THE ASSAY OF VERATRUM VIRIDE.*[1,2]

BY B. V. CHRISTENSEN[3] AND A. P. MCLEAN.[4]

In a previous paper (1) the literature dealing with the attempts to standardize preparations of Veratrum viride by chemical and pharmacological methods was reviewed. A consistent emetic response of pigeons to small doses of Veratrum was reported, and the determination of the minimum pigeon emetic dose was suggested as a possible means of estimating the potency of preparations of this drug.

The present work was undertaken to determine the suitability, accuracy and reliability of the minimum pigeon emetic dose method for estimating the potency of Veratrum viride and to determine whether or not this method is more suitable than previously proposed assay methods.

I. PIGEON EMESIS.

The method has been previously described (1) as a modification of the method proposed by Hanzlik (2, 3) for estimating the potency of digitalis. The minimum pigeon emetic dose (M. Em. D.) for a preparation of Veratrum viride was considered as the smallest amount of the drug, expressed in cc. of the tincture per Kg. body weight, which would produce emesis in at least 75 per cent of a group of eight pigeons within fifteen minutes after the intravenous injection of the diluted tincture.

In this study thirty-three assays were carried out on twenty tinctures of Veratrum viride; an average of thirty-three pigeons per assay was used. The M. Em. D. of the tinctures ranged from 0.010 to 0.090 cc. per Kg., and the mean M. Em. D. for all assays was found to be 0.0395 cc. per Kg. Since these assays were performed in the usual manner, the detailed results will not be given; however, the following dose-response chart, based upon the data obtained from all of the emetic assays, is presented.

A record was kept of the individual responses of one hundred and fifty pigeons during the course of Veratrum assays over a period of three months. An analysis of this data indicates that, although there is some variability of response as one would expect with any physiological experiments, there is not sufficient difference in the response of pigeons to Veratrum to necessitate the "standardization" of the test animals. Furthermore, a comparison with the analyses made of other assay methods showed that pigeons gave a more uniform response to emetic doses of Veratrum than dogs, cats, rabbits, mice or frogs gave to lethal doses of this drug.

Since Lieb and Mulinos (4, 5) have reported that, upon continued use for emetic assays, pigeons became susceptible to the conditions of the experiment and that spontaneous emesis would result in a high per cent of animals when saline was injected, it was decided to investigate this possibility of error under the conditions of these experiments. From time to time during the regular assay work saline was injected instead of the drug. One hundred and twenty-four saline injections were made, and in only two cases did emesis occur. This difference in results is perhaps due to the fact that Lieb and Mulinos injected pigeons with a weekly emetic dose, while in the Veratrum assays reported above the pigeons were allowed a rest period of approximately two weeks between injections, and in routine assays many animals received injections of sub-emetic doses. These results indicate that the "conditioning" of pigeons to emesis is not a great source of error under the conditions observed in these assays.

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[3] Director, School of Pharmacy, University of Florida.

[4] Instructor in Pharmacology, School of Medicine, University of Oklahoma.

Since the quantity of alcohol present in an emetic dose of the tincture was quite small (average about 0.009 cc. per animal), it would appear improbable that it would have any effect upon the emetic dose. The wide range in potency of the preparations assayed would appear to substantiate this belief; however, injections of 3 cc. of 20 per cent alcohol per Kg. were made in one hundred and seven pigeons and emesis resulted in only two cases. This is in agreement with results reported by Hanzlik (3) in connection with the pigeon assay of digitalis.

The possibility that pigeons might develop a tolerance for Veratrum or that they might become more susceptible after repeated injections was investigated. In 1937 several preparations were assayed using some pigeons for the first time and others that had been used repeatedly. No difference in response could be detected. In 1938 two tinctures were assayed on pigeons that had been used at intervals of fourteen days for three months; on the same day each preparation was assayed on birds that had not been previously used for Veratrum assays. The emetic dose of one tincture was the same with both groups; that of the second tincture was 0.005 cc. per Kg. larger

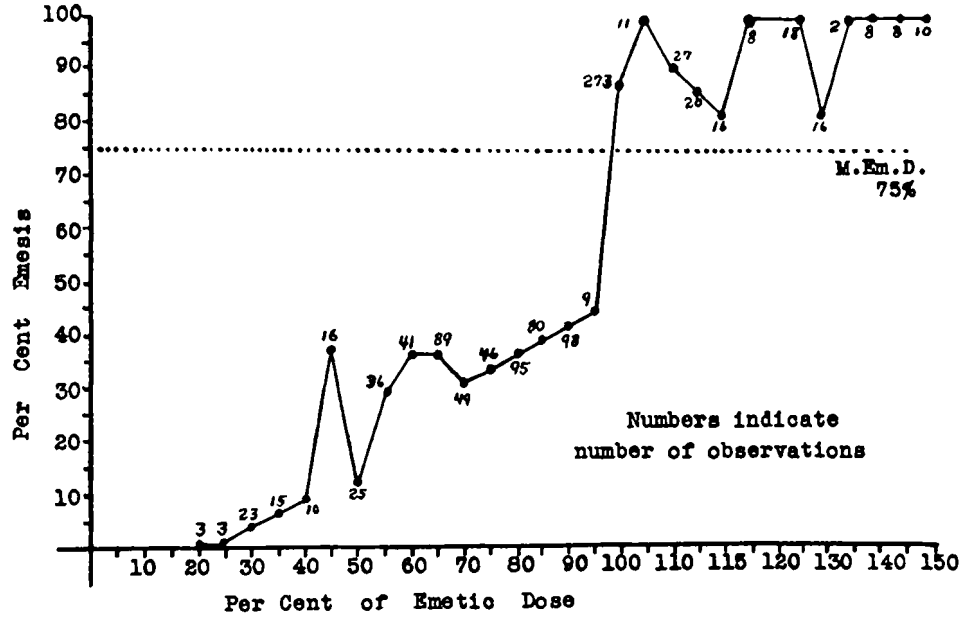

Fig. 1.—The response of pigeons to fractions of the M. Em. D. The per cent emesis represents the mean for all observations at a given dose fraction.

with the group of pigeons repeatedly used, but this difference was within the range of experimental error.

It was found that the emetic response of pigeons to Veratrum viride was quite sensitive and that differences of 10 per cent in dosage could be easily detected.

II. MINIMUM LETHAL DOSE METHODS.

Pigeons.—For the determination of the M. L. D. for pigeons, healthy adult pigeons weighing from two hundred and fifty to four hundred Gm., from which food had been withheld for from twelve to sixteen hours, were weighed to the nearest 5 Gm. and tied on a pigeon board (3). A single injection of the tincture, diluted one to three in physiological salt solution, was made into the brachial vein after which the birds were immediately removed from the board and placed in observation cages.

With a fatal dose death usually resulted within four minutes and in only three instances did death occur after fifteen minutes. In view of the rapid action of the drug, it was decided to exclude these delayed reactions and to set fifteen minutes as the maximum observation time. One hour after the injection of a sub-lethal dose pigeons were found to be markedly depressed, but in only one case did death occur after this time.

The M. L. D. for pigeons was considered as the smallest amount of the drug, expressed as cc. of tincture per Kg. body weight, which would produce death in at least 75 per cent of a group of eight pigeons within fifteen minutes after intravenous injection.

The lethal dose for twelve tinctures was determined with an average of twenty-four pigeons used for each tincture. The M. L. D. of these tinctures ranged from 0.30 to 1.05 cc. per Kg. The following dose-response chart is based upon the data obtained in these determinations:

An analysis of the above series of assays showed a very favorable consistency in the response of pigeons to fatal doses of Veratrum. It was found that for the twelve tinctures assayed the mean M. L. D. was 13.05 (A.D. ± 1.9) minimum pigeon emetic doses per Kg. This deviation of ±15 per cent shows a close relationship between the M. Em. D. and the M. L. D. for pigeons. This ratio of one to thirteen seems quite favorable from the standpoint of Veratrum toxicity.

The fact that emesis is produced by one-thirteenth the fatal dose of Veratrum is a point in favor of the emetic reaction as the end-point for the biological assay, for it would appear that the

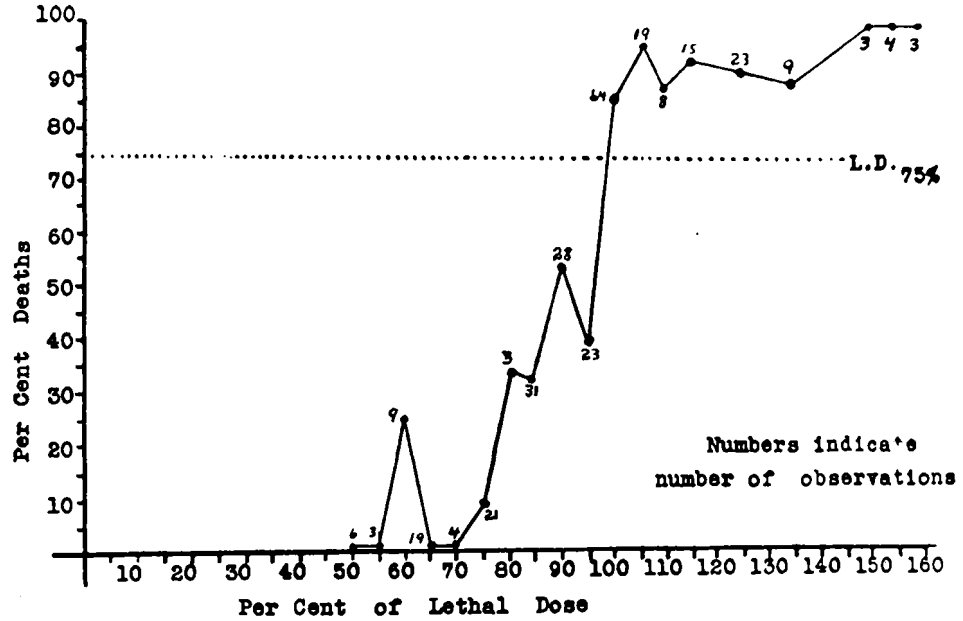

Fig. 2.—The response of pigeons to fractions of the M. L. D. The per cent deaths represents the mean for all observations at a given dose fraction.

various factors of error inherent in a biological assay would be proportionally increased with the increased dosage necessary to produce death as an end-point for an assay.

Although the minimum emetic and the minimum lethal doses of twelve tinctures of Veratrum showed close parallelism in pigeons, the M. Em. D. method is preferred for the reason that it is based upon a response to a large therapeutic dose rather than to a fatal dose. In addition, the emetic dose method is more economical as the animals can be repeatedly used.

White Mice.—The method employed was similar to those used by Rowe (6) and by Swanson and Hargreaves (7, 8). Healthy adult white mice were weighed to within the nearest 0.5 Gm. and injected intraperitoneally with freshly prepared dilutions of the tinctures, one to three, in physiological salt solution. The M. L. D. was considered as the smallest amount of the drug, expressed as cc. of the tincture per Gm. body weight, which would produce death in at least 75 per cent of a group of eight mice within one hour after the intraperitoneal injection.

Twenty-four M. L. D determinations were carried out with an average of thirty-three mice used for each assay. This dose for the fifteen tinctures used ranged from 0.0020 to 0.0080 cc. per Gm. The following dose-response chart is based upon the 784 injections made in this study:

An analysis made of six hundred and fifty mouse injections showed that mice were not as consistent in their response to Veratrum as were pigeons. Of the 130 deaths recorded from doses

below the M. L. D. only 12 per cent were from doses within 10 per cent, and 56 per cent were from doses within 20 per cent of the M. L. D. Of the recoveries from doses above the M. L. D. 53 per cent were from doses more than 20 per cent above the M. L. D.

No parallelism was found between the mouse assay and the pigeon emetic assay.

Frogs.—For several days before the M. L. D. determinations were to be made, the frogs were kept in a tank of running water at a temperature of approximately 20° C. The tincture to be used was evaporated to one tenth the original volume on a water-bath and brought to one-half the original volume with physiological salt solution. Injections of the drug were made into the ventral lymph sac, and the frogs were placed in moist jars at room temperature for observation. Over two hundred injections were made, but the method was so unsatisfactory and the response so variable that the results will not be presented.

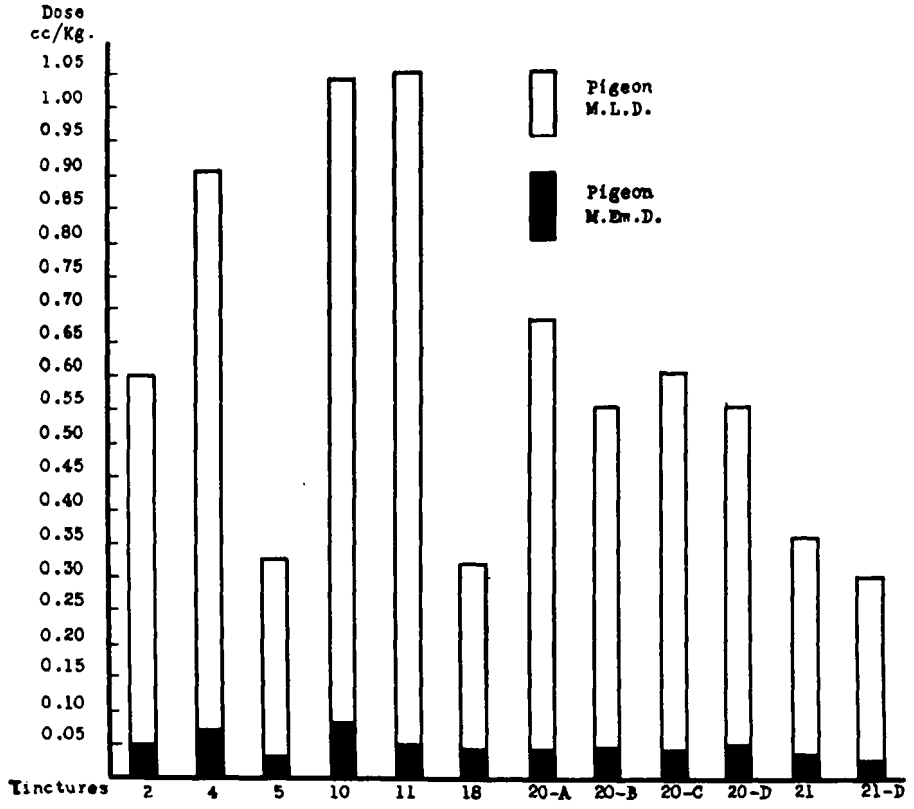

Fig. 3.—Relationship between the M. L. D. and M. Em. D. for pigeons.

Cats.—The cats were weighed and given an oral anesthetic dose of morphine-urethane. The tincture, freshly diluted with physiological saline, was injected into the femoral vein at the rate of approximately two M. Em. D. (pigeon) per Kg. per minute until the circulation failed. The characteristic effects produced by the drug are shown in Fig. 5.

Thirty-four lethal dose determinations of several tinctures of Veratrum were made but the results were exceedingly variable. An example of this variability is seen in the case of tincture No. 20-B. Seven determinations in cats gave the following doses: 18, 80, 100, 11, 33, 12 and 6 minimum pigeon emetic doses per Kg. or, expressed in volume of tincture, 0.585, 2.60, 3.25, 0.358, 1.075, 0.390 and 0.195 cc. per Kg.

Because of the inconsistency of results obtained with this method (the average of the deviations for the series of experiments was ± 71 per cent) it was concluded that the M. L. D. for cats would not furnish a satisfactory basis for the assay of Veratrum preparations.

In order to eliminate vagal activity and the influence of respiratory depression, twelve lethal dose determinations were made on cats with divided vagi, and six determinations were made on cats with artificial respiration. Approximately the same mean lethal dose and the same variability of results were obtained as in the case of normal cats.

Dogs.—The dogs were weighed, anesthetized with ether and tied on an animal board. The carotid artery was cannulated for the recording of blood pressure with a mercury manometer. The

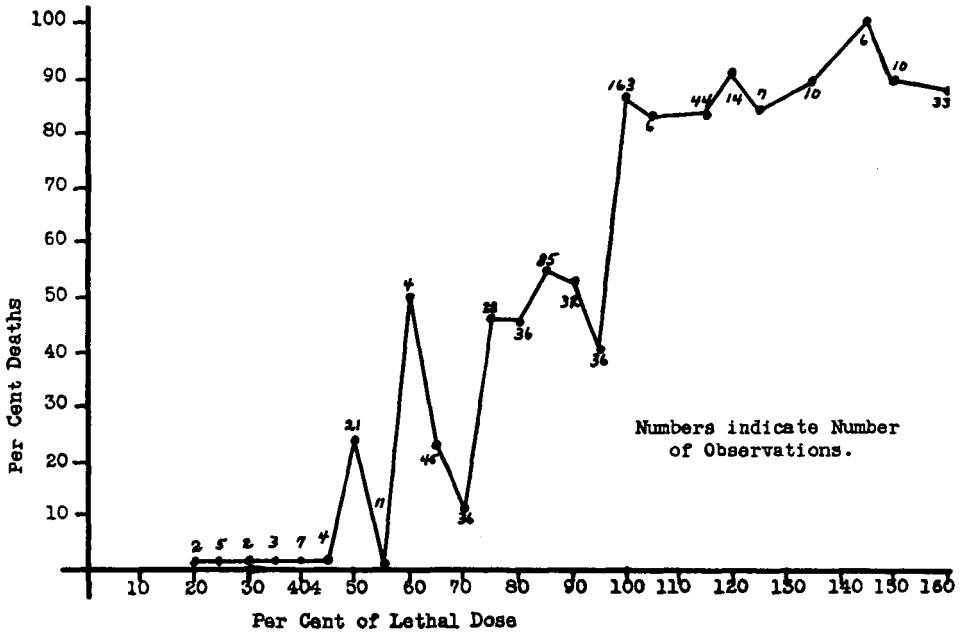

Fig. 4.—The response of mice to fractions of the M. L. D. The per cent deaths represent the mean for all observations at a given dose fraction.

injection of the tinctures, diluted with physiological salt solution, was made into the femoral vein at the rate of approximately two minimum pigeon emetic doses per Kg. per minute. Seven determinations were made, and the results were found to be as variable as with cats.

Rabbits.—The procedure followed was the same as that described for dogs except that, with rabbits, the diluted tincture was injected into the marginal ear vein. Five determinations were made, and the results were found to be more variable than with either cats or dogs.

III. THE RELATIONSHIP BETWEEN THE PIGEON EMETIC AND THE CHEMICAL ASSAY.

The alkaloidal content of several tinctures was determined by the method described by Viehoever and Clevenger (9). The results of these assays are presented in Table I.

TABLE I.—ALKALOIDAL ASSAYS OF EIGHT TINCTURES OF VERATRUM VIRIDE. FOR COMPARISON OF THE CHEMICAL AND PHYSIOLOGICAL ASSAYS, THE MINIMUM PIGEON EMETIC DOSE OF EACH TINCTURE IS GIVEN.

Tincture Number.	Total Alkaloids Per Cent.	M. Em. D. Pigeon Cc./Kg.
5	0.637	0.030
10	0.129	0.025
11	0.148	0.030
12	0.145	0.030
13	0.203	0.015
18	0.141	0.035
20-A	0.168	0.020
21-A	0.107	0.010

The above results show wide variations between the chemical and biological assays of Veratrum preparations. Tincture No. 21-A had approximately one-sixth the alkaloidal content

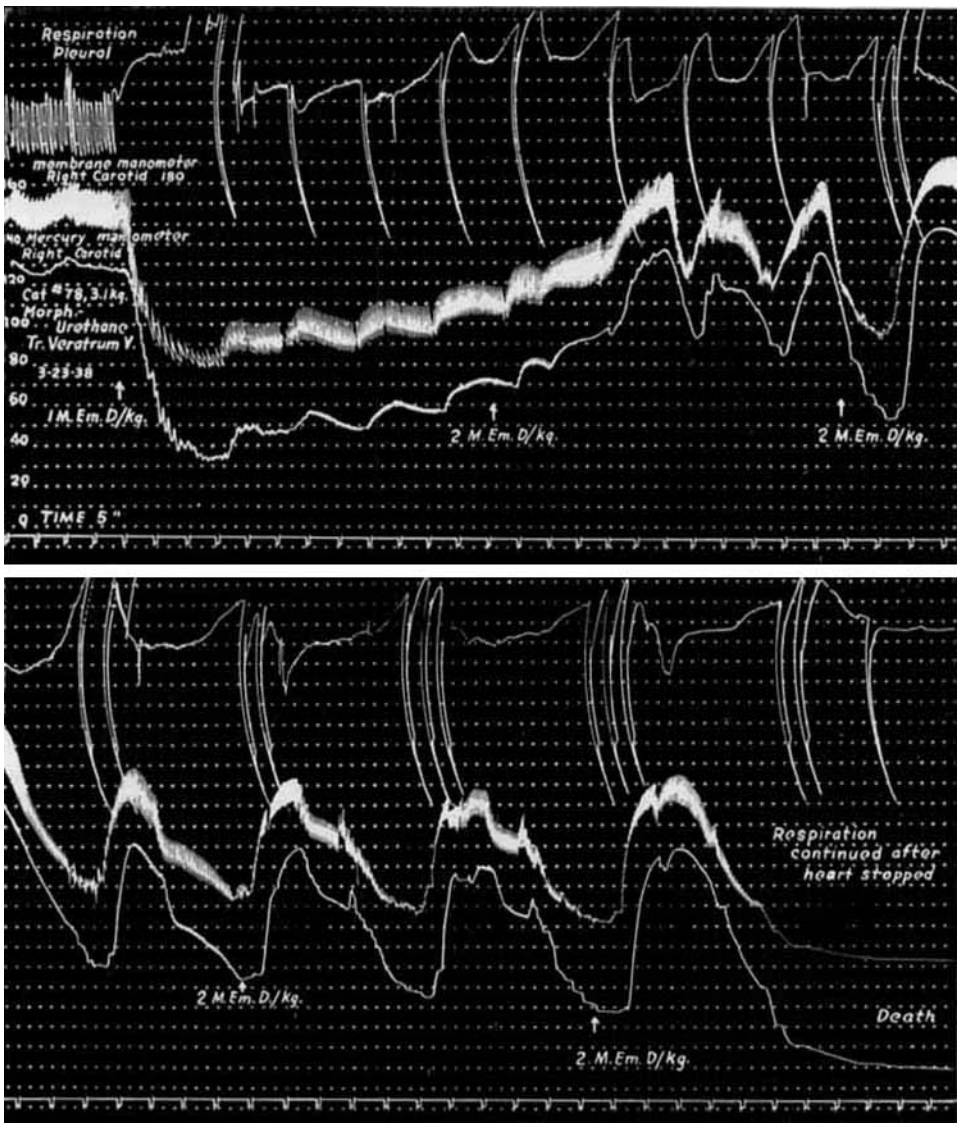

Fig. 5.—A Minimum Lethal Dose determination of a tincture of Veratrum viride on a cat.

of tincture No. 5 but possessed three times the physiological activity of tincture No. 5 as indicated by pigeon emetic assay.

IV. THE EFFECT OF p_H ON THE STABILITY OF TINCTURES.

In 1937 tinctures No. 20 and 21 were prepared from different samples of crude drug by the U. S. P. X method. Each tincture was assayed by the pigeon emetic and the mouse methods, divided into four fractions and placed in amber bottles. To the B, C and D fractions of each tincture were added increasing amounts of acetic acid and the p_H activities were determined by the

electrometric method. One year later the tinctures were again assayed and the p_H activities were determined by the same method.[1] The results of this study are presented in the following table:

TABLE II.—SUMMARY TABLE SHOWING THE RELATIONSHIP OF THE p_H ACTIVITY CHANGES TO THE PHYSIOLOGICAL ACTIVITY CHANGES OF TINCTURES OF VERATRUM VIRIDE.

Tincture Number.	p_H Activity. 1937.	1938.	M. Em. D. Pigeon Cc./Kg. 1937.	1938.	M. L. D. Mice Cc./Gm. 1937.	1938.	Change in p_H	Decrease in Em. Potency Per Cent.
20-A	5.57	6.53	0.020	0.035	0.0030	0.0030	0.96	75.0
20-B	5.53	6.31	0.020	0.0325	0.0030	0.0025	0.78	62.5
20-C	5.37	5.89	0.020	0.040	0.0030	0.0030	0.52	100.0
20-D	4.87	6.29	0.020	0.050	0.0030	0.0025	1.42	150.0
21-A	5.16	6.18	0.010	0.030	0.0025	0.0025	1.02	200.0
21-B	5.06	5.99	0.010	0.035	0.0025	0.0030	0.93	250.0
21-C	5.02	6.21	0.010	0.030	0.0025	0.0025	1.19	200.0
21-D	4.93	5.96	0.010	0.020	0.0025	....	1.03	100.0

From the results presented in Table II it appears that unbuffered tinctures of Veratrum change in p_H activity on standing regardless of whether or not the original p_H was altered by the addition of an acid.

There was little or no change in the M. L. D. of the tinctures for white mice at the end of one year. This is essentially in agreement with the reports of Swanson and Hargreaves (7, 8). According to the pigeon emetic assay, however, these preparations showed a variable decrease in potency.

V. THE RELATIONSHIP BETWEEN THE PIGEON EMETIC ASSAY AND THE CIRCULATORY EFFECTS PRODUCED IN ANIMALS.

Cats.—The cats were weighed, given an oral anesthetic dose of morphine-urethane, and tied on an animal board. The carotid artery was connected to a mercury manometer for recording the blood pressure and to a membrane manometer to show more clearly the changes in the rate and amplitude of the pulse. The drug was injected into the femoral vein in the usual manner.

In the preliminary work it was noted that small intravenous doses of a tincture of Veratrum caused a rapid and pronounced fall in blood pressure, and that there was a difference in the degree of the effects produced by the injection of equal volumes of different tinctures. In the following chart is presented the summary of results of one series of thirty-two blood pressure determinations in which a definite volume (0.005 cc. per Kg.) of several different tinctures was injected. The relationship of the blood pressure effect to the pigeon emetic assay is shown.

In a second series of twenty-one blood pressure determinations, one pigeon M. Em. D. of a tincture per Kg. was injected. Although the eight tinctures used in this series differed as much as 266 per cent in potency according to the pigeon emetic assay, the blood pressure fall produced in cats was rather consistent. There was a mean blood pressure fall of 74.16 per cent with an average deviation of ± 7.39 per cent.

The above results strongly indicate that the pigeon emetic assay gives a true measure of the circulatory activity of Veratrum preparations. The fact that cats respond more uniformly to sub-lethal doses of Veratrum indicates the desirability of an assay method for this drug based upon a sub-lethal response.

A series of blood pressure studies were made with cats under artificial respiration and with cats with vagi cut, but the results were more variable than were the results presented above. When the vagi are divided there is a fall in blood pressure, but the fall is more gradual and less pronounced.

Dogs.—The intravenous injection of one pigeon M. Em. D. of Veratrum per Kg. produced in seven dogs a mean blood pressure fall of 52.13 per cent (A.D. ± 3.95 per cent).

Rabbits.—The intravenous injection of one minimum pigeon emetic dose of a tincture per Kg. produced in five rabbits a mean fall in the carotid blood pressure of 24.52 per cent with

[1] The p_H determinations were made by Dr. A. A. Hellbaum, Department of Physiology, University of Oklahoma School of Medicine.

an average deviation of ±10 per cent. This effect was less marked and more variable than the effects produced by the same dose of this tincture in cats and dogs.

DISCUSSION AND CONCLUSIONS.

The minimum pigeon emetic dose of tinctures of Veratrum viride can be determined with an accuracy sufficient for a biological assay of this drug. Emesis is produced rapidly and furnishes a definite and easily observed end-point. Pigeons can be used repeatedly at fourteen day intervals. This assay offers the advantages of accuracy, simplicity, speed and economy.

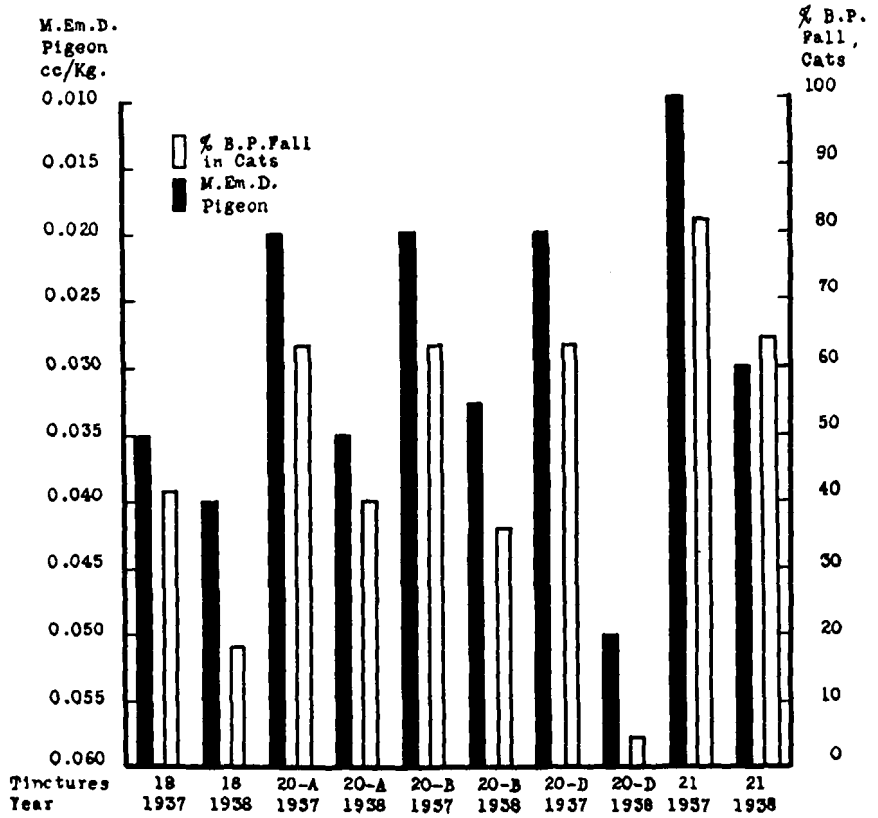

Fig. 6.—The relationship between the M. Em. D. (pigeon) and the blood pressure fall produced in cats by a dose of 0.005 cc. of tinctures of Veratrum viride per Kg.

The blood pressure response of cats and dogs to the injections of small doses of Veratrum was found to be sufficiently consistent to justify the use of this method for indicating the reliability of an assay method for Veratrum viride. The potencies of several tinctures of Veratrum as indicated by the blood pressure effects produced in cats and dogs showed a close parallelism to the potencies of these tinctures as indicated by the pigeon emetic assay.

With a sufficient rest period the pigeons do not appear to become more sensitive or to develop a tolerance for Veratrum. Variability in the response of pigeons to Veratrum does not appear to be sufficient to necessitate the "standardization" of the test animals. The presence of alcohol in the tincture does not appear to

influence the emetic dose. The "conditioning" of pigeons to spontaneous emesis was found to present no great factor of error when a fourteen-day rest period was allowed.

The minimum emetic dose and the minimum lethal dose for twelve tinctures showed close parallelism in pigeons; however, the minimum emetic dose is preferred for the reason that it is based upon a response to a large therapeutic dose rather than to a fatal dose. In addition the emetic method is more economical as the pigeons can be used over a considerable period of time. The ratio of the emetic to the lethal dose was found to be 1 to 13.

The results obtained with the minimum lethal dose determinations for mice did not parallel those obtained by the pigeon emetic assay. Mice showed greater variability than pigeons in their response to Veratrum. The minimum lethal dose for mice did not give a satisfactory measure of the physiological activity of Veratrum preparations as indicated by the blood pressure effects produced in cats and dogs.

The minimum lethal dose method for frogs gave inconsistent and unsatisfactory results. The minimum lethal dose for cats, for dogs and for rabbits showed variations of approximately 100 per cent. The wide range between the therapeutic and fatal doses of Veratrum might explain these variations obtained in lethal dose determinations. When one pigeon emetic dose of Veratrum per Kg. is administered to cats and dogs there results a blood pressure fall of 74 per cent and 52 per cent, respectively. The mean lethal dose was found to be 26 pigeon emetic doses for cats and 38 pigeon emetic doses for dogs.

The determination of the alkaloidal content gives no indication of the physiological activity of tinctures of Veratrum viride. These alkaloids are not stabilized by a p_H of from 4.87 to 5.57, for eight tinctures whose p_H activities were adjusted within this range showed variable decreases in their physiological activity as indicated by the pigeon emetic assay.

Sufficient data have been obtained on the pigeon emetic assay to justify the proposal of a biological standard for Tincture of Veratrum viride based upon the minimum pigeon emetic dose, but to avoid the necessity for changing the established dose of this drug, a standard will not be proposed until sufficient clinical data are available to establish the relationship of the minimum emetic dose to the therapeutic dose for man.

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