BIO-ASSAY OF PARATHYROID EXTRACT

by

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Presented to the Faculty of
The Graduate College in the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Doctor of Philosophy
Department of Physiology and Pharmacology

Under the Supervision of Dr. A. R. McIntyre

Omaha, Nebraska

1958

TITLE

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ACKNOWLEDGEMENT

I wish to express my gratitude to Dr. A. R. McIntyre, Chairman of the Department of Physiology and Pharmacology, who made this work possible.

I give my sincere thanks to all the members of the Department and especially to the members of my graduate committee who have given help and guidance in this study.

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INTRODUCTION

Successful extraction of the active principle of the parathyroid gland was first made in 1925 (Hansen; Collip and Clark, a). As a means of measuring the activity of parathyroid extracts Collip and Clark (1925b) devised an assay which was based on the increase in dogs' serum calcium produced by the injection of the extracts. It has been retained as the official U.S.P. method of assay for parathyroid extract though it is not precise from the standpoint of statistical analysis. One ml. of U.S.P. Parathyroid contains 100 units of Parathyroid. "Each unit represents one one-hundredth of the amount required to raise the calcium content of 100 ml. of the blood serum of normal dogs 1 mgm. within 16 to 18 hours after administration." The requirements for a valid assay, set down in the U.S.P., are essentially as follows. Ten dogs weighing between 8 and 16 kgm., but differing by no more than 5 kgm., are injected with a fixed dose of parathyroid extract under assay, which by preliminary trial, had been found to cause an increase in serum calcium of at least 2 mgm.% and not more than 5 mgm.%. Control serum samples are taken from each animal prior to injection and again 16 to 18 hours after the subcutaneous injection of the extract. The increase in serum calcium (expressed in mgm.%) per ml. of the injected preparation is determined for each dog as

well as the average of these values for all the animals used. The potency of the preparation is satisfactory if the average of 10 or more values so obtained is not less than 1 mgm.% increase in serum calcium per ml. of preparation injected. The potency was thus obtained without running a control series of injections, and the range of values from which the average was calculated has not been restricted.

Bliss and Rose (1940) tried to put this assay on a more sound statistical basis. They compared a preparation with the activity of a U.S.P. preparation, which, of course increased the number of animals used and the number of serum samples that had to be analyzed. They found that it required 20 dogs with a total of 4 serum samples per dog to produce a balanced comparative type assay which would have confidence limits of error of 79 to 126%. However, the more precise results obtained by this method seem to have been outweighed by the increase in work and number of animals required. This modification has never been added to the official assay.

The evaluation of parathyroid extract has been attempted using animals other than dogs. Biering (1950 a and b) measured the increase in serum calcium of rats. Though this method employed a smaller laboratory animal, a larger number of them was required. The rats proved to be rather insensitive to the extract used, and the actual quantities of extract required were large. In order to obtain an increase in serum calcium of 0.34 to 1.95 mgm.%, Biering administered U.S.P. equivalents of the extract ranging from 162 to 495

units per rat. To minimize the experimental error, he found it necessary to use 20 rats for assaying this material. Therefore a very large amount of extract would be required for this method.

It was observed that parathyroidectomy increased the sensitivity of rats to parathyroid extracts. Therefore, Davies et al (1954), proposed that the increase in serum calcium in parathyroidectomized rats be used as a measure of parathyroid extract activity. Using doses of parathyroid equivalent to 25, 50, and 125 units, these workers carried out cross-over assays on parathyroidectomized rats. In order to obtain limits of error of 64 to 158%, they needed 24 parathyroidectomized rats, using each animal twice.

Munson et al (1953) and Munson (1955) also proposed a serum calcium assay using parathyroidectomized rats. They found that parathyroidectomized rats maintained on a low calcium diet were more sensitive to parathyroid extract than parathyroidectomized rats maintained on a normal diet. Their experiments were not carried out to the completion of an assay but, if they used approximately 20 to 30 animals per preparation assayed, they anticipated an accuracy similar to Davies et al (1954). The increase in sensitivity, as a result of parathyroidectomy and a low calcium diet, made it possible to use doses of 5 to 20 units of parathyroid per 100 gms. of rat.

Hamilton and Schwartz (1932) reported several experiments in which normal rabbits responded to parathyroid extracts with an increase in serum calcium. They found that this increase,

following doses of 1.5 to 6.0 units of parathyroid per kgm., was directly proportional to the dose. However, no assay has been developed based on these observations.

The increase in serum calcium, following the injection of parathyroid extracts, is accompanied by an increase in urinary calcium. This response was studied by Dyer (1932, 1933), Pugsely (1932), Olsen (1934), and Truszkowski et al (1939). Although they reported a dose-response relationship for this increase in urinary calcium, none of these investigators developed an assay based on this response nor did they determine its sensitivity.

The increase in phosphorus excretion following the administration of parathyroid extract is accompanied by a decrease in serum inorganic phosphorus. Tepperman et al (1947) experimenting with rats used this decrease in serum inorganic phosphorus as a means of assaying the phosphate activity of parathyroid extracts. They showed that the reduction in serum inorganic phosphorus was directly proportional to the log of the dose of parathyroid injected. However, their assay, using this dose-response relationship, lacked a high degree of precision because of seasonal variation in the response of the animals.

Rubin and Dorfman (1953) used parathyroidectomized rats in the assay of phosphate excretion activity of parathyroid extracts and found increased sensitivity similar to that obtained where serum calcium levels were measured. They injected as little as 0.5 U.S.P. units of parathyroid into parathyroidectomized rats and were able to measure an increase

in the excretion of radio phosphorus (P^{32}), which had been administered just prior to the parathyroid injection.

Kenny et al (1954) and Munson (1955) investigated the possibility of measuring the increase in urinary phosphate of rats for assaying the phosphate excretion activity of parathyroid extracts. These workers found that when parathyroid extract was administered to either intact or parathyroidectomized rats, there was an increase in urinary inorganic phosphate. The response of the parathyroidectomized rats was more uniform than that of intact rats and was clearly demonstrated with a smaller dose of extract. They reported that the increase in urinary inorganic phosphate, resulting from the subcutaneous injection of 0.2 to 0.8 ml. of crude parathyroid extract, yielded a linear dose-response curve.

Recently mice have been used for the evaluation of the phosphate excretion activity of parathyroid extracts. Davies et al (1955) carried out cross-over assays based on the increase in urinary phosphorus collected from 192 mice, using each animal twice. The mice were injected subcutaneously with 0.1 to 3.0 U.S.P. units of parathyroid per mouse; urine was collected for the following 3.5 hours. The results of 7 assays showed the average limits of error to be 60 to 168%.

Although the principal actions of parathyroid have been thought to be calcium mobilization and phosphate excretion, there are other possible roles of parathyroid hormone which have been utilized for assay methods. For example, L'Heureux and Roth (1955) reported experiments carried out on normal

and parathyroidectomized rats in which they compared the effect of parathyroid extracts on serum citric acid levels as well as on serum calcium levels. They concluded that the evaluation of citric acid levels offered no advantage over measuring the increase in serum calcium in the same animals.

Gelhorn (1935), working with frog nerve-muscle preparations, noted an increase in the strength of muscular contraction after the administration of parathyroid extract. He suggested that this increase in strength of contraction might be used to evaluate the activity of parathyroid extracts, but no assay has yet been developed based on these observations.

Some of the procedures proposed for assaying parathyroid extracts were not developed to their fullest extent while others were thought to offer sufficient advantages to warrant their adoption as assay methods. Although any one of these proposals included a single important advantage, such as greater precision, utilization of smaller and more economical test animals, or a high degree of sensitivity of the test animals to parathyroid injection, no single method contained all these desirable features.

If all these desirable features mentioned previously could be incorporated in a single calcium and a single phosphorus assay, it would be very helpful in studies of the mechanism of action of parathyroid. This would be especially valuable if there were separate components of activity in parathyroid, as some have postulated (Munson 1955; Davies et al 1955). The indications that parathyroid extracts contain separate

components responsible for calcium and phosphate activity have resulted from recent advances in the method of extraction of parathyroid (Ross and Woods 1939; L'Heureux et al 1947; Davies and Gordon 1955). These newer methods of extraction produced extracts that were shown to have different molecular characteristics and different biological activity than extracts produced by the early methods of extraction (Hansen 1925; Collip and Clark 1925a). It has been reported that the phosphorus/calcium activity ratio of different extracts varies depending on the method of extraction. Davies et al (1955) reported that the mouse urinary phosphate activity of a hot-HCL extraction of parathyroid glands was 3.1 times rat serum calcium activity, while the phosphate activity was 2.6 times the calcium activity of an acetone-HCL extraction. Munson (1955), using parathyroidectomized rats maintained on a low calcium diet, reported the phosphate activity to be 3.6 times the calcium activity (rat urinary phosphorus activity/rat serum calcium activity) for a crude extract of parathyroid. This was the first time that a single animal species was used for comparing the separate activities of parathyroid extracts. The use of a single species of animals for such a comparison enhances the value of the study.

The need for a very sensitive assay was indicated in the work of Davies and Gordon (1955). They hoped to separate the calcium mobilization component of parathyroid extract from the phosphate excretion component by subjecting parathyroid extract to ultra-filtration. However, the yield of filtration product was very small and a complete evaluation

of the two activities was not carried out, even though they used parathyroidectomized rats for the calcium assay (Davies et al 1954) and mice for the assay of phosphate excretion activity (Davies et al 1955). Their results underline the need for more sensitive methods of assay.

The need for better methods of assaying calcium and phosphate activity of parathyroid extract is apparent. Further insight into the true mechanism of action of parathyroid will, no doubt, await the development of better methods of assay. The purpose of the work presented in this thesis was to fill this need by developing methods of assaying calcium and phosphate activities which combined high sensitivity with convenience and precision.

METHODS

The experimental results obtained in this study can be separated into two parts. The first part of the work was to develop a new means of assaying the calcium mobilization activity of parathyroid extracts and the second part of the work was to assay its phosphate activity. Mice were chosen as test animals because they would be economical to obtain and a large number of them could be handled with a minimum of animal facilities. Also, it was hoped that the use of mice would make it possible to carry out a valid assay with only a small quantity of parathyroid extract. The size of blood samples which would be available from the mice would be small. Therefore, it was necessary to choose methods of measuring serum calcium and blood inorganic phosphorus which combined precision and convenience.

Normal male white mice, ranging in weight from 18 to 24 gms. were used for both assays. All animals were maintained on a standard laboratory diet and water ad libitum prior to and during the test period. The parathyroid extract (Lilly's U.S.P. Parathyroid Extract) was administered to the mice by subcutaneous injection. The volume injected varied from 0.25 to 0.50 ml. depending on the weight of the animal. All doses of parathyroid injected were on a basis of U.S.P. units of parathyroid per gm. body weight. The different concentrations of parathyroid extract needed were made by diluting U.S.P. parathyroid extract with distilled

water. Saline dilutions were tried but did not give a true solution. Control mice were given control injections of normal saline.

All the injected mice were sacrificed after the designated interval of time by severing the spinal cord at the cervical level. The thoracic cavity was quickly opened to expose the heart. A blood sample of 0.5 ml. or more was taken from the right ventricle by means of a syringe and needle which had been rinsed with a 0.02% heparin solution to delay clotting until the blood had been removed from the syringe.

Serum for calcium determinations and blood for inorganic phosphorus determinations was accurately measured with a 0.2 ml. "Normax", calibrated to contain, pipette.

Only chemically pure reagents were used and the water used for making solutions was double-distilled. All determinations were carried out with a Beckman Spectrophotometer, Model DU, equipped with acetylene-oxygen flame attachment and photomultiplier tube.

Serum Calcium Determinations

The method of measuring the serum calcium was that proposed by Humoller et al (1956). The smaller serum samples obtained from the mice necessitated only a corresponding reduction in the volume of added solutions.

Reagents.

1). Organic diluting fluid. This was made by combining 250 ml. of redistilled isopropol alcohol with 150 ml. of double distilled water and 0.1 ml. of

Sterox SE. (a non-ionic detergent supplied by Monsanto Chemical Company, St. Louis, Mo.).

2). Control Solutions.

a). Blank. This solution was made each day just prior to its use. It consisted of 1.8 ml. of 6.2% trichloroacetic acid solution plus 8.2 ml. of organic diluting fluid to give a volume of 10 ml. Ten ml. was an adequate volume for analyzing 25 to 30 serum samples.

b). Standard calcium solution. This solution was made each day just prior to its use. It was made by combining 1.8 ml. of 6.2% trichloracetic acid solution with 8 ml. of organic diluting fluid and 0.2 ml. of mixed stock standard solution. The mixed stock standard solution contained 150 milliequivalents (meq.) of sodium, 5 meq. potassium, and 5 meq. calcium per liter. It was prepared as described by Humoller et al (1956).

Procedure.

Approximately 24 hours after the parathyroid extract injection, a blood sample was obtained and placed in a 15 ml. centrifuge tube where it clotted in approximately 30 minutes. The serum was then obtained by centrifugation. Then 0.2 ml. of serum was transferred to a test tube containing 1.8 ml. of 6.2% trichloroacetic acid solution. The pipette was washed twice with the solution of the test tube to free it of residual serum. After precipitation of the proteins the tube was centrifuged to obtain a clear supernatant fluid.