THE BIOLOGICAL ASSAY OF VEGETABLE PURGATIVES

PART I.—SENNA LEAF AND FRUIT AND THEIR PREPARATIONS

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INTRODUCTION

AS part of a general investigation on the vegetable purgatives containing anthracene derivatives, I was asked in 1947 to carry out biological assays of several of these drugs and their preparations. Several biological methods of assay have been described and reviewed by Munch[1] and Viehoever[2]. More recently, Loewe[3] used rhesus monkeys and claimed that his method is applicable to many types of laxatives. Straub and Gebhardt[4] used white mice and determined the minimum effective dose (ME) of senna infusions. The potency was expressed as the number of such doses per ml. of the infusion. Geiger[5] adopted a similar method but instead of using animal units the use of a standard (a 5 per cent. infusion of senna leaf) was introduced. The potency of a test preparation was compared with the standard by comparing the percentage of mice which produce positive response. Later, Hazleton *et al.*[6,7,8] introduced the term T.C.D. (Threshold Cathartic Dose—i.e. the dose which produces catharsis in approximately 50 per cent. of the mice), and Grote and Woods[9] introduced the use of powdered senna leaf as their standard of reference. Collier *et al.*[10] further modified the method by using the ratio of the number of unformed fæces (UFF) to the number of total fæces (TF) as the criterion of purgative activity.

The most promising method seemed to be that of Geiger as modified by others, since mice are convenient to handle and require only small amount of test material; this last point is particularly important when only small quantities of pure compounds are available for assay. Accordingly, work was commenced in 1947 on the basis of their methods; but experience soon showed that improvements were necessary for the following reasons: (a) the handling of large number of frog-jars or beakers used as mouse containers during test is inconvenient; (b) droplets of water occasionally appeared on the inside of the jars, indicating a high humidity due to bad ventilation; (c) it is impossible to use water-bulbs with the jars and previous workers have withheld drinking water during test, but this seems undesirable, since water plays a large part in purgation; (d) my experience showed that increase in dose of purgative produced a corresponding increase in the number of wet (or unformed) fæces (i.e. those which differ from the normal dry ones in being round and pasty and leaving a brown stain when placed on blotting paper). It appears therefore that a method based on a quantitative response, rather than the qualitative ("all or none") type used by Geiger and others, would give more accurate results. Collier[10] apparently had a similar idea as he determined the ratio of UFF/TF and used this value as a criterion

of purgative activity. This method, however, is tedious as it involves counting a very large number of fæces in each test.

To overcome these defects, (a) special cages were devised to avoid the use of large number of frog-jars or beakers, (b) a definite proportion of water was added to the feeds during test, and (c) counts of *wet* fæces *only* were used as criteria of activity. The resulting method described in this paper is not only free from the defects already mentioned, but is more convenient in use and gives results of a comparatively high degree of accuracy. Furthermore, this method has been found applicable to other purgatives apart from senna; Hazleton *et al.*[7] report that their method is unsuitable for cascara and aloes.

General Experimental Details

1. *The Standard.* It was decided to use the powdered crude drug as standard, wherever possible, rather than infusions, because infusions may not contain the entire activity. Moreover, owing to the unavoidable variation in preparation, successive infusions may vary in potency. According to Collier[10], an infusion made from 5 to 6 mg. of senna fruit (0·24 g./kg. body-weight) produced no response; I have found that the same dose of senna fruit given directly always produce marked response. While this may be due to my sample of senna fruit being highly active, it may also be due to the fact that the infusion (as made by Collier) does not contain all the activity. The standard, in powdered form, is kept in evacuated glass bottles in a refrigerator. To prepare a standard suspension for administration, a weighed amount of the powder is triturated in a mortar with a small quantity of boiling distilled water and made up to volume in a measuring cylinder with cold distilled water. For a mouse of 20 g. body-weight, a dose of 0·5 ml. is suitable.

2. *Design of the Cages.* The cage as shown in Figure 1 is 15 inches long, 9 inches wide, and 6 inches high. It is divided into 10 compartments with tinned plates. The outer walls were made of "window substitute" (wire gauze impregnated with transparent plastic). Each compartment has a food container made of tinned plate; these containers were connected in pairs by a ∩-shaped handle which hangs over the wall dividing two compartments. The floor and ceiling consist of loose (detachable) grids made of galvanised wire with a mesh of 1 cm. by 1 cm. Six feet are provided at the edges of each of these grids so that the bottom grid is raised about 1 inch from the table on which a sheet of white blotting paper is provided to receive the fæces. The advantages of this cage are (a) good ventilation is obtained, (b) the inconvenience of using a large number of jars is avoided and (c) the bottom grid can be easily removed (either for inspection of the adhering fæces or for washing) by inverting the cage; the top grid will now serve as a floor.

3. *The Test Animal and Diet.* White albino mice weighing not less than 18 g. are used in all tests. Owing to the variation in response of the sexes mentioned by Hazleton *et al.*[8], male mice only are used. They are housed in metal cages and fed with rat-cubes (Diet 41 supplied by the Associated London Flour Millers Ltd.). In addition, each mouse receives an unrestricted supply of fresh tap-water; green vegetables are given over

the week-ends only, thus avoiding the possible interference with the test during the week. The animals, if in good condition, can be used repeatedly after a resting period of not less than 1 week.

4. *The Test.* All food and water are withdrawn from the mice early in the morning and the animals are put singly or in pairs into each compartment of the cage. After 2 or 3 hours, the fæces are examined and any mouse having soft or wet fæces is discarded. The mice are then weighed to the nearest gramme and the weights recorded. They are evenly divided into 4 groups each of 10 mice. As will be shown, later, it is necessary to give two dose-levels each of the standard preparation and the test preparation for every assay. The dose is given into the œsophagus of the animal by means of a blunted needle attached to a 1 ml.-syringe. After dosing, the animals are kept under observation for at least 12 hours.

During this testing period, a special food made by mixing 10 parts of powdered rat-cubes and 7 parts of fresh tap-water is supplied in the food containers. This moist food has several advantages: it allows the animals a uniform intake of water in proportion to the diet; it ensures the normal working of the alimentary canal during the 12 hours of test; it, unlike the dry rat-cubes, when scattered during feeding, does not absorb water from the wet fæces. Moreover, the inconvenience of using a large number of water-bulbs is avoided.

Purgation is indicated by the excretion of wet fæces which are recognised by their somewhat rounded shape and the presence of a brown stain surrounding each on the blotting paper. They can be easily distinguished from the normal dry fæces which are elongated in shape and do not stain the paper. Counting of the wet fæces is usually started from the second hour after dosing and repeated every 1½ hours until the fifth or sixth hour. The final counting is done early in the following morning.

Relation Between Dose and Response

One would expect that an increase in the dose of purgative would result in an increase in the number of wet fæces produced; in other words, the response evoked by purgatives is "quantitative" rather than the "all or none" type such as that evoked by digitalis where the animal

TABLE I

Numbers of wet fæces produced by groups each of 10 mice

Experiment No.	Body-weight of 10 Mice	Dose mg./kg.	Number of WF per Group	Number of WF per kg. of Mouse
	g.			
30	210	280	8	38·1
	245	350	15	61·2
	198	840	25	126·3
	233	1050	34	146·0
33	328	280	19	57·9
	305	350	23	75·4
	329	840	46	139·8
	320	1050	49	153·2

either lives or dies. The following experiments were designed to prove this assumption and to investigate the relation, if any, between dose and response.

4 graded doses of powdered senna leaf suspended in distilled water were given to 4 groups each of 10 mice and the number of wet fæces (WF) produced by each group was recorded as shown in Table I.

Table I clearly shows that an increase in dose produced an increase in the number of wet fæces. To determine the relation between the dose and response, graphs were constructed to illustrate the relation of response to (a) dose (Fig. 2) and (b) logarithm of the dose (Fig. 3). To eliminate

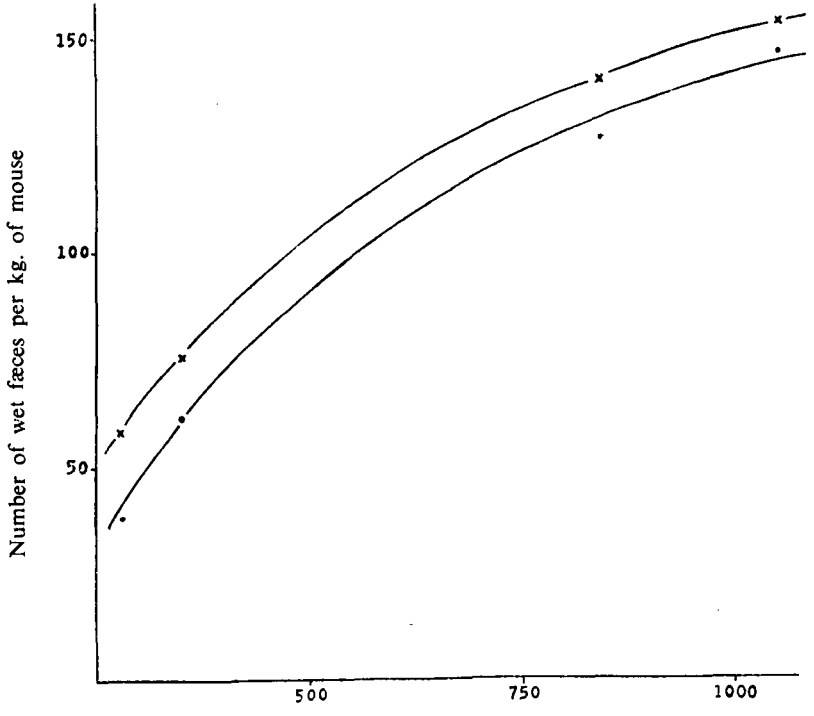

FIG. 2.—Relation of response to dose.

the variation in body-weight of different groups of mice, the response was expressed as number of wet fæces per kg. of mouse. These graphs show that the relation response/log. dose is linear, whereas response/dose is not so. This conclusion was confirmed by later experiments (using the same sample of senna leaf as above) involving 92 × 10 mice; the responses of groups of mice were averaged for each dose-level and the results are shown graphically in Fig. 4. It can be clearly seen that the response is proportional to the log. dose.

In Fig. 3, it will be noted that the log. dose/response lines representing

the same dose-levels given on different days are almost parallel. Though most subsequent experiments resulted in lines of similar slope this was not invariabley true. Hence, it is necessary always to give two dose-levels each of the test and of the standard for every assay, so that the slope of the log. dose/response line for that particular day can be determined. Furthermore, if the distance between the lines of response is unusually great or the slopes of the log dose/response lines of the standard and the test given on the same day differ to a great extent, one may suspect that either the choice of the dose-levels is unsuitable or the nature of the response is different due to different types of active constituents present in the standard and the test, or that the distribution of mice is uneven. It may be necessary, therefore, to repeat the test after due consideration.

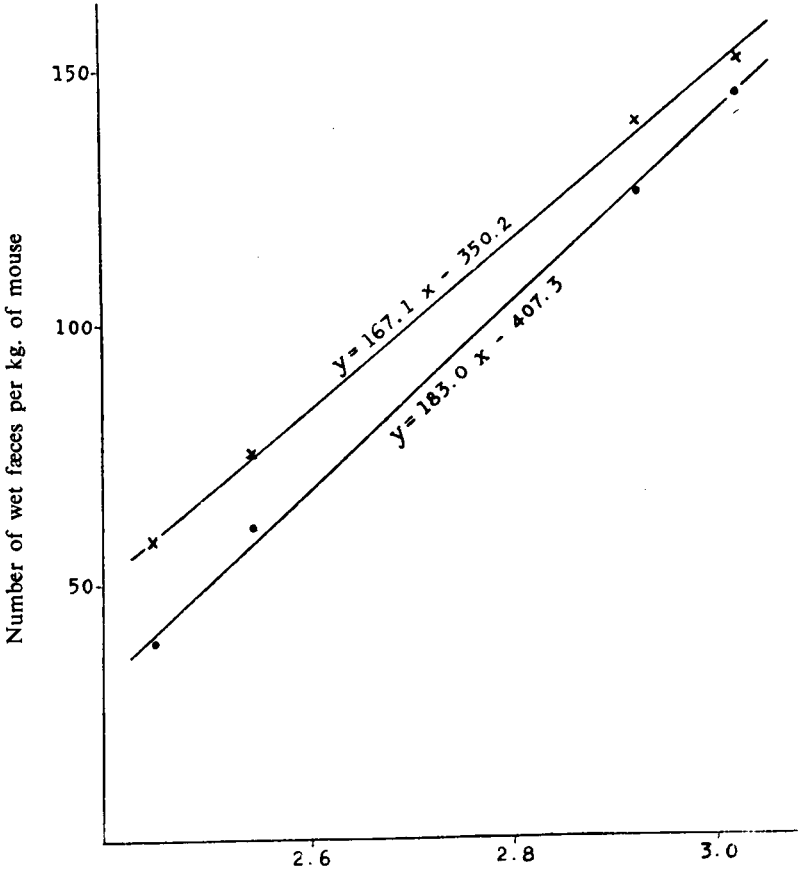

FIG. 3.—Relation of response to the logarithm of the dose.

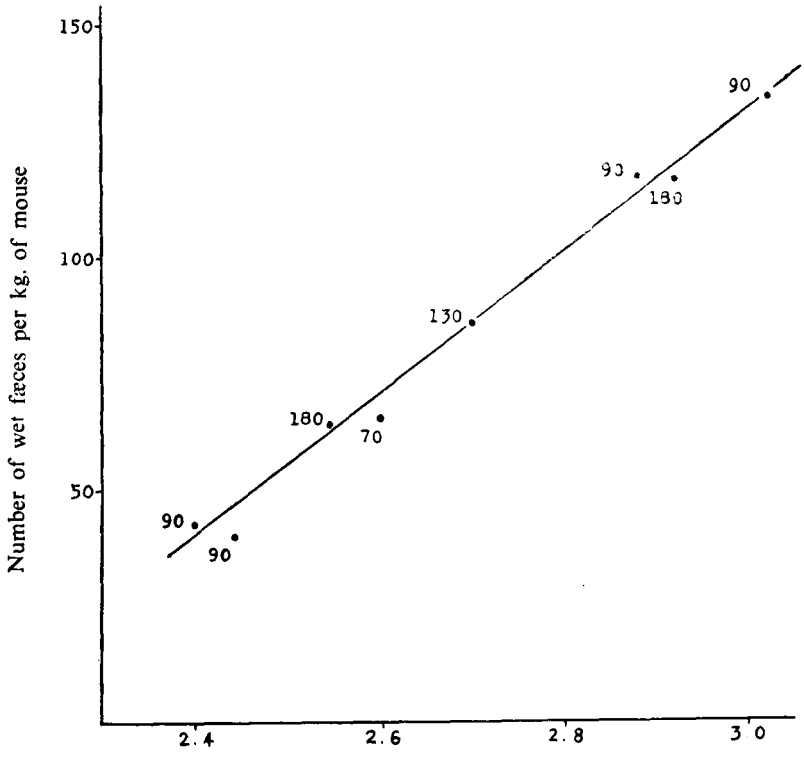

FIG. 4.—The average response/log. dose line. The figures indicate the number of mice used.

EXAMPLE OF THE METHOD: ASSAY OF DRY EXTRACT OF SENNA

A sample of dry extract of senna (E2) made in this laboratory was compared with the laboratory standard sample of senna fruit (Ps) from which the extract was made, by testing them on 4 groups each of 10 mice. In Table II are given the details of the test.

TABLE II

BIOLOGICAL ASSAY OF E2 (EXPERIMENT NO. 68)

	Body-weight of 10 Mice	Dose mg./kg.	Number of WF per Group of Mice	Number of WF per kg. of Mouse
	g.			
Ps	197	350	21	106·7
Ps	186	1050	47	252·7
E2	190	150	28	147·4
E2	185	450	51	275·7

Calculation: (At length to demonstrate the principle involved).

Tripling the dose of Ps caused an increase of 146·0 WF/kg.

Tripling the dose of E2 caused an increase of 128·3 „

Mean effect of tripling the dose ... ... = **137·15** „
Mean effect of the two doses of Ps ... ... = 179·7 „
Mean effect of the two doses of E2 ... ... = 211·55 „
Difference between the mean effects of Ps and E2 = **31·85** „

Since the number of *wet* fæces per kg. of mouse is proportional to the logarithm of the dose

$$\frac{137{\cdot}15}{\log.\ 3} = \frac{31{\cdot}85}{\log.\ r} \quad \text{or} \quad \frac{137{\cdot}15}{31{\cdot}85} = \frac{\log.\ 3}{\log.\ r}$$

where r is the ratio of the potency of the doses of Ps and E2.

Hence $\log.\ r = 0{\cdot}1109$
and $r = 1{\cdot}291$
i.e. $\dfrac{\text{potency of 150 mg. E2}}{\text{potency of 350 mg. Ps.}} = 1{\cdot}291$

Potency of 1 g. E2 = potency of 3 g. Ps.

i.e. the extract E2 possesses three times the purgative activity of the same weight of the standard senna fruit from which it was made.

Accuracy of the Method

In order to determine the accuracy obtainable by the method, doses of a laboratory standard of powdered senna leaf were given to 4 groups (1, 2, 3, 4) each of 10 mice on 9 different occasions. 2 of the 4 groups (1 and 3) each received 3 times the dose given to the other 2 groups (2 and 4). The responses of each of the 4 groups were noted. The responses of 1 high-level dose and 1 low-level dose (say groups 1 and 2) were taken to represent a potency of 100, the potency of the remaining two (groups 3 and 4) was calculated. This process was repeated by rearranging the groups so that the responses of groups 1 and 4 were taken to represent a potency of 100, and the potency of the groups 2 and 3 was calculated as before; hence, one single "assay" yielded two results. Altogether 18 such results were obtained and are recorded in Table III. The mean (M) of these 18 results was 99·44 with a standard deviation (σ) of 15·626 (or 15·714 per cent. of the mean). Limits of error (P = 0·99) for a single assay are therefore 100 ± 40·5 per cent.

Calculation:

Mean potency of B calculated $= \dfrac{1789{\cdot}94}{18} = 99{\cdot}44.$

Sum of squares of deviations from the mean $= \Sigma d^2$ 4150·832.

Standard deviation of a single determination

$$\sigma = \sqrt{\frac{4150{\cdot}832}{18\text{-}1}} = 15{\cdot}626 \text{ or } \frac{15{\cdot}626}{99{\cdot}44} \times 100 = 15{\cdot}714 \text{ per cent.}$$

Comparison of Accuracy with that of Other Methods

Previous workers used rats[11, 12], guinea pigs[13], daphnia[2], etc., for the evaluation of purgative activity but none of them gave any indication of the accuracy obtainable. Munch[1] who obtained his best results with cats claimed an accuracy of only 20 to 50 per cent. Loewe[3] using rhesus

TABLE III

ESTIMATION OF THE ACCURACY OF THE METHOD

Experiment No.	Group of Mice	Number of WF/kg. of Mouse	Potency of B calculated (A = 100)	Deviation from the Mean d	d^2
30*a*	A 1	126·3	108·93	9·49	90·0601
	A 2	33·5			
	B 3	136·6			
	B 4	38·1			
b	A 1	126·3	103·39	3·95	15·6025
	A 4	38·1			
	B 3	136·6			
	B 2	33·5			
31*a*	A 1	130·4	105·62	6·18	38·1924
	A 2	54·5			
	B 3	135·8			
	B 4	56·8			
b	A 1	130·4	102·29	2·85	8·1225
	A 4	56·8			
	B 3	135·8			
	B 2	54·5			
32*a*	A 1	173·0	89·15	10·29	105·8841
	A 2	82·0			
	B 3	187·3			
	B 4	43·1			
b	A 3	187·3	78·00	21·44	459·6736
	A 2	82·0			
	B 1	173·0			
	B 4	43·1			
33*a*	A 1	146·6	90·88	8·56	73·2736
	A 2	65·4			
	B 3	139·8			
	B 4	57·9			
b	A 1	146·6	100·54	1·10	1·2100
	A 4	57·9			
	B 3	139·8			
	B 2	65·4			
34*a*	A 1	99·2	72·91	26·53	703·8409
	A 2	41·7			
	B 3	82·7			
	B 4	25·0			
b	A 1	99·2	100·19	0·75	0·5625
	A 4	25·0			
	B 3	82·7			
	B 2	41·7			
35*a*	A 1	76·2	105·30	5·86	34·3396
	A 2	31·3			
	B 3	86·2			
	B 4	26·2			
b	A 1	76·2	117·52	18·08	326·8864
	A 4	26·2			
	B 3	86·2			
	B 2	31·3			
37*a*	A 1	119·5	100·50	1·06	1·1236
	A 2	42·9			
	B 3	109·4			
	B 4	53·6			
b	A 3	109·4	118·82	19·38	375·5844
	A 2	42·9			
	B 1	119·5			
	B 4	53·6			
38*a*	A 1	46·0	114·32	14·88	221·4144
	A 2	20·0			
	B 3	72·5			
	B 4	4·9			

Experiment No.	Group of Mice	Number of WF/kg. of Mouse	Potency of B calculated (A = 100)	Deviation from the Mean d	d^2
b	A 3	72·5			
	A 2	20·0	61·35	38·09	1450·8481
	B 1	46·0			
	B 4	4·9			
39*a*	A 1	123·3			
	A 2	22·1	112·97	13·53	183·0609
	B 3	142·3			
	B 4	27·2			
b	A 1	123·3			
	A 2	27·2	107·26	7·82	61·1524
	B 3	142·3			
	B 4	22·1			
TOTALS ...			1789·94		4150·8320

monkey as test animal claimed an accuracy of ± 15 per cent., however there is neither data nor statement as to how many monkeys are needed to achieve this accuracy.

Geiger's original method[5] involved the use of 72 mice per single assay; however, insufficient data is available to calculate the accuracy obtainable. Moreover, his method was improved by Grote and Woods who used 105 mice per single assay. Again, however, no figure for the degree of accuracy was given. This omission from the published methods of bioassay is a serious one, especially in view of the large number of mice used in each assay.

Collier[10] states that the standard deviation of his method is usually about 20 per cent. with a slope (b) of log. dose/response line of about 80 per cent. ($b^2/s^2 = 16$). The limits of error (P = 0·99) of each assay using 40 mice calculated from the formula quoted by him would be 63 and 160 per cent. However, when a ratio of $b^2/s^2 = 30$ is obtained, as he states occasionally occurred, the limits of error using 40 mice would be 71 and 141 per cent. As already stated the limits of error of the method described in this paper (P = 0·99) are 100 ± 40·5 per cent., which on the whole is a higher accuracy than that of Collier's method.

APPLICATIONS OF THE METHOD

The method was found very satisfactory when used to assay senna leaf, senna fruit and extracts and commercial preparations made from these drugs, also the pure glycosides, sennosides A and B, and the pure anthracene compounds, aloe-emodin and aloe-emodin anthranol. The method was further applied to cascara, rhubarb and to preparations of these drugs also with satisfactory results, although in some instances slight modification is necessary. It is hoped to publish details of these investigations later.

The results of many of these assays are incorporated in the paper of Dr. J. W. Fairbairn[14].

SUMMARY

1. A method for the biological assay of vegetable purgatives based on the number of wet fæces produced by groups of mice after dosing is described.

2. The relation of the number of wet fæces per kg. of mouse to the logarithm of the dose was found to be linear.

3. 40 mice divided equally into 4 groups were used in each assay. 2 groups received the standard preparation and the other 2 groups received the test preparation. The standard deviation of a single determination based on 9 such assays was estimated to be 15·7 per cent. The limits of error (P = 0·99) for a single assay are 100±40·5 per cent.

4. A special cage has been designed for this assay, and it has been found advantageous to incorporate a definite proportion of water in the diet, during test.

5. The method described is not only convenient in use but also gives a comparatively high degree of accuracy.

6. The method has been successfully applied to senna leaf, senna fruit and extracts and commercial preparations made from these drugs, pure glycosides (sennosides A and B), and pure anthracene compounds (aloe-emodin and aloe-emodin anthranol).

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