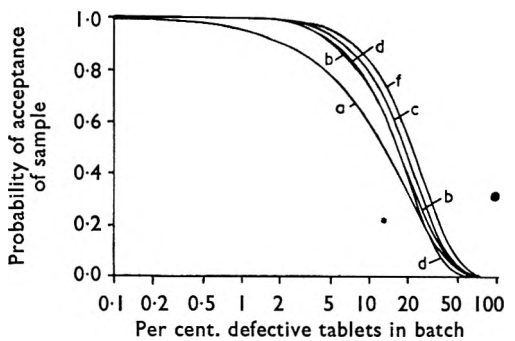

FIG. 6. Operating characteristic curves for tablet disintegration tests (a) (sample of five tablets), (b), (c), (d) and (f).

(*a*) The usual form of specification is to require that no tablet out of five (or ten, etc.) shall fail to disintegrate in the specified conditions and time. Operating characteristic curves similar to those for the weight variation test can be constructed, and some relevant data are given in Figures 5 and 6. In order to be sure that the chance of a sample from a satisfactory batch failing the test is negligible (P = 0·005), the manufacturer must work to a maximum of about 0·1 per cent. of defective tablets in the batch, with

samples of five tablets; this is a high standard. On the other hand, a batch containing as many as 10 per cent. of defective tablets has a chance of 3 in 5 of being accepted.

(*b*) To avoid rejecting a batch containing an occasional abnormal tablet, Prance, Stephenson and Taylor[22] suggested that if the first sample of five tablets is rejected, a further ten should be taken and must all pass. Figure 6 shows that the manufacturer must work to not more than about 1 per cent. of defective tablets in the batch; a sample from a batch containing 10 per cent. defective has 3 chances in 4 of passing the test.

(*c*) The test of the British Pharmacopœia 1948 has been analysed by Evers[11]. It has about the same stringency as the test of Prance, Stephenson and Taylor, although a maximum of only ten tablets is needed (*cf.* fifteen for the latter). Note: The data on which curve (*c*) of Figure 6 are based are different from those in column 4 of Table II of Evers' paper[11], since he neglected the proportion of tablets which fail on the first sample and do not qualify for a second sample, i.e., those with two or more defective in the first sample of five tablets.

Evers[11] suggested two alternative tests: (*d*) Not more than one defective tablet shall be permitted in a sample of ten tablets.

(*e*) Not more than two defective tablets shall be permitted in a sample of 20 tablets. The latter, in particular, is more discriminating than test (*c*), since it is more severe on unsatisfactory batches and less severe on satisfactory batches (see Fig. 6). However, the gain in discrimination is not sufficient to compensate for the need to expend a greater number of tablets in the sample. The suggestion by Evers that the same 20 tablets which are used for the test of uniformity of weight should be employed is not really valid, because these are normally required in the assay.

(*f*) The test of the 1955 Addendum to the British Pharmacopœia 1953 must be considered in relation to two types of tablets. Certain tablet formulations give gummy masses which fail to break up in conventional disintegration tests, and with these a guided disc may be used to assist in breaking up the residue[2]. Gummy tablets will almost always fail in the first half of the test, so that curve (*a*) of Figure 6 applies. The non-gummy tablets, however, may fail or pass the first half, and so the second half of the test, involving the use of the disc, must be considered in addition. Assuming that the presence of the disc does not facilitate rapid disintegration of a non-gummy tablet, then curve (*f*) of Figure 6 applies; a sample from a batch containing 10 per cent. of defective tablets has 5 chances in 6 of being accepted by the double test. In practice, the disc will facilitate rapid disintegration, and so even fewer batches will be rejected. This anomaly could be overcome by requiring the guided disc to be inserted in all cases, and accepting or rejecting the batch on the evidence of the first sample of five tablets. In addition, to allow for instances in which ten tablets are available for disintegration testing, it could be required that all of a sample of ten tablets (or of two samples of five tablets each) must disintegrate within the stated time with the disc fitted; if ten tablets are not available, five may be used and all must disintegrate.

(*g*) Bull[41] stressed the importance of variation from mean disintegration

time when comparing formulations; Linnell[42] has also raised this point. Satisfactory batches of tablets show a coefficient of variation of disintegration time which is less than 25 per cent., although not much information on this point is available in the literature. (Analysis of Tables III and IV of the paper by Hoyle[21] gives values of 24 and 9·8 per cent. respectively.) Where the mechanics of the disintegration test permit measurement of the disintegration times of individual tablets, it should be possible to specify a maximum permitted standard deviation (or coefficient of variation). However, to ensure rejection of those batches containing a proportion of tablets which might not disintegrate within hours or days, it would be more expedient to require that all tablets should disintegrate within twice the limit allowed for the bulk of the tablets in those tests such as (*b*), (*c*), (*d*) and (*e*) which allow a small proportion to exceed the standard disintegration time limit.

Durability

Spengler and Kaelin[43] listed eight desirable mechanical properties of tablets, namely resistance to wear, rolling, shaking, impact, rubbing, pressure, bending and indentation. Smith[44] reviewed some of the equipment available for testing some of these properties. More recent work has been published by several authors[37,45–49].

Three criteria are in use in different types of tests: (*a*) The tablet must not show any significant change when subjected to specified misusage. (*b*) The tablet must not lose more than a certain weight, or proportion of its weight, when subjected to specified misusage. (*c*) The tablet must withstand a specified stress. Adequate replication must be ensured, since the between-tablets variation is frequently large; Webster and Van Abbé[47] specify a maximum permitted coefficient of variation of loss of weight in their test.

The test of uniformity of weight of the British Pharmacopœia is to some extent a check that the tablets are mechanically sound, since (unlike the U.S.P. XV) it does not specify the use of unbroken tablets. Batches containing a high proportion of chipped or broken tablets would almost certainly be rejected by the test of uniformity of weight.

The number of tablets taken for a test of durability depends mainly on the mechanical construction of the apparatus used, although tests of type (*c*) normally involve only one tablet at a time. With tests of types (*a*) and (*b*), more tablets are required if their size is small. Since the tablets are expended in the test, and since large samples (e.g., 100 tablets) are often needed for useful results, tests of durability are more likely to be applied by a manufacturer as a form of quality control than to be included in a book of official standards.

The various criteria discussed in the two previous sections can be applied, with obvious modifications, to tests of mechanical properties. If official standards were contemplated, data showing the characteristics of large numbers of satisfactory and unsatisfactory batches of different formulations of tablets would be required before detailed recommendations could be made.

Dimensions and Weights

Several foreign pharmacopœias specify the weights and die sizes of official tablets[12,50]. The Wholesale Drug Trade Association (now the Association of British Pharmaceutical Manufacturers) has issued schedules of recommended weights and die sizes to its members for many years[51]. Smith[50] and Firth[52] have tabulated the dimensions and weights of many tablets commercially available.

Smith[50] compared possible methods of standardising these properties, and put forward specifications as a basis for discussion. If a specification were to be adopted by an official body (possibly as a first step only for tablets newly introduced into a book of standards), it might take the form of a statement either of die size[2] with a tolerance of (say) $\pm$ 0·05 cm. or of total weight with a tolerance of $\pm$5 per cent.

Conclusions

Control of the quality of tablets is required at two stages (at least) of their life-history. The manufacturer should maintain production under statistical control, in order to detect, and so be able to correct, abnormalities as soon as they develop. The consumer should inspect some or all of the tablets at the time of purchase, in order to be sure that the alleged quality has been maintained.

For the control of variation of weight by the production unit, tests based on range are probably the most useful; tests based on sequential analysis carried out by the analytical control department may be a useful supplement. For the control of variation of weight by the consumer, the test of the British Pharmacopœia, modified in one or more of the ways suggested in this paper, or (better) a test based on standard deviation, is recommended.

For the control of disintegration, the test of the 1955 Addendum to the British Pharmacopœia 1953, modified as suggested in this paper, is recommended.

There is a lack of sufficient data for detailed recommendations on standards for durability and for weights and dimensions.

Summary

1. The statistics of tests of weight variation, disintegration and durability, and standards of dimensions and weights of tablets are discussed.

2. The specifications laid down in the British Pharmacopœia are criticised, and alternatives are suggested.

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DISCUSSION

The paper was presented by MR. A. R. ROGERS.

MR. K. L. SMITH (Nottingham) said that as worded the paper meant that a sample would be failed if it had such uniformity that the upper confidence limit did not exceed the critical coefficient of variation. When considering tests based on range the author suggested that the range in weights to be observed in five tablets may be as great as five times the standard deviation which is not to be exceeded. This is the order which would indicate that there was a 95 per cent. probability that the standard deviation did exceed the critical value. To ensure, with 95 per cent. probability, that this was not so, the range in weights of five tablets should not exceed the critical standard deviation. He could not understand how

the author found that in a sample of 20 the range of the B.P. test could be 15 per cent. of the mean weight. Under the B.P. test, batches of tablets weighing five grains would be accepted with 95 per cent. probability if 4 per cent. of them had weights deviating from the mean by 5 per cent. or more. The standard deviation of such populations, if they are normally distributed, is about 2·5 per cent. of the mean weight, from which it could be calculated that the average range in weight of 20 tablets should be 9·3 per cent. On the subject of sequential sampling, he was surprised that the author considered the values calculated using other points on the operating curve for the B.P. test differed markedly from those given by him. He thought it more reasonable to calculate from points towards the extremities of the curve, and it could be shown that the points chosen by him gave an operating curve closer to that of the B.P. test than Table III. It was unfortunate that in the discussion of his paper at the Aberdeen conference a misquote led Mr. Rogers to suggest he considered that the suitability of the sequential sampling test depends on other information regarding the uniformity of the batch being available. This was certainly not the case: it stands on its own as efficiently as any equivalent procedure. Mr. Rogers suggests that the test based on sequential analysis could provide a useful supplement for the analytical control department, presumably to quality control tests. There is a good argument for the claim that where quality control charts are available these could provide sufficient evidence for everybody that the batches have the desired uniformity.

MR. E. W. RICHARDS (Upminster) suggested that manufacturers should release for publication the large amount of information in their files on tablet weights. There appeared to be no direct reference in the paper to batch size, which might be up to 10^6, and in such cases a sample of 20 tablets could only be regarded as a spot check. The tablet maker would make frequent checks during the run of a batch. It was difficult to understand the author's remark on page 1109 that the weights of pan-coated tablets were not directly proportional to the weights of active ingredient contained in them. Provided variations between tablets and batches were not so obvious as to be readily noticed by the customer, there did not appear to be a need for the uniformity test for coated tablets. The recommendation that the disc should be used in all cases was not sound. With certain formulations there was a tendency for tablets to clump together or stick to the disc itself, and the disintegration time could then be considerably longer with the disc than without it. He suggested that tests might be made, with the disc and without, and the shorter time recorded. It would be almost impossible to devise a single durability test which could be related to the behaviour of a tablet in all the hazards it met from the die to the consumer.

MR. G. R. WILKINSON (London) asked for some amplification of whether "slackness" applied to the machine rate or to the operator. With reference to the author's remark concerning one punch out of 25 in a rotary compressing machine being wrongly adjusted, he said that he had yet to find a rotary machine where the punches could be individually adjusted. A

feature of the compression coated tablet was the time delay experienced between checking and the actual adjustment of the machine to rectify any fault found. He had found that a chargehand working with a pair of calipers could produce much better control than many of the statistical methods.

MR. D. STEPHENSON (Dartford) said that many rotary tablet machines had adjustable punches. He supported Mr. Richards' comment on the disintegration test. Many tablets containing vegetable extracts tended to stick to the under side of the disc.

MR. N. J. VAN ABBÉ (Loughborough) said that from the point of view of the consumer or of the public analyst it was not the remaining length of life for the tablet which mattered. The durability test was essentially a quality control for the manufacturer and not a test for the tablets at the time of use.

MR. A. R. ROGERS, in reply, said that he felt that the figures in the paper with reference to the ratio of range to standard deviation were correct. He was prepared to stand by his figure of 15 per cent. for a sample of 10 tablets. With regard to sequential analysis tests he maintained that his figures, even as amended, were different, but perhaps in practice the difference was not very important. He agreed that the sequential analysis test could supplement other tests and that the analyst in a manufacturing concern should make control charts available to the public analyst, but it was difficult to see how a body such as the Pharmacopoeia Commission could take cognizance of such a procedure. He also agreed that it would be useful to have more of the data which were in manufacturers' files. He had emphasised that it was desirable for tablet manufacturers to keep their products under statistical control in regard to weight variation. He had not a very wide experience of the new disintegration apparatus. He agreed that it would be better to leave the durability test as unofficial. Perhaps "slackness" was a loose term; it might have been better to say "worn punches".

STUDIES ON *LEONTICE LEONTOPETALUM* LINN.

PART I. THE ISOLATION OF THE CHEMICAL CONSTITUENTS OF *Leontice leontopetalum* AND SOME PRELIMINARY OBSERVATIONS ON THE PHARMACOLOGICAL ACTION OF LEONTICINE AND PETALINE CHLORIDE

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OUR attention was first drawn to *Leontice leontopetalum* Linn. in 1951 by Dr. W. M. Ford-Robertson of the Lebanon Hospital for Mental and Nervous Disorders, Asfuriyeh, Beirut, and the present investigation was undertaken at his request. We are indebted to him for supplying us with material, and with descriptions of the use of the drug as a native remedy for the treatment of epilepsy. The following account of its use is based on these descriptions, and on discussions between one of us (P. F. N.) and Dr. A. S. Manugian, of the Lebanon Hospital.

Dr. Manugian has made a special study of epilepsy in Lebanon, and, in the course of this work, found that a preparation of the tuberous roots of a plant which grows in parts of the Lebanon has a reputation for curing epilepsy. According to the descriptions which we received the stem and leaves of the plant are succulent, and the roots resemble soft watery potatoes. The "juice" prepared from the fresh roots, is bitter and poisonous. It is this juice which is used in the treatment, which we may add, is regarded as "kill or cure". We are informed, however, that several cures of confirmed cases of epilepsy have been observed.

For treatment[1] the tuber is dug up after the aerial parts have died down, cut into small pieces, and pounded in a mortar. The "juice" is given in a dose of about one teaspoonful three times daily. This dosage is continued for three days and during this time the patient is reported to be more or less in *status epilepticus*, having convulsions which recur every two or three minutes. The "juice" is prepared from freshly collected roots for each dose. At the end of this initial treatment the patient is given large quantities of an aqueous extract prepared from the marc left after the preparation of the "juice". The marc is pounded in water, and glassfuls of the extract thus prepared are given frequently every day, usually for a period of several months. The native prescribers say that this extract stops the convulsive action of the drug, but Dr. Manugian believes that this is not an essential part of the treatment.

The present investigation was begun with a small batch of the dried roots supplied by Dr. Ford-Robertson. The plant had been tentatively identified by Mr. R. W. Highwood of the British Council in Beirut as *Leontice leontopetalum* Linn. (Berberidaceae). To confirm this identification and to examine the growing plant in its natural habitat one of us

(P. F. N.) was enabled through the generosity of the Cross Trust, to arrange a short visit to Lebanon early in 1954. Specimens of the entire plant in the flowering and fruiting condition were collected and preserved. On comparison with authentic specimens at the British Museum of Natural History the identity of the material was confirmed as *Leontice leontopetalum* Linn. The part of the plant used as a drug is variously described as a root[2], tuberous root[3–5], corm[2], tuberous stem base[6], or tuberous rhizome[7]. Our observations up to the present indicate that it is an intercalary root tuber, but this awaits confirmation from histological investigations now in progress. A description of the plant is given in Part II.

Plants of *Leontice* species which have been investigated chemically are *Leontice eversmanni* Bge.[8–13] and *Leontice alberti*[11]. They are reported to contain a number of alkaloids, of which leontamine ($C_{14}H_{26}N_2$)[8,9] leontidine ($C_{15}H_{20}ON_2$)[8–11], pachycarpine (sparteine)[10,11] lupanine[10,11] leontine ($C_{15}H_{24}ON_2$)[10,13], isoleontine[11] and taspine ($C_{20}H_{19}O_6N$)[11,12] are found in *L. eversmanii*. *L. alberti* has been less extensively investigated, but has been shown to contain methylcytisine, leontine, leontidine, and an unidentified alkaloid[10]. There has been no report of pharmacological activity either of the plants, extracts, or constituent alkaloids. On the other hand, certain of the Berberis and related alkaloids including capnaurine[14], cryptopine[15], protopine[16], corycavine[17], corycavamine[17], corytuberine[17], glaucine[18], dicentrine[19], domesticine[20], pukateine[21], and boldine[22], have been shown to exhibit convulsant activity in experimental animals. And, with the discovery by us of alkaloids in *L. leontopetalum*, it seemed reasonable to suppose that the activity of the drug resided therein. However, as described in the sequel, *L. leontopetalum* resembles the related species *Caulophyllum thalictroides* (Berberidaceae) in possessing a high content of saponin, and in view of a recent report that caulosaponin, the crystalline glycoside from *C. thalictroides* and the corresponding sapogenin (now identified as hederagenin[23]), exhibit oxytocic activity[24], the work of isolating the alkaloids from *L. leontopetalum* was combined with an investigation of the saponin. We have to report, after careful and repeated examination of both caulosaponin and leontosaponin that we have been unable to find any evidence of oxytoxic activity.

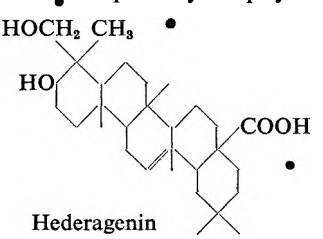
A. Chemical Examination of L. Leontopetalum

Small quantities of the drug were extracted with various solvents such as water, dilute acid and ethanol, and the resulting solutions examined qualitatively. The aqueous solution was saponaceous, and saponin was shown to be present by (*a*) the voluminous white precipitate with basic lead acetate (glycosides), (*b*) positive Molisch's reaction (carbohydrate), (*c*) reduction of Fehling's solution only after acid hydrolysis. Two alkaloid fractions were shown to be present in the ethanol extract, one extractable from ammoniacal or caustic soda solution with organic solvents. The second non-extractable fraction was freely soluble in water and presumed to be quaternary in type.

Isolation of Saponins

Several methods of isolating the saponins were examined. Water, although an effective solvent for saponin, was unsuitable, extracting much extraneous material and yielding gummy products. Three saponin fractions were obtained as powderable solids (total yield 17·3 per cent. of the drug), by extracting with absolute ethanol and fractionally precipitating the resulting extract with dry ether. It was essential to use dry solvents, and difficulties were visualised in translating the process to a large scale. Finally a modification of the method used by Power and Salway[25] for the isolation of caulosaponin from the roots of *C. thalictroides* was employed. Three crude saponins were extracted, saponin 1A, IIA, IIIA, which yielded a single pure saponin, leontosaponin. This was obtained as a colourless crystalline solid m.pt. 235–238° C. (decomp.), optically active $[\alpha]_D^{20}$ + 15·1°, soluble in water to give an almost colourless colloidal solution, slightly acidic in reaction (pH 6·8). Solutions give a copious and persistent froth, and they emulsify oils to form coarse emulsions, which soon break. Solutions readily hæmolyse red blood corpuscles yet appear to be relatively non-toxic *in vivo*; 30 mg. injected into mice (25 g.) showed no apparent effect.

Acetylation of leontosaponin with acetic anhydride and sodium acetate gave leontosaponin acetate. Comparison of the analytical data for leontosaponin and its acetate indicated that the saponin molecule incorporates a sugar chain of about six or more monosaccharide units. Support for this conclusion also comes from the low yield of sapogenin (29 per cent.) which was repeatedly obtained on hydrolysis of leontosaponin with dilute hydrochloric acid (N) in methanol. Hydrolysis proceeded smoothly under these conditions and produced much cleaner products than the more strongly acid conditions recommended by Wall and others[26] for the hydrolysis of steroidal saponins which, however, when adopted led to the isolation of a small quantity of phytosterol. The crystalline



Hederagenin

sapogenin $C_{30}H_{48}O_4$, m.pt. 333–334° C. $[\alpha]_D^{20}$ + 78°, was identified as the triperpenoid sapogenin, hederagenin[27–30], by direct comparison with authentic material, and by examination of the diacetate and dibenzoate. The sapogenin gave a yellow colour with tetranitromethane, and showed an ultra-violet absorption maximum (end absorption) at 210 mμ (ε 2860) as in authentic hederagenin (trisubstituted double bond). Evidence of the carboxyl group was obtained by titration, and by formation of a methyl ester. The proximity of the two hydroxyl groups to each other was demonstrated by the preparation of the ester acetonyl derivative.

The mother liquors from the above hydrolysis, after the isolation of sapogenin and subsequent neutralisation, gave positive reactions for reducing sugars. The presence of glucose was established by specific quantitative tests and the formation of glucosazone and glucose-*NN*-diphenylhydrazone. The solution also gave positive reactions in tests characteristic of pentose sugars. Chromatography of these solutions on paper using the solvent system *n*-butanol-ethanol-water[31] gave three spots[32], one due to traces of leontosaponin, the others attributable to glucose and arabinose, the identity of the latter being further confirmed by the preparation of arabinose-*N*-benzyl-*N*-phenylhydrazone.

Isolation of Alkaloids

Extraction of the alkaloids was severely handicapped by the high concentration of the saponin also present, and for this reason a large number of alternative extraction procedures were examined. One per cent. hydrochloric acid extracted a large proportion of the alkaloids but yielded only gummy products. Precipitation of the alkaloids with potassium mercuric iodide effectively separated them from solutions, but the complex could not be decomposed.

Maceration of the drug with ammonia or aqueous calcium hydroxide (later shown to be unnecessary), and subsequent extraction in turn with boiling 70, 90 per cent. and absolute ethanol, showed that extraction was not complete with dilute ethanol. The chloroform soluble alkaloid fraction from these extracts was gummy and gave a gummy picrate and hydrochloride. The water soluble (quaternary) alkaloid in the residual liquors was precipitated quantitatively with phosphotungstic acid. The precipitate, however, was slimy, extremely difficult to filter, and recovery of the alkaloid was poor. Mercuric chloride was found to be a more suitable precipitant, the complex being filterable and readily decomposed, although the recovered material was still impure.

The pure quaternary alkaloid was finally obtained after extraction of the chloroform-soluble alkaloids by precipitating as reineckate. The crude reineckate so obtained was readily purified by chromatography from acetone on alumina[33] and obtained as a pale pink amorphous solid, $C_{20}H_{22}O_3N\,[Cr(NH_3)_2(SCN)_4]$. Decomposition of the pure reineckate[34] gave the pure quaternary alkaloid chloride, designated petaline chloride as bright greenish-yellow scales, m.pt. 140–143° C. (decomp.), $[\alpha]_D^{20} + 11{\cdot}3°$. Petaline chloride, so obtained, has been formulated $C_{20}H_{22}O_3NCl,H_2O$, is extremely hygroscopic, readily soluble in water, methanol and ethanol, but insoluble in ether and chloroform. All attempts to obtain the chloride in a crystalline state failed, largely due to the facility with which it absorbs moisture. The formula of petaline was confirmed by conversion to a picrate, $C_{20}H_{22}O_3N,C_6H_2O_7N_3$; a platinichloride $(C_{20}H_{22}O_3NCl)_2PtCl_4$, and the reineckate already described. An iodide and a perchlorate were also prepared but these proved less suitable for characterisation purposes. Zeisal determination on the chloride and picrate indicated the presence of two methoxyl groups.

In a further attempt to purify the chloroform-soluble alkaloids, the use of ion exchange resins was not successful. Success was achieved with the precipitation of a reineckate, which when chromatographed on paper by the method of Milletti and Adembri[35] indicated the presence of two (possibly three) alkaloids, although there was considerable trailing with some of the spots. Recrystallisation from aqueous acetone, followed by chromatography from acetone on alumina, gave a small yield of a bright-red crystalline reineckate which when chromatographed on paper by the above method gave a single compact spot, $R_F = 0{\cdot}626$. Decomposition of the reineckate, however, yielded a pale brown viscous oil which failed to crystallise.

A crystalline fraction was finally isolated from the chloroform-soluble alkaloids by dissolving in acid, extracting from acid solution with ether and chloroform to remove impurities, basifying with ammonia, and then extracting with ether. A small proportion only of the total alkaloid could be extracted in this way, yielding a clear pale brown viscous oil, which partly crystallised from ethanol, to give a colourless crystalline optically inactive alkaloid, leonticine, m.pt. 118–119° C. The latter readily formed a reineckate, m.pt. 218–221° C. (decomp.), apparently identical with that obtained above. Leonticine has been tentatively formulated as $C_{20}H_{25}O_3N$. The oily residue from the ether extraction was distilled under reduced pressure, giving a colourless oily, strongly basic alkaloid, the properties of which closely resembled those reported for leontamine, the oily base from *L. evermannii*[8]. Insufficient material, however, has so far prevented complete characterisation of this fraction.

The ether insoluble fraction of the total chloroform-soluble alkaloids was precipitated as the reineckate. The latter, however, was not chromatographically homogeneous, and has not yet been resolved into its constituent fractions. Moreover, although decomposition of the reineckate gives a clear solution of the hydrochloride, concentration of the solution and extraction of the alkaloids by normal method leads to considerable decomposition. Work is proceeding on the further examination of this fraction.

Experimental

Melting points are uncorrected. Rotations were determined in 95 per cent. ethanol, unless otherwise stated, in a 1 dm. tube. Ultra-violet absorption spectra were determined in absolute ethanol on a Hilger Uvispek photoelectric spectrophotometer. We are indebted to Mr. W. McCorkindale and Dr. A. C. Syme for the microanalyses and to Mr. W. Gardiner for technical assistance.

Material. This was an authentic sample consisting of the root-tubers of *Leontice leontopetalum* Linn. (Berberidaceae), collected in the Bekaa Plain, Lebanon in the spring of 1953, sliced and dried.

Isolation of Leontosaponin

Method 1. The tuber (10 g.) in No. 80 powder was exhausted by boiling with water (3 portions each of 300 ml.). The combined extracts were

evaporated (water bath) to a syrup, filtered, and, whilst still hot, mixed, with stirring, with three times its volume of hot ethanol (60 per cent.), and again filtered. The filtrate poured dropwise into twice its volume of ethanol (90 per cent.) gave a flocculant gummy precipitate (0·68 g. after drying *in vacuo*) of crude leontosaponin. Addition of ether to the filtrate caused further precipitation of crude leontosaponin (0·25 g. after drying *in vacuo*).

Method 2. The tuber (400 g.) in No. 80 powder was exhausted by boiling with absolute ethanol (2 l.). The ethanol extract was filtered whilst still hot, and diluted by addition of excess dry ether, when crude leontosaponin was obtained as a yellow flocculent precipitate (saponin I), m.pt. 218–226° C. (decomp.) (45·7 g.). A further yield of 8·5 g. (saponin II) was obtained on concentrating the filtrate, and repeating the precipitation with dry ether. Further concentration and precipitation gave a small yield (3·3 g.) of a dark yellowish-brown saponin (saponin III), m.pt. 188–194° C. (decomp.).

Method 3. The drug (933 g.) in No. 80 powder was exhausted by boiling with absolute ethanol. The ethanolic extract was filtered whilst still hot, and on cooling deposited crude leontosaponin (70 g.) as a yellow solid m.pt. 217–221° C. (saponin I A). The filtrate evaporated to dryness gave a dark brown gummy residue, which, after the addition of water and steam distillation, was separated as a dark yellow solid (20 g.), m.pt. 212–222° C. (decomp.) (saponin II A). Repeated extraction of the aqueous filtrate with hot amyl alcohol (10 × 50 ml.), and evaporation of the solvent gave a further yield of dark yellowish brown saponin (62·6 g.), m.pt. 188–220° C. (decomp.) (saponin III A).

Purification of leontosaponin. Saponin I A was refluxed with absolute ethanol (1100 ml.), the solution filtered from the insoluble residue, and saponin reprecipitated by the addition of excess dry ether. The product was decolourised by triturating repeatedly with cold absolute ethanol, and finally crystallised from absolute ethanol to yield *leontosaponin* as a colourless crystalline solid, m.pt. 236–238° C. (decomp.) after drying *in vacuo* $[\alpha]_D^{20} + 15{\cdot}1°$. (Found: C, 51·9, 51·5; H, 8·1, 8·1 per cent.) The ethanol-insoluble residue, dissolved in methanol, precipitated with ether, and recrystallised from methanol-ether (charcoal) gave leontosaponin, m.pt. 236–238° C. (decomp.). Recrystallisation of saponins II A and III A, from ethanol similarly gave leontosaponin.

Leontosaponin acetate. Leontosaponin (0·76 g.) was heated under reflux (water bath) with acetic anhydride (10 ml.) and sodium acetate (0·56 g.) until solution was complete (30 min.) and thereafter for 2 hours. The reaction mixture was cooled, and poured onto crushed ice (2 g.), and mixture cooled in ice for 30 minutes. The solution was neutralised with NaOH (20 per cent.), and the gummy crystalline precipitate extracted with ether. The ethereal solution, dried (Na_2SO_4) and evaporated, yielded a pale cream precipitate (0·54 g.), which, recrystallised from ethanol (70 per cent.), gave *leontosaponin acetate* as an almost colourless crystalline solid, m.pt. 155–156° C. (Found: C, 55·76, 55·69; H, 6·7, 6·8 per cent.)

Hydrolysis of Leontosaponin

Method 1. Leontosaponin (1·0 g.) was refluxed with 50 per cent. aqueous ethanolic hydrochloric acid (40 ml., 4N) and benzene (16 ml., previously equilibrated by shaking with an equal volume of 50 per cent. aqueous ethanol), for four hours. The sapogenin, precipitated as a pale buff solid was refluxed with benzene (150 ml. including the above fraction) and methanolic potassium hydroxide (50 ml.; 20 per cent.) for one hour. The benzene extract was washed with water, dried (Na_2SO_4), and evaporated to yield a yellow solid (20 mg.) m.pt. 112–120° C., giving the typical colour reactions of a phytosterol. Dilution of the methanolic potassium hydroxide solution with water (50 ml.) gave a colourless crystalline precipitate (265 mg.; 26·5 per cent.) of potassium leontosapogenin.

Method 2. Crude leontosaponin (35 g.) was heated with a mixture of methanol (900 ml.) and dilute hydrochloric acid (190 ml., 3N) under reflux on a water bath for five hours. After dilution with water (200 ml.), methanol was removed by distillation, and the precipitated sapogenin (8·64 g.; 24·7 per cent.) separated by filtration. The product, recrystallised from 80 per cent. ethanol (charcoal), gave *leontosapogenin*, m.pt. 332–333° C. (slight sintering at 319°), ϵ max. 2860 at 210 mμ, $[\alpha]_D^{20}$ + 79·6° (*c*, 0·10, $[\alpha]_D^{20}$ + 80·4 (*c*, 1·0 in pyridine). Jacobs[29] gives m.pt. 332–334° C., $[\alpha]_D^{20}$ + 81° (*c*, 2·00 g. in pyridine) for hederagenin. Mixed melting point of leontosapogenin with authentic hederagenin 332–333° C. (Found: C, 76·01, 76·3; H, 10·1; 10·5; equiv. 474·1. Calc. for $C_{30}H_{48}O_4$: C, 76·2; H, 10·2 per cent.; equiv. 472·7.) The following yields of sapogenin were obtained in a series of hydroyses:—26·35; 25·2; 27·4; 28·8; 21·6; and 24·5 per cent.

Leontosapogenin diacetate. Leontosapogenin (0·47 g.) refluxed with acetic anhydride (5 ml.) for 1 hour gave leontosapogenin diacetate (from 1:1-aqueous methanol), m.pt. 173–174° C. (sintering at 157–158° C.), $[\alpha]_D^{20}$ + 66·4° (C, 2·08). Jacobs[29] gives m.pt. 172–174° C, $[\alpha]_D$ + 64° (*c*, 1·0) for hederagenin diacetate. Mixed m.pt. with hederagenin diacetate, 172–172° C. (Found: C, 73·3; H, 9·6 Calc. for $C_{34}H_{52}O_6$: C, 73·3; H, 9·4 per cent.)

Leontosapogenin dibenzoate. Leontosapogenin (2 g.), refluxed with benzoyl chloride (2 ml.) in pyridine (20 ml.) and the mixture poured into aqueous sodium bicarbonate, gave leontosapogenin dibenzoate, m.pt. 290–291° C., $[\alpha]_D^{20}$ + 114·5° (*c*, 0·1 in $CHCl_3$). Jacobs[29] gives for hederagenin dibenzoate, m.pt. 290–291°C. Mixed m.pt. with hederagenin dibenzoate, 290–291° C. (Found: 3, 77·4; H, 8·3. Calc. for $C_{44}H_{56}O_6$, C, 77·6; H, 8·3 per cent.)

Leontosapogenin methyl ester. Leontosapogenin (1 g.) was shaken with excess diazomethane in ether. The ether solution on evaporation yielded a residue which on crystallisation from aqueous methanol gave leontosapogenin methyl ester, m.pt. 236–237° C., $[\alpha]_D^{20}$ + 74·5° (*c*, 1·018). Jacobs[29] gives for hederagenin methyl ester, m.pt. 238–240° C., $[\alpha]_D$ + 75° (*c*, 1·0). Mixed melting point with hederagenin methyl ester (m.pt. 236–

237° C.), 236–237° C. (Found: C, 76·1, H, 10·3. Calc. for $C_{31}H_{50}O_4$: C, 76·5; H, 10·4 per cent.)

Leontosapogenin methyl ester diacetate. Leontosapogenin methyl ester (0·177 g.) refluxed with acetic anhydride (4 ml.) for 1 hour yielded leontosapogenin methyl ester diacetate as needles, m.p. 193–194° C., $[\alpha]_D^{20}$ + 63° (c, 0·564). Van der Haar[27] gives for hederagenin methyl ester diacetate, m.p. 193° C., $[\alpha]_D^{20}$ + 62° (in absolute ethanol). Mixed m.p. with hederagenin methyl ester diacetate, 192–193° C. (Found: C, 73·8; H, 10·0. Calc. for $C_{35}H_{54}O_6$: C, 73·6 H, 9·5 per cent.)

Acetonyl leontosapogenin methyl ester. A solution of leontosapogenin methyl ester (74 mg.) in acetone (3 ml.) with 3 drops of concentrated hydrochloric acid slowly deposited platelets of acetonyl leontosapogenin methyl ester, m.p. 250–251° C. (from absolute ethanol), alone or mixed with acetonyl hederagenin methyl ester. (Found: C, 77·5; H, 10·3 Calc. for $C_{34}H_{54}O_4$: C, 77·5; H, 10·3 per cent).

Isolation and identification of sugars formed by hydrolysis of leontosaponin. The aqueous filtrate remaining after the separation of leontosapogenin (above) was neutralised with ammonia and decolourised by boiling with activated charcoal. The colourless filtrate gave a positive reaction in Molisch's test for carbohydrates, reduced both Fehling's solution and Barfoed's reagent, gave a blue-green colour in Bial's test (conc. hydrochloric acid and ethanolic orcinol-ferric chloride), and a cherry-red colour in the aniline test (glacial acetic acid and aniline), both the latter reactions being indicative of pentose sugars.

The solution (5 ml.) heated with phenylhydrazine hydrochloride (4 g.) and sodium acetate (0·6 g.) in a boiling water bath for 10 minutes deposited a crystalline osazone from hot solution, m.p. 201–204° C., and crystalline form characteristic of glucosazone. On further heating the reaction mixture gave a crystalline product, showing a variety of crystalline forms, some characteristic of arabinosazone.

The solution (2 ml.) was treated with a small quantity of lead acetate and boiled. Dilute ammonia solution (5 ml.) was added and the solution boiled again, when a salmon-pink precipitate was obtained, indicative of glucose.

The solution was spotted on Whatman No. 1 filter paper and chromatographed using the solvent system *n*-butanol-ethanol-water, and upward development for 15 hours[31]. After drying, the paper was sprayed with ·3 per cent. *p*-anisidine hydrochloride[32], and showed three spots against a pink background, (*a*) yellow R_F = 0·434, (*b*) brown-red R_F 0·259 and (*c*) yellowish-brown R_F 0·214. Control experiments with leontosaponin and known sugars gave the following comparative R_F values, leontosaponin, 0·434 (yellow), arabinose, 0·265 (brown-red), and glucose, 0·213 (yellowish-brown).

The crude sugar residue (3·3 g.) obtained by evaporation of the solution from the hydrolysis, was dissolved in 50 per cent. ethanol (14 ml.). *N*-Benzoyl-*N*-phenylhydrazine (4·09 g.) in absolute ethanol (33 ml.) was added, and the mixed solution left for 24 hours, when a crystalline precipitate was obtained of arabinose-*N*-benzyl-*N*-phenylhydrazone, m.p. 173·5–

174° C. (from 75 per cent. ethanol), undepressed on admixture with authentic arabinose-*N*-benzyl-*N*-phenylhydrazone. (Found: C, 65·6; H, 6·8; N, 8·5. Calc. for $C_{18}H_{22}O_4N_2$: C, 65·4; H, 6·7; N, 8·5 per cent.) The mother liquors were refluxed with solution of formaldehyde (10 ml.; 40 per cent.) on a water bath for one hour, and the oily formaldehyde -*N*-benzyl-*N*-phenylhydrazone extracted with ethyl acetate. The aqueous solution, which gave reactions for hexoses, but not pentoses, was evaporated to dryness. The residue in water (1 ml.) was refluxed with *NN*-diphenylhydrazine (0·66 g.) in ethanol (17 ml.) to yield glucose-*NN*-diphenylhydrazone, m.pt. 159–160·5° C., undepressed on admixture with authentic glucose-*NN*-diphenylhydrazone. (Found: C, 62·6; H, 6·6; N, 8·1. Calc., for $C_{18}H_{22}O_5N_2$: C, 62·4; H, 6·4; N, 8·1 per cent.)

Isolation of the Alkaloids

(*a*) The drug (100 g.) in No. 60 powder was exhausted by percolation with 1 per cent. hydrochloric acid (1830 ml.). A portion of the percolate (610 ml.) was made alkaline with ammonia, and completely extracted with chloroform. The combined chloroform extracts were washed with water, dried (Na_2SO_4), and evaporated to a dark brown gummy extract (0·213 g.; 0·64 per cent.). A further portion of the percolate (610 ml.) was treated with solution of potassium mercuri-iodide (30 ml.), when the alkaloid complex was precipitated as a dark-brown amorphous solid (0·614 g.). The latter (0·2 g.) was heated on a water bath with saturated aqueous sodium carbonate (30 ml.) for 30 minutes. After filtering, the solution was neutralised with hydrochloric acid, basified with ammonia and extracted with chloroform. The combined chloroform extracts, washed with water, dried (Na_2SO_4) and evaporated, yielded a trace of pale brown alkaloid (15 mg.). Similar treatment with 20 per cent. aqueous sodium hydroxide yielded only traces of alkaloid insufficient for further investigation.

(*b*) The drug (5 kg.) was moistened with dilute solution of ammonia, macerated with ethanol for 24 hours, and percolated to exhaustion with ethanol (10·5 l.). The percolate was concentrated to 3 l., cooled, filtered, acidified with dilute sulphuric acid to pH 4·0, filtered, concentrated to remove ethanol and again filtered. The filtrate was made alkaline with ammonia, and extracted completely with chloroform. The combined chloroform extracts were washed with water, dried (Na_2SO_4), and evaporated to a dark gummy solid (22·85 g.; 0·46 per cent).

The crude base (6·43 g.) was dissolved in dilute hydrochloric acid, filtered, the solution poured onto a column of Zeo-Karb 225 (30 g.) and the column washed with water until the eluate was free from acid. Dilute hydrochloric acid failed to displace the alkaloids, but elution with concentrated hydrochloric acid (65 ml.) gave a solution, without resolution of the components, which when basified with ammonia and extracted with chloroform gave a dark-brown gummy residue of alkaloids (4·79 g.).

The crude base (54 mg.) suspended in water (3 ml.) and treated with saturated aqueous ammonium reineckate, gave a pale pink amorphous reineckate m.pt. 180–182° C. (decomp.) (from aqueous acetone). The

reineckate, in acetone was spotted on Whatman No. 1 filter paper, and chromatographed using the solvent system pyridine-water (1:4) and upward development for 16 hours[34]. After drying, the papers were sprayed with a solution of potassium bismuth iodide in dilute acid, and showed evidence of three spots with R_F values about 0·92, 0·62, 0·50, though with considerable tailing.

The ammoniacal solution (1 l.) remaining after the isolation of chloroform-soluble alkaloids was acidified to pH 2·0 with hydrochloric acid and treated with saturated aqueous mercuric chloride (2 l.), when the mercuric chloride complex of the quaternary alkaloid was obtained as an amorphous brown precipitate (39·1 g.). The latter was suspended in ethanol (95 per cent.) and hydrogen sulphide passed into the solution. The solution, filtered from mercuric sulphide, and evaporated gave crude petaline chloride as a dark-brown powderable solid (19·85 g.; 0·4 per cent.).

(*c*) The drug (14·106 kg.) in No. 80 powder was completely extracted by boiling with industrial spirit, and the extract concentrated to give a dark brown soft extract (5·4 kg.). 3·5 kg. of the crude extract was treated with dilute sulphuric acid (5 l.), filtered from precipitated saponin, basified with ammonia, and extracted with chloroform. The combined chloroform extracts were washed with water, dried (Na_2SO_4), and evaporated to give a dark-brown crude alkaloid (66·5 g.).

The ammoniacal solution remaining after the isolation of chloroform-soluble alkaloids was acidified with dilute sulphuric acid to Congo red, and treated with saturated aqueous ammonium reineckate solution to precipitate crude petaline reineckate (216 g.). The crude reineckate (137·4 g.) was dissolved in dry acetone (1 l.), filtered from the pale brown insoluble residue (saponin, 50 g.) and the solution chromatographed on a column of alumina (1 kg.) previously washed with dry acetone (500 ml.). Development of the chromatogram with dry acetone, yielded after elution of a small yellow band, a solution of the pure reineckate. Careful evaporation of the acetone eluate below 40° C. gave petaline reineckate m.pt. 179–181·5° C. as a pale pink solid, (49·95 g.). (Found: C, 44·77; H, 5·2 equiv. wt., 678·2; 679·5. $C_{20}H_{22}O_3N$ $[Cr(SCN)_4(NH_3)_2]$ requires C, 44·80; H, 4·4 per cent.; equiv. wt., 642·8.) R_F when chromatographed on paper using pyridine-water (1 : 4), as described above, 0·68. The reineckate, dissolved in acetone (1 l.) was titrated with silver sulphate solution (0·6 per cent. w/v) until no further precipitate of silver reineckate was formed. Sulphate ions were displaced by quantitative precipitation with barium chloride, the solution filtered, the precipitate repeatedly washed with acetone, and the combined filtrate and washings evaporated (below 60°) to yield *petaline chloride*, m.pt. 140–143° C. (decomp.), $[\alpha]_D^{20}$ + 11·3° as pale grenish-yellow scales (26·6 g.; 100 per cent). (Found, C, 63·35; H, 6·8; N, 3·8; OMe, 16·9. $C_{20}H_{22}O_3NCl \cdot H_2O$ requires C, 63·4; H, 6·65; N, 3·9; OMe, 17·2 per cent.)

Petaline picrate.—Petaline chloride (481 mg.) in water, treated with saturated aqueous sodium picrate gave *petaline picrate*, m.pt. 165·5–166° C. (decomp.) (from absolute ethanol). (Found C, 56·43; H, 4·4; OMe 11·4. $C_{26}H_{24}O_{10}N$ requires C, 56·52; H, 4·4 OMe 11·2 per cent.)

Petaline chloroplatinate. Petaline chloride (100 mg.) in water (10 ml.) acidified and treated with 5 per cent. aqueous platinic chloride gave *petaline chloroplatinate*, m.pt. 197–198° C. (decomp.) (from absolute ethanol). (Found: C, 44·34; H, 4·1; Pt, 18·18. $(C_{20}H_{22}O_3NCl)_2PtCl_4$ requires C, 45·42; H, 4·2; Pt, 18·47 per cent.)

Leonticine reineckate.—The crude chloroform-soluble alkaloid (1·8 g.) in ethanol (100 ml.) treated with saturated aqueous ammonium reineckate, gave a crystalline reineckate m.pt. 218–221° C. (decomp.) after recrystallisation and chromatography from acetone on alumina. R_F when chromatographed on paper using pyridine-water (4:1) as described above 0·625.

Leonticine and leontamine.—The crude chloroform-soluble alkaloid (10 g.) was dissolved in dilute sulphuric acid (300 ml.), extracted first with ether (rejected), and then with chloroform (rejected), basified with ammonia and extracted with ether. The combined ethereal extracts were washed with water, dried (Na_2SO_4), and evaporated to yield a pale brown viscid oil, which partly crystallised to yield *leonticine* as a colourless needles, m.pt. 118·5–119·5° C. (from 80 per cent. methanol) $[\alpha]_D^{20}$ + 0°. (Found: C, 73·34; H, 7·59; N, 4·44, 4·50. $C_{20}H_{25}O_3N$ requires C, 73, 36; H, 7·7; N, 4·27 per cent.) The oily residue was distilled to yield a colourless oily base, b.pt. 155–160° C. (bath)/4 mm., n_D^{20} 1·5115. (Found: C, 74·73; H, 11·4; N, 12·24. Calc. for $C_{14}H_{26}N_2$ C, 75·61; H, 11·8; N, 12·6 per cent.) Orekhov and Konovalova[8] give for leontamine, b.pt. 118–119° C./4 mm., n_D 1·5113.

B. Preliminary Pharmacological Investigations

A previous examination by Dr. G. Brownlee of King's College, London, of crude base extracts of *Leontice leontopetalum* indicated the presence of pharmacologically active compounds, and prompted the present preliminary investigation of the quaternary and tertiary bases subsequently isolated.

Intravenous Administration of Petaline Chloride in Mouse

Freshly prepared solutions in normal saline were administered to mice of 25 g. body weight. At doses below 0·05 mg. per mouse recovery from injection was immediate and no functional disturbance was observed. Administration of 0·05 mg. per mouse produced symptoms of sedation and slowed respiration; recovery was complete in approximately 3 minutes and the animals showed no subsequent disorder.

In a group of 10 mice receiving 0·075 mg. per mouse there was one immediate death from respiratory failure, the heart continuing to beat for about 45 seconds after the cessation of respiration; two mice recovered after a period of sedation and reduced respiratory activity lasting $4\frac{1}{2}$ minutes; the remaining 7 mice showed acute respiratory distress with irregular shallow respiratory movements together with varying degrees of stimulation of the central nervous system from slight twitchings of the

extremities to co-ordinated clonic spasms. In the latter, recovery was slow but was complete in all instances in 9–10 minutes after which the animals appeared normal.

Administration of a dose of 0·08 mg. per mouse in a group of 8 mice resulted in death from respiratory failure within 30 seconds of injection in 7 mice, with no convulsive activity; the remaining animal recovered slowly and exhibited mild convulsive activity. In all groups receiving doses of 0·1 mg./mouse and above, death from respiratory failure immediately followed injection.

It is concluded that the Mean Lethal Dose of petaline chloride in mouse is approximately 3·1 mg./kg.

Intravenous Administration of Petaline Chloride in Rabbit

From a preliminary investigation it was apparent that the rabbit is much more tolerant of petaline chloride than the mouse. Accordingly a dose of 20 mg. was injected intravenously in a female rabbit of 1·802 kg. body weight. This amount produced no apparent effect in the rabbit and after an interval of 5 minutes a further 10 mg. was injected; the rabbit immediately showed respiratory depression accompanied by mild clonic convulsive movements of the limbs. Within 2 minutes both convulsive and respiratory movements ceased though the heart continued to beat for a further 1 minute. The animal did not recover.

A second female rabbit of 2·725 kg. body weight was injected slowly until symptoms of respiratory failure were produced (after injection of 40 mg.). The animal showed convulsive movements of the limbs as in the previous case and about 1 minute after the termination of the injection respiration ceased, the heart continuing to beat. After a period of apnœa lasting 1 minute respiration recommenced spontaneously and the animal recovered rapidly, resuming its normal activity in 7 minutes. After 48 hours the animal was again injected with 40 mg. petaline chloride; on this occasion only slight respiratory depression was noted and after an interval of 5 minutes, a further 5 mg. was administered. The effects previously observed were repeated and, as spontaneous respiratory movements did not recommence, artificial respiration was attempted; by this means the heart was maintained for a period of 3 minutes after which it gradually slowed and stopped.

It is concluded that the effects of petaline chloride are similar in both mouse and rabbit, and though in the latter the Mean Lethal Dose is much greater, being approximately 15·6 mg./kg.

The Effect of Petaline Chloride on the Frog Rectus Abdominis muscle Preparation

The prepared muscle was mounted in an isolated organ bath of 5 ml. capacity and maintained at 15° C. In all cases the spasmogen used was 5·0 μg. of acetylcholine in oxygenated frog Ringer. Between the recording of contractions the muscle was washed with 3 changes of Ringer's fluid

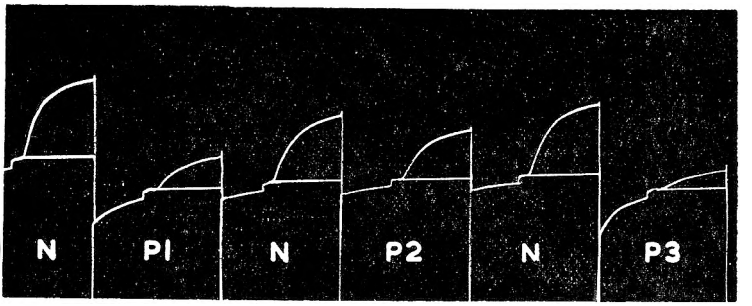
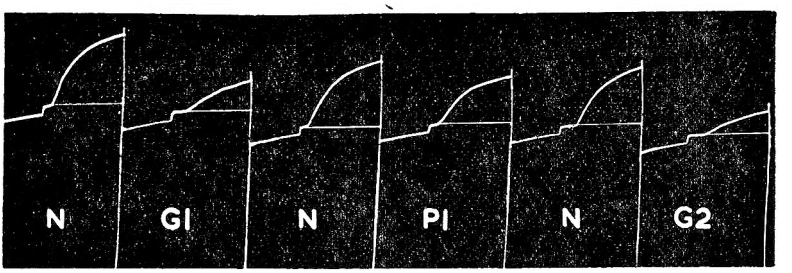


FIG. 1. Response of frog rectus abdominis muscle to acetylcholine 5·0 μg.
N. Normal contraction
P1. Contraction after application of petaline chloride 100 μg.
P2. ,, ,, ,, ,, ,, ,, 33 μg.
P3. ,, ,, ,, ,, ,, ,, 200 μg.



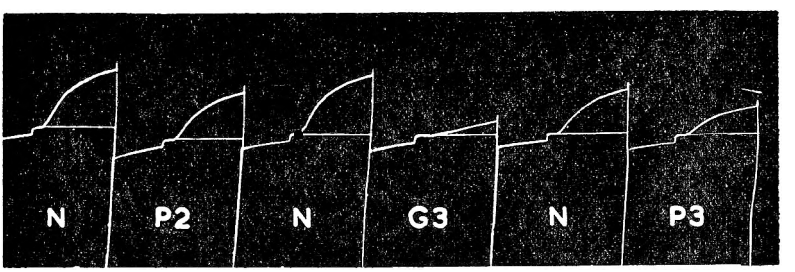


FIG. 2. Response of frog rectus abdominis muscle to acetylcholine 5·0 μg.
N. Normal contraction.
P1. Contraction after application of petaline chloride 25 μg.
P2. ,, ,, ,, ,, ,, ,, 33 μg.
P3. ,, ,, ,, ,, ,, ,, 50 μg.
G1. Contraction after application of gallamine triethiodide 25 μg.
G2. ,, ,, ,, ,, ,, 33 μg.
G3. ,, ,, ,, ,, ,, 50 μg.

and rested for a period of 4 minutes. The antagonist drugs (petaline chloride and gallamine triethiodide) were added to the bath for a period of 3 minutes before the addition of the acetylcholine. Contraction was recorded for a period of 1 minute.

Typical inhibitory effects of petaline chloride are shown in Figure 1, and compared in Figure 2 with gallamine triethiodide. Mean dose response values to petaline chloride and gallamine triethiodide plotted from a number of observations on the frog rectus muscle are shown in Figure 3. They indicate the relative potency of petaline chloride to gallamine triethiodide to be 0·643 : 1.

Addition of physostigmine (50 μg.) to the bath antagonised the blocking action of petaline chloride.

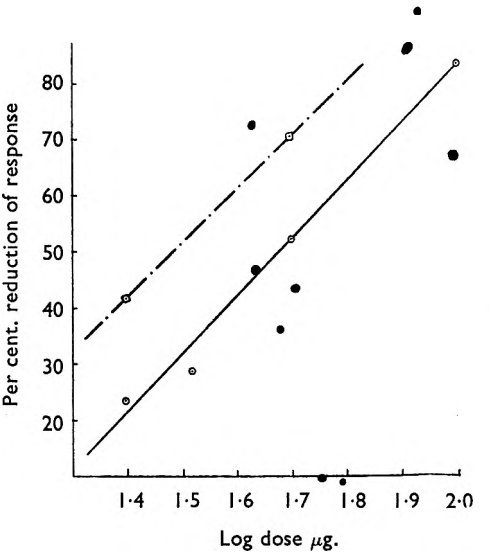


FIG. 3. Inhibition of response of frog rectus abdominis muscle to acetylcholine (5 μg.).
— · — Gallamine
——— Petaline

Intravenous Administration of Leonticine in the Mouse

Solutions of leonticine in 0·05 per cent. aqueous tartaric acid (pH 5·2) were administered to mice of 25 g. body weight.

With doses of 0·5 mg./mouse or less recovery from injection was immediate and no symptoms were observed. Administration of 0·66 mg./mouse produced in all cases evidence of respiratory and cardiac embarrassment; respiration was shallow and slow and the heart rate decreased appreciably. Recovery was protracted, normal activity being resumed after about 15 minutes. In no case was there any convulsive activity though in the majority of individuals there was evidence of increased sensitivity to stimulus, the animal being irritable to touch.

In a group of 8 mice which received 0·7 mg./mouse, 2 instances of complete respiratory failure followed immediately by cardiac failure were recorded; in 4 mice there was slow recovery after an initial period of coma during which the animals were markedly cyanosed. In the remaining 2 mice the period of coma was preceded by relatively powerful clonic spasms of the limbs which lasted for 45 seconds; one of these animals subsequently recovered slowly but in the other the period of coma was terminated by respiratory and cardiac failure.

All groups receiving 0·8 mg./mouse or above showed almost immediate respiratory failure followed in a few seconds by stoppage of the heart. It is concluded that the Mean Lethal Dose of leonticine in mouse is approximately 33·0 mg./kg.

Summary

1. Certain of the chemical constituents of *L. leontopetalum* Linn. have been isolated. The saponin, leontosaponin, yields hederagenin, glucose and arabinose on acid hydrolysis.

2. A small quantity of a crystalline alkaloid, leonticine, and of a colourless oily alkaloid (possibly identical with leontamine) have been obtained from the ether-soluble fraction of the total chloroform-soluble alkaloids.

3. Precipitation of the ammoniacal liquors remaining after the extraction of the chloroform-soluble alkaloids with ammonium reineckate yields a water-soluble alkaloid, petaline chloride, $C_{20}H_{22}O_3NCl,H_2O$.

4. Petaline chloride acts as a central nervous depressant in both mouse and rabbit. It also shows anti-acetylcholine activity on isolated skeletal frog muscle.

5. The effect of leonticine in the mouse appears to be similar to that of petaline chloride, although it is significantly less potent, and bulbar paralysis is preceded by active clonic spasms.

We wish to thank Dr. W. M. Cumming and Mr. D. J. Duff of British Dyewood Co. Ltd. for facilities and help with the large scale extractions. We wish also to thank the Cross Trust and the McCallum Bequest for financial help, and the Pharmaceutical Society of Great Britain for an educational grant to one of us (J. McS.).

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DISCUSSION

The paper was presented by MR. J. McSHEFFERTY.

DR. W. MITCHELL (London) said he did not think that alcohol was always the best solvent for drug extraction, because one often extracted too much foreign material. A good general method for alkaloids was to damp the powdered drug with lime and water and then extract with a water-immiscible solvent, such as benzene. He was aware that in the case under discussion petaline would be lost, but other alkaloids might have been extracted in a purer form. Again, the old method of crystallising alkaloids as neutral or acid citrates, tartrates, or oxalates might have been worth while, and fractional extraction of the hydrobromides with chloroform might have produced results. In purifying the saponin he wondered whether the initial purification by alcohol was really necessary, and whether it could have been omitted.

MR. J. J. LEWIS (Glasgow) asked whether the authors had tested the alkaloids for cardiac activity. He also wondered what method they had used for testing for oxytocic activity, and whether they had compared compounds and extracts for anti-convulsive activity against electrically induced convulsions using known anti-convulsive drugs. It would also be interesting to know on what part of the nervous system the drugs were acting. Again, why did the authors use gallamine as a comparison substance and not tubocurarine? When petaline was injected into the rabbit it would be useful to know whether there was any evidence of curareform activity.

MR. N. J. VAN ABBÉ (Loughborough) said it would be interesting to know what sort of cure was claimed for the material.

DR. F. HARTLEY (London) referred to the hydrolysis of leontosaponin and said that the choice of a weaker acid than was often used with steroidal saponins seemed to require a longer time for hydrolysis than four hours in method 1, and five hours in method 2. As the sapogenin yield was in part used as support for the analytical data for the carbohydrate side chain it would be worth checking that eight hours gave no significant addition to the yield.

MR. J. McSHEFFERTY, in reply, said that they had tried moistening the drug with lime and extracting with solvents such as benzene, but this did not extract the alkaloid. Alcohol was the best solvent for the extraction of the alkaloidal fraction. He had endeavoured to separate the tertiary basic fraction by formation of tartrates, but it was not as successful as

the method adopted. The saponin was not very soluble in boiling alcohol, and a large volume had to be used. No test had been made for cardiac activity. The method used for testing oxytocic activity employed the isolated uterus of the guinea-pig. He had no detailed information of the part of the central nervous system on which the alkaloids acted. Both gallamine and tubocurarine were used, and it was found that the former gave the best comparable results with petaline. A number of hydrolyses had been carried out using different conditions and periods of time, and the yields quoted for the particular time were approximately the maximum yields obtainable. The yield was not significantly raised by continuing hydrolysis for another eight hours.

MR. P. F. NELSON, in reply, said that treatment with the drug was regarded as a kill or cure.

DR. J. B. STENLAKE, in reply, said that cardiac activity had not been investigated. Oxytocic activity was measured on the isolated organ and compared with ergometrine.

STUDIES ON *LEONTICE LEONTOPETALUM* LINN.

PART II. HISTORY, SOURCES AND MACROSCOPICAL CHARACTERS OF THE PLANT *L. leontopetalum*

BY P. F. NELSON AND F. FISH

From the School of Pharmacy, The Royal College of Science and Technology, Glasgow

Received June 28, 1956

IN a previous communication[1] attention was drawn to the use in the Middle East of *Leontice leontopetalum* Linn. (Berberidaceae) as a drug and a report of the chemical constituents of the dried root and their pharmacological action was given. A number of inadequate descriptions of the plant, or parts of the plant, appear in the literature, particularly in older works[2–15], but there is no complete accurate description of the entire plant. The present communication gives a detailed description of the macroscopical appearance of the plant, illustrated by means of photographs and line drawings. Brief notes on the history of the drug, and the geographical distribution of the plant are given. The materials used consisted of samples given by Dr. W. M. Ford-Robertson and his colleagues in 1951, 1952 and 1953 and of specimens collected by one of us (P. F. N.) in Lebanon in 1954.

HISTORY

In his work *The Greek Herbal of Dioscorides*, Gunther[16] mentions two drugs, Leontopetalon and Chrusogonon. The former is there identified as *Leontice chrysogonum* and the latter as *L. leontopetalum*, but this latter identification cannot be definite since the inner part of the root of Chrusogonon is said to be strongly red, whereas that of genuine *L. leontopetalum* is yellow. Lovell[17] in his *Compleat Herball* of 1665 makes similar mention of these plants. Linnaeus[2,3] and Jussieu[4] gave brief descriptions of the plant *L. leontopetalum* and it was further described by various later authors[5–15], of whom Loudon[5] mentions its introduction into Britain in 1597. It has been used as a soap substitute[11,18,19], a snakebite antidote[11], a corrective for overdoses of opium[18] and as a remedy for epilepsy[11,13,15]. As indicated in a previous communication[1] the present study was commenced as a result of the current use of the root in the Middle East as a native remedy for the treatment of epilepsy.

GEOGRAPHICAL DISTRIBUTION

The plant has been reported to inhabit an area including those countries bordering the eastern part of the Mediterranean[5] from Italy[3,7,15], Greece[3,7] and Turkey[3,6,7,15,18], through Lebanon[13], Syria[7,11,13], Israel[7,11,13], Jordan[13] and Iraq[7] to north-eastern parts of Egypt[13]. It has also been seen on the island of Cyprus[7,20].

All the specimens which we have studied were collected in the Bekaa Plain in Lebanon, although in a brief visit to Syria the plant was also

observed growing in a pass through the Anti-Lebanon Mountains. The Bekaa Plain lies at an altitude of 500 to 1000 metres between the Lebanon and Anti-Lebanon mountain ranges; it is about 12 kilometres from west to east and some 48 kilometres from north to south. The plant was found to grow abundantly in wheat fields at an altitude above 650 metres and specimens were observed growing in fields on the sides of the surrounding mountains up to 1050 metres. Post[13] reported that it was to be found near Sidon which is at sea level, but none was observed by us in this area. However, it was reported that in Cyprus it had been seen growing at about sea level[20].

Extremes of climate are experienced in the Bekaa Plain, the fields being under snow for much of the winter while the summer is very hot with little or no rainfall. The land is extensively cultivated both in the heavy red loam which is found in most areas and in the denser clay which also occurs. According to the local farmers, the plant will not grow in fields where water lies for any length of time. There were many other fields where the plant did not grow, but all of these appeared to have a shallow top soil and to be very stony, whereas the swollen roots usually grow at a depth of 20 to 30 cm.[13]. The majority of plants in any particular field were found to be roughly at the same stage of development, although sometimes both flowering and fruiting plants were observed growing side by side. In addition a few plants bearing leaves only were found. It was observed that the plants growing at lower altitudes were more advanced, presumably because of the somewhat higher temperature.

Macroscopical Characters

The plant is a perennial herb with large compound leaves, a compound inflorescence of yellow flowers and a large intercalary root tuber. The inflorescence grows to a height of about 29 to 45 cm. while the foliage leaves usually lie close to the surface of the ground both in plants with, and without an inflorescence.

Inflorescence. (Figs. 1, 2, 3.) The compound inflorescence is a panicle composed of about 8 to 18 racemes. The mature panicle has a maximum diameter of about 22 to 28 cm. and occupies about one-half of the total height of the aerial part of the plant. The lateral racemes arise from the axils of small foliage leaves which vary greatly in shape and size and have a phyllotaxis of 3/8. The lateral racemes vary considerably in length on any one plant, becoming smaller towards the apex of the inflorescence and they also bear varying numbers of flowers. A typical plant examined had 17 lateral racemes varying in length from 7 to 18 cm. each bearing between 9 and 30 flowers. The apical raceme also varies from plant to plant, and as a rule bears more flowers than any of the lateral racemes, usually from about 28 to 48.

Stem. (Fig. 4.) The upright stem is cylindrical in shape and about 0·6 to 1·2 cm. in diameter, the underground portion being white to very pale green in colour, while the aerial stem is pale green with a purplish zone at ground level. The surface of the stem has very faint longitudinal striations. The cut transverse surface of the stem shows a thin

epidermis, a narrow cortex and a zone of lignified pericycle surrounding a large central area containing several irregular rings of collateral vascular bundles, which lie more or less radially and surround a central pith. Each of the inner vascular bundles has a separate well defined pericycle, and here, as in the outer zone of pericycle, very large spaces occur. In

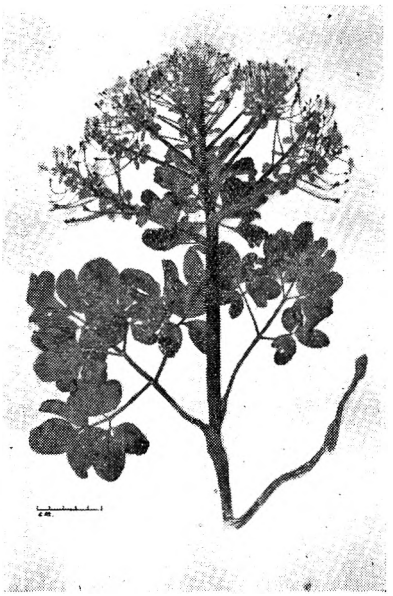

FIG. 1. *Leontice leontopetalum* flowering top (pressed specimen) × 1/6.

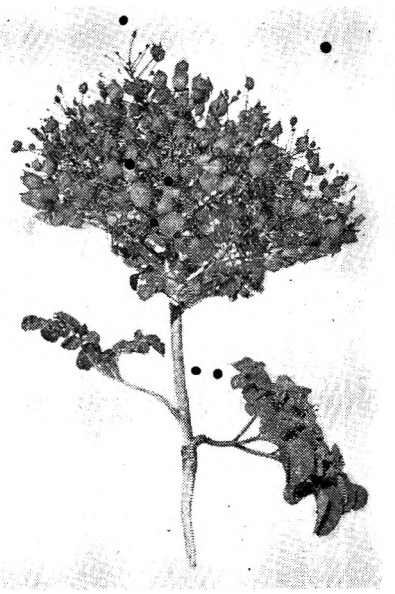

FIG. 2. *Leontice leontopetalum* fruiting top (fresh specimen) × 1/6.

longitudinal section these appear as wide tubes. The pith may become slightly lignified, and according to Solereder[10] it retracts in older stems to leave a central cavity.

Leaves. (Fig. 3, E & F.) The leaves may be conveniently divided into types, large and small, the latter being those leaves which subtend the racemes.

Most plants have two to four large leaves, but sometimes five or six such leaves are found. Several of these usually arise around the base of the stem, the remainder arising just below or above ground level. The leaf is biternatisect, often triternatisect and sometimes showing an even greater degree of dissection in the apical group of leaflets, especially in those arising at the base of the stem. The leaves are about 12 to 60 cm. long by 9 to 25 cm. broad and the leaflets are about 1·5 to 8 cm. long by 0·8 to 4·2 cm. broad. The long, stout rachis is somewhat flattened dorsiventrally and is vaginate at the base, the sheath bearing well marked longitudinal striations. Most of the leaflets are asymmetrical and vary from ovate to oval to obovate and are occasionally subcordate. The apex of the leaflet is often somewhat recurved and is usually retuse with a small mucronate projection of the lamina at the end of the main vein.

Leontice leontopetalum

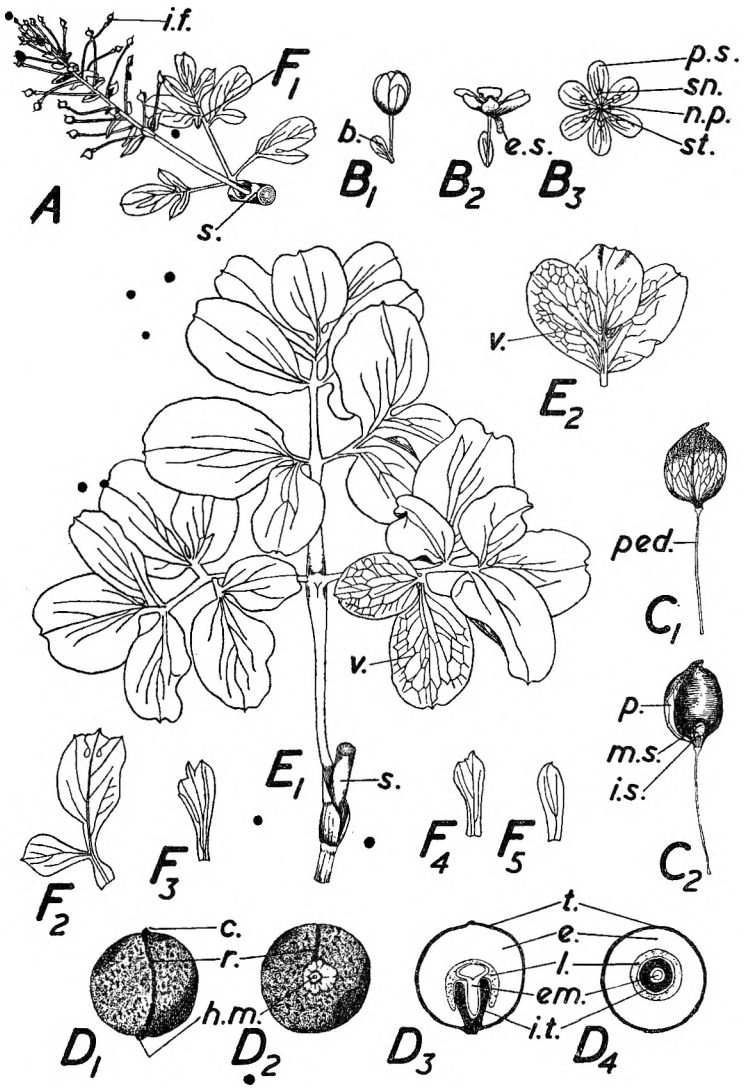

FIG. 3. *Leontice leontopetalum* aerial parts:—*A*, lateral raceme × ¼; *B*, flower × ¾: 1, unexpanded, 2, expanded, side view, 3, expanded, from above; *C*, fruit × ½: 1, entire, 2, with part of pericarp removed; *D*, seed × 2: 1, entire, side view, 2, entire, from below, 3, longitudinal section, 4, transverse section through lower third; *E*, large foliage leaf × ¼: 1, entire, upper surface, 2, apical leaflets, under surface; *F*, small foliage leaves × ¼: 1–5, series showing variation in degree of dissection.

b., bract; *c.*, chalaza; *e.*, endosperm; *em.*, embryo; *e.s.*, extra sepal; *h.m.*, hilum and micropyle; *i.f.*, immature fruit; *i.s.*, immature seed; *i.t.*, infolding of testa; *l.*, laconose region of endosperm; *m.s.*, mature seed; *n.p.*, nectariform petal; *p.*, pericarp; *ped.*, pedicel; *p.s.*, petaloid sepal; *r.*, raphe in shallow groove; *s.*, stem; *sn.*, stamen; *st.*, stigma; *t.*, testa; *v.*, detailed venation.

The margin of the leaflet is usually entire but may bear one or two small indentations. The leaflets are sessile on the branches of the rachis and the lamina is asymmetrical at the base. The venation is pinnate but sometimes there is a vein lying parallel to the midrib without apparently joining it at the base. The lateral veins leave the midrib at an acute angle and often anastomose close to it; there are many smaller veinlets forming a network, those towards the outside anastomosing about 2 to 3 mm. from the margin. The main veins are slightly depressed on the upper surface and raised on the under surface. Both surfaces of the leaflets are smooth, of a somewhat waxy texture and bluish-green colour. If any part of the rachis lies below the ground it is usually white to pale green in colour with a purplish zone at ground level.

The small leaves occur on the upper part of the stem and bear in their axils the racemes which form the leafy panicle. They vary greatly in size, shape and degree of dissection, those nearest the apex of the panicle being smaller and without any dissection. The largest of these leaves, those subtending the first of the racemes, are from about 6·1 to 8·2 cm. long by about 7·0 to 9·3 cm. wide, while the smallest are only about 1·5 to 2·5 cm. long by about 0·3 to 1·2 cm. wide. The largest leaves are ternatisect with the leaflets sometimes further dissected and the degree of dissection decreases in the smaller leaves as illustrated in Figure 3, F 1-5.

Racemes. (Fig. 3, A.) The racemes are borne on slender cylindrical stems arising in the axils of the small foliage leaves. These stems bear numerous bracts which are oval, ovate or oblong with a rounded apex, entire margin and a wide sessile base. They are from about 0·4 to 2·8 cm. long by about 0·3 to 0·9 cm. wide. In the axils of these bracts the yellow flowers are borne.

Flowers. (Fig. 3, B.) The flowers are borne on green, cylindrical pedicels which widen slightly at the base and at the receptacle. The length of the pedicel varies greatly, increasing as the flower and the fruit develop, becoming up to 6 cm. and more in the mature fruit. The flat receptacle bearing the flowering parts is circular in surface view.

The flowers are hermaphrodite, cyclic and trimerous. According to Bailey[15] flowers of the genus *Leontice* may have 3 to 9 petaloid sepals with 6 petals which are often reduced to nectaries and Bentham and Hooker[6] state that the sepals may be from 6 to 9 and petaloid, the outer being smaller, and the 6 petals are very small and nectariform with a truncated apex. The perianth of *Leontice leontopetalum* consists of six yellow, obovate, petaloid sepals, about 8 to 10 mm. long, arranged in two whorls of three, with a small, yellow nectariform petal attached to the base of each sepal. In addition there is often a smaller seventh sepal to the outside. This extra sepal is about 6 to 7 mm. long, and is yellowish green in colour with a darker green central vein. It lies immediately external to one of the outer whorl of sepals and is often recurled in the open flower. There are six free stamens with filaments which often broaden out below the anther. The ovary is solitary and bulbous with a short curved style and an inconspicuous stigma with a flattened top; it is unilocular containing 2 to 4 ovules attached at the base.

Fruits. (Fig. 3, C.) After the fall of the perianth and stamens the fruit develops quickly, the mature fruit consisting of an ovoid bladdery capsule about 4 cm. long by 2·8 cm. in diameter. The thin pericarp dries on ripening, becoming membraneous, and often becomes completely detached from the pedicel leaving the seed naked. The style persists to

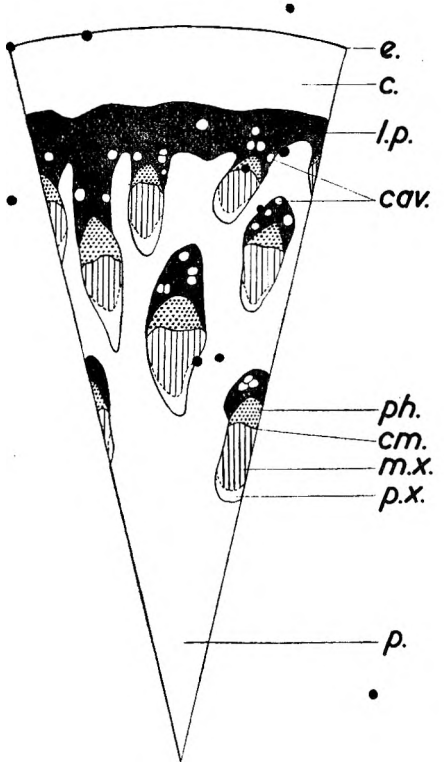

FIG. 4. *Leontice leontopetalum* stem in T.S. × 20. *c.*, cortex; *cav.*, cavities in pericycle; *cm.*, cambium; *e.*, epidermis; *l.p.*, lignified pericycle; *m.x.*, metaxylem; *p.x.*, protoxylem; *p.*, pith; *ph.*, phloem.

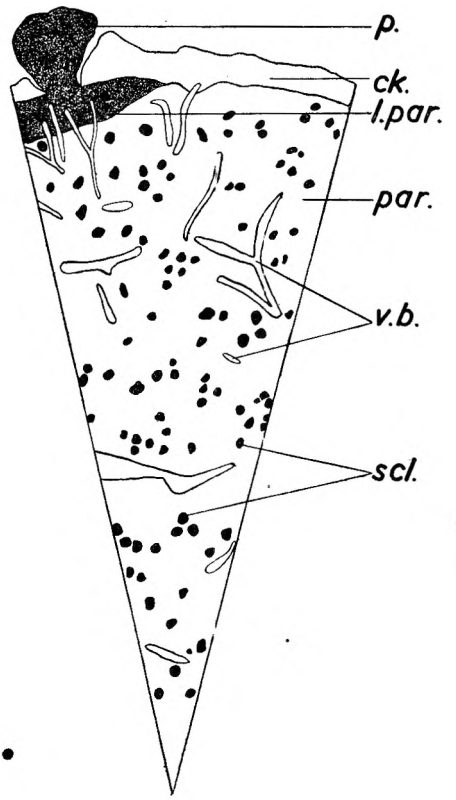

FIG. 5. *Leontice leontopetalum* root tuber in T.S. × 2½. *ck.*, cork; *l.par.*, lignified parenchyma; *p.*, protuberance; *par.*, parenchyma; *scl.*, groups of sclereids; *v.b.*, vascular bundles.

form an acute projection, often slightly to one side at the apex. The base of the capsule is rounded and joined to the pedicel by a short tube-like extension. On the adaxial side of the pericarp there is one wide vein running longitudinally from base to apex; the remaining veins are much finer and anastomose to form a network over the entire pericarp. The lower part of the capsule is green and the upper part varies from a very light brownish-purple to a fairly deep purple. The fruit usually contains only one ripe seed, with 1 to 3 immature seeds, but occasionally 2 or 3 seeds may develop fully.

Seeds. (Fig. 3, D.) The subglobular seed is borne on a short funicle

and is formed from an anatropous ovule. The greatest diameter of the seed varies from about 0·4 to 0·7 cm. The rough testa is of a dark purplish-brown colour with a whitish-violet bloom, especially evident at the base and in the shallow groove in which the raphe occurs. The hilum and micropyle lie in a light brown raised area at the base of the seed and the chalaza occurs as a slight protuberance at the apex. In longitudinal section the seed shows a narrow brown testa with a cup-shaped infolding at the base into the white, proteinous and oily endosperm which is lacunose centrally. The embryo is small with a terete radicle and two small thick incurved cotyledons. It lies in the lacunose region of the endosperm with the radicle in the cup-shaped infolding of the testa.

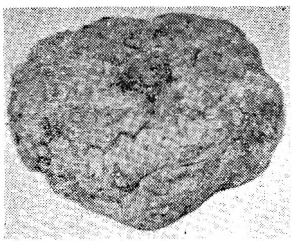

FIG. 6. *Leontice leontopetalum* root tuber × 1/3.

Root Tuber. (Figs. 5, 6.) The tuber varies greatly in size and shape but is usually irregularly circular to oval in outline and somewhat flattened dorsiventrally. The maximum size of tubers of flowering plants is about 23 cm. in diameter by about 10 cm. in thickness, while the smallest tubers from such plants are about 7 cm. in diameter by about 5 cm. thick. Externally it is covered with a thick brown cork with small protuberances from which fine white rootlets arise. The main stem is attached more or less centrally to the upper side of the tuber and some of the foliage leaves arise around the stem at this point. In the dormant tuber this point is often difficult to determine though it may be seen as a protuberance with some slight remains of the aerial parts. Similarly the position of the rootlets is marked by smaller protuberances in the cork which becomes scaly and exfoliates in places. The cut surface of the fresh tuber is bright creamy-yellow in colour. The cork is thick, lignified and irregular due to the presence of the many protuberances. These are composed of large lignified cells and groups of such cells also occur in some places internal to the cork. The bulk of the tissue is composed of starchy parenchyma with groups of large yellow sclereids and with vascular bundles in transverse, oblique and longitudinal view, scattered throughout. In the growing tuber there is a layer of actively dividing meristematic tissue about 1·5 to 3 cm. internal to the cork, but this layer is not discernible in the dormant tuber.

In Lebanon the tubers are dug up for use as a drug after the aerial parts have died down and only as required for immediate compounding, therefore the drug used is the fresh dormant tuber. Some of the earlier specimens received by us had been partially dried and were considerably attacked by mould during transit, therefore arrangements were made to have the freshly collected tubers washed, cut into slices and dried rapidly in the sun. This proved satisfactory for the prevention of obvious deterioration.

The dried drug, obtained in this way, consists of irregular slices about 0·5 cm. thick. Externally there is a thick brown cork and the remainder

of the drug is light brown in colour and consists of starchy parenchyma with the vascular bundles appearing as points or striations and the groups of sclereids as protuberances. Often there are dark brown areas lying internal to the cork and these consist of lignified parenchyma. The texture is hard and brittle; the odour is quite pleasant but the freshly fractured drug and the powder are sternutatory; the taste is bitter.

Summary

1. Notes on the history and geographical distribution of the plant *Leontice leontopetalum* Linn. have been recorded.

2. The macroscopical characters of the entire plant in the flowering and fruiting condition have been described and illustrated.

3. A description of the collection and drying of the root tubers in Lebanon has been given.

4. The macroscopical characters of the dried drug have been described.

The authors gratefully acknowledge the help of Mr. W. T. Stearn of the British Museum (Natural History) for help and advice on the collection and identification of specimens, of Dr. W. M. Ford-Robertson of the Lebanon Hospital for Mental and Nervous Disorders, Asfuriyeh, Beirut, his wife and colleagues for hospitality and much assistance in the collection and supply of specimens, of the Cross Trust through whose generosity one of us (P. F. N.) was able to visit Lebanon, and of Professor J. P. Todd, Dr. Blodwen Lloyd and Mr. G. Rattray of the School of Pharmacy, The Royal Technical College, Glasgow, for encouragement and advice.

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DISCUSSION

The paper was presented by MR. P. F. NELSON.

DR. T. E. WALLIS (London) said with reference to the structure of the stem, it was typical of Berberidaceae to find the peculiar arrangement of the vascular tissues. It might be mistaken for that of a monocotyledon. As to the question of precise identity of the sclerenchyma associated with the isolated bundles, it seemed a little unlikely that it should be pericyclic. So far as the shape of the leaflets was concerned, he took it that those which were not asymmetrical were terminal ones. There was no definite statement on the number of carpels present in the ovary. The statement was made that the ovary was "solitary" and it was difficult to be certain what that meant; it did not state whether there was one carpel or more.

DR. J. M. ROWSON (London) asked the authors to say a little more about the small protuberances on the root tuber. The emergence of sclerenchymatous matter in the cork was referred to, and when the authors studied the detailed anatomy no doubt they would again reflect on the morphology.

MR. P. F. NELSON, in reply, said on the question of the pericycle, he had consulted some of his botanical colleagues about the matter and they suggested that it was the correct term. The term "solitary" should be "single", with reference to carpel. The symmetry of the leaflets was difficult to determine because they were shrivelled up, and began to shrivel almost as soon as they were picked. The protuberances occurred where the rootlets arose in the following spring when the plant started to grow.

RESEARCH PAPERS

THE INFLUENCE OF SOAPS ON THE BACTERICIDAL ACTIVITY OF A SPARINGLY WATER-SOLUBLE PHENOL

By H. Berry and A. Briggs

From the Department of Pharmaceutics, School of Pharmacy, University of London

Received March 15, 1956

The fact that soaps modify the bactericidal activity of phenols has been known for many years, but it is only comparatively recently that attempts have been made to determine the exact extent of this modification and the manner by which it is effected.

As the published results of the earlier workers appeared to be contradictory, Bean and Berry[1–4] investigated the mechanism of the reaction. Taking into account the present theories of the micellar structure and properties of aqueous solutions of soaps[5] they determined the bactericidal activity of benzylchlorophenol and chloroxylenol in aqueous solutions of potassium laurate, using solutions containing a constant benzylchlorophenol or chloroxylenol to potassium laurate molar ratio. This activity was shown to be related to the concentration of the phenol in the soap micelles and independent of the total concentration in the solutions.

Alexander and Tomlinson[6] investigated the effect of soaps on the bactericidal activity of phenol. At soap concentrations above the critical, they were able to show a relation between the time for complete disinfection and the concentration of phenol in the aqueous phase. They did not observe any increase in activity at the higher soap concentrations, as did Bean and Berry[3], though it must be noted that Alexander and Tomlinson used solutions containing a constant concentration of phenol. Similarly Allawala and Riegelman[7] found that the LT99 of iodine dissolved in solutions of a non-ionic detergent was related to the concentration of iodine freely dispersed in the aqueous phase and was not necessarily dependent upon the total amount of iodine present.

This paper is a continuation of the work originated by Bean and Berry and is an investigation into the effect of different soaps on the bactericidal activity of a sparingly water-soluble phenol (benzylchlorophenol). Of the few soaps more than slightly soluble at 20° C., three were chosen, namely: potassium laurate, a straight chain saturated soap, potassium oleate, a monoethenoid soap, and potassium ricinoleate, a hydroxymonoethenoid soap. It was also intended to study the effect of potassium elaidate, the trans-isomer corresponding to potassium oleate, but it was found to be only slightly soluble at room temperature, thus precluding its use.

The solutions investigated were those containing (*a*) a constant benzylchlorophenol to soap molar ratio and varying concentrations of soap and (*b*), a fixed soap concentration and varying benzylchlorophenol to soap molar ratios.

EXPERIMENTAL

Materials

The phenol. The phenol used was benzylchlorophenol (5-chloro-2-hydroxy-diphenyl methane), m.pt. 48·5° C. water solubility 1 in 8650 at 20° C.

The Soap Solutions. Potassium laurate and potassium oleate solutions. These were prepared by neutralisation of lauric acid (acid value 278·8, iodine value 0·04, m.pt. 39·2° C.) and oleic acid (acid value 198·3, iodine value 88·10, n_D^{20} 1·4610). The resulting stock solutions of 0·1M potassium laurate and 0·2M potassium oleate were stored under nitrogen in glass-stoppered bottles, and diluted with carbon dioxide free water as required. The former stock solution had a pH of 9·9 and the latter a pH of 10·0 (glass electrode).

Potassium ricinoleate solution. A sample ricinoleic acid of high acid value (179, theoretical value 188) was prepared from castor oil by the method recommended by Berry and Cook[8]. This sample was used for the preparation of a 0·0307M stock solution of potassium ricinoleate which had a pH of 8·6 (glass electrode) and was stored under nitrogen. The phenol and soap mixtures were prepared as required with carbon dioxide free water and stored under nitrogen.

Culture media. The peptone water contained 2 per cent. Oxoid peptone and 0·5 per cent. sodium chloride, the pH after the final autoclaving being between 7·1 and 7·3.

The peptone water agar used for the cultivation of the test organism contained 1 per cent. Oxoid peptone, 0·5 per cent. sodium chloride and 2 per cent. New Zealand agar.

Test organism. A 24 hour culture of *Escherichia coli*, Type I (formerly Lister Institute No. 5933) in tryptic digest broth was freeze-dried in sterile tubes and stored at 4° C. Tubes of freeze-dried culture were reconstituted at approximately monthly intervals and cultivated on peptone water agar slopes. Four slopes were prepared from each freeze-dried culture and incubated at 37° C. for 24 hours. Afterwards three of them were sealed with paraffin wax and the fourth used to start a series of subcultures, four slopes being prepared each day. The daily slopes were incubated at 37° C. for 24 hours and only slopes from the 4th to the 14th subculture were used in the experiments.

The Solubility of Benzylchlorophenol in Water and Aqueous Solutions of Potassium Laurate, Potassium Oleate and Potassium Ricinoleate, at 20° C.

Solubility in aqueous solutions of potassium laurate. The method consisted of preparing a series of solutions of benzylchlorophenol in potassium laurate, such that each solution contained a smaller proportion of benzylchlorophenol to potassium laurate than the preceding one. Each solution was then diluted with freshly boiled and cooled distilled water until precipitation of the benzylchlorophenol occurred.

Figure 1 shows the relation between the weight of benzylchlorophenol solubilised per ml. of solution and the concentration of the potassium

laurate solutions, whilst in Figure 3 the solubility, expressed as number of molecules of benzylchlorophenol solubilised per molecule of soap, is plotted against potassium laurate concentration.

Solubility in aqueous solutions of potassium oleate. The critical concentration of potassium oleate has been recorded[9] as 7–12 $\times$ 10^{-4}M and the results of McBain and Merrill[10] indicate that the micelles are fully formed at 0·016M. The range over which the micelles are increasing in size occurs, therefore, at a very low soap concentration. Attempts to determine the solubility of benzylchlorophenol in the way used for potassium laurate, failed because of the small quantity of benzylchlorophenol crystallising out. The method used consisted of accurately weighing a fixed quantity of benzylchlorophenol into a series of wide-mouthed, screw-capped bottles and adding gradually increasing volumes of the appropriate strength of potassium oleate solution to each one. The bottles were then heated to about 60° C. and shaken to dissolve the benzylchlorophenol. When cool, each solution was seeded with a crystal of benzylchlorophenol and, after being maintained at 20° C. for five days, examined for crystals.

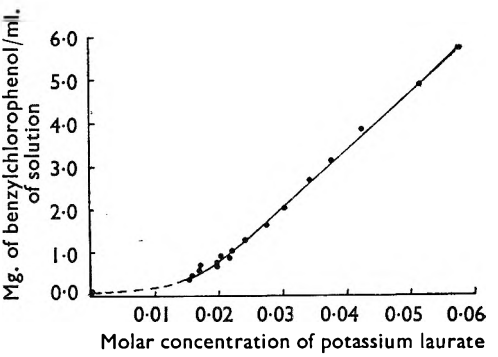

FIG. 1. The solubility of benzylchlorophenol in solutions of potassium laurate.

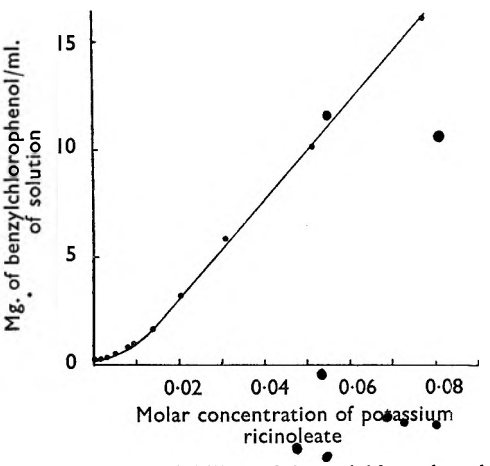

FIG. 2. The solubility of benzylchlorophenol in solutions of potassium ricinoleate.

The relation between the solubility, expressed as number of molecules of benzylchlorophenol solubilised per molecule of soap, and the concentration of potassium oleate is shown in Figure 3.

Solubility in water and aqueous solutions of potassium ricinoleate. As a spectrophotometer became available for use the solubility of benzylchlorophenol in water and aqueous solutions of potassium ricinoleate was determined spectrophotometrically.

Water solubility. The E (1 per cent. 1 cm.) of benzylchlorophenol,

at 283 mμ, was 104·3. The solvent used to prepared the standard solutions and the final dilution for testing contained 4 per cent. v/v ethanol and 0·05 N hydrochloric acid. The solubility was found to be 1 in 8650.

Solubility in aqueous solutions of potassium ricinoleate. The $E^{1}_{1\,cm.}$ (1 per cent. 1 cm.) of benzylchlorophenol at 283 mμ, in the presence of varying concentrations of potassium ricinoleate, 50 per cent v/v ethanol and 0·05 N hydrochloric acid was 113·4. The large amount of ethanol was necessary to dissolve the precipitated ricinoleic acid.

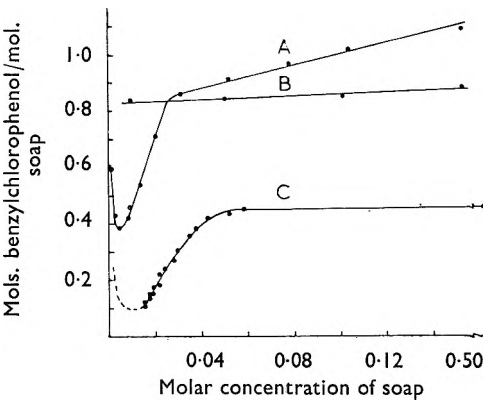

FIG. 3. The solubility of benzylchlorophenol in solutions of: A, potassium ricinoleate prepared from acid having an acid value of 179; B, potassium oleate and C, potassium laurate.

Saturated solutions in varying strengths of potassium ricinoleate were prepared, the supernatant liquid filtered and a portion of the filtrate diluted to give a concentration suitable for measurement. The diluent contained 50 per cent. v/v ethanol and 0·05 N hydrochloric acid.

Figure 2 shows the relation between the weight of benzylchlorophenol solubilised and the concentration of potassium ricinoleate, whilst in Figure 3 the solubility, expressed as number of molecules of benzylchlorophenol solubilised per molecule of soap, is plotted against potassium ricinoleate concentration.

The Determination of Bactericidal Activity

The method used was similar to that of Berry and Bean[11]. Five ml. quantities of the phenol and soap solution were used and the inoculum consisted of five drops of a suspension of *E. coli*. The suspension was previously adjusted to contain 2×10^9 organisms per ml., and was delivered by means of a standard dropping pipette made by the method described by Withell[12].

With any phenol and soap solution each experiment was repeated six times, where possible, simultaneously, or failing that on the same day, with as short a time interval as possible between each. The mean death-time was calculated from the individual observed times, adopting the convention that if no growth was obtained after a given period of exposure in all replicates, then any growth thereafter was neglected.

The Bactericidal Activity of the Soap Solutions

The contribution made by the soap solutions themselves towards the bactericidal activity of the benzylchlorophenol and soap solutions was assessed by determining the death-time of *E. coli* in various concentrations of the soaps. The results obtained are shown in Table I.

TABLE I

THE MEAN DEATH-TIME OF *E. coli* IN AQUEOUS SOLUTIONS OF POTASSIUM LAURATE, POTASSIUM OLEATE AND POTASSIUM RICINOLEATE

Soap	Concentration of soap (Molar)	Mean death-time (Minutes)	Time of determination
Potassium laurate	0·06	41·6	During work
	0·08	25·7	
	0·10	17·7	
	0·15	8·3	
	0·20	4·8	
Potassium oleate	0·20	300+	At beginning of work
	0·20	300+	At end of work
Potassium ricinoleate	0·307	240	At beginning of work
	0·307	300+	At end of work

Both potassium oleate and potassium ricinoleate made no contribution to the bactericidal activity of the benzylchlorophenol and soap mixtures used but potassium laurate was toxic. An exponential relation was obtained when the concentration of this soap was plotted against the mean death-times and extrapolation of the curve showed that the death-time of *E. coli* would be about 100 minutes in 0·04 M potassium laurate. Thus the contribution made by potassium laurate to the mixtures is small at the soap concentrations used.

The Bactericidal Activity of a Saturated Aqueous Solution of Benzylchlorophenol

A saturated solution of benzylchlorophenol was prepared in sterile distilled water and the mean death-time at 20° C. found to be 75 minutes.

The Bactericidal Activity of Benzylchlorophenol in Aqueous Solutions of Soap

These experiments were arranged to show the influence on the bactericidal activity of the following changes in the composition of the solutions. (i) Maintaining a constant benzylchlorophenol/soap molar ratio whilst increasing the concentration of soap from below to well above the critical concentration. (ii) Maintaining a constant concentration of soap and varying the benzylchlorophenol/soap molar ratios.

(*a*) *The Bactericidal Activity of Benzylchlorophenol in Aqueous Solutions of Potassium Laurate*

The results obtained when solutions containing 0·0653 molecules of benzylchlorophenol per molecule of potassium laurate were used are shown in Figure 4 where the mean death-times are plotted against soap concentration, the points being superimposed on the solubility curve. The concentration of potassium laurate was varied from 0·0065 to 0·12 M, that is, from just below to well in excess of the critical concentration.

The effect on the bactericidal activity of keeping constant the potassium laurate concentration and varying the benzylchlorophenol/potassium laurate molar ratio is shown in Figure 5. Concentrations of 0·025, 0·04 and 0·075 M potassium laurate were used.

(*b*) *The Bactericidal Activity of Benzylchlorophenol in Aqueous Solutions of Potassium Oleate*

It was impossible to carry out experiments using benzylchlorophenol/potassium oleate solutions of constant molar ratio owing to the large changes in death-time that occurred with change in soap concentration. The investigation was restricted to the effect on the bactericidal activity of keeping constant the concentration of the soap and varying the benzylchlorophenol to potassium oleate molar ratio. The results are shown in Figure 6.

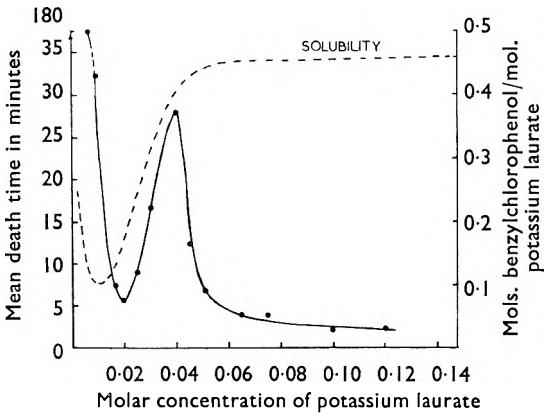

FIG. 4. The bactericidal activity against *E. coli* of solutions with constant benzylchlorophenol/potassium laurate molar ratio (0·0653) and increasing potassium laurate concentration, and the relation of the activity to the solubility of benzylchlorophenol.

(*c*) *The Bactericidal Activity of Benzylchlorophenol in Aqueous Solutions of Potassium Ricinoleate*

Two series of solutions were used containing benzylchlorophenol in the ratio of 0·383 and 0·331 molecules per molecule of potassium ricinoleate. The latter molar ratio was used to show the effect on bactericidal activity of solutions having a potassium ricinoleate concentration below the critical value (0·005 M). Had this been attempted with the former ratio it would have necessitated the use of unstable solutions in the region of 0·005 M potassium ricinoleate concentration. The results are shown in Figure 7.

The effect of varying the benzylchlorophenol/potassium ricinoleate molar ratio at soap concentrations of 0·025, 0·04 and 0·075 M potassium ricinoleate was also investigated. The results are shown in Figure 8.

DISCUSSION

The Solubility of Benzylchlorophenol

The weight of benzylchlorophenol solubilised per ml. of potassium laurate and potassium ricinoleate solution has been plotted against soap concentration in Figures 1 and 2 respectively. The curves are of similar shape. As the soap concentration is increased from zero to approximately 0·005 M potassium ricinoleate and 0·01 M potassium laurate there is but little increase in the solubility of benzylchlorophenol. Above these concentrations the weight of benzylchlorophenol solubilised increases sharply with increase in soap concentration due to the formation of micelles.

Figure 3 shows the solubility of benzylchlorophenol expressed as the number of molecules solubilised per molecule of soap, plotted against soap concentration. The benzylchlorophenol/potassium oleate curve could not be completed as the method used was not sufficiently sensitive to determine solubilities at very low soap concentrations. The critical concentration of potassium oleate has been recorded as $7\text{–}12 \times 10^{-4}$M. The curves plotted in Figure 3 indicate that the critical concentration of potassium ricinoleate is 0·005 M and potassium laurate 0·01 M.

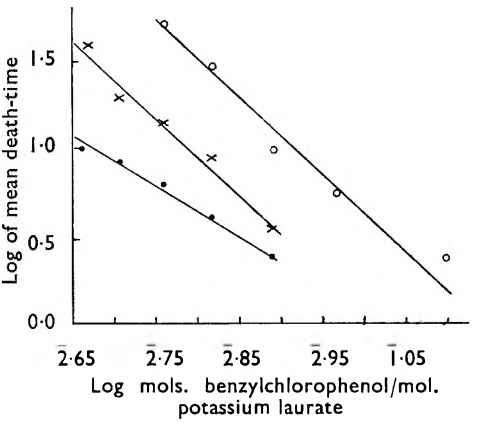

FIG. 5. The effect on the death time of *E. coli* of varying the benzylchlorophenol/potassium-laurate molar ratio in solutions with constant potassium laurate concentration.
×——×, 0·025 M potassium laurate.
○——○, 0·04 M " "
●——●, 0·075 M " "

The curves (Fig. 3) describing the solubility of benzylchlorophenol in solutions of potassium laurate and potassium ricinoleate are, except for the initial decrease at low soap concentrations, of the same general shape as those described by Hartley[13], McBain, Merrill and Vinograd[14], and McBain and Johnson[15] for the solubilisation of water insoluble substances—usually dyes—by aqueous soap or synthetic detergent solutions. Only occasionally has this initial decrease been noted[16,17] and then it has usually been attributed to the suspending action of the soap solution. One exception is the work of Heller and Klevens[18] who determined the solubility of ethylbenzene in potassium laurate solutions and obtained a curve very similar in shape to those in Figure 3 for the solubility of benzylchlorophenol in potassium laurate and potassium ricinoleate. Both ethylbenzene and benzylchlorophenol have approximately the same water solubility. Failure to demonstrate this initial fall in the solubility curve is probably due to the fact that water-insoluble substances were used or that the method was not sufficiently sensitive to detect solubilisation at concentrations below the critical.

The Bactericidal Activity of Solutions containing a Constant Benzylchlorophenol/Soap Molar Ratio and Varying Concentrations of Soap

It is seen from Figures 4 and 7 that when solutions containing a constant molar ratio of benzylchlorophenol to potassium laurate or potassium ricinoleate are used, changes in the death-time curve and changes in the slope of the solubility curve can be correlated.

Over the first part of the curves, where the death-time decreases rapidly as the soap concentration is increased up to the critical, the saturation of the solution increases whilst the interfacial tension decreases, reaching

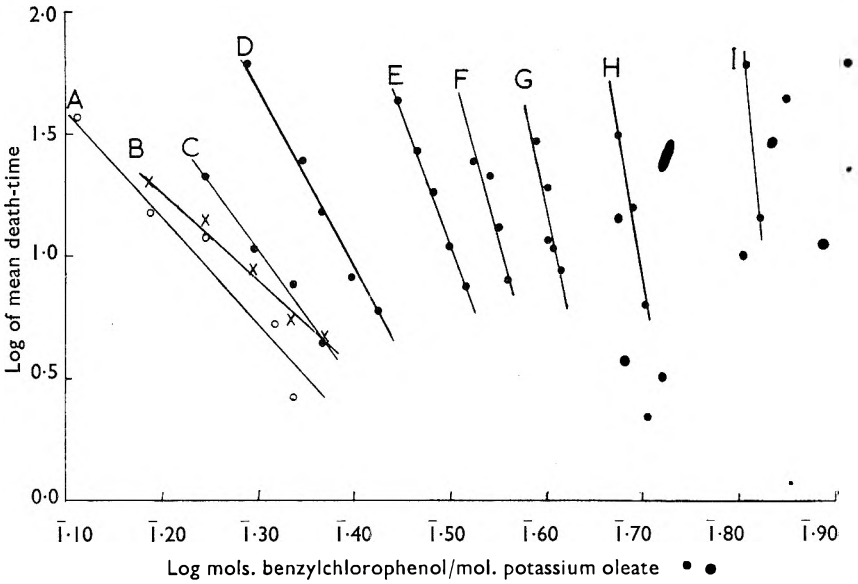

FIG. 6. The effect on the death-time of *E. coli* of varying the benzylchlorophenol/potassium oleate molar ratio in solutions with constant potassium oleate concentration of A, 0·10 M; B, 0·075 M; C, 0·05 M; D, 0·025 M; E, 0·01 M; F, 0·0075 M; G, 0·005 M; H, 0·0025 M and I, 0·001 M.

a minimum at the critical concentration. This decrease in interfacial tension will favour an increase in the adsorption of potassium laurate and benzylchlorophenol causing the concentration of benzylchlorophenol at the bacterial surface to be much higher than in the bulk solution, thus leading to a decrease in the death-time. This surface active effect of soap solutions has been noted by previous workers[3,6,19], and put forward as the cause of the increase in bactericidal activity of soap-phenol solutions in the pre-micellar range of soap concentrations. A phenomenon which has not previously been reported is that, over the pre-micellar range of soap concentration, the saturation of the solution is actually increasing. Thus the decrease in death-time will be due to both these factors.

The second part of the curve commences at approximately the critical micellar concentration and both the solubility and death-times increase rapidly. The interfacial tension remains approximately constant at all concentrations above the critical and therefore changes in bactericidal activity can no longer be ascribed to changes in interfacial tension. It is at the critical concentration that the micelles begin to form and they increase rapidly in size over the range of soap concentration now under consideration. The rate of increase in size is much more rapid than the rate of increase of benzylchlorophenol concentration and therefore the total saturation of the solutions will decrease. Bean and Berry[3] related the activity of the solutions in this range of soap concentration to the percentage saturation of the micelles. It has been shown[6], however, that

the activity of phenol-soap solutions is related to the concentration of phenol in the aqueous phase rather than that in the micelles. These two views are not at variance as in any given solution an equilibrium will be set up between the concentration of benzylchlorophenol in the micelles and that in the surrounding water. Variation of the concentration of phenol in one phase will be accompanied by a similar variation in the other phase. Thus the decrease in saturation of the solutions over the second part of the curve will cause a decrease in the concentration of benzylchlorophenol in the aqueous phase and will explain the increase in the death-times that are obtained.

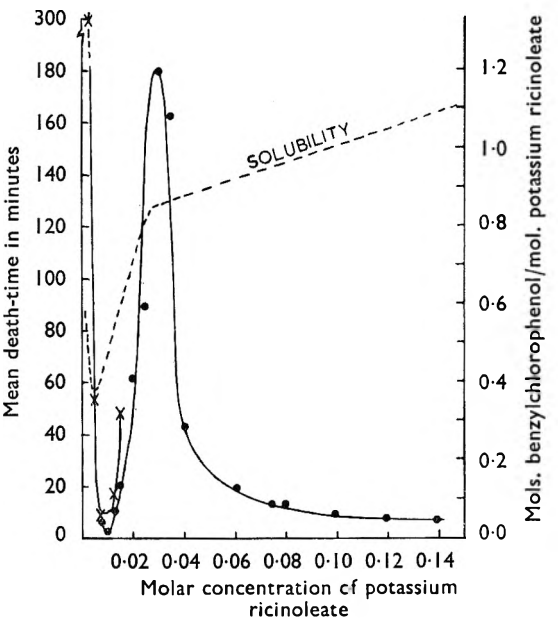

FIG. 7. The bactericidal activity against *E. coli* of solutions with constant benzylchlorophenol/potassium ricinoleate molar ratio and increasing potassium ricinoleate concentration, and the relation of the activity to the solubility of benzylchlorophenol.
●——● 0·383 Mol. benzylchlorophenol/mol. potassium ricinoleate.
×——× 0·331 Mol. benzylchlorophenol/mol. potassium ricinoleate.

The death-time curve reaches a peak at a point which coincides approximately with the change of slope of the solubility curve—the point at which the rapid increase in the number of molecules of benzylchlorophenol solubilised per molecules of soap ceases, and becomes but a relatively slight increase with increasing soap concentration. Above this concentrations the death-times commence to decrease rapidly, finally reaching an approximately constant value at the higher soap concentrations. Thus, over the third part of the curve, the percentage saturation of the solution is decreasing slightly with increasing soap concentration, whilst the death-time falls rapidly before attaining a constant value.

The reason for this rapid decrease in death-time is not apparent. If one accepts that it is the benzylchlorophenol in the aqueous phase that determines the bactericidal activity, then as the solution is becoming less saturated with respect to benzylchlorophenol, it would be expected that the death-time would continue to increase. Results in accordance with this expectation were obtained by Alexander and Tomlinson[6] who investigated the effect of various concentrations of Aerosol MA on the bactericidal activity of a 0·5 per cent. solution of phenol. They stated that, where the

soap is not bactericidal, then the death-time will continue to increase until activity is abolished. These workers were, however, using a constant percentage of phenol, whereas in this work the amount of benzylchlorophenol was increased in proportion to the increase in soap concentration.

The rapid decrease in death-times cannot be due to the toxicity of the soaps as it has been shown that they possess little or no activity at the concentrations concerned. It is significant that the percentage saturation of the solution decreases only slightly with increasing soap concentration. The amount of micellar material per unit volume will, however, increase in ratio to the increase in soap concentration. Thus as the soap concentration is increased, there will be an increasing number of micelles, of approximately the same degree of saturation, available to replace the benzylchlorophenol adsorbed from the water by the bacteria. This might be expected to lead to a more rapid replenishment of the water with benzylchlorophenol and hence to shorter death-times.

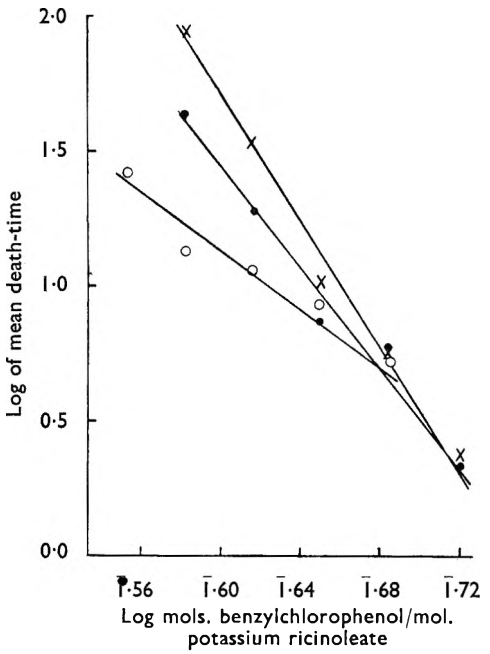

FIG. 8. The effect on the death-time of *E. coli* of varying the benzylchlorophenol/potassium ricinoleate molar ratio in solutions with constant potassium ricinoleate concentrations of: ×——×, 0·25 M; ●——●, 0·04 M; ○——○, 0·075 M.

The death-time falls to an approximately constant value at the higher soap concentrations. This would seem to indicate a limiting factor in the reaction between the soap-phenol solution and the bacteria, this being in all probability the rate of diffusion of the benzylchlorophenol from the micelle to the bacterial cell via the water.

It has already been stated that it was impossible to carry out experiments using benzylchlorophenol/potassium oleate solutions of constant molar ratio owing to the large changes in death-time that occurred with change in soap concentration. Some indication of the effect can, however, be obtained from the results shown in Figure 6. For a constant molar ratio of benzylchlorophenol to potassium oleate, for example 0·2, the death-time increases as the soap concentration is decreased from 0·10 to 0·025 M, as it does with the corresponding decrease in soap concentration for benzylchlorophenol/potassium laurate and benzychlorophenol/

potassium ricinoleate solutions (Figs. 4 and 7), over soap concentration ranges of 0·12 to 0·04 M and 0·14 to 0·03 M respectively.

The Bactericial Activity of Solutions Containing a Fixed Soap Concentration and Varying Benzylchlorophenol/Soap Molar Ratios

The results obtained by using solutions where the soap concentration was kept constant whilst the proportion of benzylchlorophenol to soap was increased are shown in Figures 5, 6 and 8 where the logarithms of the mean death-times are plotted against the logarithms of mols. benzylchlorophenol/mol. soap. A linear relation was obtained in every case, although the slope of these lines varied with soap concentration. Thus, once the soap concentration has been fixed, the mean death-time becomes a function of the concentration of benzylchlorophenol. It is to be expected that the slopes of the lines would not be similar as each soap concentration would affect the mechanism of the bactericidal reaction in a different manner. In the pre-micellar range the composition of the interfacial film would be changing, whilst above the critical concentration the partition coefficient for benzylchlorophenol between micelles and water probably changes with increasing soap concentration, as also will the structure of the micellar material and the interfacial film.

Comparison of the Effect of Different Soaps

The percentage saturation of the three soaps by benzylchlorophenol required to kill a standardised suspension of *E. coli* in ten minutes, has been calculated from the experimental results for three different soap concentrations and tabulated in Table II.

TABLE II

THE PERCENTAGE SATURATION OF POTASSIUM LAURATE, POTASSIUM OLEATE AND POTASSIUM RICINOLEATE BY BENZYLCHLOROPHENOL REQUIRED TO KILL THE STANDARDISED SUSPENSION OF *E. coli* IN TEN MINUTES

Soap	Percentage saturation		
	At 0·025 M	At 0·04 M	At 0·075 M
Potassium laurate	25	22	10
Potassium oleate	30	25	22
Potassium ricinoleate ..	56	51	44

It is realised that had a different death-time been used at which to make the comparison, different values for the percentage saturation of the solutions would have been obtained. A similar difference between the soaps would still have been illustrated.

From the above figures it is evident that each soap influences the bactericidal activity of benzylchlorophenol to a different degree. The amount of benzylchlorophenol needed to bring about a given effect increases in the order potassium laurate, potassium oleate, potassium ricinoleate. Conversely, equally saturated solutions of these soaps would show differing bactericidal activity. This variation between soaps is attributed to the difference in the structure of the soap molecules and its

influence on the structure of the micelles formed in aqueous solutions. It also indicates that the affinity of these micelles for benzylchlorophenol varies from soap to soap, thus influencing the degree of saturation of the aqueous phase and hence the bactericidal activity of the solution.

Summary

1. The solubility of benzylchlorophenol in solutions of potassium laurate and potassium ricinoleate, when expressed as molecules of benzylchlorophenol solubilised per molecule of soap, decreases as the soap concentration is increased up to the critical. Thereafter the shape of the solubility curve is similar to those previously reported for the solubility of water-insoluble dyes in aqueous solutions of soaps.

2. When mixtures containing a constant benzylchlorophenol to soap molar ratio and varying soap concentration are used, changes in the bactericidal activity can be correlated with changes in the slope of the solubility curve.

3. The effect of varying the benzylchlorophenol to soap molar ratio has been determined at various constant soap concentrations.

4. It has been shown that the three soaps used each influence the bactericidal activity of benzylchlorophenol to a different degree. For a given weight of benzylchlorophenol the highest level of activity is obtained when it is dissolved in potassium laurate solution, the lowest in potassium ricinoleate solution.

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CONTRIBUTION TO THE KNOWLEDGE OF THE HAEMOLYTIC ACTIVITY OF *HYDROCOTYLE VULGARIS* L

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The use of *Centella asiatica* (L.) Urban in the treatment of leprosy has been reported, and from plants growing in Madagascar, Bontems[1] isolated the crystalline glycoside, asiaticoside. Its aglycone is the monobasic triterpene asiatic acid, which belongs to the α-amyrin series[2] and is therefore closely related to other triterpenoid saponin aglycones. Despite this close affinity asiaticoside appeared to have no haemolytic properties[3].

The investigations of Lythgoe and Trippet[4] have shown that plants of *C. asiatica* occurring in Ceylon contained a glycoside that was not identical with asiaticoside. The centelloside they isolated from the fresh Ceylonese plant differed from asiaticoside not only in its aglycone moiety, but also in its sugar components.

The investigations carried out by these authors into the exclusively tropical *C. asiatica* prompted research on how far *Hydrocotyle vulgaris*, occurring exclusively in Europe, might possess corresponding glycosides. Both genera belong to the tribe Hydrocotyleae, sub-family Hydrocotyloideae of the family Umbelliferae, and are, therefore, botanically closely related. The aims of the work were to ascertain whether asiaticoside is present or not in *H. vulgaris*, applying the method of Bontems; should a glycoside other than asiaticoside be present, to investigate the most suitable method of extraction, isolation and chromatographical examination together with an estimation of haemolytic activity.

The Examination of *Hydrocotyle vulgaris* for Asiaticoside and for Haemolytic Activity

Commencing with 1500 g. of the entire fresh plant, including subterranean organs, of *H. vulgaris*, the extraction method of Bontems[1] was followed. About 600 mg. of a white amorphous solid was isolated which on ignition yielded a residue of about 20 per cent.; the isolate was slightly soluble in water, less so in acid and, on shaking, the aqueous solution gave much persistent froth; it possessed haemolytic activity and, after hydrolysis, glucuronic acid was split off. These facts led to the assumption that acid saponins are present. Subsequent investigations yielded no evidence of the presence of asiaticoside in this mixture of saponins.

Throughout the remainder of the work to be described in this paper the whole plant, including subterranean organs, of *H. vulgaris*, dried *in vacuo* over P_2O_5 and reduced to coarse powder (No. 20 U.S.P.), has been used for extractions.

The haemolytic index (HI) of the material was determined by the method of Runge[5], using dilutions in geometric gradation. Digitonin was initially

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used as a standard but this was later replaced by a purified saponin preparation from the plant itself. Two extraction methods were used: (i) One g. of powdered drug was mixed with 0·4 g. of washed sand, triturated with ethanol 60 per cent. v/v, transferred to a percolator, covered with more solvent, allowed to stand 15 to 20 hours and then percolated with further solvent until, in about 2 hours, 50 ml. of percolate was obtained. Ten ml. of percolate was evaporated to dryness at 70 to 80° C. (it has been shown that this temperature has no influence on the haemolytic action), the dry residue was taken up in 10 ml. of phosphate buffer pH 7·4, warming to 70° C. if necessary, and centrifuging off any undissolved material. The haemolytic index of this solution was then estimated according to Runge's method. (ii) One g. of powder was continuously extracted for 6 hours with chloroform, the extract being rejected. The powder was then extracted with methanol for 7 to 8 hours and the extract adjusted to 50 ml., 10 ml. of which was then evaporated to dryness at 70 to 80° C. and treated as in (i) above. Both methods of extraction yielded practically equal haemolytic index values, and the second method is regarded as more suitable.

This second method of extraction was applied to samples of senega root and sarsaparilla root as well as to *H. vulgaris*. The haemolytic index values for the three drugs are shown in Table I, and it will be seen that the activity of *H. vulgaris* is greater than that of sarsaparilla, and is approximately equal to that of senega and for this reason a more detailed study of the saponins present in the plant was undertaken.

TABLE I

HAEMOLYTIC INDEX FOR 3 DRUGS

Drug	Haemolytic index
Senega root	1925
Sarsaparilla root	1117
Hydrocotyle vulgaris herb ..	1811

ISOLATION OF SAPONIN FRACTIONS

The isolation of saponins from vegetable material using barium hydroxide, magnesium oxide or lead acetate has been shown to result in considerable losses[6]. The method used by Bontems[1] employs lead subacetate and it was rejected on this account. To effect the isolation, a method of continuous extraction with different organic solvents was investigated using successively light petroleum (40° to 60° C.), ether, chloroform, ethyl acetate and methanol, the introduction of ethyl acetate being based on the observation of Rosenthaler[7] that prosapogenins in aqueous medium are easily extracted by shaking with this solvent.

A preliminary experiment was set up with 1 g. of the drug, and continuous extraction, successively using the different solvents for varying times, was applied. The weight of material extracted by each solvent was determined after drying at 80° C. to constant weight and the whole was taken up in 10 ml. phosphate buffer pH 7·4 (if necessary by heating to 70° C.), after which its haemolytic index was determined according to the

method described above. The index was calculated on the basis of the weight of solid (HI_S) and also in relation to the original weight of drug (HI_{Dr}). The HI_S values give some indication of the purity of the extracted haemolytically active substances, the higher the value the more pure the extract.

The results are shown in Table II where it will be seen that repeated extractions were made by both ethyl acetate and methanol. Extraction with chloroform, light petroleum and ether produced very little or no loss of haemolytic activity and since chloroform gave a better extraction of colouring materials it is preferable as a decolourising solvent. Ethyl acetate appeared to extract one group of haemolytically active constituents during the first two or three hours of continuous extraction; more prolonged extraction yielded only a slow and incomplete further extraction. Methanol rapidly extracted the haemolytic activity remaining after ethyl acetate treatment.

The above investigation shows that powdered *Hydrocotyle vulgaris* may be decolourised by the action of chloroform practically without loss of haemolytic activity; from this material one fraction of saponins may be isolated by continuous extraction with ethyl acetate; subsequent treatment of this partially exhausted powder with methanol yields further saponin fractions. This method of isolation was next applied on a larger scale and the two fractions were examined chromatographically on paper both before and after electrodialysis as follows: 250 g. dry powdered drug was mixed with an equal weight of washed sand, made into a paste with chloroform, divided amongst five Soxhlet extractors (Quickfit EX/83/200) and extracted with chloroform for 20 hours, the extract being rejected. The powdered drug was re-dried and extracted with ethyl acetate for 5 to 6 hours, the ethyl acetate extract being reserved. The drug was re-dried and extracted with methanol for 14 hours, the extract being reserved.

The ethyl acetate extract, on standing, deposited a precipitate on the walls of the flask; the solution was decanted, the solvent evaporated almost to dryness under reduced pressure and the residue dissolved in the minimum quantity of methanol 90–98 per cent. The precipitate in the first flask was dissolved in about 50 ml. of warm methanol 80 per cent. v/v and the two solutions were then combined and evaporated at 60° C. to a volume of 30–40 ml. This was allowed to stand for 24 hours in a closed vessel at room temperature, after which it was decanted from any precipitate which formed. The clear solution thus obtained was slowly diluted with 200 ml. ether with continuous mechanical stirring, was allowed to stand at 0° C. for 24 hours, after which the ether layer was decanted leaving a viscous residue. This saponin residue was diluted to about 20 ml. with water and precipitated by adding 4 ml. of 4N sulphuric acid dropwise. After standing at 0° C. for 24 hours the precipitate was centrifuged and washed twice with a little water. It was again centrifuged and was "impure ethyl acetate saponin"; 40 mg. (corresponding to 20 mg. dry substance) was dissolved in 0·4 ml. of methanol and used for paper chromatographic investigation.

The remainder of the impure ethyl acetate saponin fraction was

TABLE II

RESULTS OF SUBJECTING THE DRUG TO EXTRACTION WITH SOLVENTS

Solvent	No.	Experimental series I				Experimental series II			
		a	b	c	d	a	b	c	d
		Extd., hrs.	Residue on drying per cent. at 80° C.	HI_{Dr}	HI_S	Extd., hrs.	Residue on drying per cent. at 80° C.	HI_{Dr}	HI_S
Light petroleum	1	2	1·68	0	0	2	n.d.*	n.d.	n.d.
Ether	2	—	—	—	—	2	0·91	0	0
Chloroform .. (1st)	3	—	—	—	—	4	3·14	20	637
,, .. (2nd)	4	—	—	—	—	—	—	—	—
Ethyl acetate .. (1st)	5	2	6·16	312	5077	3	8·51	316	3714
,, ,, .. (2nd)	6	—	—	—	—	1	1·61	50	3106
,, ,, .. (3rd)	7	—	—	—	—	1	0·72	35	4862
,, ,, .. (4th)	8	—	—	—	—	—	—	—	—
,, ,, .. (5th)	9	—	—	—	—	—	—	—	—
Methanol .. .. (1st)	10	4	17·79	650	3654	3½	15·12	648	4285
,, ... (2nd)	11	—	—	—	—	1	3·50	149	4257
,, ... (3rd)	12	—	—	—	—	2	1·91	60	3141
,, ... (4th)	13	—	—	—	—	3	1·03	50	4913
,, ... (5th)	14	—	—	—	—	4	0·82	0	0

Solvent	No.	Experimental series III				Experimental series IV			
		a	b	c	d	a	b	c	d
		Extd., hrs.	Residue on drying per cent. at 80° C.	HI_{Dr}	HI_S	Extd., hrs.	Residue on drying per cent. at 80° C.	HI_{Dr}	HI_S
Light petroleum	1	—	—	—	—	—	—	—	—
Ether	2	—	—	—	—	—	—	—	—
Chloroform .. (1st)	3	6	6·06	20	331	6	5·55	20	360
,, .. (2nd)	4	1	0·29	0	0	—	—	—	—
Ethyl acetate .. (1st)	5	6	11·08	453	4090	—	—	—	—
,, ,, .. (2nd)	6	1½	1·56	60	3847	—	—	—	—
,, ,, .. (3rd)	7	2½	1·24	34	2743	—	—	—	—
,, ,, .. (4th)	8	2	1·05	29	2762	—	—	—	—
,, ,, .. (5th)	9	3	1·66	60	3615	—	—	—	—
Methanol .. .. (1st)	10	6	13·68	648	4735	6	31·52	1944	6172
,, ... (2nd)	11	1½	0·60	0	0	2	0·79	29	3671
,, ... (3rd)	12	—	—	—	—	—	—	—	—
,, ... (4th)	13	—	—	—	—	—	—	—	—
,, ... (5th)	14	—	—	—	—	—	—	—	—

* n.d. = not determined.

dissolved as completely as possible with very little 0·5N NaOH until just alkaline to phenolphthalein. This solution was filtered through an IG4-crucible and dialysed by means of electrodialysis, using "Ultra-cellafilter, allerfeinst" membranes, a platinum gauze electrode as anode and a silver gauze electrode as cathode. Electrodialysis was continued until the current at 200 volts amounted to only 6–8 milliamp. The contents of the middle chamber were then evaporated in an oven at 70° C. The residue on drying was dissolved in 5 ml. methanol; after storing at 0° C. for 24 hours it was filtered through an IG4-crucible and, after washing the filter with 2–3 ml. methanol, the filtered solution was slowly diluted under mechanical stirring with 120 ml. ether, when the saponins were precipitated. After storing for 20 hours the precipitate was filtered off, washed with ether and dried at 70° C. for 25 minutes. This dried precipitate, yield 250–300 mg., is the saponin fraction provisionally indicated as "dialysed ethyl acetate saponin." Twenty mg. of this fraction

was dissolved in 0·4 ml. methanol and used for paper chromatographic examination.

The methanol extract may contain precipitated material, but this can be rejected if the volume is more than 175 ml. in each flask. The filtered methanol extract was evaporated under reduced pressure (15 mm. Hg.) to 80 ml. which was then slowly diluted with 650 ml. ether with mechanical stirring, and was stored for 24 hours at 0° C. The viscous residue left after decanting the ether layer was diluted to 300–350 ml. with water at 60° C. After cooling, 25 ml. of 4N sulphuric acid was added dropwise with mechanical stirring. On standing at 0° C. for at least 24 hours the saponin precipitated was separated by centrifuging and was washed twice with 150 ml. of 1 per cent. sulphuric acid. About 40 mg. (corresponding to 20 mg. dry substance) of this "impure methanol saponin" was dissolved in 0·4 ml. methanol and used for paper chromatographic investigation.

The remainder of the impure methanol saponin was dissolved in a small quantity of N or 2N caustic soda until the resulting solution was just alkaline to phenolphthalein. Undissolved residue was separated by centrifuging and the clear supernatant liquid was again acidified with 4N sulphuric acid to Congo red, and the above treatment was repeated. This alkaline solution was filtered through a IG4-crucible and the filtrate, diluted with water to 70 ml., was subjected to electrodialysis until the current at 200 volts amounted finally to only 8–10 milliamp. The contents of the middle chamber were evaporated at 70° C. and the residue on drying dissolved in 30 ml. methanol, stored at 0° C., filtered and diluted with 300 ml. ether with mechanical stirring. After standing for 24 hours the precipitate was collected, washed with ether and dried for 25 minutes at 70° C. The white-yellow powder, "dialysed methanol saponin," was obtained in a yield of 3–3·5 g. A solution of 20 mg. of this dialysed methanol saponin in 0·4 ml. methanol was used for paper chromatographic investigation.

The Paper Chromatographic Investigation of Saponin Fractions

Heftmann and Heyden[8] have demonstrated that steroid sapogenins and their acetates were specifically detectable after paper chromatography by spraying the chromatogram with a 2–10 per cent. suspension of rat or guinea-pig blood cells. In 1954 Fiedler[9] made use of citrated ox blood which had been diluted with physiological sodium chloride solution in the ratio 1 : 8.

Method. Whatman No. 1 filter paper was used, spots containing 75–450 μg. being applied at intervals of 2·5 cm. The ascending method of paper chromatography according to Williams and Kirby[10] was employed, the development taking place at 20° C. in an airtight container. When the solvent had travelled about 28 cm. the chromatogram was removed and the solvent front marked.

After investigating the solvents applied by Fiedler, some introduced by Partridge[11] and Jermyn and Isherwood[12] for the paper chromatography of

sugars were found to give a maximal separation of the saponin fractions. These were:

(*p*) benzene/*n*-butanol/pyridine/water..	-1:5:3:3 (upper layer)
(*q*) ethyl acetate/pyridine/water ..	-2:1:2 („ „)
(*r*) phenol/ammonia solution 10 per cent./water (trace HCN).. ..	-75:10:15 (homogeneous layer)

Solvent *p* appeared to be suitable for routine investigation, while solvent *r* gave fine chromatograms but was not suitable for the examination of the "impure ethyl acetate saponin," as two components (Component-S_4 and Hydrocotyle-saponin A_2, see below) could not be separated.

The chromatograms obtained with the solvents *p* and *q* were dried for 40 minutes at 70–80° C. and then sprayed with citrated ox blood diluted with the phosphate buffer pH 7·4 according to Runge in the ratio 1:7. The chromatograms obtained with solvent *r* were first dried at 70° C. for one hour; after that they were exposed to the air for 20 hours before spraying.

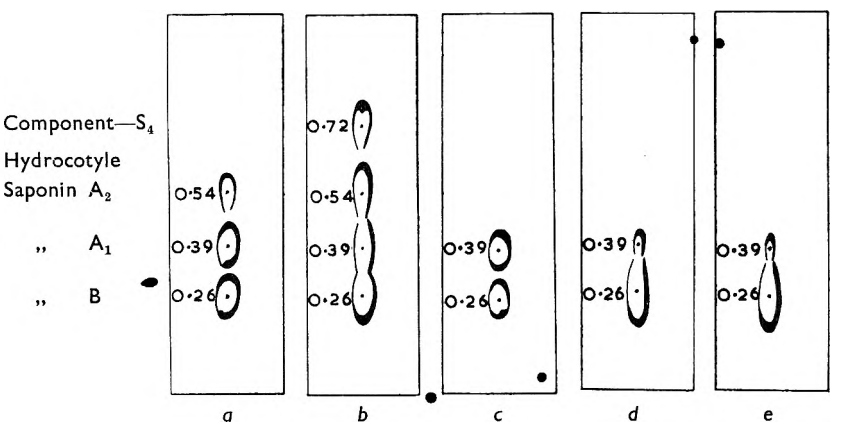

FIG. 1. Chromatograms obtained, using solvent *p*, with the ethyl acetate and methanol fractions of *H. vulgaris*.

a. Impure ethyl acetate saponin.
b. Impure ethyl acetate saponin (high concentration).
c. Dialysed ethyl acetate saponin.
d. Impure methanol saponin.
e. Dialysed methanol saponin.

The chromatograms obtained using solvent *p* with the ethyl acetate and methanol fractions of *H. vulgaris* are shown in Figure 1, *a*–*e*. It was found that the impure ethyl acetate saponin normally shows 3 haemolytically active substances, which are believed to be saponins. These were therefore provisionally called as follows:

with $R_F = 0{\cdot}54$, Hydrocotyle-saponin A_2
with $R_F = 0{\cdot}39$, „ „ A_1
with $R_F = 0{\cdot}26$, „ „ B

If high concentrations were used it was sometimes possible to distinguish a fourth substance with $R_F = 0{\cdot}72$, which was provisionally called Component-S_4. After spraying with the reagent, the spots of Hydrocotyle-saponin A_2 and Component-S_4 appeared first.

After electrodialysis of the impure ethyl acetate saponin, only Hydrocotyle-saponins A_1 and B were observed. Hydrocotyle-saponin A_2 and Component-S_4 passed through the membrane but it seems probable that

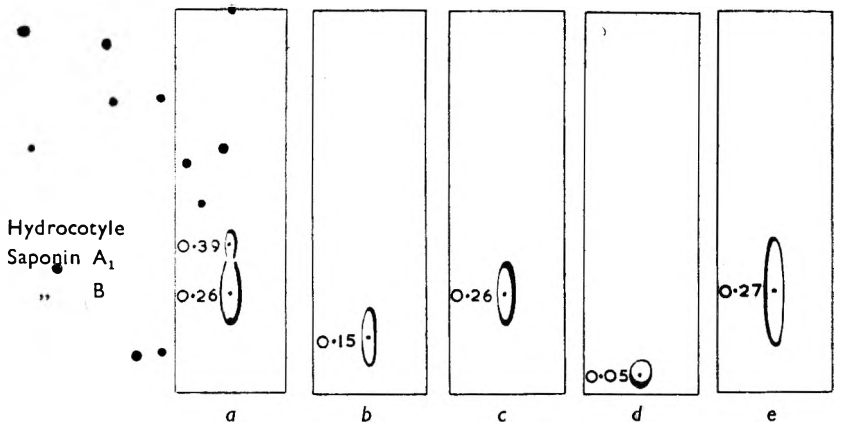

FIG. 2. Chromatographical comparison, using solvent *p*, of:

a. Methanol total extract of *H. vulgaris*.
b. Methanol total extract of senega root.
c. Saponin fraction obtained according to Bontems' isolation method.
d. Quillaia saponin.
e. Digitonin.

they are saponins, since the aglycone of Hydrocotyle-saponin B and of some commercial saponins were found again in the solvent front of the solvent *p*; they may be crystalline saponins, which pass through the membranes more quickly than amorphous saponins.

Both the impure and the dialysed methanol saponin fractions behaved in the same manner and appeared to consist of Hydrocotyle-saponin B with a small amount of Hydrocotyle-saponin A_1. The comparatively large yield (3–3·5 g.) of dialysed methanol saponin means that from a quantitative point of view Hydrocotyle-saponin B forms the most important haemolytically active component of *H. vulgaris*.

Using solvent *p*, the haemolytically active substances of *H. vulgaris*, obtained by continuous extraction with methanol of 1 g. of drug for 8 hours after a 6-hour extraction with chloroform, were compared chromatographically with (i), a methanol extract of a senega root prepared as for *H. vulgaris*; (ii) a solution in methanol of the saponin preparation obtained according to the isolation method of Bontems (50 mg./ml.); (iii) a solution of quillaia-saponin in water (50 mg./ml.); (iv) a solution of digitonin in methanol (50 mg./ml.). These chromatograms are shown in Figure 2, *a–e*, from which it will be seen that the methanol total extract of *H. vulgaris* gave, in high concentrations, only 2 components, namely:

Hydrocotyle-saponin A_1 and B with the regular R_F values 0·39 and 0·26. The methanol total extract of senega root yielded one spot, senegin, with $R_F = 0{\cdot}15$. The saponin preparation obtained according to Bontems' isolation method showed one spot with R_F value = 0·26 which appeared to be identical with Hydrocotyle-saponin B. The quillaia-saponin appeared as a fine, nearly round spot and had a R_F value = 0·05. Digitonin in low concentrations gave less fine, elongated spots with R_F values that ranged between 0·25 and 0·30. It thus appears that other saponins may be distinguished from those occurring in *H. vulgaris* when examined by paper chromatography using solvent *p*.

Summary

1. A systematic investigation of *Hydrocotyle vulgaris* using small quantities of the dried herb has shown the presence of two different saponin fractions, which were isolated by continuous extraction with ethyl acetate and with methanol.

2. Using paper chromatography four haemolytically active substances can be detected. A dilution of citrated ox blood with the phosphate buffer pH 7·4 according to Runge in the ratio 1 : 7 serves as a specific detection of the saponins on the paper.

3. The herb possesses haemolytic activity equal to that of senega root.

4. Two methods of extraction are given for determining the haemolytic index of the dried herb.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Chloramphenicol, Colorimetric Determination of. F. M. Freeman. (*Analyst*, 1956, **81**, 299.) The method depends upon the formation of a stable red colour resulting from interaction of the nitro group with a dimethylformamide-acetone solution in the presence of tetraethylammonium hydroxide. To 10 ml. of dimethylformamide is added 0·2 ml. of 0·1 per cent. solution of acetone in dimethylformamide and 0·1 ml. of a 25 per cent. solution of tetraethylammonium hydroxide in water. After mixing, 0·1 to 0·15 mg. of chloramphenicol in dimethylformamide is added, the tube shaken and the absorption at 520 mμ measured after 10 minutes using a 1-cm. cell and a reagent blank. D. B. C.

Chlorinated Organic Pesticides, Separation and Identification of. L. C. Mitchell. (*J. Ass. off. agric. Chem., Wash.*, 1956, **39**, 484.) A procedure is described by which the following chlorinated pesticides may be identified and separated by paper chromatography: Aramite (2(*p-tert.*-butylphenoxy)*iso*-propyl-2-chloroethylsulphite), captan (*N*-trichloromethylmercapto-4-*cyclo*hexene-1 : 2-dicarboximide), dieldrin(1 : 2 : 3 : 4 : 10 : 10-hexachloro-6 : 7-epoxy-1 : 4 : 4a : 5 : 6 : 7 : 8 : 8a-octahydro-1 : 4-endo, exo-5 : 8-dimethanonaphthalene), Gamma benzene hexachloride, Spergon (chloranil : 2 : 3 : 5 : 6-tetrachloro-1 : 4-benzoquinone), and tritisan (pentachloronitrobenzene). The solvent systems recommended are acetic anhydride 20 per cent. in ether as immobile solvent and *n*-heptane as mobile solvent, or refined soyabean oil 5 per cent. in ether as immobile solvent and 95 per cent. ethanol as mobile solvent. The two systems did not separate the 6 pesticides in the same order. With acetic anhydride-*n*-heptane the ascending order was captan, Spergon, Aramite, gamma benzene hexachloride, dieldrin and tritisan; with oil-ethanol it was tritisan, Spergon, dieldrin-gamma benzene hexachloride, Aramite and captan. Several chromogenic agents were used and the relative merits of each are discussed. The R_F values for the pesticides in either of the solvent systems are given and it is shown that the 6 compounds can be identified in mixtures by the order of separation and the distances from one another. B. A. W.

Lobeline, Determination of. E. Steinegger and F. Ochsner. (*Pharm. Acta Helvet.*, 1956, **31**, 65.) Lobelia herb may contain up to 20 bases, and chromatographic separation of the lobeline is essential. The total alkaloids are first determined, and this operation may be carried out with 1·2 g. of the drug if 0·01N solutions are used. For the chromatography 9 ml. of formamide is shaken with anhydrous ammonium formate and treated with 20 ml. of acetone. The excess of formate is filtered off, and 1 ml. of concentrated formic acid is added. The paper is saturated with this mixture, and hung up to drip and evaporate. The mobile phase is composed of 9 volumes of chloroform and 1 volume of benzene. A test is run with pure lobeline, followed by a little of the mixture, as the presence of other compounds modifies the R_F value. This spot is made visible with Dragendorff's reagent. The lobeline to be determined is extracted, using the method of the authors (*Pharm. Acta Helvet.*, 1955, **30**, 345) and the extinction is determined at 245 mμ. A blank is done

from a strip of the chromatogram cut out at the same height. Examples of results obtained are given below:

	L. salicifolia	*L. inflata*
Total alkaloids per cent. ..	1·388	0·342
Lobeline per cent.	0·17	0·03

G. M.

Nux Vomica Tincture, Simplified Assay for. M. Scott, A. Taub and C. Piantadosi. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 232.) The method depends upon the treatment of a sample of tincture with aluminium oxide which absorbs pigments, followed by absorption of the alkaloids on an ion exchange resin, subsequent elution and estimation of strychnine and brucine by ultra-violet spectrophotometry. 10 g. of aluminium oxide is stirred with 5 ml. of tincture and 5 ml. of ethanol (70 per cent.) for 5 minutes, allowed to stand and filtered. The aluminium hydroxide is washed with quantities of ethanol (70 per cent.) and the washings mixed with the filtrate and made up to 50 ml. 10 ml. of this solution is adjusted to pH 9–10 with ammonia solution, mixed with 10 ml. of specially prepared and purified resin (Amberlite IRC–50(H)), the liquid filtered off and the resin washed. The alkaloids are extracted from the resin by heating at 80° C. with successive quantities of 25 ml. of 0·1N hydrochloric acid in ethanol (70 per cent.) and the extracts diluted to 100 ml. This solution, after suitable dilution with water is clarified by filtration through a porous crucible and the absorbance determined at 255 and 264 mμ, using a blank prepared as above, taking 5 ml. of ethanol (70 per cent.) instead of the tincture. The quantity of strychnine and brucine present is calculated by means of simultaneous equations. The method is rapid and gives results in agreement with the U.S. National Formulary assay. G. B.

***Rauwolfia serpentina* Preparations, The Chemical Evaluation of.** J. Carol, D. Banes, J. Wolff and H. O. Fallscheer. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 200.) The following method is recommended for the determination of reserpine and rescinnamine, the constituents of *Rauwolfia serpentina* which are chiefly responsible for the hypotensive and sedative effects of the drug. Preparations of powdered root, extracts and tablets are extracted and the fraction containing reserpine and rescinnamine is separated by partition chromatography on a Celite column using an ethanolic citrate buffer solution as the immobile solvent and a mixture of *iso*-octane, chloroform, water and ethanol as the mobile solvent. The solution containing reserpine and rescinnamine is evaporated and hydrolysed in alcoholic sodium hydroxide solution by heating on a water bath for 20 minutes. Trimethoxybenzoic and trimethoxycinnamic acids are extracted from the solution by acidifying and shaking with chloroform, and the light absorption of the chloroform solution is determined at 270 and 300 mμ. The quantities of trimethoxybenzoic and trimethoxycinnamic acids, and hence of reserpine and rescinnamine are calculated after determination of the absorbancies of solutions of the pure acids in chloroform. The method appears to be satisfactory for commercial preparations, although in one case interference was caused by the dye from the coating of a tablet and in another by an emulsifying agent present in the preparation. For the identification of *Rauwolfia serpentina* preparations, two methods of ascending paper chromatography are proposed. One requires the use of a mixture of heptane, carbon tetrachloride and formamide as mobile solvent in an atmosphere of ammonia. The other, which permits the separation of alkaloids of relatively high R_F

value, depends on the use of a mixture of benzene, *iso*-octane and formamide. A large number of fluorescent spots are obtained in these tests, and no other species of *Rauwolfia* produces all of them. G. B.

Tyrothricin, Nephelometry in Assay of. S. Leclercq. (*J. Pharm. Belg.*, 1956, **11**, 33.) *Streptococcus faecalis* M19 or *Staphylococcus aureus* 209P or Oxford may be used as the test organism, special precautions being necessary to ensure that the culture employed is in the logarithmic growth phase. Solutions of a standard preparation of tyrothricin are prepared to contain 0·033, 0·05 and 0·075 μg./ml. in a mixture of propylene glycol 43·75 ml., water 43·75 ml. and ethanol (95 per cent.) 12·5 ml. Similar dilutions are prepared from the sample under examination, and tubes of the test culture are inoculated with 1 ml. of the tyrothricin solutions. 8 tubes are inoculated for each concentration of the standard, and 6 for each concentration of the unknown. The tubes are incubated at 37° C., 2 tubes of each concentration of the standard being used as pilot tubes, the turbidity being measured at intervals until the change in turbidity is proportional to the logarithm of the concentration and the slope of the turbidity/log. concentration graph is sufficient to give a high precision. Growth in all tubes is stopped by adding 2 drops of formaldehyde solution. The turbidities in the tubes are measured, and the concentration of tyrothricin calculated. The assay is sensitive and good precision may be achieved. It is not affected by the usual quantities of quaternary ammonium compounds such as cetrimide which may be present in pharmaceutical preparations of tyrothricin. G. B.

ORGANIC CHEMISTRY

Cephalosporin C, Degradation of. E. P. Abraham and G. G. F. Newton. (*Biochem. J.*, 1956, **62**, 658.) Cephalosporin C was rapidly hydrolysed at pH 12 with loss of activity and of the absorption maximum at 260 mμ. Back titration after 2 hours at pH 12 showed that two new acidic groups had been formed. The solution gave a positive nitroprusside test, but titration failed to reveal the presence of a new group with a pK indicative of a simple thiol. Alkaline hydrolysis with barium hydroxide (0·3N; 100° C.; 2 hours) followed by paper ionophoresis, showed the formation of glycine and α-aminoadipic acid. Cephalosporin C treated with cephalosporinase gave an acid, with loss of the absorption maximum at 260 mμ. Hydrolysis in N hydrochloric acid at 105° C. gave carbon dioxide, a volatile base (2 equivalents), D-α-aminoadipic acid, and traces of glycine, whilst hydrolysis of dinitrophenylcephalosporin C gave dinitrophenyl α-aminoadipic acid, indicating the presence of a D-α-aminoadipic acid residue with a free amino group. The pK (9·8) of the amino group in cephalosporin C was very close to that of the amino group of α-aminoadipic acid, suggesting that the α-carboxyl group of this residue is also free in cephalosporin C. Hydrogenation of cephalosporin C at a palladium-charcoal catalyst gave a product which retained the ultra-violet absorption at 260 mμ, but only 10 per cent. of the antibacterial activity of cephalosporin C. Hydrolysis of this material with dilute acid at 105° C. gave α-aminoadipic acid and glycine. Treatment of the hydrolysate with bromine yielded a small quantity of substance, which when chromatographed on paper behaved as penicillaminic acid. Hydrogenolysis with Raney nickel and acid hydrolysis of the product gave D-α-aminoadipic acid, valine, L-alanine and glycine, and in this respect cephalosporin C resembles the penicillins. The dinitrophenyl and benzyl derivatives of cephalosporin C are more active against *Staph. aureus* and less active against *Salm. typhi* than the parent compounds. J. B. S.

BIOCHEMISTRY

BIOCHEMICAL ANALYSIS

Chlorpromazine, Colorimetric Estimation of. H. Leach and W. R. C. Crimmin. (*J. clin. Path.*, 1956, **9**, 164.) A rapid and sensitive method is described for the estimation of chlorpromazine in blood and urine. For extraction from urine, add 1 ml. of N sodium hydroxide to 20 ml. of urine, extract four times with 20 ml. portions of ether and wash the pooled extracts with 10 ml. 0·2N sodium hydroxide followed by 10 ml. of water. Remove the wash water completely and shake the extract vigorously with 0·1N sulphuric acid. Stand for five minutes, remove the acid extract, warm slightly and aerate to remove traces of ether. Take duplicate 4 ml. quantities for the estimation. For free chlorpromazine in blood, heat 5 ml. of oxalated blood with 5 ml. of 50 per cent. potassium hydroxide for two minutes on a boiling water bath. Cool, add 10 ml. of water, shake with 20 ml. ether and remove the ether layer. Repeat the extraction three times. Wash the pooled ethereal extracts with sodium hydroxide and water, extract with 6 ml. of 0·1N sulphuric acid and take 4 ml. of the acid extract for colour development. For extraction of total chlorpromazine in blood, heat 5 ml. of blood and 5 ml. of concentrated hydrochloric acid in a boiling water bath for five minutes. Cool, make alkaline and extract with ether as for chlorpromazine. For the estimation treat 4 ml. samples with 2 ml. of 50 per cent. sulphuric acid and mix well. Add 0·2 ml. of a solution of 2 per cent. ferric nitrate in 1·0N sulphuric acid and read the colour at 530 μ (Ilford spectrum green filter 604) against a water blank. Obtain the result from a standard calibration curve. At least 90 per cent. of chlorpromazine added to urine could be recovered down to 1·0 mg. per litre. G. F. S.

Mercury in Biological Materials, Microdetermination of. F. R. Barrett. (*Analyst*, 1956, **81**, 294.) A method is described suitable for quantities up to 20 μg. with a sensitivity of 0·5 μg. of mercury, and for handling quantities up to 20 g. of blood or tissue. A modified digestion procedure is described which ensures complete destruction of organic matter with moderate heating so that no mercury is lost. The tissue is digested with an appropriate amount of a mixture of equal parts of concentrated sulphuric and nitric acids under a cold-finger condenser for 2 hours after foaming has ceased. The bulk of fatty material is then filtered off through glass wool. Potassium permanganate is added in the form of 0·5 g. tablets and the mixture heated until a precipitate of manganese dioxide persists after boiling. It is then diluted with water and further tablets added while boiling until a pink colour persists and any fatty material has been oxidised. After decolorisation with hydroxylamine hydrochloride, the solution or an aliquot of it is extracted with a solution of dithizone in chloroform (6 mg. per litre) and the chloroform extracts washed with 0·25N sulphuric acid and then extracted with a mixture containing 10 ml. of a 40 per cent. solution of potassium bromide and 50 ml. of 0·25N sulphuric acid. This latter extract, containing mercury as K_2HgBr_4, is brought to a pH of about 6 with a buffer containing disodium hydrogen phosphate and sodium carbonate and extracted with exactly 10 ml. of dithizone reagent which is separated and filtered through a plug of absorbant cotton wool. The absorption of the solution at 490 mμ is measured. A blank must be carried through the whole process. Practical results are given which show that good recoveries of mercury added to blood and liver samples are obtained. The preliminary extraction of the metal is claimed to minimise interference from copper and other heavy metals. D. B. C.

PHARMACY

NOTES AND FORMULAE

Digitalis Leaf, Stability of. R. A. Sachs, J. D. Highstrete and M. L. Pabst. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 250.) The potency of a sample of digitalis was determined by the U.S.P. XIV pigeon method, and redetermined at intervals of 12, 18 and 24 months. The initial potency was 17·4 units/g., and there was a significant loss during the first 12 months' storage, the potency then averaging 13·5 units/g. No further loss in potency was observed during the following 12 months. The samples were stored in the form of whole leaf and in powder, in metal containers at room and refrigerator temperatures, and in paper sacks and cardboard containers at room temperature. There was no significant difference in stability between whole and powdered leaf, but samples stored in sealed metal containers retained their potency better than those stored in paper sacks or cardboard containers. There appeared to be no advantage in storing the metal containers in a refrigerator. G. B.

Erythrocyte-Glycerol Mixtures; Preparation for Transfusion. H. A. Sloviter and R. M. Tietze. (*Amer. J. med. Sci.*, 1956, **231**, 437.) Erythrocyte-glycerol mixtures which have been thawed after prolonged storage at low temperatures cannot be transfused directly because of the osmotic lysis of the hypertonic erythrocytes which would occur. A simple method is described whereby the osmotic lysis is prevented and the erythrocytes are relatively rapidly prepared for transfusion. To the thawed erythrocyte-glycerol mixture is added concentrated glucose solution (50 g. of glucose and 0·9 g. of sodium chloride per 100 ml.) in the proportion of 50 ml. of glucose solution per 100 ml. of thawed mixture. After standing for 10 minutes at room temperature the mixture is quickly diluted with twice its volume of 0·9 per cent. sodium chloride solution. The resulting suspension of erythrocytes contains approximately 4·5 per cent. glycerol and 5·5 per cent. glucose in addition to the electrolyte, and has a total osmotic pressure about 4 times that of blood plasma. *In vitro* experiments simulating the osmotic forces which occur during transfusion showed the feasibility of the method from the osmotic point of view. It was shown that 85 per cent. of the original numbers of erythrocytes remained intact after prolonged storage, processing and simulated transfusion. Transfusion studies in man are now in progress. S. L. W.

Oil-in-water Emulsions, The Formation of, with Ultrasonic Vibrations. J. Mounier, P. Blanquet, G. Piffault and G. Dallies. (*Bull. Soc. Pharm. Bordeaux*, 1955, **94**, 161.) Experiments were carried out with an ultrasonic generator tuned to 958, 576, 320 and 288 kc/s. With radiation of constant strength, an oil-in-water emulsion was formed which fairly rapidly became richer in oil until a constant value was reached. Emulsions formed at any of the above frequencies were very stable and were not destroyed by another of those frequencies. The rapidity with which the oil was emulsified was dependent on the viscosity of the oil, castor oil and liquid paraffin being less rapidly emulsified than olive and sweet-almond oils, but was independent of the chemical nature of the oil. Particles of oil in the emulsions appeared to be spherical, 0·5 to 4 μ in diameter, but larger particles appeared when high intensities of radiation were used and foaming was allowed to occur. Of the oils examined, only sweet-almond showed any chemical change during the treatment with ultrasonic radiation. In this case the iodine value fell from 75 to 51. G. B.

PHARMACOLOGY AND THERAPEUTICS

Carbutamide in the Treatment of Diabetes. A. S. Ridolfo and W. R. Kirtley. (*J. Amer. med. Ass.*, 1956, **160**, 1285.) This is a report on the compound carbutamide, 1-butyl-3-sulphanilylurea (BZ-55). Carbutamide is rapidly absorbed when administered by mouth; within 30 minutes after a single 2·5 g. dose there is an appreciable concentration in the blood. Maximum values (10–15 mg. of free sulphonamide per 100 ml. of whole blood) are reached within 3 to 6 hours, and the blood level falls slowly after 6 to 7 hours. Within 2 to 3 hours after ingestion of a dose of 2·5 g. a definite lowering of blood sugar level occurs. Excretion is relatively slow; the drug is found in the urine with approximately 66 per cent. as the free form and 33 per cent. as the acetylated form. The substance does not have an action equivalent to injected insulin; to be effective, some insulin must be present, either endogeneous or injected. Carbutamide was administered to 31 diabetic patients. Satisfactory responses were achieved with an average loading dose of 2·5 g. the first day, 1·5 g. the second day, and 1 g. daily thereafter; attempts were made to maintain a level of at least 10 mg./100 ml. of blood. This trial showed that carbutamide will effectively lower the blood sugar level in many patients with mild or moderately severe diabetes, and in some cases may enable them to dispense with insulin. Carbutamide cannot be used in the emergency treatment of a diabetic with acidosis, nor is it satisfactory in young persons with unstable diabetes; in this respect it is not an insulin substitute. Those responding favourably to the drug are those who become diabetic in maturity, are obese or overweight and have not required an excessive dose of insulin. Only one patient out of 31 showed any toxic effects, a skin rash and leucopenia, which developed after 3 months on a dose of 1 g. daily; these conditions cleared up on discontinuation of therapy. The toxicity of the drug appears to be low, but since the mechanism of action is not yet known it should be used with caution. S. L. W.

Methylserotonins as Potent Antimetabolites of Serotonin. E. N. Shaw and D. W. Woolley. (*J. Pharmacol.*, 1956, **116**, 164.) The serotonin-like and anti-serotonin activities of several alkyl-substituted serotonins were estimated *in vitro* on sheep carotid artery segments and on oestrogenic rat uteri, and *in vivo* on the blood pressure of anaesthetized dogs. Anti-serotonin activity was expressed as an inhibition index, being the amount of analogue required to prevent the effect of a unit weight of serotonin, usually measured after one minute exposure to the analogue. The inhibition indices for 2:5-dimethylserotonin were 10 for arterial segments, and 100 to 1000 for the uterus, the latter becoming 10 to 20 after 20 minutes exposure to the antagonist. The antagonist had no serotonin-like activity on the arterial segments and little on the uterus. On the blood pressure, however, about 1 mg./kg. i.v. of the dimethylserotonin caused a rise, the response to subsequent doses of serotonin being considerably modified: the pressure rise was abolished and the initial fall in pressure became very prominent and persistent, even where previously the initial fall had been absent. Large intravenous doses of serotonin protected against a subsequent injection of the unit dose, through tachyphylaxis, but compared with the anti-serotonin activity of 2:5-dimethylserotonin, the effect was short-lived, recovery being full after one hour. Orally 2:5-dimethylserotonin was about 10 times less effective than by injection. 1:5-Dimethylserotonin had marked serotonin-like activity, being 1/10 to 1/5 as active as serotonin on the uterus and 1/10 on blood pressure: anti-serotonin activity was less than that of 2:5-dimethylserotonin. The inhibition indices of other serotonin derivatives on the rat uterus

were: 1-benzyl-2:5:-dimethylserotonin, 30; 1-benzyl-5-methylserotonin, 40; 1:2:5-trimethylserotonin, 200; 2:5-dimethylserotonin on this tissue having an index of 300. The increase in potency with the benzyl compounds was associated with irreversible inhibition by the compounds, which may explain the high activity. The 1-benzyl-2:5-dimethyl derivative was also the most effective orally in antagonizing the serotonin pressor response in the dog. Toxicity of the compounds in mice was low. G. P.

Morpholinoethylnorpethidine, Analgesic and other Properties of. A. F. Green and N. B. Ward. (*Brit. J. Pharmacol.*, 1956, **11**, 32.) Determination of the analgesic action of this compound in the rat by heat and tail pressure methods have shown it to have an activity intermediate between morphine and pethidine. The compound was capable of giving as great an elevation of the pain threshold as morphine. The LD50 by the intravenous route in mice was 45 mg./kg., and the main toxic action was respiratory depression, which paralleled analgesic activity. The action of the compound on the cough reflex in cats, on rectal temperature in rabbits and on heart rate and pupil diameter in dogs also resembled those of morphine. It abolished the peristaltic reflex of the isolated guinea-pig ileum and caused defaecation in dogs. Like morphine it produced excitement in cats. The effects of the compound were antagonised by nalorphine. G. F. S.

Motion Sickness, Evaluation of Drugs for Protection Against. (*J. Amer. med. Ass.*, 1956, **160**, 755.) The results are recorded of a trial of a number of drugs against motion sickness. These included diphenhydramine, meclizine, 1-diethylamino-2-(2′-benzyl-4′-chlorophenoxy) ethane (BL-717), dimenhydrinate, cyclizine, ethopropazine, promethazine, pyrathiazine, scopolamine hydrobromide and pheniramine. Other compounds given preliminary screening included calcium pantothenate, nicotinamide, pyridoxine and thiamine, and the antihistamines *N*-benzhydryl-*N*-*m*-methylbenzyl-piperazine, buclizine (Vibazine) hydrochloride, β-diethylaminoethylphenothiazine-10-carboxylate (Transergan), phenyltoloxamine (Bristamin), and 1-methyl-4-amino-*N*-phenyl-*N*′-(2′-thenyl)-piperidine tartrate (Sandostene); antispasmodics such as benztropine (Cogentin) methanesulphonate, scopolamine methobromide (Pamine bromide); and tranquilizers such as chlorpromazine (Thorazine), reserpine (Serpasil), and the alseroxylon fraction of *Rauwolfia serpentina* (Rauwiloid). The drugs were tested on service personnel in transport ships of the same type on the Atlantic crossing during the autumn and winter months. All medicaments and a placebo were supplied in pink capsules to ensure identical appearance, and the capsules were swallowed under observation. Vomiting was the sole criterion of effectiveness, and the level of significance of each drugs' effectiveness compared with the placebo was determined by a chi-square test. Four regimens, namely, meclizine 50 mg. 3 times daily and once daily, cyclizine 50 mg. 3 times daily, and promethazine 3 times daily, by statistical analysis were more effective than the other treatments tested. Diphenhydramine and dimenhydrinate were identical with the placebo. Reserpine, alseroxylon, and scopolamine hydrobromide, especially the latter when more than a single dose was given, were responsible for numerous distressing side effects. Cyclizine gave significant but not impressive protection when given twice daily and promethazine twice daily was inferior to meclizine once daily; there was no statistical difference between the results of giving meclizine once daily and three times daily. None of the vitamins nor chlorpromazine was of any value. For long sea voyages meclizine is the drug of choice. For shorter voyages, a single dose of meclizine, cyclizine or promethazine should be equally effective. H. T. B.

Noradrenaline in the Adrenals of Young Dogs. T. Ozaki. (*Tohoku J. exp. Med.*, 1956, **63**, 241.) Extracts of the adrenal glands of young dogs were made in 4 per cent. trichloroacetic acid and the adrenaline and noradrenaline content estimated by the permanganate method. It was found that in dogs of 2–7 days there was a mean of 64 per cent. noradrenaline, in dogs of 20–30 days 42 per cent. noradrenaline, while in adult dogs a value of 18·5 per cent. noradrenaline was found. This is similar to results obtained in guinea-pigs, rabbits and cats. M. M.

Novobiocin, Laboratory and Clinical Evaluation. F. Lin and L. L. Coriell. (*Antibiotic Med.*, 1956, **2**, 268.) Data are presented that show that novobiocin is bacteriostatic in low concentration (0·19 to 50 μg./ml.), and bactericidal in higher concentration (200 to 400 μg./ml.), being intermediate in this respect between penicillin and erythromycin. Very high blood levels are obtained promptly following oral dosage, adequate levels persisting for at least 8 hours. Every one of 22 strains of *M. pyogenes* var. *aureus* isolated from patients was found sensitive to novobiocin; 19 out of the 22 were resistant to penicillin, 12 were resistant to chloramphenicol and 5 to erythromycin. Resistance to novobiocin developed in a step-like manner similar to that with erythromycin and chloramphenicol. Of 11 strains of micrococci isolated from patients, all became resistant to novobiocin following 12 serial transfers in a sublethal concentration of the drug. The occurrence of cross-resistance with erythromycin, chloramphenicol and penicillin was not demonstrated; several strains actually became more sensitive to penicillin after they became resistant to novobiocin. Novobiocin was used in 12 staphylococcal infections of the skin, respiratory tract, and bone, 3 cases of scarlet fever, and 1 case of cutaneous anthrax. All patients recovered promptly, both clinically and bacteriologically, with the exception of a case of fibrocystic disease of the pancreas with extensive bronchopneumonia. In most cases of pyodermia, abscesses, pneumonia, and osteomyelitis dramatic clinical improvement appeared after 24 hours of therapy. A dose of 5 mg./kg. repeated every 6 to 8 hours was found sufficient in most cases to maintain continuous therapeutic blood levels. The only side-effects noted were an urticarial rash in one patient and diarrhoea in another. S. L. W.

Novobiocin; Plasma and Spinal Fluid Concentrations in Man. G. M. Bayne, S. C. Strickland, J. M. Glyfe and W. P. Boger. (*Antibiotic Med.*, 1956, **2**, 166.) Novobiocin (previously called streptonivicin or cathomycin) is an antibiotic with activity against most of the commonly pathogenic Gram-positive organisms and several Gram-negative organisms. *Micrococcus pyogenes* var. *aureus*, *Corynebacterium diphtheriae*, *Streptococcus pyogenes*, *Diplococcus pneumoniae*, and *Streptococcus agalactiae* are all inhibitied by concentrations less than 5 μg./ml. and *Streptococcus faecalis* by 12·5 μg./ml. Of the Gram-negative organisms *Haemophilus pertussis*, *Pasteurella multocida*, and *Proteus vulgaris* are all sensitive at concentrations less than 10 μg./ml. From a study of 41 individuals given either single or multiple doses of novobiocin, data are presented which indicate that an initial oral dose of 2 g. of novobiocin sodium promptly produced a plasma level averaging 126·8 μg./ml., and subsequent doses of 500 mg. at 12-hourly intervals tended to maintain levels in the range of 20 μg./ml. or greater. Such levels are four or more times greater than those necessary for the *in vitro* inhibition of the common Gram-positive pathogens. In 24 individuals who received single oral doses of novobiocin up to 2 g. none of the antibiotic was assayable in the spinal fluid despite plasma levels up to 90 μg./ml. The samples of cerebrospinal fluid were obtained within 2–7 hours after administration of the dose. None of the patients studied showed any evidence of untoward side-effects with the exception of vomiting in one case. S. L. W.

Novobiocin, Studies on. H. J. Simon, R. M. McCune, P. A. P. Dineen and D. E. Rogers. (*Antibiotic Med.*, 1956, **2**, 205.) The studies reported in this paper indicate that novobiocin possesses a high degree of *in vitro* activity against the Gram-positive cocci. Most strains of staphylococci were found sensitive to low concentrations of novobiocin regardless of their sensitivity or resistance to other antibiotics. Most strains of proteus isolated directly from patients appeared moderately sensitive to novobiocin but all other Gram-negative micro-organisms studied proved resistant. The *in vitro* studies indicated a high degree of serum binding with 90 to 95 per cent. loss of novobiocin activity in the presence of 20 per cent. serum. Such binding is rapidly reversible on simple dilution, and prompt urinary excretion in high concentrations suggests that the novobiocin-protein complex is readily dissociated *in vivo*. Novobiocin at dietary levels of 100 mg./kg. was effective in the treatment of an experimental staphylococcal infection in mice; an experimental proteus infection in mice also responded to treatment. Studies in man indicated that novobiocin is rapidly absorbed from the gastrointestinal tract. High serum concentrations were frequently attained after oral ingestion of the drug, peak concentrations of 25–50 μg. occurring 1 to 6 hours after ingestion of 500 mg., with detectable serum concentrations remaining at 8 hours. Marked variation in serum concentrations and 24-hour urinary excretion was noted, suggesting variation in the degree of intestinal absorption. Ingestion of the drug on an empty stomach produced significant serum concentrations more rapidly than ingestion after meals. Novobiocin did not appear in the cerebrospinal fluid but was found in significant concentrations in pleural and ascitic fluid after an initial time lag. High urine concentrations were achieved following oral administration though only a small fraction of the dose was recovered from the urine in the first 24 hours. No evidence of hepatic, renal, or haemopoietic system toxicity was observed following administration of novobiocin to a small number of patients. S. L. W.

Oxytocin as Stimulator for the Release of Prolactin from the Anterior Pituitary. G. K. Benson and S. J. Folley. (*Nature, Lond.*, 1956, **177**, 700.) The suckling stimulus influences the mammary gland in two ways. First, suckling or other conditioned stimuli can cause the release from the neurohypophysis of oxytocin, which in turn causes the contraction of the myoepithelial cells associated with the alveoli, resulting in ejection of stored milk. Secondly, the suckling stimulus causes the release from the anterior lobe of the pituitary of prolactin which participates in the maintenance of secretion and of the functional integrity of the mammary alveolar tissue. These facts suggest that the primary effect of such impulses is to activate the neurohypophysis, thus causing a release of oxytocin which stimulates the anterior pituitary to release prolactin. In order to test this hypothesis use was made of the fact that the involution of mammae in the lactating rat, the suckling of which is prevented by the surgical removal of the nipples, is retarded provided that suckling of the intact nipples is continued. Studying the effect of injections of oxytocin on the course of mammary involution in lactating rats from which the litters were removed on the fourth day of lactation, it was found that the oxytocin treatment caused marked retardation of mammary involution. Such results indicate that the release of prolactin (and perhaps other anterior-pituitary hormones concerned in lactation) can be stimulated by treatment with oxytocin. It is unlikely that the results are due to a direct effect of oxytocin on the mammary gland. M. M.

Penicillin-Triple Sulphonamide Mixture in Urinary Tract Infections. A. W. Bohne and W. E. Chase. (*Amer. J. med. Sci.*, 1956, **231**, 389.) A clinical

and bacteriological study was conducted in 148 out-patients, of whom 98 per cent. were adult females, to compare the effectiveness of two oral preparations, a triple sulphonamide mixture and a penicillin-triple sulphonamide mixture, in uncomplicated acute and chronic infections of the urinary tract. The triple sulphonamide mixture contained sulphadiazine, sulphamerazine and sulphadimidine, 0·167 g. of each, or a total of 0·5 g./5 ml. in an alumina gel base; the second mixture contained the same amounts of the three sulphonamides plus 150 mg. of benzathine-penicillin G in 5 ml. Microscopic examination and culture of urine specimens before medication demonstrated a variety of organisms, with *E. coli* present in 65 per cent. An initial dose of one or other of the mixtures of 2 g., followed by 0·5 g. 4 times a day was given for 7 days. There was no significant difference in the effectiveness of the two mixtures. Of 71 patients treated with the triple sulphonamides 79 per cent. were freed of urinary infection; of 77 who received the penicillin-sulphonamide mixture 75 per cent. were freed of infection. Both preparations were active against the coccal infections. The triple sulphonamide agent eradicated *Proteus vulgaris* in 6 out of 7 cases. The penicillin-sulphonamide mixture was more effective in preventing secondary growth of organisms not present in pre-treatment cultures. Reactions, including headache, chills, rash, swelling and redness of the tongue occurred in only 3 cases and subsided promptly on withdrawal of medication. There was no evidence of crystalluria and no gastrointestinal disturbances. S. L. W.

Prednisone and Prednisolone in Rheumatoid Arthritis. E. W. Boland. (*J. Amer. med. Ass.*, 1956, **160**, 613.) The effects of prednisone, prednisolone and hydrocortisone were compared in 141 arthritic patients over periods of from 6 to 9 months. None of the cases was mild and the average duration had been 126 months. The drugs were discontinued in 12 cases because improvement was insignificant and/or serious complications intervened. In the moderately severe cases the maintenance doses of prednisone and prednisolone were found to be 5 to 15 mg./day by mouth, indicating that these drugs are about four times as potent as hydrocortisone. Prednisone and prednisolone were found to be interchangeable. The patients were divided into three active groups: (1) those who had never or not recently been on hydrocortisone therapy (32 patients); (2) those whose condition had previously been adequately controlled on hydrocortisone (39 patients); and (3) those whose condition had not been adequately controlled on hydrocortisone (70 patients). In (1), satisfactory levels of improvement were maintained in 19 (59 per cent.); in (2), 38 of the 39 patients maintained adequate improvement; in (3), adequate improvement was maintained in 34 (49 per cent.). Compared with hydrocortisone the advantages of the new drugs are their lack of salt and water retention and absence of potassium loss, their lesser tendency to raise blood pressure, and their ability to restore adequate levels of improvement in a significant number of patients whose arthritis has not been controlled by prolonged administration of hydrocortisone. Their disadvantages consist of a greater proclivity for gastric irritation and demonstrable peptic ulcers, ecchymotic skin lesions, and vasomotor symptoms. Qualitatively they produce the same antirheumatic response but their milligram potency is multiplied. Prednisone or prednisolone are to be preferred when salt and water retention is an actual or potential problem, and in patients who do not respond adequately to the older steroids or who escape control after their prolonged use. On the other hand, hydrocortisone should be preferred in patients with a history of peptic ulcer and gastric irritation from the new steroids. S. L. W.

Sarin and Tabun, Contributions to the Pharmacology of. C. Heymans, A. Pochet and H. van Houtte. (*Arch. int. Pharmacodyn.*, 1956, **104**, 293.) In the anaesthetized dog the anticholinesterases Sarin and Tabun induced bronchospasm, laryngospasm, bradycardia and other manifestations of parasympathetic activity, convulsions and muscular fasciculation. Death resulted from bronchospasm or laryngospasm or from cardiac arrest. Sarin depressed respiration, whereas Tabun caused transient hyperpnoea. Either hypotension or hypertension was produced by both drugs, depending upon whether the main effect was peripheral and hypotensive or central and excitatory on the vasomotor centres. Section of the vagus nerves had no effect on bradycardia or bronchospasm induced by the drugs. Injection of either drug into the circulation of the perfused isolated head, connected to its trunk only by the vagus nerves, did not cause bradycardia; so that, unlike acetylcholine, Sarin and Tabun have no stimulant action on the cardioinhibitory centre. Similarly the two anticholinesterases had no action on carotid chemoreceptors, other than to cause a fleeting hyperpnoea, caused by the cyanide in the Tabun molecule. Ganglion-blocking agents such as tetraethylammonium, hexamethonium and Pendiomid diminished or depressed transitorily the bradycardia induced by Sarin or Tabun. Residual bradycardia was blocked by atropine. The convulsions and muscular fasciculations were also blocked by the ganglion-blocking agents and atropine. Most of the parasympathomimetic actions of Tabun or Sarin were blocked by atropine; the miosis induced by large doses of the anticholinesterases was only influenced slightly, however, by atropine. Death in atropinized dogs was brought about by central or peripheral respiratory collapse. G. P.

Spermine and Spermidine, Pharmacology of. C. W. Tabor and S. M. Rosenthal. (*J. Pharmacol.*, 1956, **116**, 139.) The toxicity of spermine, spermidine, their enzymatic oxidation products and some related aliphatic mono- and diamines was studied in mice and rats. Injected intravenously into unanaesthetized rats, spermine and spermidine in a dose of 0·15 mM/kg. produced a transient histamine-like fall in blood pressure. The same dose given intraperitoneally to rats and mice caused gasping and laboured abdominal respiration (suggestive of bronchoconstriction). In these animals renal insufficiency developed within three to eight days. Like other nephrotoxic agents spermine exerted a diuretic action in mice. Among the other amines examined, nephrotoxic activity was shown by ethyleneimine, bromoethylamine, ethylenediamine and propylenediamine. The results suggested that spermine, spermidine and the above amines owe their nephrotoxic action to formation of reactive and unstable amino-aldehydes. Enzymatic degradation of spermine by beef plasma amine oxidase gave intermediately, spermidine, and finally NH_3, H_2O_2, putrescine, and an amino-aldehyde. The degradation products had no nephrotoxic action in mice. Also, if spermine and amine oxidase were injected separately no significant renal injury resulted. However, injection of the degradation products of spermine directly into the renal artery of a rabbit caused renal damage which indicates that damage after i.v. injection may be the result of degradation of spermine in the high concentration in which it accumulated in the renal tubular epithelium; destruction elsewhere may be the basis for the protection with amine oxidase described above. Amine oxidase had no protective action in renal damage caused by chloroform, mercuric chloride, polymyxin B and alloxan. Spermine or spermidine had no toxic action *in vitro* on spermatozoa, but amine oxidase added with, or incubated previously with, spermine or spermidine increased toxicity greatly. Bactericidal action and to a less degree trypanocidal

(ABSTRACTS *continued on p.* 1175.)

BOOK REVIEW

REMINGTON'S PRACTICE OF PHARMACY, Eleventh Edition, 1956. Edited by Eric W. Martin and E. Fullerton Cook. Pp. xii + 1707 (including over 1000 illustrations and Index). Interscience Publishers, Ltd., London, 135s. The Mack Publishing Company, Easton, Pa., U.S.A. $18.00.

This is an astonishing publication. It is described as a "treatise on the manufacturing, standardising and dispensing of pharmaceutical products with biological and chemical properties, tests for purity, assays, uses and doses: also a guide to the legal obligations of the pharmacist and the professional services rendered in helping to maintain community health. A textbook and reference guide for pharmacists, physicians and other medical scientists". Even this description is too modest. It does not refer to veterinary medicines, horticultural products including insecticides, or first aid. It does not suggest that here is to be found detailed information on how to run the pharmaceutical side of a drug store from the pricing of prescriptions to a list of items that should be kept in the Baby Department, neither does it indicate that the book contains a fascinating account of American hospital pharmacy and of the organisation and administration of the hospital service in that country. But all this and more is contained in the 1700 large pages and 100 chapters of this encyclopaedic work with an index comprising some 20,000 entries.

There is no doubt that individually the editors, Dr. E. W. Martin, of the University of Pennsylvania, and Dr. E. Fullerton Cook, formerly chairman of the U.S.P. Revision Committee, the four associate editors and more than 200 assistant editors and contributors have done a first-class job and produced a book which contains a vast amount of information likely to be needed by all who practise or hope to practise pharmacy. But that is not to say that all pharmacists need the whole of this massive compilation.

Remington's "Practice", which first appeared in 1885, was originally compiled by a teacher as a comprehensive single-volume textbook for students of pharmacy, and 70 years ago the student's needs could be covered in a single volume of reasonable size. In spite of the vast increase that has taken place in the intervening period in the range of the various subjects included in the term pharmacy the original plan has been followed and even extended in this eleventh edition.

Obviously a book on pharmacy for students must deal comprehensively with what we know as "pharmaceutics" and this the new Remington does adequately, in quality as well as quantity. On the other hand there are several subjects, for example organic, inorganic and analytical chemistry, biological assays and legal matters, still coming within the broad ambit of "pharmacy", which the student could probably learn better from one of the many available textbooks in each of these subjects rather than from a "potted" version of all, however well the potting is done. In any event the student does not want to pay for information he will not need until he is actually in practice.

For the practising pharmacist the comprehensiveness and "reference" character of Remington will be attractive, although the appeal to British pharmacists will, of course, be diminished by the fact that the book is based on American practice. In some respects the book overlaps the standard reference books which he will already possess, and there is much that no pharmacist, whatever his nationality or the branch of the profession in which he is engaged, will need when once his student days are over.

However, the defects of the book are those of its qualities, particularly its comprehensiveness. Too much has been attempted and unfortunately the attempt has succeeded. It is impossible to meet the needs of widely differing kinds of reader and still give full value to each. So large and heavy a publication is not really a practical proposition from the user's point of view and it seems essential in future to issue the book in more than one volume. If the contents of each volume could be selected with the main kinds of user in mind, the student would certainly welcome something more portable and the pharmacist would not have to pay for information he does not require. Yet for those who have the money to spare and want a single reference work on all the subjects that go to make up modern pharmacy, Remington is probably the book of choice.

H. TREVES BROWN.

(ABSTRACTS *continued from p.* 1173.)

action of these amines followed a similar pattern. Some related amines showed no such toxicity. Some sulphhydryl compounds inhibited the toxic action. Spermine had an anti-heparin action and an effect on blood coagulation similar to that of protamine. G. P.

APPLIED BACTERIOLOGY

Mercury Salts, Value of, as Disinfectants and Fungicides for Inanimate Surfaces. L. F. Ortenzio, L. S. Stuart and J. L. Friedl. (*J. Ass. off. agric. Chem., Wash.*, 1956, **39**, 476.) The authors report results obtained when six mercury salts were tested for bactericidal and fungicidal activity. The bactericidal tests were made in accordance with the Association of Official Agricultural Chemists use-dilution method, using *Salm. choleraesuis* and *M. pyogenes* var. *aureus* as the test organisms and using U.S.P. thioglycollate broth as the subculture medium. Tests for fungicidal activity employed the A.O.A.C. fungicide test method, using *Trichophyton interdigitale* and *Streptomyces scabies* as test organisms. The mercurials were found to have much greater fungicidal than bactericidal activities. Thus 1–20 dilutions of mercuric chloride and mercuric potassium iodide were required to kill the two bacteria whereas dilutions of 1–4500 and 1–3500 respectively were effective against the spores of *T. interdigitale*. Phenylmercuric salts were also found to be fungicidal in high dilution. The mercury salts were used in aqueous solutions containing low but unspecified concentrations of acetic acid. Investigations with *Streptomyces scabies* were made in order to determine the behaviour of organisms intermediary between bacteria and fungi. A standardised conidiospore suspension was killed within 10 minutes by 1–80 phenol and by 1–5000 mercuric chloride, indicating that *Streptomyces* are similar to fungi in their sensitivity to mercurials. It is concluded that mercurials might possess value in decontaminating premises or articles carrying causative agents of fungal infections. Although the bacteriostatic and fungistatic activities of mercurials are well-established, the authors point out that, where pyogenic and enteric bacteria are concerned, claims of disinfecting benefits cannot be justified unless the formulation contains bactericidally active chemicals other than mercury salts. B. A. W.

LETTER TO THE EDITOR

Statistics of Therapeutic Trials

SIR,—I regret to have to draw your attention to certain misrepresentations and distortions of facts in Dr. M. Weatherall's review of my book *Statistics of Therapeutic Trials* in the *Journal of Pharmacy and Pharmacology*, 1956, 8, 223. After having carefully considered the matter, I must ask you to publish this letter in your Journal as correction of the above-mentioned misrepresentations.

The reviewer makes the following statements:

"Most of the chapters deal with trials in specific diseases. . . ."

"This sort of difficulty seems to arise from trying to avoid general treatment of the logic and mathematics of therapeutic trials. . . ."

". . . general principles are discarded in favour of a series of examples. . . ."

I submit that these statements are untrue, and that it is very difficult to understand how a responsible reviewer could arrive at such views. A glance at the Table of Contents must have shown him that he was in error.

As regards the first of these statements: contrary to what the reviewer says, 16 chapters comprising over 200 pages deal with the general principles of significance testing and with the logic and methodology of therapeutic trials, as against 13 chapters comprising 150 pages which deal with specfic diseases. As the reviewer's second and third statements show, he has turned a completely blind eye to the content and import of 200 pages out of 350, and all his arguments are affected by that inexplicable omission.

As regards the second statement: what I say in the Preface about an alternative approach to medical statistics, viz. to start with the medical situation and try to allocate to certain typical situations the appropriate statistical methods, does not mean that general treatment has been avoided. On the contrary. Had the reviewer only read as far as p. 8 he would have seen that the medical concepts of "outcome," "duration" and "course" of disease are introduced in such a way as to enable the general principles of qualitative, quantitative and bi-variate statistics to be developed in their legitimate order and in such detail as required for their sensible application in medical statistics.

The third of the reviewer's statements is the premiss for certain obvious conclusions. Since the premiss is not true, it would seem futile to go into greater detail about the conclusions. Only in so far as the reviewer complains here about being quite bewildered, it must be pointed out that somebody who misses over 200 pages of general theory *would* find the application confusing. Otherwise he would not, since it is one of the characteristic features of my book that it is just the painstaking development of the methodology of therapeutic trials in the general part which has enabled me to define clearly the position, with regard to these principles, of any of the multifarious statistical methods encountered in the special part, and in medical literature in general.

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Canynge Hall,
Whatley Road, Bristol.

October 28, 1956.

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