THE SEPARATION OF ALKALOIDS BY PARTITION PAPER CHROMATOGRAPHY

PART I. THE SOLANACEOUS ALKALOIDS

PART II. THE WATER-INSOLUBLE ALKALOIDS OF ERGOT

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REFERENCES to the separation of alkaloids on columns by partition chromatography have been made by Catch *et al.*[1] and by Partridge *et al.*[2,3]. The theory and use of buffered columns have been previously described[4,5,6]. In the separation of the penicillins, buffered filter paper has been used to obtain suitable paper chromatograms[7] while the difficulty of obtaining absolute R_F values for such separations has been noted[8]. The separation of water-soluble alkaloids of ergot has been achieved by paper chromatography using unbuffered paper[9]. Conditions for the separation of certain alkaloids and mixtures of alkaloids on unbuffered paper have also been described[10].

The present writers have successfully used buffered filter paper for the separation of certain of the strongly basic solanaceous alkaloids and of the feebly basic water-insoluble alkaloids of ergot. The method of separation on buffered paper should prove to be a general method for the separation of micro amounts of mixed acids or bases, provided the substances do not dimerise in either phase. Separations are only to be expected where the dissociation constants and/or partition coefficients differ from each other.

PART I. THE SOLANACEOUS ALKALOIDS

From an analytical viewpoint mixtures of solanaceous alkaloids seemed suitable for an investigation of the possibility of employing paper chromatography in the separation of alkaloids. The actual nature of the alkaloids is known, the number is not large and some information is available as to their solubilities in various solvents. If separations were obtained the method would prove useful as a means of qualitative control of assay processes for the hyoscine content of solanaceous alkaloids. If work on a larger scale using columns were contemplated it would also prove useful as a guide to those solvents which would be most successful for use in such assays, and to the best *p*H to employ. If the hyoscine content of *Atropa Belladonna* had been found to be reasonably high (about 10 per cent of total alkaloid), it was hoped to elaborate a quantitative method by use of Allport's colorimetric method as developed by James and Roberts which will detect quantitatively 20 to 30 μg. of solanaceous alkaloids. In fact the amount of hyoscine in the *Atropa Belladonna* available was less than 10 per cent. of the total alkaloid and therefore this line of investigation had to be abandoned.

EXPERIMENTAL

(a) *Detection of Alkaloids.* For the detection of solanaceous alkaloids on buffered paper chromatograms a solution of 0·5 per cent. of iodine in aqueous potassium iodide would detect solanaceous alkaloids in concentrations greater than 20 μg. This reagent stains the paper and the alkaloid only shows up when the iodine has been allowed to evaporate at room temperature (about half an hour). The colour of the complex alkaloidal iodides formed fades and the developed spots are thus transient in colour. The position of the alkaloids may, however, be shown up permanently by use of Macheboeuf and Munier's modified Dragendorff reagent[10] which will detect solanaceous alkaloids down to 5 μg. Spraying these solutions is not particularly effective and it is best to run the paper through the reagent solution as though developing a photographic film.

(b) *Preliminary Separations with Buffered Papers.* Previous workers using buffered papers have prepared the paper by soaking in the buffer solution, pressing the soaked paper with blotting paper to remove surplus buffer, drying in air and keeping in a damp atmosphere 1 hour before use[7].

Using Whatman No. 1 paper treated in this way with phosphate buffer (Sørçnson) of *p*H 7·4 and developing with butanol saturated with water, it was found that atropine, hyoscine and apoatropine gave spots of very different R_F. With paper buffered at *p*H 6·8 hyoscine gave an R_F only slightly larger than atropine, and on running a mixture they were not completely separated. One of the merits of the aqueous iodine solution was that it gave different colours with the various alkaloids, and in this experiment the hyoscine was coloured red-brown, while the atropine was blue-grey and apparently just separated. The modified Dragendorff's reagent showed no such distinction in colour between the two, and in fact with its greater sensitivity showed that the hyoscine and atropine were overlapping. Paper with no buffer showed atropine and hyoscine running at the same rate, tailing badly, and not separable. At *p*H 8·0 the R_F of all three alkaloids was greater and very similar to those at *p*H 9·0. The alkaloids were applied as a 1 per cent. solution in chloroform or ethanol, in a volume of about 0·01 ml. giving a spot on the paper of about 1 sq. cm. area. Smaller spots of high concentration can overload the buffer.

(c) *Preparation and Choice of Paper.* Marked changes in the initial dryness of the paper, of the time of soaking in buffer solution (0 to 17½ hours), of drying of soaked paper (3 to 24 hours), and of equilibration of the buffered paper with the vapour phase (0 to 24 hours) were found to give no significant variation in the paper chromatograms obtained. It is, however, important that the buffered paper be not too dry prior to equilibration with the vapour phase.

As wet buffered papers are fragile and easily torn, a hardened paper, Whatman No. 54, was tried, but was not satisfactory, nor was a thicker paper, Whatman No. 100, found to be suitable. Whatman paper No. 1 was found to give more satisfactory chromatograms than No. 4. This seems to be due to the fact that No. 4 paper runs faster and the equilibra-

tion of partition of alkaloid between the moving organic phase and the stationary aqueous phase is not as complete as with the slower running paper No. 1.

(d) *Development with Water-miscible Solvents.* Runs with water-miscible solvents such as ethanol 95 per cent. and 70 per cent. and acetone 95 per cent. and 70 per cent. did not separate mixed alkaloids on buffered paper. In ethanol 70 per cent. and acetone 70 per cent. both the salts and the free bases have a high solubility, and in fact both these solvents have been used in extracting the alkaloids from the crude drug, so, as might be expected, the alkaloids run near the solvent front on these chromatograms. If the butanol is not saturated with water no separations result, and even if not quite saturated the spots of alkaloid were more diffuse.

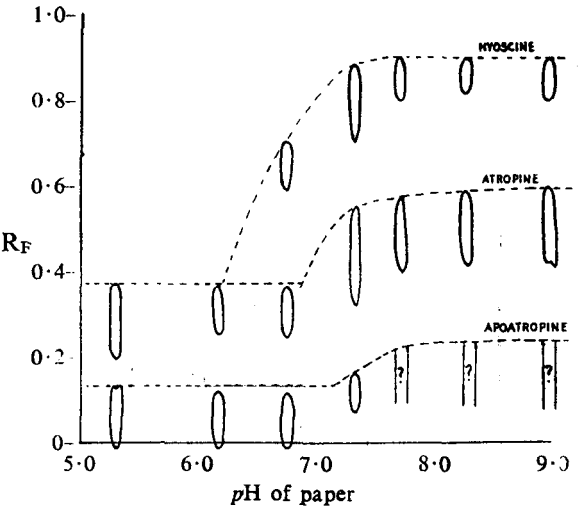


FIG. 1. Appearance of paper chromatograms of atropine, apoatropine and hyoscine at different *p*H values. Buffer, M/15 phosphate; solvent, butanol.

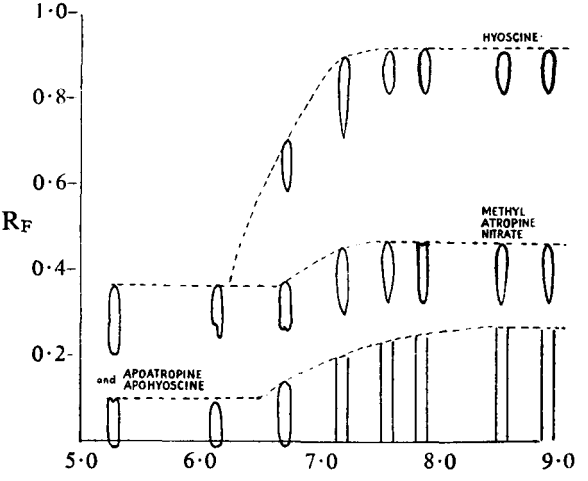


FIG. 2. Appearance of paper chromatograms of apoatropine, apohyoscine, atropine methyl nitrate at different *p*H values. Buffer, M/15 phosphate; solvent. butanol.

(e) *Effects of Change of* p*H and of Type of Buffer.* In making comparative runs with papers soaked in buffers of different *p*H it seemed prudent to prepare them all under similar conditions and run them at the same time by the descending method so that any differences due to variation of temperature, etc.,

would be avoided. Upward runs (see also Part 2) were found to give very compact spots, but it was found more convenient to do comparison runs at varying *p*H, by running downward.

If 8 different buffer strengths were used, 8 strips of buffered paper, 3 × 45 cm., were prepared and run at the same time. The preparation of the buffered papers was standardised, i.e., the papers were soaked in the buffer solution, taken out and, after shaking off surplus buffer, allowed to air-dry for 10 hours. The papers were then left in the bell jar used for the chromatographic separations and allowed to equilibrate with the vapour phases before commencing the run. With Whatman No. 1 paper the equilibration was for 24 hours and the run was started in the evening, whereas with Whatman No. 4 it was left to equilibrate 14 hours overnight and the run was started next morning. Two similar series of runs were made using Whatman No. 4 paper and butanol saturated with water. In the first case a mixture of apoatropine, atropine and hyoscine was used (Fig. 1). In the second case a mixture of apoatropine, apohyoscine, methyl atropine nitrate and hyoscine was used (Fig. 2). The resulting chromatograms are represented diagrammatically with the abscissae in terms of R_F and the ordinates of *p*H. The relative size and shape of the alkaloidal spots are recorded at the appropriate *p*H.

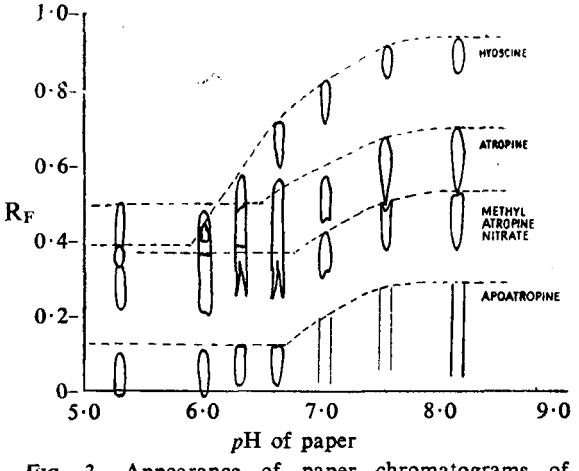


FIG. 3. Appearance of paper chromatograms of apoatropine, atropine methyl nitrate, atropine and hyoscine. Buffer, M/15 citrate; solvent, butanol.

The buffer used was M/15 phosphate buffer (Sørenson, 1909 to 1912).

Some characteristic features were noted. The hyoscine spots began to move faster at a lower *p*H than the other alkaloids, between 6·0 and 6·5. They tended to show maximum concentration at the front. Atropine began to move faster at a slightly higher *p*H between 6·5 and 7·5. Apoatropine and apohyoscine were not separated and gave elongated trailing spots above *p*H 7·5. This probably was due to adsorption or slow equilibrium of partition between the aqueous and the organic phase.

Similar graphs were obtained for separations using other buffers such as citric acid and disodium hydrogen phosphate (McIlvaine, 1921) and also on replacing the citric acid with tartaric or maleic acids. Citrate buffers gave a very satisfactory series of paper chromatograms (Fig. 3), the change of R_F with change of *p*H being more gradual than with phosphate buffer. Tartrate runs were not so good, while maleates ran

badly for comparative purposes. The maleate salts, excepting those of apoatropine were apparently much more soluble in aqueous butanol than were the salts of the other buffer acids used.

Several series of runs were made using various buffer solutions at varying concentrations both stronger (4 M/15) and weaker (M/30) than the M/15, and it was found that optimum results were obtained with M/15 buffer, as it gave a reasonable variation of R_F values over a suitable range of *p*H (about 2 *p*H units).

(f) *Effect of Change of Solvent.* Using butanol alone, atropine and methyl atropine nitrate ran very close together. Methyl atropine nitrate is soluble in polar solvents and insoluble in non-polar ones, so it was thought that better separations would be obtained by using 80 ml. of *n*-butanol with 20 ml. of chloroform and shaking up with 100 ml. of water to saturate the organic phase. On buffered paper satisfactory separations of hyoscine, atropine, methyl atropine nitrate and apoatropine were obtained at *p*H values above 6·5. Similar results were obtained using citrate, tartrate and maleate buffers, but in these cases the apoatropine tended to run faster than the methyl atropine nitrate and thus these two alkaloids were not separated.

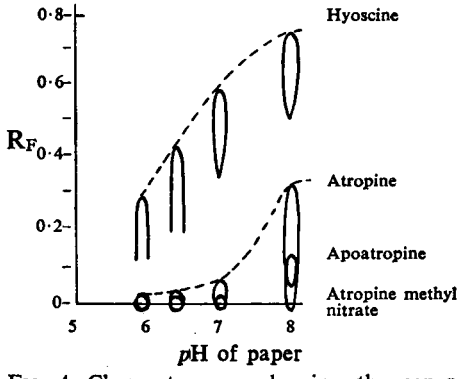


FIG. 4. Chromatograms showing the separation of hyoscine, atropine, apoatropine and atropine methyl nitrate at different *p*H values using ether as solvent in M/15 phosphate buffer.

Experiments were also made with a variety of volatile solvents, including chloroform, light petroleum, carbon tetrachloride, benzene and ether. A small bell-jar 12″ × 6″ with a ground-glass lid was used so that the volume in the apparatus and the area of possible escape of vapour were much reduced. It was found that by pleating the paper it was possible to use full-size strips, 3 × 45 cm. These solvents did not give good separations, but hyoscine in chloroform showed a very steep rise of R_F with increasing *p*H and above *p*H 7·0 it ran very close to the solvent front. With light petroleum (30° to 60°C.) none of the alkaloids moved appreciably, the bases being insoluble in this solvent. Carbon tetrachloride gave a more gradual shift of hyoscine R_F with change of *p*H than does chloroform, and the other alkaloids, atropine, etc., were only slightly soluble between *p*H 6 and *p*H 8. Benzene gave long trailing diffuse spots even with hyoscine. Ether gave good separations of hyoscine and atropine, though the spots of alkaloid were rather large. Atropine is relatively insoluble in this solvent compared with hyoscine (Fig. 4).

As atropine and hyoscine are only slightly soluble in light petroleum

and atropine is not very soluble in carbon tetrachloride, whereas hyoscine is very soluble in chloroform, various mixtures of these solvents were tried, as such mixtures might be used on buffered columns to elute rapidly hyoscine and yet leave the atropine on the column. Such a column prepared from buffered kieselguhr will take a heavy load of alkaloid without break through. 100 ml. of a mixture of equal parts by volume of chloroform. carbon tetrachloride and light petroleum (30° to 60°C.) was shaken with 100 ml. of water to saturate the organic phase and used as eluant on phosphate buffered papers (Fig. 5). This solvent mixture gave very wide separations of hyoscine from the other alkaloids present (apoatropine, methyl atropine nitrate and atropine).

Similar results were obtained with two other such mixtures—one containing 100 ml. of a mixture of equal parts by volume of carbon tetrachloride and chloroform saturated by shaking with 100 ml. of water and the other being a mixture of light petroleum (30° to 60°C.) and chloroform 60 ml., shaken with 90 ml. of water to saturate.

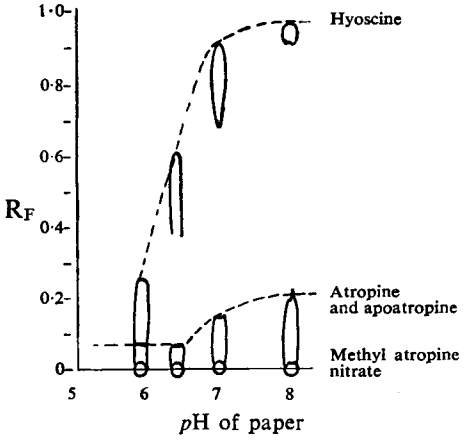


FIG. 5. The separation at different *p*H values of hyoscine, atropine, apoatropine and atropine methyl nitrate using equal parts of chloroform, carbon tetrachloride and light petroleum (30° to 60°C.) as solvent.

(g) *Separations using Extracts, etc.* Some experiments were made with old extracts of belladonna. In all cases atropine (or *l*-hyoscyamine) was found and hyoscine not detected, and thus could only be present in small proportions (less than 10 per cent. of the total alkaloids). Variation of method of preparation of the alkaloidal solution prior to application on the paper was made as follows (a) extracting with chloroform in the presence of ammonia and evaporating until the chloroform solution had a concentration greater than 1 per cent. of total alkaloid (b) extract applied directly and (c) extract with ammonia added to liberate the free base. Using paper treated with phosphate buffer of *p*H 8·0 and eluting with water-saturated butanol the R_F and appearance of the detected atropine spots were identical for (a), (b) and (c).

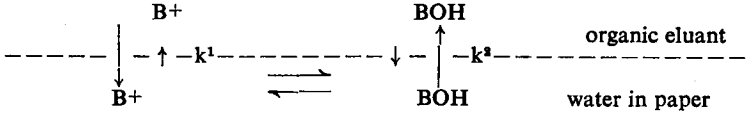
The use of an extract directly (b) raised the question as to whether the alkaloid may be applied initially as a salt. (The alkaloid in the extract is presumably present as malate.) When atropine sulphate was used the front had the same R_F as atropine but tailing took place. This presumably only applies to salts of strong acids the anions of which have been previously reported as causing trouble in chromatography. Similarly an initial spot of mixed atropine sulphate and hyoscine hydrobromide gave tailing, though the R_F of the front in each case is the same as that

of the free base. In some cases using old extracts which may well have been overheated in preparation a spot of alkaloid was left on the starting line even at a *p*H as alkaline as 8·0 and thus would not be apoatropine but might be belladonnine. A very faint mark at the solvent front was also observed in certain cases (possibly tropine). Some runs using dry extract of hyoscyamus extracted with chloroform in the presence of ammonia showed only atropine (or hyoscyamine) to be present.

A sample of leaf of *Datura Stramonium* gave an alkaloidal residue containing a proportion of hyoscine (about 20 per cent. ± 5), mixed with atropine. In all separations the identity of individual alkaloids was established by running a marker composed of a mixture of known alkaloids, and no reliance was placed on interpolation from R_F values alone. The use of solutions of extracts directly on paper led to trouble due to the amount of resin, etc. present. This extraneous material renders solutions with a concentration of 1 per cent. and over very tacky and viscous and so unsuitable for direct application to the paper. Adsorption chromatography with alumina as the adsorbent may be used for the separation of the alkaloids of *Atropa Belladonna* from the bulk of non-alkaloidal matter present in galenicals, as used in the assays of Merz and Frank[11] and by Brownlee[12]. During the course of experiments using alumina columns (Brockman grade 1) it was noticed that the extracts tried showed strongly adsorbed bands of brown pigments at the top of the alumina columns, whereas tinctures prepared by the percolation process gave much more delicately coloured bands varying from pale buff to green. Heating of tincture residues above 80°C. for half an hour changed the appearance of their adsorption chromatograms and led to the appearance of brown strongly adsorbed bands. Tinctures from two different manufacturers gave adsorption chromatograms similar to those of a tincture made by the official process. Two different samples of a third manufacturer's tinctures showed brown strongly adsorbed bands and bright green eluates which could be explained by decomposition of leaf pigments at some stage by heat, and possibly addition of pigment such as chlorophyll in order to mask the brown pigments.

Discussion

The equilibrium between alkaloid ion (B^+) and alkaloid free base (BOH) is governed primarily by the *p*H of the buffered paper. The developing liquid is organic and normally the alkaloid ion (B^+) will be slightly soluble while the free base (BOH.) will be freely soluble thus



It is apparent that the equilibria set up are very complex and governed by the nature of the organic solvent, the relative concentration of the two phases, the distribution coefficients of the alkaloid and the degree

of dissociation and amount of hydrolysis of the various alkaloidal salts. The *p*H range of separations of a given alkaloid will thus, to a limited extent, vary with the nature of the system and of the solvent.

A comparison of the curves obtained for the titration of hyoscine in solution in (a) distilled water, (b) 17 per cent. ethanol, (c) water saturated with butanol, using 0·05 N hydrochloric acid gave curves which were very similar in appearance. The titration curve for atropine in the presence of ethanol (17 per cent.) is also shown (Fig. 6). It will be seen from the

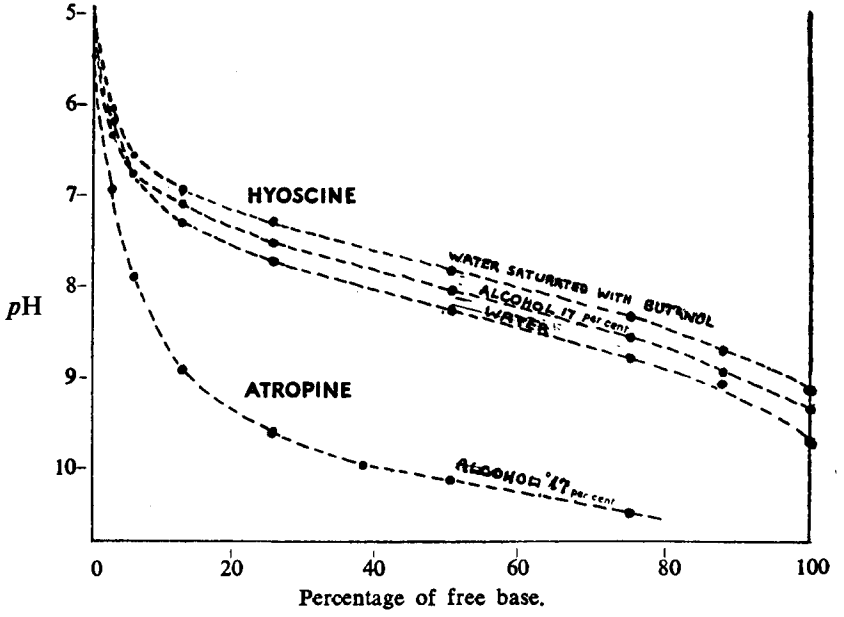


FIG. 6. Titration Curves of hyoscine and atropine with 0·05N hydrochloric acid.

graph that in water saturated with butanol about 50 per cent. of hyoscine will be present as free base at *p*H 7·7· whereas atropine will be 91 per cent. salt. Therefore, with continuous extraction at this *p*H the bulk of the hyoscine would pass into the organic phase. Thus in the paper chromatograms the hyoscine R_F becomes greater on changing from *p*H 6·0 to *p*H 7·5 (*cf.* Fig. 1). Atropine salts do not change to 50 per cent. free base until a *p*H of about 9·0 is reached and the R_F of atropine becomes greater on going from *p*H 7 to 8·3 (*cf.* Fig. 1).

Of the solvents used only ether and butanol gave reasonable spots and this is probably due in part to the fact that both need an appreciable amount of water to saturate them. The dissolved water may be important in two inter-related ways. In the first place it may facilitate partition of the alkaloids between the two phases and in the second place it may assist the solvent to run in the matrix of the paper with no tendency to run superficially along the surface. Those solvents which do not dissolve much water, such as chloroform, benzene, carbon tetrachloride, etc., gave tailing spots of atropine and it has previously

been remarked by other workers that such solvents cause adsorption effects.

The separations with ether on paper showed the same order of separation (hyoscine nearer the solvent front than atropine) as that found by Evans and Partridge[2] in their separations of solanaceous alkaloids on buffered kieselguhr columns. The effects of change of *p*H of the buffer in these columns was also similar to that observed on paper chromatograms using ether to develop (Fig. 2). Paper chromatography might therefore be used for pilot runs with micro amounts of alkaloids to discover the optimum conditions for separation of mixtures of alkaloids prior to using kieselguhr columns for separation of large amounts of the alkaloids.

Part II. Water-insoluble Alkaloids of Ergot

The problem of devising suitable methods of assay for ergot and its preparations has engaged the attention of numerous workers. The present official method of assay originally proposed by Hampshire and Page[13] is based on the widely different partition coefficients of the water-soluble and the water-insoluble alkaloids between ether and weakly alkaline water. This does not, however differentiate between the physiologically active and the inactive alkaloids, since the colorimetric assay lacks specificity, and any alkaloid (or degradation product) containing the indole nucleus gives the colour reaction with dimethylaminobenzaldehyde reagent.

Pharmacological methods such as those of Broom and Clarke for the estimation of the water-insoluble alkaloids are tedious and liable to a considerable margin of error. Assay methods specific for ergometrine in the presence of other alkaloids have been devised[14,15], the most recent method being that of Foster *et al.*[16], who separated ergometrine by paper partition chromatography. Partition ratios for ergometrine and ergotoxine between a buffered aqueous phase and ether or chloroform phases have been previously studied[17]. A graphic method of studying separations of mixtures by immiscible solvents with special reference to ergometrine and ergometrinine has been given[18].

Experimental

Separation of Alkaloids. A solvent system of *n*-butanol-acetic acid-water which is successful for separating ergometrine and ergometrinine on unbuffered filter paper is useless for the separation of the water-insoluble alkaloids of the ergotoxine and ergotamine groups, since the partition is so much in favour of the moving organic phase that all the alkaloids move with the solvent front. In preliminary experiments ether and chloroform were found to be the most suitable solvents, but practical difficulties were encountered due to their volatility. The alkaloids are insoluble in light petroleum and also in light liquid paraffin. The possibility of using *n*-butanol to which had been added varying proportions of these solvents in order to obtain a suitable organic phase was tried, but was not successful since badly tailing spots resulted. This was probably due to adsorption of the alkaloid on the paper. Finally,

the solvents selected were ether or chloroform, used with buffered Whatman No. 1 filter paper. The water-insoluble alkaloids of ergot are weakly basic, and hence in order to obtain a useful range of partition ratios between the aqueous and organic phases it is necessary to employ buffer solutions of greater hydrogen ion concentration than in the separation of stronger bases, e.g., the solanaceous alkaloids. At *p*H values higher than 6, ergotoxine, ergotinine, ergotaminine and ergotamine will be present almost entirely as the free bases, and the R_F values will tend towards unity so that the scope of the separations will be greatly reduced.

The experimental work is discussed below under two headings.

A. Qualitative—use of buffered filter paper.

B. Quantitative—use of buffered filter paper and buffered cellulose columns.

A. *Qualitative.*

Chromatography of pure alkaloids. Whatman No. 1 filter paper, buffered as previously described in this paper was used for the following work:—

Buffer solutions—0·1M citric acid—0·2M disodium hydrogen phosphate (McIlvaine, 1921), 0·1M tartaric acid—0·2M disodium hydrogen phosphate and 0·1M maleic acid—0·2M disodium hydrogen phosphate.

Solvents—Anæsthetic ether B.P., saturated with water, was used unless otherwise stated. Solvent ether B.P. gave higher R_F values, due to its alcohol content.

Alkaloids—Ergocristinine (ergotinine), ergotoxine (prepared from ergotoxine ethanesulphonate), ergotamine (prepared from ergotamine tartrate), ergotaminine (prepared from ergotamine by heating with methyl alcohol), lysergic and isolysergic acid.

The alkaloidal bases were prepared by dissolving the appropriate salt in aqueous acetone and precipitating the base by the addition of freshly prepared sodium bicarbonate solution. The base was rapidly filtered off, washed with water until free of alkali and then dried over phosphorus pentoxide.

For the addition of the alkaloid to the paper, solutions were prepared, either in 90 per cent. ethanol or chloroform, and approximately 0·01 ml. of the solution containing from 10 to 30 μg. was applied to the starting line of the buffered filter paper. The time of the run was from 3 to 5 hours for the organic solvent to move 25 to 30 cm. from the starting line. The chromatographic chamber (bell-jar) had to be carefully sealed to avoid undue loss of solvent as such loss resulted in very variable R_F values and the rate of flow showed considerable fluctuation. The alkaloidal spots were located by viewing the paper under a suitable source of filtered ultra-violet light. Although the solvent rapidly evaporates from the paper, the position of the solvent front was readily seen since a certain amount of fluorescent filter paper extractive passed along with the solvent front and a fluorescent band thus marked the front.

The effect of *p*H on the relative R_F values is shown in Figures 7 to 12.

The R_F values were measured from the front of the spots. Very similar results were obtained with the citrate or tartrate buffer, in conjunction with ether saturated with the aqueous phase. The maleate-phosphate

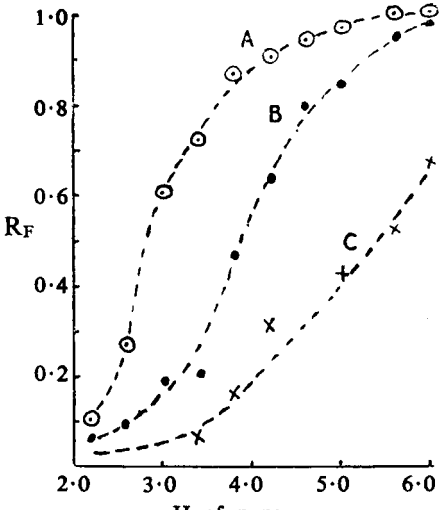


FIG. 7.

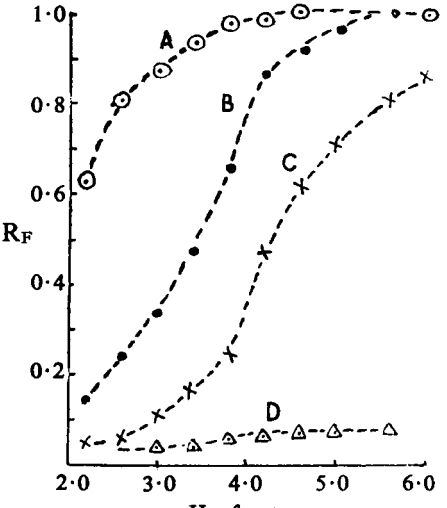


FIG. 8.

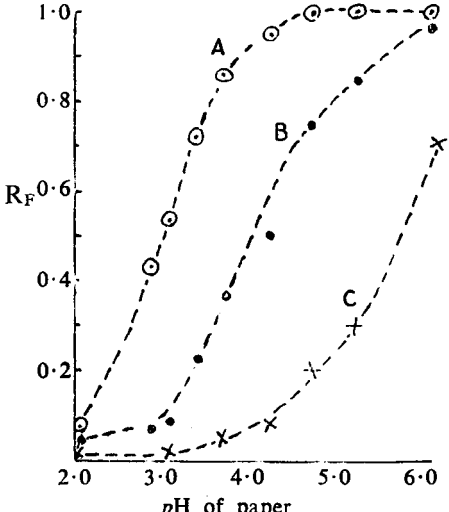


FIG. 9.

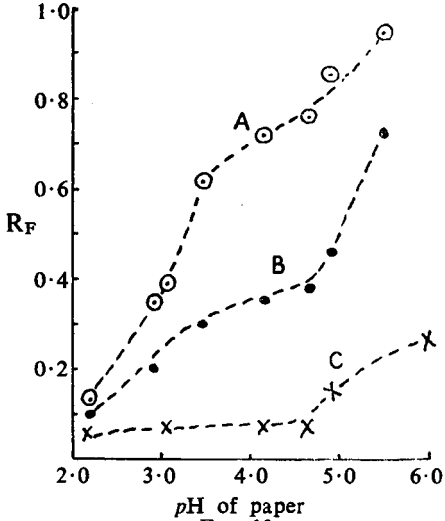


FIG. 10.

FIGS. 7-10. Effect due to change of *p*H, solvent and buffer solutions on the separation of ergocristine (A), ergotoxine (B), ergotamine (C) and *iso*-lysergic acid (D). FIG. 7, citrate-phosphate buffer and ether/water; FIG. 8, citrate-phosphate and chloroform/0·1M citric acid; FIG. 9, tartrate-phosphate and ether/water; FIG. 10, maleate-phosphate and ether/water.

buffer (Fig. 4) gave results unlike either the citrate or tartrate buffers since the curves showed considerable distortion and the presence of the maleate buffer completely quenched the fluorescence of the alkaloid on the paper, but on "wetting" the paper with ether or alcohol the fluorescent spot appeared. From Figures 7 to 11 it is seen that the alkaloids are widely separated at *p*H 3 to 4, and this would appear to be the optimum *p*H for maximum separation. In Figure 12 there is not complete separation of ergotoxine and ergotaminine and the spots overlap. A secondary effect due to change in *p*H was the change in size of the alkaloidal spot. The area of the spots was at a minimum when the alkaloid was running as the free base or as the salt. In later experiments it was found that the spots were more compact when the method of ascending chromatography[20,21] was used. The elongation of the spots appears to be due, partially at least, to incomplete equilibration of alkaloid between aqueous and organic phases.

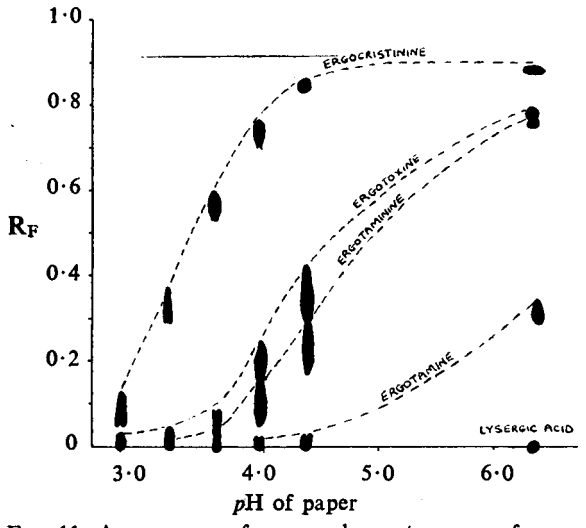


FIG. 11. Appearance of paper chromatograms of ergocristinine, ergotoxine, ergotaminine, ergotamine and lysergic acid at different *p*H values. Buffer, citrate-phosphate; solvent, solvent ether/0·1M citric acid.

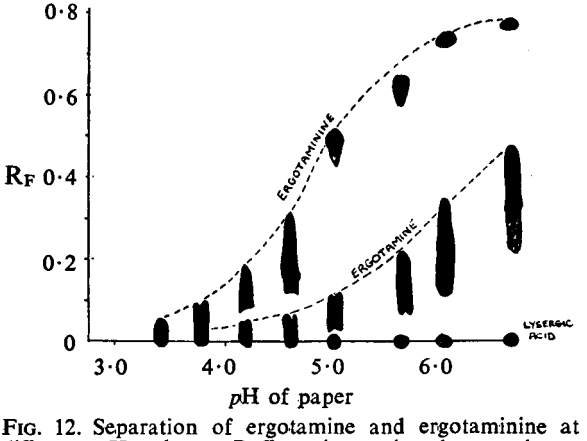


FIG. 12. Separation of ergotamine and ergotaminine at different *p*H values. Buffer, citrate-phosphate; solvent, solvent ether/0·1M citric acid.

The use of *d*-camphorsulphonic acid for resolution of racemic bases into their optical antipodes is well known and, although ergocristine-ergocristinine are not optical antipodes since a structural change is involved[25], it is of interest to record that filter paper soaked in 1 per

cent. *d*-camphorsulphonic acid used in conjunction with ether (saturated with water) brought about a separation of ergotoxine and ergocristinine. In this case the ergotoxine had a greater R_F value than ergocristinine. This method showed no advantage over the buffered filter paper and in fact tailing of the spots was greater. Ergotoxine ran as a single spot, and there was no evidence of it being resolved into its components. However, work is being continued with a view to studying the chromatography of the ergotoxine complex. The identification of the amino acids produced by hydrolysis of the polypeptide portion of the molecule has been used to detect the presence of ergocornine, ergokryptine and ergocristine[15].

Chromatography of deteriorated alkaloids. Ergocristinine in citrate-phosphate buffer *p*H 2·2 was stored for 25 days in the dark at room temperature. The alkaloid was separated by making the solution alkaline and extracting with ether. The solution was concentrated and spotted on to the paper. Two spots from the deteriorated solution ran with the same speeds as control spots of ergotoxine and ergocristinine. Thus, this method is a very simple one for the demonstration of the interconversion of the alkaloids.

An ergotoxine solution in 50 per cent. ethanol, several months old, was examined in the same way and a spot of similar R_F value to ergocristinine was present, although above *p*H 3·8 the R_F values diverged slightly. Whether this divergence was due to ergokryptinine or ergocorninine requires further investigation. A green fluorescent elongated spot was also visible under ultraviolet light. The substance appeared to be less basic than the alkaloids since the R_F values over the same range of *p*H showed much less variation. This is in the course of investigation.

B. *Quantitative.*

Methods for the detection of spots and estimation of amount of alkaloid present. In quantitative estimations not only must the alkaloids be located but sufficiently accurate means must be found for their estimation either *in situ* or after removal from the paper. In the case of ergot alkaloids, their detection is easy since they fluoresce in ultraviolet light, and less than 1 μg. per sq. cm. can be detected on unbuffered filter paper although the sensitivity of this test is slightly reduced when using buffered paper. The following methods were considered for estimation of the alkaloids:—

1. *In situ.* (a) Foster[16] has used the method of matching the fluorescence of ergometrine spots with a series of standard spots. The error was ± 20 per cent. and may be even greater in the case of diffuse spots.

(b) For quantitative estimation of amino-acids and sugars the relationship between area of the spot and concentration of the substance has been utilised. This method was not found satisfactory for estimation of ergotoxine, and probably the insensitivity of this method may be due to the use of buffered filter paper which has a greater capacity than unbuffered paper, although the same difficulty has been found with ergomentrine[16].

(c) The formation of coloured derivatives on paper and matching with a series of standard spots has also been employed. A coloured compound can be produced by spraying the alkaloidal spots with a solution of *p*-dimethylaminobenzaldehyde in a solution of butanol and hydrochloric acid as used for detection of sugar on paper[22]. The paper is heated at 90°C. for 5 minutes and a blue colour indicates alkaloid. Also dilute solution of iodine and the modified Dragendorff's reagent used for detection of the solanaceous alkaloids in Part I of this paper, gave coloured derivatives but all these tests suffer from the disadvantages mentioned in (a).

2. *After removal from paper.* Once the alkaloids are in aqueous solution they may be estimated by normal colorimetric procedure using the dimethylamino-benzaldehyde reagent of the B.P. 1948. From the qualitative experiments it was found advisable not to exceed a load of 30μg. in 0·05 ml. on the buffered paper if tailing was to be minimised, so that a satisfactory colorimetric assay should estimate from, says, 5 to 40 μg. of ergotoxine. It was found that the most practical way to achieve this sensitivity, was to evaporate the alkaloidal solution to dryness and apply the colour test to this residue. It was possible to estimate from 5 to 40 μg. The experimental details of the colorimetric assay are given below.

Experimental

Preparation of Calibration Curve for Colorimetric Assay. A 0·1025 per cent. solution of ergotoxine in 50 per cent. ethanol was prepared. 1 ml. of this was diluted to 50 ml. with 95 per cent. ethanol so that the solution contained 20·5 μg./ml. The solution was transferred to a 2-ml. burette and volumes from 0·25 ml. to 1·5 ml. were placed in small Pyrex evaporating dishes. The alcohol was removed by evaporation, 0·5 ml. of tartaric acid solution (1 per cent.) was added, followed by 1·0 ml. of dimethylamino-benzaldehyde reagent of the British Pharmacopœia. The contents of the dish were mixed and after 10 to 20 minutes the solution was transferred to the micro cells (capacity 0·5 ml.) of the Spekker absorptiometer. The filters used were Chance glass filters H455, OG1 and OR2 on the left-hand side and filter OR2 on the right-hand side. 2 volumes of dimethylamino-benzaldehyde reagent added to 1 volume of tartaric acid solution, was used in the blank cell. The calibration curve is shown in Figure 13.

Extraction of alkaloid from filter paper. Considerable time was spent in finding a suitable method for eluting the alkaloid from the paper. From preliminary experiments the most obvious solvent to try was 95 per cent. ethanol. Attempts to obtain quantitative recoveries by macerating filter paper containing alkaloid with 95 per cent. ethanol, were not successful and the most consistent results were obtained by irrigation of the buffered filter paper with 50 per cent. ethanol. The solvent extracted less extraneous material from the filter paper than did 95 per cent. ethanol. The method finally adopted was to cut out a strip of filter paper containing the alkaloidal spot—one end was cut to a point and the strip was suspended with the straight edge dipping into a glass trough of 50 per cent.

ethanol. The solvent travelled down the strip and dripped into a small Pyrex dish below. It was found most convenient to use spots that could be included on a strip of paper approximately 6 cm. × 3 cm. To prevent evaporation of the solvent from the filter paper, the apparatus was enclosed in a glass jar. The strips were left to irrigate for 2 to 3 hours when about 1 to 2 ml. of eluate was collected. The strips were washed with an additional 0·5 ml. of 50 per cent. ethanol to ensure no residual alkaloidal solution at the tip or edges of the paper. Complete elution of the alkaloid was checked by viewing the extracted strips under ultraviolet light. The alkaloidal solution was then evaporated using gentle heat, and the colour test applied. The coloured solutions were filtered through a No. 3 sintered glass micro filter before transferring them to the Spekker cells. To check the efficiency of this extraction method, 0·01 ml. volumes of 50 per cent. ethanol containing from 11 μg. to 43 μg. of ergotoxine were added to buffered filter paper and the alkaloid extracted as described above, within a few hours of its being added. The results are summarised in Table I.

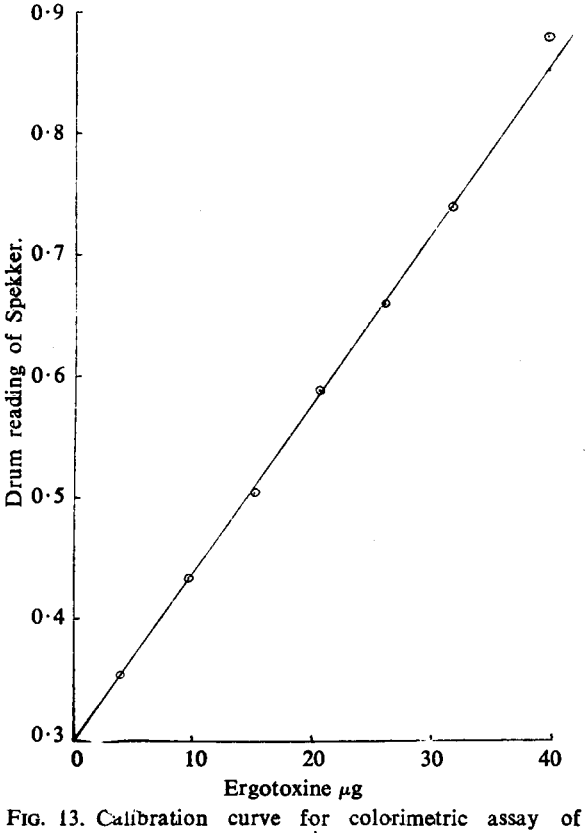


FIG. 13. Calibration curve for colorimetric assay of ergotoxine.

The 9 per cent. loss of alkaloid was considered to be due possibly to one or more of the following factors:—(a) decomposition of alkaloid on the paper, (b) interference of the colour test by filter paper extractive, (c) incomplete elution of alkaloid. Investigating these factors in turn, it was found that no detectable decomposition of ergotoxine had occurred on buffered filter paper (citrate-phosphate *p*H 2·6 to 3·8) after 64 hours storage at room temperature. The effect of material extracted by the 50 per cent. ethanol from the filter paper alone was found not to decrease

the colour intensity. This was tested by extracting strips of buffered filter paper with 50 per cent. ethanol for 3 hours. Quantities of 9·2 μg. of ergotoxine in 0·01 ml. volumes of 50 per cent. ethanol were added to the extracts and assayed. Compared with controls (containing no extractive) it was found that the effect of the extractive was to give a slightly higher average figure for the assay. All solutions were carefully filtered through a No. 3 sintered glass filter prior to colour measurement. Incom-

TABLE I

RECOVERY OF ERGOTOXINE FROM BUFFERED FILTER PAPER (CITRATE-PHOSPHATE *pH* 3·8)

Amount of Alkaloid Added (μg.)	Percentage Recovery
43·0	90, 84, 91, 90
17·0	96
14·5	93
12·3	89, 90, 92
11·0	94

AVERAGE RECOVERY=90·9 per cent. (standard deviation=3·2 per cent.)

plete elution of the alkaloid from the paper was originally considered unlikely since the extracted filter paper strips showed no fluorescence when viewed under ultraviolet light. However, on further inspection of these strips, a very faint fluorescence was just visible at the lower edge adjoining the tip of the strip. Whether this was due to a significant amount of alkaloid has not yet been determined. Since the simple method of extraction gave a recovery of 91 per cent. (standard deviation 3 per cent.) this was considered satisfactory for the present work.

The losses of ergotoxine after chromatography on buffered citrate-phosphate paper (ether as solvent) ranged from 12 to 23 per cent. in the preliminary experiments. These losses were thought to be due to tailing of the spots on the paper. A small amount of alkaloid may thus lie outside the visually detectable fluorescent area and a significant loss might easily result when only the fluorescent spot is cut out and eluted. This view tends to be confirmed by the fact that ergometrine, which runs as a compact spot using the chromatographic method of Foster *et al.*, was recovered with only a 10 per cent. loss. 17·3 μg. of ergometrine was added to the paper, and after chromatography the average recovery for 4 assays was 88·5 per cent. (standard deviation 2·2 per cent.). It has been previously mentioned in this paper that the ascending method of chromatography gave more compact spots than the descending method. If tailing of the spots is the main course of the loss of alkaloid, then the ascending method should be more suitable. The apparatus used has the advantages: (a) simplicity, and (b) the volume of the vapour phase is kept to a minimum, which is of importance when using volatile solvents. The use of an ascending method in test tubes has been previously described[23], and this has been modified by the authors.

Apparatus. Pyrex test tubes 19 cm. long and 2·9 cm. bore were each fitted with a rubber bung slit on the lower surface to hold a filter paper strip 2·6 cm. wide and shaped as shown in Figure 14. The lower end of the strip was cut away, leaving a strip C, 2 to 5 mm. wide, which dipped into the solvent at the bottom of the tube. The width of this strip C and the depth to which it was immersed in the solvents determined the rate of flow of the solvent upwards. The alkaloid was applied as a spot on the line A-B. A suitable width for C was 2 to 3 mm., and when immersed 2 cm. in ether saturated with water the solvent front travelled 10 cm. above the starting line in 2 to 3 hours. A series of tubes may be run simultaneously and their temperature controlled by immersing them in a tank of water. For the equilibration of the buffered strips with water vapour, a similar set of tubes were used, containing about 5 ml. of water saturated with ether. The strips held by the rubber bungs were folded just below the line A-B and mounted in the tube so that the paper was suspended above the water. After 2 to 6 hours the bungs and damp papers were removed and transferred to the Pyrex tubes containing the solvent for the ascending run. The alkaloidal spots were quite compact, although if the ergotoxine exceeded 20μg. in 0·01 ml. a small residual ghost spot was observed at the starting line. The addition of 2 to 5 per cent. ethyl alcohol to the ether before it was saturated with water tended to reduce these ghost spots. They may be due to the alkaloid being retained in the matrix of the paper whilst the solvent tends to run on the surface. It seems probable that the increased water content of the ether-alcohol phase would give better penetration into the matrix of the paper. This point has been previously mentioned in Part I of this paper.

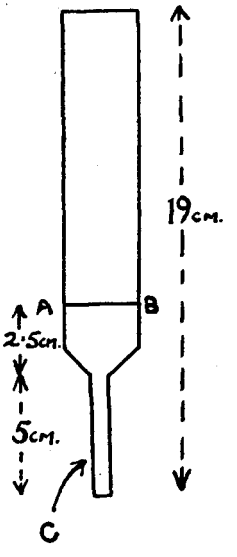


FIG. 14. Shape of filter paper used for the ascending method of paper chromatography.

The recoveries of various alkaloids are shown in Table II; 0·01 to 0·02 ml. of alkaloidal solution (50 per cent. ethanol) was added to the paper in each case. From the data in Table II, the average recovery of ergotoxine (extracted from the strips containing the ghost spot on the starting line and the ergotoxine spot) was considerably higher than in the case when the strips cut out for extraction did not extend back to the starting line. It would appear that the low recoveries are due to tailing of the ergotoxine, although this was not visible under ultraviolet light.

Buffered Cellulose Columns. Buffered kieselguhr columns have been used for separation of the solaneaceous and pomegranate alkaloids[2], but these have not been found satisfactory for the separation of the water-insoluble alkaloids of ergot. This may be due to the rate of flow of ether

through such columns being too rapid for complete equilibration of alkaloid between organic and aqueous phase. Buffered cellulose columns were found most satisfactory, and the data obtained from filter paper chromatography was found to be applicable to separations on these columns.

For preparation of the columns, 4 g. of cellulose* was mixed with 1·0 ml. of citrate-phosphate buffer and the powder packed dry into a glass tube of 1 cm. bore, into the lower end of which was fused a No. 3 sintered glass filter. The alkaloid was added to the column in the fol-

TABLE II

SEPARATION OF MIXTURES OF ERGOTOXINE AND ERGOCRISTININE BY PAPER CHROMATOGRAPHY

(USING CITRATE-PHOSPHATE BUFFERED PAPER, ASCENDING METHOD)

Solvent	pH of Paper	Ergocristinine			Ergotoxine		
		Added	Recovered	Percentage Recovery	Added	Recovered	Percentage Recovery
		μg.	μg.		μg.	μg.	
AE5	3·8	—	—	—	20·2	18·4*	91
AE2	3·8	—	—	—	20·2	19·0*	94
AE2	4·6	—	—	—	20·2	18·8*	93
AE5	3·8	—	—	—	13·2	10·5	80
AE5	3·8	—	—	—	13·2	11·8	96
AE5	3·8	13·2	11·0	83	12·5	10·5	84
AE5	3·8	13·2	11·0	83	12·5	9·8	79
AE5	3·8	13·2	12·0	91	12·5	11·5*	92
AE5	3·8	8·8	7·2	82	—	—	—
AE5	3·8	17·5	15·5	89	—	—	—
AE5	3·8	13·1	11·7	89	16·7	13·0	78
AE5	3·8	—	—	—	16·7	14·0	84

* " ghost spot " at starting line. The figure in the Table is recovery obtained by extracting the strip containing both ghost spot and ergotoxine spot.

AE2—Anæsthetic ether containing 2 per cent. of ethanol and saturated with water.

AE5— " " " 5 " " " " " " "

With solvent AE5 the ergocristinine was present as a band close to the solvent front.

lowing way:—1 ml. of alcoholic solution of alkaloid (200 to 500 μg./ml.) was transferred to a small glass dish and sufficient ether extracted kieselguhr added to absorb the liquid—it was gently warmed to remove the alcohol, and the powder transferred to the top of the column. The dish was rinsed with chloroform and further amounts of kieselguhr added. This was repeated a second time, the powder in each case being packed on to the top of the column as before. The chloroform must be completely removed from the kieselguhr at each stage. Failure to do this resulted in rapid movement of ergotoxine down the column due to the high solubility of the alkaloid in this solvent. The column was then

* Solka Floc purified by method of Cambell *et al.*[24] 8 g. of this cellulose was percolated with 100 ml. of ether before use.

developed with ether saturated with water, and the assay of the alkaloid in the eluent was done as follows:—

For the separation of a mixture of ergotoxine and ergocristinine the first portion of the eluate (containing ergocristinine) was collected in 5 ml. fractions. Ergotoxine, which was eluted much more slowly, was collected in fractions of 5 to 25 ml. To increase the rate of elution of ergotoxine the addition of 5 to 10 per cent. of ethanol to the ether was used. The collected fractions were then evaporated to dryness at room temperature. 1 volume of 1 per cent. tartaric acid solution (usually 2 ml), followed by 2 volumes of dimethylamino-benzaldhyde reagent was added, and the colour measured as before. The preliminary results obtained for the recoveries of ergotoxine and ergocristinine are given in Table III.

TABLE III

SEPARATION OF ERGOTOXINE AND ERGOCRISTININE ON BUFFERED CELLULOSE COLUMNS (CITRATE-PHOSPHATE BUFFER *p*H 3·4)

Ergotoxine (μg.)		Ergocristinine (μg.)	
Added	Recovered	Added	Recovered
182	196	0	—
234	225	0	21
234	242	200	200
484	461	196	181

DISCUSSION

The separations achieved indicate the scope of this method as a basis for the analysis of mixtures of ergot alkaloids. In this paper the number of alkaloidal separations is limited by the availability of the pure alkaloids, and as further alkaloids are available this method will be extended. In chromatography on buffered paper the separation of ergometrine-ergometrinine has not yet been studied. From published partition coefficients it would appear to be necessary to use paper buffered at *p*H 7 and above, to obtain a satisfactory partition between ether and the buffer. In the range *p*H 2 to 5 using ether as solvent these alkaloids together with the lysergic acids remain stationary whilst the water-insoluble alkaloids are widely separated. Thus from two chromatograms the presence of the chief ergot alkaloids may be detected rapidly and with a minimum of material. Ergotoxine and ergotamine can be identified when both are present and the method is more rapid than that of Foster, which depends on identification of the characteristic aminoacids produced by hydrolysis of these water-insoluble alkaloids. If the quantitative recoveries of the alkaloids from the paper chromatograms can be improved, then a very attractive method for both qualitative and quantitative work is available. The buffered cellulose column method offers certain advantages over the paper method, since it is operative over a

much wider range of alkaloid loads and it is at least as accurate as that of the paper method. The two methods are complementary since the information gained from a qualitative run on paper is a valuable guide to the positions of the alkaloids on the columns where they occupy the same relative positions and modifications of solvents and buffer systems can be made with the minimum amount of material.

For column work it is advantageous to collect small successive volumes (say 5 ml.) of eluate for assay, and then plot the curve of alkaloid content against the number of fractions as Partridge has done[2]. The curves so obtained will indicate whether or not complete separation of the alkaloids has been achieved. The alternative method of viewing the column in ultra-violet light, and collection of the fractions containing the fluorescent zones as they moved off the column was found not to be reliable since alkaloid was often detected in the eluate before and after the fluorescent band was at the base of the column. A certain amount of fluorescent material was also extracted from the cellulose which interfered with the fluorescence test for presence of alkaloids in the eluate.

A paper has recently been published[19] on a method of counter current extraction, based on the partition of the ergot alkaloids between buffer solutions and an organic phase. Partition between ether or benzene and phosphate buffers was the basis of the process, and the results obtained agree closely with those of the present authors.

We are indebted to Dr. G. E. Foster, of the Wellcome Chemical Works, for supplying specimens of egocristinine and lysergic and *iso*-lysergic acids. Part of this work was carried out by one of the authors (J.E.C.) during the tenure of an Allen and Hanbury's Fellowship.

Summary, Part I

1. Alkaloids may be detected on buffered paper chromatograms by a solution of 0·5 per cent. iodine in 1 per cent. potassium iodide. A more sensitive reagent is the modified Dragendorff's reagent of Mächebœuf and Munier[10].

2. Buffered paper was found satisfactory for the separation of certain mixtures of alkaloids such as atropine, hyoscine, and apoatropine which may be separated by development with butanol on phosphate or citrate buffered papers.

3. The effect of change of *p*H, type of buffer and solvent used, have been investigated and graphs are shown for some of the separations obtained.

4. Certain solvent mixtures of chloroform, carbon tetrachloride and/or light petroleum (30° to 60°C.) showed R_F values for hyoscine near to unity while the atropine was only slightly soluble in the moving organic phase.

Summary, Part II.

1. A method for the separation of certain water-insoluble alkaloids of ergot by partition chromatography has been described.

2. The effect of change of *p*H, type of buffer and solvent used, have

been investigated and graphs are shown for some of the separations obtained.

3. Results are given for the quantitative estimation of alkaloids by extraction from paper chromatograms and subsequent colorimetric assay. The ascending method of paper chromatography was found most suitable for quantitative methods using filter paper.

4. A quantitative method for the separation of mixtures of ergotoxine and ergocristinine on buffered cellulose columns has been described.

5. In the systems used no resolution of ergotoxine into its components was obtained.

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DISCUSSION

The paper was presented by MR. J. CARLESS.

DR. W. MITCHELL (London) asked the authors whether they were aware of any other work on *Atropa Belladonna* in which hyoscine had been properly identified, as, from his own experience, he doubted whether *Atropa Belladonna* contained hyoscine. The green and brown bands obtained with tincture of belladonna on columns of alumina might be due to added colour. In his experience, chlorophyll, when adsorbed on alumina, underwent very considerable changes, and was difficult to elute.

DR. J. M. ROWSON (London) said that the work under discussion agreed with the results of his own previous work. Had the authors investigated hyoscyamus leaf as well as the commercial dry extract?

DR. G. FOSTER (Dartford) asked whether extracts from species of *Duboisia* had been examined. The detection of ergocristinine when it seemed that it should have been ergocorninine rather implied that those two alkaloids ran together and had the same R_F value. The use of ergo-

toxine ethanesulphonate by the authors as the source of their ergotoxine implied that the material would contain 90 per cent. of ergocornine and 10 per cent. of ergocristine. Had the authors examined the amino-acids obtained by the hydrolysis of the ergotoxine which they had used in their experiments ?

MR. CARLESS, in reply, said that the amino-acid hydrolysis showed the presence of both ergocornine and ergocristine in his sample of ergotoxine. Some difficulty was experienced in the detection of ergokryptine, because in the only chromatogram which was run problems were encountered in the separation of phenylalanine and leucine, which were characteristic of ergocristine and ergokryptine respectively.

MR. WOODHEAD, also in reply, was of the opinion that hyoscine was definitely present in *Atropa Belladonna*. On the question of added colour he suggested that the leaf might have been overheated when it was dried. There was definitely a green pigmentation, but he was being charitable when he suggested that it was chlorophyll. It was of interest that the tincture showed some racemisation of hyoscyamine to atropine. They had not examined any species of *Duboisia* but had examined an old sample of duboisine sulphate and suspected that it was completely hydrolysed to tropine, as it did not behave like any of the other solanaceous alkaloids.

PROFESSOR H. BRINDLE (Manchester) added that work had been carried out by Dr. Rowson's process, although it had been modified so that a complete separation of hyoscine from atropine and hyoscyamine had been effected. The process, which was very laborious, depended upon a separation at a definite *p*H (8·3 to 8·5). On the average, hyoscine formed 2 to 3 per cent. of the total alkaloids.