INDUCED HYPERPARATHYROIDISM IN THE RAT

Thesis for the Degree of M. S.
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by

BEVERLY BUCKNER

AN ABSTRACT

Submitted to the College of Science and Arts
Michigan State University of Agriculture and
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the requirements for the degree of

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Department of Physiology and Pharmacology

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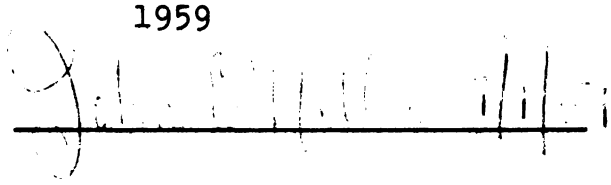
Approved by 

ABSTRACT

Hyperparathyroidism was induced in Long-Evans hooded rats by feeding a calcium deficient, vitamin D-free diet. After eight days on the dietary regime, a parathormone-like activity in the blood sera was assayable in thyroparathyroidectomized rats. The assay rats responded to the injected serum by an elevation in their serum calcium. Graded doses of injected serum produced graded elevations in the serum calcium levels. By comparing the serum calcium rise in the assay animals, produced by injected serum, to the serum calcium rise, produced by injected standard "Parathormone", 10 to 43 units of parathormone-like activity could be estimated.

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to my brother

EDWARD ADAMS BUCKNER

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INTRODUCTION

Much of the work that has been reported on the parathyroid glands has been devoted to assaying glandular extracts or to detecting the changes in animals caused by the excessive presence or lack of the hormone.

In the detection of parathyroid malfunction, the effects of too much or too little hormone are sometimes obvious. However, some of these same effects are associated with other diseases. It would be advantageous to demonstrate that a circulating blood agent is the cause of a specific condition associated with hyperparathyroidism.

This thesis describes a method of detecting parathormone-like activity in the blood of rats with induced hyperparathyroidism.

REVIEW OF LITERATURE

According to Turner, 1955, anatomically, the location of the parathyroid glands was discovered in 1880 by Sandstrom and functionally distinguished from the thyroid gland by Gley in 1891. Collip (1925) and Hanson (1925), working independently, succeeded in preparing extracts of bovine parathyroids. In 1925, Collip presented a very complete survey of experimental work and observations up to that time. He also coined the word "parathormone" to refer to parathyroid hormone and developed a method of assaying parathyroid extracts using dogs. In the same year, Felix Mandl removed a parathyroid adenoma from a patient with generalized fibrocystic osteitis and demonstrated that Von Recklinghausen's disease of the bone is the result of hyperparathyroidism. In subsequent observations, he associated this disorder with specific abnormalities of the blood and urine calcium and phosphorus levels. Mandl's work led to the elucidation of much of our knowledge of parathyroid physiology (Gordan, 1958).

Theories of parathyroid hormone action

Parathyroid hormone activity is reflected in changes in the calcium and phosphorus metabolism. In parathyroid deficiency states, abnormally low concentrations of serum calcium are found in conjunction with an elevation of the renal threshold to phosphorus excretion. There is also a

reduction in the amount of phosphate excreted in the urine. Administration of parathyroid hormone induces an increase in serum calcium and a slight reduction in serum phosphate ion concentration. The exact mechanism responsible for these effects remains controversial.

Thompson and Collip (1932) hypothesized that the direct action of parathormone is to stimulate release of calcium from bone. The changes in serum and urinary calcium and phosphorus levels are secondary to the bone action. This hypothesis has been called the bone theory in oppcsition to the renal theory expounded by Albright, et al. (1929), and Albright and Ellsworth (1929). This group suggests that the primary action of the hormone is on the electrolyte balance of body fluids, enhancing their ability to dissolve bone salts, and, thereby, inducing bone dissolution as a secondary phenomenon. The renal theorists maintain that the primary site of hormonal action is the kidney, where the renal threshold for phosphorus is lowered. The resulting hyperphosphaturia leads to a hypophosphatemia, which, in turn, causes the blood to become unsaturated with respect to calcium phosphate. As a result calcium, in excessive quantity, enters the serum from the gastro-intestinal tract and bones.

The renal theory implies that excessive parathormone leads to a continuously inverse correlation between serum calcium and phosphorus and that parathormone would be

inactive in the absence of kidneys. However, Greep (1948) notes that a quantitative action of parathormone in nephrectomized animals has frequently been reported. For example, Stewart and Bowen (1951) were able to elicit quantitative responses to parathormone in bilaterally nephrectomized dogs. Collip, et al. (1934), obtained the characteristic affect of the parathyroid hormone on the bones of rats after bilateral nephrectomy. On the other hand, Levinsky and Davidson (1957) found that infusion of one kidney of a chicken with parathormone gives a unilateral increase in phosphate excretion. No changes in the glomerular filtration rate or plasma phosphorus concentration occurred. He concluded that parathormone acts directly on the renal tubule. Tweedy et al. (1947), using radioactive phosphorus, P^{32}, reported that the prompt action of 5 units of parathyroid extract in promoting urinary excretion of P^{32} is evidence for the kidney action of parathormone. Monahan and Freeman (1944) reported that parathyroid extracts do not produce hypercalcemia when administered to bilaterally nephrectomized dogs, cats, and rats. Ligation of ureters or renal blood vessels also effectively stop hypercalcemia. When the ligatures are cut, the usual hypercalcemia results. Harrison and Harrison (1941) were able to demonstrate that parathyroid extract administered to dogs produced a decreased reabsorption of phosphate in the proximal kidney tubules. In humans, altered tubular

reabsorption of phosphorus is used as a criterion for diagnosis of parathyroid malfunction (Chambers et al., 1956).

Jahan and Pitts (1948) studied the effect of parathyroid hormone on renal tubular reabsorption of inorganic phosphorus over a range of plasma concentrations from 0.9 to 5.4 mM/liter. They found the same rate of reabsorption of phosphorus in normal and parathormone treated dogs. The rate of calcium reabsorption was greater following parathormone treatment due to increased filterable plasma calcium which increased the quantity of calcium presented to the tubules. These workers concluded that the hypercalcemia and hypercalcuria produced by parathormone administration are dependent upon extrarenal actions and not on any specific depression of renal tubular reabsorption of either calcium or phosphorus. According to Smith (1955) the exact mechanism of calcium reabsorption has not been clearly worked out. Fay et al. (1942) studied the phosphate-to-creatinine ratios over a wide range of plasma concentrations in normal, parathormone treated and parathyroidectomized dogs. They infer that a lack or excess of the hormone produces no demonstrable effect upon the capacity of the kidney to excrete and, hence, to reabsorb phosphate.

The bone theory to which Thomson and Collip subscribe was supported by Bodansky et al., 1930; Bodansky and Jaffe, 1931; Jaffe, et al., 1931; Jaffe, et al., 1932;

in a series of papers on experimentally induced hyperparathyroidism and the subsequent syndrome of osteitis fibrosa in guinea pigs. Selye (1932, A and B) published two papers supporting the bone theory. He reported that dissolution of bone salts from the organic matrix is responsible for lesions associated with excessive parathyroid hormone. Pugsley and Selye (1933), by injecting parathormone into rats, were able to correlate cellular changes in bone with serum and urinary calcium changes. The animals responded to injections of parathormone by formation of numerous osteoclasts in the bones by the second day of treatment. By the ninth to twelfth days, osteoclasts disappeared; osteoblastic activity resumed and the blood and urinary calcium returned to normal levels. Continued injections led to increased osteoblastic activity which resulted in large deposition of calcium in the bones, a condition referred to as "marble bone", and no further increase in serum calcium level was produced.

Elliott and Freeman (1956) reported evidence for an effect of parathyroid hormone on the citric acid cycle in the metabolism of the rat, rabbit, and guinea pig. Talmage et al. (1957) employed a citric acid peritoneal lavage to demonstrate a possible means by which bone salts could be deposited and reabsorbed. They suggested that one function of parathormone is to release citric acid immediately from bone cells for localized dissolution

of bone. Thus, an elevation of calcium level in the fluid compartment is produced to maintain the normal blood level.

Stewart and Bowen (1952) achieved a biological separation of calcium-mobilizing and phosphorus-excreting activities of the parathyroid gland extracts by using a formaldehyde inactivation of the calcium factor. They were able to show phosphorus-excreting activities in extracts of the thymus and spleen when extracted in the same way as the parathyroid glands. This would suggest that the phosphorus-excreting activities are non-specific and that extraction methods could account for some of the wide variations in the reports of workers studying effects of various extracts on the tubular reabsorption of phosphorus.

Chemical Nature of Parathyroid Hormone

Numerous methods of making extracts from parathyroid glands have been reported. The first extracts, thought to be protein in nature, were prepared by Hanson (1925) and Collip (1925) from bovine parathyroids by hot, dilute hydrochloric acid extraction. Ross and Wood (1942) attempted to purify their extract with ammonium sulfate fractionation, and L'Heureux *et al.* (1947) used an acetone precipitation. Davies *et al.* (1955) extracted a hormonally active material with 80% acetic acid. Their work also demonstrated that hydrochloric acid-extracted and acetone-extracted materials may have different

chemical properties. Ross and Wood (1942) reinforced the concept of the parathyroid hormone being protein in nature by their studies using pepsin digestion, ultraviolet absorption spectrum and stability to electrodialysis. Two protein fractions were obtained by ultracentrifugation. One had a molecular weight between 500,000 and 1,000,000 and the other between 15,000 and 25,000. They associated activity of the hormone with the protein of lower molecular weight.

A comparison of the isoelectric points of parathyroid extracts, as reported in the literature, demonstrates the heterogenous nature of the hormone. Collip's purest extract precipitated sharply at pH 4.8. Tweedy and Torigoe (1932) reported an isoelectric point of pH 5.8. Allardyce (1932) reported two points, one at pH 4.8 approaching from the acid side, and the other at pH 6.8 from the alkaline side; Ross and Wood (1942) found their material to be soluble in acids up to pH 4.5 to 5.0 and insoluble in alkali up to pH 10.5 to 11.0. Rasmussen and Westall (1957) obtained a partially purified hormone by means of hydrochloric acid extractions, acetone fractionation, ultrafiltration, and displacement chromatography on an exchange resin. They presented evidence that active materials obtained by hydrochloric acid and acetic acid extraction have different chemical properties. Rasmussen (1957) has named the hydrochloric acid-extracted active substance "parathormone A" and the

acetic acid-extracted active substance "parathormone B". By means of zonal electrophoresis on polyvinyl chloride and ultracentrifugation, he has calculated the molecular weight of the "parathormone B" to be approximately 10,000 and the nitrogen content 16.4%. Rasmussen and Westall (1957) could not detect parathormone activity in lipid extracts of the parathyroid gland but did find 80% of the activity in nitrogenous residues.

According to Greep and Kenny (1955), Gordon has demonstrated that phosphate activity and calcium-mobilizing activity migrated identically in starch electrophoresis preparations.

Stewart (1957) chemically identified a large, acidic, non-protein molecular species in parathyroid extracts. However, no biological assays were performed.

The complex, heterogeneous nature of available parathyroid extracts makes variations between the work of different investigators understandable. It appears also that the dualistic nature of the hormone is directly related to different proteins now comprising glandular extracts or to different groups attached to the same molecule.

The purification of parathyroid hormone is essential before a stable, uniform standard can be established. Elimination of the nonspecific activity in glandular extracts is necessary before extracts can be compared and the most sensitive assays selected for evaluation of unknown material.

Laboratory Induction of Hyperparathyroidism

Several diseases, other than those of parathyroid malfunction, result in a secondary disturbance of calcium and phosphorus metabolism (Gordan, 1958). For this reason blood and urinary calcium and phosphorus levels in an animal may be diagnostically misleading. Of the many tests for hyperparathyroidism, no one test is pathognomonic for the disease (Chambers et al., 1956). It would be of diagnostic significance, therefore, to show the presence of high levels of circulating parathormone-like substances in the blood of hyperparathyroid animals.

Induced, or secondary, hyperparathyroidism can result from 1) low calcium diet, 2) pregnancy, 3) lactation, 4) rickets, and 5) osteomalacia (Guyton, 1956). Calcium-restricted diets in normal animals or in pregnant or lactating animals seem to be the most logical choice of methods to induce parathyroid hyperfunction (Greep, 1948).

Boda and Cole (1954) reported that milk fever in dairy cattle due to parathyroid insufficiency could be alleviated by conditioning the parathyroid gland prior to lactation. This could be accomplished by reducing the calcium or by increasing the Ca:P ratio in the diet. Luce (1923) demonstrated that rats fed a diet deficient in calcium developed a consistent enlargement of the parathyroid glands. This enlargement was due to hyperplasia, not hypertrophy, of the cells. No correlation

was noted between the sex or weight of the rats, nor was there a corresponding cellular change in the thyroid gland.

Bauman and Sprinson (1939) encountered enlarged parathyroid glands while autopsying rabbits which had been fed a carrot-oats diet contained a Ca:P ratio of 0.5 as compared to a ratio of 4 in the regular stock diet. Rabbits fed the carrot-oats diet for three months developed parathyroid glands weighing 30 to 50 mgs. as compared to a normal weight of 10 mg. Serum calcium and phosphorus levels were followed, and a reaction to the diet could be detected after the first week. The cells of enlarged glands were 50% larger than normal parathyroid cells and exhibited increased lipid content. The presence of increased parathormone in the blood of the dietary animals was assayed by the method of Hamilton and Highman (1936).

Ham et al. (1940) and Carnes, et al. (1942) have conducted experiments which show that conditions leading to decreased serum calcium levels induce hyperplasia of the parathyroid glands of rats. Ham et al. (1940) concluded that it is the hypocalcemia and not hyperphosphatemia that is the stimulus to parathyroid gland enlargement. Stoerk and Carnes (1945) reported finding in mature rats a close direct proportionality between the logarithm of dietary Ca:P ratio and the serum calcium concentration. An inverse proportionality exists between

the logarithm of the dietary Ca:P ratio and the volume of the parathyroid gland.

Crawford et al. (1957) developed a calcium-deficient, vitamin D-free diet which caused enlarged, hyperemic glands in rats. Rats fed this diet, but with added vitamin D, did not show grossly abnormal glands. The kidneys, bone, and parathyroid glands of their animals were removed for histological study. No abnormalities were found in the kidneys, except for an increased size in the group receiving the calcium-deficient diet with added vitamin D.

Since pregnancy and lactation alone can cause secondary hyperparathyroidism, animals in either condition fed a calcium-deficient diet undergo extreme calcium deprivation. The calcium deficiency stress on a pregnant rat is indirect in that the calcium for fetal development comes from the maternal bones and not from a direct drain on the calcium absorbed from the dietary source (Bodansky and Duff, 1941).

Methods of Assay

A standard unit of parathyroid hormone has not been established; consequently, the potency of unknown preparations is defined in terms of response or by comparison with a standard which is defined in terms of response.

Collip and Clark (1925) originally defined a unit of parathyroid hormone as 1/100th of that amount of an extract required to raise the serum calcium level of a dog

weighing 20 kg. by 5 mg./100ml. Hanson (1928) suggested reducing the size of the unit to 1/100 of that amount causing a rise of 1 mg./100 ml. in serum calcium of parathyroidectomized dogs.

The United States Pharmacopoeia (15th revision) defines a unit of parathyroid hormone as 1/100th of that amount required to cause a rise of 1 mg./100 ml. in the serum calcium of normal dogs within 16 to 18 hours after administration of a parathyroid hormone preparation.

Methods of assaying parathyroid hormone, according to Thorp (1950), may be grouped as follows:

1. Methods based on elevation of serum calcium.
2. Antagonism of magnesium anesthesia by the rise in the serum calcium produced by parathyroid hormone.
3. Methods based on fall of serum phosphorus.
4. Methods based on excretion of calcium in urine.
5. A method based on action of parathormone on hypodynamic muscle.

An additional group developed since 1950 is:

6. Methods based on increase in urinary phosphorus excretion.

Group 1: Serum Calcium Rise. The Clark and Collip (1925) assay involves measuring the rise in serum calcium levels in groups of 10 dogs of approximately 10 kg. weight. Normal serum calcium levels of the dogs are determined, followed by subcutaneous injections of the hormone

preparation. Fifteen hours later, serum calcium levels are again determined. Miller (1938) reported that the linear relationship between dose and response was poor and highly variable. Bliss and Rose (1940) analyzed the variances in dose-response relationship and reported that computations from responses to dose within the same dog were more consistent than responses to dose from among different dogs. They concluded that the final serum calcium determination was a more reliable criterion for evaluation of parathormone response than using the initial serum calcium and its subsequent rise following parathormone injections.

Hamilton and Schwartz (1932) described a test using rabbits by which they could determine an approximation of parathyroid hormone in biological substances. Hamilton and Highman (1936) used this method to detect abnormal amounts of parathyroid hormone in human blood. They withdrew 30 ml. of blood from a patient and injected it intramuscularly into the legs of a rabbit, which was then given 0.1 ml. of $CaCl_2$, by gastric tube at 1, 3, and 5 hours. Normally, the serum calcium level rose only after the first dose of $CaCl_2$, but in the presence of abnormally large levels of parathormone, the serum calcium remained elevated after the later doses of $CaCl_2$. Large variations in normal serum calcium levels and in the response to parathormone encountered in rabbits limit the usefulness of this assay (Dyer, 1935).

Tweedy and Chandler (1929) reported that parathyroidectomized rats exhibited a greater increase in serum calcium levels in response to parathormone injections than intact animals. A two- to three-fold increase in response was evident 17 hours after subcutaneous or intraperitoneal injection of 20 units of parathormone. Davies et al. (1954) cauterized the parathyroid glands in their assay rats and determined the combined serum calcium and magnesium levels by chelation just before and 21 hours after injecting parathormone. Muson (1955) increased the sensitivity of parathyroidectomized test rats further by feeding them a calcium-deficient diet prior to injecting parathormone.

Group 2: Calcium Antagonism to Magnesium Narcosis. Magnesium narcosis was extensively studied in a wide variety of experimental animals by Meltzer and Auer (1905). In 1935, Simon suggested an assay for parathormone based upon calcium-mobilizing activity of parathormone and its subsequent prevention of magnesium narcosis. An optimal dose of parathormone would mobilize sufficient calcium ions to antagonize injections of magnesium sulfate and prevent narcosis in a large number of mice. Unknown material could then be assayed by comparison with standard preparations. Dyer (1935) extended this assay method and suggested that a useful criterion of narcosis is the ability of a mouse to right itself when turned on its back. This assay has not been analyzed statistically.

Group 3: Fall of Serum Phosphate. Tepperman et al. (1947) developed an assay based upon the measurable fall in the serum inorganic phosphate in rats three hours after subcutaneous injection of parathormone. The relation between decreased serum inorganic phosphate and the logarithm of the administered dose of hormone was substantially linear over the range 12.5 to 100 U.S.P. units. Albino male rats were maintained on a standard diet for two weeks prior to the experiment. Pre-injection blood samples were collected from a cut tail vein. Following this, hormone preparations were injected subcutaneously in 0.5 ml. quantities on either side of the lumbar region. All the injection material was made up to a total volume of 1 ml. with 0.9% NaCl. Three hours after injection blood samples were again collected and analysed for inorganic phosphate. Fed rats were found to be more suitable for assay purposes than fasted rats. Since the initial serum inorganic phosphate level influenced the extent of decrease, the findings were adjusted to the mean value of 9.15 mg%. The logarithmic ratio of the unknown and standard potencies were then calculated.

Davies and Gordon (1953) adopted the method of Tepperman et al. (1947), but substituted thyroparathyroidectomized rats for intact animals, and reported that 2 U.S.P. units produced a decrease of 35% in the serum inorganic phosphate levels in the spring and a decrease of 15% in the fall of the year. Twelve rats were tested

at each dose level and blood samples were collected one and one-half hours after the injection of parathormone. No loss of sensitivity was detected up to 24 days after parathyroid surgery.

Group 4: Increase in Urinary Calcium Excretion. Dyer (1932) and Pugsley (1932) studied the effect of parathormone on urinary excretion of calcium. Dyer suggested using parathormone-induced urinary calcium increases in rats as an assay method. In 1933 he devised an assay utilizing rats fed a high-calcium diet. The high-calcium diet enhanced the response of rats to parathormone.

Truszkowski et al. (1939) modified the rat urinary calcium assay. Daily urinary calcium determinations were performed over a seven-day period until normal fluctuations could be established. Extreme variations in normal urinary calcium levels make this assay difficult to standardize. A rat unit was proposed as 1/10th of that amount which would give a total rise in urinary calcium of 1 mg.

Group 5: Parathormone Action on Hypodynamic Muscle. Gellhorn (1935) employed the increased sensitivity of hypodynamic muscle to calcium ions as the basis of an assay for parathyroid hormone. The abdominal aorta of a pithed frog was perfused with a phosphate buffered Ringer's solution containing various dilutions of parathormone. The gastrocnemius muscle was connected to an isotonic lever of a kymograph and stimulated regularly until fatigue was evident as judged by a reduction in the height

of contraction to 50% of the starting height. Parathormone in a 1:250 dilution not only increased the height of contraction of the fatigued muscle but also decreased the recovery time of a previously fatigued muscle. Dilutions as high as 1:1000 of parathormone were still capable of producing a weak effect.

Group 6: Increase in Urinary Phosphate. Tweedy et al. (1947) reported that thyro-parathyroidectomized rats, two to three hours after surgery, are sensitive to 2.5 to 5 units of parathormone. Their studies were conducted using radioactive phosphorus, P^{32}; and they concluded that the prompt action of such a small dose was good evidence for the renal theory of parathormone activity.

Stoerk and Silber (1949) discovered that an injection of 20 units of parathormone into parathyroidectomized rats produced a maximum increase in urinary phosphate excretion. No additional effect was obtained by additional amounts of hormone. These workers concluded that the influence of parathormone in the tubular reabsorption of phosphate is essentially an "all or none" effect. This effect cannot be demonstrated in intact animals with adequately functioning kidneys. This conclusion was based on the lack of response when 20 to 160 units of parathormone were injected into normal rats.

Davies and Gordon (1953) parathyroidectomized rats, and injected them subcutaneously with 3 units of parathormone. The urine from three animals was pooled for each

sample. Three units of parathormone produced a maximum increase in urinary phosphate excretion. However, they encountered marked variations between animals and seasonal fluctuations in urinary phosphate levels.

Davies et al. (1955) published a urinary phosphate assay employing saline-loaded mice. Sixteen mice were maintained in each metabolism cage and their urine output was pooled for sampling. In order to stimulate urine excretion, 1 ml. of 0.9% NaCl per 5 gm. of body weight was injected intraperitoneally into each mouse. Urine collections were started 15 minutes after parathormone injection and continued for 3 1/2 hours. By using 550 mice at each dose level, they were able to show a high correlation between the dose given and the milligrams of phosphate excreted per hour. The number of animals necessary to substantiate this assay limits its application. Davies (1957) used this method to assay benzoid acid extracts of urine from normal, hypoparathyroid and hyperparathyroid patients. A phosphorus unit (P.u.) for this work was set as 1 ml. of "Parathormone" (E. Lilly and Co.) equals 100 P.u. The "Parathormone" was labelled as containing 100 U.S.P. units of calcium activity/ml. In a series of five normal patients, urine extracts averaged 60 P.u./24-hour urine sample with a range of 47 to 72 phosphorus units. The amount of parathormone in urine of hypoparathyroid patients was too low to estimate. In four hyperparathyroid patients, there was a distinct increase in

parathyroid hormone in urine. The average phosphorus unit was 121/24-hour urine sample and the range, 103 to 146, was markedly higher than that of the normal group.

To the author's knowledge, there are two reports in the literature of attempts to detect parathormone-like activity in the blood of animals. (Hamilton and Highman, 1936 and Bauman and Sprinson, 1950). The assay method employed by both of these groups was a qualitative test on rabbits, which has been criticized by Dyer (1935) for its lack of sensitivity.

This thesis presents a sensitive assay method for the detection of parathormone-like activity in the blood of hyperparathyroid rats.

METHODS AND PROCEDURES

In order to produce secondary hyperparathyroidism in the animals used in this project, the diet of Crawford et al. (1957) was adopted with a few minor changes (Table I). This diet was calculated as containing 0.001% calcium and 2.4% phosphorus in contrast to a normal diet of 0.8% calcium and 0.4% phosphorus. The calcium-deficient, vitamin D-free diet of Crawford et al. will be referred to as the C-D diet.

Blood samples from animals with a well established induced hyperparathyroidism should contain sufficient parathormone to produce hypercalcemia when injected into parathormone-sensitive animals. Since thyroparathyroidectomized rats are two to three times more sensitive to injected parathormone than intact animals (Tweedy and Chandler, 1929), the assay method of Davies et al. (1954), with slight modifications by Rasmussen (1957) was chosen to test the blood samples. As the test animals were sacrificed, blood samples were collected and the parathyroid glands, kidneys, and in some instances other soft tissues were removed for histological studies. Tissues were fixed in 10% neutral formalin and processed through a butyl alcohol series. Hemotoxylin and eosin was used throughout for staining.

Inducing Hyperparathyroidism

To insure that the animals on the C-D diet did not

TABLE I.

EXPERIMENTAL DIET OF CRAWFORD, ET AL., 1957

BASIC DIET	GMS.
Casein	625
Glucose	1900
Corn Oil	125
Cystine	20
MINERALS	
Na_2HPO_4	404
NaCl	47
$MgSO_4 \cdot 7H_2O$	100
NaI	0.3
$CuSO_4 \cdot 5H_2O$	0.4
ZnCl	0.09
$MnSO_4 \cdot 4H_2O$	3.2
$FeC_6H_5O_7 \cdot 3H_2O$	30
KCl	143
$NaHCO_3$	182
VITAMINS	
Thiamin Hydrochloride	0.04
Riboflavin	0.12
Pyridoxine	0.04
*Na Pantothenate	0.25
Menadione	0.5
Nicotinic Acid	0.5
Inositol	10.0
p-aminobenzoic Acid	20.0
Tocopherol	0.3
Choline Dihydrogen Citrate	20.0
Vitamin A	50000 units
INGREDIENTS ADDED	
Folic Acid	0.01
Vitamin B_{12}	0.02
Wood flock	to 10% of total diet

*Calcium pantothenate was substituted for the sodium compound.

have access to calcium depositions on the equipment, the cages, watering tubes, and feed jars were scrubbed with a dilute solution of hydrochloric acid. In addition, distilled drinking water was supplied to all the animals on the calcium-deficient regime.

The C-D diet was fed ad lib. to the test rats in two duplicate experiments. The first experiment was completed before the second was started. The second experiment was to confirm and define in more detail the results obtained from the first experiment. The animals were divided into the following four groups:

Group I

Ten 150 gm. Long-Evans hooded, female rats were placed on the C-D diet. On the 10th day of the experiment and every five days thereafter, two C-D dietary rats and a control were killed. Two rats were allowed to remain on the calcium-deficient diet for 40 days, at which time one died and the other was sacrificed for blood and histological studies.

Marked calcifications were noted in the kidneys of the rats maintained on the C-D diet for 10 days. In order to establish the time at which the calcifications first appeared, 20 rats were employed in the second experiment, and two were sacrificed every two days after the start of the experiment. Male rats, 200 to 230 gms., and female rats, 180 to 200 gms., were killed in pairs.

Group II

Two pregnant females were maintained on the C-D diet from the day of estimated implantation, or the 8th day, until the day before expected parturition.

Group III

Two lactating rats were maintained on the C-D diet from the second day of lactation until weaning, the 21st day. The litters were sexed and reduced to 6 pups each at the start of the experiment. A higher proportion of female pups was retained because of their more uniform and slower growth rate as compared to male pups. After the 11th day of lactation, a pup was killed every two days for histological sectioning.

Group IV

The control rats were maintained on the nutritionally adequate stock diet developed by Drs. Ullrey and Miller of the Animal Husbandry Department, Michigan State University, and were supplied with tap drinking water. In all other respects they were treated in the same manner as the C-D dietary rats.

After six to ten days on the diet, hyperexcitability of the animals was apparent. Tetany, however, was never obvious. Without the addition of wood flock to the diet, the rats exhibited a marked diarrhea. The animals rejected the diet for the first few days and weight loss was evident in all the experimental animals.

Blood Assay for Parathormone-like Substances

The blood samples were collected from the orbital sinuses of the rats by the method of Halpern (Stone, 1954) with heparinized capillary tubes. This method was especially effective when serial samples were needed for analysis. Exsanguination frequently yielded 5 to 6 ml. of blood free from hemolysis. As soon as clotting occurred, the serum was separated from the clot and frozen until needed. Serum calcium levels were determined on 0.1 ml. of serum by colorimetric titration with E.D.T.A.* using ammonium purpurate as an indicator (Wilkinson, 1957). The serum inorganic phosphorus levels were determined by a modification of the molybdivanadate method of Simonsen et al. (1946) on 0.1 ml. of serum. Weichselbaum's modification of Kingsley's biuret method (1946) was used to determine the total protein content of the serum.

The animals used in the blood assay were male, albino rats weighing 115 to 130 gms. They were thyroprarthyroidectomized 3 to 4 days prior to the experiments (Davies et al., 1954). Hoskin and Chandler (1925) showed by serial sections of rat neck regions that less than 10% of adult animals have accessory glands, so no search was made to determine if they were present. Animals with calcium levels below 8.5 mg.% prior to the experiments were

*Disodium ethylenediaminetetraacetate, a chelating agent sold under the trade names of "Versene" or "Sequestrene".

considered parathyroidectomized. Injections of parathormone and blood serum were made subcutaneously over the lumbar region in 0.5 ml. units. A blood sample was drawn just before injection of the material and again 18 hours later (Rasmussen, 1957).

RESULTS

Normal serum calcium levels determined on a series of control animals averaged 9.8 mg.% (S.E. ± 0.43); serum inorganic phosphorus levels averaged 5.2 mg.% (S.E. ± 0.32); and the total serum proteins averaged 6.2 gms.% (S.E. ± 0.26). Data summarized by Spector (1956) reported normal rat whole blood calcium levels of 12.2 mg.% (10.8 - 14.4), a normal plasma inorganic phosphorous of 5.9 mg.%, and a plasma total protein of 6.3 gms.%. The methods used were not reported. Peterson and Beatty (1958) reported a value of 6.45 (S.E. 0.09) gms.% for total protein levels in albino rats using paper electrophoresis.

In order to evaluate the serum parathormone-like activity and to express it in terms of "Parathormone" equivalence, a standard "Parathormone"* preparation was injected subcutaneously into 15 thyroparathyroidectomized rats, three at each dose level of 10, 15, 20, 40, and 80 units. The results are tabulated in Table II. These data are plotted as calcium rise in mg.% against the logarithm of the dose in Figure I. By standard statistical analysis the slope of the line is $Y = -3.64 + 3.75 \log X$. The 95% confidence limits for this experiment are marked on either side of the line.

In a further study of the parathormone-like activity in the serum of hyperparathyroid rats, the sera

*The "Parathormone" used in this experiment was generously supplied by the Eli Lilly Company, Indianapolis, Indiana. It contained 100 U.S.P. units per ml.

TABLE II.

SERUM CALCIUM RISE IN ASSAY ANIMALS* AFTER INJECTION OF STANDARD "PARATHORMONE".

Rat No.	Weight gms.	Dose U.S.P. units	Base Ca. ** mg.%	18-hour Ca. *** mg.%	Rise in Ca. mg.%	Av. Ca. Rise mg.%
16	111	10	6.8	7.0	0.2	0.13
17	117	10	7.0	7.0	0	
34	101	10	7.8	8.0	0.2	
36	100	15	7.0	7.5	0.5	0.56
27	130	15	6.3	7.0	0.7	
38	107	15	6.5	7.0	0.5	
16	90	20	8.0	8.8	0.8	1.16
3	125	20	7.8	8.9	1.1	
12	117	20	7.4	9.0	1.6	
20	110	40	6.0	9.0	3.0	2.53
2	124	40	7.7	10.3	2.6	
15	118	40	6.8	8.8	2.0	
19	110	80	6.0	10.0	4.0	3.60
18	111	80	7.3	10.8	3.5	
20	117	80	7.5	10.8	3.3	

*Thyroparathyroidectomized rats.

**Determined 3 hours prior to "Parathormone" injections into assay animal.

***Determined 18 hours after injection of "Parathormone" into assay animal.

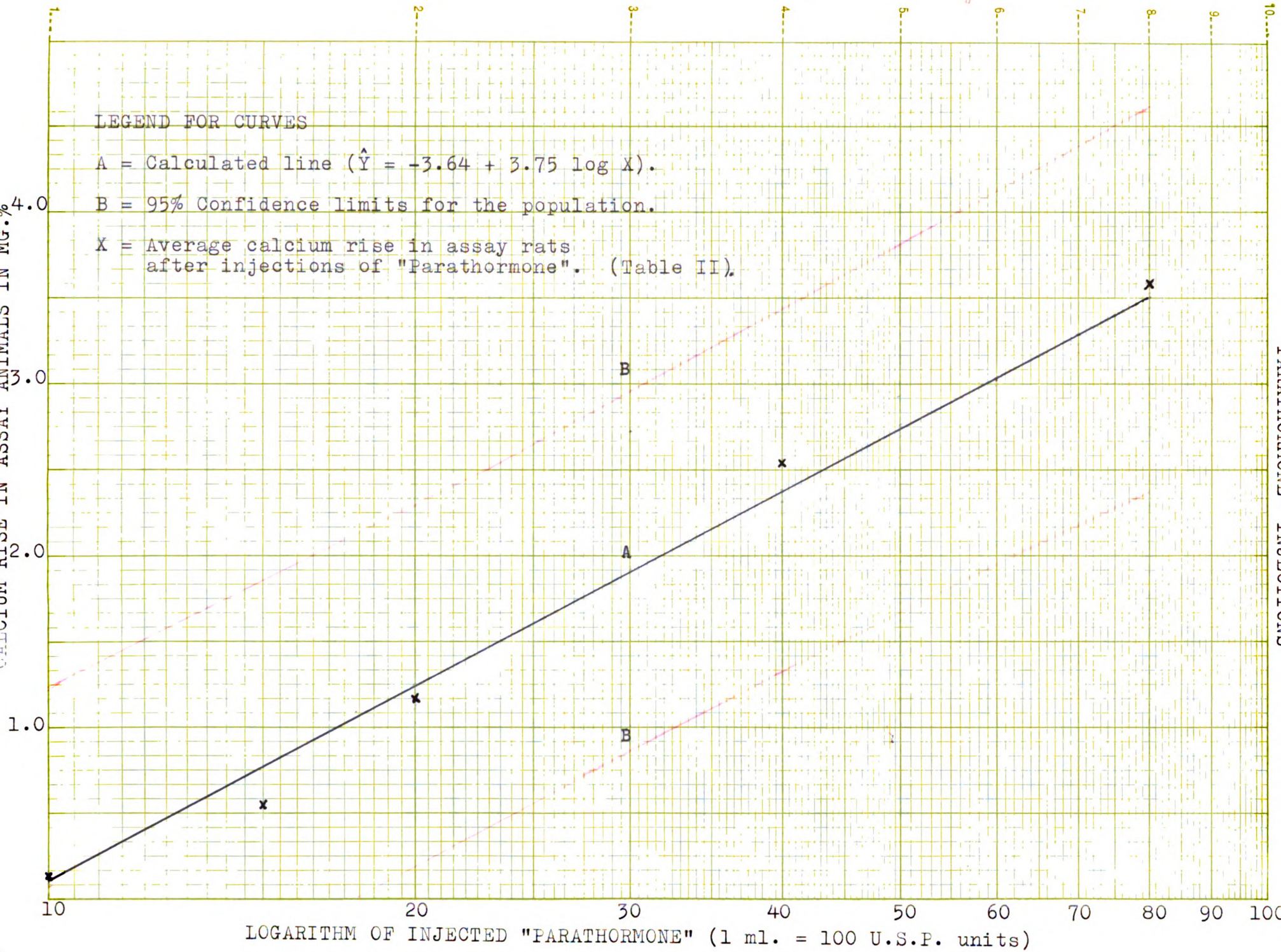


FIGURE I. RESPONSE OF ASSAY ANIMALS TO STANDARD "PARATHORMONE" INJECTIONS

from four animals on the C-D dietary regime were tested at graduated dose levels. The sera were injected into thyroparathyroidectomized test animals and the serum calcium rise produced was compared with the standard "Parathormone" curve and a "Parathormone" equivalent estimated. The results of this experiment are listed in Table III.

Serum calcium levels were determined daily on the animals fed the C-D* diet the first ten days of the experiment and at approximately two-day intervals thereafter up to the 20th day. The rats maintained serum calcium levels in the 9 to 11 mg.% range, except for two periods, one at the fifth day and the other between the 12th and 15th days. (Figure 2). Serum inorganic phosphorus levels were determined approximately every other day throughout the experiment (from Figure 2). Serum inorganic phosphorus increased from the second day and continued rising, except for a mild regression between the 15th and 18th days. An abrupt rise between the 12th and 15th days coincided with a transient drop in the serum calcium curve. The total serum protein curve remained relatively constant at approximately 6.1 gms.%. Two rises to 6.8 gms.% occurred, one on the 7th day and the other for a four-day period from the 14th to the 18th days. The second rise in total serum protein

*C-D diet refers to the calcium-deficient, vitamin D-free diet of Crawford *et al*. (1957) as found in Table I.

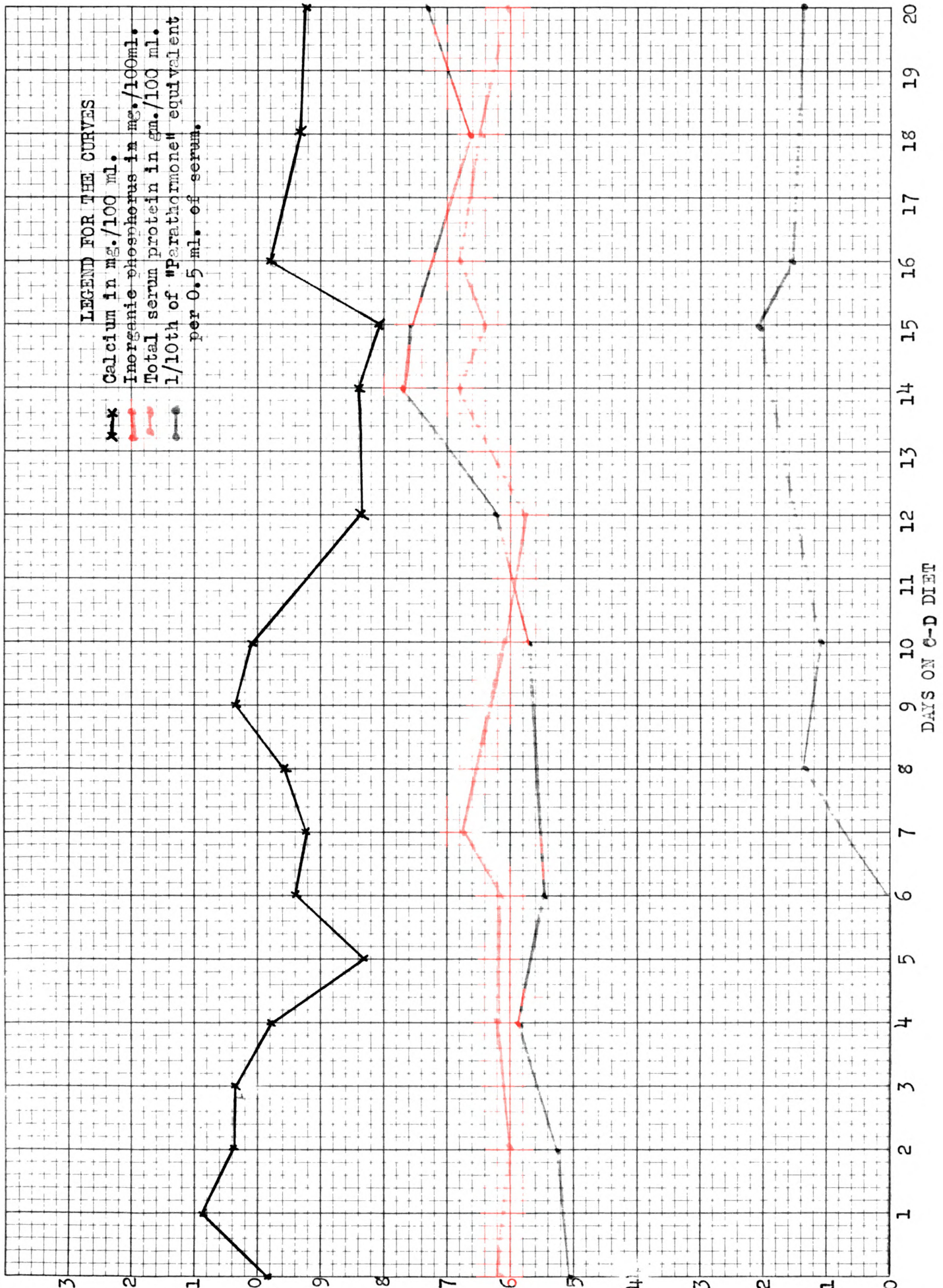
TABLE III.

SERUM CALCIUM RISE IN ASSAY ANIMALS AFTER INJECTION OF GRADED DOSES OF SERUM FROM THE TEST ANIMALS

Assay Rat No.	Days Test Animals on C-D Diet	Dose of Serum ml.	Base Ca* mg.%	18-hour Ca** mg.%	Ca Rise mg.%	"Parathormone" Equiv. U.S.P. units***
21	6	0.25	7.3	6.0	0	0
22	6	0.50	7.5	6.0	0	0
23	6	1.00	7.5	7.3	0	0
15	8	0.60	7.0	7.7	0.7	14.5
16	8	0.90	7.0	8.0	1.0	17.5
24	16	0.25	7.8	8.0	0.2	11.5
25	16	0.50	6.0	6.8	0.8	15.5
26	16	1.00	7.0	8.5	1.5	23.5
1	29	0.25	6.6	7.5	0.9	16.25
2	29	0.50	7.5	9.2	1.7	26.5
3	29	1.00	7.3	9.8	2.5	43.5

*Determined on thyroparathyroidectomized rats 3 hours prior to injection of test serum.
**18 hours following injection of test sera into assay rat.
***In sera of test animal.

FIGURE 2. COMPOSITE CURVE



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levels coincided with the second rise in the serum inorganic phosphorus curve and a drop in the serum calcium curve.

Parathormone-like activity was not detected in 0.5 ml. serum samples until the test animals had been on the C-D diet eight days. At that time the first elevation in the serum calcium level in assay animals was produced by serum injection. A comparison of the serum calcium curve and the "Parathormone" equivalence curve demonstrates that the first parathormone-like activity appears soon after the initial decrease in the serum calcium levels (Figure 2).

The data from which the curves in Figure 2 are constructed are tabulated in Table IV.

In order to rule out the possibility that the calcium content of the injected serum might be sufficient to cause a rise in the serum calcium level of thyroparathyroidectomized test animals, a normal serum containing 9.8 mg.% calcium was injected into three test animals. The following table shows the results of this experiment:

Rat No.	Dose of serum ml.	Base Ca mg.%	18-hour Ca mg.%	Ca rise mg.%
11	1.0	8.4	8.4	0
6	0.5	6.3	6.0	0
4	0.5	8.2	8.3	0.1

TABLE IV.

TABULATION OF SERUM CALCIUM, INORGANIC PHOSPHORUS, TOTAL SERUM PROTEIN AND "PARATHORMONE" EQUIVALENTS IN HYPERPARATHYROID RATS.

Days on C-D Diet	Calcium* mg.%	Inorganic Phosphorus* mg.%	Total Serum Protein* gm.%	"Parathormone" Equiv./0.5 ml.
0	9.8 (S.E.±0.43)	5.2 (S.E.±0.32)	6.2 (S.E.±0.26)	0
1	10.8	--	--	--
2	10.3	5.2	6.0	0
3	10.3	--	--	--
4	9.7	5.8	6.2	0
5	8.3	--	--	--
6	9.3	5.4	6.2	0
7	9.2	--	6.8	--
8	9.5	--	--	15.5±1.06**
9	10.3	--	--	--
10	9.9 (S.E.±0.29)	5.7	6.1 (S.E.±0.31)	10.8±1.08
12	8.3	6.2	5.8	--
14	8.4	7.7	6.8	--
15	8.0	7.6	6.5	21.0±1.04
16	9.8	--	6.8	15.5±1.06
18	9.3	6.7	6.6	--
20	9.2 (S.E.±0.24)	7.3	6.0	14.5±1.06
***29	7.5	--	--	26.5±1.04

*The figures shown here are the average figures of two determinations. If three to seven determinations were made, the mean and standard error are reported.

**This figure is the ninty-five percent confidence limit for any assay rat of the correct weight used in this assay.

***Data for this animal are not shown on Figure 1.

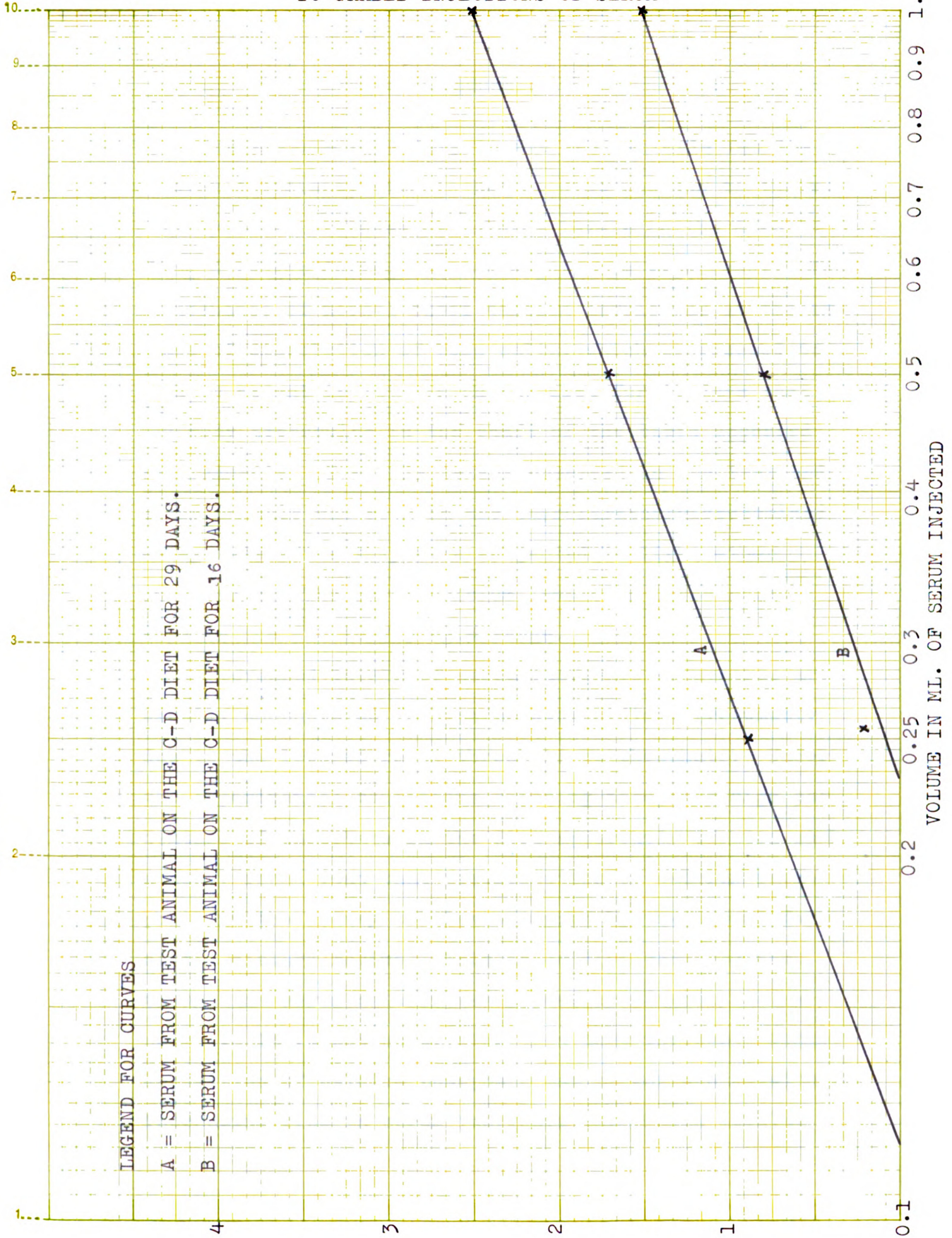
Since Wilkinson (1957) reports the experimental error in this method to be ±3%, there is no significant rise in the serum calcium level of the test animals after the injection of normal serum.

If the serum calcium rise produced in the assay rats is plotted against logarithm of the dose of serum in ml. for animals on the diet 16 and 29 days, three point straight line curves result (Figure 3). Thus, it is possible to show a graded response to the parathormone-like activity in the serum of rats with induced hyperparathyroidism.

Since the rats subjected to the C-D dietary regime exhibited a measurable parathormone-like activity in their blood, serum studies on pregnant and lactating rats maintained on the C-D diet, the second and third choices for inducing hyperparathyroidism, are not included in this work.

A deposition of calcium in the kidney tubules of the experimental rats could be demonstrated from the second day of the dietary regime by means of histological sectioning, special staining with silver nitrate and comparison with decalcified sections. Investigation of urinary pH revealed that a constant alkaline condition (av. pH 7.5) prevailed.

FIGURE 3. RESPONSE OF ASSAY ANIMALS TO GRADED INJECTIONS OF SERUM.



DISCUSSION

The absence of tetany in the test animals fed the calcium-deficient, vitamin D-free diet was due to their ability to maintain serum calcium levels in the 8 to 11 mg.% ranges during the course of this experiment. The maintenance of normal blood calcium levels was at the expense of dissolution of bone salts, which progressed to such an extent in animals on the diet for 15 days that the bony trabeculae in the shaft of the tibia appeared as fragments. The only tetany observed in the test and assay animals was seen in the thyroparathyroidectomized assay rats after they were subjected to light ether anesthesia. The serum calcium levels in these animals were in the 6.0 to 7.5 mg.% range.

Decreased levels of serum inorganic phosphorus are usually encountered in hyperparathyroidism, presumably due to the lowered renal threshold to phosphate. Under the experimental conditions existing here, the high percentage of phosphate in the diet apparently created such a heavy renal load that precipitation of calcium phosphate in the kidney tubules occurred by the second day of the dietary regime. This deposition occurred even though the serum calcium was normal or slightly below normal. It is not surprising then that increased kidney deposition of calcium phosphate can be correlated with an increasing rise in serum inorganic phosphorus levels.

Urinary pH determinations revealed that the diet produced a constant state of alkaline urine, a condition conducive to calcium phosphate precipitation.

Parathormone-like activity in the blood could not be detected in the test animals until after the serum calcium levels fell below 9.0 mg.%. The ability of the parathyroid gland to respond rapidly to the initial decreased calcium level is reflected in the return by the next day of the calcium level to the normal range. However, two days elapsed from the initial calcium level decrease before a parathormone-like activity could be detected in the sera. This activity dropped off insignificantly by the tenth day, but was elevated to a new peak five days later. During the five days between parathormone-like activity determinations, the serum calcium level decreased once again to the 8 to 9 mg.% range and remained there 3 days. This second calcium decrease accounts for the new peak in parathormone-like activity. The serum calcium levels of the test animals rose at a slower rate in response to the second increase in parathormone-like activity, possibly because the pool of available bone calcium was near depletion. As mentioned before, histological sections of the tibia show a marked decrease in bony spicules. One animal fed the diet 29 days exhibited a serum calcium of 7.5 mg.% and a parathormone-like activity of 26.5 units/0.5 ml. of serum. Evidently, even though the amount of

parathormone-like activity continues increasing, a point is reached beyond which the calcium reserve is insufficient to maintain normal blood calcium levels or the effectiveness of parathormone is reduced. The level of parathormone-like activity did not return to the detectable level after the serum calcium level returned to the normal range but remained at 10 to 15 "Parathormone" equivalent units. An extension of the dietary period and daily sera analyses would be advantageous in plotting the exact cyclic activity.

The lowest level of parathormone-like activity detected in a 0.5 ml. dose of serum was 10.5 units in an animal maintained on the diet 10 days. The highest level of parathormone-like activity detected was 43.5 units in a 1.0 ml. serum sample from an animal which had been on the diet 29 days. It was impossible to perform all the chemical tests and all the serum dose level assays on all the animals; it would be of interest to rerun this assay comparing higher dose levels of serum (1.0 ml.) which should give the same curve slope but show increased activity.

This assay method is not sensitive enough to detect normal parathormone activity, and therefore the detectable parathormone-like activity curve lags behind the return of serum calcium levels to the normal range after an increase in parathyroid activity. When an assay is developed which is sensitive enough to detect serum

parathormone in normal concentrations, it should then be possible to correlate the parathormone-like activity curve with a concomitant rise in serum calcium levels.

In order to ascertain whether or not the general blood picture remained stable except for the changes noted in calcium and inorganic phosphorus levels, total serum protein was chosen as the criterion. Total serum protein levels remained relatively constant except for two marked rises, which, in view of the diuretic effect of phosphate, are not unexpected. However, the total serum protein rise encountered between the 12th and 18th days appears to correlate with a sudden serum inorganic phosphorus rise and a serum calcium decrease.

Other hormones, in abnormal concentrations, are known to have secondary effects on the parathyroid glands. For example, in Cushing's disease, abnormal utilization of protein from the bony matrix causes large quantities of calcium and phosphorus to be released with a subsequent depression of the parathyroids. Thyroxin, sex hormones, growth hormone and perhaps insulin could cause a secondary effect on the parathyroids by affecting the concentrations of calcium and phosphorus in the extracellular fluid or by changing bone matrix deposition or absorption. Any action by thyroxin in this assay was eliminated by the removal of the thyroid glands at the time of parathyroidectomy. A certain amount of thyro-active material is supplied by the meat products in the standard

stock diet. Injections of serum from normal animals did not produce any calcium-elevating effects in the assay animals.

Even though the test animals maintained a normal or slightly decreased serum calcium level and exhibited hyperphosphatemia, histological sections of the parathyroid glands presented an appearance of hypertrophy and bone sections showed the bone changes characteristic of increased parathormone activity. The detection of parathormone-like activity in the serum of the test animals is added evidence for increased activity of the parathyroid glands. It remains to be seen whether or not, by blood fractionation, normal and abnormal levels of parathormone-like activity can be concentrated and detected and so indicate the critical levels for diagnosis of hyperparathyroidism.

SUMMARY

Normal rats fed a calcium-deficient, vitamin D-free diet are able to maintain their serum calcium levels in the 9 to 11 mg.% range over a twenty-day period, except for mild decreases to the 8.0 to 9.0 mg.% range on the fifth and twelfth to fifteenth days. The initial decrease in serum calcium level is followed on the eighth day by the first measurable parathormone-like activity in the blood of these animals. The parathormone-like activity was detected by measuring the rise in serum calcium levels in the blood of thyroparathyroidectomized rats following the injection of serum from the hyperparathyroid rats.

A comparison of the rise in serum calcium produced by the injection of unknown serum and the curve derived from injections of graduated doses of standard "Parathormone" makes it possible to estimate the "Parathormone" equivalence of the unknown serum. Injections of graded doses of sera from hyperparathyroid rats yielded graded parathormone-like responses. Injections of serum from normal animals did not produce significant increases in the serum calcium level of thyroparathyroidectomized rats.

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