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A PLASMA CALCIUM ASSAY FOR PARATHYROID HORMONE, USING PARATHYROIDECTOMIZED RATS

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Many attempts have been made to work out a convenient method for the assay of parathyroid hormone. That this, however, is still to be achieved is indicated by the fact that most investigators who have attempted to purify the hormone have tested their preparations by measuring the increase of serum calcium in dogs as originally described by Collip & Clark (1925). A partially successful attempt to break away from this tradition was made by Tepperman, L'Heureux & Wilhelmi (1947), who utilized the fall in serum phosphate of normal rats as a measure of parathyroid activity. However, the recent preparation by Stewart & Bowen (1952) and Davies & Gordon (1953*b*) of gland extracts devoid of effect on plasma calcium, but still capable of stimulating the renal excretion of inorganic phosphate, demonstrates the need for separate methods, dependent on changes in either calcium or phosphate, to measure the two types of activity.

The present paper reports an attempt to work out a test based on changes in the plasma calcium level of parathyroidectomized rats. Unfortunately, the normal rat is known to be relatively resistant to injected parathyroid hormone. Thus Biering (1950), who described a method in which serum calcium changes in normal rats were measured, found it necessary to use approximately 1000 U.S.P. units of hormone to secure an increase of 3 mg Ca%. Rather large amounts of hormone were also employed by Pugsley (1932), Dyer (1933), Olsen (1934), Truszkowski, Blauth-Opieńska & Iwanowska (1939), who all measured increases in the urine calcium of normal rats. Fortunately, Tweedy & Chandler (1929) observed a noticeable increase in the sensitivity of the plasma calcium response in rats after parathyroidectomy, and this has been taken advantage of in the present work. Such animals, unless kept on a low calcium high phosphate diet, rarely show signs of tetany (Shelling, 1932) and

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are eminently suitable for parathyroid assays, except for their wide initial variation in serum calcium level. Not unexpectedly, the response of individual animals to injected parathyroid hormone has been found to depend markedly on their initial plasma calcium level, but it has been possible to find a simple means of correcting for such differences during calculation of the results.

METHODS

Rats and diet

The rats used were hooded (Norwegian) animals, weighing 200–250 g. Males were generally used, though no differences due to sex were detectable. They were bred at the National Institute for Medical Research and maintained there on the stock cube diet no. 41 (Bruce & Parkes, 1949). Rats received food and water *ad lib.* throughout the experiment, except for certain experiments on starving rats. The pair of parathyroids lying embedded in the thyroid were destroyed by cauterization (Davies & Gordon, 1953*a*). No attempt could be made to locate and remove accessory parathyroids, but according to Hoskins & Chandler (1925) these were only present in less than 10% of rats whose neck regions they examined. Parathyroidectomized rats were used from 3 to 21 days after operation since no changes in sensitivity could be detected during this period. Most experiments were started 10 days after the operation. Individual rats were used for one experiment or assay only. Pugsley (1932) and Dyer (1932) had encountered immunity in rats receiving repeated doses of hormone. A preliminary experiment showed that parathyroidectomized rats, given four injections of hormone totalling 300 units over 12 days, were slightly less responsive to the last than to an identical first dose.

Hormone

Standard hormone was 'Parathormone' supplied by the Eli Lilly Co. Hormone doses are expressed as U.S.P. units. Parathyroid extracts assayed were HCl-acetone preparations of ox parathyroid glands, made according to L'Heureux, Tepperman & Wilhelmi (1947). All injections were given subcutaneously.

Plasma calcium estimations

0·6 ml. samples of blood were obtained by heart bleeding from rats under light ether anaesthesia, using a heparinized syringe. The plasma was drawn off from the red blood cells as soon as possible.

(*a*) *Titrations with sodium versenate*

This method, used for all assay estimations, estimates plasma calcium + magnesium. It is based on the observation by Schwarzenbach and his associates (Schwarzenbach & Ackermann, 1947; Biedermann & Schwarzenbach, 1948) that Eriochrome T forms a pink-coloured complex at pH 11 with Ca^{2+} and Mg^{2+} and that the addition of sufficient ethylenediaminetetraacetate (versenate) to chelate all the divalent ions present results in the appearance of a blue colour. The method below is a modification of that of Betz & Noll (1950).

Solutions. (1) Sodium versenate (stock solution): 4 g sodium ethylenediaminetetraacetate (versenate) +0·86 g A.R. NaOH in 800 ml. distilled water. Diluted 5 times for use.

(2) Buffer solution (stock solution): 10 g A.R. NaOH; 5 g A.R. sodium sulphide; 40 g A.R. borax ($Na_2B_4O_7 \cdot 10H_2O$). Dissolved in distilled water and made up to 1 l. Diluted 10 times for use.

(3) Indicator: 0·1% solution of Eriochrome T (Gurrs Ltd.) in ethanol.

(4) Standard calcium carbonate solution: 1·0 g of A.R. calcium carbonate was dissolved in a little diluted HCl and diluted to 1 l. (stock solution, 1 ml. = 400 μg Ca). The standard solution was a 1/20 dilution of the stock solution (1 ml. = 20 μg Ca).

Method. 0·1 ml. of plasma was pipetted into a tube containing 0·5 ml. distilled water, duplicate estimations being made for each plasma. 1 ml. of diluted buffer and 2 drops of indicator were then added. Only one tube was prepared at a time from freshly poured out buffer, since pH changes

occurred on standing. Diluted versenate was run in from a Conway microburette, with shaking, until the last trace of pink colour had been discharged and a pure blue colour had replaced it. Additions near the end-point were made very slowly, leaving time for the colour changes to develop. All colours were observed under a 60 W electric light bulb a few inches from the titrating vessel. A daily check of the titration value for 10 μg calcium was made and since small differences were observed, these were corrected when calculating the serum results.

(*b*) *Accuracy of versenate method*

A calibration curve over a range of 4–14 μg Ca was constructed. Calcium carbonate solutions, containing either 1 or 2 mg Ca% were used. Samples were run out from a Conway microburette, made up to 0·6 ml. with distilled water and estimated as above. On plotting the results, a straight line was obtained. The range of accuracy was free from −7 to +1%, there being an average error of 3%. The greatest error occurred with samples of 4 and 6 μg Ca.

A recovery experiment for added calcium carbonate in the presence of 0·1 ml. plasma gave the results shown in Table 1. These results may be compared with those of Clark & Collip (1925) who stated that their macro-oxalate method estimated serum calcium with an error of not more than 2%.

TABLE 1. Calcium recovery experiment using the versenate method

Added Ca (μg)	Plasma Ca + Mg (μg)	Total Ca + Mg found (μg)	Total Ca + Mg found, expressed as % total Ca + Mg present
0	9·20	—	—
2	9·20	11·6	103
4	9·20	13·4	102
6	9·20	15·3	101
8	9·20	17·6	102
10	9·20	19·5	102

(*c*) *Comparison of versenate and oxalate titrations*

A small-scale experiment was carried out to compare plasma calcium + magnesium estimated by the versenate method and plasma calcium estimated in 0·1 ml. plasma by the micro-oxalate method of King, Bain & Pash (1951). Seven parathyroidectomized rats were bled, injected with 50 units of Parathormone and re-bled 21 hr later. All plasma samples were estimated by the two methods. The average rise in plasma calcium by the oxalate method was 1·2 mg/100 ml. Assuming that the versenate titration measured the same increase in calcium together with a rise in plasma magnesium, the latter amounted to a rise of only 0·1 mg Mg, which was regarded as negligible. The conclusion that the serum magnesium is not at a higher value 21 hr after the injection of parathyroid hormone is in accord with the work of Collip & Clark (1925), Schlotz (1931) and Greenberg & Mackey (1932), who report that Parathormone injection in dogs causes a very slight and transient (2–6 hr) rise in serum magnesium. Our experiment would seem to indicate that the change measured by the versenate method can be accounted for by a rise in plasma calcium only. Hence, in the rest of this work responses to hormone will be referred to as rises in plasma calcium. Since in the assays it is only required to measure some regular response which has a proportional relationship to the dose of hormone given, if more extensive work did demonstrate a rise in plasma magnesium as well as a rise in plasma calcium 21 hr after injecting parathyroid hormone, the present assay method would still remain valid.

RESULTS

Time curve

The time at which the maximum plasma calcium response to injected hormone occurred in parathyroidectomized rats was first investigated. Table 2 shows the increases found at various times after injection of 60 units of Para-

thormone. The animals were bled just prior to injection at 12 noon, and then again after the stated time. As a result of this experiment, 21 hr was adopted as a satisfactory and convenient time for the development of the response to the hormone and was used in the rest of this work, except on certain occasions where the hormone was injected in two doses. A later experiment indicated that the same rise in plasma calcium was to be expected whether the hormone was given in a single injection at 12 noon or was injected as two half doses at 10 a.m. and 5 p.m. and responses measured 24 hr later (10 a.m.).

TABLE 2. Time curve : response of groups of four parathyroidectomized rats to 60 units of Parathormone at various intervals after injection

Group	Time after injection (hr)	Average response (y). Increase in mg Ca/100 ml. plasma	Average z value, $z=\frac{1}{2}$ initial plasma Ca + Mg level $+y$
1	3	0·9	5·7
2	6	1·3	5·9
3	12	1·6	6·5
4	18	1·8	5·6*
5	24	1·7	6·7
6	30	1·2	6·2

* In group 4 the average initial plasma calcium + magnesium level was 7·5 ml./100 ml., which was 2·0 mg/100 ml. below the average for the other groups. Therefore it seems likely that in this case the initial level correction has overcompensated for this very low level.

Sensitivity of normal and parathyroidectomized rats to parathyroid hormone

An experiment was carried out to confirm the finding of Tweedy & Chandler (1929) that after parathyroidectomy rats give larger increases in plasma calcium in response to injected hormone. Six rats (♀) were parathyroidectomized, and then 11 days after the operation were bled and each injected with 50 units of Parathormone. For comparison six rats (♂, but of similar weight) were bled and each injected with 200 units. The average increases measured after 21 hr were 1·70 mg Ca% for the operated rats and 1·85 mg Ca% for the normals. A t test showed there to be no significant differences between these results ($P>0·7$); thus it can be concluded that parathyroidectomy very markedly increases the sensitivity of rats to injected hormone.

Relationship of increase in plasma calcium + magnesium to dosage of hormone

This was investigated with four groups of four rats of equal total weight, used 10 days after parathyroidectomy in a test lasting for four consecutive days. Each rat was bled at 10 a.m. and injected with Parathormone at 10 a.m. and 5 p.m. on days 1 and 3. On days 2 and 4 each animal was bled at 10 a.m. On day 1 the groups received totals of 20, 37, 67 and 123 U.S.P. units of hormone per animal respectively. On day 3 the groups were exchanged so that those which had received 123 units now received 20 units etc. The average increases of plasma calcium for each dose for the two days shown in Fig. 1 indicated

a straight-line relationship between increase in plasma calcium (y) and log. dose of hormone. Since it was found that there was a dependence of response on initial plasma calcium + magnesium level (i), the results were later expressed as z values, z being the response corrected for the varying initial plasma calcium + magnesium levels of the rats used (see 'Interpretation of Results' later). The log. dose–z curve was also a straight line.

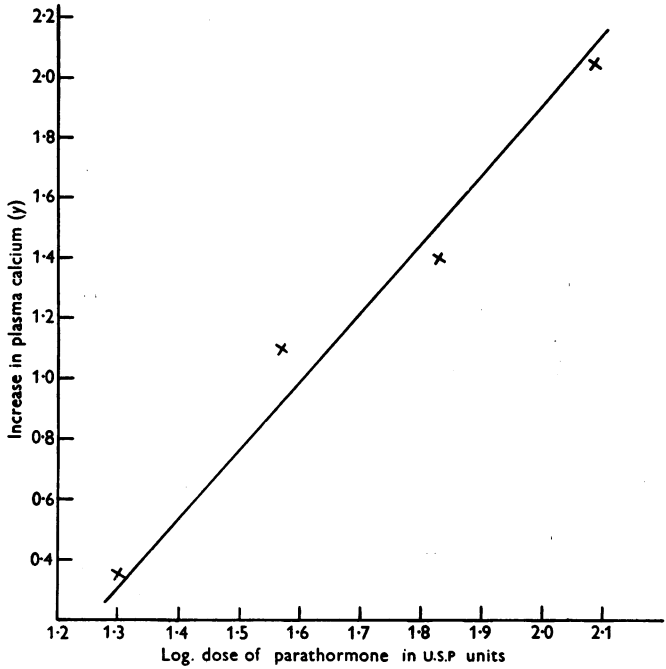

Fig. 1. Log. dose–response curve. Cross-over test using parathyroidectomized rats (details in text).

The dose-response relationship was re-investigated three more times over a period of 9 months. The variation was generally considerably less using z rather than y as a measure of the response. This was shown in four experiments in which average λ using $y = 0{\cdot}66$, and average λ using $z = 0{\cdot}49$, where λ = index of precision = $\sqrt{}$(error variance) $\div$ slope of log. dose-response line. Log. dose–z curves were always straight lines, but the slope varied on each occasion, indicating the necessity of including animals on standard hormone in each assay.

Fig. 2, which includes results obtained with Parathormone both in assays and in dose-response experiments, shows that from May–August 1953 persistently low slopes for the Parathormone log. dose–response (z) lines were obtained. Unfortunately, no assay results are available for these months, as solutions which possessed negligible parathyroid activity were being assayed at that

time. If this seasonal effect re-occurs, it might be necessary at such times to use more animals to obtain the limits of error claimed for the present assay.

In this connexion, the use of starved parathyroidectomized rats was investigated, since Olsen (1934) affirmed that starved normal rats showed a larger urine calcium response to parathyroid hormone than fed animals. Our work was carried out from September to November 1953. In four experiments, each using twenty-four parathyroidectomized rats, the response to 20–125 units

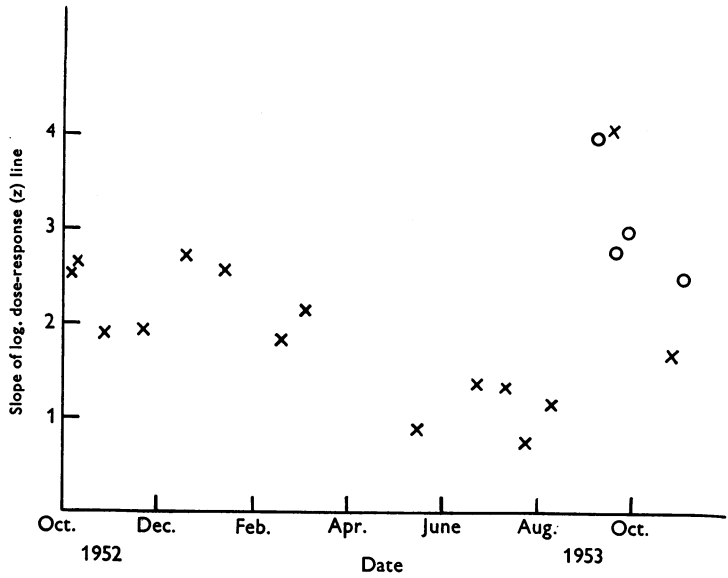

Fig. 2. Slope of log. dose–response lines for 'Parathormone' over a 13-month period. ×, fed rats; ○, starved rats.

of Parathormone was measured. Fed and starved animals were either used simultaneously in a cross-over test, or the whole group was first used while starving and then while feeding or vice versa. Satisfactory slopes (1·66–4·01) for the log. dose–z lines were obtained with both categories of rats. In no case was the slope or the index of precision significantly different when comparing the results from fed and starved animals.

Assay method

Having established the general conditions under which increase in plasma calcium might be expected to provide a measure of the activity of injected Parathormone, seven assays of unknown parathyroid extract were carried out. In each the responses to three levels of the standard preparation were compared with those obtained from three levels of the unknown. The experimental design was the triplet cross-over assay as described by Smith (1950), and

TABLE 3. Dose ratios in triplet cross-over plasma calcium assay. The parathyroidectomized rats received the first dose on day 1 of the assay and the second after an interval of 2–4 days

Rat nos.	1st dose	2nd dose
1–4	20 U.S.P. units of Parathormone	6·25 *d* mg unknown preparation
5–8	50 U.S.P. units of Parathormone	2·5*d* mg unknown preparation
9–12	125 U.S.P. units of Parathormone	*d* mg unknown preparation
13–16	*d* mg unknown preparation*	125 U.S.P. units of Parathormone
17–20	2·5*d* mg unknown preparation*	50 U.S.P. units of Parathormone
21–24	6·25*d* mg unknown preparation*	20 U.S.P. units of Parathormone

* d = the unit dose of unknown preparation.

dosage ratios and number of rats used etc. are shown in Table 3. The time schedule of injections and bleeding was as described above, except that for the first two assays the dose was divided into two halves which were injected at 10 a.m. and 5 p.m. In the first three assays 1 clear day was left between the two halves of the assay, but in later work this was lengthened to 2 days because it was found that the average initial plasma calcium level was not quite regained after the shorter interval. The results obtained are given in Table 4, suitably corrected for the varying initial plasma calcium + magnesium levels of the rats (see below).

TABLE 4. Calcium assays of unknown parathyroid hormone preparations

Assay no.	Preparation	Date of test	No. of rats used*	Slope of log. dose–z lines for Parathormone	Potency†	U.S.P. units/mg	Limits‡ of error expressed as % of potency
I	D/77	2. xii. 52	18 × 2	1·97	0·92	12	59–165
II	D/91	30. xii. 53	24 × 2	2·74	0·30	1·0	62–144
III	D/96 (test 1)	12. i. 53	18 × 2	2·50	0·86	11	73–135
IV	D/96 (test 2)	16. ii. 53	24 × 2	1·82	1·07	11	56–191
V	E/6	2. iii. 53	24 × 2	2·16	0·60	7·5	58–157
VI	E/106§ (test 1)	9. ix. 53	24 × 2	3·99	0·48	5·9	62–147
VII	E/106§ (test 2)	30. ix. 52	24 × 2	3·02	0·30	3·8	40–177

* Including rats receiving standard hormone.
† Doses of Parathormone the same but doses of unknown different in each assay.
‡ Limits were calculated at the 5% level.
§ Starved rats.

Interpretation of results

In all the experiments already mentioned, and in other preliminary observations, dependence of response on initial plasma calcium + magnesium concentration was always noticeable for each dose level.

The responses to 125 units of Parathormone in the assays I–III described above were used to devise a new expression (z) for the response, which unlike the observed response (y) would not depend on the initial plasma calcium + magnesium level (i). For this condition to be fulfilled it is necessary for z to be a constant at any one dose level. All the y values were plotted against i: the

regression line (line A) is shown in Fig. 3. The interdependence of i and y was shown since the correlation was $-0{\cdot}91$ ($P<0{\cdot}001$). The slope of line A was $-0{\cdot}503$; hence the equation of the line was $y=-0{\cdot}5\ i+$a constant. This constant depends on the size of the response. If this constant is now referred to as z and the equation rewritten as $z=y+0{\cdot}5i$, this satisfies both the conditions that z is a measure of the response and that z is independent of i. The fact that z bears no relationship to i is shown by plotting z against i (Fig. 3, line B) using the same data as for line A. The correlation between i and z was $-0{\cdot}013$ which is not significant ($P>0{\cdot}1$).

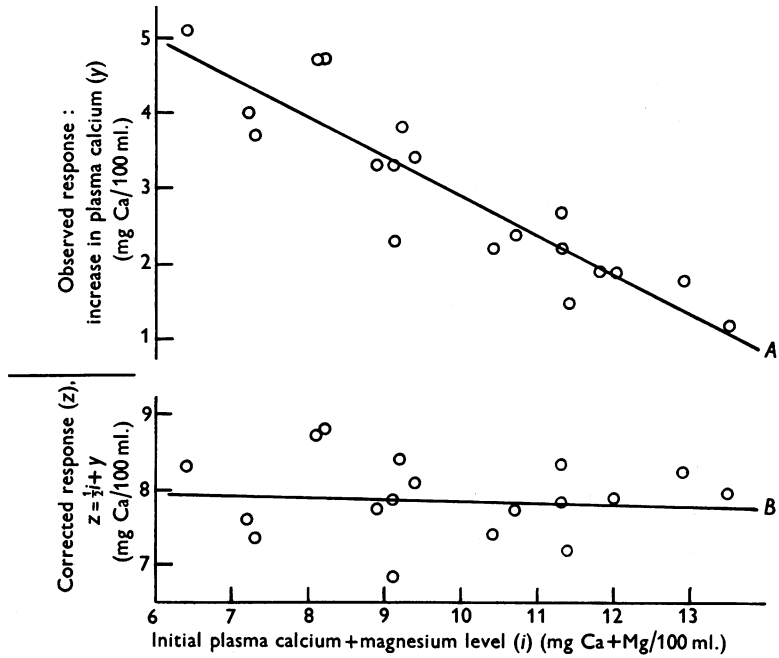

Fig. 3. Correlation of response to 125 U.S.P. units of 'Parathormone' with initial plasma calcium + magnesium level, using 20 parathyroidectomized rats. A, regression line of increase in serum calcium (y) on initial plasma calcium + magnesium level (i); B, regression line of corrected response (z) on initial plasma calcium + magnesium level.

The validity of adopting the expression $z=y+0{\cdot}5i$ for other doses of Parathormone and for other hormone extracts has been investigated. All responses to 20 units of Parathormone in the same three assays (I–III) were analysed and this gave rise to results similar to those obtained with the responses to 125 units. Thus there was a high correlation between i and y ($-0{\cdot}834$, $P<0{\cdot}001$), no significant correlation between z and i ($+0{\cdot}195$, $P>0{\cdot}1$) and the slope of the regression line of y on i was $-0{\cdot}44$ which is sufficiently close to $-0{\cdot}5$ to be regarded as confirmation of the expression

derived above. Again, when an attempt was made to establish the equation $z=y+0{\cdot}5i$ for various other hormone extracts, by considering the responses at all dose levels in each of the three assays I–III, y on i, regression lines having the following slopes (b) were obtained:

$$\left.\begin{aligned} \text{I}\, b &= -0{\cdot}543 \\ \text{II}\, b &= -0{\cdot}449 \\ \text{III}\, b &= -0{\cdot}537 \end{aligned}\right\} \text{ i.e. average } b = -0{\cdot}51$$

Since it seems possible that from time to time the slope of such lines might vary, it is necessary to redetermine the numerical coefficient of z for each series of assays. In point of fact, the equation $z=y+0{\cdot}5i$ has held for all assays reported here using either fed or starved rats.

DISCUSSION

Although Hanson as long ago as 1928 recommended the use of parathyroidectomized dogs for the assay of Parathormone the present work would appear to be the first in which the response of a parathyroidectomized animal has been investigated as the basis for an assay method. Comparison with normals indicated that in such animals similar increases in plasma calcium could be obtained by injection of approximately one quarter the amount of hormone. The animals' plasma calcium + magnesium levels were found, however, to vary widely (Fig. 3) and it is thus not surprising that it was possible to demonstrate an inverse relationship between plasma calcium + magnesium level and response to hormone. In this respect the parathyroidectomized rat appears to differ both from normal rats (Biering, 1950) and parathyroidectomized dogs (Collip & Clark, 1925) in neither of which was it possible to demonstrate this relationship. It is interesting to note that Davies & Gordon (1953*a*) have reported marked dependence of both fall in plasma inorganic phosphate and rise of urine phosphate on initial plasma or urine phosphate level in the parathyroidectomized rat. Although in theory differences in response due to varying initial plasma calcium + magnesium levels can be eliminated by proper assay design, in practice using a limited number of animals unless each response is appropriately corrected to compensate for this factor, the answer obtained is likely to be as much a reflexion of initial calcium level as it is of the effect of the hormone itself. As explained above an expression (z), which measures only the real effect of the hormone freed from dependence on initial plasma level, has been obtained.

Since the observed responses, at any one dose level, were proportional to half the initial plasma calcium + magnesium concentrations, the possibility arises that response is dependent on initial calcium-ion concentration, providing that this is about half the total plasma calcium in parathyroidectomized as in normal rats. However conflicting reports have been published on this

point (Thomson & Collip, 1932) and in the present work no estimations of ionized calcium were made.

The effectiveness of using the expression z in calculating results is shown by a comparison of the curves obtained by plotting both z and y against log. dose (Fig. 4). First, it was found that the log. dose–response lines for the standard and the test animals deviated less from parallelism using z rather than y as

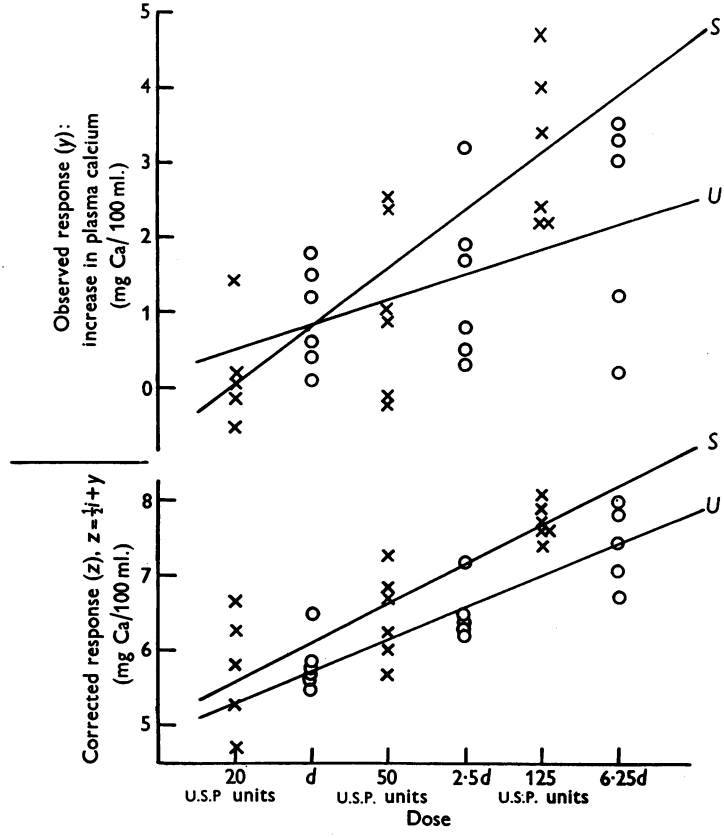

Fig. 4. Comparison of observed responses (y) and corrected responses (z) in Assay III. ×, doses of Parathormone, Line S; o, doses of unknown preparation, line U; d, the unit dose of unknown preparation.

a measure of the response. This is illustrated for one assay in Fig. 4. Although in other assays the improvement in parallelism was not so striking, it always occurred to some extent, and hence the use of z aided the accuracy of the assay. Secondly, it will be seen that the spread of experimental values at each dose level was less using z, i.e. index of precision (λ) ($\lambda = \sqrt{}$(error variance) ÷ slope of log. dose–response line) was smaller (0·23) using z than when using y (0·36). The rectification of this source of error was particularly noticeable at the

higher dose levels, where the experimental values on the y curve lie in a wide scatter while those for z deviate less from the average value. The dependence of response on initial level could have been overcome by analysing the results using co-variance, but the use of z has a very similar effect and leads to a simpler calculation of potency.

In order to reduce the rather considerable error due to day-to-day changes in the responsiveness of the animals, the assay design described above was chosen in which equal numbers of animals receive standard and test doses. By the use of this triplet cross-over design some of the error due to the differences between animals is obviated. The whole of this error could theoretically be removed by repetition of doses until each group of animals had received every dose level. However, the advantage thus gained might not be realized in practice owing to changes in plasma calcium caused by haemodilution or to any loss of sensitivity towards the hormone. The percentage limits of error obtained in the seven assays are given in Table 4. These assays were carried out in the autumn and winter. It has been mentioned that the limits of error might be slightly wider in assays undertaken in the summer if the decreased responsiveness to injected hormone encountered from May to August 1953 re-occurred.

TABLE 5. Comparison of calcium assays by various other workers

Authors	Type of animal	Total no. of animals	Times used in the assay	Potency	Limits* expressed as % of the potency
Bliss & Rose (1940)	Normal dogs	20	4	1·30	79–126
Bliss & Rose (1940)	Normal dogs	36	2	1·13	79–126
Biering (1950)	Normal rats	20†	1	1·24	83–121

* Limits were calculated at the 5% level.
† All on unknown preparation.

Assay results obtained by Biering (1950) with normal rats, and Bliss & Rose (1940) with dogs, are shown in Table 5. In comparing the various assays, it must be noticed that the average potency (0·65) in the parathyroidectomized rat assays deviated more from 1·0 than the potencies in the other assays. Had the dose levels been chosen a little differently so that the potency was nearer 1·0, narrower limits of error would have been obtained in our assays. In examining the claim of Biering to have obtained 83–121% limits by the use of only twenty animals, it must be noticed that all the animals received the test substance and the result was read off from a previously established dose–response line. However, Biering noted the existence of day-to-day variation in the reactivity of the rat, and to minimize this he recommended that experimental observations be spread over as long a period as possible. Nevertheless, his log. dose–response line was constructed from work on ninety-eight rats carried out during 1 month (June–July 1948) and the length of experi-

mental period required to give the limits of error claimed is not clearly indicated. It would appear that these narrow limits are obtained because the author is in fact using one set of values from a 3-point test in which the slope of the experimental log. dose–response line happened to be identical (3·228) with the slope of the standard line (3·214). It seems unlikely that this would occur persistently and yet by performing assays with one dose of unknown only, it would have to be assumed that this identity of slope for both the standard and the unknown always existed.

The slope of Biering's standard log. dose–response line (3·2) is similar to the average slope (2·6) of the Parathormone log dose–response (z) line in the present assays. It is probable that this low slope partially explains the rather wide limits of error in the parathyroidectomized rat assays. Much higher slopes, 4·1 and 7·35, were found by Bliss & Rose (1940) who analysed the data of Miller's (1938) dog assays. Despite these high values they found that twenty dogs each used 4 times in a Latin square arrangement gave limits of error only a little better than the best assay reported here with the parathyroidectomized rats in which eighteen animals were used twice.

Thus, the chief advantages of the present method over the dog method would appear to be: (1) the use of the cheaper and more convenient test animal; (2) using the same number of animals nearly the same level of accuracy can be obtained in a much shorter period, i.e. in 1 week instead of 1 month as by Miller; (3) less hormone is required. Compared with the method of Biering the advantages would seem to be the saving of hormone, and a more valid estimate of potency, resulting from the simultaneous use of animals receiving both standard hormone and an unknown extract.

SUMMARY

1. A triplet cross-over assay for the calcium activity of parathyroid hormone, using twenty-four parathyroidectomized rats and based on the increase in plasma calcium 21 hr after injection of hormone, is described.

2. It is shown that there is a dependence of response on initial plasma calcium + magnesium level. An expression (z) is derived for the response which corrects for the varying initial plasma calcium + magnesium values of the rats.

3. Seven assays are reported, five using fed and two using starved rats. Average limits of error are 64–158%. Since the rats showed decreased responsiveness in the summer, slightly wider limits of error may be expected during this period. Two of the unknown solutions were re-assayed: the potencies thus found for each were similar to those obtained at first.

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