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The In Vitro Inactivation of Parathyroid Hormone

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THE IN VITRO INACTIVATION OF PARATHYROID HORMONE

by

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LIFE

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CHAPTER I

INTRODUCTION

It is generally held that the primary factor regulating the secretion of the parathyroid hormone is the concentration of ionized calcium in the blood (Talmage and Toft, 1961). A decrease in the serum calcium level results in an increase in the secretory activity of the parathyroid gland, whereas a rise in serum calcium concentration decreases the secretion. Direct evidence for this arose from the experiments of Patt and Luckhardt (1942) who perfused the isolated thyroparathyroid apparatus of a dog with calcium-deficient blood. When the perfusate was given intravenously to a second parathyroidectomized dog, it caused hypercalcemia, hyperphosphaturia and hypophosphatemia. Several years later, Copp and Davidson (1961) reported that perfusion of the isolated thyroparathyroid apparatus of a dog with ethylenediaminetetraacetic acid, a calcium chelating agent, would cause a systemic hypercalcemia. More recently, Dale et al. (1965) transplanted the parathyroid gland into the anterior chamber of the eye of parathyroidectomized rats and demonstrated the presence of a systemic hypercalcemia when the glands were perfused with solutions low in calcium. Recent studies on the direct measurement of hormone concentrations in the blood by radioimmunoassay confirm this viewpoint (Sherwood et al. 1966, Care et al. 1966 and Aurbach and Potts, 1967).

The concentration of this hormone in the circulation has been documented for both animals and humans. Rats which were maintained on a calcium-deficient diet were found to have 20-64 U.S.P. units of parathyroid hormone-like activity per ml. of blood as measured by the Munson (1955) bioassay method (Buckner and Neltor, 1960). No parathyroid hormone activity could be demonstrated in the blood of control animals. Normal human plasma, fractionated with cold 95% ethanol, has been found to contain 40 U.S.P. units of parathyroid hormone-like activity per 100 mls of blood. The physiological activity was found to be present in the β and γ globulin and lipoprotein fractions. No activity could be found in normal human plasma which was not fractionated (Reichert and L'Heureux, 1961). The concentration of parathyroid hormone in normal human plasma has been more recently estimated to be 0.3-1.5 milliunits/ml. by a radioimmunoassay technique (Berson et al. 1963).

The rate of secretion of parathyroid hormone has been measured in several laboratories. Normal calcium levels can be maintained in parathyroidectomized dogs by continuous infusion of 0.1 U.S.P. unit/kg/hr of parathyroid extract (Copp, 1960). Observing that the average duration of effect of a dose of parathyroid extract necessary to obtain normal calcium levels in thyroparathyroidectomized rats was ten hours, Premachandra and Blumenthal (1961) calculated the average parathyroid hormone

secretion rate to be 20 U.S.P. units/kg/hr. The rate of secretion was calculated to be 0.4-1.7 milliunits/kg/minute by Melick et al. (1965). They measured the rate of disappearance of the injected hormone from the extracellular fluid and assumed that the rate of secretion equaled the rate of disappearance. Aurbach (1965) states that the rate of secretion in rats is 0.5-1.0 U.S.P. units/kg/hr.

Whatever process operates for the removal of parathyroid hormone from the circulation, it is rapid (Munson, 1955). After the removal of the parathyroid glands by cautery, serum calcium levels fall from a normal level of 9 mg% to 6-8 mg% in two hours and to 5 mg% in four hours (Munson et al. 1953). Injected purified parathyroid hormone is also rapidly destroyed as noted by the rapid fall, after an initial increase, in serum calcium levels in parathyroidectomized rats which have been injected with purified extract (Rasmussen and Westfall, 1956). Urinary inorganic phosphate excretion in rats is also reduced within the first hour after parathyroidectomy (Talmage and Kraintz, 1954). Beutner and Munson (1960) reported that the rate of inorganic phosphate excretion decreased within 25-52 minutes following parathyroidectomy and continued to do so for three to five hours.

Using iodine-131 labeled parathyroid hormone, the half-life of the hormone has been calculated to be 22.3 minutes in intact animals, and 24.3 minutes in parathyroidectomized animals. Labeled hormone that

was given in excess to parathyroidectomized rats had a half life of 27.6 minutes, indicating that whatever system is operating to remove parathyroid hormone from the blood under normal conditions is not working to full capacity in this situation (Melick et al. (1965).

The question arises, what mechanism is responsible for this rapid elimination of the hormone? There are indications that some of the hormone may be stored in the kidney. Using dog kidneys and an extraction procedure similar to that used to prepare parathyroid extract from parathyroid tissue, a substance with parathyroid hormone-like activity could be extracted from the normal dog kidney with a potency equivalent to 18-40 U.S.P. units. No activity could be extracted from the kidneys of dogs which had been thyroparathyroidectomized 24-48 hours before use (Orimo et al. 1966).

Possibly, the hormone is excreted unchanged in the urine. Parathyroid hormone-like activity has been extracted with benzoic acid from 48 hour specimens of human urine (Davies, 1958). The method of assay used here measured the urinary phosphate excretion in intact rats after the injection of test material. Using a commercially available parathyroid hormone preparation as a standard having 100 "phosphate units of activity", she found that the average activity in urine from five normal subjects was 60.4 "phosphate units". Urine specimens from four hyperparathyroid subjects with uremia had an average of 121.2 "phosphate

units" of activity, and from four subjects with hypoparathyroidism, the urinary activity was too small to be measured, i.e. less than "30 phosphate units".

Twenty-four hour specimens of human urine were assayed for parathyroid hormone activity by Fujita et al. (1961) who measured ^{32}P urinary excretion in parathyroidectomized rats after administration of the test substance. Ten to thirty "units" of parathyroid hormone-like activity were found in the specimens of three out of five normal subjects. No activity was demonstrated in the urine specimens of the other two normal patients. Values above 30 "units" were found in six out of seven untreated patients with hyperthyroidism. These levels declined to normal after treatment. Elevated levels were also found in one case of hyperparathyroidism, three cases of hypertension and one case of nephrolithiasis. In two cases of hypoparathyroidism, no activity could be demonstrated in the urine. Kleeman (1961) stated that he also has been able to extract a substance with parathyroid hormone-like activity from human urine.

In a report by Eliel et al. (1965) twenty-four hour urine specimens of seven out of eight normal patients were found to contain 20-37 U.S.P. units of activity. No activity could be detected in similar specimens from four hypoparathyroid patients. After one patient was given a calcium-deficient diet for six days, no change in the parathyroid hormone

activity of the urine was detected. High concentrations of dietary calcium given to two patients over a four day period resulted in an excretion of 80 U.S.P. units per day.

Assuming that the average secretion rate of parathyroid hormone in the human is approximately the same as in the dog, 0.1 U.S.P. unit/kg/hr., it can be calculated that a seventy kilogram man should secrete about 170 units of parathyroid hormone in a twenty-four hour period. If the activity found in the urine is due to parathyroid hormone, one can account for 30 of the 170 units. The remaining 140 units must be inactivated or excreted by some other mechanism.

Kenny (1957) suggested that phosphate ions play a role in reducing the activity of parathyroid hormone in vitro. When parathyroid extract was incubated with phosphate ions in a concentration of 4 mgP/ml, there was a significant reduction in the activity of the extract as demonstrated by injection into parathyroidectomized rats. Injections of the same quantities of phosphate and extract simultaneously but separately at different subcutaneous sites failed to reduce the calcium-mobilizing activity.

The in vivo experiments of Davis and Talmage (1960) suggested that the liver is the main organ site for inactivation. Rats with parathyroid glands transplanted into the tip of the spleen could not maintain calcium levels during peritoneal lavage as well as intact animals or animals with

glands transplanted into the testes. It was concluded that the hormone was partially inactivated during its passage from the spleen through the liver.

However, in vitro studies done by Orimo et al. (1965a, 1965b) with tissue slices incubated with parathyroid hormone showed that the kidneys possessed the greatest ability to inactivate the hormone as measured by bioassay. Rat plasma also possessed some inactivating power, while other tissues incubated - liver, skeletal muscle and spleen - did not inactivate the hormone. Based on evidence that boiled kidney tissue had less ability to inactivate the hormone, and that two enzyme inhibitors, Trasylol and epsilon-aminocaproic acid, also decreased the inactivation power, it has been proposed that the inactivating system is enzymic in nature. Since the hormone was not absorbed by the kidney slices and since high concentrations of calcium ions which presumably decrease tissue permeability decrease the inactivating ability of the kidney slices, the enzyme system was presumed to be localized within the kidney tissue per se. Kidney slices of dog with proximal tubular damage brought about by subcutaneous injections of sodium tartrate did not inactivate the hormone. Slices of kidneys with distal tubular damage from mercurous chloride treatment retained the ability to inactivate parathyroid hormone. These findings indicated that the proximal tubule was probably the site of inactivation.

The site of inactivation of other protein and peptide hormones is well documented. Schizume and Irie (1957) suggest that the liver is the main site of inactivation of MSH. The thyroid gland itself has been implicated as the organ of inactivation of TSH (Rawson et al. 1942) (Siedlin, 1940); the gonads (Siedlin, 1940) and the liver (Hall, 1950) for gonadotropic hormone. Insulin is inactivated mainly by insulinase in the liver (Mirsky and Broh-Kahn, 1949) (Mirsky and Perisutti, 1953) (Tomizawa, 1962). Both the liver (Geschwind and Li, 1952) and the kidney (Richards and Sayers, 1951) are said to inactivate ACTH. The kidney is also considered to be the site of inactivation for ADH (Thorn and Willumson, 1963) (Smith and Sachs, 1961) (Dicker, 1954) as is the liver (Birnie, 1952) (Eversole et al. 1949) (Heller and Urban, 1935). The liver is also considered the site of inactivation for glucagon (Kakuicki and Tomizawa, 1964).

Statement of the Problem

As has been noted, the exact mechanism for the elimination of parathyroid hormone from the body is uncertain. There have been reports that at least some of the hormone is excreted unchanged in the urine. The spleen and liver have been implicated as inactivators of parathyroid hormone in the body.

Recently, in vitro experiments have indicated that total kidney

slices of the rat are the main inactivators of parathyroid hormone - liver, skeletal muscle and spleen having little ability to do so. There is also evidence that the rapid inactivation by kidney slices is due to an enzyme system located in the proximal tubules.

The work described in this thesis was designed to study further the inactivation of parathyroid hormone. Differences in inactivating ability between kidney cortex and medulla and among microsomal, mitochondrial and supernatant fractions of rat kidney homogenates were looked for in order to localize the inactivating system. The effects of removal of either the thyroparathyroid apparatus, the parathyroid gland, or the thyroid gland on the inactivating process were also investigated.

CHAPTER II

MATERIALS AND METHODS

The procedure that was followed in this study involved three main parameters: 1) preparation of the tissue whose inactivating ability was to be tested, 2) incubation of the tissue with a commercially available and standardized parathyroid hormone preparation, and 3) determination of residual parathyroid hormone activity after tissue incubation.

The experimental procedure was performed over a three day period. On the first day, animals for bioassay were prepared by removing the thyroparathyroid apparatus from rats which had been on a calcium-deficient diet two to three days previously. On the second day, kidney tissue was prepared, either from intact rats or from rats whose thyroid or parathyroid glands, or both, had been surgically removed as required for the specific experiment planned. The tissue was incubated with parathyroid extract and after a desired time interval, aliquots of the aqueous phase of the incubation medium were injected subcutaneously into the bioassay animals from which blood samples had been removed three to four hours before injection. On the third day, a second blood sample was taken from the animals under test and the calcium levels of both initial and final samples were determined.

Surgical Techniques

The procedure of thyroparathyroidectomy was patterned after that

of Farris and Griffith (1949). Surgical thyroparathyroidectomy was done in lieu of parathyroidectomy by surgery or cautery to lessen the chance of release of the hypocalcemic agent, thyrocalcitonin (Hirsch et al. 1963; Talmage et al. 1965; and Tashjian, 1965, 1966).

The animals were anesthetized in two ways. In experiments one through eight, the animals were placed in an ether desiccator and removed sixty seconds after they had lost their 'righting sense'. In later experiments, nine through twenty, the animals were given an intraperitoneal injection of 50 mgs of sodium pentobarbital in aqueous solution, a dose which lightly sedated the animals. In both cases, the anesthetized animals were taped to a board equipped with an apparatus for holding an ether face mask and a magnifying glass. All animals were supplemented with ether throughout the operation. An incision was made from the hyoid bone to the sternum. Underlying salivary glands, lymph nodes and connective tissue were separated and retracted with hemostats, laying bare the trachea and the thyroparathyroid apparatus. With the magnifying glass in place, the thyroparathyroid apparatus was teased off the trachea with tweezers, care being exercised not to injure the recurrent laryngeal nerve. Inevitably, one of the thyroidal blood vessels would be broken. Blood was absorbed with cotton swabs. When bleeding ceased, a secondary exploration of the area was done to insure total removal of thyroidparathyroid tissue. Particular attention was paid

to the area behind the cartilage of the larynx. When satisfied that the desired tissue had been removed and that bleeding had become minimal, underlying structures were brought into approximate position by light massage and the wound was closed with surgical clips. Throughout the operation, a clean, but not sterile technique was used. Total operating time depended upon the degree of bleeding and ranged between seven to fifteen minutes. After the operation, the animals were maintained on a calcium-deficient diet until used.

To determine the effect of removal of either the parathyroid gland or the thyroid gland on the inactivation process of the kidney, euthyroid-aparathyroid and athyroid-euparathyroid animals were prepared by a transplant procedure which was based on the work done by Russell and Gittes (1959) and Brooks (1962).

Rats were anesthetized with the sodium pentobarbital solution, injected intraperitoneally, and were maintained under anesthesia with ether. The same procedure as that used for thyroparathyroidectomy was followed until the thyroparathyroid apparatus was bared. Then an incision was made through the skin on the posterior thigh of the lower left extremity and a 'pocket' made with tweezers either in the fascia covering the hamstring muscles or the muscles themselves. The two pearly white parathyroid glands were then located on the cephalo-ventral surface of the deep red thyroid gland. One gland was firmly held

between the tips of tweezers, and, it was dissected from the thyroid gland with another pair of pointed tweezers. This gland was placed in cold buffer and a second gland located, if possible, and dissected in the same manner. Whatever remained of the thyroparathyroid apparatus was removed, bleeding was stopped, and the incision closed as before. The parathyroid glands were taken out of the cold buffer and placed in the 'pocket' in the hamstring muscles as close as possible to nearby blood vessels. The 'pocket' was closed by manipulation and the wound closed with surgical clips.

Basically, the same procedure was utilized for thyroid transplants, except that after the parathyroid glands were teased from the thyroid, the former were discarded and one lobe of thyroid tissue was transplanted into the 'pocket'. All transplanted animals were then placed in cages and given stock rat chow and water ad libitum.

Even though all parathyroid tissue was probably removed, not all of the tissue was autotransplanted. It did not seem necessary to transplant all of the parathyroid tissue, however, because it has been shown that in the rat, the parathyroid glands operate at only a small fraction (5-25%) of their capacity (Rosof, 1934). Also, the animals with one parathyroid gland removed can still maintain normal serum calcium levels (Tweedy and Chandler, 1929). It was reasoned, therefore, that one gland would be sufficient to keep the animals euparathyroid. After

the operation, the calcium level of the serum was determined at various time intervals. A serum level of 9.5 mg% or above indicated a satisfactory transplantation of the parathyroid glands. A gain in weight in a healthy looking animal was used as the criterion for successful thyroid transplants.

Brooks (1962) maintains that because of wide fluctuations of the serum calcium levels in thyroparathyroidectomized rats, the only way to establish whether or not a transplanted gland is functioning is to remove the graft and be able to demonstrate normal histological structure six to twelve days after the operation while the animal in the meantime would revert back to the parathyroid-deficient state. This was not done in this study.

The criterion for a functioning thyroid transplant, as stated by Brooks (1962) is a normal protein-bound iodine. This also was not determined in these experiments. However, if a difference in the inactivating ability could be found between kidney tissue from a rat with a parathyroid transplant and a rat with a thyroid transplant, these differences could then possibly be explained by a functioning parathyroid gland alone or a functioning thyroid gland alone.

Preparation of Tissue for Incubation

Rats were killed by a stunning blow followed by immediate decapi-

tation. An inital incision was made in the abdomen with a scalpel and the wound enlarged with scissors. Both kidneys were removed by grasping the renal artery, vein and ureter with tweezers and snipping the kidney attachments with scissors. Kidneys were placed in cold buffer. After a sufficient number of kidneys had been collected in the above manner, they were taken to a room maintained at 4 ± 2^{o} C. where they were decapsulated and placed in fresh cold buffer. Kidney slices were prepared with the use of a cold Stadie-Riggs tissue slicer and placed in fresh cold buffer. The first two slices were rejected. From 0.5-1.0 gram of tissue slices were obtained from the kidneys of one animal.

For the preparation of cortical and medullary slices, the kidneys were removed as before and taken to the cold room for decapsulation. Here, the kidneys were bisected, enabling a clear pearly white medullary area to be distinguished from a muddy brown cortical area. The two areas were separated with a scalpel. These separate sections were sliced and the slices placed into cold buffer.

For the preparation of microsomes, mitochondria and supernatant fractions, rat kidneys were removed as above, decapsulated, placed in fresh buffer and minced with a scalpel. The mixture was decanted, and 9 mls of fresh buffer was added to each gram of kidney tissue. The mixture was then homogenized in a Potter-Elvehjem homogenizer. The

10% kidney homogenate was transferred to 50 ml centrifuge tubes and centrifuged at 2,600 r.p.m. in a refrigerated centrifuge (International Equipment Corporation, Model PR-2) for fifteen minutes with a low speed head (No. 823). The fluid portion containing mitochondria, microsomes and supernatant was decanted into 25 ml centrifuge tubes and centrifuged at 4,100 r.p.m. in the refrigerated centrifuge for fifteen minutes with a high speed head (No. 290). The fluid portion, containing microsomes and the supernatant fractions was decanted into centrifuge tubes. The precipitated mitochondria were resuspended in buffer, washed and re-centrifuged as above. The microsomal and supernatant fractions were centrifuged at 40,000 r.p.m. for one hour in the ultracentrifuge (Beckman/Spinco, Model L). The supernatant was decanted. The precipitated fraction containing the microsomes was resuspended in 9 mls of buffer as were the mitochondria. Two mls of the above solutions of supernatant, microsomes or mitochondria, each equivalent to 200 mg of kidney tissue, were used in each incubation flask.

Incubation of Tissue with Parathyroid Extract

A water bath maintained at 37° C. equipped with a shaker and thermostat was used for the incubations (Elmac Engineering Co., Chicago, Illinois). The desired number of Erlenmeyer flasks of 25 ml capacity were placed in the bath and two mls of Robinson's buffer, pH 7.4, was

pipetted into each flask. Two hours later, 80-100 mg of kidney slices, weighed with a Roller-Smith balance were added to each flask and allowed to stand for one hour. Sixty U.S.P. units (0.6 ml) of a parathyroid extract (Injection Parathyroid, Lilly) was injected into each flask with a 1.0 cc tuberculin syringe. After two hours, two mls of fluid were drawn into a 2 cc syringe and injected subcutaneously into an animal for bioassay.

When incubations were done with microsomes, mitochondria or supernatant, no buffer was added to the flasks. Instead, two mls of the preparation, equivalent to 200 mg of kidney tissue, containing either supernatant, microsomes or mitochondria, were incubated for two hours. At the end of this time, parathyroid extract was added and two hours later, two ml aliquots of the fluid were assayed as before. The choice of the length of time of the incubation of parathyroid extract with kidney slices (two hours), the pH of the medium (7.4), and the weight of the kidney tissue used (80-100 mg) were based on the work of Orimo et al. (1965a) who stated these parameters as optimal in his studies on inactivation of the hormone.

Robinson's buffer was used as the medium in the incubations for several reasons. It not only has the composition of extracellular fluid of the rat but also it has been shown that the medium will maintain a constant oxygen consumption of the tissue with a minimum of swelling

and nitrogen loss (Robinson, 1949), factors upon which the viability of the tissue is judged (Aebi, 1952). Of importance is the concentration of calcium ions in the buffer. It has been indicated (Thorn and Willumson, 1963b) that calcium ions in the fluid surrounding the cells of tissue slices are essential to maintain the permeability of the cell membrane. Without the necessary concentration of calcium ions, the hormone would not be able to penetrate the kidney slice. It has also been shown that calcium ions prevent the release of enzymes from the tissue into the medium (Krebs et al. 1963).

To prepare Robinson's Buffer, the following solutions are mixed: 464 ml of 0.154 M NaCl, 16 ml of 0.154 M KCl, 12 ml of 0.110 M $CaCl_2$, and 4 ml of 0.154 M $MgSO_4$. To this mixture are added 50 mls of a phosphate buffer prepared by bringing 150 ml of 0.20 N NaOH to pH 7.4 with 2.0 M H_3PO_4 and diluting to 200 ml with water. This buffer was added immediately before use. The ionic concentrations of Robinson's Buffer are as follows: sodium, 145 meq/L; chloride, 140 meq/L; sulphate, 2 meq/L; potassium, 5 meq/L; calcium, 5 meq/L; magnesium, 2 meq/L; and phosphate, 8 meq/L.

Bioassay of Parathyroid Hormone Activity

A method to evaluate the activity of parathyroid extract was based on bioassay methods of Munson (1955), Davies et al. (1954) and Causton

et al. (1965). This method of evaluation of parathyroid activity depends upon the effect of parathyroid extract in increasing the serum calcium levels of thyroparathyroidectomized male rats of the Sprague-Dawley strain 18-20 hours after the subcutaneous administration of the test material.

Male albino rats of the Sprague-Dawley strain, 140-160 grams in weight, were obtained from Abrams Small Stock Breeders, Chicago, Illinois. After receipt from the supplier, the rats were maintained on a calcium-deficient diet and placed in suspended wire mesh cages in an air-conditioned room maintained at 75 $\pm$ 5° C. Water was given ad libitum. On the following two or three days, the thyroparathyroid apparatus was removed surgically. A blood sample was taken from the tail blood vessels (about one ml) twenty to twenty-four hours after the operation - the pre-injection blood sample - and the material to be tested - incubated parathyroid extract - was injected subcutaneously. Eighteen to twenty hours after the injection, a second tail blood sample was obtained - the post-injection blood sample. The serum calcium content of each sample was determined and the activity of the hormone reported in terms of the increase or decrease in the serum calcium level after the injection of the test material. Injected test material, 60 U.S.P. units of parathyroid extract which had been incubated with various tissue slices and two mls of medium, which could raise the

serum calcium level of thyroparathyroidectomized animals equal to that of control animals receiving 60 U.S.P. units of parathyroid extract incubated with medium only, was considered to be fully potent. Injected material which could not raise the serum calcium level of thyroparathyroidectomized rats was considered to be devoid of any parathyroid hormone-like activity. Test material giving a rise in the serum calcium level between these two values was said to have a potency between 100% and 0%, the potency being approximately proportional to the rise in serum calcium.

Approximately 20% of the animals operated upon would die about eighteen hours post-operatively; usually they exhibited very low serum calcium levels and sometimes a tetanic state. Why some of the animals showed these very low calcium levels was never determined. Accessory parathyroid tissue in the surviving animals was considered. However, Kenny (1962) and Goldsmith et al. (1965), working with thyroparathyroidectomized dogs did not believe that the presence of accessory parathyroid tissue is responsible for differences in observed survival times. They suggested differences in sensitivity to vitamin D, absorption of calcium from the gut, various renal effects or some unknown mechanism as possible etiologies.

No animal was used for bioassay more than twice. When used for the second time, the animals received the test material no earlier than

four days after the administration of the first test sample.

Animals have not been used twice in bioassay procedures used by others, because it has been reported that repeated injections of hormone decrease the sensitivity of the animal to the hormone. Dyer (1932) states "there is some evidence that rats do not respond well to a second injection of hormone". No further information was given pertaining to this observed decrease in sensitivity. Rats injected with 10 U.S.P. units daily in divided doses have been found to become refractory to the extract after ten days (Pugsley, 1932). Since injections would be given twice in this study with a four day interval between injections and less than sixty U.S.P. units of hormone per injection, it was necessary to determine what effect repeated injections had on the test animals when subjected to the conditions of these experiments. To do this, 60 U.S.P. units of parathyroid extract was incubated for two hours with buffer and then injected into previously thyroparathyroidectomized animals which had never received injections of parathyroid extract and also into animals which had received injections of 60 U.S.P. units of parathyroid extract and buffer four days previously. Animals which received only one injection of parathyroid extract exhibited an increase in the serum calcium level of 2.3 mg%; animals which received a second injection of parathyroid extract exhibited an increase of 2.8 mg%. Thus in the experimental method used in this work, the repeated injections had

little effect on the sensitivity of the animal to parathyroid extract.

Two lots of parathyroid extract, both donated by Eli Lilly and Company, through the courtesy of Dr. A. S. Ridolfo, were used in this study. One lot, No. 6057-738758, bore an expiration date of November 1, 1960, and the other lot No. 9GZ15A an expiration date of November 1, 1967. To determine what difference in potency, if any, there would be between these two lots, 60 U.S.P. units of "out-date" parathyroid extract and of "fresh" parathyroid extract were each incubated with two mls of buffer for two hours and injected into bioassay animals. The "out-dated" hormone raised the calcium level of nine assay animals an average of 2.6 ± 0.8 mg% as compared to an average of 3.1 ± 1.3 mg% for 23 assay animals injected with fresh extract. Since the difference between these two is not significant ($p \approx 0.7$), both lots of extract have about equal potency and the average value of the thirty-two assays, 2.9 ± 0.3 mg%, was used as the basis for comparison in further experiments. Thyroparathyroidectomized rats injected with 60 U.S.P. units of untreated parathyroid extract should increase the serum calcium level to about 2.9 mg% under the conditions of the assay. The inability of injected material to raise the serum calcium level to this value was taken to mean a loss of activity; the degree of loss of activity being approximately proportional to the relative increase in the serum calcium level.

Determination of Serum Calcium

To determine the concentration of ionizable calcium in the serum, the method of Ashby and Roberts (1957) was used. The basis for this determination rests on a specific property of calcein, an iminoacetate derivative of fluorescein. At pH above 12, this compound will fluoresce under long-wave ultraviolet light only in the presence of free calcium. The procedure consists of adding a known amount of EDTA in excess of that necessary to complex all the calcium present in the sample. Cyanide is added to complex Cu^{++} or Fe^{++} ions present. Magnesium ions are replaced by calcium ions in the course of the titration. The solution is back titrated to the appearance of fluorescence, indicating that all the EDTA has become complexed with calcium and that there is some additional calcium to combine with the calcein. Water blanks (calcium free solutions) and a standard calcium solution are titrated along with the samples and the concentration of calcium in the serum is calculated from the following equation:

$$\text{mg\% calcium} = \text{mg\% standard} \times \frac{\text{microliters for unknown}}{\text{microliters for standard}}$$

where: microliters unknown = microliters of titrant required to titrate blank minus microliters of titrant required to titrate unknown sample

microliters standard = microliters of titrant required to titrate blank minus microliters of titrant required to titrate standard

Blood samples, about one ml, are taken from the tail vessels of the rat directly into a premarked micro test tube. The sample is placed in a refrigerator and allowed to clot while subsequent samples are taken. The sample is centrifuged for five minutes in a Beckman/Spinco Microfuge Model 152. Using Beckman micropipettes, 30 µl of serum and 150 µl of 0.002 M EDTA are pipetted into each of three Beckman micro-titration cups. One drop (circa, 60 µl) of calcein indicator, one drop (circa, 30 µl) of 1% NaCN solution and one drop (circa, 60 µl) of 1.0 N NaOH are added to each cup. The sample is then titrated with a known standard calcium solution (20.0 mg%) under an ultraviolet lamp with a Beckman/Spinco Microtitrator Model 153. The end point is reached when the green fluorescence no longer increases with the addition of 0.3 - 0.5 µl of standard calcium solution.

Preparation of Solutions

Sodium Cyanide Solution (1%)

Dissolve 1 gm NaCN in doubly-distilled water and dilute to 100 mls.

Calcein Solution (concentrated)

Dissolve 0.25 g powdered indicator in 4.0 ml of 1.0 N NaOH and dilute to 100 mls with doubly-distilled water.

Calcein Solution (diluted)

Dilute 0.5 ml concentrated calcein solution to 25 ml with doubly-distilled water. This is the solution used in the titration.

Standard Calcium Solution

Oven dry 0.25 g $CaCO_3$ at 100° C., dissolve in 30 ml of 2 N HCl and dilute to 500.0 ml with doubly-distilled water.

Standard EDTA

Add 0.375 g EDTA to 1000.0 ml of CO_2 free water.

Sodium Hydroxide Solution (1.0 N)

Dissolve 4 g NaOH pellets in doubly-distilled water and dilute to 100 mls.

In order to substantiate the use of the above equation, a calcium standard curve was prepared. Eight solutions of known concentrations were prepared by diluting a known stock solution of calcium. These solutions were titrated as outlined above.

Two series of titrations were done, the results compared and averaged. The standard titration curve data are given in Table I. Since the number of milligrams of calcium titrated is directly proportion-

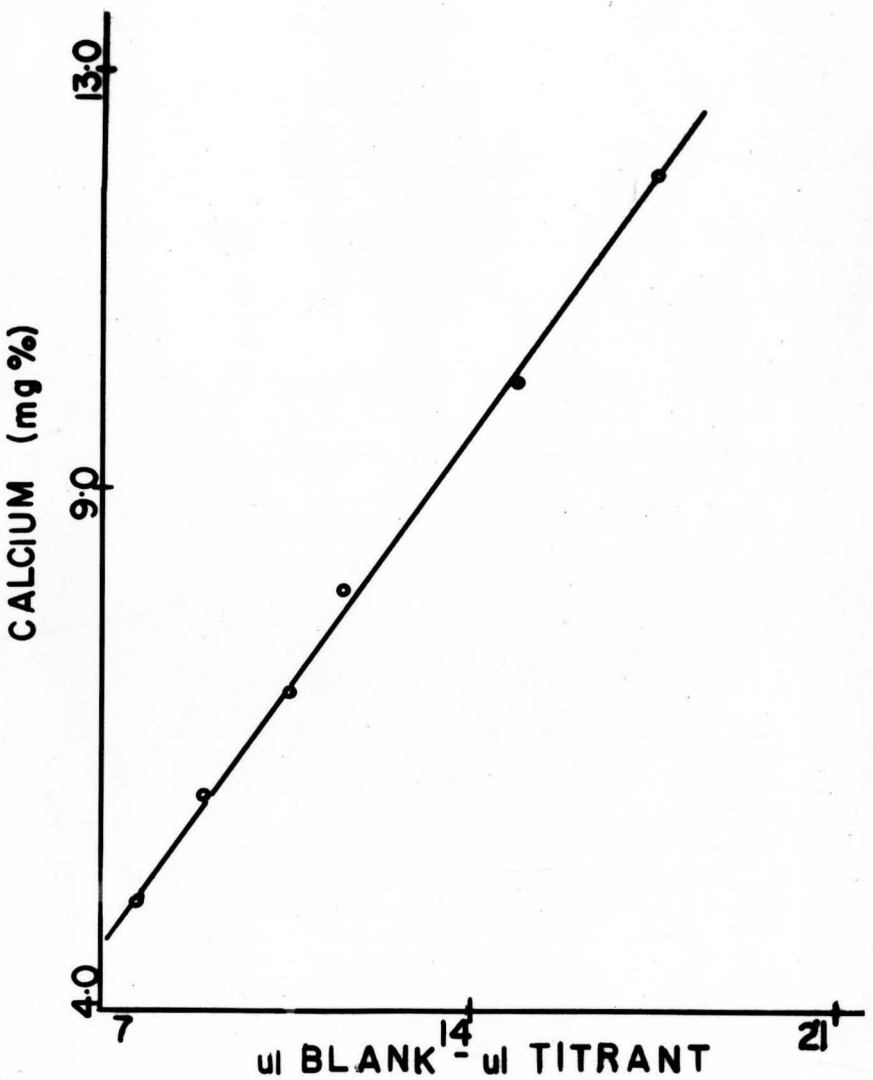
TABLE I

STANDARD TITRATION CURVE DATA FOR CALCIUM DETERMINATION

Calcium (mg%)	Number of Samples	Microliters for unknown
5.1	6	7.7
6.1	6	8.9
7.1	6	10.5
8.2	6	11.5
10.2	7	14.7
12.2	7	17.2
14.3	7	20.8
16.3	6	23.9

al to the number of microliters added, the linearity of the above equation was established. This is shown in Figure 1.

To assure that an acceptable technique for determining serum calcium levels had been accomplished, a series of recovery experiments were performed. One rat was exsanguinated, the blood allowed to clot and then centrifuged. Serum was added to eighteen different micro-beakers in sets of three, making six sets. In four of the six sets, 10 μl of a solution of known concentration of calcium was added. The fifth set received an added 10 μl of the same serum. The sixth set received 10 μl of doubly distilled water. Results of the titrations are shown in Table II. The recovery was 100 $\pm$ 10% and taken as being satisfactory.



STANDARD CURVE FOR CALCIUM DETERMINATION

FIGURE 1

TABLE II

RECOVERY OF ADDED CALCIUM TO SERUM SAMPLES

Solution Added to 10 µl Serum	Calcium Added as $CaCO_3$ (mg x 10^7)	Total Calcium Present (mg x 10^7)	Total Calcium Observed (mg x 10^7)	Percent Recovery
none	none	9.0	---	---
10 µl of 6.1 mg% $CaCO_3$	6.1	15.1	16.0	106%
10 µl of 8.2 mg% $CaCO_3$	8.2	17.2	18.1	110%
10 µl of 12.2 mg% $CaCO_3$	12.2	21.2	21.5	101%
10 µl of 16.3 mg% $CaCO_3$	16.3	25.3	23.8	93%
10 µl of serum	9.0	18.0	16.6	92%

CHAPTER III

EXPERIMENTAL RESULTS

Experiment 1: The Incubation of Parathyroid Extract with Kidney Slices from an Intact Rat.

Orimo et al. (1965a) reported that most of the calcium-mobilizing ability of parathyroid extract was lost after incubation with kidney slices from an intact rat. To confirm these results, kidney slices from one rat were incubated with 60 U.S.P. units of parathyroid extract and two ml of buffer for two hours in each of six different flasks. As shown in Table III, the average serum calcium increase in the bioassay animals was 1.1 ± 0.8 mg% which is significantly less than that of the control animals receiving 60 U.S.P. units of parathyroid extract, 2.9 ± 0.3 mg% ($P < 0.001$), indicating a loss of parathyroid hormone-like activity. This finding correlates with that of Orimo et al. (1965a) in implicating the kidney as a site of inactivation of parathyroid extract.

Experiment 2: The Incubation of Parathyroid Extract with the Medium that Had Been Incubated Previously with Kidney Slices -- Effect of Soluble Factor(s).

To determine whether the system for inactivation is present within the kidney slices per se or comprises a soluble factor or factors which may be released into the medium, the kidney from one rat was incubated

TABLE III

EFFECT OF TOTAL KIDNEY SLICES FROM AN INTACT RAT ON THE INACTIVATION OF PARATHYROID EXTRACT

Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
8.6	8.5	-0.1
6.5	7.6	+1.1
7.1	9.7	+2.6
8.7	9.1	+0.4
8.6	10.4	+1.8
8.3	8.9	+0.6
Mean $\pm$ Standard Deviation*		1.1 $\pm$ 0.8

*The standard deviation was determined by the use of the equation:

$$S.D. = \pm \sqrt{\frac{\leq (x - \bar{x})^2}{n - 1}}$$

with the medium for two hours. The medium, which then would contain soluble factor(s) if present, was then transferred to clean Erlenmeyer flasks. The kidney slices were discarded. Parathyroid extract was added to the medium and incubated for two hours. Two mls of the fluid portion were then injected into three thyroparathyroidectomized rats.

The results in Table IV show an average increase in the serum calcium of bioassay animals of 1.8 ± 1.6 mg%. Compared with the control value of 2.9 ± 0.3 mg%, the difference is insignificant ($P \approx 0.15$), indicating that the inactivating system is present in the kidney slices and for the most part is not released into the media during the incubation.

Experiments 3 and 4: The Incubation of Parathyroid Extract with Cortical and Medullary Kidney Slices from an Intact Rat.

In an attempt to further localize the site of inactivation of the antidiuretic principle by kidney slices, Thorn and Willumson (1963), incubated four separate zones of kidney with ADH and found that the "papillary" zone - the medullary part of the kidney - possessed the greatest power of inactivation.

In this laboratory, four zones could not be established in kidney slices, but two zones could be clearly seen, the cortical zone and the medullary zone. Histologically, the cortical zone contains the greater number of glomeruli, distal and proximal tubules, and the papillary zone,

TABLE IV

EFFECT OF SOLUBLE KIDNEY FACTOR (S) ON THE INACTIVATION OF PARATHYROID EXTRACT

Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
6.9	7.5	+0.6
8.7	9.8	+1.1
4.9	8.7	+3.6
Mean $\pm$ Standard Deviation		1.8 $\pm$ 1.6

the greatest concentration of collecting ducts.

The kidneys from two animals, A and B, were used and the cortex separated from the medulla as best as could be established with the naked eye. Slices of each were incubated separately for two hours in two mls of buffer with 60 U.S.P. units of parathyroid extract and the fluid portions then injected into nine previously thyroparathyroidectomized rats. The average increase in the serum calcium level of animals that had been injected with parathyroid extract that had been incubated with cortical kidney slices was 1.6 ± 1.2 mg% whereas those injected with parathyroid extract incubated with medullary slices showed an increase of 0.8 - 1.2 mg% (Tables V and VI). This difference is not significant ($P \approx 0.3$). On the basis of these data, one cannot designate either the cortex or the medulla as the more important site of inactivation for parathyroid extract. These data do, however, substantiate the data from Experiment 1. Kidney slices do inactivate the extract since both of the average increases in the serum calcium levels observed in Experiments 3 and 4 were significantly lower than that of the control animals ($P < 0.01$ and $P < 0.001$ respectively).

TABLE V

EFFECT OF KIDNEY CORTEX FROM AN INTACT RAT ON THE IN-ACTIVATION OF PARATHYROID EXTRACT

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	8.4	8.1	-0.3
Kidney A	5.6	9.0	+3.4
Kidney A	8.3	8.3	+0.0
Kidney B	7.2	9.4	+2.2
Kidney B	7.0	9.5	+2.5
	Mean $\pm$ Standard Deviation		+1.6 $\pm$ 1.2

TABLE VI

EFFECT OF KIDNEY MEDULLA FROM AN INTACT RAT ON THE IN-ACTIVATION OF PARATHYROID EXTRACT

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	7.3	9.3	+2.0
Kidney A	5.6	8.5	+2.9
Kidney B	8.4	8.7	+0.3
Kidney B	8.4	6.5	-1.9
	Mean $\pm$ Standard Deviation		+0.8 $\pm$ 1.2

Experiments 5 and 6: The Incubation of Parathyroid Extract with Supernatant, Microsomes and Mitochondria from the Kidney of an Intact Rat.

Working with the differential centrifugation products of tissue homogenates, Dicker (1956) has found that the supernatant fraction of the kidney is the most potent in the inactivation of ADH. Geschwind and Li (1952) have determined that the supernatant from differential centrifugation of kidney tissue is the most potent in the inactivation of ACTH.

Following procedures of differential centrifugation similar to those employed by these investigators, two mls of either supernatant, mitochondria or microsomes prepared from kidney homogenates were incubated with 60 U.S.P. units of parathyroid extract for two hours and the test material was injected into thyroparathyroidectomized rats.

The results of the bioassay of two series of experiments are given in Tables VII, VIII and IX. The letters A and B refer to results from Experiments 5 and 6 respectively.

The average increase in serum calcium levels shown by thyroparathyroidectomized rats receiving parathyroid extract which had been incubated with supernatant is 0.8 ± 0.4 mg%, with microsomes 2.0 ± 1.9 mg%, and with mitochondria is -0.5 ± 1.3 mg%. This indicates that the microsomes have little or no ability to inactivate the extract ($P \lessapprox 0.2$) and that both the supernatant and mitochondria do inactivate the extract ($P < 0.001$). It appears that the mitochondrial fraction of kidney tissue

TABLE VII

EFFECT OF SUPERNATANT FROM KIDNEY HOMOGENATES ON THE INACTIVATION OF PARATHYROID EXTRACT

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	8.1	8.3	+0.2
Kidney A	6.4	7.5	+1.1
Kidney A	6.7	7.2	+0.5
Kidney B	7.9	8.5	+0.6
Kidney B	7.0	8.4	+1.4
Kidney B	8.9	9.8	+0.9
	Mean $\pm$ Standard Deviation		0.3 $\pm$ 0.4

TABLE VIII

EFFECT OF MICROSOMES PREPARED FROM KIDNEY HOMOGENATES ON THE INACTIVATION OF PARATHYROID EXTRACT

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	7.8	6.9	-0.9
Kidney A	7.1	5.8	-1.3
Kidney A	5.9	7.6	+1.7
Kidney A	7.9	8.2	+0.3
Kidney B	7.7	9.2	+1.5
Kidney B	7.0	9.9	+2.9
Kidney B	7.6	13.2	+5.6
Kidney B	5.5	9.3	+3.8
Kidney B	7.8	12.1	+4.3
Kidney B	7.5	9.6	+2.1
Mean $\pm$ Standard Deviation			2.0 $\pm$ 1.9

TABLE IX

EFFECT OF MITOCHONDRIA PREPARED FROM KIDNEY HOMOGENATES ON THE INACTIVATION OF PARATHYROID EXTRACT

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	7.9	5.4	- 2.5
Kidney A	6.0	5.8	- 0.2
Kidney A	6.9	7.6	+ 0.7
Kidney A	8.0	4.8	- 3.2
Kidney B	6.2	5.4	- 0.8
Kidney B	5.9	7.7	+ 1.8
Kidney B	8.5	8.1	- 0.4
Kidney B	7.1	7.6	+ 0.5
Kidney B	8.7	8.4	- 0.3
Kidney B	8.5	7.8	- 0.7
Mean $\pm$ Standard Deviation			- 0.5 $\pm$ 1.3

possesses considerable ability to inactivate the extract ($P < 0.01$).

Experiment 7: The Incubation of Parathyroid Extract with Kidney Slices from a Thyroparathyroidectomized Rat.

The effect of the removal of the thyroparathyroid apparatus on the inactivation system in the kidney was then sought. A rat which had been thyroparathyroidectomized about two weeks previously was used and its kidney tissue was incubated for two hours with 60 U.S.P. units of parathyroid extract in two mls of buffer. The fluid portion was then injected into four thyroparathyroidectomized rats. The results of the bioassay are shown in Table X. The average increase in the serum calcium, 2.3 ± 0.6 mg%, is not statistically different from that of animals receiving 60 units of untreated parathyroid extract ($P \approx 0.5$). This indicates that the hormone has not been inactivated by the kidney from a thyroparathyroidectomized rat.

Experiments 8 and 9: The Incubation of Parathyroid Extract with Cortical Kidney Slices and Medullary Kidney Slices from a Thyroparathyroidectomized Rat.

To further substantiate the indications from Experiment 7 and again to attempt to find a difference in the inactivation ability between kidney cortex and medulla, two experiments were performed similar to Experi-

TABLE X

EFFECT OF KIDNEY SLICES FROM A THYROPARATHYROIDECTOMIZED RAT ON THE INACTIVATION OF PARATHYROID EXTRACT

Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
5.5	7.1	+1.6
6.1	8.2	+2.1
6.3	8.8	+2.5
6.3	9.3	+3.0
Mean ± Standard Deviation		2.3 ± 0.6

ments 3 and 4 except that the cortical and medullary slices were obtained from the kidney of a rat that had been thyroparathyroidectomized about two weeks previously. The results are shown in Tables XI and XII. Both the rise in serum calcium from animals which had been injected with parathyroid extract incubated with cortical kidney slices, 2.5 $\pm$ 1.9 mg%, and that from parathyroid extract incubated with medullary slices, 2.6 $\pm$ 0.2 mg%, were not significantly different from the rise in serum calcium of control animals indicating that the extract had not been inactivated. This data further substantiates the previous observation that kidney slices from a thyroparathyroidectomized rat can no longer inactivate parathyroid extract. There is no difference between the inactivating ability of the kidney cortex and kidney medulla.

Experiment 10: The Incubation of Parathyroid Extract with Kidney Slices from a Thyroparathyroidectomized Rat with Autografted Thyroid Tissue.

As indicated by Experiments 7, 8 and 9, kidneys from thyroparathyroidectomized animals could not inactivate the hormone. It was thought, therefore, that either the parathyroid gland, the thyroid gland or both the parathyroid and thyroid glands must necessarily be present in an animal to maintain the parathyroid hormone inactivating system of the kidney. To test this hypothesis, the thyroparathyroid apparatus was removed from three animals, A, B and C and half of the thyroid tissue

TABLE XI

EFFECT OF KIDNEY CORTEX FROM A THYROPARATHYROIDECTOMIZED RAT ON THE INACTIVATION OF PARATHYROID EXTRACT

Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
8.0	10.2	+2.2
5.1	10.4	+5.3
7.5	9.5	+2.0
7.6	8.3	+0.7
Mean ± Standard Deviation		2.5 ± 1.9

TABLE XII

EFFECT OF KIDNEY MEDULLA FROM A THYROPARATHYROIDECTOMIZED RAT ON THE INACTIVATION OF PARATHYROID EXTRACT

Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
6.7	8.5	+1.8
7.2	10.2	+3.0
4.4	7.6	+3.2
5.9	8.4	+2.5
Mean ± Standard Deviation		2.6 ± 0.2

was transplanted into the hamstring muscles. These animals were considered to be euthyroid but aparathyroid. Fifteen days after the autotransplant, the kidneys from these animals were removed and incubated with 60 U.S.P. units of parathyroid extract in two mls of buffer for two hours and the media injected into ten thyroparathyroidectomized rats. Results of the bioassay are shown in Table XIII. The increase in the serum calcium level is 2.9 ± 1.4 mg% which is not significantly different from that of the control level of 2.9 ± 0.3 mg% ($P \approx 0.5$). Assuming that the autotransplanted thyroid gland was functioning, these results indicate that the kidneys from an euthyroid-aparathyroid animal can no longer inactivate the hormone. It seems that the thyroid is not responsible for the functioning of the kidney inactivation process.

Experiment 11: The Incubation of Parathyroid Extract with Kidney Slices from a Thyroparathyroidectomized Rat with Autografted Parathyroid Tissue.

Since the thyroid did not appear to be responsible for maintaining the inactivation system in the kidney, the effect of a functioning parathyroid gland in the absence of the thyroid was sought. Three thyroparathyroidectomized animals, A, B, and C with autografted parathyroid tissue which exhibited serum calcium levels of about 9.5 mg% fifteen days after the operation were killed and their kidneys incubated with 60

TABLE XIII

EFFECT OF KIDNEY SLICES FROM A EUTHYROID-APARATHYROID RAT ON THE INACTIVATION OF PARATHYROID EXTRACT

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	3.9	5.9	+ 2.0
Kidney A	4.7	6.3	+ 1.6
Kidney A	3.9	7.5	+ 3.6
Kidney B	3.8	6.7	+ 2.9
Kidney B	4.8	8.2	+ 3.4
Kidney B	4.5	7.3	+ 2.8
Kidney C	4.6	7.9	+ 3.3
Kidney C	2.3	7.8	+ 5.5
Kidney C	4.7	8.1	+ 3.4
Kidney C	5.7	6.0	+ 0.3
Mean $\pm$ Standard Deviation			2.9 $\pm$ 1.4

units of parathyroid extract in two mls of media for two hours and the media injected into nine thyroparathyroidectomized rats. Results of the bioassay are shown in Table XIV. The increase in serum calcium, 0.4 $\pm$ 1.0 mg%, is significantly lower than that of animals receiving 60 units of parathyroid extract ($P < 0.002$) indicating that the parathyroid extract had been inactivated when incubated with the kidney slices. Assuming that the autotransplanted parathyroid glands were functioning, it may be surmized that the secretion of the parathyroid glands is necessary to maintain the inactivation process of the kidney.

Experiment 12: The Incubation of Parathyroid Extract with Kidney Slices from a Rat Which Had Been Thyroparathyroidectomized Two Days Previously.

Previous experiments have indicated that kidneys taken from animals which had been thyroparathyroidectomized showed a difference in the ability to inactivate parathyroid extract compared to kidneys taken from intact animals. To more clearly define this difference by determining how the length of time between thyroparathyroidectomy and the removal of the kidney for incubation studies affected the inactivating system, Experiments 12 through 20 were initiated. The essential procedure was to take kidneys from animals which had been thyroparathyroidectomized a designated number of days previously and incubate them with para-

TABLE XIV

EFFECT OF KIDNEY SLICES FROM A EUPARATHYROID-ATHYROID RAT ON THE INACTIVATION OF PARATHYROID EXTRACT

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	8.5	10.0	+ 1.5
Kidney A	7.1	8.3	+ 1.2
Kidney B	8.6	10.5	+ 1.9
Kidney B	6.9	6.3	- 0.6
Kidney B	8.5	9.1	+ 0.6
Kidney C	7.6	8.9	+ 1.3
Kidney C	8.3	7.9	- 0.6
Kidney C	8.1	8.5	+ 0.4
Kidney C	7.9	8.1	+ 0.2
Mean $\pm$ Standard Deviation			0.4 $\pm$ 1.0

thyroid extract. The test material was then assayed as before.

For Experiment 12, kidneys were taken from three animals (A, B and C in Table XV) which had been thyroparathyroidectomized two days previously and incubated with 60 U.S.P. units of parathyroid extract and 2 mls of medium for two hours. When the fluid portion was injected into nine bioassay animals the average change in the serum calcium level was -2.5 ± 1.2 mg%, indicating a definite inactivation of parathyroid extract ($P < 0.01$). This degree of inactivation is also greater than that obtained with kidney tissue taken from an intact animal, 1.0 ± 0.9 ($P < 0.01$).

Experiment 13: The Incubation of Parathyroid Extract with Kidney Slices from a Rat Which Had Been Thyroparathyroidectomized Four Days Previously.

One animal which had been thyroparathyroidectomized four days previously was killed, its kidneys removed and sliced. The slices were incubated for two hours with 2 ml of medium and 60 U.S.P. units of parathyroid extract. Two mls of supernatant were then injected into nine bioassay animals.

The average rise in the serum calcium level in this bioassay was found to be -1.8 ± 1.0 (Table XVI); Again a definite inactivation of the extract was noted when these data were compared to that of the control

TABLE XV

BIOLOGICAL ASSAY OF PARATHYROID EXTRACT INCUBATED WITH KIDNEY SLICES FROM A RAT THYROPARATHYROIDECTOMIZED TWO DAYS PREVIOUSLY

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	5.4	5.3	- 0.1
Kidney A	7.5	4.9	- 2.6
Kidney A	7.9	3.8	- 4.1
Kidney B	7.7	5.5	- 2.2
Kidney B	7.1	4.6	- 2.5
Kidney B	6.8	4.8	- 2.0
Kidney B	8.9	6.2	- 2.7
Kidney C	8.3	6.7	- 1.6
Kidney C	8.2	3.9	- 4.3
	Mean $\pm$ Standard Deviation		- 2.5 $\pm$ 1.2

TABLE XVI

BIOLOGICAL ASSAY OF PARATHYROID EXTRACT INCUBATED WITH KIDNEY SLICES FROM A RAT THYROPARATHYROIDECTOMIZED FOUR DAYS PREVIOUSLY

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	5.2	3.6	- 1.6
Kidney A	5.3	4.7	- 0.6
Kidney A	8.1	5.7	- 2.4
Kidney A	8.2	4.5	- 3.7
Kidney A	4.9	4.1	- 0.8
Mean $\pm$ Standard Deviation			- 1.8 $\pm$ 1.0

level of 2.9 ± 0.9 ($P < 0.01$).

Experiment 14: The Incubation of Parathyroid Extract with Kidney Slices from a Rat Which Had Been Thyroparathyroidectomized Five Days Previously.

Continuing in the progression, two animals which had survived thyroparathyroidectomy for five days were killed and slices of their kidneys were incubated with parathyroid extract and 2 mls of medium for two hours.

When two mls of the fluid portion of the incubation mixture were injected into nine bioassay animals, the average change in the serum calcium level was -0.9 ± 2.8 (Table XVII), a significant inactivation of parathyroid extract ($P < 0.01$).

Experiment 15: The Incubation of Parathyroid Extract with Kidney Slices from a Rat Which Had Been Thyroparathyroidectomized Seven Days Previously.

Kidneys were taken from two animals seven days post-operatively and incubated for two hours with two mls of medium and 0.6 cc (60 U.S.P. units) of parathyroid extract. Two mls of the supernatant were injected into each of eight bioassay animals.

The change in the serum calcium level was found to be 0.3 ± 1.9

TABLE XVII

BIOLOGICAL ASSAY OF PARATHYROID EXTRACT INCUBATED WITH KIDNEY SLICES FROM A RAT THYROPARATHYROIDECTOMIZED FIVE DAYS PREVIOUSLY

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	8.7	5.6	- 3.1
Kidney A	6.0	3.8	- 2.2
Kidney A	7.7	8.5	+ 0.8
Kidney A	6.4	5.7	- 0.7
Kidney A	3.2	5.3	+ 2.1
Kidney A	7.8	3.6	- 4.2
Kidney A	7.5	4.1	- 3.4
Kidney B	5.0	4.1	- 0.9
Kidney B	7.2	3.6	- 3.6
	Mean $\pm$ Standard Deviation		- 1.6 $\pm$ 2.1

mg% (Table XVIII), again showing a loss of parathyroid activity of the incubated extract ($P < 0.01$). This figure also begins to establish a trend shown in the three preceding experiments of a sharp increase in the inactivating activity of the kidney slices from a rat thyroparathyroidectomized two days previously with a gradual return of activity to the level of intact animals.

Experiment 16: The Incubation of Parathyroid Extract with Kidney Slices from a Rat Which Had Been Thyroparathyroidectomized Eight Days Previously.

Two mls of medium, 60 U.S.P. units of parathyroid extract and kidney slices from three animals which had been thyroparathyroidectomized eight days previously were incubated for two hours. When the fluid portion was injected into eleven bioassay animals, the average change in the serum calcium level was 0.5 ± 1.0 mg% (Table XIX).

This indicates that the kidney tissue from an animal which had been thyroparathyroidectomized for eight days can inactivate parathyroid extract, and to a degree greater than can kidney tissue from an intact animal.

TABLE XVIII

BIOLOGICAL ASSAY OF PARATHYROID EXTRACT INCUBATED WITH KIDNEY SLICES FROM A RAT THYROPARATHYROIDECTOMIZED SEVEN DAYS PREVIOUSLY

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	6.8	7.8	+ 1.0
Kidney A	6.7	9.3	+ 2.8
Kidney A	8.9	8.7	- 0.2
Kidney A	8.9	11.0	+ 2.1
Kidney B	8.8	6.7	- 2.1
Kidney B	8.4	7.7	- 0.7
Kidney B	8.5	6.3	- 2.2
Kidney B	5.7	7.4	+ 1.7
Mean $\pm$ Standard Deviation			+ 0.3 $\pm$ 1.9

TABLE XIX

BIOLOGICAL ASSAY OF PARATHYROID EXTRACT INCUBATED WITH KIDNEY SLICES FROM A RAT THYROPARATHYROIDECTOMIZED EIGHT DAYS PREVIOUSLY.

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	7.3	8.1	+ 0.8
Kidney A	6.9	8.7	+ 1.8
Kidney A	5.0	4.9	- 0.1
Kidney A	5.9	5.9	0.0
Kidney B	5.6	6.2	+ 0.6
Kidney B	7.1	7.2	+ 0.1
Kidney B	6.6	7.5	+ 0.9
Kidney B	7.1	9.3	+ 2.2
Kidney C	7.8	6.7	- 1.1
Kidney C	7.1	6.3	- 0.8
Kidney C	5.4	6.6	+ 1.2
Mean $\pm$ Standard Deviation			+ 0.5 $\pm$ 1.0

Experiment 17: The Incubation of Parathyroid Extract with Kidney Slices from a Rat Which Had Been Thyroparathyroidectomized Eleven Days Previously.

Kidneys were taken from four animals, A, B, C, and D, which had been thyroparathyroidectomized for eleven days. Slices were made and incubated with 60 U.S.P. units of parathyroid extract and two mls of medium for two hours. Two mls of the supernatant were injected into each of 15 bioassay animals.

The average increase in the serum calcium level was $+1.4 \pm 1.4$ mg% (Table XX), which is significantly different from that of control animals (2.9 ± 0.3) ($P < 0.01$), indicating that kidney tissue taken from an animal which had been thyroparathyroidectomized for eleven days could still inactivate the extract, but not to the extent that kidney tissue from animals which had been thyroparathyroidectomized two days previously could do so.

Experiment 18: The Incubation of Parathyroid Extract with Kidney Slices from a Rat Which Had Been Thyroparathyroidectomized Fifteen Days Previously.

Kidney slices prepared from two animals which had been thyroparathyroidectomized fifteen days previously were incubated with 60 U.S.P. units of parathyroid extract and two mls of medium for two hours

TABLE XX

BIOLOGICAL ASSAY OF PARATHYROID EXTRACT INCUBATED WITH KIDNEY SLICES FROM A RAT THYROPARATHYROIDECTOMIZED ELEVEN DAYS PREVIOUSLY

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	7.1	7.7	+ 0.6
Kidney A	5.9	6.3	+ 0.4
Kidney A	7.0	9.8	+ 2.8
Kidney A	6.3	7.9	+ 1.6
Kidney B	7.1	7.5	+ 0.4
Kidney B	7.1	8.0	+ 0.9
Kidney B	6.4	7.4	+ 1.0
Kidney B	8.0	7.7	- 0.3
Kidney C	8.4	8.5	+ 0.1
Kidney C	4.3	9.5	+ 5.2
Kidney C	8.3	8.7	+ 0.4
Kidney C	7.4	8.3	+ 0.9
Kidney D	5.4	7.3	+ 1.9
Kidney D	5.3	8.4	+ 3.1
Kidney D	8.9	10.7	+ 1.8
Mean $\pm$ Standard Deviation			+ 1.4 $\pm$ 1.4

and the supernatant injected into nine bioassay animals.

The average rise in the serum calcium level of 0.6 ± 1.4 mg% observed here (Table XXI) indicates that the extract has been inactivated when compared with that of control injections ($P < 0.01$).

Experiment 19: The Incubation of Parathyroid Extract with Kidney Slices from a Rat Which Had Been Thyroparathyroidectomized Eighteen Days Previously.

Two animals, which had been thyroparathyroidectomized eighteen days previously, were killed and their kidney tissue incubated with two mls of medium and 60 U.S.P. units of parathyroid extract for two hours. Two mls of supernatant were injected into each of seven bioassay animals.

The average rise in the serum calcium level, 1.0 ± 0.4 mg%, (Table XXII) indicates that the extract had been inactivated by this kidney tissue and that the effect observed was comparable to that obtained with kidney tissue from intact animals.

Experiment 20: The Incubation of Parathyroid Extract with Kidney Slices from a Rat Which Had Been Thyroparathyroidectomized Twenty-Seven Days Previously.

Kidneys were taken from two animals which had been thyropara-

TABLE XXI

BIOLOGICAL ASSAY OF PARATHYROID EXTRACT INCUBATED WITH KIDNEY SLICES FROM A RAT THYROPARATHYROIDECTOMIZED FIFTEEN DAYS PREVIOUSLY

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	7.8	8.5	+ 0.7
Kidney A	6.8	5.9	- 0.9
Kidney A	5.9	5.5	- 0.4
Kidney A	6.7	7.6	+ 0.9
Kidney B	8.3	8.9	+ 0.6
Kidney B	5.2	4.4	- 0.8
Kidney B	6.1	7.3	+ 1.2
Kidney B	6.0	8.4	+ 2.4
Kidney B	6.7	8.2	+ 1.5
Mean $\pm$ Standard Deviation			+ 0.6 $\pm$ 1.4

TABLE XXII

BIOLOGICAL ASSAY OF PARATHYROID EXTRACT INCUBATED WITH KIDNEY SLICES FROM A RAT THYROPARATHYROIDECTOMIZED EIGHTEEN DAYS PREVIOUSLY

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	4.7	6.0	+ 1.3
Kidney A	5.4	6.5	+ 1.1
Kidney A	6.8	6.8	0.0
Kidney A	6.1	7.2	+ 1.1
Kidney B	8.3	10.4	+ 2.1
Kidney B	5.3	6.6	+ 1.3
Kidney B	6.7	6.7	0.0
Mean $\pm$ Standard Deviation			+ 1.0 $\pm$ 0.4

thyroidectomized 27 days previously and incubated with two mls of medium and 60 U.S.P. units of parathyroid extract for two hours. Two mls of the fluid were injected into each of eight bioassay animals.

The average rise in the serum calcium was found to be 1.0 ± 0.9 mg% (Table XXIII). A definite inactivation of the extract was noted under the conditions of this experiment. The increase in serum calcium observed was comparable to that obtained upon incubation of kidney tissue from intact animals under the same conditions.

TABLE XXIII

BIOLOGICAL ASSAY OF PARATHYROID EXTRACT INCUBATED WITH KIDNEY SLICES FROM A RAT THYROPARATHYROIDECTOMIZED TWENTY-SEVEN DAYS PREVIOUSLY

Sample	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%
Kidney A	7.2	7.7	+ 0.5
Kidney A	5.3	5.3	0.0
Kidney A	7.2	6.6	- 0.6
Kidney A	6.3	7.5	+ 1.2
Kidney A	5.1	6.7	+ 1.6
Kidney B	5.4	7.6	+ 2.2
Kidney B	6.0	7.8	+ 1.8
Kidney B	5.4	6.9	+ 1.5
Mean $\pm$ Standard Deviation			1.0 $\pm$ 0.9

CHAPTER IV

DISCUSSION AND CONCLUSION

For purposes of discussion, a Summary, Table XXIV, has been included.

Experiments performed in this laboratory implicate the kidney as the organ of inactivation of parathyroid extract as determined by in vitro studies. The system involved is localized within the kidney and is not released into the medium during the incubation. These observations are in accord with those of Orimo et al. (1965a, 1965b).

In order to further localize the inactivating system in the kidney, Orimo et al. (1965b), by selectively damaging either the proximal tubules or the distal tubules, has shown that the presence of functioning proximal tubules are necessary for the inactivation of parathyroid extract in vitro. Since the greatest concentration of proximal tubules is found in the kidney cortex, and the medulla is composed mostly of collecting ducts, one might expect that cortical slices would be the more powerful inactivator. However, no significant difference could be found between the two zones in the work reported here. Both cortical and medullary slices inactivate the extract about equally.

Dicker (1956), working with the differential centrifugation products of tissue homogenates, has found that the supernatant fraction of the kidney is the most potent in the inactivation of ADH. Geschwind and Li

TABLE XXIV

SUMMARY OF BIOLOGICAL ASSAY DATA FOR THE IN VITRO INACTIVATION OF PARATHYROID EXTRACT BY KIDNEY TISSUE

Test System	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%	Number of Animals
Control	6.2	9.1	$+2.9 \pm 0.3$	32
Intact Kidney	7.9	9.0	$+1.1 \pm 0.8$	6
Soluble Factor(s)	6.9	8.8	$+1.8 \pm 1.6$	3
Intact Kidney Cortex	7.3	8.9	$+1.6 \pm 1.2$	5
Intact Kidney Medulla	7.4	8.2	$+0.8 \pm 1.2$	4
Kidney Supernatant	7.4	8.2	$+0.8 \pm 0.4$	6
Kidney Microsomes	7.2	9.2	$+2.0 \pm 1.9$	11
Kidney Mitochondria	7.4	6.8	-0.5 ± 1.3	10
Kidney from Thyroparathyroidectomized Rat	6.1	8.4	$+2.3 \pm 0.6$	4
Kidney Cortex from Thyroparathyroidectomized Rat	7.3	9.8	$+2.5 \pm 1.9$	4

SUMMARY, CONTINUED

Test System	Initial Serum Calcium, mg%	Final Serum Calcium, mg%	Change in Serum Calcium, mg%	Number of Animals
Kidney Medulla from Thyro-parathyroidectomized Rat	6.0	8.7	+ 2.6 ± 0.2	4
Kidney from Euthyroid-Aparathyroid Rat	4.2	7.2	+ 2.9 ± 1.4	10
Kidney from Euparathyroid-Athyroid Rat	7.9	8.5	+ 0.4 ± 1.0	9
Kidney from Thyroparathyroidectomized Rat:				
Two Days Postoperatively	7.5	5.1	- 2.5 ± 1.2	9
Four Days Postoperatively	6.3	4.5	- 1.8 ± 1.0	5
Five Days Postoperatively	6.8	4.9	- 1.6 ± 2.1	9
Seven Days Postoperatively	7.8	8.1	+ 0.3 ± 1.9	8
Eight Days Postoperatively	6.5	7.0	+ 0.5 ± 1.0	11
Eleven Days Postoperatively	6.9	7.5	+ 1.4 ± 1.4	15
Fifteen Days Postoperatively	6.6	7.2	+ 0.6 ± 1.4	9
Eighteen Days Postoperatively	4.8	5.6	+ 1.0 ± 0.4	7
Twenty-Seven Days Postoperatively	7.0	8.0	+ 1.0 ± 0.9	8

(1952) have determined that the supernatant fraction of kidney homogenates is the most potent in the inactivation of ACTH. For the parathyroid hormone, both the supernatant and the mitochondrial fraction are implicated, the mitochondrial fraction being the more potent of the two.

Preliminary experiments seemed to indicate that the kidney from a thyroparathyroidectomized animal could not inactivate parathyroid extract. If this were so, it was assumed that a substance from either the parathyroid gland or the thyroid gland was necessary to maintain the inactivation process of the kidney. By transplanting either thyroid or parathyroid tissue into the hamstring muscles of thyroparathyroidectomized rats, animals were made euthyroid-aparathyroid and euparathyroid-athyroid. The kidney from a euparathyroid-athyroid animal could inactivate the extract; a euthyroid-aparathyroid animal could not. This implied that some secretion from the parathyroid gland was necessary to maintain the inactivation process of the kidney.

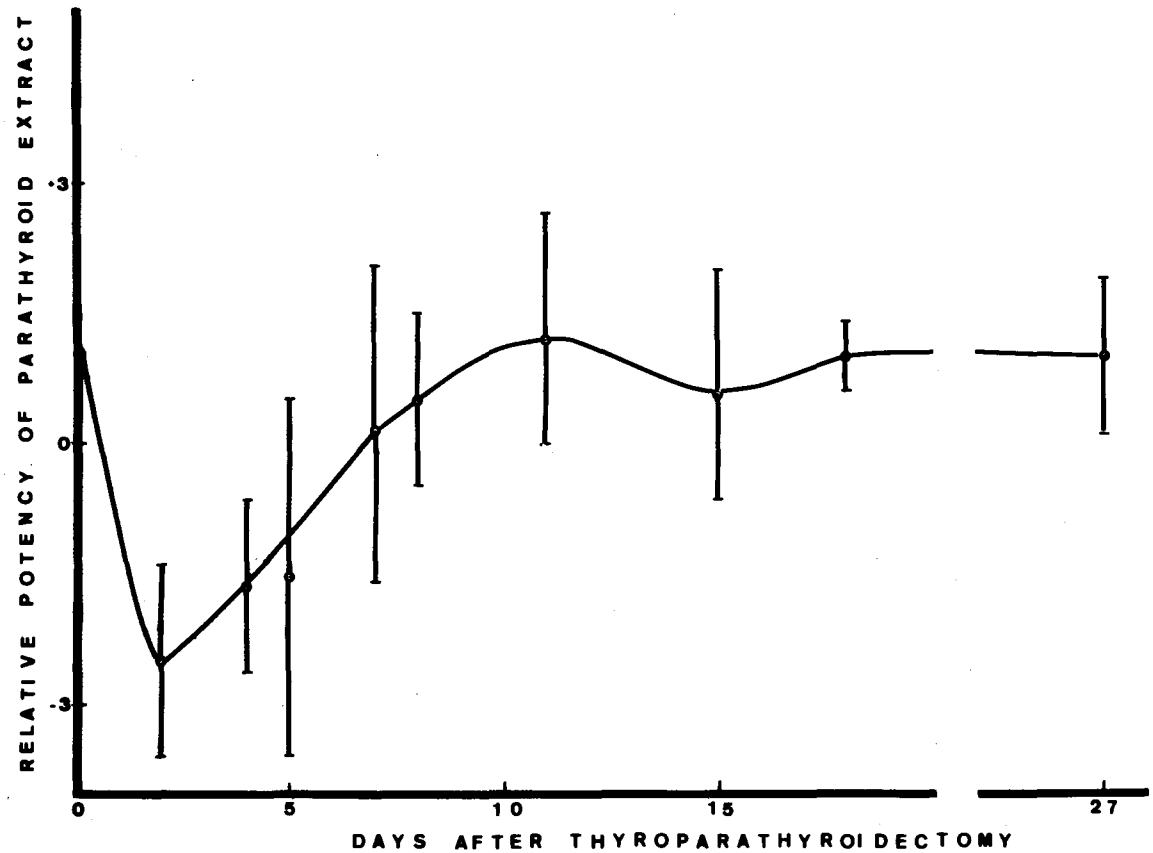
However, the above experiments were not definitive because of the lack of sufficient experimental data in the pilot experiments and in the non-exacting criteria used for successful parathyroid and thyroid transplants. A series of experiments were then designed to determine what influence the interval of time between the removal of the thyroparathyroid apparatus and the removal of the kidney for use in the in vitro studies had on the inactivation.

The data are summarized in Figure 2. The ordinate is the average change in the serum calcium level shown by the bioassay animals which had been injected with parathyroid extract that had been incubated with kidney slices taken from an animal that had been thyroparathyroidectomized the designated number of days shown on the abscissa. The control values given by parathyroid extract not incubated with kidney slices can increase the serum calcium level of bioassay animals 2.9 ± 0.3 mg%.

Intact animals, on day zero, can inactivate the extract as shown by the average increase in serum calcium of 1.0 mg%, a difference of about 2 mg% when compared to the control group. Kidneys from animals which had been thyroparathyroidectomized two days prior to removal of their kidneys had a greatly enhanced ability to inactivate the extract as shown by the steep decline in the Figure. As the time interval between thyroparathyroidectomy and removal of the kidney for use increased, however, there is a gradual return to the level of which an intact animal's kidneys can inactivate the extract. This leveling is complete at about 10 days after thyroparathyroidectomy and continues to at least 27 days after thyroparathyroidectomy.

This seems to indicate that after the removal of the thyroparathyroid apparatus, and therefore the secretion of parathyroid hormone, the enzyme system necessary for its inactivation is increased in either quantity or potency. In about one to two weeks, however, the inactiva-

FIGURE 2



INFLUENCE OF THYROPARATHYROIDECTOMY UPON PARATHYROID HORMONE INACTIVATION BY KIDNEY TISSUE

ting system gradually returns to the level of that of an intact animal. After two weeks, there does not seem to be any loss in the inactivating ability of kidneys from thyroparathyroidectomized rats.

That parathyroid hormone is inactivated by the kidney may in some way be related to the occurrence of osteitis fibrosa generalisata associated with parathyroid dysfunction. As early as 1948, it had been postulated that there were two types of primary hyperparathyroidism, one "with" and one "without" bone disease (Albright and Reifenstein, 1948). They suggested that this difference was related to the dietary intake of calcium; patients with a high intake of calcium not having the bone lesion and those with a low calcium intake presenting osteitis fibrosa generalisata. However, a systemic dietary survey showed that there was no difference in the calcium intake between patients with bone disease and those without (Dent et al., 1961).

Another theory proposed was that the parathyroid gland secretes two different hormones, one acting on bone to produce osteitis fibrosa and a raised alkaline phosphatase and having nephrotoxic action, and another hormone having a calcium raising and phosphorus lowering action (Dent, 1962). This, however, has not been substantiated.

Perhaps the severity of the hyperparathyroidism could be a relevant factor. The greater the concentration of parathyroid hormone in the blood, the greater the degree of bone damage. In an autonomous

functioning parathyroid system, the level of circulating parathyroid hormone would be expected to be greater if the mechanism for removal of the hormone was not working efficiently, i. e. in the presence of renal disease. This may in part be substantiated by work done by Dent (1962). Of eight patients with bone disease and nephrocalcinosis, only three had normal renal function which was defined as a plasma urea level greater than 50 mg%. All three patients had marked renal tubular dysfunction as shown by a vasopressin-resistant diabetes insipidus and renal aminoaciduria. However, nine out of ten patients with nephrocalcinosis and without bone disease had normal renal function. There seems to be a correlation between renal dysfunction and bone disease, but not necessarily a correlation between the presence of nephrocalcinosis and bone disease. If renal dysfunction, as determined, would parallel the ability of the kidney to inactivate parathyroid hormone, then the presence of osteitis fibrosa could be explained on the basis of high levels of parathyroid hormone secondary to an impaired process of inactivation by the kidney. This would be substantiated by comparing parathyroid hormone concentration in patients with bone disease and in patients without bone disease.

In patients who have hyperparathyroidism secondary to renal disease proven by autopsy, all have osteitis fibrosa. However, only 30% of patients with primary hyperparathyroidism, even of long duration,

have osteitis fibrosa (Gilmore, 1947). Again, based on work presented in this thesis, this may in part be due to a diseased kidney not being able to inactivate circulating parathyroid hormone thereby causing higher levels of parathyroid hormone in the blood resulting in osteitis fibrosa.

Another significant corollary between renal damage and increased level of parathyroid hormone comes from the work of Fujita et al. (1962). Parathyroid hormone like phosphaturic activity was scareely detectable in normal rabbit sera, but was significantly elevated in rabbits made hypertensive by clamping the left renal artery. This increased level of activity may be due to the inability of the clamped kidney to inactivate circulating parathyroid hormone.

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ABSTRACT

Abstract of the thesis entitled "THE IN VITRO INACTIVATION OF PARATHYROID HORMONE" submitted by Robert C. Lukesh in partial fulfillment of the requirements for the degree of Master of Science, June 1968.

Parathyroid extract was incubated aerobically for two hours at 37° C with rat kidney slices and with supernatant, microsomal and mitochondrial fractions of kidney homogenate from normal animals and with kidney slices from rats that had been thyroparathyroidectomized surgically two, four, five, seven, eight, eleven, fifteen, eighteen and twenty-seven days previously. Slices of rat kidney from normal animals inactivated the extract. The mitochondrial fraction of kidney homogenates from normal rats possessed the greatest ability to inactivate the extract, although some ability was also shown by the supernatant fraction. Kidneys from rats that had been thyroparathyroidectomized two days before use showed a marked increase in the ability to inactivate the extract. This enhanced ability was also shown to a degree by kidneys from rats which had been thyroparathyroidectomized four and five days before use. Kidneys from animals thyroparathyroidectomized eleven, fifteen, eighteen and twenty-seven days before use showed no difference in the inactivating ability from that observed with the kidney tissue from normal animals.

APPROVAL SHEET

The thesis submitted by Robert C. Lukesh has been read and approved by the undersigned member of the faculty of the Loyola University Stritch School of Medicine who served as the director of the research program.

The final copies have also been examined and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

May 24 1968
Date

Maurice V. J. Heusey
Signature of Advisor