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BIOASSAY OF PHENOLPHTHALEIN USING THE RHESUS MONKEY.*

BY K. A. BARTLETT[1] AND R. H. HERBINE.[1]

Being interested in laxatives in general, and especially phenolphthalein, we were very desirous of having some satisfactory method for evaluation of laxative action. A review of the literature showed that no satisfactory chemical assay was available and that all animals tried in connection with bioassays were not satisfactory, with one exception. The work of Fleig (1), back in 1908, suggested that it would be interesting to try experimental work on the monkey and twenty-six years later the work of Williams, Abramowitz and Killian (2) which, to our knowledge, is the first real work utilizing the monkey, showed definite promise of the development of a satisfactory bioassay. However, this work was not carried on to the point of establishing a definite assay method.

In view of the monkey showing more possibility than any other animal, we decided to further study the monkey in the hope that we might be able to develop a satisfactory bioassay method for laxatives. Our work over the past two years has enabled us to develop a satisfactory method for determining the relative potency of various samples of phenolphthalein. While this work has been conducted particularly in connection with phenolphthalein, we have found in our experiments that the rhesus monkey responds to Epsom Salts, Milk of Magnesia, Cascara, Castor Oil and Aloes. The method which has been evolved and which we are using at the present time follows.

We are using animals not commonly used in drug assays and believe it desirable to go into more detail concerning this method than would normally be considered necessary.

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Animals.—Our colony consists of twenty-three rhesus monkeys, thirteen males and ten females. Although we use the name "rhesus" which is commonly used in describing this type monkey, the specific name is Macaca mulatta (Zimmermann) (3).

Our animals are of various ages and weights, ranging from estimated ages of one and a half to five years old and from 1.5 to 5 Kg. in weight.

When new animals are purchased, they are isolated for a month and very closely observed. Almost half of the animals which we have purchased were rejected during this isolation period and we have had no deaths or signs of ill health in any of the animals which we have accepted and added to our regular colony. The presence of tuberculosis is the reason for the rejection of the majority of the animals during this isolation period. All the animals in our regular colony have gained considerable weight. Some of the older animals have more than doubled their weight. This, in itself, is an excellent criteria of their good health and contentment.

We have never purchased any monkeys which were entirely free from intestinal parasites. A few of the animals had only round worms. Most of them, however, had both round worms and hookworms. We originally thought that test animals used for this type work should be entirely free from all intestinal parasites and although we have spent considerable time in attempting to free these animals of these parasites, we have not been able to find an anthelmintic which is one hundred per cent effective against hookworms. However, we do have some animals which originally had only round worms and are now entirely free from these intestinal parasites, and we have observed that the results obtained on these worm-free animals are no more significant than those obtained upon the infested animals. There is, therefore, a question as to the importance of this point.

We have been unable to observe any difference in results obtained from either sex. If future experience bears out this observation, we feel that females will probably predominate because it has been our experience that females are more friendly, less nervous and easier to handle. We have been unable to observe any abnormal effects on administering phenolphthalein to females during the menstrual period. However, as our females become older this observation may be reversed.

Housing and Care.—The monkey laboratory is completely isolated from the rest of the plant. It is kept under lock and key at all times and only the immediate attendants are admitted. The animals are at no time teased, scolded or annoyed in any way.

The cages are 30" square, 42" in height and each is equipped with a swing, perch and removable front and sides. The bottoms are of expanded metal, the openings of which are large enough to allow the droppings to fall through into a removable metal tray.

A temperature of 75–85° F. is maintained whenever possible. Our cages are comparatively large and because of their size and their being equipped with a swing and perch, it is not necessary to have a so-called exercising cage.

The laboratory is so situated that it is entirely exposed on all four sides and although there is an abundance of light and fresh air, the animals are at no time exposed to direct sunlight. However, in our opinion, our diet adequately makes up for a lack of direct sunlight. On this question of direct sunlight, it is interesting to note that Mr. Otto Heinzer, caretaker of the Department of Pathology, Harvard Medical School has kept four rhesus monkeys in good health for a period of eight years in an indoor laboratory cage 5′ x 4′ x 3′. The diet used was very similar to the one we are using.

Diet.—The morning (9 A.M.) and evening (5 P.M.) meals each day consist of giving each animal approximately 4 fluidounces of milk-egg mixture (2 eggs per quart of milk prepared with Klim Powdered Whole Milk according to label directions). The noon meal is varied as follows:

Sat., Sun. & Mon.	Tues. & Wed.	Thurs. & Fri.
1 banana	1 banana	1 banana
1/4 head cabbage (med.)	1 small apple	1 small apple
1 small apple	spinach & carrot tops	1/2 head lettuce (med.)
	1/2 carrot	
	1/4 orange	

The food given at the noon meal is sprinkled with about one teaspoonful of bone meal and blood meal (equal parts). Every morning with the milk, each animal receives one White's Cod

Liver Oil Concentrate Tablet. The amounts of food are only tentative and we vary these amounts to suit the individual monkey. We try not to overfeed the animals but endeavor to give them just enough food to satisfy them and no more. Occasionally between feedings several grains of dried corn are given to the animals. With this diet the animals normally have stools of a putty-like consistency.

Technique.—We have tried several methods of administering the phenolphthalein doses to the animals. The one which we have found to be most satisfactory is to deposit the weighed amounts of phenolphthalein in the center of a small, $^3/_8$", cube of apple. In administering this cube of apple containing phenolphthalein, we find it only necessary to hold the apple cube in front of the cage. The animal will readily take and eat the entire cube. A cube of plain apple is given almost immediately after.

We have found that two doses can be administered to the animals each week. At the present time we administer doses on Mondays and Thursdays. All doses are administered immediately following the morning milk feeding. Stool examinations are made every hour after administration for six hours and also a twenty-four hour reading is made, noting the consistency of the stools.

Our results are either positive or negative, being based on passage of stools which are noticeably different (more fluid) then the normal stools which have a putty-like consistency. Sometimes these stools are very watery and without any form, and, on the other hand, they may be soft and though they do not have any definite form they do not run or spread as do the watery ones. Therefore, a negative reaction consists of the passage of no stools or of a stool of normal consistency within twenty-four hours, and a positive reaction consists of the passage of a soft stool or a watery, unformed movement within twenty-four hours.

Standardization of Animals.—The first goal of our work was to standardize all the test animals, that is determine the minimum (threshold) dose which will produce the desired effect. All dosages are based on the body weight of the animals—allowances are made of any gain in weight from week to week. In establishing this minimum effective dose, the animals were first given 1.0 mg. per Kg. of body weight of phenolphthalein, increasing the dosage gradually thereafter until the minimum effective dosage was reached (see Table I).

TABLE I.—DETERMINATION OF THRESHOLD DOSES OF INDIVIDUAL MONKEYS.

Animal Number.													Threshold Dose.
2	1.5	2.0	2.5	3.0	3.0	3.5	3.5	3.5	3.5	3.5	3.5	3.5	*3.5*
	N	N	N	W	N	N	W	W	S	S	W	W	
4	2.0	3.0	3.5	3.5	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	*4.0*
	N	N	W	N	W	W	S	S	S	W	S	W	
7	1.0	2.0	3.0	4.0	5.0	6.0	6.5	7.0	7.0	7.0	7.0	7.0	*7.0*
	N	N	N	N	N	N	S	W	W	S	S	S	

All doses are in mg. per Kg. of body weight.
N = normal.
S = soft.
W = watery.

Each animal was standardized following the above procedure and Table II shows the standardization figures for the individual animals.

A sample of phenolphthalein has been set aside by us and we are arbitrarily recognizing this as standard reference phenolphthalein.

A number of assays were then conducted using our standardized monkeys and the standard phenolphthalein in the following manner. Nine standardized monkeys were divided into three groups of three animals each, one group was given their respective threshold doses, another group 75% of their respective threshold doses and the third group received 125% of their respective threshold doses. Stool observations were made in accordance with the procedure previously outlined. The results of each assay (potency index[1]) were calculated by adding the percentage

[1] Potency Index—Comparative laxative response obtained in an assay in terms of response produced by standard phenolphthalein (taken as 100).

TABLE II.—INDIVIDUAL THRESHOLD DOSES.

Animal No.	Sex.	Mg. per Kg. of Body Weight.
1	F	2.5
2	F	3.5
3	F	3.0
4	F	4.0
7	M	7.0
8	M	3.6
9	M	3.5
10	M	6.0
11	M	6.5
13	M	2.5
16	M	5.5
17	F	2.5
19	F	4.5
20	F	3.0
23	M	1.8

positive reaction of the three groups together and dividing by two. The following potency indices were obtained:

105.0, 75.0, 105.0, 105.0, 105.0, 120.0, 90.0, 120.0, 100.0, 100.0, 93.7, 83.3; average, 100.08.

By applying interpretative mathematics (4) to these results we obtain the following biometric expressions which indicate the accuracy of the method.

True Mean, 100.08; Average Deviation, 9.83; Probable Error, 8.31; Chance to be taken (5), 1 in 100.

Applying these expressions to the accepted formula

$$N = \left(\frac{K \times PE}{Diff.}\right)^2$$

we find that the degree of accuracy is 10.58%. This means that we are able to detect a variation of 10.58% or more in potency when using nine animals, and that the chance of error is 1 in 100.

PROPOSED METHOD OF ASSAY.

Animals.—The colony should contain at least twenty-one rhesus monkeys (macaca mulatta) of either sex and weighing between 1 and 5 Kg. Each animal should be housed in an individual cage and all animals should be fed exactly the same diet. Before conducting assays, all animals must be individually standardized with a standard reference sample of phenolphthalein. (Although only eighteen animals are used in conducting an individual assay, it is advisable to maintain at least three additional animals.) In the interests of accuracy, periodic re-standardization of animals is suggested.

Procedure.—Immediately following the morning meal, give reference phenolphthalein to nine animals and the unknown to an additional lot of nine animals. Three animals from each group are given their threshold dose (standardization dose), three are given 75% of their threshold dose and three are given 125% of their threshold dose. The doses are given in a small $^3/_8$" cube of apple.

Examine the individual cages at hourly intervals for six hours, and at twenty-four hours, noting the consistency of stools. A negative reaction consists of the passage of no stool or of a stool of normal consistency within twenty-four hours. A positive reaction consists of the passage of a soft stool or of a watery unformed movement within twenty-four hours.

Interpretation.—Add the percentage of positive reactions obtained in the three groups of animals receiving reference phenolphthalein and divide by two to obtain the potency index. If this value is not 100 make proper adjustments in interpretation. Similarly add the percentage of positive responses with the unknown at the three dosage levels and divide by two to obtain the

potency index of the unknown. Divide the potency index of the reference standard phenolphthalein by that of the unknown to determine their relative potency.

Results not falling within the range of error previously determined for the reference standard can only be considered indicative of the unknown potency. Adjustments of the unknown dosage level are then necessary and the assay re-run at this adjusted level.

DISCUSSION.

Subsequent reports will be presented covering the assays of unknown samples of phenolphthalein and other laxative substances.

We believe that the rhesus monkey is as near the ideal test animal for laxatives as is possible to find. This is borne out by the fact that the mg. per Kg. doses required by the monkey when calculated on the basis of the human are practically identical.

CONCLUSIONS.

1. A procedure for the bioassay of phenolphthalein using the rhesus monkey has been developed and found satisfactory.
2. Individual rhesus monkeys require different threshold doses of phenolphthalein.
3. This bioassay method might be found applicable to all other laxatives.

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PHARMACEUTICAL USES OF THE GLYCOLS AND THEIR DERIVATIVES.*

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The use of the glycols and their derivatives in Pharmacy is of comparatively recent origin but is increasing rapidly or was, until the occurrence of the "Elixir of Sulfanilamide" tragedies, a little more than a year ago. While it is a fact that ethylene glycol was prepared by Wurtz (1) in 1859 and that its possibilities as a substitute for glycerol were called attention to by a number of investigators over the next fifty or sixty years, little, if any, consideration was given to it or to the glycols prepared later (2) until after the World War. Prior to this time, alcohol was the solvent generally used by pharmaceutical manufacturers. It was cheap, there were no burdensome restrictions on its use and it was deemed to be as satis-

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