THE PARTITION CHROMATOGRAPHY OF ALKALOIDS

PART III.—THE ALKALOIDS OF *PUNICA GRANATUM*

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Received June 21, 1950.

THE systematic study of anthelmintics appears to have been hitherto totally incommensurate with the importance of helminthiasis, which in various forms affects an estimated 800 million people[1]. In particular, traditional remedies have been subjected to little critical examination. The difficulties encountered in the *in vitro* culture of many parasitic worms[2] and in testing anthelmintics are amongst the important reasons for this. Recently progress has been made in the development of *in vitro* tests, one of which[3] employs the liver fluke and would appear to be of value in estimating the comparative activities of anthelmintics, particularly against trematode and cestode parasites.

Preparations of the root of *Punica Granatum* have long been in use as tænifuges and the so-called "pelletierine tannate" of the Pharmacopœia is an example of such a preparation which is intended to contain the active constituents of this drug. A thorough search of the literature has revealed only the work of von Schroeder[4] in 1884, who showed that pelletierine was toxic to tapeworms; this alkaloid has, as a consequence, been considered to be the active component of the mixture of alkaloids present in *Punica Granatum*. It therefore appeared of interest to investigate the separation of the alkaloids of pomegranate, their relative proportions in the crude drug and in commercial samples of "pelletierine tannate" and to obtain more information on their relative activities as anthelmintics.

Four alkaloids of *Punica Granatum* have been isolated and characterised, namely, pelletierine, methyl*iso*pelletierine, *pseudo*pelletierine and *iso*pelletierine[5]. Because of the inaccessibility of experimental material and since a partial fractionation of pomegranate alkaloids by fractional liberation of the bases from their sulphates has already been effected[6,7,8], it was considered that an extension of the methods described in previous parts of this series of communications would provide a simple means of separating these alkaloids.

EXPERIMENTAL

Fractionation of "Pelletierine Tannate."—The conditions applicable to the partition chromatographic separation of pomegranate alkaloids were investigated, using two commercial samples of "pelletierine tannate," one purchased in England, the other in France.

The " pelletierine tannate " was assayed; the total bases were liberated with sodium hydroxide, collected in chloroform, recovered as their sulphates in an excess of standard acid and, after evaporating the chloroform, their titre was determined by back titration of the excess of acid, using bromocresol green as indicator.

Because it was found in *ad hoc* experiments that some of the basic material co-distilled with chloroform during evaporation of a chloroform solution of the alkaloids, and that aqueous sodium hydroxide caused their resinification, a special procedure was adopted for the preparation of the alkaloids for chromatographic separation. The " pelletierine tannate " was dissolved in N sodium hydroxide and the mixed bases were immediately extracted with chloroform; from the chloroform solution, the alkaloids were collected in a small excess of N sulphuric acid and the aqueous solution was concentrated under reduced pressure to a small volume. This solution was saturated with sodium phosphate, and 4N sodium hydroxide, equivalent to the N sulphuric acid used in extracting the alkaloids, was added. The resulting solution was absorbed on twice its weight of kieselguhr, and this material was packed on top of a previously prepared chromatographic column.

The partition chromatographic columns were similar to those described in Parts I and II[9,10] and consisted of kieselguhr on which was distributed phosphate buffer. The technique for the elution and recovery of the alkaloids was the same as that previously described. Throughout these experiments, we have found the method of packing chromatographic columns described by Martin[11] to be extremely useful. By systematic experiments, using quantities of mixed bases equivalent to about 30 to 40 mg. of *pseudo*pelletierine, a study was made of the optimum conditions for fractionation on a column containing 5 ml. of 0·5 M phosphate buffer distributed on 10 g. of kieselguhr. The homogeneity of each alkaloid fraction was checked by repeating the process on a column containing a buffer of lower *p*H value and a higher proportion of kieselguhr and buffer to alkaloid.

Although kieselguhr is usually regarded as having a low adsorptive capacity for alkaloids, it was found that even after development of the column with a chloroform solution of ammonia, some bases were retained on the column. After extrusion of the column, the alkaloids could still not be desorbed by shaking with N sodium hydroxide and chloroform. Accordingly the adsorbed alkaloids were recovered as their sulphates. It was found that the major portion of the alkaloids which were adsorbed by kieselguhr could be submitted to chromatographic separation on a column consisting of phosphate buffer distributed on " Pyrex " glass in No. 60 powder; 7 g. of the glass supported 1 ml. of buffer.

For the identification of the alkaloids, and for their isolation in quantities sufficient for comparison of their anthelmintic activity, the small-scale experiments which afforded the maximum enrichment of the different fractions were repeated on a larger scale. In order to obtain sharp separations in these experiments, it was found necessary to employ

a column built up of two units as described by Claesson[12]. By this means, quantities of mixed alkaloids equivalent to up to 5 g. of *pseudo*-pelletierine were fractionated.

The procedures described in Parts I and II were adopted for the identification of the alkaloids as their picrates and reineckates in the eluate fractions corresponding to peaks in the graphs.

Fractionation of the alkaloids of Punica Granatum.—The materials available consisted of four samples (20 to 90 g.) of museum specimens of pomegranate root bark, the bark (190 g.) and wood (300 g.) of fresh young roots from Poona, bark (180 g.) of old roots from Poona and fresh whole roots (770 g.) from Hong Kong.

The crude drug, dried at below 60°C. and in moderately coarse powder, was mixed with 30 per cent. of its weight of calcium hydroxide, moistened with water, and kept for 4 hours. After packing in a percolator, the drug was macerated for 24 hours with alcohol (70 per cent.) and percolated with alcohol (70 per cent.). The percolate was acidified with dilute sulphuric acid, precipitated calcium sulphate was removed, the filtrate was concentrated to a small volume, filtered to remove resin and washed with chloroform. Alkaloids liberated by sodium hydroxide were collected in chloroform as rapidly as possible. The total titre of the alkaloids in the chloroform, and hence also the proportion of alkaloids in the drug, were calculated from the titre of an aliquot portion. An equivalent of N sulphuric acid was shaken with the chloroform in order to recover the mixed alkaloids as an aqueous solution of their sulphates. This solution was treated in the manner described earlier for the preparation of the alkaloids derived from " pelletierine tannate " for chromatographic separation. Chromatographic separation and identification of the alkaloids were effected in the same way as outlined in the description of the fractionation of " pelletierine tannate."

Results

In the course of experiments designed to yield information on the conditions necessary to achieve separation of the alkaloids, the effect of changes in the *p*H value of the phosphate buffer, of the quantity of mixed alkaloids placed on the chromatographic column, the dimensions of the column and the rate of flow of eluting solvent were studied. The results obtained revealed no new feature additional to those recorded in Part I, and are therefore not described in detail here.

Alkaloids of " Pelletierine Tannate."—Figure 1 shows the separation of the alkaloids from " pelletierine tannate " under the empirically determined optimum conditions at *p*H 6·8, using ether for the elution of alkaloids corresponding to peaks 1A and 1B and chloroform for alkaloids corresponding to peak 1C. From an examination of Figure 2, it can be seen that no further fractionation was achieved when the alkaloids corresponding to Figure 1, peaks 1A and 1B, were refractionated at *p*H 6·5 (curve 2A, 2B), and those corresponding to Figure 1,

peak 1C, were refractionated at *p*H 5·9 (curve 2C). The course of the fractionation of alkaloids equivalent to 5 g. of *pseudo*pelletierine on the Claesson multiple column is shown in Figure 3 (curve 3B, 3C) and in curve 3B′, 3C′, the fractionation of alkaloids equivalent to 0·6 g. of *pseudo*pelletierine from the second sample of "pelletierine tannate" is shown.

Figure 4 refers to the fractionation of the alkaloids from the English sample of "pelletierine tannate" which are adsorbed by kieselguhr, but

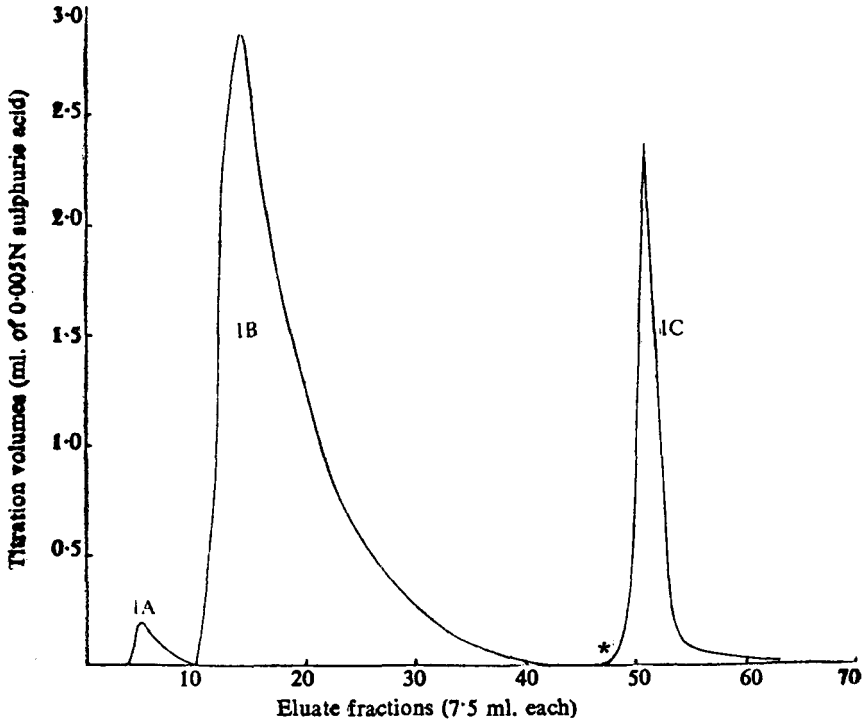

FIG. 1. Separation of alkaloids from "pelletierine tannate" equivalent to 30 mg. of *pseudo*pelletierine. Column, 10 g. of kieselguhr with 5 ml. of 0·5M phosphate buffer, *p*H 6·8.
* Chloroform used as eluant.

may be submitted to partition chromatography on phosphate buffer—glass powder columns. From curve 4D, 4E, it is seen that only a partial separation of two bases can be achieved. The phosphate buffer—glass powder partition chromatogram of the kieselguhr-adsorbed alkaloids from "pelletierine tannate" of French origin is summarised in Figure 5. This material contained one major basic component (curve 5D). Curve 5E refers to authentic pelletierine isolated from the crude drug and passed through a partition chromatographic column under identical conditions; comparison of curves 5D and 5E indicates that the two bases corresponding to the major peaks are not identical.

The characters of derivatives prepared from eluate fractions corresponding to peaks on the curves representing the different partition chromatograms are given below:—*Peaks 1B, 2B, 3B:* Picrate, needles from water, m.pt. 252°C. (with decomposition); Hess[13] records the same m.pt. for *pseudo*pelletierine picrate.

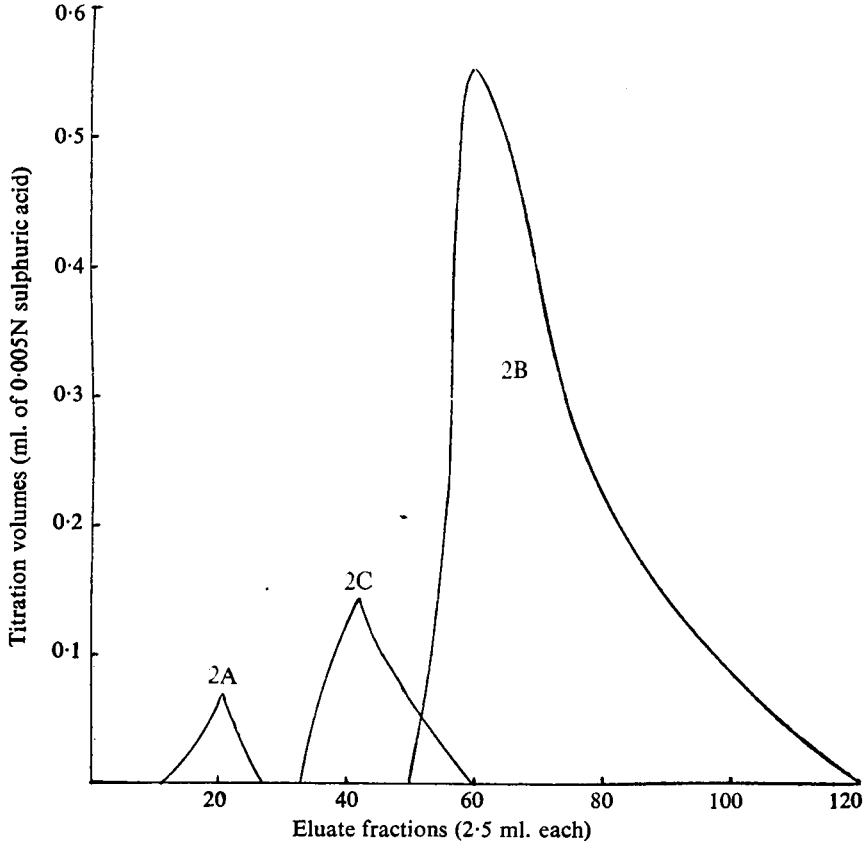

FIG. 2. 2A, 2B—Refractionation of alkaloids corresponding to 1A, 1B at *p*H 6·5; eluant, ether. 2C—Refractionation of alkaloid corresponding to 1C at *p*H 5·9; eluant, chloroform. Columns, 10 g. of kieselguhr with 5 ml. of 0·5M phosphate buffer.

Peaks 1C, 2C, 3C: Picrate, rosettes from absolute alcohol, m.pt. 154°C.; methyl*iso*pelletierine isolate by Hess[13] had identical m.pt.

Peaks 4D, 5D: Picrate, insoluble amorphous powder, m.pt. 208°C. (with decomposition), which decomposed when recrystallisation was attempted; Reineckate, leaflets from aqueous acetone, m.pt. 240°C. (with decomposition); found SCN, 49·6, 49·2 per cent.; *Z*, 150, 154.

Peak 4E: Picrate, after two recrystallisations from water separated as rosettes, m.pt. 150°C., depressed to 128° to 130°C. by methyl*iso*pelletie-

rine picrate. Hess[14] describes pelletierine picrate as having m.pt. 150° to 151°C.

A further fraction of basic material which was not eluted from the phosphate buffer—glass powder columns by chloroform failed to yield any solid derivative, even after extensive further fractionations.

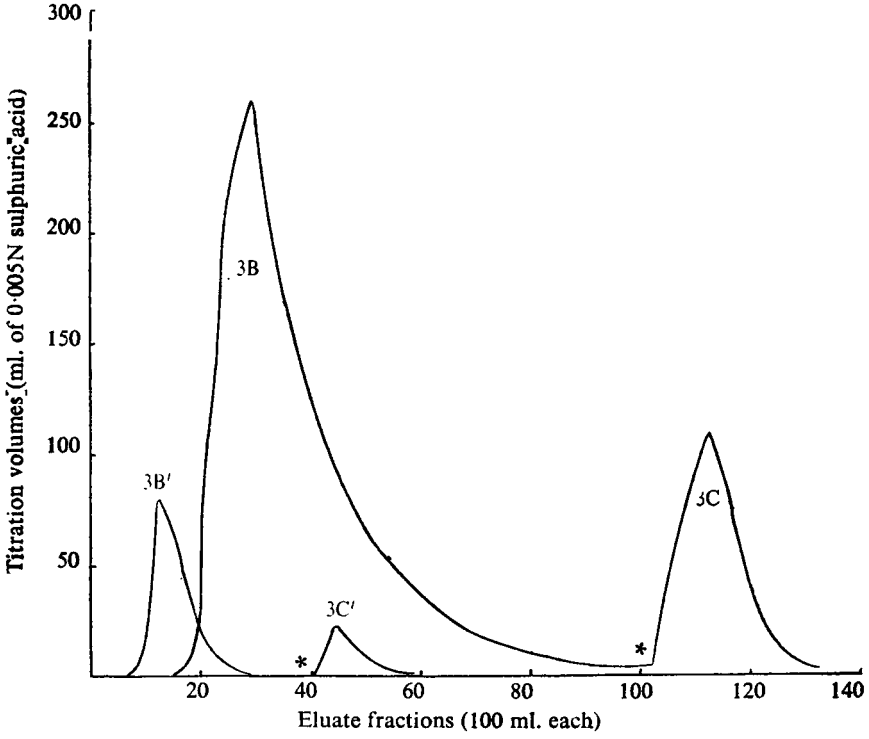

FIG. 3. 3B, 3C—Separation of alkaloids from "pelletierine tannate" equivalent to 5 g. of *pseudo*pelletierine. Column, 650 g. of kieselguhr with 325 ml. of 0·5M. phosphate buffer, *p*H 6·8. 3B′, 3C′—Separation of alkaloids from "pelletierine tannate" equivalent to 0·6 g. of *pseudo*pelletierine. Column, 200 g. of kieselguhr with 100 ml. of 0·5M phosphate buffer, *p*H 7·0.

* Chloroform used as eluant.

TABLE I

ALKALOIDS OF "PELLETIERINE TANNATE"

Sample	Peak 2A* bases per cent.	Peaks 3B, 3B′, ψ-pelletierine per cent.	Peaks 3C, 3C′ methyl*iso*-pelletierine per cent.	Peak 4E, pelletierine per cent.	Unidentified bases* per cent.	Total bases† per cent.
English "pelletierine tannate" ...	0·85	63	14	4·1	18	9·2
French "pelletierine tannate" ...	0·70	64	16	nil	19	10·3

* Calculated as ψ-pelletierine. † Calculated as ψ-pelletierine in original "pelletierine tannate."

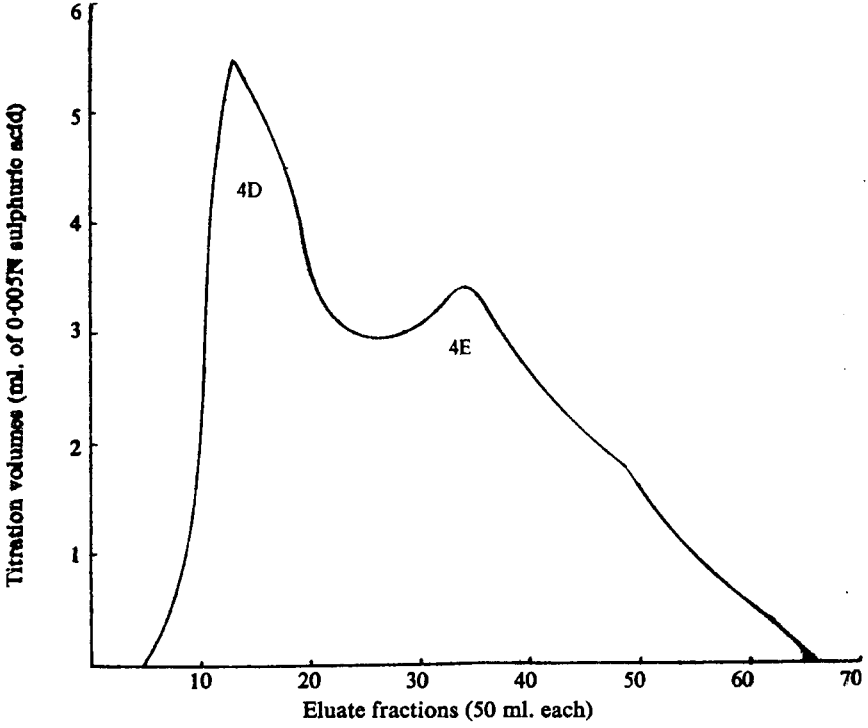

FIG. 4. Fractionation of English "pelletierine tannate" alkaloids equivalent to 250 mg. of *pseudo*pelletierine. Column, 560 g. of glass powder with 80 ml. of 0·5M phosphate buffer, *p*H 7·4; eluant, chloroform.

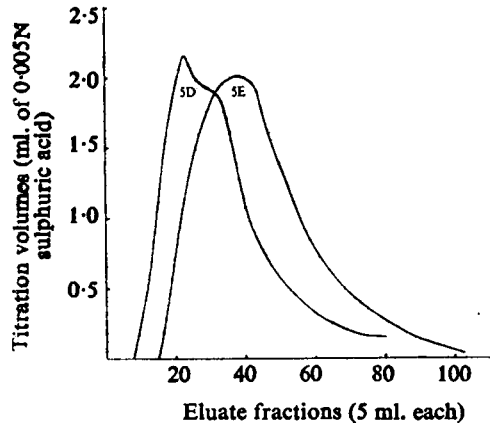

FIG. 5. 5D—Fractionation of French "pelletierine tannate" alkaloids equivalent to 75 mg. of *pseudo*pelletierine. 5E—Partition chromatogram of pelletierine (75 mg.). Columns, 140 g. of glass powder with 20 ml. of 0·5M phosphate buffer, *p*H 7·3; eluant, chloroform.

The quantitative data obtained from the two samples of "pelletierine tannate" are summarised in Table I.

Alkaloids of Punica Granatum.—A typical curve obtained with a phosphate buffer—kieselguhr column showing the course of fractionation of the alkaloids extracted from the fresh bark of the roots of *Punica Granatum* is given in Figure 6.

Alkaloids adsorbed by the kieselguhr afforded a single peak (Figure 5, curve 5E) after recovery and passage through a phosphate buffer-

glass powder column. The characters of the alkaloids in the eluate fractions are summarised below:—

*Peak 6B: pseudo*pelletierine picrate, needles from water, m.pt. 252°C. (with decomposition); *Reineckate*, plates from aqueous acetone, m.pt. 216°C. (with decomposition); found SCN, 49·3 per cent.; $C_9H_{15}ON$, $H[Cr(SCN)_4(NH_3)_2]$ requires SCN, 49·2 per cent.

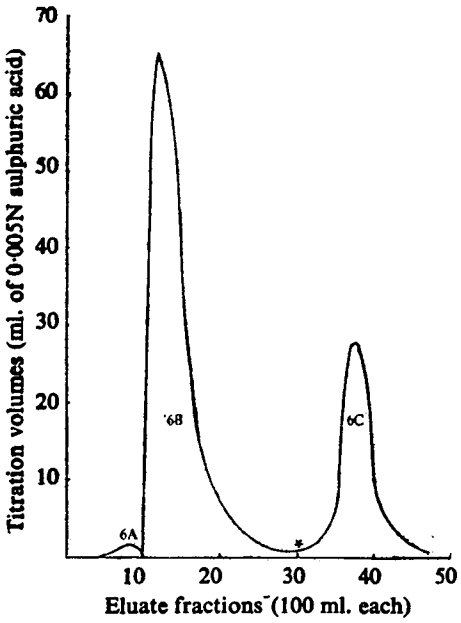

FIG. 6. Separation of alkaloids from root bark of *Punica Granatum* equivalent to 600 mg. of *pseudo*pelletierine. Column, 160 g. of kieselguhr with 80 ml. of 0·5M phosphate buffer, *p*H 7·0. Chloroform used as eluant.

Peak 6C: methyl*iso*pelletierine picrate, rosettes from absolute alcohol, m.pt. 154°C.; *Reineckate*, leaflets from aqueous acetone, m.pt. 228°C. with decomposition); found SCN, 49·0 per cent. $C_9H_{17}ON, H[Cr(SCN)_4(NH_3)_2]$ requires SCN, 48·9 per cent.

Peak 5E: pelletierine picrate, rosettes from water, m.pt., 150°C.; *Reineckate*, clusters of needles from aqueous acetone, m.pt., 254°C. with decomposition); found SCN, 50·6 per cent. $C_8H_{15}ON$, $H[Cr(SCN)_4(NH_3)_2]$ requires SCN 50·5 per cent.; sulphate $[\alpha]_D^{18°C.}$ −10·5° (c=11·8 in water).

The quantitative results obtained with the samples of crude drug are summarised in Table II.

Biological activities of the alkaloids of Punica Granatum.—The activities of pelletierine, *pseudo*pelletierine and methyl*iso*pelletierine against

TABLE II

ALKALOIDS OF *Punica Granatum*

Sample	Total Alkaloids* per cent.	Peak 6A* per cent.	ψPelletierine per cent.	Methyl*iso*pelletierine per cent.	Pelletierine per cent.	Recovery of total alkaloids per cent.
1. Old bark	0·10	trace	58	24	17	71
2. "	0·10	trace	27	41	32	96
3. "	0·23	1·6	25	36	38	91
4. "	0·19	0·4	38	33	28	90
5. Fresh bark of young roots ...	0·38	nil	32	36	32	82
6. Fresh wood of young roots ...	0·115	nil	21	32	47	76
7. Fresh bark of older roots ...	0·44	1·4	54	20	25	84
8. Fresh whole roots	0·39	0·5	22	35	42	95

* Calculated as ψ-pelletierine.

the liver fluke were determined by Drs. M. R. A. Chance and T. E. Mansour*, to whom we are greatly indebted for permission to quote their results (Table III); a fuller account of this work will be reported elsewhere.

TABLE III

ACTIVITIES OF ALKALOIDS OF *Punica Granatum* AGAINST LIVER FLUKE

Alkaloid	Maximum dilution causing	
	cessation of movement	visible interference with movement
Pelletierine sulphate	1 : 8000	1 : 64,000
Methyl*iso*pelletierine sulphate	1 : 4000	1 : 16,000
*Pseudo*pelletierine sulphate	1 : 2000	1 : 4000

DISCUSSION OF RESULTS

The results described in this communication provide evidence additional to that reported in Parts I and II of the effectiveness of partition chromatography as a means of fractionating mixtures of alkaloids. No new factors governing the enrichment of the fractions were observed. The high adsorptive capacity of kieselguhr was contrary to expectation since this material is frequently used in the clarification of liquids during the isolation of alkaloids[15]. Adsorption during partition chromatography is usually undesirable since it tends to cause spreading of the bands of material undergoing partition and thereby hinders separation[16].

Powdered glass provides a satisfactory means of overcoming this difficulty. Its capacity as a carrier of the stationary aqueous phase is about one-third of that of kieselguhr as a volume-weight ratio but as a volume-volume ratio, which is of greater practical importance in partition chromatography, the two carriers have about equal capacity. Powdered glass has the additional advantage that it is easily prepared in a standard and reproducible form, whereas kieselguhr is an extremely variable material. Experiments on the applicability of different grades of kieselguhr in partition chromatography will be described in a forthcoming communication.

The proportion of total alkaloids in pomegranate root and root bark varies widely; in agreement with the findings of Goodson[7] and of Ewers[17], we find that fresh samples generally contain a higher concentration of total alkaloids than older samples. Although the bark of pomegranate root has been traditionally used as the source of the alkaloids, the sample of wood examined by us was found to contain a useful proportion of alkaloids. From the restricted number of samples examined, there appears to be no regularity in the relative proportions of pelletierine, *pseudo*-pelletierine and methyl*iso*pelletierine; the highest proportion of pelletierine relative to the other alkaloids was found in the fresh sample of wood of the root.

* Dept. of Pharmacology, The Medical School, Birmingham.

For the characterisation of pelletierine, *pseudo*pelletierine and methyl-*iso*pelletierine, the properties of the picrates were in good agreement with those recorded in the literature. Unfortunately authentic specimens of these alkaloids were not available for the determination of mixed melting-points. The characters of the Reineckates provided further confirmation of the identity of these alkaloids, particularly of pelletierine and methyl-*iso*pelletierine which afford picrates of similar melting-point. Hess[13] reported the isolation of *iso*pelletierine in 0·0015 per cent. yield from pomegranate root bark. We failed to find this alkaloid; attempts to characterise the alkaloid corresponding to peak A in Figure 6 as *iso*-pelletierine were unsuccessful.

The optical activity of pelletierine has been the subject of controversy. Hess and Eichel[8] found no optically active base in pomegranate root bark, whereas the results of Tanret[18] and Goodson[7] showed that the bark contains *l*-pelletierine. Hess and Eichel[19] resolved racemic pelletierine and for the *l*-sulphate record $[\alpha]_D^{18°C.}$ - 5·33°; whereas Tanret[18] reported - 30·3° as the specific rotation of the sulphate of pelletierine isolated from the bark. We now find that the sulphate of pelletierine extracted from the root and isolated by partition chromatography has $[\alpha]_D^{18°C.}$ - 10·5° (c = 11·8 in water).

The two samples of " pelletierine tannate " examined by us bore little resemblance in composition to the mixed alkaloids extracted from the crude drug; one contained only a minor proportion and the other was apparently devoid of pelletierine. The unidentified basic material, which bore some resemblance to pelletierine in its behaviour on a chromatographic column and in its equivalent weight, may possibly have been a polymer of pelletierine formed during preparation of the " pelletierine tannate." It is unlikely that this material has any connection with the suggested bicyclic tautomeride of pelletierine[20]. The failure of " pelletierine tannate " accurately to represent the total alkaloids of the crude drug has previously been reported by Tanret[6] and Goodson[7], who ascribe this feature to the preferential precipitation of *pseudo*pelletierine and methyl*iso*pelletierine tannates during manufacture. In the process for the large-scale manufacture of " pelletierine tannate " given by Schwyzer[21], such preferential precipitation would be impossible. However, in Schwyzer's process, loss of pelletierine due to formation of a polymeric resin during maceration of the drug with 2·5N sodium hydroxide would doubtless take place, and some loss of volatile alkaloid would occur during evaporation of the ether-chloroform solution of the bases.

The biological results recorded in Table III may be regarded as an indication of the relative activities of pelletierine, *pseudo*pelletierine and of methyl*iso*pelletierine as anthelmintics (see Chance and Mansour[3]). Tanret's[6] and Goodson's[7] observations and those reported here indicate that " pelletierine tannate " is a relatively inefficient form of presentation of the anthelmintic alkaloids present in *Punica Granatum*. The assay process of the Pharmacopœia, which is designed to determine total bases in " pelletierine tannate " affords no evidence of the proportion of active

alkaloids in this material. It is apparent that pelletierine or one of its salts, isolated from the crude drug, would provide the most satisfactory anthelmintic preparation derived from pomegranate. For its isolation, fractional distillation or exploitation of the adsorptive capacity of kieselguhr under conditions similar to those described here would appear to be suitable. A preparation of the sulphates of the total alkaloids of pomegranate by the method described in the experimental section would be more likely to contain the active alkaloids than the " pelletierine tannate " at present available. We observed that aqueous solutions of these alkaloidal sulphates undergo no decomposition on storage for 6 to 7 months. A general study of the pharmaceutical aspects of this subject was not possible, owing to the great difficulty of obtaining even small quantities of the crude drug.

We are greatly indebted to Professor J. E. Driver, Mr. F. Fish, Dr. J. W. Fairbairn and Mr. S. G. Naravane for their assistance in procuring samples of the crude drug.

SUMMARY

1. The application of partition chromatography to the separation of the alkaloids of *Punica Granatum* and of " pelletierine tannate " has been studied.

2. Different samples of *Punica Granatum* contain widely different proportions of pelletierine, *pseudo*pelletierine and methyl*iso*pelletierine. Commercial samples of " pelletierine tannate " may contain little or no pelletierine, which exhibits the highest activity of the pomegranate alkaloids against the liver fluke.

3. Powdered glass has been found suitable as a carrier for the stationary phase of partition chromatographic columns.

This communication is abstracted from a thesis submitted by one of us (J. C.) in partial fulfilment of the requirements for the degree of Master of Pharmacy in the University of Nottingham.

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The paper was read in abstract by Mr. J. Chilton.

There was no discussion.