THE EVALUATION OF BELLADONNA HERB*

PART I. THE QUANTITATIVE DETERMINATION OF SEED IN POWDERED HERB

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BELLADONNA Herb B.P. consists of the leaves, or leaves and other aerial parts, of *Atropa belladonna* L., or of *Atropa acuminata* Royle ex Lindley, or of a mixture of both species, collected when the plants are in flower and dried. It contains not less than 0·30 per cent. of the alkaloids of Belladonna Herb, calculated as hyoscyamine.

Routine microscopical examination in this department of commercial samples of powdered belladonna herb revealed apparently excessive amounts of fragments of belladonna seed, which suggested that the drug was not being collected during the flowering period.

It has been shown by Kuhn and Schäfer[1] that, in all parts of the plant, the alkaloidal content increases rapidly in spring, reaching a maximum when the first flower-buds form. As flowering commences, the alkaloidal content falls rapidly, then rises again, and finally falls with the ripening of the fruit. Stems do not share in the second rise. With the flowering shoots and leaves, the second maximum is more marked than the first. The proportion of hyoscyamine in total alkaloid shows a similar curve with the two maxima.

Methods for ascertaining the amount of seed present in commercial material would be useful, and experiments were made, 1, to determine the number of testa cells per g. of belladonna seed, 2, to determine the number of testa cells present in a known weight of powdered belladonna herb, and hence the proportions by weight of each, and 3, to determine the maximum percentage of seed which might be expected in the flowering herb.

RESULTS

Mathematical Determination of Number of Testa Cells per g.

A mathematical method was devised to measure the surface area of the seed. Early in the 19th century Cauchy proved that the mean area of the projections of a convex body on to planes of all orientations is one-quarter of its surface area. The fraction is one-half for each plane element of surface, each projected area being double covered. Thus the surface area of a convex body can be accurately determined by projection on to the facets of a regular body with an infinite number of facets.

For convenience calculations were made to determine the error involved in reducing the number of facets to a small finite number. It was found that the cube, octahedron, dodecahedron, and icosahedron gave such

* This paper forms part of a Thesis to be submitted by one of us (R. G. A.) for the Degree of Master of Science of the University of Manchester.

good results that it was unnecessary to pursue the matter any further. The mean square error for these finite values was computed (Lighthill, personal communication) as in Table I.

Moran[2] calculated that, using the dodecahedron and icosahedron respectively, minimum values for the surface area would be 91·8 per cent. and 95·7 per cent. of the true area, and maximum values would be 107·9 per cent. and 104·8 per cent.

It will be noted, from Table I, that the mean square error for three mutually orthogonal directions is only 10 per cent., and that this can be reduced to 4 per cent. by the use of six planes. It was considered convenient and adequate to use the planes of the dodecahedron for practical application, and the following procedure was adopted. A projection of the seed was made in an arbitrary "initial direction", and then in five others all making an angle $\tan^{-1}(2)$, that is 63° 26′, with the initial direction, and equally spaced round it. Then the estimated area is four times the mean projected area, or two-thirds of the sum of the projected areas.

TABLE I

Number of planes	Projection on to faces of	Mean square error per cent.
3	Cube	10·16
4	Octahedron	7·52
6	Dodecahedron	3·96
10	Icosahedron	2·44

The practical details of the method are as follows. A microscope with low-power objective is set up in a dark room so that projections of the object can be made at a known magnification, calculated from the use of a micrometer slide.

A cork is cut in such a way that it has five equal lateral faces, each inclined at an angle of 26° 34′ to a fine pin which passes through the principal axis of the cork (Fig. 1, *a* and *b*).

The seed is placed on a glass slide on the microscope stage, and its outline projected and drawn. While still in this position, the seed is then impaled on the pin by pressing the pin in a direction normal to the glass slide.

The cork is turned on to the first face, and the pin pushed so that the seed is almost touching the slide. The outline is projected and drawn, and the procedure is repeated for the other four faces.

The areas of each of the six projections are determined by using a planimeter or by other means, and their sum, multiplied by two-thirds, and divided by the square of the linear magnification, gives the actual surface area of the seed. The result does not take into account any concavities in the seed, but these are not normally present in belladonna seed.

To test the method, larger regular objects of known surface areas ranging from 25·0 to 99·1 sq. cm. were projected at the given angles, using a point source of light and a screen. The per cent. errors on the known values for 17 objects were as follows, − 5·2; − 5·1; − 4·2; − 2·5; − 1·8; − 1·6; − 1·5; − 0·7; + 1·4; + 2·0; + 2·4; + 2·9; + 3·2; + 3·2; + 3·9; + 4·4; + 5·5.

Results for the surface areas of seeds, determined by the projection method, are shown in Table II.

TABLE II

SURFACE AREAS OF SEEDS BY PROJECTION

Sample	No. of seeds measured	Range (sq. mm.)	Mean	Standard deviation
1. Department stock	27	4·44–6·43	5·40	0·52
2. Harrow, 1952	20	5·14–7·47	6·19	0·59
3. "var. *lutea*", Chelsea, 1955	20	5·51–7·43	6·68	0·41
4. Chelsea, 1955	20	5·30–9·10	6·61	0·89
5. Jodrell Bank (from unripe fruit), 1954	20	5·10–7·67	6·68	0·63
6. Jodrell Bank (from ripe fruit), 1954 ..	60	6·75–9·20	7·91	0·55
7. Commercial sample	20	4·84–7·95	6·19	0·93
8. Commercial sample	20	4·72–6·48	5·48	0·53
9. Pharm. Soc. Exptl. Ground	20	5·49–7·69	6·70	0·61
All seeds	227	4·44–9·20	6·66	1·07

Determination of Number of Testa Cells in Unit Area

The testa of belladonna seed includes a single layer of cells which are characteristic in appearance. Counts were made of $\frac{1}{4}$ sq. mm. areas of the testa of the dry seed, using an Ehrlich eye-piece. One count was made on each seed, the flattest part of the seed being chosen. Portions

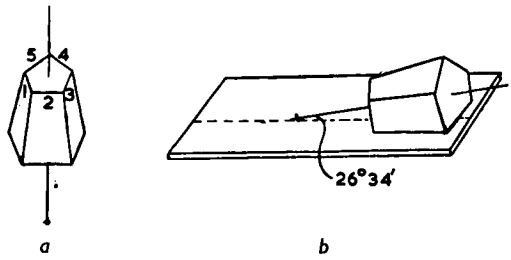

FIG. 1. *a*. Showing the cut cork. *b*. Cork on the slide.

of cells adjoining two adjacent sides of the field were included in the count, while portions of cells adjoining the other two sides were ignored (Table III). From these results were derived figures for the number of testa cells in unit weight (Table IV).

Determination of Number of Testa Cells per g. by Lycopodium Method

Wallis[3] has shown that the spores of the club-moss, *Lycopodium clavatum*, are uniform in size, and that 1 mg. of the powder contains approximately 94,000 spores. This figure was used as the basis of a second method of determining the number of testa cells per g. of belladonna seed.

Prepare a filter by folding a $2\frac{1}{2}$ in. diameter circle of closely-woven glass cloth into a cone, and support this in a glass funnel. Into the filter place about 0·2 g., accurately weighed, of belladonna seed, reduced to No. 60 powder.

TABLE III

NUMBER OF TESTA CELLS IN UNIT AREA ($\frac{1}{4}$ SQ. MM.)

Seed batch	No. of seeds	Range	Mean	Standard deviation
1	60	16–33	23·6	3·8
2	40	16–22	19·2	2·2
3	40	17–27	21·7	2·4
4	40	15–26	19·7	2·8
5	40	15–23	18·9	2·1
6	60	15–24	20·6	1·8
7	40	17–27	21·1	2·5
8	40	18–27	21·6	2·4
9	40	18–28	21·3	2·1
All seeds	400	15–33	21·0	2·9

Pour Schultze's Maceration Fluid, heated to near boiling point, slowly through the filter until the testa is bleached to a light brown and begins to disintegrate. At this point, pour about 10 ml. of hot solution of chloral hydrate (50 g. in 20 ml. water) through the filter, a few ml. at a time. Transfer the residue carefully to a small test-tube, using a small brush made from about 20 strands of stiff wire, diameter approximately 0·3 mm., fixed in a glass holder, and successive small volumes of solution of chloral

TABLE IV

NUMBER OF TESTA CELLS PER G.

Seed batch	Mean weight per 100 g.	Number of testa cells per g. (mean surface area × No. of testa cells per sq. mm. × No. of seeds per g.)
1	0·101	504,000
2	0·116	411,000
3	0·111	522,000
4	0·108	481,000
5	0·098	514,000
6	0·117	558,000
7	0·065*	802,000
8	0·066*	716,000
9	0·129	443,000
Mean	0·101	550,100
Standard deviation	0·022	127,900

* Most of the seeds of these two batches were unusual in being strongly flattened and occasionally biconcave in shape; endosperm was scanty. Both batches were taken from commercial herb received on two different occasions from the same supplier.

hydrate to a total volume of about 7 ml. Shake vigorously, and add 0·1 ml. of a suspension, in a suitable medium, containing about 0·05 g., accurately weighed, of lycopodium in 10 ml.

Place a small drop of the mixed suspension on a microscope slide, and cover with a $\frac{5}{8}$ in. cover slip. Count the total number of spores in the area bounded by the cover slip by scanning in strips equal in width to the field of view. Count the total number of testa cells by the same method, but with the assistance of polarised light. Include all testa cells of which the major portion is judged to be present; ignore those of which less than half is judged to be present.

Results obtained by this method on seed of Batch 1 are shown in Table V. To test the accuracy of this suspension method, further counts

were made using dry lycopodium powder, measured to an accuracy of 0·0001 g.; comparable results were obtained.

Change in Weight of Seeds on Powdering

Since the calculation of the number of testa cells per g. by the projection method was derived from the whole seed, it was necessary to determine whether any change in weight occurs on powdering.

TABLE V

NUMBER OF TESTA CELLS PER G. BY LYCOPODIUM METHOD

(Seeds—Batch 1; 10 mounts per macerate)

Macerate	Total weight of seed g.	Total of lycopodium	Spores	Testa cells	Testa cells/g.		
					Range	Mean	Standard deviation
1	0·314	0·1 ml. ≡ 53,600 spores	607	1807	356,600 to 696,000	521,940	91,300
2	0·247	do.	964	2218	375,500 to 648,800	496,010	82,600
3	0·205	do.	778	1197	358,400 to 537,400	410,740	54,700
4	0·226	do.	1688	3376	361,700 to 597,400	479,840	73,300
5	0·204	0·0026 g.	7485	2584	341,600 to 495,400	421,740	47,300
6	0·389	0·0069 g.	10,161	2513	321,000 to 589,600	424,110	89,000
7	0·233	0·0021 g.	7766	3382	259,000 to 489,900	385,470	71,600
8	0·205	0·0035 g.	5835	1436	300,900 to 469,500	403,100	52,400
Summation of macerates ..					259,000 to 648,800	446,390	85,400

Accurately weighed quantities of seed in No. 60 powder were exposed to the atmosphere, suitably shielded from dust, and re-weighed after three weeks.

Seventy separate weighings were made of seed from 15 different batches. 55 lost in weight under the conditions of the experiment; the maximum percentage loss was 4·22 and the mean 2·2 per cent. 11 samples increased in weight, but in only three of these was the increase more than 1 per cent. Gains and losses were evenly distributed within the individual batches. Hence it would appear that the powdering had no significant effect, and the value of the number of testa cells per g. as determined on the intact seed was equally applicable to the powder.

Determination of Number of Testa Cells per g. of Commercial Belladonna Herb

Seeds can be separated from commercial herb, or from powders coarser than No. 10, by sifting through a No. 10 sieve, and the percentage present may then be determined by weighing.

In powders finer than No. 10, it was found necessary to "concentrate" the testa cells by removal of as much extraneous material as possible, in order to obtain sufficient for each mount. The method finally adopted was maceration under carefully controlled conditions of time and temperature, such that the testa cells were not affected to any noticeable degree. The resulting cellulosic material was then dissolved out, again under carefully controlled conditions, and filtered rapidly through a sintered glass filter, on which the testa cells, isolated almost completely, were retained. The details of the method are as follows. Insert a clean No. 2 sintered glass filter, diameter 2½ in., into a suitable flask in which the pressure can be reduced to ¼ atmosphere or less. Evenly distribute over the surface of the filter about 1·2 g., accurately weighed, of the sample, in powder not coarser than No. 60, and add one or two small crystals of potassium chlorate. Cover the powder with 25 ml. of a mixture of equal parts of nitric acid and water, heated to 70° C., and allow the reaction to proceed, without negative pressure, for about half a minute, or until bleaching just commences. At once reduce the pressure, wash the residue with two successive quantities of about 10 ml. of hot water, and partially dry by suction for a few minutes.

Transfer the residue to a 50 ml. beaker containing 25 ml. of a saturated solution of zinc chloride in hydrochloric acid, heated to 45° C. Maintain this temperature, with constant stirring, for about one minute, or until the solution begins to become clear. Without delay, transfer the contents of the beaker to the sintered glass funnel, and reduce the pressure fully. Immediately the liquid has passed through, wash the residue with two successive quantities, each of about 10 ml. of hot solution of choral hydrate.

Apply positive pressure momentarily to loosen the residue from the filter; transfer to a 50 ml. beaker, with the aid of the wire brush described previously and successive minimum quantities of solution of chloral hydrate, to a total of about 5 ml.

Add 0·1 ml. of the lycopodium suspension previously described, and stir thoroughly with a glass rod. Determine the relative numbers of spores and testa cells as before, and hence the number of testa cells per g. of the commercial sample.

To test the accuracy of the method, direct counts were made as follows:

A few mg. of the powdered herb, accurately weighed to 0·0001 g. on microscope slides, were evenly distributed in a drop or two of a hot, saturated solution of chloral hydrate in equal parts of hydrochloric acid and water. A ⅞ in. cover slip was applied and the total number of testa cells counted with the assistance of polarised light. The acid in the mountant dissolves the calcium oxalate which tends to be troublesome under polarised light.

Table VI compares results obtained by both methods.

TABLE VI

DETERMINATION OF NUMBER OF TESTA CELLS PER G. IN COMMERCIAL POWDERED BELLADONNA HERB

	By lycopodium method					By direct counting					
Batch	No. of macerates	Total No. of mounts	Range	Mean	S.D.	No. of mounts	Total testa cells	Total weight of powder (g.)	Range	Mean	S.D.
B	3	9	33,600 to 59,100	40,480	8980	11	1415	0·0262	33,880 to 86,700	56,870	18,000
H	3	30	2860 to 10,100	5130	2135	20	918	0·1570	3480 to 10,100	6040	1560
J	3	32	6050 to 28,330	13,060	4750	12	981	0·0662	8610 to 25,160	15,650	4,110
K	3	30	2200 to 14,800	5330	2670	20	1302	0·2131	4420 to 8450	6,170	1,180

Determination of Amount of Seed in whole Herb

Belladonna plants from various geographic sources were cut late in the season, and the aerial parts air-dried for one month. The seeds were separated from the fruits, air-dried, and weighed, and the remainder of the dried herb was also weighed (Table VII).

In an attempt to obtain upper limits for the percentage of seed, plants were included which had only one or two flowers remaining; in the second part of Table VII are shown results for some plants which had completely fruited.

DISCUSSION

The mathematical method described for the measurement of the surface area of belladonna seed has been shown to be accurate, and is suitable for the determination of the surface area of irregular convex objects of microscopic dimensions, or of larger objects. The method may conveniently be used in branches of science where it is necessary to estimate the number of discrete particles regularly distributed over the surface of such an object. The number of testa cells in unit area of belladonna seed was determined by direct counting, and the number per g. was estimated to be about half a million.

The accuracy of these estimates are supported by quantitative lycopodium determinations on the powdered seed. In initial experiments on the powdered seed, counting of the spores and cells was rendered difficult by refraction of light by the globules of fixed oil, which had proved resistant to de-fatting, except on prolonged exposure. The reduction of the seed to number 60 powder, and the use of hot solution of chloral hydrate, reduced this difficulty. The polyvinyl alcohol medium described by Hall and Melville[4] was used as a vehicle for the lycopodium suspension. Polarised light is very valuable in the counting of the testa cells; it should, however, be used with discretion, as non-refractive cells sometimes occur, particularly if the maceration has been allowed to proceed too far.

In the examination of commercial samples of powdered belladonna herb, it was found necessary to "concentrate" the testa cells in order to obtain sufficient for each mount. Removal of extraneous cellulosic material was accomplished by a method depending on the differential solubilities of each in a zinc chloride reagent. The dissolved cellulose produces a thick gel-like solution which is difficult to filter, and, as success depends upon leaving the testa cells in contact with this reagent for a minimum of time, it is essential to use relatively small amounts of powder and reagent, a clean filter with a large filtering area, and low pressures.

The above method may be adapted for the isolation from vegetable material of such resistant tissues as cork cells, or pollen grains.

The results obtained by this method for the sample B (Table VI) indicated that about 10 per cent. of seed was present in the powdered herb; this, at first, seemed a high proportion, but determinations on various samples of whole herb produced values as high as 16·6 per cent. (Table VII).

Information is insufficient at present for a "Limit of seed" to be suggested.

TABLE VII

PERCENTAGE OF SEED IN BELLADONNA HERB

Batch	Source and date	Per cent. of seed
1	University Experimental Grounds. Late August. Age of plant not known	16·6
2	Pharmaceutical Society's Experimental Grounds. Late August. Age not known	13·8
3	University Experimental Grounds. Late August. 1st year plant	4·0
4	Chelsea Physic Garden. Early September. Age not known	5·2
5	do.	11·9
6	Cheshire. Early October. 1st year plant	4·2
7	do.	5·9
8	Jodrell Bank, Mid-October. 1st year plant. No flowers	11·0
9	do.	14·3
10	do.	8·7
11	do.	14·8
12	do.	18·5
13	do.	13·7
14	do.	17·5

SUMMARY

1. A mathematical method is described for the determination of the surface area of objects of irregular shape, and applied to the measurement of the surface area of belladonna seeds.

2. From these results, and from direct counting of the testa cells in unit area, the number of testa cells per g. of belladonna seed is calculated. These figures are compared with those obtained by quantitative lycopodium methods.

3. A method is described for the separation of testa cells of belladonna seed from other cellulosic material in the powdered herb. This enables a quantitative estimate to be made of the amount of seed present in the powdered herb. The proportion of seed present in the dried entire herb, collected when in flower, was also determined.

For providing materials and facilities, the authors are indebted to Dr. J. M. Rowson, Curator, the Museum of the Pharmaceutical Society; Mr. W. G. MacKenzie, Curator, Chelsea Physic Garden; Dr. Adela Erith, University of Reading; the staff of the Manchester University Experimental Grounds. Thanks are also due to Professor M. J. Lighthill, F.R.S., Department of Applied Mathematics, Manchester University, for confirming and enlarging upon the mathematical approach.

REFERENCES

1. Kuhn and Schäfer, *Pharm. Zentralh.*, 1939, **80**, 151, 163.
2. Moran, *Ann. Math.*, 1944, **45**, 793.
3. Wallis, *Analyst*, 1916, 357–374.
4. Hall and Melville, *J. Pharm. Pharmacol.*, 1951, **3**, 936.

DISCUSSION

The paper was presented by MR. R. G. ATKINSON.

The CHAIRMAN said that the immediate object of the paper seemed to be to determine the amount of seed in the herb to ensure that it was not collected at a time when it did not contain enough atropine. It would seem that an alkaloidal assay of the material would have been a more direct method.

DR. T. E. WALLIS (London) said that belladonna seeds were rather flat, and he wondered whether the authors could have devised a simpler method of measuring the surface area. The method designed for determining the total surface area of convex bodies was checked by examining other regular objects in order to see whether the method worked. The authors did not describe the objects or give their shape, and it would be interesting to have more information about them. The methods described for estimating the amount of seed in the powder were difficult to follow, and it would have been valuable if complete details of at least one experiment could have been given. He would have thought the shape of the epidermal cells could have been identified for counting purposes without using polarised light. The specimens from Jodrell Bank were all first year plants and all contained a high proportion of seeds. Other first year plants mentioned in Table VII contained 4 to 6 per cent. of seeds as against a figure of up to 18 per cent. in the former group. It seemed remarkable that there should be such a large difference.

DR. J. W. FAIRBAIRN (London) observed that the standard deviation obtained with the whole seeds was 10 per cent. of the mean, which was good. When the lycopodium method was applied the standard deviation was about 22 per cent. of the mean, which was fairly wide; but when the method was applied in practice to a powdered herb the standard deviation was very wide indeed. In Table VI, sample K, the standard deviation

was about 50 per cent. of the mean, which meant that even if one did 25 determinations, which would take a very long time, there would still be an error of about $\pm$ 25 per cent. It was possibly due to the fact that the herb was bound to contain seeds at all stages of maturity; very slight variations in the oxidation process would lead to greater or less destruction of the cells of immature seeds. It was difficult to understand why the authors (in Table VII) collected plants so late in the season. One would assume that they desired to know what was the normal amount of seed present in B.P. belladonna, which should be collected when in flower. In London, belladonna flowered by early June. It would have been helpful to have a typical belladonna herb as a standard. The commercial samples HJK, in Table VI, contained from 1 to 3 per cent. of seeds, which was a fair amount, but those cited in Table VII were artificially high.

DR. J. M. ROWSON (London) agreed with the Chairman that determination of the total alkaloids and the proportion of hyoscyamine would have given an answer rather more readily than the lycopodium method. He pointed out that no reference to the original publication by Cauchy had been given. He asked the authors the number of counts made in Table V and whether they were based on 25 fields or more. He agreed with Dr. Wallis about the use of irregular bodies to test the projection method, and asked whether the authors would be able to state the accuracy of their value by that method compared with the first projection. The Jodrell Bank values for seed content seemed very high for one year plants. His own one year plants at Manchester did not produce many seeds. It would be interesting to know how the authors' method would behave for seeds of varying degrees of maturity.

MR. R. G. ATKINSON, in reply, said that he had hoped to find some relation between the amount of seed present at various stages and the alkaloidal content. However, it was rather more complex than was anticipated, depending on the age of the plant and the various conditions of growth. The maximum amount of seed, 16·6 per cent., which was found on material which was flowering, compared very closely with the 18·5 per cent. of Jodrell Bank with no flower, and it seemed doubtful that it would be possible to fix a limit on seed. It had been found that most of the seeds were convex. There were very few concavities. Regular bodies—cylinders of maximum surface area 90 sq. cm.—which could be measured relatively easily were chosen. The projection method in theory gave a mean of about 5 per cent. with a latitude of about 10 per cent. on either side. It had been found that polarised light was very useful as an adjunct to determine the number of cells. Maceration tended to reduce the refractibility to polarised light, and it was necessary to exercise care in its use. It was possible with practice to acquire the technique of passing the material through the filter without much loss of the testa material. The work of Cauchy was described to him by an expert in mathematics, but he was unable to find a reference in the literature. It would be seen from Table V that ten counts were taken for each of the macerates. The belladonna seeds where endosperm was found lacking were unusual.