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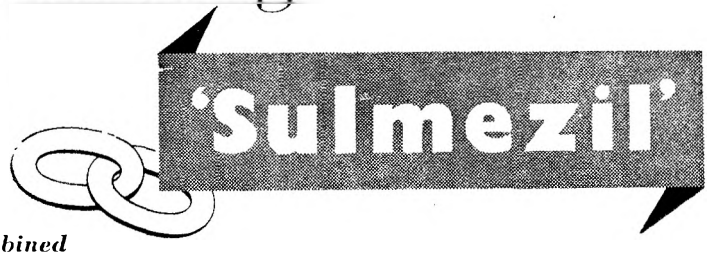

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REVIEW ARTICLE

NON-AQUEOUS ACID-BASE TITRATIONS IN PHARMACEUTICAL ANALYSIS

By Per Ekeblad and Kurt Erne

Apotekens Kontrollaboratorium, Stockholm K, Sweden

In recent years a great number of papers have appeared, dealing with different types of non-aqueous titrations and complete bibliographies on the subject have been published by Riddick[1,2]. This review will treat of the non-aqueous titration of Brönsted acids and bases, a subject which has become of great importance in the analysis of pharmaceutical preparations.

The Brönsted acids and bases are protolytes, proton donors and proton acceptors respectively. An acid A and its corresponding base B are related by the formula:—

$$A \rightleftharpoons B + H^+$$

The strength of an acid or a base is indicated by the K_a value of the dissociation constant of the acid in aqueous solution.

In the titration of an acid A_1 with a base B_2 or *vice versa* a solvent should be used, where the equilibrium

$$A_1 + B_2 \rightleftharpoons B_1 + A_2$$

lies so far to the right, that a large change in the hydrogen ion potential takes place at the end point. The equilibrium constant in a given solution depends on the K_a values and the electrical charge of the protolytes, the ionic strength and the dielectric properties of the solution. The protolytic properties of the solvent have a levelling effect on the strength of dissolved protolytes. Thus perchloric acid and the weaker hydrochloric acid have the same acid strength in aqueous solution because both react completely with the solvent, giving rise to the weaker acid H_3O^+. In the same way diethylaniline and the stronger base guanidine in acetic acid solution are of the same strength, both being protolysed to give the base CH_3COO^-. From a practical point of view, the choice of solvent is influenced by the requirements that it should not be too dangerous or too disagreeable to handle, that the solubility of the titrated samples should not be too low, that suitable electrodes or colour indicators should be available for indicating the end-point and that stable titrant solutions can be prepared.

Titration of Bases

The non-aqueous titration of weak bases with perchloric acid is the part of non-aqueous titrimetry that has been until now, most thoroughly dealt with. The method permits a rapid determination of different types of compounds common in the pharmacy of today: amines and heterocyclic nitrogen compounds, amino-acids, alkali and organic salts of weak acids and of hydrogen halides.

Acetic acid is the solvent most commonly used in the non-aqueous titration of bases. The fundamental work on the method was carried out

by Conant, Hall and Werner[3,4,5,6,7] in the years 1927 to 1930. Some years later Kolthoff and Willman[8,9] published important results from studies of the acid strength of different cations in acetic acid.

Titration with acetous perchloric acid in different organic solvents was studied by Fritz[10]. In recent years Pifer and Wollish[11,12,13] have recommended a solution of perchloric acid in *p*-dioxane as being more generally useful than the solution in acetic acid. The levelling effect of acetic acid on the strength of bases can be overcome by titration in aprotic solvents. Fritz[14] performed differential titration of bases of different strength in acetonitrile solution with perchloric acid in dioxane as the titrant.

Spengler and Kaelin[15] applied the acetous perchloric acid titration to many pharmaceutical problems, and gave titration curves for many pharmaceutical compounds. The preparation of the reagents and the role of acetic anhydride were discussed. The application of the method to pharmaceutical preparations has also been discussed by Auerbach[16] and in this journal by Beckett, Camp and Martin[17].

The method is inserted in the Collection of monographs of this laboratory[18]. Details of the technique used have been published[19,20,21].

Reagents

The acetic acid used as solvent in the titration should have a water content not exceeding 0·1 to 0·2 per cent. The perchloric acid solution (usually 0·1 N) is prepared from 70 per cent. aqueous solution, which is diluted with acetic acid. Acetic anhydride is added *after the dilution* to eliminate the water. Excess of acetic anhydride should be avoided if easily acetylated bases are to be titrated.

For back titration Spengler and Kaelin[15] used a 0·1 N sodium acetate solution prepared by dissolving standard sodium carbonate in acetic acid. This solution was also used for the standardisation of the perchloric acid solution. Due to the acidic property of the sodium ion in acetic acid, sodium acetate is a weaker base than the strong organic bases in this solvent. A strong, organic base, not sensitive to acetic anhydride, is recommended for the standard base solution. Guanidine[22] and triethylamine[19] have been used. The former is more easy to handle. It can be used in the form of carbonate or acetate.

Seaman and Allen[23] introduced potassium hydrogen phthalate for standardisation of the acetous perchloric acid. The low ionic strength during the titration, due to the precipitation of potassium perchlorate, gives an extremely sharp end-point.

The standard base solution is best standardised against the perchloric acid. The standard solutions in acetic acid are stable for a long time.

Though many acid-base indicators give colour changes in acetic acid, crystal violet, introduced by the pioneers of the method[6], is still the most commonly used. α-Naphthol-benzein and benzoylauramin were found to have no advantage over this indicator[22]. Crystal violet gives a series of colour changes around the "neutral point" of the acetic acid system and some practice is required if good results are to be obtained with this indicator. In the titration of potassium salts, the end-point is indicated by

the first change from violet to blue, in the titration of strong organic bases the change is from blue to green and in the titration of weaker bases the change is from bluish green to greenish yellow.

A Ciba-dye, Blue BZL, which has a colour change from blue to red at the "neutral point" has been successfully used in our laboratory in the titration of nikethamide and stronger bases. For weaker bases neutral red[19] (colour change red—blue) is recommended. For potentiometric indication of the end-point chloranil[3] or hydroquinone[24] electrodes can be used in cases where the solutions do not contain acetic anhydride or other compounds, which react with the electrode. The glass electrode is most suitable. A calomel electrode or a silver—silver chloride electrode[10] can be used as reference electrode. Reproducible and stable potentials are best obtained with a calomel electrode, connected to the solution by the lithium chloride bridge used by Hall and Conant[3].

APPLICATIONS

Organic bases: As shown by Hall[7], the basic strength of amines and heterocyclic nitrogen compounds in acetic acid is, in most cases, linear to their strength in aqueous solution. Organic bases with $K_a = 10^{-6}$ or less are strong bases, that is, they give completely ionised acetates in acetic acid solution. Bases with K_a values up to 10^{-3} often are strong enough to be titrated visually. Among the weak bases titratable in acetic acid can be mentioned phenazone and *iso*propylphenazone, nikethamide, nicotinamide and phenylcinchonic acid. Many bases, monovalent by titration in aqueous solution are divalent in acetic acid, for example quinine and procaine.

For the titration of tertiary amines in mixtures with primary and secondary amines, Blumrich and Bandel[25] used acetic anhydride. By boiling with acetic anhydride the primary and secondary amines are acetylated and lose their basic character, and the tertiary amines can be titrated. The method can be used on compounds containing a tertiary and a primary or secondary nitrogen. Examples of this are procaine and tetracaine. On account of the weaker basic properties of the aromatic amino groups in these compounds and the precipitation of diperchlorate during the titration, visual indication of the end-point is not sharp. After boiling with acetic anhydride, giving an acetylated aromatic amine, the strongly basic tertiary aliphatic nitrogen can be titrated in acetic acid. The same procedure has been used for titration of the pyridine nitrogen in isoniazid.

Amino-acids: The potentiometric and visual titration of amino-acids in acetic acid was described by Nadeau and Branchen[22]. The amino-acids are usually very slowly soluble in acetic acid. Heating can cause acetylation and decomposition[20]. Toennis and Callan[26] showed that the amino-acids could be dissolved in a small amount of formic acid and the solution diluted with acetic acid before titration. The final solutions should contain less than 2 per cent. of formic acid. The same procedure can be used for electrolytes, which are difficult to dissolve in acetic acid and sensitive to heat, for example thiamine hydrochloride.

Salts: The low dielectric constant for acetic acid favours the titration of anion bases. Whereas the weakly basic caffeine can hardly be titrated, anion bases of approximately the same strength, e.g., the picrate and the dihydrogen phosphate ion, are titrated as strong bases. Higuchi and Concha[27] titrated salts of such strong acids as nitric acid. Sulphates can be titrated to acid sulphates, but not to sulphuric acid. The halogen ions are too weakly basic to be titrated directly. As a great deal of the pharmaceutically useful organic bases are used as chlorides or bromides, a great widening of the field of non-aqueous acidimetry was made when Pifer, Wollish and Schmall[12] introduced mercuric acetate as a means of removing halogen ions in acetic acid solution. The mercuric acetate reacts with haloid ions giving mercuric halide and an equivalent amount of acetate ions. The mercuric acetate and halide have no basic properties in acetic acid.

In a recently published review article[28], the same authors mention that they have succeeded in titrating sulphates in acetic acid after removing the sulphate ions by heating with mercurous acetate. No details have yet been given.

Today, it is possible to titrate acidimetrically the salts of most of the commonly used acids, except perchloric and sulphonic acids, provided that the acid strength of the cation in acetic acid is not too great. The salts of organic bases, themselves not too weak to be titrated, can be assayed in this way. As to the inorganic cations, it is of special importance to have a rapid method for the assay of the alkali salts.

Titration of Acids

The pioneer in this field was Folin who as early as 1910 estimated fatty acids in non-aqueous solvents by titrating with sodium ethoxide[29]. La Mer and Downs seem to be the first to apply potentiometry to the determination of acids in aprotic systems[30]. In 1948 Moss, Elliot and Hall[31] published an important account of the determination of phenolic compounds in resins. They used sodium aminoethoxide in ethylenediamine as titrant and antimony-antimony electrodes as indicating system. Of fundamental importance to the further analytical development of the method has been the work of Fritz and co-workers who since 1950 have issued a series of papers concerning the non-aqueous titration of both weak acids[32,33,34,35,36], and bases. As titrant Fritz used alkali methoxide in benzene-methanol, while the solvent was varied according to the acid strength of the compound to be titrated.

Solvents

Selection of a proper solvent is essential in non-aqueous titrations. Especially important factors are the basicity and the dielectric properties of the solvent. Increased basicity of the solvent enhances the acidic properties of a dissolved acid and a low dielectric constant of the solvent depresses the ionisation and thereby augments the acid strength or base strength of dissolved protolytes[37].

The solvent power of such a solvent may, however, be poor and the addition of a more polar medium is required. There is evidence that such

mixed solvents (e.g., benzene-methanol) are superior to pure solvents in solubilising effect and sharpness of the indicator change or the potential break at the equivalence point. No solvent with such general applicability for weak acids as glacial acetic acid has for weak bases, has yet appeared. A widely used mixed type solvent is benzene-methanol introduced by Fritz[32]. Dimethylformamide has shown itself to be a very useful solvent[32,34,36], among its advantages is the freedom from odour, and it does not absorb carbon dioxide as eagerly as the more basic solvents. A drawback however is the risk of hydrolysis of the amide linkage, especially in the presence of water, with subsequent liberation of acid. For the most weakly acidic compounds butylamine, pyridine and ethylenediamine seem to be the most suitable solvents.

Titrants

Several alkaline titrants have been used in non-aqueous solvents: alkali hydroxides, alkoxides[1,32], aminoalkoxides[31] and amides[38]; as well as quaternary ammonium bases and—for extremely weak acids—triphenylmethyl sodium[39]. Although none has gained absolute dominance like acetous perchloric acid, sodium methoxide in benzene-methanol has found a rather wide application. There are some interferences however. Water behaves like a weak acid and should be absent. Likewise the need for protection against carbon dioxide is obvious. There is also some risk of atmospheric oxidation of alcoholic alkoxide solutions to acidic compounds[40]. An inert atmosphere in the storage bottle and in the titration vessel tends to minimise the influence of these last mentioned factors. In some instances lithium methoxide may be preferable because of its greater solubility in organic solvents and in other cases the more strongly basic potassium analogue may offer some advantages[28].

Indicator systems

Some acid-base indicators known from aqueous titrimetry have been used with success in non-aqueous media. Here may be mentioned thymol blue, thymolphthalein and phenolphthalein and also azo violet (*p*-nitrobenzene azoresorcinol) and *o*-nitroaniline. Among these thymol blue especially has found a wide acceptance. The colour change is from yellow through green to blue. By comparison with potentiometric determinations the exact colour shade at the equivalence point may be established.

For that purpose several electrode couples have been used, e.g., glass-calomel[35], antimony-antimony[31] antimony-glass, antimony-calomel[32]. In some instances such systems operate successfully but often the electrode response is slow and unstable. Electrode systems in non-aqueous media have been investigated at the National Bureau of Standards[41,42,43], but much work remains to be done in this field. Until now there has appeared no electrode system that functions satisfactorily in solvents of low dielectric constant.

Most of the methods published have employed an alkali methoxide titrant. The procedure used in this laboratory (which follows Fritz

closely) for the determination of barbiturates and other weak acids may serve as a guide to the technique.

Reagents

0·1 M Sodium methoxide

Dissolve 3 g. of freshly cut sodium metal in 50 ml. of dry methanol protecting the vessel from carbon dioxide, add 100 ml. of methanol and then 750 ml. of benzene. The solution is stored in alkali resistant glass and is protected from carbon dioxide.

Thymol blue

0·3 per cent. solution in methanol.

Procedure

To 10 to 20 ml. of a suitable solvent add 2 drops of thymol blue and bubble nitrogen gas through for 10 minutes. Then titrate to a blue colour with the methoxide. Add the sample corresponding to about 1 m-equiv. and titrate to the same colour change. During the titration nitrogen gas is led into the solution whereby the latter is both stirred and protected from carbon dioxide and moisture. The titrant is standardised in the same manner against benzoic acid. The titre is rather sensitive to temperature on account of the high thermal expansion of benzene-methanol and, therefore, frequent restandardisation is advisable.

With this technique it is possible to determine a great many substances of importance in pharmacy. Carboxylic acids and other moderately acidic compounds may be titrated in neutral or basic solvents but the sharpest end-points are obtained in mixed solvents such as benzene-methanol. Thymol blue is a good indicator[31].

In "sulpha" drugs the amide hydrogen is sufficiently acidic to permit of ready titration in dimethylformamide with thymol blue as the indicator[34,36]. The same technique applies to the barbiturates[36] but butylamine and pyridine[44] are also good solvents.

The acidic moiety of ammonium salts and salts of aliphatic amines is amenable to titration in dimethylformamide or ethylenediamine (thymol blue or azo violet)[33]. Likewise negatively substituted phenols possess sufficient acidic character to be titrated in dimethylformamide, azo violet being a suitable indicator. Phenol and alkyl phenols are weaker acids and require a more basic solvent such as ethylenediamine (*o*-nitroaniline)[32,35].

Numerous other compounds of very slight acidity such as enols, mercaptans and imides may be titrated in butylamine or ethylenediamine[32]. In all these cases the water content of the system should be kept at a minimum because of the acidic properties of water. Besides, as the solvents often contain acidic contaminants, it is a good rule always to neutralise the solvent prior to the titration of the sample.

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RESEARCH PAPERS

THE EFFECT OF IMMUNE SERUM ON HÆMAGGLUTINATION BY RICIN

By E. G. C. Clarke

From the Department of Physiology, Royal Veterinary College, London

Received April 1, 1954

The agglutination of red blood cells by ricin has been studied intensively since it was first noted by Stillmark[1]. Ehrlich[2], who discovered that an animal can be immunised against the toxic effects of ricin, showed that the agglutination of rabbit blood is inhibited by the addition of serum from an immunised goat, the inhibition of agglutination running parallel to the neutralisation of toxicity. Müller[3] found that immune rabbit serum prevented the agglutination of rabbit blood, but that the blood of an immune animal is agglutinated as easily as ordinary blood. He suggested that this apparent discrepancy was due to the difference in concentration of the serum. Kraus[4] showed that normal rabbit serum inhibited the agglutination of rabbit red cells by ricin, but that normal goat serum had no such action. Fraenkel[5] also noted the inhibitory action of normal serum, but found that it varied in intensity from species to species. Miessner and Rewald[6] made a somewhat similar observation, but found that it was impossible to generalise. Thus, while normal goat serum had little or no effect on the action of ricin on rabbit or bovine blood cells, in the case of dog cells it promoted agglutination, and with guinea-pig cells it had an inhibitory action. They also noted that immune serum has no anti-agglutinating effect unless it is mixed with the ricin solution and allowed to stand for 15 minutes before the red cells are added. di Macco[7] found that the addition of guinea-pig serum in optimum quantity promotes the agglutination of sheep red cells by ricin, too much having an inhibitory effect. Similarly Guest[8] showed that a trace of rabbit serum increases the agglutinating power of ricin towards goat red cells.

Moriyama[9,10] noted that normal rabbit serum inhibits the agglutination of horse red cells by ricin, but promotes the agglutination of bovine cells. On the other hand, anti-ricin serum in optimum concentration promotes the agglutination of equine red cells.

Karel[11] found that both rabbit and guinea-pig serum decreased the agglutinating power of ricin in respect to guinea-pig red cells, while Kabat, Heidelberger and Bezer[12] noted that a trace of normal rabbit serum increased the agglutinating power of crystalline ricin towards human red cells (group O). They also state that anti-ricin sera may be standardised by assay of their inhibition of the hæmagglutinating power of ricin.

The above summary contains a mass of inconsistencies and contradictions. This is partly because different workers have used widely

different experimental conditions, and partly because no adequately controlled experiments, in which the effect of immune serum is compared with the effect of normal serum of the same species of animal, have been carried out. The work described was undertaken to find whether there is a specific anti-agglutinin in anti-ricin serum, or whether the observed effects are due to some substance normally present.

Experimental

Materials. (1) Ricin. This was prepared by the method of Osborne, Mendel and Harris[13]. In view of the observation of Kabat *et al.*[12] that ricin loses nine-tenths of its agglutinating power on crystallisation, no attempt was made to crystallise it. Its lethal dose for a 25 g. mouse was about 0·5 μg. All solutions were in physiological saline.

(2) Serum. This was obtained by immunising 3 goats and some dozen rabbits. No significant difference was noticed in the effects of serum from different animals of the same species. Goat serum X, which was used in the following experiments, neutralised 200 mouse lethal doses (i.e. 100 μg.) per ml. Rabbit serum P neutralised 25 mouse MLD (12·5 μg.) per ml. All sera were preserved with 0·5 per cent. of phenol.

(3) Red blood cells. Fresh oxalated blood from several animal species was centrifuged, and the red cells washed 3 times with 20 times their volume of physiological saline solution. 2 ml. of the packed cells were suspended in 100 ml. of saline solution.

Technique of agglutination tests.

12 rows, each of 8 test-tubes 3 in. $\times$ $\frac{1}{4}$ in., were used. To each tube in the first row was added 1 ml. of physiological saline solution; to each tube in the second row 1 ml. of ricin solution of 2·5 mg./l. concentration; to each tube in the third row 1 ml. of a ricin solution of twice this concentration namely, 5·0 mg./l.; to each tube in the fourth row, 1 ml. of ricin solution of twice the last concentration, and so on, the concentration for the twelfth row being 2·56 g./l. In the case of animals whose red cells are difficult to agglutinate, concentrations 16 times as great as these were used. To the first tube in each row 1 ml. of saline solution was now added; to the second tube in each row 1 ml. of serum diluted with saline solution to a concentration of 0·01 per cent.; to the third tube in each row, 1 ml. of a serum solution of 4 times this concentration, and so on, the concentration for the last tube in each row being 41 per cent. The tubes were shaken and allowed to stand at room temperature for 15 minutes. To each tube was then added 0·5 ml. of a 2 per cent. suspension of red blood cells. The tubes were stirred and incubated at 37° C. for two hours, when the result was read.

Results

Complete agglutination, with clear supernatant liquid, is shown by +++; almost complete agglutination, but with supernatant liquid opalescent, is shown by ++; and partial agglutination, as compared with

the controls, by +. The results obtained with dog red blood cells and normal goat serum are shown in Table I. There is no increase in the agglutinating power of ricin with increases of serum. Indeed, with quantities more than 1 per cent. inhibition takes place, more ricin being needed to bring about complete agglutination. The results with immune goat serum, shown in Table II, are very different. Small additions of serum cause an increase in the agglutinating power of ricin, the maximum, 32-fold, occurring with an addition of 0·064 per cent. of serum. This effect decreases with increasing volumes of serum, until finally an inhibitory action is noticed. Other experiments, in which quantities of

TABLE I

RICIN: NORMAL GOAT SERUM AND DOG RED BLOOD CELLS

Ricin	Serum per cent.							
p.p.m.	0	0·004	0·016	0·064	0·256	1·02	4·1	16·4
512	+++	+++	+++	+++	+++	+++	+++	+++
256	+++	+++	+++	+++	+++	+++	+++	+
128	+++	+++	+++	+++	+++	+++	++	—
64	++	++	++	++	++	+	—	—
32	+	+	+	+	—	—	—	—
16	—	—	—	—	—	—	—	—

There is no increase in the agglutinating power of ricin with increase of serum. When 1 per cent. is present inhibition takes place. (Concentrations of ricin above 512 and below 16 p.p.m. have been omitted.)

TABLE II

RICIN: IMMUNE GOAT SERUM AND DOG RED BLOOD CELLS

Ricin	Serum per cent.							
p.p.m.	0	0·004	0·016	0·064	0·256	1·02	4·1	16·4
265	+++	+++	++−	−++	+++	+++	+++	+++
128	+++	+++	++−	−++	+++	+++	+++	++
64	+	+++	++−	+++	+++	+++	+++	+
32	—	+	++	+++	+++	+++	+++	—
16	—	—	++	+++	+++	+++	—	—
8	—	—	+	+++	+++	++	—	—
4	—	—	—	+++	++	—	—	—
2	—	—	—	++	+	—	—	—
1	—	—	—	+	—	—	—	—
0	—	—	—	—	—	—	—	—

There is an increase in the agglutinating power of ricin with increase of serum with a peak at 0·064 per cent. followed by inhibition.

serum up to 64 per cent. of the total volume were added, showed that at higher concentrations the effect of normal and immune serum is exactly the same, the inhibitory action increasing with increasing percentage of serum.

The results shown in Tables I and II are depicted graphically in Figure 1. A, where the minimum concentrations of ricin necessary to bring about complete agglutination are plotted logarithmically as ordinates against the logarithm of the volumes of serum present as abscissæ. On the same figure are shown the results for dog red cells with normal and immune rabbit sera, which exhibit exactly the same effects as goat sera.

Figures 1. B and 1. C show the results obtained with cat and guinea-pig red cells, respectively. They show the same phenomena noticed with

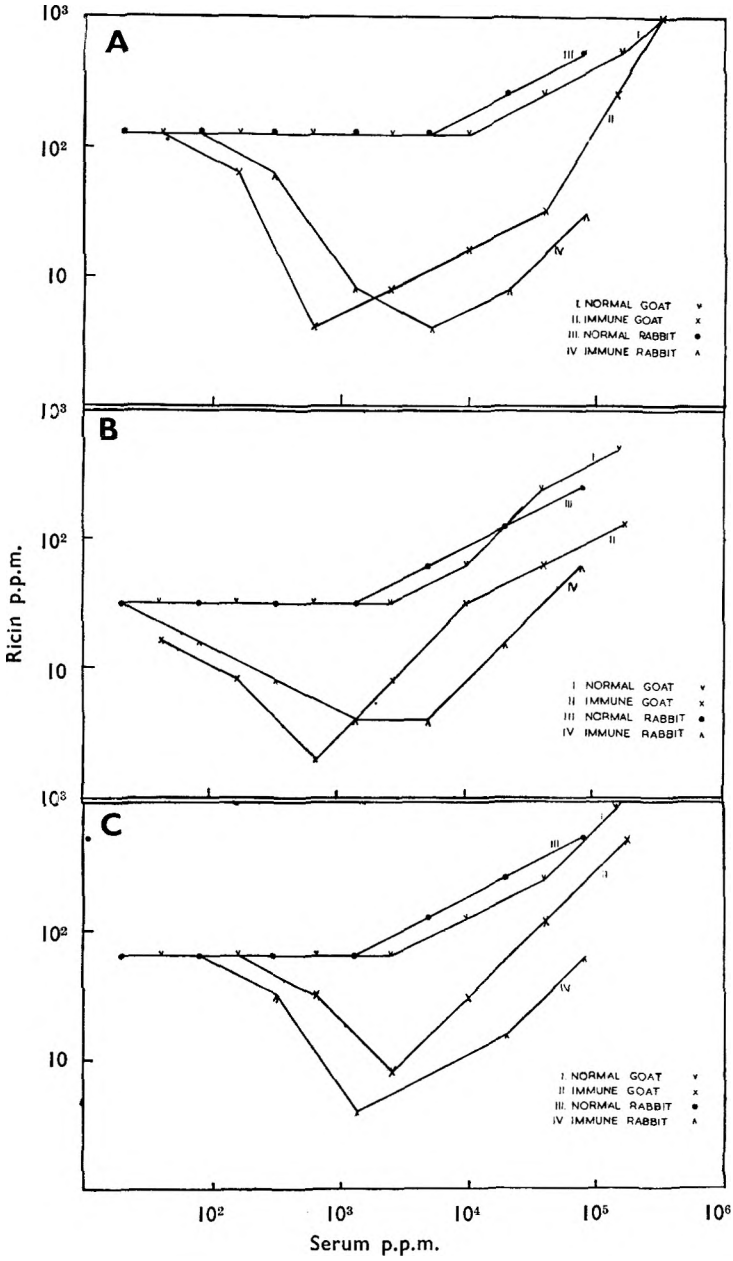

FIG. 1. A. The effect of concentration of goat and rabbit serum on the agglutination of dog red cells by ricin. There is promotion of agglutination only with immune sera at optimum concentration; both normal and immune sera show inhibition at high concentrations. B. The effect is similar with cat red cells and C with guinea-pig red cells.

The ordinates are the logarithms of the minimum concentration of ricin needed for complete agglutination. The abscissæ are the logarithms of the volumes of serum present.

dog cells, that is inhibitions at higher concentrations with both normal and immune sera, and marked promotion of agglutination with immune sera at optimum concentration. With rabbit cells (Fig. 2. A), rabbit serum behaves in the same way. With goat sera, however, although there is a similar picture with concentrations of sera up to 1 per cent. above this figure complete agglutination takes place even in the control tubes without ricin, owing to the presence in goat serum of some natural agglutinin for rabbit red cells.

With red cells which are less easy to agglutinate than the foregoing, somewhat different results are obtained. In the case of horse red cells (Fig. 2. B) both normal and immune rabbit sera have exactly the same effect, increasing the agglutinating power of ricin slightly when added to an extent of about 0·25 per cent. and inhibiting it when added in larger quantities. Normal and immune goat sera show a similar but slightly greater promotive action over a somewhat greater range, the effect being more pronounced for normal serum. Neither shows any inhibitory action when added in quantities of up to 8 per cent.

With bovine red cells results are very similar. Normal and immune goat sera, and normal rabbit serum promote agglutination when added in quantities between 0·1 and 4 per cent., above which the effect diminishes. With immune rabbit serum, however, this promotive action increases for increasing additions of serum up to 30 per cent.

In the case of goat red cells, Figure 2. C, an increase in the agglutinating power of ricin was observed for both normal and immune sera, the optimum quantities being 1 to 2 per cent. No inhibition of agglutination was noticed for additions of sera up to 16 per cent., though the graph suggests that further additions might cause it.

It was found impossible to agglutinate sheep red cells with ricin solutions of the concentrations employed (1·5 per cent.). Addition of serum, however, caused agglutination to take place over a narrow range for both rabbit and goat sera, the effect being the same for both normal and immune.

It will be seen from these experiments that in all cases immune serum causes a definite increase in the agglutinating power of ricin, this effect being greatest for additions of serum of the order of 1 per cent. Normal serum has a similar effect with blood of some species, while with that of other species it is without effect when present in small quantities. Inhibition only occurred in certain cases and then only when larger quantities of serum were added. In no case was the inhibition caused by immune serum greater than that caused by normal serum under the same conditions.

In order to determine whether this somewhat unexpected result was due to experimental conditions, a further series of experiments was carried out with dog red cells and rabbit sera under varying conditions. The following results were obtained:

1. *Red cell suspension.* Experiments similar to those described were carried out, but with 8 per cent., 4 per cent. and 1 per cent. suspensions of red cells instead of the 2 per cent. suspension employed previously.

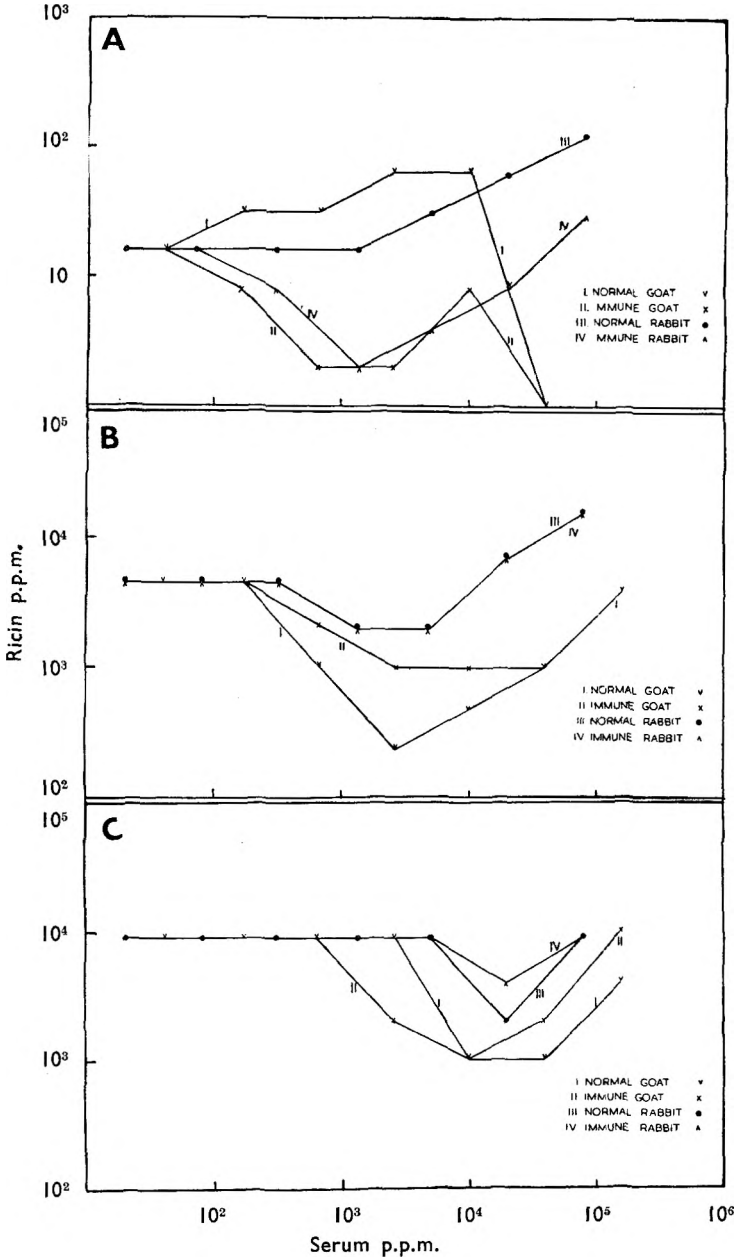

FIG. 2. A. The effect of concentration of goat and rabbit serum on the agglutination of rabbit red cells by ricin. There is promotion of agglutination only with immune sera at optimum concentration. Both normal and immune rabbit sera show inhibition at high concentrations. Above 1 per cent. goat serum agglutinates rabbit cells. B. The effect on horse red cells. Both normal and immune rabbit and goat sera first increase and then inhibit ricin agglutinating power. C. The effect on goat red cells. Both normal and immune rabbit and goat sera first increase and then inhibit ricin agglutinating power. The ordinates are the logarithms of the minimum concentrations of ricin needed for complete agglutination. The abscissæ are the logarithms of the volumes of serum present.

It was found that the observed results were independent of red cell concentration.

2. *Order of mixing.* In view of the statement of Miessner and Rewald[6], referred to above, that no anti-agglutination was noticed unless the immune serum was mixed with the ricin solution and allowed to stand before the red cells were added, a series of experiments was carried out as follows.

A. Ricin and serum were mixed and stirred, and incubated at 37° C. for half an hour, then red cells added.

B. Ricin and red cells were mixed and stirred, and incubated at 37° C. for half an hour, then serum added.

C. Serum and red cells were mixed and stirred, and incubated at 37° C. for half an hour, then ricin added.

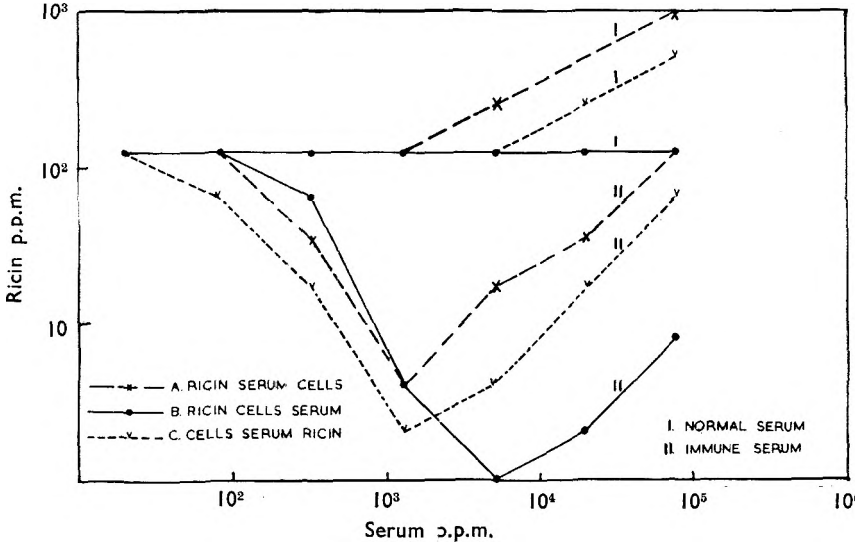

FIG. 3. The effect of the order of mixing the components upon the promotion of the agglutination of dog red cells by ricin in the presence of normal and immune rabbit serum. The ordinate is the logarithm of the minimum concentration of ricin needed for complete agglutination. The abscissa is the logarithm of the volumes of serum present.

The results are shown graphically in Figure 3. The inhibitory effect is greatest when ricin and serum are mixed and the cells added later, but the promotive effect of the immune serum appears to be least under these conditions. However, when the ricin and red cells are mixed first the promotion is at a maximum and the inhibition apparently least. Both effects have an intermediate value if the ricin is added last.

Use of goat serum instead of rabbit serum produced similar results. These results may be explained by postulating the presence of two factors in serum—an "inhibitory" factor, present in both normal and immune serum and a "promotive" factor, present in immune serum only. In experiment A the inhibitory factor has neutralised some of the ricin

before the red cells are added. Hence less agglutination takes place in the tubes containing the higher proportions of serum. In B the ricin has been adsorbed by the red cells, and subsequent addition of serum does not enable the inhibitory factor to neutralise it. In C the ricin, added last, is divided between the red cells and the serum, so that only a limited quantity is neutralised. It would appear that the promotive factor is independent of the order of mixing, as the apparent differences in intensity of its action are, in fact, due to the inhibition which takes place.

3. *Time and temperature.* As previous workers have employed temperatures ranging from 4° to 37° C. and have read the results after intervals varying from 10 minutes to 24 hours, it seemed essential to find the effect of varying these two quantities. The usual experiment was made at (*a*) 4° C., (*b*) 20° C., (*c*) 37° C. and (*d*) 45° C. Readings were taken after 0·5, 1, 1·5, 2, 4, 6 and 24 hours. Some typical results are shown in Figure 4. A and B. Agglutination took place more quickly at the higher temperatures, but in the absence of serum, eventually proceeded to a greater extent at lower temperatures. Thus at 37° C. in the control tubes without serum, 128 p.p.m. of ricin caused complete agglutination after 2 hours, no further increase being noticeable after 24 hours. At 4° C. however, it required 512 p.p.m. to cause complete agglutination after 4 hours, but after 24 hours, the value had fallen to 16 p.p.m.

In all cases agglutination was first noticed in tubes containing the larger quantities of serum, but these were also the first to reach equilibrium, i.e., the state at which no further agglutination took place as time went on. With both normal and immune sera, the concentration of ricin to cause complete agglutination in the tube containing the largest volume of serum employed (8 per cent.) was independent of temperature. In the case of immune serum, the same was true of the promotive effect at optimum concentration.

4. *Hydrogen ion concentration.* As agglutination by ricin is entirely dependent on the ions present, not taking place at all in the absence of electrolytes (Rona and Gyorgy[14]), it is impossible to investigate the effects of change in *p*H by adding buffer solutions, as one is unable to say how much of the observed effect is due to any small change in H-ion concentration and how much is due to any large changes in say, phosphate-ion concentration. By adding to each tube 1 drop of 0·01N hydrochloric acid or 0·01N sodium hydroxide, it was possible to bring the *p*H to 5·5 or 7 respectively, further additions of acid or alkali causing hæmolysis of the red cells. It was found that agglutination increased slightly with increasing acidity and diminished with alkalinity, but that these small changes in *p*H had no noticeable effect on either the inhibitory or promotive factors.

5. *Specificity.* Experiments were carried out with normal and immune anti-ricin sera and dog red cells, but with solutions of the phytotoxins abrin (from *Abrus precatorius*) and robin (from *Robinia pseudacacia*). No promotive effect was noticed, but there was some inhibition of

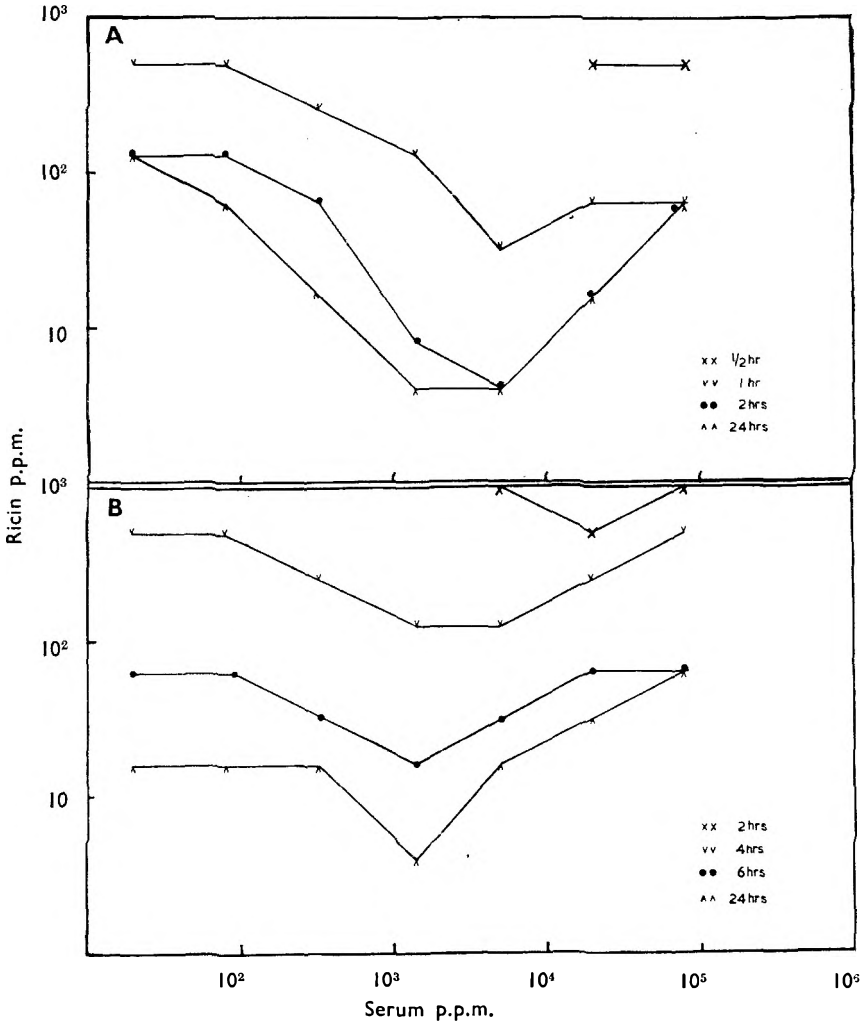

FIG. 4. A. The effect of contact time on the observed effect of agglutinating dog red cells by ricin in the presence of immune rabbit serum at 37° C. B. The effect of contact time on the observed effect of agglutinating dog red cells by ricin in the presence of immune rabbit serum at 4° C. The ordinates are the logarithms of the minimum concentration of ricin needed for complete agglutination. The abscissæ are the logarithms of the volumes of serum present.

agglutination with both normal and immune sera. This suggests that the promotive effect is specific, the inhibitory effect not.

DISCUSSION

It will thus be seen that, so far from there being a specific anti-agglutinin in anti-ricin serum, there appears to be a factor which, with the red cells of certain species at any rate, exerts exactly the opposite effect, bringing about a definite promotion of agglutination. Experiment showed that

this factor appeared in the blood at about the same time as the antitoxin and increased at a roughly parallel rate. It was found impossible to separate these two factors by procedures such as fractional precipitation with ammonium sulphate and similar reagents; any procedure which concentrated one factor concentrated the other and any method which destroyed the one destroyed the other. Both the promotive factor and the antitoxin could be clearly demonstrated in phenolised serum that had been stored at room temperature for 10 years. This would suggest that the promotive factor is some property of the antitoxin itself, the optimum concentration at which it exerts its maximum effect being akin to the zone phenomena encountered in precipitin reactions. So far no satisfactory explanation of ricin agglutination has been given, although several workers (Gunn[15], Northrop and Freund[16]), have suggested that it is a colloidal phenomenon. If this is so, one may possibly liken the "inhibitory" and "promotive" effect to the "protection" and "sensitisation" of one colloid by another. It would seem, however, that a complete explanation of these effects must await the elucidation of the mechanism of agglutination by ricin.

Summary

1. The literature dealing with the subject is briefly reviewed.
2. It was found impossible to demonstrate the presence of any specific anti-agglutinin in the serum of either goats or rabbits immunised against ricin.
3. Large additions of both normal and immune sera inhibit agglutination of the red cells of most species of animals tested.
4. When added in an optimum quantity of approximately 1 per cent. immune serum greatly increases the agglutinating power of ricin towards the red cells of dog, cat, rabbit and guinea-pig.
5. With the blood of horse, ox, goat and sheep, both normal and immune sera exert a slight promotive effect when added in optimum quantity.
6. The action of immune serum on dog red cells was investigated under varying conditions and the promotive action shown to be specific to ricin and independent of red cell concentration and of *p*H. It is also independent of temperature, provided sufficient time is allowed for equilibrium to be reached.
7. It is concluded that one cannot explain these phenomena until more is known of the mechanism of agglutination by ricin.

Acknowledgment

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FACTORS INFLUENCING THE ACTIVITY OF THROMBIN PREPARATIONS

By D. Maxwell Bryce*

From the Laboratories of S. Maw Son and Sons, Barnet

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Introduction

It has been the practice for some years in this laboratory to assay preparations of thrombin by comparing their activity in plasma with that of a laboratory standard. The purpose of this paper is to record certain of the observations and experiments which have been made from time to time to illuminate the assay.

Thrombin

Thrombin is a comparatively recent addition to the range of animal substances used in medicine and surgery. It is a protein formed in shed blood from an inactive precursor by the action of thromboplastin in the presence of ionic calcium and accelerator globulin (also known as factor V or labile factor) and it brings about the conversion of fibrinogen into insoluble fibrin, the physical basis of the blood clot. The reaction is almost certainly enzymic since it has been demonstrated using highly purified reagents that thrombin is able to convert at least 10,000 times its weight of fibrinogen into fibrin[1] and that the yield is independent of thrombin concentration over a wide range[1,2].

Thrombin of commerce is a partially concentrated serum protein fraction, or it may be obtained *via* prothrombin. It contains buffering salts in varying amount and its activity seldom exceeds 60 National Institute of Health (U.S.A.) Units (originally Iowa Units), per mg. Preparations containing up to 1400 units of activity per mg. dry weight have been prepared[3] and shown to be electrophoretically heterogeneous[4], so it is clear that the actual enzyme is present only in very small amount. The commercial enzyme is stable for many years in the absence of moisture and is comparatively stable in cold aqueous solution provided that micro-organisms are not present in large numbers.

Clotting

When thrombin is added to a fibrinogen-containing solution, fibrin is formed at a rate depending on the concentration of the reactants and the experimental conditions, and the reaction continues until the whole of the fibrinogen has been converted. At a certain point in the course of the reaction the system is observed to clot when the quantity of fibrin formed is sufficient to give rise to a gel capable of supporting its own weight. This is a largely arbitrary point and generally occurs quite early in the process when only a fraction of the fibrinogen has reacted.

*Present address: Fletcher, Fletcher and Company, Ltd., London, N.7.

It cannot be uniquely defined since observation of the physical changes in the system merely serves to demonstrate the essential continuity of the process. Further the clot characteristics vary with circumstances, for example, *bovine plasma gives an opaque, elastic precipitate, which* readily forms a web-like network when agitated, whereas sheep plasma gives rise to a translucent, friable gel. It is therefore essential in carrying out experimental work on clotting systems to define the characteristics of the end-point used at the beginning of the work and to adhere to this.

The Isolated Reaction: Thrombin: Fibrinogen.

A number of workers[2,5,6] have studied the kinetics of the isolated thrombin : fibrinogen reaction in dilute solution, generally by gravimetric determination of the fibrin yield as the reaction proceeds. These studies have shown that in low concentrations the reaction is practically of the first order with respect to both thrombin and fibrinogen, and as would be expected from consideration of the Langmuir equation for heterogeneous catalysis there is a gradual change to zero order for fibrinogen as the concentration increases to an optimal range. Thereafter further substrate actually decreases the rate[5,7]. In solution of ionic strength 0·15 and with 1 unit/ml. of thrombin, the clotting time is almost constant from 0·05 to greater than 0·3 per cent fibrinogen, whereas with 0·1 units of thrombin per ml., there is a well-marked optimum near 0·1 per cent[5].

Therefore assuming constant experimental conditions and optimal fibrinogen concentration, rate of reaction may be taken as solely dependent on the enzyme concentration. If rate comparisons may be made by comparing clotting times, these should approximate to a linear function of the enzyme dilution. This or the converse that reciprocal clotting time approximates to a linear function of thrombin concentration has been generally confirmed[8,9,10].

This state of affairs may be represented mathematically by the formula,

$$t_c = b\,[\text{thrombin}]^{-1} + c$$

where t_c = clotting time

b = slope of the regression line of clotting time upon thrombin dilution.

c = a constant, and

$[\text{thrombin}]^{-1}$ = reciprocal thrombin concentration.

In all but very fast systems c may be taken as zero.

$$\text{therefore } \log t_c = \log b - \log [\text{thrombin}]$$

and in any particular system b is a constant so that for all practical *purposes there should be a straight line relationship between log clotting* time and log thrombin concentration and it should be of unit slope. This relationship has the virtue of stabilising the clotting time variance within quite wide limits, and it is also of more general applicability. There are limits to the usefulness of the thrombin concentration : reciprocal clotting time relationship. Astrup and Darling[11] have shown that it does not hold good for impure preparations of fibrinogen, neither does it hold for certain other published data[2] in which the mean slope of the log : log

curve is −0·67. The fibrinogen here used had a mean clottable protein content of 97 per cent. and the thrombin was a physically activated high potency preparation. The physical agency was prolonged exposure to the effect of cold (Seegers *et al.*[4]). We have little experience of the use of solutions of fibrinogen for assay purposes but the above derived relationship fits much published data well. In plasma the reaction is complicated by the presence of other proteins and more especially of thrombin inhibitors. Waugh and Livingstone[6] have shown that non-clottable protein did not influence the course of the reaction, and despite the presence of antithrombin, we find that the log : log relationship holds generally, provided that the clotting times are not less than about 12 seconds. The slope of the regression line of log t_c upon log thrombin concentration is generally about − 0·5 to − 0·7.

The Effect of Temperature on the Reaction in Plasma.

The effect of temperature on the rate of reaction between sheep thrombin and fibrinogen in oxalated bovine plasma, prepared from blood containing 0·16 per cent. of potassium oxalate plus 0·04 per cent. of oxalic acid, was investigated with the aid of a stopwatch, a test-tube, and a water bath.

0·1 ml. of plasma was pipetted directly into the bottom of a scrupulously clean test-tube and after temperature equilibration in the water bath an equal volume of a solution of sheep thrombin in water at the same temperature was blown in and mixed immediately. The system was maintained in the water bath until about one second before it clotted, this having been determined by previous trial, and clotting was detected visually.

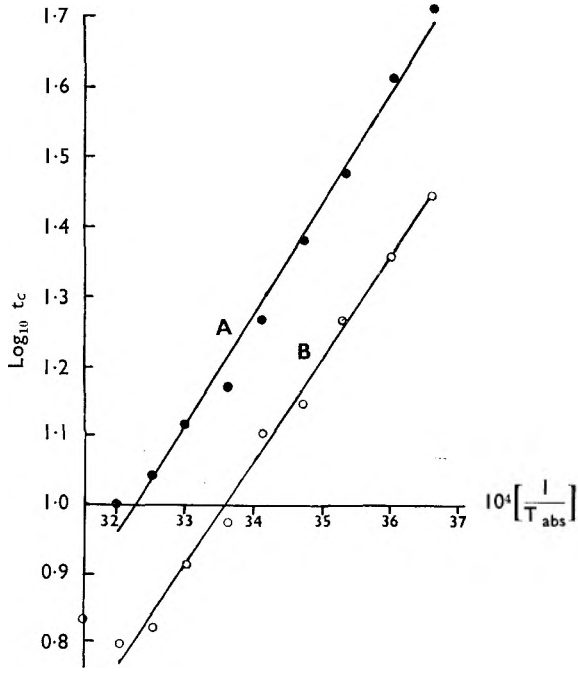

FIG. 1. Effect of temperature on the rate of reaction between thrombin and fibrinogen in oxalated plasma. A, 5 N.I.H. units/ml. B, 10 N.I.H. units/ml. Each point is the mean of not less than 3 determinations.

Two sets of determinations were made, in the one case with a thrombin concentration of 5 and in the other of 10 units per ml. The results are

shown graphically in Figure 1 as a plot of log. clotting time (in seconds) against reciprocal absolute temperature (1/°K). It will be seen that the points are in good agreement with the Arrhenius equation lying very close to a straight line within the range 0° to 40° C. This is by no means always the case with enzyme reactions[12]. Above 40° C., further increase in rate is offset by thermal denaturation of the enzyme. The best fitting straight line has been superposed on the points. The values of E calculated from these slopes are 7·2 and 6·7 kcal., but since the exact nature of the reaction has not yet been established it is not possible to attribute a precise meaning to this parameter.

The Effect of Various Added Substances.

Many substances affect the activity of thrombin preparations, the most important of which have been investigated by various workers[5,13,14,15,16] in buffered solutions of fibrinogen. The effects here considered are those which particularly relate to assay processes and in all cases the substrate employed was citrated or oxalated plasma, and the enzyme concentration was large enough to cause clotting in under half a minute.

Salt Effects.

The most important of these is that due to the presence of sodium salts. Sodium citrate, sulphate and chloride all retard the reaction. In general there was a practically straight line relationship between molar strength and clotting time. The molar concentration of each salt which was the minimum observed to produce marked prolongation of the clotting time and the added maximum salt concentration which produced no evident prolongation in plasma respectively were as follows:

Sodium chloride 0·010 M and 0·005 M
Sodium sulphate 0·008 M and 0·002 M
Sodium citrate 0·005 M and 0·002 M

These figures were determined by consideration of the results obtained in a series of randomised block experiments.

TABLE I

THE EFFECT OF ADDED SODIUM SULPHATE ON THE REACTION BETWEEN BOVINE THROMBIN AND CITRATED BOVINE PLASMA

Molar concentration	Additional ionic strength	Clotting times (seconds)			
0·008	0·024	16	17·5	19	19
0·004	0·012	12	15	16	17
0·002	0·006	14	15	15	14
0·001	0·003	15	14	14	15
0·000	*0·000*	*14*	*14*	*15*	*15*

Table I shows a typical set of results; in the majority of instances the clotting time of the system without added salt fell within the range 10 to 20 seconds. The shortest was 9, and the longest 26 seconds.

In addition to the general salt effect shown by the above mentioned salts, certain other salts were found to be capable in low concentration

of exerting an accelerating effect which had previously been observed only in the case of calcium chloride in fibrinogen solutions[2]. These were salts of divalent metals or of transition elements in the divalent state.

The experiment carried out was to mix 0·2 ml. of citrated sheep plasma with 0·1 ml. 0·1M solution of the salt in distilled water. 0·1 ml. of a solution of bovine thrombin in distilled water (of the order of 10 units per ml.) was blown into the mixture from a pipette, and mixing was effected by agitation without the introduction of air bubbles. Table II shows the results obtained.

TABLE II

Added solution	Clotting time (seconds)	Observation
H_2O	16	
$MnSO_4$	10	
$CoCl_2$	10	
$BaCl_2$	8	Very fine precipitate
H_2O	17	
$MnSO_4$	11	
$CoCl_2$	11	
$BaCl_2$	8	Very fine precipitate
$CaCl_2$	8	
H_2O	17	
$CaCl_2$	8	
$CaCl_2$(0·2M)	8	

The precipitate was presumably barium citrate which has a solubility in water of 0·04 per cent. at 18° C.

It is evident that the four cations examined fall into two classes. Cobaltous and manganous cause acceleration to 10 to 11 seconds and barium and calcium cause greater acceleration to 8 seconds. Furthermore this figure was not decreased by doubling the calcium concentration. That this effect is not brought about by initiation of the blood clotting mechanism is shown by the fact that the recalcification time of plasma—0·2 ml. of plasma plus 0·1 ml. of 0·1M calcium chloride—was 8·25 minutes. Barium, cobalt and manganese in corresponding tests were devoid of fibrinogenic action.

Further experiments were carried out in an endeavour to throw some light on the mechanism of the acceleration. Tables III and IV show the relationship between concentration of calcium chloride and clotting time. The maximum rate is attained in the presence of about 0·03 M calcium chloride. In higher concentrations the salt becomes a potent inhibitor. It is interesting to note that Häusler and Schnetz[17] record nickel and cobalt as inhibitors of blood clotting in concentrations greater than 0·003 N.

The smaller variance observed in Table IV is probably associated with formation of calcium oxalate which adsorbed on to the deposited fibrin thus renders the progress of the coagulation particularly lucent.

Various hypotheses would account for this effect but they are of two types only: firstly, those in which there is no change in the mechanism of the reaction, the acceleration being accountable in terms of concentration changes, or, secondly, those in which the course of the reaction is modified with a change associated with the entropy or energy characteristics of

the enzyme-substrate complex. In the former case it could be that either the divalent ions were removing an inhibitor of the forward reaction by combination with it, the segregation being complete before addition of the thrombin, or it could be that calcium is an essential component of the normal mechanism, so that the effective concentration of enzyme is increased by its addition. A further possibility is that removal of the inhibitor occurred at a slower rate so as to complicate the kinetics without actually interfering with the reaction steps. In view of the nature of the results this was not considered likely, and by finding out if there was any marked change in the kinetics it was hoped to throw some light on the mechanism.

TABLE III

THE EFFECT OF CALCIUM CHLORIDE ON THE RATE OF FORMATION OF FIBRIN IN CITRATED SHEEP PLASMA, MEASURED AS CLOTTING TIMES IN SECONDS

Molar concentration of calcium chloride in system					
0·05	0·04	0·03	0·02	0·01	0
9	9	7	8	17	19
8	7	8	11	18	20
9	9	8	12	18	21
10	9	8	11	16	23
36	34	31	42	69	83

TABLE IV

THE EFFECT OF CALCIUM CHLORIDE ON THE RATE OF FORMATION OF FIBRIN IN CITRATED BOVINE PLASMA MEASURED AS CLOTTING TIMES IN SECONDS

Molar concentration of calcium chloride in system					
0·05	0·04	0·03	0·02	0·01	0
14	13	12	12	15	23
15	14	11	11	16	21
14	13	12	12	14	21
43	40	35	35	45	65

Rough determinations of the value of E, the "activation energy," in the presence of 0·03 molar barium and calcium chloride were calculated from experimentally determined temperature coefficients. They were found to be less than the values for thrombin alone and were in good agreement (6·5 and 6·4 kcal.)—but though 0·2 kcal./mol. reduction in "activation energy" would be more than adequate to double the rate of reaction, other factors remaining unchanged, it is not possible to draw any conclusion due to the marked differences between the two values found for thrombin alone. As will be seen from Figure 2, in each case the points in the lower temperature range fell on a straight line, but with calcium chloride the bend towards the optimum commenced at 25° C. It may well be that this premature curvature was an artefact due to the nature of the experimental technique and the shortness of the clotting times. The matter demands fuller investigation.

Specific Effects

Astrup has presented evidence[18] that the specific effects which undoubtedly occur in the thrombin:fibrinogen reaction[19] are attributable solely to the substrate. But even if this be regarded as established, the possibility also exists that the antithrombins of plasma may be specific in some degree. Several experiments were carried out to determine the magnitude of specific effects in plasma.

In the first two experiments carried out, a statistically confounded 3 factor design was used in order to reduce the block size, with sheep and bovine thrombin each at 4 levels[20] (about 10, 5, 2·5 and 1·25 units/ml. final concentration) and with citrated sheep plasma (0·56 per cent. sodium citrate in blood) and oxalated bovine plasma (0·16 per cent. potassium oxalate plus 0·04 per cent. oxalic acid in blood). The experiments were carried out by two different workers who used the same plasmas but different thrombin solutions and it was evident from consideration of the results represented graphically, that while sheep thrombin had the same activity in sheep and bovine plasma, bovine thrombin was more active than sheep thrombin in bovine plasma when present in higher concentration. The graphs also demonstrate the essential rectilinearity of the log clotting time : log thrombin concentration relationship. Statistical analysis of the results however shows in the one case a quadratic component of the concentration relationship which was just significant at the 5 per cent. level and no other significant effect except the differences due to potency in the thrombin solutions used, and in the other case no quadratic component, a significant plasma effect and a significant interaction between dose and thrombin type. Unfortunately the rigid derivation of the thrombin : plasma : dose interaction was not possible since this was confounded with the sum of squares due to block differences.

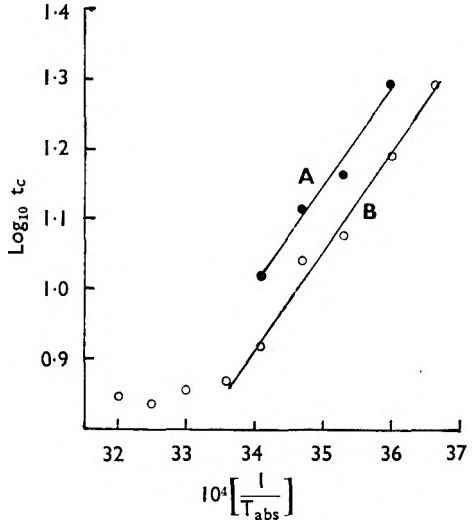

FIG. 2. Effect of temperature on the rate of reaction between thrombin and fibrinogen in oxalated plasma in the presence of calcium and barium. A, 0·05 M barium chloride. B, 0·025 M calcium chloride.

In a further experiment using ox and sheep thrombin each at three levels and oxalated and citrated ox and sheep plasma in a factorial design with three replications, a highly significant plasma effect was found as well as a distinct anticoagulant effect, however there was no significant interaction of any kind, though the random variance was only about 50 per cent. greater than in the confounded block experiment. An identical experiment in the presence of optimal calcium gave significant plasma and anticoagulant effects. In neither of these experiments was the quadratic component of the regression of log clotting time on log concentration significant.

It was evident therefore that interactions between ox and sheep preparations are not generally striking, but can occur. The possibility

of major interspecific effects was however demonstrated quite incidentally in an attempt to relate the "blood clotting dose" of the British Pharmacopœia, or M.R.C. Unit, as we prefer to call it, to the National Institute of Health Unit. The N.I.H. standard was of bovine origin obtained from the National Institute of Health, Bethesda, Maryland, and the M.R.C. preparation was of human thrombin kindly assayed and supplied by Dr. Kekwick of the Lister Institute. Three assays, each satisfying the internal requirements for validity were carried out by three workers, two of whom used bovine plasma as substrate and one a solution of bovine fibrinogen. The two workers who used plasma obtained mutually consistent results which differed markedly from those of the third worker who used fibrinogen. But the fibrinogen data agreed with past experience of the relative magnitudes.

The Effect of Glycerol and Acacia and the Interactions of these with Salts.

These experiments were carried out in randomised blocks generally with each factor at 2 or 3 levels. Glycerol was investigated as it is frequently used as a means of preserving thrombin as a solution in "deep-freeze," and acacia as it is used to increase the stability of dilute solutions of enzymes. Before use the solution of acacia was passed through a column of *biodeminrolit* (mixed bed deionising resin) so that in fact a solution of arabinic acid was used the *p*H of which was found to be 3·0.

The results of the experiments carried out are shown in Tables V, VI, VII and VIII.

TABLE V

THE EFFECT OF SODIUM CHLORIDE, CALCIUM CHLORIDE AND ARABINIC ACID ON THE CLOTTING OF CITRATED SHEEP PLASMA BY THROMBIN

0·1 ml. of each reagent was added to 0·4 ml. of citrated sheep plasma then 0·1 ml. of bovine thrombin (10 μ/ml. in water) was blown in and mixed quickly

	0·019 M sodium chloride			
	0·025 M calcium chloride	Water	0·025 M calcium chloride	Water
	Clotting times in seconds			
0·25 per cent. arabinic acid	20 17	35 29	16 15	28 25
Water	24 20	58 48	22 17	48 50

TABLE VI

ANALYSIS OF VARIANCE OF LOG METAMETERS DERIVED FROM THE RESULTS IN TABLE V

Item	Sum of squares	
Blocks	158·1	
A (arabinic acid) ..	1072·6	
N (NaCl)	158·1	(p 1—0·1 per cent.)
C ($CaCl_2$)	3957·6	($p < 20$ per cent.)
A × N	10·6	
A × C	248·1	($p < 0·1$ per cent.)
N × C	1·6	
A × N × C	1·6	
Error (7 d.f.)	59·4	∴ mean square 8·5

TABLE VII

THE EFFECT OF SODIUM CHLORIDE, CALCIUM CHLORIDE AND GLYCEROL ON THE CLOTTING OF OXALATED BOVINE PLASMA BY THROMBIN

0·1 ml. of each of the reagents + 0·1 ml. of water was introduced into the bottom of a clean 3 × ½ in. soda glass tube and 0·5 ml. of plasma was blown in and mixed quickly

		Full strength bovine thrombin		2/3 strength bovine thrombin	
		0·04 M sodium chloride	Water	0·04 M sodium chloride	Water
		Clotting times in seconds at 24° C.			
0·025 M calcium chloride	1 per cent. of Glycerol	19	11	22	13
	Water	16	9	21	12
Water	1 per cent. Glycerol	36	19	51	23
	Water	29	18	42	21

TABLE VIII

ANALYSIS OF VARIANCE OF LOG. METAMETERS DERIVED FROM THE RESULTS IN TABLE VI

Item	Sum of squares
N (NaCl)	2730·1
C ($CaCl_2$)	3164·1
G (Glycerol)	138·1
T (Thrombin)	430·6
N × C	18·1
N × G	7·6
N × T	14·1
C × G	0·6
C × T	3·1
G × T	7·6
Residual (5 d.f.)	48·4
	∴ Mean square 9·7

Therefore only the main effects are significant.

From these results the following inferences may be made.

Acacia has a very marked accelerating effect even when present only to the extent of 0·25 per cent., but this is much reduced in the presence of calcium and conversely calcium has a reduced effect in the presence of acacia. It was not at first appreciated that the *p*H of the arabinic acid used was as low as 3 so that a first experiment on the effect of acacia, sodium chloride and calcium chloride served merely to demonstrate the protection of thrombin against hydrogen ions by inorganic salts.

Glycerol was found to have a marked decelerating effect when present to the extent of 1 per cent., but no interaction between glycerol and salts was detected.

SUMMARY

1. The nature of the relationship between thrombin concentration and clotting time is discussed. The rectilinear log:log relationship holds good generally.

2. The effect of temperature on the rate of reaction in dilute plasma is found to be in agreement with the Arrhenius equation and a value for the "activation energy" is calculated.

3. The temperature optimum for thrombin in plasma appears to be between 40° and 45° C.

4. The general retarding effect of salts is noted, and the specific effect of calcium, barium, manganous and nickelous salts is noted and discussed.

5. The activity of thrombin in plasma may show complex dependence on the species of origin.

6. Glycerol retards coagulation, and arabinic acid accelerates it. There is a very marked interaction between the latter and calcium salts.

I gratefully acknowledge the assistance of Misses Margaret Kennedy and Freda Wilbraham in carrying out this work, and the interest and co-operation of Dr. R. Maxwell Savage.

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THE DETECTION OF THE ALKALOIDS OF *LUPINUS TERMIS* IN VISCERA

BY E. H. W. J. BURDEN, D. N. GRINDLEY and RIAD MANSOUR

From the Wellcome Chemical Laboratories, Ministry of Health, Khartoum (Sudan)

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THE seeds of the leguminous vegetable *Lupinus termis* (Family Papilionaceæ) form an important article of diet of the people of the Sudan and other Middle East countries, where they provide a valuable source of protein. The composition of the seeds has been shown to be as follows:—Moisture 3·2 per cent., oil 8·2 per cent., protein 38·9 per cent., carbohydrate 38·2 per cent., crude fibre 8·4 per cent., ash 3·1 per cent., providing a total calorific value of 392 kg. calories per 100 g. However, it has been shown by Clemo and Leitch[1] that these seeds contain the alkaloid lupanine, in the *d*- and *dl*- forms and according to Bamford[2] symptoms of poisoning occasionally arise which are attributed to the seeds when they are eaten without thorough washing. The fact that these seeds contain a bitter principle is of course well-known to those who include this material in their diet, but the alkaloid is readily soluble in water, and the bitter principle may easily be removed by soaking the seeds and washing thoroughly before cooking.

The properties of the lupin alkaloids, in particular lupanine, do not appear to have been very fully reported from the point of view of the toxicologist, and the purpose of the present investigation is to be able to detect its presence in post-mortem specimens, and to know exactly at what stage it appears when such specimens are subjected to the well-known Stas-Otto process and when the purified extracts are subsequently examined by the scheme for separation and identification for alkaloids described by Bamford[2].

The recorded colour tests for lupin alkaloids are as follows:—

SPARTEINE

(*a*) *Grant's Test*[3], as modified by Couch[4], is stated to be highly specific. A strip of filter-paper is moistened with a chloroform extract, allowed to dry, exposed to bromine vapour and then to ammonia vapour, and finally warmed. A bright pink colour is given in the presence of sparteine.

(*b*) *Jorissen's Test*[5] is said to be highly characteristic but requires about 10 mg., which is frequently impracticable in forensic work. However, a modification of this test is proposed which greatly increases its sensitivity. Take about 0·1 mg. of alkaloid in a small porcelain basin, and moisten with about 5 μl. of N sodium hydroxide. Add 2 ml. of ether and stir well with a small glass rod, add about 0·2 mg. of sulphur and again stir well. Pass hydrogen sulphide through a fine jet into the ether. A deep red turbidity is obtained in the presence of sparteine, and a red deposit remains when the ether volatilises.

(*c*) *Nascent Chlorine Test.* Bamford[2] classifies sparteine in Group VI in his scheme for identification of alkaloids using this test. When a little of the alkaloid is dissolved in a drop of concentrated hydrochloric acid with a trace of potassium chlorate, and evaporated to dryness, a "reddish purple" colour is stated to be obtained on exposing the residue to ammonia fumes. We were unable to obtain this reaction with either sparteine sulphate or with a sample of mixed alkaloids from *L. termis*, although both gave strongly positive reactions with Grant's test and with the modified Jorissen's test.

Lupinine

Chloranil Test[2]. An olive-green to brown residue is obtained in the presence of lupinine by mixing a little of the base dissolved in benzene, with a 1 per cent. solution of chloranil in benzene, and evaporating to dryness.

Preliminary Extraction and Separation of Lupin Alkaloids

120 g. of the seeds of *Lupinus termis* was ground and extracted with ethanol acidified with tartaric acid. The ethanol was removed by evaporating spontaneously at room temperature (25° C.), and the residue was extracted with acidulated water and filtered. The aqueous solution was then extracted in turn (*a*) with light petroleum to remove fatty material, (*b*) with ether, and (*c*) with chloroform after making ammoniacal. The ammoniacal chloroform extract was purified in the usual way, and the extracted alkaloids when tested gave the following results:—

General Alkaloidal Reagents

Wagner's reagent, Mayer's reagent, Marmé's reagent, Sonnenschein's reagent, Dragendorff's reagent, picric acid solution, gold chloride solution and mercuric chloride solution all gave strong reactions. Platinic chloride gave only a faint reaction.

Classification According to Bamford's Scheme

Group I—concentrated sulphuric acid—no colour.
Group II—Marquis' reagent—no colour.
Group III—Vitali's test—no colour.
Group IV—sulphuric acid-potassium dichromate—no colour.
Group V—ethanolic *p*-dimethylaminobenzaldehyde a brilliant crimson colour on warming. Residue is crimson after evaporation, turning deep orange with ammonia.

The alkaloids are therefore placed in Group V (*b*) together with nicotine, mescaline, and pilocarpine.

Further tests were made with the following results:—

Mecke's reagent—no colour.
Mandelin's reagent—no colour.
Fröhde's reagent—no colour.
Chloranil (1 per cent.)—deep brown to olive-green colour.
Nascent chlorine test—no colour.
Grant's test—positive.
Modified Jorissen's test—positive.

The acid-ether extract was also examined with the following results:—

Concentrated sulphuric acid—bright orange colour.

Marquis' reagent—strong purple-orange.

Mecke's reagent, Mandelin's reagent, Fröhde's reagent } as for sulphuric acid.

p-Dimethylaminobenzaldehyde—deep purple on taking to dryness on water bath. Brilliant violet on adding a drop of ethanol.

These results were confirmed on a larger scale by grinding and extracting 5 kg. of seed with 20 l. of acidified ethanol. The ethanolic extract was evaporated spontaneously at room temperature and the residue extracted with water. The extract was allowed to stand overnight in a separating funnel, when most of the oil (*c.* 100 ml.) separated to the surface. The lower layer was run off, and as it proved very difficult to filter, it was passed through muslin and was purified by treatment with basic and neutral lead acetate, allowed to stand, and filtered. The excess of lead was removed by treatment with hydrogen sulphide and filtration. The aqueous solution was evaporated to a small bulk by warming under a strong current of air. The aqueous solution was then given a preliminary extraction with ether, and then made alkaline to litmus with sodium hydroxide and extracted 5 times with chloroform, washing the extracts 3 times with water. The chloroform extracts were evaporated to dryness when 4·2 g. of crude alkaloids was obtained. The aqueous solution was then made alkaline to phenolphthalein and re-extracted with chloroform as before, when a further 0·07 g. of crude alkaloids was obtained.

The yellow colour remained in the aqueous (alkaline) layer, which was decolorised by treatment with animal charcoal and evaporated to small bulk. This solution gave no reactions for alkaloids.

Both extracts of crude alkaloids gave the same reactions with the general alkaloidal reagents and the colour reagents given above.

The mixed extracts were further purified by dissolving in acidulated water, extracting 3 times with chloroform, and rejecting the extracts, making alkaline to phenolphthalein with sodium hydroxide, and extracting 3 times with chloroform, and washing the combined chloroform extracts 3 times with water. The chloroform extracts were then shaken out 3 times with approximately 0·2 N hydrochloric acid. The aqueous solution was brown in colour, and was decolorised by adding 2 per cent. of animal charcoal, warming at 80° C. for 1 hour, and filtering, when a colourless solution was obtained. However, after making alkaline with sodium hydroxide, extracting with chloroform and removal of the solvent, the brown colour returned to the residue.

Partial Separation of the Mixed Alkaloids

A portion of the mixed alkaloids was extracted in turn with light petroleum (b.pt. 60° to 80° C.), benzene and acetone when the following fractions were obtained:—

Fraction I—soluble in light petroleum.

Fraction II—insoluble in light petroleum, soluble in benzene.

Fraction III—insoluble in benzene, soluble in acetone.
Fraction IV—insoluble in acetone.

Each fraction was re-extracted and redissolved with the appropriate solvents until fractions II, III, and IV were well separated. However, it was very difficult to obtain fraction I completely free from insoluble matter, even after 10 extractions.

Each fraction was found to give positive reactions with Wagner's and Dragendorff's reagents, and the following colour reactions were given:—

Fraction	I	II	III	IV
(a) Ethanolic *p*-dimethylaminobenzaldehyde evaporated to dryness	brilliant crimson	faint brown-pink	faint brown-pink	no colour
(b) Residue from above exposed to ammonia fumes	deep orange	light violet and yellow	light purple	no colour
(c) Nascent chlorine test	no colour	no colour	no colour	no colour
(d) Residue exposed to ammonia fumes	no colour	no colour	no colour	no colour
(e) Chloranil (1 per cent.)	deep brown to olive-green	no colour	no colour	no colour
(f) Modified Jorissen's test	red colour	no colour	no colour	no colour
(g) Grant's test	orange-red	no colour	no colour	no colour
(h) Solubility in water	insoluble	partially soluble	soluble	soluble

A further attempt to characterise these fractions was made by examining microscopically the precipitates formed by the general alkaloidal reagents. The results were as follows:—

Fraction		I	II	III	IV
Wagner's reagent	15 minutes	globules	amorphous	amorphous	amorphous
	1 day	globules and tufted crystals	amorphous	amorphous	amorphous
	4 days	some globules had become crystalline	amorphous	amorphous	amorphous
Dragendorff's reagent	15 minutes	amorphous	amorphous	amorphous	amorphous
	1 day	small globules	masses of minute crystals	masses of minute crystals	amorphous
	4 days	granular	masses of minute crystals	masses of minute crystals	amorphous
Mayer's reagent	15 minutes	amorphous	minute crystals	faint amorphous	faint amorphous
	1 day	globules	minute crystals	faint amorphous	faint amorphous
	4 days	globules some crystallised	minute crystals	faint amorphous	faint amorphous
Marmé's reagent	15 minutes	amorphous	amorphous	faint amorphous	very faint amorphous
	1 day	small globules	amorphous	faint amorphous	very faint amorphous
	4 days	small globules	amorphous	faint amorphous	very faint amorphous
Picric acid	15 minutes	globules	amorphous	faint amorphous	no visible precipitate
	1 day	globules	masses of minute crystals	faint amorphous	no visible precipitate
	4 days	globules	masses of minute crystals	faint amorphous	no visible precipitate
Mercuric chloride	15 minutes	globules	minute crystals	faint amorphous	amorphous
	1 day	globules	minute crystals	globules	globules
	4 days	globules	minute crystals	globules	globules

Fraction		I	II	III	IV
Gold chloride	15 minutes	amorphous	amorphous	amorphous	faint amorphous
	1 day	small triangular crystals	amorphous	amorphous	faint amorphous
	4 days	small triangular crystals	amorphous	amorphous	faint amorphous

As far as is known, only one alkaloid has so far been described in *L. termis*[1], but it is apparent that there is a complex mixture of alkaloids present. In fraction I alone, besides the probability of the presence of sparteine, there is another alkaloid which gives the brilliant reaction with *p*-dimethylaminobenzaldehyde which does not hitherto seem to have been described. All the fractions failed to crystallise even after standing for several weeks, and, in view of their obvious complexity, no efforts were made to characterise them further. Clearly there is scope for investigation here, and it is hoped to attempt to identify the alkaloids present at a later date.

Summary and Conclusions

1. An investigation of the properties of the alkaloids of the seeds of *Lupinus termis*, a common article of food in the Middle East, with the intention of detecting them chemically in toxicological specimens is described.

2. The seeds are shown to contain a complex mixture of alkaloids. However, the colour reactions described are sufficiently specific to ensure identification in specimens, although their fate in the human body is unknown.

3. A new modification of Jorissen's test for sparteine is described, making it sensitive to 0·1 mg.

We are indebted to the Director, Medical Services, Ministry of Health, Khartoum (Sudan), for permission to publish this note.

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PHARMACOLOGICAL STUDIES IN THE APOCYNACEOUS GENUS ASPIDOSPERMA MART. AND ZUCC. *ASPIDOSPERMA ALBUM* (VAHL) R. BEN. AND *ASPIDOSPERMA MEGALOCARPON* MUELL. ARG.

BY J. N. BANERJEE*† and J. J. LEWIS*

From the Pharmacology Laboratories, Pharmacy Department, University of Nottingham

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IN previous communications[1,2] the pharmacological properties of the alkaloids of *Aspidosperma oblongum* A.DC. have been discussed. The results obtained from studies of these alkaloids lead us to an investigation of the pharmacological properties of alkaloids found in the barks of other *Aspidosperma* species.

This paper deals with the alkaloids of *Aspidosperma album* (Vahl) R.Ben. and *Aspidosperma megalocarpon* Muell. Arg.

MATERIALS

The dried bark was reduced to a coarse powder and the total alkaloids extracted by classical methods. The pharmacological tests were made with the dried total alkaloid fractions, which were dissolved in 0·1 N hydrochloric acid and adjusted to *p*H 6·6 to 6·8 by addition of 0·05 N sodium hydroxide solution. Precipitation occurred if the *p*H was higher. Sodium chloride was added to the adjusted solution to give a final concentration of 0·9 per cent. for mammalian work, and 0·6 per cent. for work on frog tissues. The adjusted solution contained 10 mg. of total alkaloid per ml. and dilutions were made from this.

METHODS AND RESULTS

The pharmacological properties of the alkaloids from both barks are described together and differences indicated where they exist.

When applied to the muscle of the frog sciatic nerve-gastrocnemius muscle preparation, there was a contractural response which was reversible on washing. There was a progressive decline in the response of the muscle to indirect stimulation, and also a depression of response when the drug was applied to the sciatic nerve. Inhibition in both cases progressed to completion and was irreversible, although the muscle still responded to direct stimulation. In the frog rectus abdominis preparation the response obtained on addition to a 10-ml. bath of 10 μg. of acetylcholine chloride was inhibited by previous exposure to both alkaloids. The inhibition was proportional to the dose given (Figs. 1 and 2).

There was depression of smooth muscle tone, with inhibition of peristalsis in rat and guinea-pig ileum, and in rabbit duodenum. The tone and normal rhythmic movements of rat, guinea-pig and rabbit uterus were inhibited. The spasmogenic actions of 1-μg. doses of acetylcholine chloride on rat and guinea-pig ileum, of 10-μg. doses on

* Present address: Materia Medica Department, University of Glasgow.
† Nottingham University Research Scholar.

the rat and guinea-pig uterus, and of 2-μg. doses on the rabbit duodenum were inhibited. Inhibition of the spasmogenic actions of 1 μg. of histamine acid phosphate on guinea-pig ileum and uterus was shown. A similar effect was shown for barium chloride at a dose level of 2 mg. using rat and guinea-pig uterus, and rat, rabbit and guinea-pig ileum. On the rabbit uterus antagonism to the spasmogenic action of 10 μg. of

FIG. 1. Inhibition of the action of 10 μg. of acetylcholine chloride by:—A 0·2 mg., B 0·1 mg., C 0·05 mg., D 0·02 mg. and E 0·4 mg. of *A. album* alkaloids. Each dose given 3 minutes previous to the dose of acetylcholine.

adrenaline hydrochloride was shown. Higher doses of alkaloids were needed to produce on the uterus effects equivalent to those seen on the gut. In all cases a 50-ml. bath was used, containing for rat tissues oxygenated de Jalon's solution[3], and for the others oxygenated Tyrode's solution. The bath temperature was 36·5° to 37° C.

The rate, amplitude and tone of the frog heart *in situ* were gradually decreased by application of solutions of both drugs. After partial

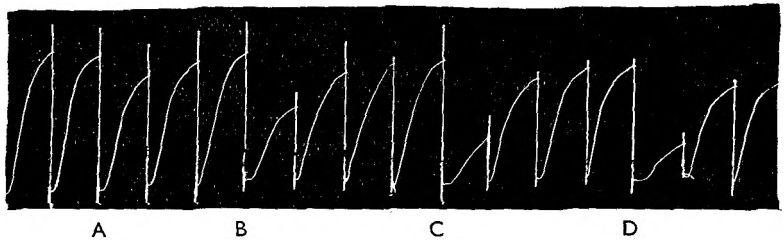

FIG. 2. Inhibition of the action of 10 μg. of acetylcholine chloride by:—A 0·1 mg., B 0·2 mg., C 0·32 mg. and D 0·4 mg. of *A. megalocarpon* alkaloids. Each dose given 3 minutes previous to the dose of acetylcholine.

cardiac depression vagal stimulation did not produce its characteristic effects. Similar effects were noted with the isolated frog's heart perfused through the aorta or sinus venosus with normal Ringer's solution or with "half-calcium" Ringer's solution, containing 1 part in 200,000 of the alkaloids. No auricular-ventricular block could be demonstrated.

The amplitude of the beat of Langendorff preparations of rabbit and kitten heart was reduced by 1 mg. of the alkaloids given as an injection into the cannula. 2 mg. administered similarly also depressed the rate. With *A. megalocarpon* alkaloids no auricular-ventricular blocking action could be seen. During the phase of recovery after a 4-mg. dose of *A. album* alkaloids a well marked but short-lived block was shown.

Similar effects were seen when the fluid perfusing the heart contained 1 in 200,000 of alkaloid. The effects were reversible. After administration of *A. megalocarpon* alkaloids (1 mg.) a well marked increase in outflow was seen. This rose from 3·3 ml. to 6·0 ml. per minute. In the isolated rabbit auricles suspended in oxygenated Ringer-Locke solution at 29° C., 1·5 mg. of alkaloids reversibly inhibited the depressant effects of 5 μg. of acetylcholine chloride.

When perfused into the abdominal aorta of the rat hind limbs preparation, solutions of *A. megalocarpon* alkaloids produced marked vasoconstriction. There was no reversal of the vasoconstrictor action of 5 μg. of adrenaline. Inconsistent results were obtained with *A. album* alkaloids which produced either marked vasoconstriction or transient vaso-dilatation followed by a return to normal tone. There was antagonism by the *A. album* alkaloids of the vasoconstrictor effects of 1 μg. of acetylcholine chloride and of the vasoconstrictor effects of 5 μg. of adrenaline hydrochloride. No vasodilator effect to administration of acetylcholine chloride was noted in this series of experiments, which numbered 10 preparations—the perfusion being carried out at room temperature. 0·5 mg. of both *A. album* and *A. megalocarpon* alkaloids dilated the blood vessels of the perfused rabbit ear. No adrenaline reversal or antagonism could be shown.

In the cat anæsthetised with ether and chloralose, administration of small doses of the drug (2 to 10 mg.) into the jugular vein caused no noticeable effects upon depth or frequency of respiration. The blood pressure was depressed after each administration, the magnitude of depression being proportional to the dose of alkaloid given. No reversal of the pressor response to doses of 5 μg. of adrenaline hydrochloride could be shown. 4-mg. doses of the alkaloids showed no protecting power in mice weighing 20 to 21 g. against the effects of lethal doses of adrenaline hydrochloride[4]. The depressor response to 5 mg. of alkaloids in the spinal cat was less marked than in the anæsthetised animal. No modification in this preparation of the pressor effects of adrenaline (1 to 2 μg.) could be shown by *A. megalocarpon* alkaloids but there was very marked potentiation of the pressor effect when *A. album* alkaloids (0·5 mg./kg.) were added.

Injection of 6 mg. of the alkaloids into the ventral lymph sac of frogs caused paralysis, loss of reflexes and death in about 50 per cent. of frogs. No convulsions were seen. With the *A. megalocarpon* alkaloids, paralysis was preceded by restlessness. In mice weighing 20 to 21 g. intraperitoneal injection of 8 mg. of the total alkaloids caused the following symptoms. 15 minutes after the injection the animals became quiet and sleepy with closed eyelids; frequent washing movements were noted; soon after there were convulsions; these alternated with periods of quiet; 6 out of 10 mice died; the others recovered within 3 to 4 hours. Similar symptoms were observed in rats at a dose level of alkaloid of 400 mg./kg.

When tested by the method of Sollmann[5] as modified by Bülbring and Wajda[6] a graded local anæsthetic activity was shown. To rule out the possibility of a generalised toxic effect upon nervous tissues or of a

neuromuscular block, immediately after sensory paralysis to the highest concentration of acid the sciatic nerve was exposed and stimulated, when the gastrocnemius muscle was found to respond normally.

No antimalarial activity was shown when the alkaloids (at 3 mg. dose level) were tested by the method of Rollo[7] against *Plasmodium berghei* in mice. When tested *in vitro* against *Entamœba histolytica* 1 part in 20,000 of the alkaloids showed some anti-amœbic activity (Table I).

TABLE I

In vitro ANTI-AMŒBIC ACTIVITY OF *A. album* AND *A. megalocarpon* ALKALOIDS

Compound and concentration	Viable amoebæ per 100	
	Incubation with drug for 24 hours	Incubation with drug for 48 hours
Emetine—		
(*a*) 1 : 200,000	4/100	3/100
(*b*) 1 : 400,000	12/100	30/100
(*c*) 1 : 800,000	40/100	many
A. megalocarpon alkaloids—		
(*a*) 1 : 20,000	24/100	3/100
(*b*) 1 : 200,000	—	many
A. album alkaloids—		
(*a*) 1 : 20,000	18/100	10/100
(*b*) 1 : 200,000	—	many

There was depression of up to 3° C. of the rectal temperature in mice weighing 23 to 25 g. when 2·0 mg. of alkaloids was given intraperitoneally.

Discussion

Unlike the alkaloids of *Aspidosperma oblongum* the alkaloids of the barks of *Aspidosperma album* and *Aspidosperma megalocarpon* do not reverse the response to adrenaline on the blood pressure of the chloralosed cat although an inconsistent effect was seen on the blood vessels of the rat hind limbs preparation using *A. album* alkaloids. There was, however, antagonism to the stimulant action of adrenaline on the virgin rabbit uterus, and potentiation of the pressor response by album alkaloids to adrenaline on the spinal cat. There was no protection in mice against lethal doses of adrenaline. Antagonism to acetylcholine, histamine and barium were shown but there was no evidence that these were specific effects. A local anæsthetic action was shown using the frog lumbar plexus method of Sollman[5] as modified by Bülbring and Wajda[6]. An *in vitro* anti-amœbic action has been shown.

Preliminary qualitative chemical tests have indicated that the alkaloids contained in the above barks differ from those of the bark of *Aspidosperma oblongum* which appears to contain indole derivatives.

Summary

1. The alkaloids of the barks of *Aspidosperma album* and *Aspidosperma megalocarpon* non-specifically antagonise the actions of acetylcholine, histamine and barium.

2. An *in vitro* anti-amœbic action has been shown.

3. There is no evidence of reversal of the pressor effects of adrenaline.
4. The *A. album* alkaloids potentiate the pressor response to adrenaline in the spinal cat.

We thank Mr. D. B. Fanshawe, Conservator of Forests, British Guiana for the barks; Dr. L. G. Goodwin for supplying us with a strain of *Plasmodium berghei* and for advice; and Mr. E. Wilmshurst for much help and advice in tests of anti-amœbic activity. We are especially indebted to Mr. G. E. Trease, Director of Pharmaceutical Studies, University of Nottingham, who aroused our interest in this work, for his help and advice.

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SOME OBSERVATIONS ON THE EFFECT OF DIFFERENT DRYING METHODS ON THE GLYCOSIDE CONTENT OF THE LEAVES OF *DIGITALIS LANATA*

By R. M. Dash and M. L. Frith

From the Pharmacy Department, University of Sydney, Australia

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There is little information available on the effect of different drying methods on the differential glycoside content of the leaves of *Digitalis lanata*. This has been due largely to the absence of adequate methods for differential analysis, most methods being dependent on a biological assay of total cardiac activity. For the determination of the component cardiac glycosides, a method using paper chromatography and fluorescence photography has been devised[1], and was used in this investigation of the genuine glycosides, digilanids A, B and C.

Method

A batch of leaves of *D. lanata* was collected in the early morning. Drying of the leaves was commenced on the same day, using the following methods:—(1) Low temperature vacuum drying; (2) sun drying; (3) hot air oven at 100° C.; (4) hot air oven at 70° C.; and (5) hot air oven at 50° C.

In low temperature vacuum drying the leaves were maintained at 5° to 10° C. under vacuum (0·01 mm. mercury) and took about 18 hours to dry. Drying was considered to be complete when the leaves had become brittle. The sun-dried leaf was exposed on a steel mesh in the open for 7 days, being left out overnight and subject to some light rain on the 5th day. Mild to warm weather prevailed, with a shade temperature of 70° to 75° F. Oven drying at 70° and 100° C. was completed within 3 hours and that at 50° C. within 7 hours. Approximately 2·3 g. of each sample of dried leaf, previously broken up in a mortar, were placed in 30 ml. of solvent, chloroform and methanol 1 : 1, for extraction. A moisture determination was carried out on each sample, using the Karl Fischer method.

Extraction

The leaf was shaken for 3 hours with the 30 ml. of chloroform-methanol solvent, followed by filtration on a Buchner funnel. This was repeated twice, shaking the residue with 10 ml. of solvent for 1 hour. The combined filtrates were evaporated to small bulk under reduced pressure, at a temperature not exceeding 30° C., and then adsorbed by diatomaceous earth. Chlorophyll and other impurities were next removed by Soxhlet extraction of the powder with light petroleum (low-boiling) in which the cardiac glycosides are insoluble. The glycosides were redissolved from the diatomaceous earth with chloroform-methanol, followed by filtration and evaporation to very small bulk under reduced pressure. The extract was finally adjusted to 10 ml. in 80 per cent. ethanol.

Chromatography

A small volume of the extract, usually 0·01 ml., was run on a chromatogram. The starting line was about 1 inch from the lower edge. The solvent used was benzene, ethyl acetate and water, 16 : 84 : 50, the aqueous layer being the stationary phase. To the organic layer, the mobile phase, 1 to 2 per cent. of ethanol was added. This strength increases the R_F value sufficiently to bring the digilanid C completely out of the spot.

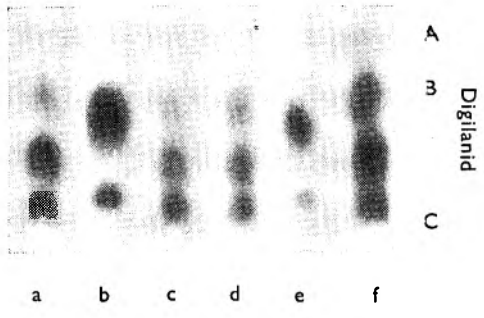

Fig. 1. The analysis of 2 plant samples by visual comparison from the photographic record.

a. 0·01 ml. of extract from sun-dried plant.
b. 5·0 μg. of digilanid B, 10·0 μg. of digilanid C } standards.
c. 0·005 ml. of extract from sun-dried plant.
d. 0·005 ml. of extract from plant dried at 50° C.
e. 2·5 μg. of digilanid B, 5·0 μg. of digilanid C } standards.
f. 0·01 ml. of extract from plant dried at 50° C.

Two tanks were used during the investigation, a glass one 6 × 8 × 14 inches high, and a wooden one with glass windows, 11 × 11 × 17 inches high. The wooden tank, being larger, was more difficult to equilibrate and required a much greater volume of solvent for saturation. Complete saturation of the atmosphere in the tank with both phases is important for a clear separation of the digilanids. This was achieved by hanging strips of filter paper down the sides of the tank and dipping into both solvents. Whatman No. 1 paper was used throughout, placing 5 spots on a 6-inch wide paper and 7 spots on an 8-inch wide paper. The system was allowed to equilibrate overnight and then run usually for about 3½ to 4 hours at 22° to 24° C.

After development, in which the ascending method was used, the chromatogram was allowed to dry in the air for at least 1 hour, and then sprayed with a mixture of chloroform and trichloracetic acid 2·5 : 1. Approximately 15 to 20 g. of spray was used for each chromatogram, best results being obtained for digilanids B and C if the spray was freshly prepared. The chromatogram was immediately heated in a hot air oven for 10 minutes at 110° C. The digilanid B and C treated in this way yield a bright blue fluorescence in ultra-violet light. Digilanid A, however, appears as a yellow spot. It was observed that variations in temperature during heating appeared to lessen the intensity of the fluorescence, although the temperature itself was not critical. Heating for 5 to 10 minutes at temperatures ranging from 105° to 115° C. has been found satisfactory.

Known amounts of standard solutions of digilanids A, B and C are run on each chromatogram. The estimation of each glycoside in the plant sample is obtained by comparing the size and intensity of the unknown spot with that of the appropriate standard. Comparisons are made

directly from the chromatogram, and also by examination of photographs of the chromatograms made under ultra-violet light using the method described in the paper of Silberman and Thorp.[1]

The intensity of the fluorescence of digilanid B is much greater than that of an equivalent amount of digilanid C. Usually two concentrations of digilanids B and C were run on each chromatogram, 2·5 and 5 μg of digilanid B, and 5 and 10 μg of digilanid C. A preliminary investigation of each plant sample is necessary to ascertain the extent to which the extract must be diluted to contain glycosides within these limits. Above these limits the fluorescence is too strong to allow accurate comparison, and below the fluorescence is too weak. It was thought that traces of plant pigment might potentiate the fluorescence of the plant glycosides. This possibility was eliminated by adding known amounts of digilanids B and C to spots of plant extracts whose digilanid content had been previously estimated. The fluorescence produced compared well with that of the standard digilanids run concurrently.

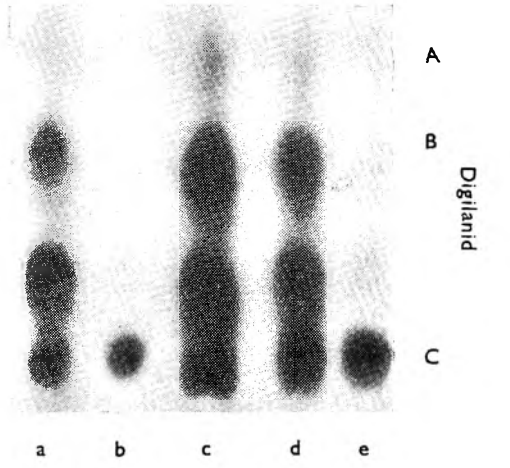

FIG. 2. Photographic record of a chromatogram showing the estimation of digilanid C in a plant sample.

a. 0·005 ml. of extract from plant dried at 100° C.
b. 5·0 μg. of digilanid C.
c. Plant sample corresponding to 0·01 ml. of the extract from the plant which was oven-dried at 100° C.
d. 0·005 ml. of the above extract and 5·0 μg. of digilanid C added.
e. 10·0 μg. of digilanid C.

Digilanid A could not be estimated quantitatively. A blue fluorescence is given by a substance in the plant extract which travels at the same rate at the yellow-fluorescing digilanid A standard. The plant extract and standards were run on a system of horizontal chromatography using benzene, ethyl acetate and *n*-butanol 12 : 78 : 5 on formamide-impregnated paper. This plant substance again travelled at the same rate as the digilanid A standard and therefore we have assumed that this plant substance is digilanid A, the blue colour perhaps being due to traces of plant pigment present.

In each plant extract a considerable amount of an unknown substance was found which appeared on the chromatogram between digilanids B and C. Digoxin, gitoxin and digitoxin travel to the solvent front in the ascending system, and as no other pure samples of digitalis glycosides are obtainable we were not able to investigate this unknown substance.

RESULTS

The results are summarised in Tables I, II and III.

TABLE I

No.	Extract	Moisture per cent.	Digilanid B		Digilanid C	
			μg. per 0·01 ml.	Percentage of anhydrous leaf	μg. per 0·01 ml.	Percentage of anhydrous leaf
1	Low temperature vacuum dried ..	6·00	6	0·27	24	1·065
2	Sun-dried	8·00	2 to 3	0·12	23 to 24	1·165
3	Oven at 100° C. ..	6·08	3	0·13	13 to 14	0·624
4	Oven at 70° C. ..	3·65	3	0·13	20 to 21	0·903
5	Oven at 50° C. ..	5·48	3 to 4	0·16	17 to 18	0·802

This table shows the number of mg. of digilanids B and C in 10 ml. (μg. in 0·01 ml.) of the extract, corresponding to approximately 2·3 g. of dried leaf. The digilanids B and C have been calculated as percentages of the anhydrous leaf. The apparent inconsistencies in the number of μg. per 0·01 ml. and the percentage of digilanids calculated on anhydrous leaf are due chiefly to the differences in weight of leaf taken for each extract.

TABLE II

Extract	Mean	Limits of error P = 0·95	Error per cent.
1	1·065	1·091–1·039	± 2·4
2	1·165	1·211–1·119	± 4
3	0·624	0·660–0·588	± 6
4	0·903	0·940–0·866	± 4
5	0·802	0·847–0·757	± 5

Table II shows the statistical analysis of the results obtained for digilanid C. The percentage error shown indicates only that error due to observation or variations in chromatographic technique; it does not include error due to sampling or extraction procedure.

TABLE III

No.	Extract	Digilanid A
1	Low temperature vacuum dried	10 x
2	Sun-dried	2·5 x
3	Oven at 100° C.	less than 1·25 x
4	Oven at 70° C.	5 x
5	Oven at 50° C.	6 x

Table III shows the digilanid A content of the plant extracts compared with the vacuum-dried extract. The arbitrary figure of 10 x was taken for the freeze-dried extract, as comparison with the digilanid A standard was not possible.

The figures shown in Table I represent the mean value for each extract, estimated independently by 3 observers from a series of chromatograms. Each extract was run approximately 12 times, excluding the preliminary investigation.

A statistical analysis of the results obtained for digilanid C was made and is shown in Table II. It indicates only the errors due to observation and variations in spraying and heating techniques in duplicate chromatograms.

As the fluorescence of the plant digilanid A could not be compared with that of the digilanid A standard, the plant extracts were compared with the vacuum-dried extract, and the results are shown in Table III.

DISCUSSION

The method of low temperature vacuum drying preserved the digilanids better than any other method. Of the three glycosides, digilanid A was the least stable, being almost completely decomposed by oven-drying at 100° C., and decomposed to a considerable extent by sun-drying. Oven-drying at 50° and 70° C. also resulted in some decomposition. Digilanid B was also considerably decomposed by all methods of drying, other than

low temperature vacuum drying. Oven-drying at 50° C. resulted in slightly less decomposition than oven-drying at 70° and 100° C. Sun-drying resulted in slightly more decomposition than any of the other methods. Digilanid C was apparently unaffected by sun-drying, and a subsequent experiment on a fresh batch of leaf confirmed this result. The digilanid C content in the sample dried at 50° C. appeared to be lower than in that dried at 70° C. Whilst the sample heated at 70° C. took 3 hours to dry, that heated at 50° C. took 7 hours, and the exposure to the lower temperature for a longer period may account for the decrease in digilanid C content. It has been assumed in these estimations that the standard digilanids are pure. However, on careful examination of the chromatograms, traces of impurities have been observed in the digilanid C standard. Therefore whilst the results are still comparative, the potency of the digilanid C in these extracts may be slightly overestimated.

Summary

1. A batch of leaves of *Digitalis lanata* was dried by 5 different methods.

2. The leaf samples were extracted and the digilanid B and C content estimated by paper chromatography and fluorescence photography. A comparative estimate indicating the decomposition of digilanid A was also made.

3. The digilanids were decomposed least in leaves which were dried at a low temperature *in vacuo*, although sun-drying was equally effective for the preservation of digilanid C.

The authors wish to thank Professor R. H. Thorp and Dr. H. Silberman for their interest and helpful suggestions in this work, Messrs. Sandoz of Basle for their donation of digilanids A, B and C, and Mrs. R. H. Thorp, who cultivated the *D. lanata* used in this experiment.

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THE COLORIMETRIC ESTIMATION OF GITOXIGENIN IN PRESENCE OF DIGITOXIGENIN

By D. H. E. Tattje

From the Laboratory of Pharmacognosy, State University of Groningen, Holland

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For the investigation of gitoxigenin in presence of digitoxigenin different reagents are known[1,2,3,4]. The first to indicate a reagent for qualitative purposes was Kiliani[2]; it consists of a mixture of 100 ml. of concentrated sulphuric acid and 1 ml. of a 5 per cent. aqueous ferric sulphate solution. This reagent was applied by Kraft[5], Windaus and Schwarte[6] and Cloetta and Fischer[7]. The reagent of Kiliani is not satisfactory for the quantitative estimation of gitoxigenin in presence of digitoxigenin. The reaction develops too quickly and moreover the reagent gives too deep a colour with digitoxigenin. When concentrated sulphuric acid is used without ferric chloride[3] the time required for obtaining a colour with gitoxigenin is rather long, namely 24 hours, and digitoxigenin also gives a colour. The proportion of the extinctions of equal quantities of gitoxigenin and digitoxigenin is 5 to 7.

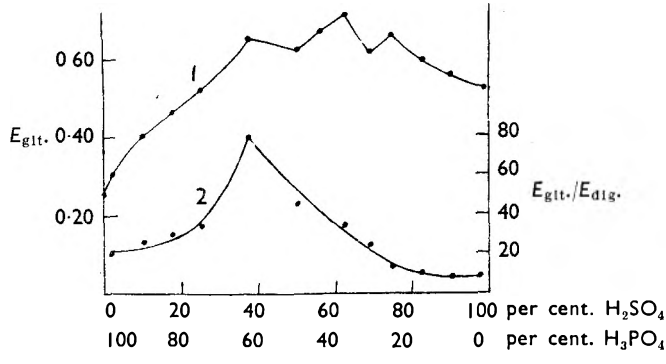

Fig. 1.

It has been proved by some workers[4,8] that concentrated phosphoric acid reacts with gitoxigenin under certain conditions. We therefore investigated the influence of phosphoric acid on the reagent of Kiliani. 1 g. of a 5 per cent. solution of ferric chloride in phosphoric acid was added to a mixture of concentrated sulphuric acid with syrupy phosphoric acid (85 per cent.). The composition of the reagent was varied and the results of the reaction with gitoxigenin are given in Figure 1.

The upper graph (1) shows the maximal extinctions of the colour which, after a shorter or longer lapse of time, develops by the reaction of 10 ml. of reagent with 0·1 mg. of gitoxigenin. The absorption was determined at a wavelength of 5800 Å.

The lower graph (2) represents the proportion of the extinction of equal quantities of gitoxigenin and digitoxigenin ($= E_{glt}/E_{dig}$), obtained

by varying the composition of the reagent. The time of reaction with the digitoxigenin is the same as that in which gitoxigenin attained its maximal value using the reagent of the same composition. From the two curves it can be seen that the most favourable results are obtained when the concentration of phosphoric acid is 62·5 per cent. w/w.

In many cases the absorption spectrum of the colour obtained by the influence of the reagents on gitoxigenin was determined. In the experiments represented in Figure 2 we used for this purpose the reagent of the following composition:—concentrated sulphuric acid, 37·5 per cent. w/w; phosphoric acid, 62·5 per cent. w/w; hydrated ferric chloride, 0·05 per cent. w/w, added as a 5 per cent. w/w solution in the phosphoric acid. The measurements were carried out with a spectrophotometer (Bleeker, Holland).

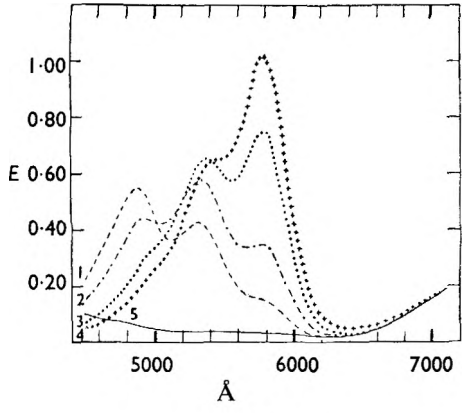

FIG. 2.

The extinctions were measured every 50 Å or, if necessary, every 25 Å. The spectral band width was 40 Å. For the estimation, 0·1600 mg. of gitoxigenin was mixed with 10 ml. of reagent and the absorption measurements were made in a 0·50 cm. cuvette against a blank of reagent only.

Figure 2 shows that at first (curve 1) a colour is formed with a maximal absorption at about 4900 Å. In curves 2 and 3 the maximum is altered, and the final result in curve 4 is that a peak is formed at 5800 Å. After a longer or shorter time, the colour begins to fade. In Figure 2 the absorption spectrum of the reaction product of 0·20 mg. digitoxigenin with the reagent is also reproduced (curve 5). This is quite different from curve 4.

McChesney, Nachod, Auerbach and Laquer[3], who used pure concentrated sulphuric acid as reagent, recommend a wavelength of 5250 Å.

The influence of the quantities of ferric chloride and of water also of the temperature was determined, and it was also ascertained for how long the colour keeps its maximal extinction value. Table I shows the influence of ferric chloride. Of the reagents examined the sulphuric acid and phosphoric acid concentrations have been kept constant and only the percentage of ferric chloride varied. To shorten the time required for

attaining the maximal extinction, the temperature at which the reaction occurs must be raised by heating in a water bath at 65° C.

Table I shows there is an optimal concentration of ferric chloride. As the proportion E_{git}/E_{dig} always is approximately 80, the concentration of 0·05 per cent. of hydrated ferric chloride can be recommended as the most favourable one.

TABLE I

INFLUENCE OF FERRIC CHLORIDE ON THE EXTINCTION

H_2SO_4 per cent.	H_3PO_4 per cent.	$FeCl_3.6H_2O$ per cent.	Time minutes	Temperature °C.	λ max. Å	$E_{0·1\ git.}$	$E_{0·4\ dig.}$	$E_{git.}/E_{dig.}$
37·5	62·5	—	30	65	5800	0·44	0·02	85
37·5	62·5	0·005	11	65	5800	0·61	0·03	80
37·5	62·5	0·050	9	65	5800	0·65	0·03	85
37·5	62·5	0·250	3	65	5800	0·58	0·03	80

In Table II the influence of water and of phosphoric acid on the reagent is shown. The quantities of ferric chloride and sulphuric acid were kept constant and the phosphoric acid was partly substituted by water.

TABLE II

INFLUENCE OF THE QUANTITY OF WATER ON THE EXTINCTION

H_2SO_4 per cent.	H_3PO_4 per cent.	$FeCl_3.6H_2O$ per cent.	H_2O per cent.	Time minutes	Temperature °C.	λ max. Å	$E_{0·1\ git.}$	$E_{0·4\ dig.}$	$E_{git.}/E_{dig.}$
37·5	62·5	0·050	—	9	65	5800	0·65	0·03	85
37·5	57·5	0·050	5	6	65	5800	0·625	0·035	70
37·5	52·5	0·050	10	7	65	5800	0·51	0·03	35
37·5	47·5	0·050	15	7	65	5800	0·37	0·07	20
37·5	42·5	0·050	20	8	65	5800	0·14	0·025	12

The extinction of the reaction product of 0·1 mg. of gitoxigenin with the reagent is continually reduced as the quantity of water is increased. The presence of a relatively small percentage of water only slightly affects the extinction. Small fluctuations in the percentage of water in the sulphuric and phosphoric acids used therefore have no significant influence on the application of the reagent.

It is demonstrated in Table III that the temperature plays an important part in the velocity of colour formation.

TABLE III

INFLUENCE OF TEMPERATURE ON THE VELOCITY OF THE REACTION

Time	Temperature °C.	λ max. Å	$E_{0·1\ git.}$
9 hours	20	5800	0·65
42·5 minutes	50	5800	0·64
25 "	55	5800	0·64
14 "	60	5800	0·63
9 "	65	5800	0·65

The time of reaction is much shortened by rise of temperature and small differences of temperature have a relatively great influence, Moreover, when the heating time is too short, the extinction reading is too

low, but a little extra heating time is without effect. It is therefore necessary to proceed very accurately regarding both time and temperature.

Finally, the length of time for which the extinction of the colour remains constant was investigated. Table IV shows that this is about 2 hours.

TABLE IV

INFLUENCE OF THE TIME ON THE EXTINCTION

Extinctions	0·57	0·57	0·57	0·57	0·57	0·57	0·57	0·57	0·56
Measured after minutes ..	5	15	30	45	60	75	90	105	120

CONCLUSION

For the estimation of gitoxigenin in presence of digitoxigenin concentrated sulphuric acid or a mixture of this acid and a small quantity of ferric chloride is less suitable than a reagent containing syrupy phosphoric acid. A concentration of 62·5 per cent. of phosphoric acid gives the best results. The ratio of the extinctions of equal quantities of gitoxigenin and digitoxigenin is in that case approximately 80. An optimal value of 0·050 per cent. was found for the concentration of the ferric chloride. Therefore a reagent of the composition given on page 477 is recommended. Addition of water to the reagent is not advantageous, though small quantities have but little influence. The reaction must be carried out at a raised temperature. As the duration of heating is dependent on the temperature, the heating must be always carried out in the same way.

The estimation is carried out as follows:—10 ml. of reagent is mixed with a quantity of gitoxigenin or of a mixture of gitoxigenin and digitoxigenin. When solution is complete the mixture is warmed for 9 minutes in a water bath at 65° C. The vessel is then cooled for 1 minute in running water and kept for another 5 minutes in water at room temperature. The absorption is measured in a 0·50-cm. cuvette against a blank of reagent only at a wavelength of 5800 Å. The spectral band width is 50 Å. As the peak at about 5800 Å is a very narrow one (Fig. 2), it is advisable to measure at 5750 Å with a broader spectral band width. The colour obtained remains constant for 2 hours.

The graph obtained by plotting the colour densities against gitoxigenin concentration between 0 and (at least) 1·6 mg. per cent. was a straight line; k (1 mg. per cent. of gitoxigenin) = 1·30. The reaction therefore obeys the Lambert–Beer law.

SUMMARY

1. A new reagent, consisting of a mixture of concentrated sulphuric acid, syrupy phosphoric acid and ferric chloride is recommended for the colorimetric estimation of gitoxigenin in presence of digitoxigenin. Various experiments showed that the most favourable results are achieved if the composition is the following:—concentrated sulphuric acid, 37·5 per cent. w/w; syrupy phosphoric acid (85 per cent.), 62·5 per cent. w/w; and hydrated ferric chloride, 0·050 per cent. w/w.

2. The red colour originating from the interaction of this reagent with gitoxigenin must be measured at 5750 Å.

3. The extinction of the colour obtained with gitoxigenin is approximately *80 times as great as that of an equal quantity of digitoxigenin* and keeps a constant value for at least 2 hours.

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THE PREPARATION OF TWO BIS-DIAZO DYES FOR INTRAVENOUS INJECTION

H. S. GRAINGER, THELMA CARR and G. F. SOMERS

From the Pharmaceutical Department, Westminster Hospital, London, S.W.1 and the School of Pharmacy, University of London

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TRYPAN red and vital red, two closely-related bis-diazo sulphonated dyes, originally prepared by Ehrlich, are used clinically for the estimation of blood volume and as spirochæticides. Trypan red has been shown to protect animals against herpes fibrilis, psittacosis and equine encephalomyelitis[1], and both trypan red and vital red are claimed to have a therapeutic value in the treatment of amyotrophic lateral sclerosis in humans. Apart from a statement by Aird[2], which mentions the use of Seitz filtration, we have found no information on methods of preparing solutions of these dyes for injection purposes. From a practical point of view, the pharmacist prefers a heat sterilisation procedure to methods involving filtration and aseptic distribution. It seemed important therefore to investigate whether these solutions could be autoclaved without destruction of the dyes or altering their toxicity. We have examined solutions of trypan red and vital red before and after autoclaving and compared them with solutions which have been sterilised by passing through a bacteria proof filter.

METHODS

Preliminary experiments indicated that 4 per cent. solutions of both dyes were required for the toxicity tests. Solutions were prepared by dissolving the dyes in water for injection with the aid of heat, followed by filtration through a No. 1 Whatman filter paper and then through a No. 3 sintered glass filter. This process was common to all solutions. Some were then autoclaved without further treatment, some were sterilised by passing through a 5/3 sintered glass filter and some were centrifuged before autoclaving. The concentration of dye in the final solutions was determined colorimetrically in a photoelectric colorimeter previously calibrated with a standard solution of the dye which had been clarified by centrifuging at 3,000 r.p.m. for 30 minutes. The *p*H values of the solutions were also measured before and after autoclaving in order to detect any possible decomposition involving the sulphonyl groups. The trypan red solution had *p*H 6·2 and the vital red solution *p*H 6·5.

The samples were tested for acute toxicity by the intravenous route in mice, the person doing the toxicity tests being unaware of the respective treatments the different solutions had received. Albino mice weighing between 16 and 24 g., which had been deprived of food overnight, were randomised into groups of 10 mice. The groups were injected intravenously at ascending dose levels with the solutions previously warmed to 37° C. and the mortalities observed over 24 hours, from which the LD50's together with estimates of their limits of error were calculated[3].

Results

The colorimetric determinations showed that there was no loss in colour through autoclaving and the *p*H values of the solutions remained constant. The solutions could therefore be safely autoclaved for 30 minutes, at 10 lb. without affecting the dye. Filtration of the solutions through a No. 5/3 sintered glass filter resulted in the loss of dye, due to adsorption on the glass filter bed. Solutions for the toxicity tests were therefore made on the strong side and adjusted to the correct strength before being sterilised. Similarly with centrifuging, a loss of dye occurred due to the removal of undissolved particles if insufficient time was allowed for the dye to go into solution. Solutions which had been passed through a No. 3 filter, but not through a 5/3 filter showed a very fine deposit of solid particles on the bottom of the ampoule after a week's storage. Solutions sterilised by 5/3 filtration and which had been centrifuged did not show this deposit. The deposit was of a dirty orange hue and was insoluble in water, dilute sodium hydroxide or dilute hydrochloric acid and may be presumed not to be undissolved dyestuff.

The results of the toxicity tests are shown in Tables I and II. The solutions of both trypan red and vital red showed no real difference in toxicity after the different treatments so that the solutions for therapeutic use could be sterilised by filtration or by heating in an autoclave.

TABLE I

Toxicity in mice of 4 per cent. trypan red solutions

Solution	LD50 ml./20 g.	Limits of error P = 0·95
1. Autoclaved	0·38	93 to 107 per cent.
2. Centrifuged before autoclaving	0·46	96 to 104 ,,
3. Filtered through 5/3 sintered glass	0·40	95 to 105 ,,
4. Filtered through 5/3 sintered glass before autoclaving	0·42	95 to 105 ,,

TABLE II

Toxicity in mice of 4 per cent. vital red solutions

Solution	LD50 ml./20 g.	Limits of error P = 0·95
1. Autoclaved	0·37	89 to 111 per cent.
2. Centrifuged before autoclaving	0·36	96 to 104 ,,
3. Filtered through 5/3 sintered glass	0·38	92 to 108 per cent.

Discussion and Conclusions

The solutions used in these investigations were at a concentration of 4 per cent. though they are normally used clinically at a concentration of 1 per cent. The weaker solution is easier to prepare, but we strongly advise filtration through a No. 5/3 sintered glass filter to ensure the absence of subsequent deposition of solid particles. Loss of dye is liable to occur during filtration procedures due to adsorption on the glass and this should be allowed for by making the initial solutions on the strong side and adjusting the final concentration from the result of colorimetric estimations before the final ampouling. For small volumes centrifuging

provides an alternative method of clarification but again losses of dye can occur if complete solution is not ensured. Final sterlisation can be carried out in the sealed ampoules by autoclaving at 10 lb. for 30 minutes without destruction of the dye or an increase in toxicity.

Summary

1. Aqueous solutions of vital red and trypan red can be sterilised by autoclaving for 30 minutes at 10 lb. without a change in colour or an increase in toxicity.
2. Solutions should be clarified by filtration through a No. 5/3 sintered glass filter or by centrifuging.
3. Loss of dye can occur through incomplete solution or by adsorption to the glass filter.
4. The concentration of the dye in the final solution should be determined colorimetrically and suitably adjusted prior to autoclaving in the final containers.

References

1. Wilson-Hurst, Peters and Melvin, *Brit. J. Pharmacol.*, 1952, 7, 455.
2. Aird, *Arch. Neurol and Psych.*, 1948, **59**, 779.
3. Finney, *Probit Analysis*, 2nd Ed., Cambridge University Press, 1952.

Correction.

THE FAILURE OF BROMAZINE HYDROCHLORIDE TO AFFECT THE OUTPUT AND COMPOSITION OF RESPIRATORY TRACT FLUID

By Eldon M. Boyd and R. Noreen Hicks

This Journal, 1954, **6**, 43.

Fig. 1, p. 47. Values for chloride content of respiratory tract fluid shown in the Figure must be multiplied by 0·176 to obtain the correct concentrations.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Erythromycin, Infra-red Determination of. W. H. Washburn. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 48.) Samples were assayed by determining the absorption of a chloroform solution containing about 30 mg. of erythromycin per ml. Absorption bands having maxima at 7·29, 9·02, 9·88 and 10·46 μ were investigated, and the results obtained from measurements at 10·46 μ approached most closely a straight line relationship between concentration and absorption, and gave results agreeing most consistently with the microbiological assays, using *B. subtilis* as test organism. The results, calculated from a standard curve prepared from measurements on pure erythromycin, were reproducible within 1 per cent. G. B.

Essential Oils, Oxidimetric Determination of, in Drugs. H. Flück and F. Hoffman. (*Sci. Pharm.*, 1953, **21**, 3–8.) The method is based on distillation of the drug with brine, and heating the distillate with chromic acid-sulphuric acid in a sealed tube, followed by titration of the excess of chromate. The factor used must be obtained by experiments with known quantities of the essential oil in question. The method is carried out on quantities of 0·05 to 1 g. of the drug. The authors treat in detail the various factors involved in the light of over 3000 determinations which they have carried out. Owing to the range of the results (up to ± 12 per cent.) the method is not suitable for standardisation of drugs, but is valuable when only a small quantity of material is available. G. M.

Morphine and Codeine, Spectrophotometric Determination of. W. A. Clarke and A. J. McBay. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 39.) Quantitative determinations of codeine and morphine may be carried out rapidly by measuring the ultra-violet absorption at 285 mμ of solutions of *p*H 2. Alternatively, solutions of *p*H 12 may be employed, measurements being made at 298 mμ for morphine or 284 mμ for codeine. Results calculated from absorption data are as accurate as those obtained by using a standard preparation and making a calibration curve. The shift of the absorption maximum of morphine from 285 mμ in acid solutions to longer wavelengths in the presence of alkali serves to distinguish morphine from codeine, the ratio absorption 285 mμ/absorption 310 mμ being greater than 1 for codeine and less for morphine, and the proportion of the alkaloids may be calculated by using 2-component system equations.
G. B.

Phenobarbitone Preparations, Ultra-violet Spectrophotometric Determination of. L. N. Mattson. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 22.) The determination depends on measurements of the optical density of solutions in borate buffer, *p*H 9·5, at 240 mμ and calculation of the quantity of phenobarbitone from the datum $E^{1\ \text{per cent.}}_{1\ \text{cm.}} = 459$. Tablets were powdered, shaken with buffer solution, allowed to stand and filtered. An aliquot quantity of the filtrate was further diluted and the optical density measured against a blank consisting of buffer solution. The method was found to

be rapid and to give results in satisfactory agreement with the U.S.P. method. Stearates did not interfere. Elixir of phenobarbitone was assayed by determination of the absorption at 240 mμ, using a blank of elixir base to compensate for the absorption due to amaranth. For the assay of capsules of ephedrine sulphate and phenobarbitone, the sample was dissolved in a 2 per cent. solution of sodium hydroxide, the ephedrine being extracted with ether and titrated with sulphuric acid. Since the alkali changes the absorption characteristics of phenobarbitone, results calculated from the optical density of the alkaline solution of phenobarbitone were erroneous. More accurate results were obtained by dissolving a sample in borate buffer, measuring the absorption at 240 mμ and correcting for the absorption due to ephedrine sulphate, calculated from the value $E^{1\text{ per cent.}}_{1\text{ cm.}} = 3{\cdot}32$.

G. B.

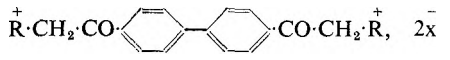
Total Nitrogen, Estimation of, using the Conway Micro-diffusion Cell. E. I. Short. (*J. clin. Path.*, 1954, **7**, 81.) Satisfactory recoveries are achieved from urea solution, serum and urine, with samples as small as 0·05 ml. The sample is heated gently for 5 minutes with 0·5 g. of potassium sulphate, 0·5 ml. of sulphuric acid and 0·03 ml. of a saturated solution of copper sulphate, allowed to cool and 0·3 ml. of a 0·06 per cent. solution of selenium dioxide added. The mixture is boiled gently for 1 hour and 0·375 ml. of a 40 per cent. solution of sodium hydroxide is added and water to 10 ml.; 0·5 or 1·0 ml. of this solution is placed in the outer compartment of a Conway micro-diffusion cell, the inner compartment of which contains 2·0 ml. of a solution prepared by dissolving 5 g. of boric acid in a mixture of 200 ml. of water and 10 ml. of ethanol, adding methylene blue/methyl red indicator solution and diluting to 1000 ml. with water. The lid, coated with white soft paraffin, is placed on the dish, 1·0 ml. of a saturated solution of potassium metaborate added to the outer compartment through a small aperture which is immediately closed and the liquids in the outer compartment mixed by gentle rotation. After a minimum period of 2 hours, required for complete absorption of the liberated ammonia by the boric acid solution, the liquid in the central compartment is titrated with 0·02N hydrochloric acid, to a faint but definite pink colour, using a micrometer syringe which delivers 0·001 ml. with an accuracy of ± 0·00005 ml. G. B.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

***Digitalis purpurea* and *Digitalis lutea* L., Effect of Freeze-drying on the Glycosidal Content of.** F. P. Cosgrove and E. P. Guth. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 90.) Leaves were collected from first and second year plants of *Digitalis purpurea* and second and third year *D. lutea* and dried either at 50° C. for 36 hours or from the frozen state before being ground to powder. The drying methods yielded powders of comparable moisture content. With the exception of those derived from second year plants of *D. purpurea*, freeze-dried samples showed a lower content of total glycosides than oven-dried samples when tested by a modified Knudson-Dresbach colorimetric method. There was no difference in biological activity between oven- and freeze-dried samples as assessed by the frog heart method. It is suggested that heat and enzymatic action break down primary glycosides into secondary glycosides and aglycones during oven drying, the latter giving rise to more colour in the Knudson-Dresbach method. G. B.

ORGANIC CHEMISTRY

Cholinesterase Inhibitors, A New Series of. J. P. Long and F. W. Schueler. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 79.) A series of 11 diphenylamine derivatives was prepared, of the type indicated below, where R contains a quaternary ammonium group and x is Cl, Br or I.



The majority of the compounds were obtained by dissolving $\alpha\alpha'$-dibromo-4:4'-biacetophenone in boiling dioxan, adding the required tertiary amine, allowing to stand for 30 minutes, recrystallising and drying *in vacuo*. The pyridyl and α- and β-methyl pyridyl compounds were as potent inhibitors of bovine erythrocyte cholinesterase as neostigmine bromide (manometric method). Gut stimulation parallelled the *in vitro* cholinesterase inhibiting activity. The more potent cholinesterase inhibitors produced marked potentiation of the depressor response to acetylcholine. Since the derivatives varied widely in activity, a potent anticholinesterase (R = pyridyl), a compound of low potency (R = dimethyl-β-hydroxyethylammonium) and a substance of intermediate activity (R = *N*-ethylpyridyl) were selected for testing on the isolated heart. In this case the potentiation of acetylcholine appeared to run parallel to *in vitro* anticholinesterase activity. G. B.

Streptothricin. Preparation, Properties and Hydrolysis Products. H. E. Carter, R. K. Clarke, Jr., P. Kohn, J. W. Rothrock, W. R. Taylor, C. A. West, G. B. Whitfield and W. G. Jackson. (*J. Amer. chem. Soc.*, 1954, **76**, 566.) Streptothricin is a basic antibiotic with an empirical formula of about $C_{20}H_{34}N_8O_9$ possessing high antibacterial activity. It is tribasic and titration data indicate the presence of three groups with *p*Ka' values of 7·1, 8·2 and 10·1. Analysis disclosed the absence of *O*-methyl, *C*-methyl and *N*-methyl groups. Streptothricin gave positive Pauly, biuret and ninhydrin tests, and amino groups were liberated during hydrolysis, indicating the probability of a peptide structure. The products of acid and alkaline hydrolysis were investigated. On hydrolysis, it yielded ammonia, carbon dioxide and three ninhydrin-positive components, none of which gave significant ninhydrin-carbon dioxide values. One of these products was identified as β:ϵ-diaminocaproic acid. A large scale carbon chromatogram is described which conveniently affords pure sulphates or hydrochlorides of streptothricin or streptomycin. A. H. B.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

***Streptomyces erythreus*. Isolation of a Second Crystalline Antibiotic from.** C. W. Pettinga, W. M. Stark and F. R. Van Abeele. (*J. Amer. chem. Soc.*, 1954, **76**, 569.) The application of paper chromatographic methods to aid in the isolation and fractionation of erythromycin gave evidence that more than one antibiotic substance could be produced by certain strains of *Streptomyces erythreus*. When grown on a variety of media, these strains were shown to produce erythromycin B in addition to erythromycin. The isolation of erythromycin B was accomplished by use of chromatography on powdered cellulose and countercurrent distribution. It is similar to erythromycin in most of its physical and

chemical properties. Electrometric titration in 66 per cent. dimethylformamide-water showed a titratable group with a *p*Ka′ of 8·5. The molecular weight is 736± 36; specific rotation at 25° C. (2 per cent. in ethanol) was − 78° and m.pt. 191 to 195° C. The infra-red absorption spectrum in methanol is recorded. The microbiological spectrum of erythromycin B is similar to that of erythromycin; however, erythromycin B is only about 75 to 80 per cent. as active. A. H. B.

BIOCHEMICAL ANALYSIS

Ascorbic Acid and Trace Elements, The Effect of, on Vitamin B_{12} Assays. E. M. Stapert, E. B. Ferrer and L. Stubberfield. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 87.) Solutions of cyanocobalamin (vitamin B_{12}) were mixed with ascorbic acid, traces of copper, etc., and assayed microbiologically by a modification of the U.S.P. method. The results were confirmed by saturating the solutions of vitamin B_{12} with ammonium sulphate, extracting with *n*-butanol, separating the butanol solution, extracting with water and calculating the vitamin B_{12} content from the absorption at 361 mμ. None of the added substances alone affected the potency of vitamin B_{12}, but combinations of ascorbic acid with traces of copper, molybdate, fluoride or manganous ions caused some loss of potency. Copper was effective at a concentration of 0·1 p.p.m. and the loss was greater in solutions containing an appreciable proportion of hydroxocobalamin (vitamin B_{12b}). Potassium cyanide and sodium diethyldithiocarbamate, which form complexes with copper, prevented the destruction of vitamin B_{12} activity. G. B.

Catechol Amines in Suprarenal Glands, Estimation of. R. Cass and G. B. West. (*Arch. int. Pharmacodyn.*, 1953, **95**, 283.) Extraction of catechol amines from guinea-pig suprarenals by three methods (acidulated ethanol, 5 per cent. trichloracetic acid, or 0·01 N hydrochloric acid) and estimation biologically, colorimetrically and chromatographically yield values for the total catechol amine content of 121 μg./g. of suprarenal weight. Of this only 3 per cent. is noradrenaline. Both estimates are considerably less than those found by von Euler and Hokfelt (*Brit. J. Pharmacol.*, 1953, **8**, 66) and suggest a real difference between British and Swedish guinea-pigs. It was found that the total catechol amines could be suitably estimated biologically by all three extraction methods. Chromatographic estimation could be used for adrenaline estimation in hydrochloric acid extracts or noradrenaline estimation on concentrated acid ethanol extracts. The noradrenaline content of the concentrated acid ethanol extracts could also be estimated colorimetrically. Hydroxytyramine, dihydroxyphenylalanine, dihydroxyphenylserine, *p*- and *m*-hydroxyphenyl-ethanolamine and tyramine were not detected in any extract. G. P.

Iron and Copper in Serum, Determination of. S. Ventura and J. C. White. (*Analyst*, 1954, **79**, 39.) A method is described for the determination of iron and copper in single samples of serum; the metals are liberated from the proteins with 6 N hydrochloric acid and are determined photometrically as the 2:2′-dipyridyl-ferrous complex and as copper diethyldithiocarbamate, the latter being extracted into a mixture of ether and amyl alcohol. Details of the various stages, including the effect of hydrochloric acid concentration, the precipitation and separation of the proteins, and the formation of the iron and copper complexes are given. Recoveries of iron added to serum ranged from 96·9 to 101·0 per cent.; in the case of copper 93·0 to 101·0 per cent. recovery was obtained.

R. E. S.

CHEMOTHERAPY

Ganglionic Blocking Agent, Synthesis and Evaluation of a New. F. W. Schueler and H. H. Keasling. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 98.) β-Bromopropionyl chloride was heated with ethylene bromohydrin under a reflux condenser and the product, after allowing to stand overnight and removing hydrogen chloride by means of a current of air, was purified by distillation *in vacuo*, and allowed to stand with a large excess of triethylamine. β-Triethylammonium-(β′-trimethylammoniumethyl) propionate dibromide, $(C_2H_5)_3\overset{+}{N}\cdot CH_2\cdot CH_2\cdot CO\cdot OCH_2\cdot CH_2\cdot\overset{+}{N}(C_2H_5)_3$, $2\overset{-}{Br}$, was filtered off and air dried until free from the odour of ether or triethylamine. The substance shows some chemical resemblance to both hexamethonium and muscarinic compounds and might be expected to act as a ganglionic blocking agent, free from side effects such as constipation and mydriasis due to depression of parasympathetic tone. In pharmacological experiments, the substance antagonised ganglion stimulation due to tetramethylammonium or to acetylcholine liberated through reflex stimulation, and did not depress heart rate or amplitude nor affect the coronary flow. Depression of the contraction of isolated intestine or of responsiveness to acetylcholinic stimulation was not observed. The muscarine-like activity seemed to be only sufficient to overcome the loss in parasympathetic tone which accompanied the ganglion-blocking properties. G. B.

PHARMACY

NOTES AND FORMULÆ

Neomycin Sulfate (*New and Nonofficial Remedies, J. Amer. med. Ass.*, 1954, **154**, 338.) Neomycin is an antibiotic isolated from culture media upon which *Streptomyces fradiæ* has grown. It is not inactivated by exudates, enzymes or gastro-intestinal secretions. Neomycin sulphate is a thermostable, polybasic compound soluble in water but insoluble in organic solvents. It is stable for at least 2 years at room temperature. Solutions retain their potency for at least one year at room temperature although the colour may darken; storage in refrigerators is recommended. Neomycin sulphate is very stable and very active in alkaline solution. It is active against Gram-positive and Gram-negative organisms. Of the former it appears more active against staphylococci than streptococci. It has a wider range of activity than bacitracin, penicillin, or streptomycin, and is sometimes effective against pseudomonas and proteus infections; resistant strains have not yet been demonstrated clinically. It is used locally as an ointment or a solution in the treatment or prevention of infections of the skin and eye, including impetigo, wounds, burns, ulcers, conjunctivitis, blepharitis and sty. Local therapy may be supplemented by administration of sulphonamides or penicillin. Blood levels of 0·2 mg. per ml. or more may produce serious kidney damage. Neomycin should not, therefore, be given parenterally or in high and prolonged oral dosage. It may be used in divided total daily oral dosage not exceeding 6 to 10 g. for one to three days for suppression of the bacterial flora in surgery of the large colon and anus. It has a mild laxative effect after oral administration but is otherwise usually well tolerated. G. R. K.

PHARMACOGNOSY

***Digitalis orientalis*, A Preliminary Phytochemical Investigation of.** D. H. Gregg and O. Gisvold. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 106.) An aqueous extract of the leaves of *Digitalis orientalis* was treated with methyl *iso*butyl ketone and the extract separated into ether-insoluble and ether-soluble fractions. The ether-soluble material was separated by chromatography on an aluminium oxide column, using chloroform, chloroform/ethanol mixture and ethanol as solvents. Acetyldigoxin, amorphous *digitalinum verum* and other amorphous glycosides were obtained from the various fractions. Solvent mixtures were devised for the separation of digoxin, acetyldigoxin and lanatoside C by ascending or descending paper chromatography, but no satisfactory separation of digitoxin, acetyldigitoxin and acetyldigoxin was effected. A suitable test for glycosides may be carried out as follows. Dry the glycosical material on filter-paper, spray with a 1 per cent. solution of hydrogen chloride in methanol to which has been added 0·5 ml. of a 10 per cent. ferric chloride solution per 100 ml. Dry rapidly under an infra-red lamp, under conditions which avoid charring of the paper. A blue-grey colour indicates the presence of glycosides. This test detects the sugar moiety, giving a positive result for digitoxose, sucrose, fructose and sorbose, but if the genin is present the spot fluoresces in ultra-violet radiation. The fluorescence test is sensitive to about 0·6 μg. of glycosides. By the application of a periodate-benzidine test it was deduced that the glucose residue of lanatoside C and the terminal glucose digitoxose residue of digitoxin, digoxin and gitalin are in the pyranose form and that the acetyl group in the cardiac glycosides of digitalis is on the terminal digitoxose residue. G. B.

PHARMACOLOGY AND THERAPEUTICS

Adrenal Steroids, Corticotrophin and Growth Hormone, Effect of, on Resistance to Experimental Infections. E. H. Kass, M. M. Lundgren and M. Finland. (*J. exp. Med.*, 1954, **99**, 89.) Large doses of adrenocorticotrophin or of cortisone depress resistance to infections. Experiments are reported on the survival of mice injected with a blood culture of a mouse virulent strain of pneumococcus or with mouse adapted influenza A virus and treated with cortical hormones. Cortisone, in doses of 5 mg. for 5 days, diminished the LD50 of pneumococci about tenfold. Administration of a single dose of 5 mg. depressed resistance significantly, and the effect persisted between 3 and 6 days suggesting a slow rate of absorption. Corticotrophin, in doses from 0·3 to 20 mg., had no significant effect on resistance or survival time. Hydrocortisone in the form of free alcohol depressed resistance, but the effects of the acetate were variable, probably due to variable rates of hydrolysis in the tissues. Growth hormone in large doses acted like cortisone and when given with cortisone did not overcome the effect. Similar results were obtained with influenza virus, cortisone and hydrocortisone depressed resistance while corticosterone had possibly no effect. The rate of multiplication of viral particles in infected mouse lungs, when determined by titration in embryonated eggs, showed a rapid multiplication during the first 2 days followed by a distinct fall by the sixth day. The cortical hormones did not alter the rate of accumulation of the virus, but the decline in the titre values was slower than with the controls. G. F. S.

Aminopentamide, Pharmacological Activity of. J. B. Hoekstra, D. E. Tisch, N. Rakieten and H. L. Dickison. (*J. Pharmacol.*, 1954, **110**, 55.) Aminopentamide, DL-αα-diphenyl-α-dimethylaminovaleramide (centrine), is a potent antispasmodic with atropine-like properties. On the isolated guinea-pig and rabbit ileum strips it has approximately half the activity of atropine and 1/5 the activity of papaverine in relaxing spasms induced by acetylcholine, barium chloride and histamine. In dogs, it decreases the tone and activity of the stomach and intestinal tract and relaxes spasms produced by acetylcholine, arecoline and pilocarpine. It does not completely block the action of histamine. Aminopentamide is nearly as effective orally as intravenously or intramuscularly. On the colon of the normal dog it is more effective and of longer duration than either atropine or banthine. Contractions of the bladder are also reduced and the spasmogenic actions of histamine arecoline and acetylcholine blocked. It is slightly less active than atropine in blocking the vasodepressor responses to acetylcholine and arecoline, and only 1/4 as active as atropine in blocking the effects of vagal stimulation of the heart. Aminopentamide has less mydriatic effect and is less effective than atropine in antagonising the stimulant action of pilocarpine on salivary flow. Daily doses up to 50 mg. have been given to dogs over 2 months without producing any gross or microscopic abnormalities.

G. F. S.

Atropine and Methylatropine, Ganglionic Blocking Action of. L. D. Fink and P. Cervoni. (*J. Pharmacol.*, 1953, **109**, 372.) Methylatropine, a quaternary nitrogen compound, has been compared with atropine and tetraethylammonium for ganglionic blocking activity. Experiments were carried out in anæsthetised cats, transmission through the cervical ganglion following preganglionic stimulation being determined by measuring the response of the nictitating membrane. The drugs were given intravenously and the dose determined which caused a 50 per cent. decrease in the contraction of the nictitating membrane. In 5 experiments this dose was 0·19 mg./kg. for methylatropine, and 1·0 mg./kg. for tetraethylammonium, methylatropine being therefore approximately 5½ times as active as tetraethylammonium on a weight basis and approximately 8 times as active on a molar basis. The duration of action was from 3 to 6 minutes for each compound. With atropine large doses reduced the response to postganglionic stimulation and corrections were necessary for this. It was found that 3·9 mg./kg. of atropine was equal to 0·8 mg./kg. of tetraethylammonium or, atropine had one-fifth the activity of tetraethylammonium and one-fifteenth to one-twentieth the activity of methylatropine. Methylatropine, tetraethylammonium and atropine blocked the carotid sinus vasopressor reflex produced by bilateral occlusion of the common carotid arteries, and also from the ganglionic stimulating drug 1 : 1-dimethyl-4-phenylpiperazium iodide. Again methylatropine was the most potent and atropine the least potent compound.

G. F. S.

***Caulophyllum thalictroides*, a Pharmacological Study of a Crystalline Glycoside of.** H. C. Ferguson and L. D. Edwards. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 16.) Oil was removed from powdered rhizomes and roots of *Caulophyllum thalictroides* (blue cohosh) by extraction with ether, and the residue extracted with hot ethanol. The solution was evaporated to a reddish-brown extract which was mixed with pumice, sand and water and filtered to extract alkaloids. The residue, dissolved in ethanol was decolourised with charcoal and the solution evaporated. The product was treated with cold dehydrated ethanol to remove impurities and recrystallised from ethanol to give white crystals of a

glycoside, melting point 255° to 256°C. An aqueous solution, prepared with the aid of sodium hydroxide was used for pharmacological tests. It was shown to cause construction of the coronary vessels of the rat's heart and of the carotid arteries of cattle and hogs. The substance exhibited a spasmogenic action on the isolated intestine of the rat, guinea-pig, mouse and rabbit and a pituitary-like effect on the rat uterus. Concentrations greater than 0·01 per cent. caused hæmolysis in the rabbit. The LD50 by intravenous injection was 11·8 mg./kg. for mice and 20·3 mg./kg. for rats. The substance was very irritant when injected into the ear or instilled into the cornea of the rabbit. G. B.

Chloramphenicol, Blood Dyscrasias Associated with. R. Hodgkinson. (*Lancet*, 1954, **266**, 285.) Details are given of 31 instances of blood dyscrasias after the administration of chloramphenicol, of which 28 were aplastic anæmia and 3 granulocytopenia. In 26 of the 28 patients with aplastic anæmia there were hæmorrhagic manifestations such as severe epistaxis, hæmoptysis, bleeding from the gums, hæmatemesis, spontaneous bruising, and petechial hæmorrhages. Reduction of the red-cell, white-cell and platelet counts occurred; in 22 persons the sternal marrow was examined and showed scanty red-cell and white-cell precursors with an increased proportion of lymphocytes. 24 of the 31 instances have so far proved fatal, 4 have recovered and 3 are being maintained by blood transfusions, The immediate cause of death in many cases was internal hæmorrhage; liver damage, except for fatty degeneration in 2 persons, was mentioned in only one post-mortem report. A heavy total dose for an adult, assuming treatment for a week, would be 26 g. Of 19 adults in the series, 16 received a total of more than 26 g. and the average quantity taken by the 14 patients whose total dose is known was more than 60 g. 3 received less than 26 g., one receiving only 1 g. of chloramphenicol and 75 mg. of pyribenzamine one week before the onset of aplastic anæmia. Of the 11 children in the series, 5 received more than double the maximum total dose that should be given having regard to age. In 8 of the 11 treatment was maintained for more than 24 days. The author suggests that chloramphenicol should not be given for chronic conditions, that the total dosage should not exceed 26 g. in adults or the equivalent of 100 mg./kg. of body weight daily for 7 days in children, and that the length of treatment should not exceed 10 days. H. T. .B.

Chlorpromazine, Anti-emetic Activity of. E. D. Brand, T. D. Harris, H. L. Borison and L. S. Goodman. (*J. Pharmacol.*, 1954, **110**, 86.) Chlorpromazine, 10-(γ-dimethylaminopropyl)-2-chlorophenothiazine, is a new drug related to promethazine, but it shows no antihistamine properties and is used for sedation. In dogs it has been found to be effective in antagonising apomorphine-induced vomiting, while promethazine is inactive. It protects against vomiting induced by morphine and ergot, but it is ineffective against intravenous copper sulphate, lanatoside-C, veratrum and oral copper sulphate. In cats chlorpromazine does not prevent vomiting induced by apomorphine, lanatoside-C and intravenous copper sulphate. In dogs therefore it blocks only drugs affecting the medullary emetic chemoreceptor trigger-zone, whereas it does not in cats. The results appear to be due to a species difference. G. F. S.

Chlorpromazine, Mechanism of Therapeutic Action. P. Decourt. (*Thérapie*, 1953, **8**, 846.) Chlorpromazine has two important pharmacological actions, an adrenergic blocking action and a "narcobiotic" action (this term being introduced by the author to embrace the direct depressant effects of the drug

on cellular metabolism). Comparative pharmacology on the one hand and analysis of the multiple effects of the drug in mammals on the other show this narcobiotic action to be the one mainly—perhaps exclusively—concerned in the therapeutic action of chlorpromazine. The comparative studies were carried out on the seed of a higher plant, *Lepidium sativum*, on a unicellular organism of the genus *Infusoria*, *Tetrahymena piriformis*, on a microscopic fungus, *Sterigmatocystis nigra*, and on some *Streptococci*. In each case there was a striking correlation between the narcobiotic action of members of the phenothiazine group of drugs and their relative central depressant action in man. Similarly 933F (piperoxane) and 883F (prosympal) which have an adrenergic blocking action equal to that of chlorpromazine, have a much feebler narcobiotic action. Conversion of the tertiary base to the corresponding quaternary compound greatly diminishes the narcobiotic action. The link between the lower organisms, where the narcobiotic effects were measured by their immersion in a solution of the drug, and mammals, where the drug was injected, was provided by the dogfish, *Scyllium canicula*. Here the drug was administered by both means and results comparable with both groups obtained. In higher animals such as the mammal where central co-ordination involves a great number of cellular elements, even a slight narcobiotic effect can be sufficient to involve complex nervous networks such as the reticular formation and the hypothalamic centres in what appears at first sight to be a selective depression. In actual fact the fractional depression of each neurone summates with its successors to raise the excitatory threshold. This explains why conditioned reflexes are more easily depressed by chlorpromazine than are simple reflexes. The narcobiotic action is suggested as being related to an anti-diastatic action, but evidence is not conclusive. G. P.

Chlorpromazine, Studies on the Adrenergic Blocking Properties of. P. Marquardt. (*Thérapie*, 1953, **8**, 787.) In the cat, chlorpromazine in a dose sufficient to cause complete reversal of the vasopressor response to intravenous adrenaline, has little effect on the action of noradrenaline. The reversal of adrenaline is one criterion of the degree of activity of a drug to be used in "hibernation anæsthesia" since this condition appears to depend mainly on a blocking of the emergency sympathetic reflexes recruited in the body during the induced hypothermia. The ideal dose of chlorpromazine in man would be one where there is blockade of the adrenaline vasopressor response without reversal. Where there is reversal of the response there is an acute danger of circulatory collapse. G. P.

Cortisone Acetate and *p*-Aminobenzoic Acid in Long Term Treatment of Rheumatoid Arthritis. L. L. Wiesel and A. S. Barritt. (*Amer. J. med. Sci.*, 1954, **227**, 74.) The synergistic action of sodium or potassium *p*-aminobenzoate and cortisone acetate, both compounds being given orally, was investigated in 31 patients during periods of a year or more. 23 of the patients were given large doses of cortisone acetate alone for 1 to 2 weeks at the commencement of treatment after which the dose was reduced to 37·5 mg. daily in 3 divided doses. As soon as relapse was obvious, 30 to 45 ml. of a 10 per cent. solution of sodium or potassium *p*-aminobenzoate was given 1 hour before each dose of cortisone. In 22/23 patients this combined treatment was as effective as the large doses of cortisone acetate, while in the other patient improvement was much greater on cortisone alone. The remaining 8 patients were given the combined treatment only and their improvement was satisfactory. 2 patients developed a toxic drug

rash due to the *p*-aminobenzoate after 12 and 13 months respectively and treatment had to be discontinued. Some gain in weight occurred but the age-height-weight ratios remained within normal limits and, rarely, there was a mild rounding of the face, but no other signs of hypercorticoidism were encountered; no euphoria occurred. Patients have been maintained on the combined treatment for periods up to 3 years without any of the serious side effects of cortisone becoming apparent. In severe cases maximal improvement was not reached until treatment had continued for 60 to 90 days. The cause of the synergism is under investigation; it appears to be connected with the inhibiting effect of *p*-aminobenzoic acid on the destruction of cortisone by liver tissue. H. T. B.

Diphenhydramine, A New Group of Sedatives Related to. W. Weidmann and P. V. Petersen. (*J. Pharmacol.*, 1953, **108**, 201.) A series of 37 compounds, closely related to diphenhydramine, have been studied for sedative, convulsant, spasmolytic and toxic properties. Sedative action was determined in mice, the movements of which were measured in an activity cage and the dose producing sedation in 50 per cent. of the animals (SD50) was determined. Convulsant action and toxicity were also determined in mice, and spasmolytic action on the isolated guinea-pig ileum. For the antihistamine antagonists the SD50 could be determined only for promethazine, since diphenhydramine mepyramine and tripelennamine showed only excitatory actions which apparently masked any sedative actions. The compounds showed qualitatively similar properties to diphenhydramine but quantitatively there were considerable differences. They showed a considerable loss of antihistamine activity *in vitro*, stronger sedative actions, and much stronger spasmolytic activities against barium chloride. A comparison between the sedative and spasmolytic effects showed no systematic relationship between the members of the series and there was no relationship between the sedative effect and histamine antagonism. G. F. S.

Disodium Calcium Ethylenediamine Tetra-acetate, Enhancement of Lead Excretion by. M. Rubin, S. Gignac, S. P. Bessman and E. L. Belknap. (*Science*, 1953, **117**, 659.) Ethylenediamine tetra-acetic acid (versene), a synthetic chelating agent, forms strong un-ionised soluble chelate complexes with cations, especially those of the di- and trivalent types. In animals and humans, when administered intravenously in large doses over short periods, it lowers the systemic calcium level, forming a calcium chelate. The calcium in this complex may be displaced by other metals and is non-toxic. This paper reports that the administration of the calcium complex causes a marked enhancement of lead excretion in patients with acute lead poisoning. G. F. S.

Isoniazid; Tubercle Bacilli Resistant to. D. A. Mitchison. (*Brit. med. J.*, 1954, **1**, 128.) Eight pairs of sensitive and resistant strains of tubercle bacilli were obtained from the sputum of 8 patients in the Medical Research Council (1952) trials of isoniazid. The sensitive strains were obtained before treatment with isoniazid, and the resistant strains were obtained during or up to 3 months after treatment with isoniazid. The virulence and response to treatment of these strains were then investigated on guinea-pigs. It was found that the higher the degree of resistance of a strain the lower was the virulence to guinea-pigs. Treatment with isoniazid in a dose comparable to that used in man (i.e. 4 mg./kg./day by subcutaneous injection) protected animals infected with sensitive strains, and partially protected animals infected with a resistant strain growing on Lowenstein medium containing isoniazid 0·2 μg./ml. but not on

1 μg./ml. It did not protect animals infected with resistant strains growing on 1 μg./ml. and, indeed, may have increased the virulence of some of these strains. S. L. W.

Nalorphine Hydrobromide, Treatment of Acute Heroin Intoxication. M. Strober. (*J. Amer. med. Ass.*, 1954, **154**, 327.) A 22-year-old mulatto woman suffering from the effects of an unknown overdose of heroin was comatose on admission to hospital, with respiratory arrest and pin-point pupils. She was promptly placed in a respirator but no improvement occurred. The patient appeared lifeless. She was then given 5 mg. of nalorphine intravenously. Within one minute she began to breathe, her pupils dilated, and she rapidly became conscious and sat up. She then became belligerent. G. R. K.

α-(2-Piperidyl)benzhydrol Hydrochloride, A New Central Stimulant, Pharmacological Studies on. B. B. Brown and H. W. Werner. (*J. Pharmacol.*, 1954, **110**, 180.) Central stimulation by α-(2-piperidyl)benzhydrol hydrochloride is characterised with small doses by a marked reduction in reaction times to environmental stimuli and by continuous very rapid, but highly co-ordinated movements in experimental animals. With increasing doses duration of hyperactivity is increased more than is the degree of activity. Lethal intravenous doses and oral doses cause tremors and convulsions, and death occurs by respiratory depression during convulsions, with the exception of oral administration in dogs and subcutaneous administration in rats, where death occurs suddenly in the state of hyperactivity. The new stimulant differs from amphetamine in inducing hyperactivity without signs of increased irritability and anorexia. Also toxic doses of amphetamine cause depression after first convulsing; this depression was absent after convulsions with the new drug. The drug antagonises light to moderate degrees of depression with barbiturates, but not lethal barbiturate depression which in some cases was increased so that death occurred earlier. Here it again differs from amphetamine which has little analeptic action with light degrees of barbiturate depression and from leptazol which antagonises lethal barbiturate depression. Also, in contrast to leptazol and amphetamine, lethal doses of the new stimulant are not antagonised to any appreciable extent by the barbiturates. The drug has little vasopressor activity. In the low decerebrate cat there is a stimulation of the righting reflexes. G. P.

Primidone (Mysoline) and Phenobarbitone, Effects of Liver Damage and Nephrectomy on Anticonvulsant Activity of. E. W. Swinyard, D. H. Tedeschi and L. S. Goodman. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 114.) Tests for anticonvulsant potency were carried out by the method of maximal electroshock seizures in rats. The anticonvulsants were administered orally as a 10 per cent. suspension in gum acacia solution, so as to permit tests to be carried out at the time of maximum liver damage, 36 to 48 hours after the injection subcutaneously of 2 ml./kg. of a 50 per cent. solution of carbon tetrachloride in arachis oil, or 12 hours after nephrectomy. Liver damage increased the anticonvulsive potency by an average of 50·7 per cent. for primidone (5-phenyl-5-ethylhexahydropyrimidine-4 : 6-dione) and 63·7 per cent. for phenobarbitone, and increased the duration of action fourfold for primidone and threefold for phenobarbitone. Nephrectomy markedly increased the effect and duration of action of primidone, but did not significantly alter the effect of phenobarbitone. It is suggested that in the rat, both liver and kidneys are important in the degradation and elimination of primidone, whereas the liver rather than the kidneys is reponsible for the removal of phenobarbitone. G. B.

NEW APPARATUS

A NEW APPARATUS FOR THE B.P. TABLET DISINTEGRATION TEST

BY W. E. FIELD

From the Analytical Department, Crookes Laboratories, Ltd. London

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Under the heading "Disintegration Test" the British Pharmacopœia states as follows:—

"*Disintegration Test.*—Five tablets are used for the test. Place each tablet in a test-tube, 6 in. long and 1 in. in internal diameter, containing sufficient water heated to 37° C. to fill the tube almost completely, so as to leave about half an inch of air space when the tube is closed. Close the tube, place it in a water bath maintained at 37° C., and repeatedly invert it at such a speed that the tablet travels through the water without striking the ends of the tube; the time required for the tablet to dissolve, or to disintegrate, or to soften throughout so that it disintegrates on a slight touch, is not more than 15 minutes, unless otherwise stated in the monograph. All five tablets should comply with the test. If one tablet fails to comply, the test may be repeated, using five tablets from the same sample; all must comply with the test."

In practice, the inversion of 5 tubes in a water bath at 37° C. presents certain difficulties. If the inversion is carried out by hand, the test is tedious in any case and if required for appreciably longer than the 15 minutes, it becomes very monotonous besides being a waste of time. The number of tests that can be carried out simultaneously is restricted and individual differences arise between operators. If the inversion is performed mechanically, a water bath is not very suitable as a medium for maintaining the temperature of the tubes. No standard apparatus is available and various devices, e.g., vacuum jacketed tubes have been used in the past. The apparatus described below and illustrated in the diagram is simple to construct and has proved very satisfactory for routine use.

The tubes are mounted radially on clips, on a rotating disc in a shallow cupboard maintained at 37° C. by a thermostat and heated by warm air. The cupboard is enclosed by a perspex panel and the progress of the disintegration can be followed all the time through this observation door. The disc is rotated by a fast running motor geared down to rotate the disc at a speed which allows the tablets to fall slowly through the water and the method of inversion ensures that the tubes turn over evenly and that the tablets are not knocked about. In the model shown, 10 tubes, equivalent to 2 tests, can be mounted together and this number can be greatly increased by increasing the diameter of the disc and placing the clips closer together. The other end of the motor armature carries a suction fan which takes the air through the short tube shown

projecting on the right, and this air is pumped through a half-inch metal duct into the bottom of the cupboard. The short tube contains a 250-watt element which heats the air as it passes through and which is automatically cut out through the action of the thermostat in the cupboard when the temperature of the air inside reaches 37° C. In this way the temperature of the air inside the cupboard is maintained between 36° and 38° C.

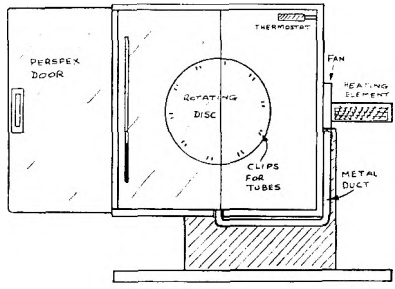
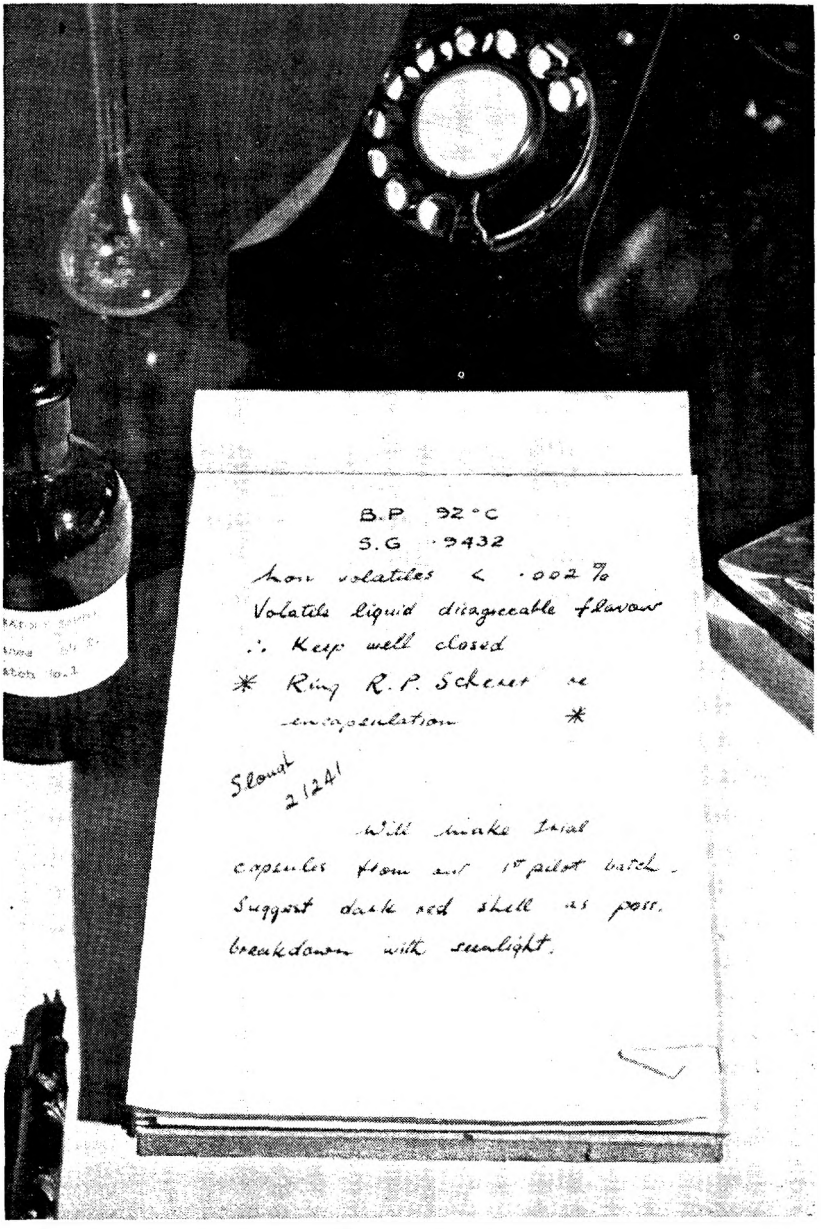


FIG. 1.

In use the cupboard is allowed to attain a steady temperature and test tubes with B.24 ground glass stoppers containing the tablets are filled with water at 37° C., leaving a small air space. The motor is then switched off, the tubes placed in the clips and the apparatus restarted. The progress of the disintegration can then be followed continuously or a timing clock can warn the operator after any desired interval of time. Tests on tablets with abnormally long disintegration times, or special tests, for instance, in the examination of enteric coatings or hardened gelatin capsules, can be continued for hours without further attention and any tube can be removed quickly at any time to allow closer examination of the contents. The results obtained are reproducible and agree with those obtained by manual methods.

I wish to thank the Directors of Crookes Laboratories Ltd. for permission to publish this paper.



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