RAUWOLFIA ALKALOIDS: FRACTIONATION BY COUNTERCURRENT DISTRIBUTION*

BY R. P. BANERJEE, M. L. CHATTERJEE AND H. F. HÄUSLER†

From the Department of Pharmacology, School of Tropical Medicine, Calcutta

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INTEREST in the therapeutic value of rauwolfia has prompted many investigations to find newer active principles therein. A recent review[1], in cataloguing the alkaloids so far reported from different varieties of rauwolfia, lists more than two dozen alkaloids isolated from *R. serpentina* alone though a few of them, it is stated, may be identical. The catalogue may be further extended by including those found in other varieties of rauwolfia, namely *R. canescens*, *R. caffra*, *R. vomitoria*, and *R. heterophylla*.

In isolating and studying the properties of the alkaloids, investigators have used as starting materials drugs collected from widely scattered regions of the world. In the earlier work of Siddiqui and collaborators[2–5], *R. serpentina* roots from Behar were used leading to the isolation of five crystalline alkaloids, namely ajmaline, ajmalinine, ajmalicine, serpentine and serpentinine. Van Italie and Steenhauer[6] used an African variety of the same drug to isolate rauwolfine. Later work on *R. serpentina* Benth has produced many more alkaloids including reserpine[7], sarpagine[8], rauwolfinine[9], reserpinine[10], rauhimbine, and isorauhimbine[11]. Chatterjee and Bose[12] have found serpine in *R. serpentina* roots of the Cochin (S. India) variety where ajmaline is reported to be entirely absent. A strongly active alkaloid, rescinamine, was reported by Klohs and his colleagues[13] in the oleoresin fraction.

In addition to those mentioned above other alkaloids have been isolated or reported to be present in *R. serpentina* from different sources and further additions must be expected.

Two principal preparations have been applied therapeutically: (i) the total alkaloids consisting of an alcoholic percolate of the roots, modified or adjusted to proper alkaloid concentration and (ii) reserpine, alone or in combination with other hypotensive and sedative drugs.

The pharmacology of reserpine has been worked out in great detail by Bein[14], Plummer and others[15], and Gaunt and others[16]. Several workers have studied the pharmacology of other individual alkaloids like ajmaline[17–19], serpentine[20–21], rauwolfinine[22], serpine[23] and rescinamine[24]. Notwithstanding these studies, the pharmacology of the total alkaloidal preparation is far from being completely understood. The "total alkaloids" is a mixture of many components and the effect of administering the total alkaloids is naturally dependent on an interplay of actions

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† WHO Professor of Pharmacology 1954–55, Head of the Pharmacological Institute, University of Graz, Austria.

of the individual alkaloids some of which incidentally may possess antagonistic properties. An understanding of the pharmacology of the total alkaloids is not easy in view of the difficulty of obtaining the pure alkaloids.

Understanding of the pharmacology of rauwolfia would be hastened if it were possible to know what specific alkaloids occur in a variety of rauwolfia and in what proportions. Not all the catalogued alkaloids would be found in any single variety harvested from a specific locality. The present investigations were undertaken with a view to exploring the possibility of the recently introduced technique of countercurrent distribution to achieve the ends stated above. Experiments described here concern the soluble alkaloids only, and exclude those found in the oleoresin fraction.

TECHNIQUE

The technique of the countercurrent distribution as developed by Craig and his collaborators has already found many applications in organic and biological chemistry in testing the purity of preparations and particularly in isolating individual components from a mixture of closely related entities. Based on the fundamental postulation of Nernst[25] on the specificity of distribution coefficient for a solute between two immiscible solvents, the method has grown in the last few years into a valuable fractionation tool with wide applicability[26]. Fried and others[27] have employed the technique, using a small scale apparatus, for the fractionation of germidine and germitrine, new alkaloids from *Veratrum viride*. Dorfman and others[28] report about the use of countercurrent extraction in the isolation of reserpine from the oleoresin fraction of *Rauwolfia serpentina*. Some other profitable applications of the technique include the isolation of metabolites of quinine in a pure form[29].

Apparatus

A cylindrical unit consisting of a circular array of stainless steel tubes described by Craig and others[30] has since been discarded in favour of all glass systems. A medium sized glass train of 80 units was designed and built, following essentially the same design for equilibration cell and rocker unit used by Craig in recent work. In trial experiments a mixture of known amino-acids was quantitatively separated by distribution between *n*-butanol and 5 per cent hydrochloric acid[31].

Material and Methods

Ground roots (2 kg.) of *R. serpentina* Benth (Dehra Dun) were exhaustively percolated by 95 per cent ethanol in the cold. The combined percolate was concentrated under reduced pressure and the resulting syrupy mass poured dropwise into a mechanically stirred volume (500 ml.) of 2 per cent hydrochloric acid. The stirring was continued for two hours and the dark brown solution was filtered off. The insoluble material including the resinous mass was again treated with 200 ml. of 2 per cent hydrochloric acid for one hour with constant stirring and filtered. The combined filtrates were made alkaline (pH 11·5) with caustic soda and

completely extracted with chloroform (tested with Meyer's reagent). The deep brown combined chloroform extract was extracted again with 2 per cent hydrochloric acid to remove colour and non-alkaloidal impurities followed by alkalization of acid extract and finally extraction with chloroform.

Preliminary Distribution Studies

An aliquot of the chloroform extract was concentrated to 20 ml. and 10 ml. portions put into the first two tubes of the countercurrent distribution apparatus. After being distributed between chloroform (lower phase) and 0·2M phosphate buffer pH 4·7 (upper phase) using 34 transfers

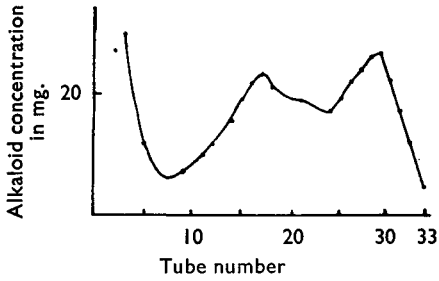

FIG. 1. Showing the alkaloid content (in mg.) of countercurrent tubes plotted against corresponding tube no. Solvents: chloroform and 0·2M phosphate buffer pH 4·7.

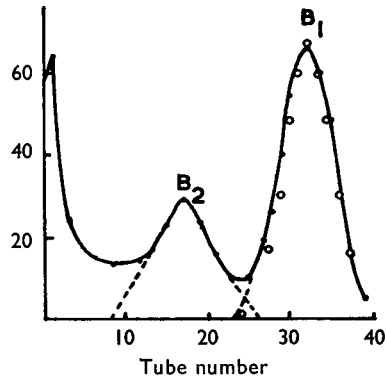

FIG. 2. Showing the countercurrent distribution of tentative fraction A between chloroform and 0·2M phosphate buffer pH 5·6. White circles represent points calculated for ajmaline.

the alkaloid distribution pattern was worked out using gravimetric method of analysis.

The concentration of solute in chloroform phase was determined by directly weighing the residue from 2 ml. aliquots after a wash with dilute ammonia water, using small glass crucibles described by Craig[32].

Alkaloid concentration in the aqueous phase was determined by alkalizing an aliquot, extracting into chloroform, and gravimetric determination of the washed chloroform layer using similar containers for evaporation.

A plot of the alkaloid content against tube number results in a distribution pattern exemplified by Figure 1. Besides yielding expected evidence of heterogenity in the system, this distribution pattern helps to plan future experiments. The material contained in the band represented by tube Nos. 24 to 33, and 8 to 23, for example, can be taken out and subjected to further enquiry using other solvents.

Tentative Fractionation by Extraction with Buffer

Based on the preliminary distribution experiments the following procedure was adopted. A chloroform solution of the total alkaloids

represented by the original chloroform extract is repeatedly extracted in a separating funnel with portions of 0·2M phosphate buffer of pH 4·7 to dissolve a major part of the alkaloids. The combined chloroform extracts are designated tentative fraction A. The chloroform residue is further extracted repeatedly, using a 0·2M phosphate solution having pH 3·0 (prepared by adding 0·2M phophoric acid to 0·2M potassium biphosphate until the resulting pH is 3·0) when further alkaloids are brought into the aqueous phase to make the tentative fraction B. The residue from this step[2] contains other alkaloids and is designated tentative fraction C.

Countercurrent Distribution of Tentative Fractions using Different Buffers

The material in fraction A, transferred to a small volume of chloroform, is again distributed between chloroform and 0·2M phosphate buffer pH 5·6 using 39 transfers. Analysis of the resulting alkaloid distribution pattern (Fig. 2) reveals the presence of two different components. A

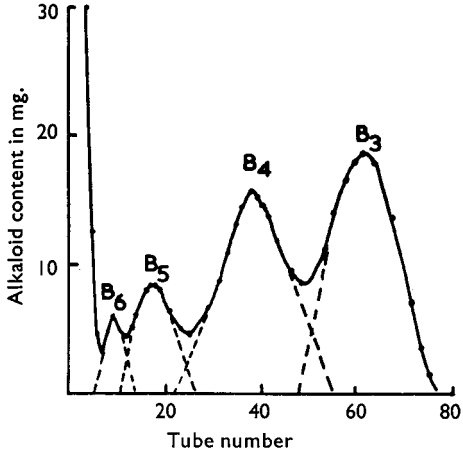

FIG. 3. Showing the countercurrent distribution of tentative fraction B between chloroform and 0·2M phosphate (pH 3·6).

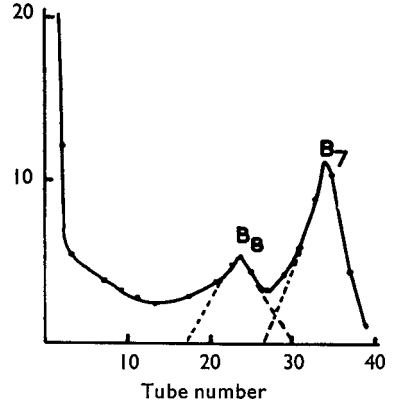

FIG. 4. Showing the distribution of tentative fraction C between chloroform and 0·2M phosphate (pH 1·8).

small amount left over in the first few tubes represents other components having small K-values for buffer (4·7)-chloroform system but carried over in minute quantities. This small amount is therefore mixed with tentative fraction B which by similar manipulation is countercurrently distributed between chloroform and a phosphate solution of pH 3·6 when a pattern represented by Figure 3 is revealed on analysis. The presence of four components is made obvious on examination of the distribution pattern. Countercurrent distribution of tentative fraction C (together with left-over from fraction B) between chloroform and 0·2M phosphate solution of pH 1·8 resolves the fraction into a further two components (Fig. 4). The residue from this operation constitutes a considerable proportion (about 60 per cent) of the starting material (tentative

fraction C). It shows copious precipitation with Meyer's reagent, and is amenable to further fractionation.

Results and Discussion

By operations like the above more than eight clear fractions are obtained from the original material. The fractions may be recovered quantitatively by permitting the operation to continue with a larger number of transfers until the peaks completely disengage from each other.

Identity of Countercurrent Fractions

The identity of the isolated alkaloids may be established chemically or by reference to the known partition ratios where pure alkaloids are available. A knowledge of these constants enables the positions of their maxima in hypothetical countercurrent distribution to be calculated by making use of the equation[26]:

$$N = \frac{n.K.r}{K.r + 1}$$

where N is the position of the maximum,
n = total number of transfers,
K = partition ratio,
r = ratio of volume of upper and lower phases.

Only some rauwolfia alkaloids are available in pure form. A specimen of pure ajmaline which was available gave a value of 4·6 for partition constant between 0·2M phosphate buffer of pH 5·6 and chloroform. Using the above equation the calculated position for maximum corresponds exactly to the experimentally found maximum for the fraction B_1.

An assessment of the purity of one countercurrent fraction could however be made, taking the pure sample of ajmaline as guide. Employing the experimentally determined value of 4·6 for K, the theoretical distribution curve for ajmaline could be calculated using the equation[26]

$$y = \sqrt{(2\pi nK/[K+1]^2)}\ \exp\left(-\frac{x^2}{2nK/(K+1)^2}\right)$$

where y = the fraction of substance in a given tube,
K = partition ratio,
n = number of transfers,
x = distance from the maximum of the tube in question.

Since the position of maximum in actual distribution coincided with the position of experimentally determined value of N for ajmaline, for example tube 32, other points in the theoretical distribution pattern for ajmaline are obtained from the above equation by calculation. Figure 2, where the calculated points for ajmaline have been shown together with the experimental curve for fraction B, indicates good agreement. The countercurrent fractions would therefore make excellent starting materials for the preparation of the respective alkaloids in a pure form.

Assay of Preparations

The alkaloid distribution curves may be used to assay the total individual alkaloidal content. A complete separation of the peaks by a large number

of transfers may be dispensed with and a situation represented by Figures 2–4 may suffice. In the estimation of plasma or serum components by electrophoretic techniques, it is often permissible to exercise some judgement for the construction of individual protein peaks by extrapolation in these regions where adjacent peaks might overlap. In the same way the alkaloid distribution patterns obtained in the above experiments may be drawn, but always keeping in mind the requirements for quantitative representation. The experiment depicted in Figure 4 concerned with fractionation of tentative fraction C was, for example, made with material obtained from 0·6 kg. of dried roots in order to have sufficient mass to start with, while 0·3 kg. roots would be found more than adequate for the treatment of tentative fraction A. Where making quantitative interpretation it would of course be necessary to reduce the curves to proper scale as in Figure 5. The proportion of each component in the total preparation is obtained by dividing the area under the corresponding peak by the total area covered, a procedure familiar in quantitative paper electrophoresis. Calculation, in this manner of the percentage of fractions B_1, B_2, etc., gives the following values: B_1 46; B_2 20·5; B_3 10·1; B_4 9·38; B_5 2·74; B_6 1·0; B_7 3·13; B_8 1·61 per cent.

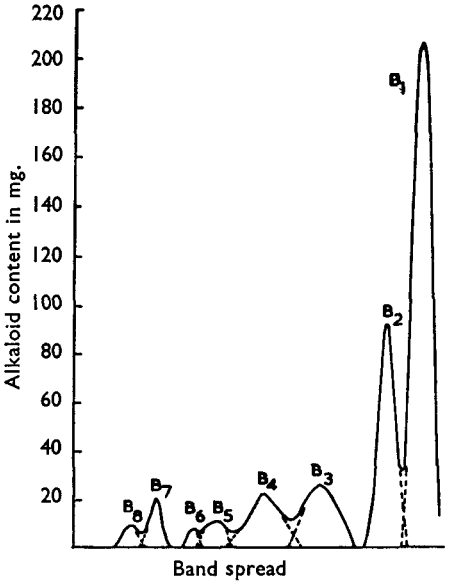

FIG. 5. Showing the quantitative pattern as would result following fractionation by countercurrent distribution of 4090 mg. total alkaloids.

The quantitative assessment of alkaloid distribution in varieties of *Rauwolfia serpentina* harvested from different regions and under different conditions is of interest and economic significance. It is intended here only to point out the possibility of applying the above technique to the problem.

The Character of the Fluorescence in Ultra-violet Light. Preparations of total rauwolfia extracts always show a very strong fluorescence. Some earlier work[33] has been done on the assumption that the fluorescence intensity in dilute solution could be taken to be a measure of the alkaloid concentration and hence measurement of fluorescence could be employed for the estimation of microquantities of alkaloids in urine or other biological materials. It is interesting to find here that the assumption does not hold good for a preparation of total alkaloids. It was considered worth while to follow the fate of the fluorescence as the fractionation of the alkaloid mixture by countercurrent distribution progressed. The

fluorescence intensity of a diluted (1 : 50) portion of the upper (buffer) phase was measured in a Lumetron Fluorescence meter against a quinine standard (1·2 μg./ml.) and plotted against corresponding tube no. in Figure 6 in which is plotted the relative fluorescence intensity of upper phase against its alkaloid content. The fluorescence though fractionating at the same time, shows maxima in regions not always corresponding to the alkaloid maxima except in two cases, i.e., B_3 and B_7. While it is probable that B_3 and B_7 may be fluorescing alkaloids, it is evident that the rest of the fluorescence distribution throughout the countercurrent train cannot belong to any principal alkaloid and must belong to other material having little relative mass. For example, in the case of B_1, very high intensity of the fluorescence in the upper phase to the right of the alkaloid maximum has not been able to distort the weight distribution curve to any appreciable extent from its symmetrical nature. This holds good for several other regions. These findings call for caution in employing the observation of fluorescence under ultra-violet for the purpose of locating and assigning R_F values to these alkaloids on paperchromatograms as practised by Pillay and others[34].

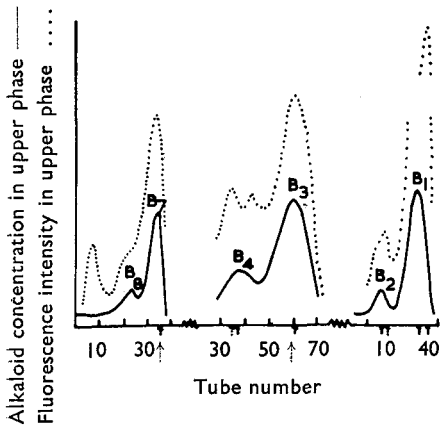

FIG. 6 (not to scale). Where the fluorescence intensity of upper phases (in arbitrary units) has been compared with the alkaloid concentration (in mg.) in the same. The presence of several fluorescence maxima are apparent, but while two of them coincide with the alkaloid concentration maxima for B_3 and B_7, others do not.

SUMMARY

1. A crude preparation of total alkaloids of *Rauwolfia serpentina* Benth yields on countercurrent distribution between chloroform and selected phosphate buffers more than eight fractions containing different components in nearly pure form. These fractions are convenient starting points for the preparation of pure crystalline materials.

2. Countercurrent distribution of total alkaloids in the above manner permits the assay of different preparations.

3. The strong greenish-blue fluorescence usually associated with total extract is composite in nature. While evidence has been obtained for the presence of two fluorescent alkaloids, most of the fluorescence appears to be non-alkaloidal in origin and associated with a molecule of little comparative mass.

4. The pharmacological evaluation of countercurrent fractions may aid in unravelling the intricacies of the pharmacology of total alkaloids.

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