

## SCIENTIFIC SECTION

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### THE DOSE-RESPONSE RELATIONSHIP IN THE U. S. P. XI PARATHYROID ASSAY.\*

BY LLOYD C. MILLER.

A monograph covering the assay of Parathyroid Extract was included for the first time in the 11th revision of the United States Pharmacopœia. In brief, the method calls for determination of the rise in serum calcium produced by a suitable dose of the material in normal male dogs of a specified weight. The question of what constitutes a suitable dose arose in applying the method in connection with the regulatory work on bioassay drug products under the Federal Food and Drugs Act. This paper records the results of our investigation on this phase of the method.

The method is based on the work of Collip and Clark (1), who published their results in 1925 on the responses of normal dogs to parathyroid hormone. They found that the proportion was fairly satisfactory between the amount of extract administered and the serum calcium rise. So far as the writer is aware, no confirmation of this work on normal dogs has been published. In 1935, Dyer (2) showed that with the rabbit as the test animal he was unable to obtain proportional rises in the serum calcium in response to graded doses of an active parathyroid preparation.

In view of the work of Collip and Clark (1) and of the wording of the pharmacopœial method, the inference seems clear that the following relationship should hold between dose and response:

$$\frac{\text{Mg.}\% \text{ rise in serum calcium} \times 100}{\text{Cc. of extract}} = \text{U. S. P. XI units per cc. of extract.}$$

The factor 100 arises from the definition of the pharmacopœial unit. A unit represents one-hundredth of the amount of extract required to raise the calcium level of the blood serum of normal dogs by 1 mg. per 100 cc. of serum. No dosage has been suggested for the assay in the pharmacopœial monograph, but in practice most manufacturers stipulate that the dose they select in standardizing their extracts causes a rise in serum calcium between 4 and 6 mg.%. It has been the object of the present investigation to determine the responses to several parathyroid extracts when each was tested at various dosage levels. The results indicate that the selection of the dose is of considerable importance in arriving at the true potency of the test material.

#### EXPERIMENTAL.

The dogs were kept on a diet of commercial dog biscuit supplemented with cod liver oil to insure an adequate vitamin D intake. The calcium content of this diet was about 2.2%. The

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phosphorous content was approximately 1.2%, so that the Ca:P ratio was about 2:1. In compliance with the pharmacopœial requirements the dogs were all healthy, mature males weighing between 10 and 12 Kg. Each test covered a 24-hour period which began with the drawing of the initial blood samples between 9:00 and 10:00 A.M. The dogs were fed as usual about 1:00 P.M., and the test material was injected subcutaneously between 4:00 and 5:00 P.M. The next day the second blood samples were drawn between 9:00 and 10:00 o'clock, which was 16 to 17 hours after injection of the extract. All the test material was U. S. P. Parathyroid Extract prepared, presumably, under the Hanson patent by well-known commercial firms. The labeled concentration was 100 U. S. P. units per cc. Thus the serum calcium rise to be expected was 1 mg. % for each cc. of the extract injected. The serum calcium was determined in duplicate by the Clark-Collip procedure, which has been proposed for U. S. P. adoption (3). This method has given closely reproducible results, and the claim for its accuracy has been verified, since duplicates usually agree within  $\pm 1\%$  of the mean value. Even though an error of  $\pm 1\%$  in estimating 10 mg. of calcium in 100 cc. of serum seems small, it can give rise to an error of  $\pm 5\%$  in the determination of an increase of 4 mg. per 100 cc. With smaller increases in serum calcium, the limits of possible error are proportionately greater, hence the desirability of aiming for increases of 4 to 6 mg.%. All the normal serum calcium levels of the dogs used in this study have been between 10 and 12 mg.%. This represents greater uniformity in the normal levels than has been reported in most parathyroid studies and perhaps is a reflection of the rigid requirements for the animals laid down by the Pharmacopœia.

RESULTS.

Table I presents the results obtained by varying the dosage of the same parathyroid extract, sample No. 5988. The apparent potency varies from 75 to 222 U. S. P. XI units per cc., depending on the dose used. This discrepancy arises from the fact that the serum calcium increase caused by 2 cc. of extract is about as great as that produced by 6 cc. Four other samples have been tested over the same dosage range with similar results.

TABLE I.—DOSE-RESPONSE RELATIONSHIP, PARATHYROID EXTRACT No. 5988.

Dog No.	Dose Cc.	Mg. % Serum Calcium Rise.		U. S. P. XI Units Per Cc. of Extract.
		Average.		
30	1.25	2.62	} 1.94	155
28	1.25	1.62		
31	1.25	1.58		
30	2.00	4.49	} 4.44	222
33	2.00	4.04		
30	2.00	4.79		
32	3.00	4.18	} 4.35	145
31	3.00	3.98		
33	3.00	5.50		
33	3.00	3.69	} 3.82	96
25	4.00	3.48		
30	4.00	4.21		
28	4.00	4.69	} 4.51	75
25	4.00	2.91		
26	6.00	5.96		
32	6.00	4.79	} 4.30	
25	6.00	3.05		
31	6.00	4.43		
28	6.00	4.30		

In Table II are data obtained with another extract. It will be seen that the relationship between smaller doses and their respective responses approaches the

theoretical straight-line proportionality. In spite of the great variations in the individual tests, the average responses to doses of 1 to 3 cc. of this extract are fairly proportional. However, the 3-cc. dosage level marks the point where the theoretical proportionality between dose and response begins to fail. It is true that 4 cc. of this extract elicits a response that is almost equivalent, in terms of U. S. P. XI units per cc. of extract, to the response obtained with the smaller doses. There is little question, however, that the average increase of 5.25 mg. % of serum calcium obtained with this dose represents the maximum rise obtainable with this extract, since the response to 6 cc. shows that an increment of 50% in dosage did not cause any greater serum calcium rise.

From Table II it appears further that the "suitable dose" must be confined to rather narrow limits. The upper limit is defined by the plateau above which the serum calcium cannot be raised. This is represented by increases of 5 to 6 mg. % above normal in the dogs of our colony. It is necessary, therefore, to select a dose that is known, by previous trial, to produce a submaximal response. Otherwise, a serious error can arise in that the indicated activity will be lower than the actual potency. The lower limit of the suitable dose is defined by the greater variation and possibility of error associated with smaller serum calcium increases, such as 1 to 2 mg. %. It is almost needless to point out that a larger number of dogs should be used in each assay if the results are to be based on smaller calcium increases, unless the error in the calcium determination and the animal variation can be reduced. Our experience indicates that the most reliable results are to be obtained when the dose employed produces an average serum calcium rise of 3 to 4 mg. %.

The possibility of extending the limits of this working-range by varying the maintenance diet has been tested experimentally. Four dogs maintained on dog biscuit were treated with a given lot of parathyroid extract in doses of 2 and 6 cc. on three occasions, each at least 1 week apart. These dogs then received raw beef heart exclusively for 10 days, and their responses were determined to doses of 2 and 4 cc. of the same extract. As may be seen from Table III there was practically no difference in the character of the responses of these dogs attributable to the change in diet. These results were confirmed on another group of four dogs, which first received another lot of extract while on a meat diet, and then again received the same extract after being shifted to dog biscuit. The average responses of these animals while on a meat diet were serum calcium rises of 4.22 and 5.64 mg. % to doses of 2 and 6 cc. of extract, respectively. The average responses while on a dog-biscuit diet were 3.84 and 5.52 mg. % to doses of 2 and 6 cc., respectively. There are two reports in the literature relating to the effect of diet on the response of dogs to the parathyroid hormone which offer some basis for controversy; these are, however, based on experimental work which is not strictly comparable. Al-lardyce (4) ascribed superiority to a diet of meat when he found that four normal dogs receiving the same amount of hormone at weekly intervals showed somewhat less variation in response when on a meat diet than when on a cereal diet. Regnier and Simonnet (5), working with parathyroidectomized dogs, concluded that changing alternately from a diet of meat to one of bread did not influence the responses of their dogs to daily administration of the hormone. Our results on normal dogs confirm the experience of the latter authors.

As indicated before, the dogs were fed at the usual daily feeding hour on the days of the tests; this was 3 to 4 hours prior to the injection of the test material. In view of the relatively slow action of the hormone, it was felt that alimentary absorption would be complete considerably before the peak of the response to the hormone was reached. Furthermore, there would be less question of a lack of calcium for mobilization during the height of the effect when the animals were fed as usual. It seemed advisable, however, to determine whether the time of feeding influenced the relationship between dose and response. In tests varying the time of feeding with respect to the time of injection of the extract, dogs fasted up to 28 hours prior to and during the effect of the injection gave results of the same character as those given in Table II. Thus the use of fasting dogs does not simplify the problem of choosing a dose which will produce a proportional and uniform response.

TABLE II.—DOSE-RESPONSE RELATIONSHIP, PARATHYROID EXTRACT No. 6033.

Dog No.	Dose Cc.	Mg. % Serum Calcium Rise.		U. S. P. XI Units per Cc. of Extract.
			Average.	
25	1.0	1.25	1.69	169
28	1.0	2.15		
32	1.0	1.53		
33	1.0	.50		
34	1.0	1.89		
37	1.0	1.58		
38	1.0	2.90	3.02	202
25	1.5	1.64		
30	1.5	2.88		
31	1.5	3.70		
33	1.5	3.30		
35	1.5	3.08		
36	1.5	3.23	3.42	171
38	1.5	3.30		
25	2.0	2.10		
28	2.0	3.32		
30	2.0	3.32		
30	2.0	3.02		
33	2.0	2.70	4.83	161
36	2.0	4.70		
37	2.0	5.18		
38	2.0	3.99		
28	3.0	4.55		
28	3.0	5.35		
31	3.0	4.65	5.25	131
25	4.0	3.95		
26	4.0	5.40		
30	4.0	6.95		
31	4.0	4.72		
31	6.0	5.14		
32	6.0	4.88		
33	6.0	5.36		

Table IV shows the responses, expressed in U. S. P. units per cc. of extract, of Dogs 25 and 28 to similar doses of the same extracts. The results obtained with

TABLE III.—DOSE-RESPONSE RELATIONSHIP AND VARIATION IN DIET,  
PARATHYROID EXTRACT No. 6069.

Dog Biscuit.					Beef Heart.				
Date (1937).	Dog No.	Dose Cc.	Serum Calcium Rise, Mg. %.	U. S. P. XI Units per Cc. of Extract.	Date (1937).	Dog No.	Dose Cc.	Serum Calcium Rise, Mg. %.	U. S. P. XI Units per Cc. of Extract.
6-15	28	2	4.01	200	8-27	28	2	4.87	244
7-27	28	2	4.49	224	9-3	30	2	2.26	113
8-3	30	2	4.64	232	8-27	31	2	2.80	140
7-27	31	2	4.50	225	9-3	32	2	4.88	244
6-15	32	2	3.01	150					
8-3	32	2	3.41	172					
				200 (Av.)					185 (Av.)
8-3	28	6	5.05	84	9-3	28	4	5.08	127
6-15	30	6	6.30	105	8-27	30	4	3.91	98
7-27	30	6	4.70	78	9-3	31	4	4.72	118
6-15	31	6	5.92	99	8-27	32	4	5.00	125
8-3	31	6	5.72	95					
7-27	32	6	5.05	84					
				91 (Av.)					117 (Av.)

Dog 28 are entirely typical of those obtained with all the other dogs in the colony, except Dog 25. The latter appeared to be hyposensitive to parathyroid hormone. Wherever a comparison is possible, it will be seen that the result obtained on Dog 25 is lower than that obtained at the same dosage on Dog 28. The greatest rise in serum calcium obtained at any time with Dog 25 was less than 4 mg.%. In each of the eleven other dogs of the colony, occasionally serum calcium increases of 5 to 6 mg.% were obtained following administration of 4 to 6 cc. of extract. There was no apparent reason why this one dog was slightly more limited in its capacity to respond to the hormone. This may be looked upon as simply an example of biological variation for which some provision might well be made in the official monograph. Despite its somewhat lower sensitivity, this animal showed quite a uniform gradation in response. Following doses of 1, 1.5, 2 and 4 cc. of sample No. 6033, it responded with serum calcium rises of 1.25, 1.64, 2.10 and 3.95 mg.%, respectively. The figures for potency corresponding to these responses are 125, 109, 105 and 99 units per cc., which indicate agreement within the experimental error of the calcium determination.

TABLE IV.—RESPONSES OF DOGS 25 AND 28 TO FOUR PARATHYROID EXTRACTS,  
APPARENT U. S. P. POTENCY AT THREE DOSAGE LEVELS.

Extract No.	Dose of Extract.					
	2 Cc.		4 Cc.		6 Cc.	
	Dog 25.	Dog 28.	Dog 25.	Dog 28.	Dog 25.	Dog 28.
5976	125	200	81	119		
5988		170	{ 87 73	117	51	72
6017		195	92	123	{ 55 63	106
6033	105	166	99			

This table also gives some idea of the response to repeated doses of the same extract in the same dog. On two different occasions Dog 25 received a given dose of the same extract. The corresponding duplicate responses agree rather well with each other. Actually, however, this is simply another manifestation of the general observation that with doses of 2 to 6 cc. the rise in serum calcium is around 4 to 5 mg.%. Hence any remarks regarding the accuracy of the method will have to pertain to responses which are known to be submaximal.

The conclusion to be reached from these results is that the U. S. P. XI method for Parathyroid Extract is satisfactory for purposes of standardization and certainly is wholly suitable for indicating an absence of parathyroid activity. Certain precautions must be observed: (1) The dose selected must be shown to produce significant but submaximal increases in the serum calcium of the dogs used; (2) for satisfactory accuracy, more than the minimum of five dogs must be used unless precautions are taken to standardize the reactions of the animals. Such standardization might well be accomplished by adopting as a reference standard a stable powder prepared by methods already published (6) and distributed by the Board of Trustees of the U. S. Pharmacopœial Convention.

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## CONSTITUENTS IN CASCARA SAGRADA EXTRACT.

### 3. THE LIPIDS AND GLYCOSIDES.\*<sup>1</sup>

BY MELVIN W. GREEN, C. G. KING AND GEORGE D. BEAL.<sup>2</sup>

The most characteristic compounds, from a pharmaceutical point of view, in cascara bark, senna, frangula, rhubarb and aloes are polyhydroxyanthraquinones. The quinones are present largely as glycosides and it has been generally thought that such glycosides were of major physiological importance. This view has led Beal and Tumminckatti (1), Daels (2) and others to make a thorough study of analytical methods for determining free and combined anthraquinones. Sipple, King and Beal (3) have recently isolated a rhamnoside of emodin from cascara bark, but the compound did not possess striking physiological activity. In fact there is little evidence that the anthraquinones account for the greater part of the purgative activity of the above types of drugs. The present investigation has been directed toward finding further information concerning the active principles in cascara bark, using as a guide a method of assay that was developed in a preliminary part of the study (4).

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