THE BIOLOGICAL ASSAY OF VEGETABLE PURGATIVES

PART II—RHUBARB AND ITS PREPARATIONS

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BIOLOGICAL methods of assay for senna and its preparations have been reviewed and described in Part I[1]. The most important worker on the biological assay of rhubarb has been Fühner[2], who introduced white mice as the test animal and stated that the purgative activity on mice and man runs parallel. The method involves the administration of purgatives by means of a pipette or in the form of pills and recording the minimum effective dose. His method was later improved by Loewe and Fauvre[3] in that the dose was administered with the aid of a stomach tube and, for obtaining more uniform results, a fasting period of 18 hours before administering the dose was suggested; during this period water only is given. However, these methods are based on the assumption that the response is "all or none," whereas, as already stated[1], it is quantitative. Furthermore no standard is given simultaneously with the test substance so that the commonly experienced variation in biological responses of different batches of animals at different times is not taken into account. Therefore, the minimum effective dose is certainly not a reliable measure of purgative activity. We have also noted that a fasting period of 18 hours for mice is too long as some of the animals became weak after prolonged fasting. A period of 3 hours during which no food is given as described in Part I[1] is adequate to evacuate the stomach contents. In the present work, therefore, the method for the biological assay of senna was tried to see whether it is also applicable to the evaluation of rhubarb.

RELATION BETWEEN DOSE AND RESPONSE

Experiments were carried out to determine whether a similar technique as that described for the assay of senna would give a linear log dose/response line. Graded doses of powdered rhubarb suspended in distilled water were given to 4 groups of 10 mice each. The number of wet fæces produced by each pair of mice was recorded as shown in Table I. All the mice used were of similar body weight so that the total weights of each of the 4 groups are similar. The numbers of wet fæces recorded in Table I are "per pair" and not "per kg. of mice" as in Part I[1], in order that whole numbers may be used for the statistical analysis as shown later.

Table I clearly shows that an increase in the dose of rhubarb produced an increase in the number of wet fæces as with senna. A graph was then constructed by plotting response against the log dose (Fig.

1, A). The graph shows that, except for the response to the lowest dose (16 mg. per pair of mice), the relation between response and log dose is

TABLE I

NUMBER OF WET FÆCES PRODUCED BY PAIRS OF MICE AFTER ADMINISTERING RHUBARB

Pair of mice (No.)	Dose in mg. per pair of mice				Totals
	16 mg.	24 mg.	36 mg.	54 mg.	
1	3	5	9	15	32
2	6	4	12	12	34
3	7	11	7	10	35
4	7	11	16	9	43
5	4	15	13	22	54
Totals	27	46	57	68	198
Averages	5·4	9·2	11·4	13·6	—

linear. This drop in response at low dose might be explained by the assumption that it approaches the threshold dose below which the animals do not respond or by the fact that rhubarb contains tannin and its astringent action becomes apparent when the dose is small. This latter assumption agrees well with the general clinical experience that when small doses of rhubarb are given the astringent action predominates and masks the purgative effect[4,5]. However, the result of statistical analysis as shown in Table II indicates that the deviation from linearity of the log dose-response line is not highly significant compared with the variation of random sampling. This experiment was therefore repeated and similar results were obtained (Fig. 1B).

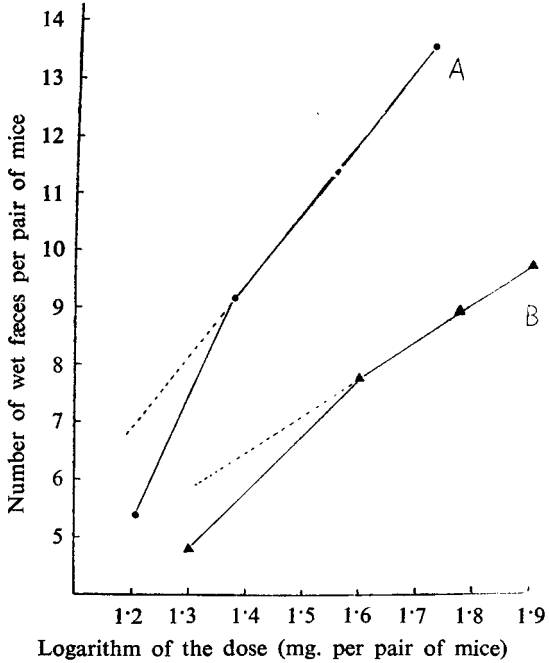

FIG. 1.—Relation of response to the logarithm of the dose.

Though the depression in response at low dose as described above is considered insignificant statistically, the astringent action of the tannin

in rhubarb should always be borne in mind. Pending the isolation of the pure purgative principle of rhubarb, the inter-relation between the effects of purgative and astringent principles of rhubarb is difficult to assess. To make sure of the results of the assay being independent of the tannin effect and also to avoid the threshold dose at which normal response may not be obtainable, it is advisable to give 3 dose levels of both standard and unknown in each assay. If the relation between response and log dose is a straight line in both cases, then the results can be relied upon; if on the other hand the response shows an undue depression at lower dose level the test should be repeated after suitably adjusting the doses.

Analysis of Variance. This is calculated by the usual procedure (Finney[6] and Emmens[7]) and the results are listed in Table II.

TABLE II

ANALYSIS OF VARIANCE FOR THE DATA OF TABLE I

Source of variation	Degrees of Freedom	Sum of Squares	Mean Square	*F*	*P*
Between doses	3	183·40	—	—	—
Regression	1	179·41	179·41	12·36	<0·01
Deviation from regression ...	2	3·99	1·99	0·14	>0·1
Between pairs	4	82·30	20·58	1·42	>0·2
Within doses and pairs (error)	12	174·10	14·51	—	—
Total	19	439·8	—	—	—

NOTE.—The mean squares are obtained by dividing the sums of squares with their corresponding degrees of freedom. The mean square for variation "within doses and pairs" (Error) 14·5 has been compared with the three sums of squares above it in Table II, whence we get the series of variance ratio, *F*, listed in the table and the corresponding probabilities, *P*, associated with them.

From Table II we may conclude that:

(a) the regression of the log dose upon the number of wet fæces is significant and there is no significant deviation from this regression, therefore, a linear relationship between log dose and response can be assumed:

(b) the variation between pairs is not significantly greater than that within them. This proves that separation of this item from the error sum of squares in the analysis of variance is unnecessary.

EXAMPLE OF THE METHOD: BIOLOGICAL ASSAY OF R_{EX}.

A quantity of the laboratory standard rhubarb (R_s^*) was exhaustively extracted with ether and methylal and dried. This exhausted rhubarb (R_{ex}) was assayed against the laboratory standard rhubarb (R_s) using 6 groups of 10 mice each. In Table III are given the details of the assay.

Analysis of Variance. As the previous analysis and similar analysis on subsequent experiments showed that there were no significant varia-

tion between pairs compared with that within them when the pairs were taken at random, the calculation for the variance "between pairs" is

TABLE III

NUMBERS OF WET FÆCES PRODUCED BY PAIRS OF MICE AFTER ADMINISTRATION OF RHUBARB, R_S AND R_{EX}

Pairs of mice (No.)	Dose of R_s (mg./pair)			Dose of R_{ex} (mg./pair)		
	80 mg.	40 mg.	20 mg.	80 mg.	40 mg.	20 mg.
1	10	8	4	13	9	2
2	11	13	5	13	5	1
3	16	13	12	12	5	0
4	24	11	4	15	3	3
5	20	7	4	12	9	1
Dose Totals	81	52	29	65	31	7
Preparation Totals	—	162	—	—	103	—
Dose Averages	16·2	10·4	5·8	13·0	6·2	1·4
Preparation Averages	—	10·8	—	—	6·87	—

therefore omitted and combined into the variance attributable to random sampling (Error). The rest of the calculations were carried out in the usual way and the results listed in Table IV.

TABLE IV

ANALYSIS OF VARIANCE OF THE DATA OF TABLE III

Source of variation	Degree of Freedom	Sum of Squares	Mean Square	*F*	*P*
Between doses	5	727·37	—	—	—
Difference between preparations	1	116·03	116·03	10·68	<0·01
Linear regression	1	605·00	605·00	55·65	<0·001
Departure from parallelism	1	1·80	1·80	0·17	>0·2
Curvature of combined curve	1	4·27	4·27	0·39	>0·2
Difference of curvatures ...	1	0·27	0·27	0·03	>0·05
Within doses (error)	24	260·80	10·87	—	—
Total	29	988·17	—	—	—

Factorial Analysis of the Variance "Between Doses." In order to examine the possible curvature of the log dose-response line and to determine how well the supposed relationship between log dose and response fits the data, and also to examine the exact nature of discrepancies, an analysis of the variance attributable to "between doses" was carried out, as these factors are of great importance as to the validity of the assay.

This assay was so designed that the log doses are equally spaced. It is possible, therefore, to make use of the polynomial coefficients for log

dose, which are small whole numbers bearing the same relation to each other as that between the differences of each log dose from the mean log dose. The scheme of the factorial analysis follows the standard pattern and is shown in Table V.

TABLE V

FACTORIAL ANALYSIS OF THE LOG DOSE-RESPONSE RELATION OF TABLE III USING POLYNOMIAL COEFFICIENTS

Source of variation	Polynomial Coefficients						Divisor	Sum of Products
	S_1	S_2	S_3	T_1	T_2	T_3		
Difference between preparations	1	1	1	−1	−1	−1	30	59
Linear regression	1	0	−1	1	0	−1	20	110
Departure from parallelism	1	0	−1	−1	0	1	20	−6
Curvature of combined curve	1	−2	1	1	−2	1	60	16
Difference of curvatures ...	−1	2	−1	1	−2	1	60	4
Dose Totals	81	52	29	65	31	7	—	—

From the above analysis, it may be concluded that:

(a) the difference in potency between the preparations, R_s and R_{ex} is significant;

(b) the linear regression between log dose and response is beyond doubt and there is no significant departure from parallelism between the two separate curves;

(c) the curvature of the combined curve is not significant and there is no significant difference in curvatures of the two separate curves.

Thus the linearity and parallelism of the log dose-response line and the validity of the assay are established.

Calculation of the Relative Potency. Having ascertained the validity of the assay, the relative potency of the Standard and Test Preparations and its fiducial limits may be calculated by use of the polynomial coefficients of the source of variation attributable to "Linear regression" as listed in Table V. The method of calculation is fully described by Emmens[7] and Finney[6]. However, in the present type of assay with restrictions in design, the following methods of calculating relative potency may be used, which are, in our opinion, simpler and quicker for routine calculations, both giving the same results:

Method A. As stated earlier, the relation between log dose and response has been proved to be a straight line (Fig. 2). A formula for such a line is given by the equation[6]:

$$Y = \bar{y} + b(x - \bar{x})$$

Where $\bar{x}$, $\bar{y}$ are the mean values of x (log dose) and y (response) and b, known as regression coefficient is given by

$$b = \frac{S\{(x - x)(y - \bar{y})\}}{S(x - \bar{x})^2}$$

the sum of products of deviations divided by the sum of squares of

deviation for x. The regression coefficient is an average slope obtained from consideration of all the points, and points whose x is very different from $\bar{x}$ are giving greater weight than those whose x is near to $\bar{x}$ in forming the average. However, when the three log doses for each preparation are equally spaced (i.e., the log medium dose = the mean of the three log doses) as designed in this assay, the above consideration is no longer necessary and, furthermore, the medium-dose response has no effect on the slope of the regression line, though it does influence its position; the slope is determined by the highest- and lowest-dose responses only. The calculation of the average slope (regression coefficient) therefore can be simplified and the following method be used:

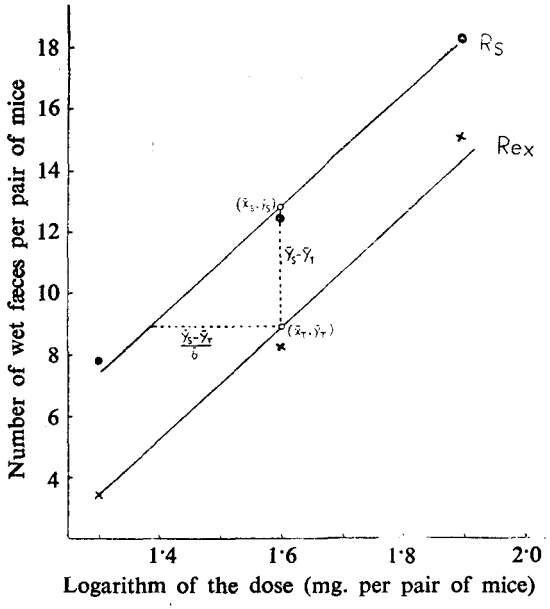

FIG. 2.—Biological assay of R_{ex}. Positions marked with o are the mid-points, $(\bar{x}_s, \bar{y}_s)$, and $(\bar{x}_T, \bar{y}_T)$, of the regression lines, R_s and R_{ex}.

The difference between the highest and lowest log doses of $R_s = 0{\cdot}6020$.

The difference between the highest and lowest log doses of $R_{ex} = 0{\cdot}6020$.

The difference between the highest and lowest responses of $R_s = 10{\cdot}4$ WF/pair.

The difference between the highest and lowest responses of $R_{ex} = 11{\cdot}6$ WF/pair.

The *average slope* of the two lines R_s and R_{ex} is

$$b = \frac{10{\cdot}4 + 11{\cdot}6}{0{\cdot}6020 + 0{\cdot}6020} = 18{\cdot}28$$

The estimate of relative potency is the ratio of equally effective doses, or the antilogarithm of the difference between log doses that produce equal responses. The difference between equally effective log doses (i.e.,

the x-values, or in other words, the horizontal distance between the two regression lines), M, can be obtained from the following equation:

$$M = \bar{x}_s - \bar{x}_T - \frac{\bar{y}_s - \bar{y}_T}{b}$$

Since $\bar{x}_s - \bar{x}_T = 0$,

$$M = \frac{\bar{y}_s - \bar{y}_T}{b} = \frac{10.8 - 6.87}{18.28} = 0.2150$$

$$= \log 1.641$$

i.e., potency of 1 g. R_s = potency of 1·64 g. R_{ex},
or potency of 1 g. R_{ex} = potency of 0·61 g. R_s.

Method B. This method follows the same pattern as that described in the previous article for the "4-point" assay of senna[1]; with rhubarb, however, a "6-point" assay is used and the highest dose is four times that of the lowest.

When the dose of R_s is quadrupled, the response is increased by	10·4	WF/pair
When the dose of R_{ex} is quadrupled the response is increased by	11·6	WF/pair
Mean effect of quadrupling the dose	=11·0	WF/pair
Mean effect of the three doses of R_s	=10·8	WF/pair
Mean effect of the three doses of R_{ex}	= 6·87	WF/pair
Difference between the mean effects of R_s and R_{ex}	= ·93	WF/pair

Let r = the ratio of the potency of the doses of R_s and R_{ex},

$$\frac{11.0}{3.93} = \frac{\log 4}{\log r}.$$

Hence $\log r = 0.2151$
and $r = 1.641$

i.e. $\dfrac{\text{potency of 1 g. } R_s}{\text{potency of 1 g. } R_{ex}} = 1.641$,

or potency of 1 g. R_{ex} = potency of 0·61 g. R_s.

Application of the Method and its Accuracy

The method as described in this paper has been successfully applied to various samples of rhubarb and their preparations, some of these results have been reported by Fairbairn and Lou[8]. Our experience with repeated assays showed that the method has similar limits of error to that already described for senna[1], i.e., the coefficient of variation for a single assay is usually about ± 15 per cent.

Summary

1. The biological assay of senna already described (Lou[1]), which was based upon the number of wet fæces produced by groups of mice, has been successfully applied to rhubarb and its preparations.

2. For rhubarb and its preparations, a " 6-point " assay is advocated. With suitable restriction in the design of the assay, it is possible to calculate the potency by simple methods.

3. An example of the assay and the subsequent calculation is given.

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