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The Relationship of Phosphate to the Function of the Calcitonins

by

James William Kennedy, III

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LITERATURE REVIEW

The existence of a hypocalcemic hormone was postulated in 1961 by Copp and his coworkers (Copp and Cameron, 1961). This new concept was based on the presence of a fast acting hypocalcemic factor in commercial extracts of bovine parathyroid glands. Because it lowered blood calcium levels, the hormone was named calcitonin (Copp et al., 1961). Further experiments (Copp et al., 1962) substantiated the existence of such a factor but did not define its exact origin. In these experiments the isolated thyro-parathyroid complex of a dog was perfused via the thyroid artery with solutions of different calcium concentrations. The venous perfusate was then returned to the systemic circulation of the dog and assayed for hypocalcemic activity. High calcium perfusion resulted in the release of a hypocalcemic factor from the thyro-parathyroid glands. These observations were confirmed in similar experiments in an independent laboratory (Kumar et al., 1963), but still no distinction was made between the thyroid and the parathyroid glands as a source of the hormone. Subsequently, Copp associated the "calcitonin concept" with the parathyroids, postulating that one gland produced two antagonistic hormones to precisely control plasma calcium levels.

Meanwhile, Munson's laboratory discovered the source of the hypocalcemic hormone using completely different experiments (Hirsch et al., 1963). While studying the differences in the rate of fall of plasma calcium in the rat following parathyroidectomy by cautery and surgical excision, these workers observed that cautery caused the calcium levels
to fall more rapidly. Thus, this group suggested that there was a hypo-
calcemic factor in the thyroid which was released as a result of cautery. 
Further investigation led to the isolation of a calcitonin-like principle 
from the rat thyroid gland (Hirsch et al., 1964). This hormone was named 
thyrocalcitonin to show its gland of origin. The exact relationship 
between Copp's calcitonin and the new thyrocalcitonin of Munson's group 
was not understood at that time; however, after many experiments and 
conferences, calcitonin and thyrocalcitonin were shown to be the same 
hormone (Munson et al., 1968). While this work established the existence 
of a hypocalcemic principle from the thyroid, no attempt was made to 
clarify the physiological role of the new hormone.

Talmage's laboratory supplied the first proof of the importance of 
thyrocalcitonin as a hormone preventing hypercalcemia (Talmage et al., 
1964, 1965). Their experiments showed that rats with functional thyroids 
maintained lower plasma calcium levels following a high calcium challenge 
than did thyroidectomized animals. These observations defined a physio-
logical role of the hormone.

Relationship of calcitonin to the ultimobranchial gland

Following the proof that thyrocalcitonin originated from the mammalian 
thyroid gland, it was shown that the ultimobranchial body of the chicken 
contained an extractable hypocalcemic factor (Tauber, 1967; Copp et al., 
1967a, 1967b). These observations revived Copp's contention that calcio-
tonin was extrathyroid in origin (Copp et al., 1968a). Furthermore, 
whole or extracted chicken thyroids which had been carefully removed and 
cleaned of adjacent tissue, failed to produce hypocalcemia (Kraintz and
Fuil, 1967; Urist, 1967). Subsequently, it was shown that the ultimobranchial glands of many vertebrates, including turkey (Copp et al., 1967a, 1967b), pigeon (Matthews et al., 1968), lizard (Moseley et al., 1968), bonyfish (Copp and Parkes, 1968), and cartilaginous fish (Copp et al., 1967a, 1967b; Urist, 1967) had very potent extractable hypocalcemic principles.

Attempts to relate the hormones between different species at a physiological level have failed. There is some evidence that porcine thyrocalcitonin is hypocalcemic at pharmacological levels in the catfish *Scturus melan* (Louw et al., 1967). However, another investigation showed no hypocalcemic effect of porcine hormone in the teleost fish *Fundulus heteroclitus* (Pang and Pigford, 1967). There is inconclusive evidence that porcine thyrocalcitonin is hyperphosphatemic in the European eel *Anguilla anguilla* (Chan et al., 1968). Because chicken ultimobranchial glands contain a large amount of calcitonin (Copp and Parkes, 1968), the chicken should be very sensitive to both its own ultimobranchial calcitonin and to porcine thyrocalcitonin. Urist (1967) showed, however, that the chicken does not respond to either hormone, even in massive doses. To date, there is no strong evidence of mammalian thyrocalcitonin or ultimobranchial hormone affecting any lower vertebrate. Conversely, ultimobranchial hormones are very potent in mammals, and the Canadian salmon serve as a commercial source of calcitonin (Copp and Parkes, 1968).

Further relationships of the ultimobranchial gland to calcitonin and calcium metabolism have been shown in a series of experiments with the leopard frog *Rana pipiens*. Robertson has reported a cell cycle in the ultimobranchial gland which lasts the whole year. The gland is active
in the summer and dormant in the winter when the frog is not eating (Robertson, 1967). The parathyroid gland on the other hand shows opposite changes; the glands are active in the fall and winter and degenerate in the spring (Romeis, 1926; Waggener, 1929). This observation led Robertson to the conclusion that the ultimobranchial gland is physiologically important in the control of calcium metabolism (Robertson, 1967). In another series of experiments, the ultimobranchial glands of frogs maintained in high calcium water hypertrophied (Robertson, 1968a) and subsequently lost their secretory granules (Robertson, 1968b). Robertson further related glandular activity to hypercalcemia because even after denervation or transplantation of the glands the same responses were observed (Robertson, 1968c). Robertson also proposed that a hormone from the ultimobranchial gland in frogs inhibits renal calcium excretion, stimulates osteoblastic activity, suppresses proliferation of osteoclasts, and suppresses the removal of calcium from the vertebral lime sacs (Robertson, 1969).

There was considerable evidence in favor of Copp's postulate that calcitonin was extrathyroid in origin. However, Godwin (1937) had provided evidence that the embryonic ultimobranchial gland becomes incorporated into the thyroid gland in mammals. Likewise, Pearse showed that cells of ultimobranchial origin were well dispersed extrafollicular cells in the thyroid (Pearse, 1966, 1968). Pearse named these cells "C"-cells, to stand for calcitonin cells. Foster et al. (1964) subsequently demonstrated that the "C"-cells showed secretory activity in hypercalcemic dogs. In addition, there is a depletion of secretory granules in rat thyroid "C"-cells following calcium chloride injection (Matsuzawa, 1967),
treatment with Vitamin D$_2$ (Ericson, 1968) or treatment with parathyroid extract (Cameron, 1968).

There are valid reasons for differences of opinion as to whether the name of the hormone should be calcitonin or thyrocalcitonin. For the purpose of this paper, the name thyrocalcitonin will be used to mean the hormone from the mammalian thyroid. The name calcitonin will be used to mean the ultimobranchial hormone of lower vertebrates. The hormones will be generally referred to as the calcitonins.

**Chemistry of the calcitonins**

The chemistry of the calcitonins is well known. In only nine years the amino acid sequences of the porcine and human thyrocalcitonin, as well as salmon calcitonin, are known and the porcine molecule has been synthesized (Bell et al., 1968; Potts et al., 1968; Neher et al., 1968). Mammalian thyrocalcitonin has a molecular weight of 3585 and contains 32 amino acid residues (Potts et al., 1968). Purification and separation of a homogenous peptide has been accomplished by gel filtration on Sephadex G-75 followed by preparative polyacrylamide gel electrophoresis. These steps resulted in a peptide which formed a single band on analytical gel electrophoresis and yielded a 50,000 fold purification from the starting material based on specific activity (Potts et al., 1967).

Chicken ultimobranchial calcitonin (Moseley et al., 1968) has been purified by gel filtration to the extent that the molecular weight is estimated to be 4500. The amino acid sequence of chicken calcitonin is not known, however, salmon calcitonin has been extensively studied (Copp, 1970).
Physiological effects of thyrocalcitonin on calcium

Since this paper describes the relationship of phosphate to the calcitonins, this review of the calcium effects of these hormones will be limited to only those points which are pertinent to the general understanding of their physiology. For further references covering the chemistry and physiology of the hormones, the reader is referred to three recent review articles (Hirsch and Munson, 1969; Copp, 1969, 1970).

Both in vivo and in vitro studies on the effects of the calcitonins have, for the most part, been limited to studies on bone metabolism. Differential labeling of bone with radionucleotopes of calcium (superscript 45Ca), strontium (superscript 85Sr), and phosphorus (superscript 32P) has aided in understanding what part of bone is acted upon by these hormones. The stable bone fraction or "deep bone" is parathyroid hormone sensitive and is not labeled in the first 24 hours after administration of the isotope. However, by 7 days after administration of the isotope, it contains most of the label. In the first 24 hours after isotope injection, the freely exchangeable fraction or "bone surface" is selectively labeled. Studying the effect of parathyroidectomy (Talmage and Elliot, 1958) and parathyroid hormone administration (Clark and Geoffry, 1958; Woods and Armstrong, 1956) on bone determined this distribution. Large specific activity (superscript 45Ca/superscript 40Ca) changes followed parathyroid treatment if the label was recently administered; however, if stable bone was labeled, the movement of radio-calcium and total calcium was the same with little change in specific activity.

Using a bone surface superscript 45Ca label, Milhaud showed that plasma superscript 45Ca specific activity falls in control rats while remaining the same in
thyrocalcitonin treated animals (Milhaud et al., 1965). This indication that thyrocalcitonin decreases the removal of calcium from stable bone; i.e., inhibits bone resorption, was confirmed by Johnson and Diess (1966).

Klein et al. (1967) showed that injections of porcine thyrocalcitonin to rats during peritoneal lavage with low calcium rinse resulted in a suppression of removal of $^{32}$P from bone after both an 18 hour and a 21 day label. In these experiments, not only deep bone resorption but also exchange with the bone surface was suppressed.

Definitive proof of thyrocalcitonin's action on bone was again from Klein and Talmage (1968). Their experiments showed that thyrocalcitonin, given during lavage, suppressed the release of hydroxyproline from bone.

Finally, perfusion experiments using isolated cut tibia showed that thyrocalcitonin causes an increased retention of calcium by bone (Parsons and Robinson, 1968). This experiment is significant because it ruled out any relationship between soft tissue and thyrocalcitonin's action on bone.

In vitro experiments with cultured bones have further stressed the action of these hormones on bone. Porcine thyrocalcitonin, as well as salmon and chick calcitonin, strongly inhibit both spontaneous and parathyroid hormone induced (Gaillard, 1961) bone resorption in cultured bones (Aliapoulios et al., 1966; Freidman and Raisz, 1965; Freidman et al., 1968; Raisz et al., 1968). Using a Von Kossa stain for bone salts, Aliapoulios et al. (1966) showed a retention of bone salts in both embryonic and postnatal bones after thyrocalcitonin treatment.
Physiological effects of the calcitonins on phosphate

Munson's early experiments indicated that rat thyroid extract was hypophosphatemic as well as hypocalcemic (Hirsch et al., 1964). However, this observation was largely ignored in favor of the calcium effects of the hormone. The phosphate effect of thyrocalcitonin was substantiated by Kenny (1964) in experiments using hog thyroid extracts. Low doses of extracts caused large drops in rat plasma phosphate.

Shortly after the discovery of thyrocalcitonin, several investigators began experiments attempting to relate the action of the hormone to its effects on the kidney. While no effects on renal calcium clearance could be shown (Kenny, 1964), there were many reports of a phosphaturic effect. Kenny and Heiskell (1965) observed phosphaturia following thyrocalcitonin administration to water loaded rats. This led Kenny to postulate that phosphaturia causes hypophosphatemia and the resulting decrease in bone resorption allows a fall in plasma calcium.

MacIntyre subsequently reported that in parathyroidectomized rats with a low plasma calcium (<7.0 mg%), thyrocalcitonin has no hypocalcemic effect. The hormone did, however, retain its hypophosphatemic potency (Robinson et al., 1966). In addition, the hypophosphatemic action of thyrocalcitonin in parathyroidectomized rats could be abolished by prior nephrectomy. No data were published to support these results; but still the investigators postulated that thyrocalcitonin had an effect on renal phosphate clearance independent of calcium and parathyroid hormone (Robinson et al., 1967). These studies used high doses of crude thyrocalcitonin; consequently, the results are now seriously questioned.

Thyrocalcitonin was shown to produce phosphaturia in thyroparathyroidectomized rats, leading Milhaud and Moukar (1966) to support
the postulation of a kidney effect independent of parathyroid hormone. Rasmussen et al. (1967) observed phosphaturia in thyroparathyroidectomized animals following the administration of ethylenebis(oxyethylene-nitrilo)-tetraacetic acid (EDTA), a chelating agent specific for calcium. This indicated that phosphaturia may result from hypocalcemia alone.

Large doses (2-6 MRC units) of thyrocalcitonin were reported to be both phosphaturic and calciuric in man (Ardaillou et al., 1967) but these results have not been reconfirmed. Because of the massive doses used and the crude nature of the thyrocalcitonin preparations, Zeigler et al. (1967) questioned the specificity and physiological significance of the responses.

In contrast to the reports that thyrocalcitonin causes phosphaturia, Pechet et al. (1967) showed suppression of urine phosphate loss. Rats infused with thyrocalcitonin retained phosphate. This effect could be negated by simultaneous infusion with calcium, leading Pechet to postulate that thyrocalcitonin's action was dependent on divalent cation concentration in the kidney cells.

Kenny's groups showed that physiological doses of a purer hormone preparation failed to cause phosphaturia (Clark et al., 1968). In refuting their earlier work, these investigators postulated that the observed phosphaturia was caused by diuresis resulting from treatment. Kenny's results were confirmed by Russell and Fleisch (1968). In pigs and dogs, only large doses of purified thyrocalcitonin produced phosphaturia. In addition, small doses were hypocalcemic without increasing urine phosphate, confirming Hirsch's observation that the hormone is fully active in nephrectomized rats (Hirsch et al., 1964).
Finally, a series of reports implied that the action of thyrocalcitonin required a retention rather than a loss of phosphate. Ziegler and Pfeiffer (1968) observed that the purer the thyrocalcitonin, the less the phosphaturia. This relationship held even when high dosages were used. In addition, endogenous or exogenous thyrocalcitonin markedly suppressed parathyroid hormone induced phosphaturia in the rat (Ziegler and Pfeiffer, 1968; Pechet et al., 1967).

At this point any importance of phosphate metabolism in the function of thyrocalcitonin was not obvious, except for the hypophosphatemic action of the hormone. In a series of experiments establishing a reliable bioassay for the calcitonins, Hirsch observed that an injection of phosphate (150 mmole), which was itself too small to cause a significant hypocalcemia administered before, simultaneously with, or just after the injection of the thyrocalcitonin, increased the hypocalcemic potency. This led Hirsch and Cooper (1968) to postulate that young rats were more sensitive to thyrocalcitonin because of their high plasma phosphate levels.

Hirsch's observations caused a reevaluation of some previously described experiments (Klein et al., 1967). While studying the effects of thyrocalcitonin administered during lavage on the removal of radioisotopes of calcium and phosphorus from bone, Talmage's group showed that the $^{45}$Ca:$^{32}$P ratio was different from the ratio of these isotopes in bone. Thyrocalcitonin caused a significant suppression of $^{32}$P removal from bone which was not statistically different whether the isotope was administered 18 hours or three weeks prior to lavage. Conversely, the removal of $^{45}$Ca from stable bone was suppressed, while that of the 18 hour label was not. In other words, Talmage's data implied that thyrocalcitonin affected phosphate but not calcium movement on the bone surface.
Studies to determine the relationship between the adrenal cortico-steroids and thyrocalcitonin function confirmed that phosphate levels in the plasma influenced the rat's response to the hormone (Thompson et al., 1968). Adrenalectomy had no effect on the dose response curves of either plasma calcium or plasma phosphate, nor did the removal of the gland alone affect either parameter. On the other hand, the administration of cortisone, in doses that retarded growth, resulted in a decreased sensitivity to thyrocalcitonin. These results indicate that animals with low plasma phosphate caused by cortisone treatment are less sensitive to the hypocalcemic effect of the hormone. However, the dose response curves for plasma phosphate were unchanged regardless of the treatment; i.e., cortisone decreased the hypocalcemic response to thyrocalcitonin but did not have any effect on the hypophosphatemic response to the hormone.

Attempts have been made to explain the phosphate effects of thyrocalcitonin as actions on extraosseous organs other than the kidney. In a series of unconfirmed experiments using a dog heart-lung preparation, Stahl et al. (1968) showed phosphate effects in the absence of calcium changes. This isolated soft tissue preparation had no bone and, consequently, no significant calcium phosphate pool. The infusion of thyrocalcitonin resulted in an increase in plasma phosphate levels with no accompanying changes in plasma calcium; however, the significance of this study is not understood.

Since calcium and phosphate exist in a fixed ratio in bone as hydroxyapatite, if these hormones act solely to decrease bone resorption by inhibiting parathyroid hormone the ratio of the suppression should be fixed.
This is not the case under some experimental conditions. Ruling out any extraosseous effects as unimportant for hormone action, the test animal's plasma phosphate levels, as well as phosphate movement to and from bone, are the most important factors in relation to the physiological function of thyrocalcitonin.

The dynamic state of bone is one in which parathyroid hormone is constantly affecting resorption to counteract a natural gradient of calcium and phosphate toward precipitation on bone. In addition to parathyroid hormone resorption, there is constant exchange between bone and extracellular fluid. The only thing necessary to cause hypocalcemia is an inhibition of parathyroid controlled movement of calcium away from bone so that the net movement of calcium and phosphate is toward bone rather than balanced as it is normally.

We propose that thyrocalcitonin acts to affect the movement of phosphate in bone and the results are changes in plasma calcium levels and a decrease in the removal of calcium and phosphate from bone. The purpose of this study is to further relate phosphate to the function of the calcitonins by demonstrating phosphate changes in the absence of calcium changes following hormone treatment, as well as differences in phosphate and calcium handling by bone as a result of these hormones.
GENERAL MATERIALS AND METHODS

For this study, calcitonins from three sources were used. Rat thyrocalcitonin was obtained from the thyroids of either Holtzman or Cheek Jones laboratory rats. Chicken ultimobranchial extracts and frog ultimobranchial extracts were obtained from the fresh ultimobranchial glands of the species.

For early experiments, the ultimobranchial glands of young chickens (obtained from Hendrix Grain Co., Houston, Texas) were surgically removed, ground in 0.1 N HCl and allowed to stand at room temperature for 48 hours. One ml. of the solution containing approximately 1 gland per ml. was injected into each rat for assay.

All subsequent experiments used extracts obtained by a different procedure. Immediately upon removal, rat thyroids were frozen in dry ice and acetone. Frozen thyroids were found to retain activity at -20°C for up to two weeks. Consequently, all thyroids were extracted within 10 days after collection.

Chicken ultimobranchial glands were obtained from poultry house reject chickens at Jim Dandy Poultry Co., Houston, Texas, through the courtesy of the USDA Inspector, Dr. Goodwin, D.V.M. The majority of the glands were removed from chickens with skin blemished, broken bones, or edema of the skin. Some of the birds however had avian leukos ( Holmes, 1964). This disease was not a factor in gland content of extractable calcitonin. Since all the ultimobranchials for each extraction were collected at once, there was no storage period; but the glands were still frozen immediately in dry ice and acetone.
Frog ultimobranchial glands were removed surgically from their position on the glottis constrictor muscle (Robertson and Swartz, 1964) in both *Rana catesbeiana* and *R. pipiens* male adults.* The glands in *R. catesbeiana* were easier to find and more fruitful due to their size. The glands were also frozen immediately in acetone and dry ice. It was not possible to determine stability of the frozen frog ultimobranchial glands because the amount of material was so little that loss of activity could not be risked.

Once the glands were collected, they were all treated the same way throughout extraction and preparation of the extract. The extraction is a modification of the one described by Copp and Parkes (1968). The frozen glands were kept in an ice bath and defatted with ten rinses of acetone followed by five rinses of chloroform, and five rinses of ether. The final wash was ten rinses of acetone. After complete drying and defatting, the glands were allowed to stand at room temperature in a slight vacuum until all the acetone was evaporated off. The completely dry glands were then weighed and placed into 12 ml TenBroek homogenizers. The tissue was ground in 5 ml of butanol:acetic acid:water, 75:7.5:21 (Gudmundsson et al., 1968), then 10 ml of solvent was added so that the first extraction was 15 ml solvent/gram dry weight of tissue. The homogenate was allowed to stand at room temperature for 12 hours, then cen-

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*R. catesbeiana* were bought from Louisiana Frog Co., Rayne, Louisiana. *R. pipiens* were obtained from Lemberger Frog Co., Oshkosh, Wisconsin.
trifuged at 3000 g for 15 minutes. The supernatant was decanted and
stored at -20°C. The pellets were reextracted in the homogenizers with
another 15 ml solvent/g dry weight tissue for 8 hours. The same harvest
procedure was again followed and the final extraction was a thirty minute
rinse with the same volume of solvent. The supernatants from the three
extractions (of all glands from one species) which had been combined and
stored in the freezer were allowed to thaw until just liquid and high
quality acetone at -20°C was added and the solution allowed to precipitate
for three days in the freezer. The resulting precipitate and solution
was then centrifuged in the cold at 37,000 g for one hour and the super-
natant discarded. The precipitated protein was placed in a vacuum to
evaporate off the acetone-solvent mixture that remained (approximately
15 minutes); then dissolved in a small amount of 0.1 N formic acid.
This solution and two washes were transferred to preweighed vials, freeze
dried, and the resulting powder weighed. This powder is subsequently
referred to as the "crude extract".

For further purification of the hormones, the crude extract was
redissolved in 0.1 N formic acid and chromatographed on Sephadex G-100.
The column was 1.5 X 100 cm and was packed and maintained in the cold
throughout the entire experiment. Usually 1.0 - 2.0 mg crude extract
was applied to the column in 0.5 ml of 0.1 N formic acid. The flow rate
of the column was 9 ml per hour and 3 ml fractions were collected. The
absorbance of each fraction was determined on a Beckman-DB spectrophoto-
meter at 260 nm (Frutos and Simmonds, 1958). All fractions of some runs were read at 260 nm to determine nucleic acid contamination (White et al., 1968). The fractions were then freeze dried and assayed by methods to be described. This step resulted in a four fold purification over the crude extract.

Further purification was attempted by ion exchange chromatography on carboxy-methyl cellulose (Sigma, capacity 0.7 meq/gm) (Copp and Parkes, 1968). Five mg of the crude extract was applied to a column 0.9 X 15 cm. The elutant buffer was ammonium acetate at pH 4.0. The ionic strength was increased in the elutant from 0.01 M to 0.5 M. The flow rate was 8 ml per hour and 2 ml fractions were collected. Aliquots of each fraction were neutralized and assayed for activity. This step resulted in a five fold purification over the crude extract.

All hormone preparations were administered using the same vehicle. The lyophilized powder was dissolved in 0.8% NaCl solution. The pH of the solution was adjusted to 3.8 with acetic acid and 0.2% Cohn's fraction V bovine serum albumin was added to reduce adsorption to the glass containers prior to the addition of the extracts. The doses are given for each individual experiment when they are described.

The calcitonins were assayed by a technique similar to the assay used by Cooper et al. (1967). Male Cheek Jones or Holtzman rats weighing 140-160 gm were given calcium deficient diet and deionized water for one day prior to use. Each rat served as his own control. Blood samples
were obtained, by a method to be described, prior to and one hour after the administration of either the hormone extract or the vehicle. All assays at first were run against Medical Research Council Thyrocalcitonin Research Standard B (Baghdiantz et al., 1964). When a sufficient amount of crude extract was obtained, a house standard was prepared again against MRC Standard B. All subsequent assays were against the house standard. Both calcium and phosphate plasma values were considered for latter assays but the potency was based on hypocalcemic ability. Total protein was determined by the method of Lowry et al. (1951).

Thyroxine solutions were prepared as described using L-tetiodothyronine obtained from International Chemical and Nuclear Company, divided into 5 ml aliquots and stored at -20°C. Storage time is unlimited but refreezing destroys the activity. Each batch of thyroxine was qualitatively assayed for activity by testing its ability to induce tail reorption and subsequent metamorphosis in R. catesbeiana tadpoles (Swingle, 1922; Bruice et al., 1956). Thyroxine was administered subcutaneously behind the neck each day to all animals in each experiment as stated. The dose, 5.0 μg/rat/day, was shown to be a sufficient amount to supplement thyroidectomized rats and also to completely inhibit 131I uptake (measured at 24 hours after administration) in rats with functional thyroids (Kennedy and Talmage, unpublished).

The cortisol used in these experiments was supplied through the courtesy of Dr. Webster S. S. Jee, University of Utah School of Medicine,
Salt Lake City, Utah. The cortisol was suspended in a saline solution with carboxymethyl cellulose as a vehicle. The dose administered, 5 mg/Kg rat/day administered subcutaneously behind the neck, was chosen as a result of assays in Dr. Jee's laboratory (Jee et al., 1966).

The procedures for surgery performed on test rats will be described here but actual time sequences are discussed with the individual experiments. All rat surgery was performed using ether anesthesia. Parathyroidectomy was performed through a midventral incision in the neck. After separation on the sternohyoid muscle, the two parathyroids were surgically removed from their position on the thyroid gland using jewelers' fine forceps. Thyroidectomy was through a similar incision. The thyroids were removed using coarse forceps with care being taken to avoid injury to the recurrent laryngeal nerve. Parathyroid autotransplants were performed two weeks prior to the experiments using two techniques. For early experiments the parathyroids were removed and placed in the neck musculature lateral to the sternohyoid muscle (Talmage et al., 1964). The more recent experiments used a newly developed technique developed from the procedures of Browning (Browning and Guzman, 1967). The parathyroids were removed using sterile instruments. An incision about 3 mm long was made in the cornea of the eye. The parathyroids were then placed just under the scleral coat and positioned in the center of cornea. This technique allows better vascularization with a higher percentage of takes. Also it allows visual proof of viability of the transplanted gland using a dissecting microscope.
Nephrectomy was performed at different times in the experiments as described. The surgical removal or ligation of both kidneys was through a midventral incision in the abdomen. The kidneys were exposed and the capsule and fat teased away. The adrenal glands were carefully avoided and remained in their right position in the fat. The ureter and the renal artery and vein were then ligated and the kidney cut off or returned to the peritoneum. The muscle of the body wall was sutured before the skin was clipped.

Adrenalectomy was performed through bilateral incisions just posterior to the last floating rib. The adrenals were exposed and the fat just under them clasped with mosquito hemostats. The liver and surrounding tissue was then teased away with coarse forceps and the glands pulled out with the hemostats with negligible bleeding. Again, the muscle was sutured before the skin was clipped. At the end of experiments using adrenalectomized rats, all the animals were placed in the cold room at 4°C with food and tap water ad lib. Animals were assumed to be completely adrenalectomized only if they could not survive for 24 hours (Dorfman et al., 1946). Any animal that was supposed to be adrenalectomized but did not die within 24 hours was autopsied to determine completeness of the operation and if any part of the gland remained the animal was dropped from the experiment.

Frog surgery was performed under an anesthesia specifically for cold blooded vertebrates. Enough solution of 1:1000 MS-222* in water

*MS-222 (tricain methane sulfonate) is available from Ayrs Pharmaceuti
cals on special request.
was placed in a shallow bowl to cover the frog completely. The frog was then allowed to soak for 10 minutes. This anesthetized the animal for at least 30 minutes with no side effects. Bilateral incisions were made in the throat just anterior to the coracoid bone of the pectoral girdle. The insertion of the glottis constrictor muscle was exposed and ultimo-branchial glands (one from each side) were removed with coarse forceps. Great care was taken to avoid injury to the various nerves and the aortic arches running through the area.

While the microanalysis of plasma calcium (Hill, 1962), 1965), phosphate (Fiske and Subbarow, 1925), and glucose (Cawley et al, 1959) is described elsewhere, the ultramicroanalysis is treated here because it is unique in this laboratory. The phosphate procedure was developed for this study.

Special bleeding techniques were used where possible with frogs and in all cases in rats. Rats were placed in restraining cages made from lucite tubes and their tails placed 18" from an infrared heat lamp. After gentle warming, the end of the tail was clipped and massaged with light pressure. The blood was collected in heparinized capillary tubes, each with a volume of 60 μl. The whole blood was immediately spun in a microhematocrit centrifuge for 2 min. Frog blood was obtained in one of two ways. In R. catesbeiana an incision was made in the skin of the leg and the sciatic vein exposed. Two ligatures were then placed 1 cm apart but were not tied fast. A small cut was then made in the vein and the
blood collected in capillary tubes. The ligatures were tied to stop the bleeding. The subsequent bleeds were accomplished by loosening the ligatures. Alternately in *R. catesbeiana* and routinely in *R. pipiens*, the animal was pithed and the heart and the aortae exposed. Blood could then be collected either by cannulation with teflon "i.v." tubing or by direct puncture with the capillary tube. Again the blood was immediately centrifuged.

When it was necessary to obtain serum by heart puncture, a twenty gauge needle was used. The syringe was rinsed with saline, the blood drawn and run carefully down the side of a centrifuge tube. Rat blood was allowed to clot in the cold for 10 minutes before centrifuging but frog blood had to stand overnight to give the highest yield of serum.

The ultramicro analysis of plasma calcium was a modification of the fluorometric method of Keprner and Hercules (1963). The indicator used was 3,6-dihydroxy-2,4-bis-(N,N'-di-(carboxymethyl)-aminomethyl)fluoran (Calcein) in 0.8 N KOH. Twenty µl samples of plasma were used. Determinations were on a Turner Model 111 Fluorometer.

Plasma phosphate was determined by a modification of the method of Chen *et al.*, (1956). The method was scaled down to use 20 µl plasma samples. Analysis was on 1.0 ml of supernatant from the TCA precipitate of the plasma. Spectrophotometric determinations were on a Coleman Jr. II spectrophotometer at 820 nm.

Plasma glucose was determined with glucostat reagent* (glucose

*Worthington Biochemical, Freehold, New Jersey.
oxidase). The method was modified to use 20 μl samples and the samples were incubated with the reagent mixture for 30 min at 30°C and then the reaction was stopped with HCl. Determinations were on a Coleman Autoset spectrophotometer at 400 nm.

The peritoneal lavage procedure using the stainless steel flange of Kolff and Page (1954) has been previously described (Talmage et al., 1957; Minkin and Talmage, 1963). Differences in the content of calcium and phosphate in the lavage rinse are discussed with each experiment. The level of glucose in the lavage rinse was varied in some experiments as noted but was generally 2% (composition in Appendix). Urine collection was accomplished using the metabolism cage set-up described by Talmage (Talmage and Kraintz, 1954). The animals were given a water load of 2.5% of their body weight by stomach tube. At collection times the animals were taken out of the cages and their bladders massaged. The urine volume was measured and the funnel was rinsed with distilled water until the volume of the sample was exactly 10 ml. The samples were acidified for storage.

Lavage fluid samples and urine samples were analyzed for calcium and phosphate on a Technicon autoanalyzer. Phosphate was determined spectrophotometrically (Chen et al., 1956) and calcium was determined fluorometrically (Hill, 1965).

Radioactivity was determined in samples of 45Ca and 32P by either liquid scintillation techniques on a Beckman LS-133 liquid scintillation
system (for both, either separate or together) or in a Nuclear Chicago low background gas flow counter ($^{32}$P only). Samples counted on the gas flow counter were plancheted directly into stainless planchets and dried; 100 μl of plasma and 1 ml of either lavage fluid or urine was used. Liquid scintillation counting was accomplished using two different types of fluor systems. For plasma, Bray’s solution was found to be very efficient and was used for early experiments (Bray, 1960). All later experiments have used a toluene based fluor with a Beckman solubilizer, Bio-solve BBS-3, added to take up water and protein (Newman, 1969). The standard fluor contained 4 gm PPO and 0.2 gm dimethyl POPOP per liter of fluor. Fifty μl plasma samples were pipetted into 2 ml 10% TCA and centrifuged. One ml of the supernatant was pipetted into a counting vial and 5 ml of fluor containing 20% BBS-3 was swirled in. The mixture was clear and stable for at least 48 hours after which it became progressively milky. One ml samples of lavage fluid and urine were dissolved in 5 ml of 15% BBS-3. In some cases where 20 μl of plasma were used, 5 ml of a 6% BBS-3 fluor solubilized the sample. No degradation of the fluor was noticed with samples other than TCA.

$^{85}$Sr was determined using a Nuclear Chicago solid scintillation counter. Lavage fluid samples (4 ml) or plasma was placed in a gamma counting tube and gamma radiation counted directly.

All summarization of data and statistical analysis of results was done using the Rice Research Computation Laboratories' Burrough's B-5500
Computer. The programs available converted all raw data for calcium, phosphate, and glucose into milligrams per 100 ml plasma (mg%). The averages and standard errors were then computed and groups were compared using the student's "t" test. Decay correction as well as "cross-talk" corrections (for dual isotope determinations) was computed and DPM was calculated and averaged. All DPM were normalized to a given value and all specific activities were computed for either isotope used. These data were also averaged and compared as desired using the student's "t" test.
RESULTS AND DISCUSSION

Extraction of calcitonin from the ultimobranchial gland of lower vertebrates

As a preliminary experiment, large doses of rat thyroid extract (RTE) and chicken ultimobranchial extract (CUBE) were assayed in intact fasted rats. Also tested was an extract from the ultimobranchial gland of the bullfrog (*R. catesbeiana*), which was available in minimal amounts. The extracts were prepared by grinding the respective glands in 0.1 N HCl (1 ml/gland). Each test rat received 1 ml of an extract solution whereas controls received 1 ml of saline (pH 3.8). Blood samples were taken prior to, and one and two hours after injection. The results are summarized in Table 1.

All three extracts produced the expected hypocalcemic response. This data indicates that *R. catesbeiana* ultimobranchial glands contain less extractable calcitonin than chick ultimobranchials or rat thyroids even though they are not significantly smaller. Plasma phosphate values were obtained after RTE and CUBE injections. Using calcitonins from both sources, the \( \Delta \text{Ca}:\Delta \text{P}_{1} \) (fall in calcium divided by the fall in phosphate) produced by the hormones was similar, and in both cases the ratio was less than one. In other words, the hypophosphatemia produced was greater than the hypocalcemia. Since the ratio of calcium to phosphate in bone is greater than one, it is obvious that the effects noted here could not be attributed solely to the influence of the calcitonins on the suppression of bone resorption.

One explanation might be that the dosages used produced maximal re-
responses. If true, this may account for the unexpected Ca to $P_i$ ratio.

But, experiments in which minimal doses of the hormone were utilized will prove that this is not the case.

**Purification of the calcitonins**

It might be possible that the unexpected $\Delta$Ca to $\Delta P_i$ ratios were due to the crude nature of the extracts used. The next series of experiments was designed to establish dose response curves in rats given RTE and CUBE. For subsequent experiments extracts were prepared using the butanol, acetic acid, and water solvent. Chicken ultimobranchial extract and rat thyrocalcitonin (RTCT) were assayed as previously described. In comparing the potency of the extracts to standard B thyrocalcitonin, only the plasma calcium change at one hour was considered. The results of these assays are given in Figure 1. These results show that the hypocalcemic activity per $\mu$g of CUBE is much higher than that of RTCT. While the slopes of the two curves approach zero at approximately the same weight of extract, the logarithmic portion of the CUBE curve has a more negative slope. Comparison of these dose response curves to the curve of MRC standard B thyrocalcitonin, showed 10 $\mu$g RTCT and 5 $\mu$g CUBE were equivalent to 25 MRC mU of thyrocalcitonin. This dose was the amount necessary to lower the plasma calcium in our system 1 mg% in one hour. Again, it is important to note all assays were based only on the hypocalcemic activity, allowing us to compare our results with those from other laboratories.

The hormones, purified by gel filtration, were compared to crude
extracts to determine differences in responses. Column chromatography of 2 mg samples of RTCT and CUBE on Sephadex G-100 resulted in a five fold purification. The elution patterns from two typical column runs are shown in Figures 2 and 3. The column was standardized using two molecular weight standards: Bacitracin, $M_W = 1496$ and Cytochrome C. $M_W = 12,600$. Using Blue Dextran 2000, $M_W = 2 \times 10^6$, the void volume was determined to be 49 ml. It can be seen that the CUBE contained high molecular weight components that were excluded by Sephadex G-100 while the RTCT large proteins were not eluted until 35 ml after one void volume. Beginning with the fraction preceding the one containing one void volume, all fractions of each run were assayed.

The hypocalcemic activity in the rat thyroid extract eludes off at 126 ml while the peak activity from CUBE comes off considerably faster at 95 ml. These results suggest that chicken calcitonin extracted by this method is larger, confirming the observations of Copp; however, due to its tendency to aggregate, molecular weight measurements by gel filtration are inaccurate (Copp et al., 1968b; 1970).

The elution pattern for rat thyrocalcitonin from the standardized G-100 column allows a molecular weight estimation. We estimate the molecular weight of the hormone prepared by our methods to be 3700 (Figure 4). This number corresponds to the published molecular weight of porcine thyrocalcitonin (Potts et al., 1968).

Partially purified hormones were assayed against crude extracts of
the glands and MRC standard B thyrocalcitonin, and the observed responses were not different. Therefore, we concluded that it was not necessary to use the product of gel filtration for all experiments, and subsequent experiments used either CUBE or RTCT as noted.

Purification by ion exchange chromatography resulted in approximately a 5 fold increase in specific activity. This step was not different enough from Sephadex chromatography to warrant its use. A typical elution pattern for RTCT on carboxyl methyl cellulose is given in Figure 5. It can be seen that the activity is eluted off at a low molarity and is not far enough away from the bulk of inert protein to allow large increases in specific activity. This partially purified hormone also gave the same calcium and phosphate responses as the standard and the crude extracts.

The specific activity of the rat thyroid gland is approximately 10 MRCmU/mg. This is purified by extraction to give a specific activity of 2000 MRC mU/mg or a 200 fold increase in activity. The chromatography steps resulted in only a 4 to 5 fold increase over this value. Therefore, the RTCT and CUBE routinely used, although not homogeneous, are partially purified preparations.

The effects of the calcitonins in frogs

The first series of experiments was designed to test the response of frogs to calcium injection. The experiments were analogous to Talmage's early rat experiments (Talmage et al., 1965). Two groups of R. pipiens were used: control and ultimobranchialectomized (UEX). Following a 24
hour post surgical period, frogs, maintained in deionized water baths, received 5.0 mg calcium as calcium chloride per 100 gm body weight. Animals were then sacrificed and bled from the heart at each of the intervals noted in Table 2. The data indicated that this calcium dose definitely caused a transient hypercalcemia; however, there was no statistical difference between UBX and intact frogs.

The next experiments used intact and UBX R. catesbeiana. On Day 1 all animals were injected with 20 μCi $^{85}$Sr; then on Day 12 half were UBX. The ether anesthetized animals were fitted with lavage plugs on Day 13. On Day 14 both groups were bled and then lavaged for 6 hours with a lavage rinse containing 9.0 or 12.0 mg% Ca. Following the last drain, the animals were sacrificed and bled by heart puncture. The plasma calcium results are summarized in Table 3 and the lavage fluid calcium and $^{85}$Sr changes are shown in Figures 6 and 7.

No significant difference was observed between the plasma calcium values of intact and UBX frogs given a high calcium challenge. However, the lavage treatment did cause significant hypercalcemia. The lavage fluid calcium levels rose steadily after either 9.0 mg% or 12.0 mg% calcium lavage. The lavage fluid calcium levels of intact and UBX frogs were not statistically different; conversely, thyroidectomized rats show higher levels than intact rats (Talmage et al., 1965). Based on similar experiments with rats (Klein et al., 1967), the removal of $^{85}$Sr from deep bone should be retarded in intact frogs; but these results show no difference
between UEK and intact frogs in the rate of removal of the isotope by 
lavage. These experiments demonstrated that frog ultimobranchial calcit-
tonin does not suppress the resportion of stable bone or protect the 
frog against hypercalcemia.

The last series of experiments was designed to determine whether or 
not CUBE was hypocalcemia and/or hypophosphatemic. Male R. pipiens were 
divided into three groups. Group I was sacrificed and bled at time 0. 
Then half of each of Groups II and III were injected with saline, while 
the other half were given 500 MRC mU CUBE. Group II was sacrificed 1 
hour after injection, and Group III, 2 hours after injection. Plasma 
calcium and phosphate results are shown in Figure 8.

These results show no difference in the plasma calcium levels among 
the three groups. These data are consistent with others' observations, 
showing no effect of the hormone on calcium levels of lower vertebrates 
(Urist, 1967). However, two hours after CUBE treatment, the plasma phos-
phate values are statistically different. Because these results represent 
a small number of observations, they are not definitive; but they do indi-
cate a phosphate effect without a calcium effect. As a result of these 
observations, the next experiments attempted to relate the hypophosphate-
mic action of the hormones to their overall action.

**Influence of the thyroid on the response of rats to exogenous calcitonins**

Because of the minor or negative effect of the calcitonins on plasma 
calcium levels in lower vertebrates, a study was begun to determine the
hypophosphatemic potency of the calcitonins in mammals. In the course of this work an unexpected relationship between the thyroid and the response of rats was discovered. Concurrent experiments in this laboratory were comparing thyroidectomized (TX) to thyroid intact (TI) rats, all with functional parathyroid transplants. These experiments tested the response of the two groups to CUBE and RTCT.

For the standard experiment Holtzman or Cheek Jones rats, weighing 120-130 gms, received parathyroid autotransplants. After 10 days, the efficacy of the transplants was tested by determining the plasma calcium values after an overnight fast. Only animals with plasma calcium levels above 10.0 mg% were considered to be good transplants. Following the test bleed, half the animals were thyroidectomized (TX) and thyroxine therapy was begun. Forty-eight hours after thyroidectomy following a second overnight fast, the response of 5 μg CUBE or 10 μg RTCT was tested. Plasma samples taken prior to, 30, 60, and 120 minutes after injection, were analyzed for calcium and phosphate.

The dose of extract injected was reduced so that a minimal hypocalcemic response was obtained in thyroid intact animals. The plasma calcium and phosphate responses to RTCT are shown in Figure 9. This data shows that at every time period the hypocalcemic response is significantly greater in TX than in TI rats. The hypophosphatemic response in TX animals is greater at every time period, and is significantly greater at 60 and 120 minutes. In both groups, the hypophosphatemic response is
greater than the hypocalcemic response; i.e., the $\Delta \text{Ca:}\Delta \text{PO}_4$ ratio is less than one. In addition, the TI animals show a significant hypophosphatemia, but no significant hypocalcemia, at this dose level.

Figure 10 shows the plasma calcium and phosphate responses to CUBE. In contrast to RTCT, CUBE causes a small hypocalcemia in TI rats; even though TX plasma calcium levels are significantly lower at every time period. At 120 minutes the TI group is returning to normal but the TX group is still markedly hypocalcemic. The plasma phosphate responses of the two groups are not significantly different. The $\Delta \text{Ca:}\Delta \text{PO}_4$ at 60 minutes is less than one, and in comparing the two hormones CUBE is a much more potent hypophosphatemic agent than RTCT. While the dose given was well within physiological limits based on the hypocalcemic potency, it appears to cause a maximum phosphate response. These results can also be interpreted to mean that thyroidectomy increases the sensitivity of the hypocalcemic response but not the hypophosphatemic response.

Dietary status was then studied because of its relationship to the calcitonin content of the thyroid gland and the sensitivity of the animal to exogenous hormone (Hirsch and Cooper, 1968). For these experiments, four treatments were used. Group 1 followed the standard procedure. Group 2 animals were given food and tap water (~2 mg% Ca) throughout the experiment. Group 3 animals had lab chow throughout the experiment but their drinking water was supplemented with 2% calcium lactate. Group 4 rats were maintained on low phosphate diet (Nutritional Biochemical
Corporation, Cleveland, Ohio) and deionized water for five days prior to the experiment.

The results of these experiments are summarized in Table 4. Regardless of the dietary status of the animals, thyroidectomy results in an increased sensitivity to the hypocalcemic action of calcitonin. An overnight fast does not significantly change the calcium response. Although calcium lactate supplement caused an increased sensitivity in both groups, TX animals still fell more than TI. Low phosphate diet treatment abolished the hypocalcemic response in TI animals but did not affect the TX response. Diet alterations on the other hand varied the phosphatemic responses. The administered dose was hypophosphatemic in Group 2, however, TX and TI animals were not significantly different. Calcium lactate supplement decreased the hypophosphatemic response of Group 3 but the TX rats dropped significantly. Low phosphate diet did not alter the hypophosphatemic action of RTCT.

In Groups 2, 3, and 4 parathyroid hormone secretion is depressed, reducing bone resorption. Thus an effect of RTCT on the secretion or function of parathyroid hormone can be eliminated as a reason for the responses. If the calcitonins work solely to inhibit parathyroid induced resorption, a decreased sensitivity should occur in these test situations.

In considering these results, it is important to rule out any influence from the classic hormone of the thyroid, thyroxine (T<sub>4</sub>). The standard experiment (Group 1, Table 4) is the basis for comparison. In
each experiment the animals were transplanted as usual and half were thyroidectomized. For Series I, the rats did not receive $T_4$ supplement and were tested 48 hours after thyroidectomy. In Series II, all animals (both TX and TI) received $T_4$ for the 48 hour post thyroidectomy period. Series III was designed to determine the surgical effects of thyroidectomy. The TX animals were given $T_4$ replacement, but the experiment was conducted 7 days after surgery. The results of these experiments are summarized in Table 5. These results show that varying thyroxine treatment does not change the responses to RTCT. Similarly, a 7 day post surgical period had no effect on the responses.

Although the efficacy of the transplants was tested, the effect of stress from bleeding or treatment on the ability of the transplant to maintain its function was questioned. Experiments were run, holding the animals by hand for bleeding rather than subjecting them to a heat lamp or restraining cage. To rule out the effects of stress non-injected controls were run simultaneously with the hormone injected and vehicle injected animals. The results of these experiments were no different from those of the standard experiment. Consequently, they were included in the summaries when possible.

It is apparent that the presence or absence of the thyroid affects the response of the rat to the two calcitonins. It is often assumed, perhaps erroneously, that the removal of an endocrine gland increases the sensitivity of the animal to the administration of the hormone secreted by that gland. Many bioassays of hormones are based on this idea. In
all these cases, however, the removal of the gland results in a change in the particular parameter that is assayed. In these experiments, the plasma calcium and phosphate levels do not change after the removal of the thyroid; i.e., it is possible for animals to remain eucalcemic and euphosphatemic following thyroidectomy. In addition, the increased sensitivity was not always seen in the hypophosphatemic effect of the calcitonins, and when seen was usually less in magnitude. Therefore, the change in the response of the rat to the injected calcitonins, caused by prior thyroidectomy, was not only increased sensitivity but also appeared to be a change in the ratio of the hypocalcemic response to the hypophosphatemic response.

It can be concluded from these experiments that the presence of the thyroid gland in animals with functional parathyroid transplants, affords the animal protection against hypocalcemia resulting from the injection of CUBE or RTCT. These results along with others to be described led to the postulate that the fundamental action of the calcitonins is involved with their phosphate effects (Talmage and Kennedy, 1969).

Influence of the thyroid on the response of rats to high phosphate challenge

The previous results indicate that the thyroid protects the animal against forced hypocalcemia. This is inconsistent with the classical concept of the action of the hormone. If the hormones act by controlling phosphate movement, a high phosphate challenge would allow two observa-
tions: first, whether or not the thyroid protects against high phosphate; and second, if hypocalcemia caused by phosphate challenge is greater after thyroidectomy.

For the first series of experiments, parathyroid transplanted rats were thyroidectomized and maintained on T4 replacement for 48 hours. After a 12 hour fast, the animals were bled and then lavaged and bled at one hour intervals for 6 hours. The lavage fluid contained no calcium and 10 mg% phosphate. Plasma samples were analyzed for calcium and phosphate. The results of these experiments are shown in Figure 11.

One hour of lavage with high phosphate caused an increase in plasma phosphate. The TI animals tend to maintain a lower plasma phosphate than the TX rats, although the two groups are not significantly different at any point. The plasma calcium levels fall, confirming our previous data. The TI animals were able to maintain a significantly higher plasma calcium than the TX. This series of experiments gave the expected results except the animals did not become hyperphosphatemic.

The next series of experiments was designed to "physiologically" raise the plasma phosphate levels. The rats were thyroidectomized as usual and the experiment was conducted 48 hours later. The animals were bled and then all were nephrectomized. Blood samples were then taken for 14 hours after nephrectomy. The plasma calcium and phosphate values are shown in Figure 12. These results show the typical plasma calcium curve following kidney removal. There is a transient rise in calcium from 1 to
6 hours post nephrectomy, and then a steady fall beginning between 6 and 8 hours. The increase in plasma calcium is caused by a rise in calcium bound with citrate which is no longer being excreted. The liver then takes over the majority of citrate metabolism and plasma calcium begins to fall due to the rising plasma phosphate. During the transient rise in plasma calcium, TI animals maintain a lower plasma calcium than the TX. As the calcium levels fall below normal, however, the curves reverse and the TI animals maintain a higher plasma calcium level than the TX. Following nephrectomy little phosphate can be excreted; therefore, there is a rapid rise in plasma phosphate levels. By six hours after nephrectomy, the plasma phosphate levels of TX animals were significantly higher than those of TI and they remained higher throughout the 14 hour period.

These results confirm previous data for plasma calcium changes. In addition, they add another aspect to the general concept that the thyroid protects the animals against forced hyper- or hypophosphatemia. After the administration of calcitonin the plasma calcium and phosphate levels fall, but TI animals fall less than TX. Following calcium injection TX plasma calcium levels are higher than TI (Talmage et al., 1964). Finally, when plasma phosphate is rising after nephrectomy, the TI animals go up less. These results imply that the overall gross controls of plasma calcium and phosphate levels are the calcitonins. However, they do not show that the hormones control phosphate.

The next series of experiments was designed to determine whether or
not high phosphate challenge causes the secretion of thyrocalcitonin. If so, animals pretreated with high phosphate lavage (10 mg%) should show an immediate hypocalcemic response to high calcium. The rats were prepared in the usual manner. Following 5 1/2 hours of high phosphate lavage, the animals were rinsed for three hours with fluid containing 10 mg% Ca. In the converse experiment, 3 1/2 hours of high calcium lavage was followed by 2 1/2 hours of high phosphate challenge. Figure 13 shows the results of these experiments.

As expected, high phosphate lavage caused a fall in plasma calcium; however, as shown in Figure 13A, the TI animals maintained a higher calcium than the TX. This difference became significant at 2 1/2 hours. If high phosphate causes the secretion of thyrocalcitonin, then TI lavage calcium levels should be significantly lower at the 6 1/2 hour drain. These results show in the 60 minutes following the change in lavage fluid, the calcium rises to the point that thyrocalcitonin is secreted, and at 7 1/2 hours the classical calcium effect is observed. Figure 13B shows a significant hypocalcemic response in TI animals for the first 3 1/2 hours. Since the hormone is being secreted at a high rate, the switch to high phosphate lavage has no effect except to cause a fall in lavage calcium.

These results indicate that phosphate challenge does not cause the secretion of thyrocalcitonin. However, since the results clearly show the stated "protective" response of TI animals to phosphate challenge at plasma calcium levels below 8.5 mg%, it is necessary to reevaluate the
physiological function of this family of hormones. This led to the postulate that the calcitonins cause phosphate movement toward bone and the calcium retention follows (Talmage et al., 1969; Talmage, 1969).

Experiments to determine the role of the kidney in the function of the calcitonins

The observed \( \Delta \text{Ca:} \Delta \text{PO}_4 \) ratios in experiments with injected calcitonins led us to question the concept that the hormones acted only to decrease bone resorption. Hirsch reported, however, that porcine thyrocalcitonin, as well as rat thyroid homogenates, was fully active in rats after acute nephrectomy (Hirsch et al., 1964). The experiments to be described were designed to test the hypocalcemic and phosphatemic potency of RTCT in animals with high and low plasma calcium and steadily increasing plasma phosphate following nephrectomy.

For the first series of experiments a number of sealed ampules of house standard RTCT were prepared. After an overnight fast, the rats were randomly divided into two groups, one of which received 25 MRC mU RTCT subcutaneously. The animals were bled prior to, one, and two hours after the administration of RTCT. Five hours after the first injection, the animals were again randomized into two groups and all nephrectomized. One hour after nephrectomy one group received RTCT. The same randomization procedure was followed prior to RTCT injection at 12 and 24 hours after nephrectomy; however, the animals were bled before and one hour after the injection. The animals remained without food and with deionized
water throughout the experiment. The time schedule and results are shown in Figure 14.

The fall in plasma phosphate caused by RTCT was almost constant in the animals before and after nephrectomy despite the rising plasma phosphate caused by kidney removal. The hypocalcemic effect of the hormone, however, was greater at each time of treatment. As stated earlier, during the period from 1 to 6 hours after nephrectomy there was a transient hypercalcemia; therefore, the results of the one hour injection were hard to interpret. Since the animals remain almost eucalcemic during the twenty four hour period after nephrectomy, and the dose of RTCT was constant for all injections, the only change that could explain the increased sensitivity of nephrectomized rats to RTCT was the rising plasma phosphate. This observation confirms the work of Hirsch (1968). This data proved conclusively that the kidney was not necessary for the full hypocalcemic and hypophosphatemic effects of RTCT. It did not rule out an effect of the hormone on the kidney; however, any effect must be negligible with respect to the overall action of thyrocalcitonin.

Robinson et al., (1967) reported that porcine thyrocalcitonin did not cause further hypocalcemia in parathyroidectomized rats with plasma calcium levels below 7 mg%. Their report stated, however, that the hormone preparation was hypophosphatemic in the parathyroidectomized animals and that this response could be abolished by prior nephrectomy. Our results in nephrectomized animals suggested that these observations were
erroneous. The next experiments were designed to determine the effects of RTCT in parathyroidectomized (PTX) and parathyroidectomized-nephrectomized (PTXNEPHRX) rats. For this series, the animals were fasted overnight and randomly divided into two groups. One group was given RTCT and all the animals were bled 30 and 60 minutes after the injection. Following a 2-day rest with food, the animals were fasted beginning at 5:00 p.m. and PTX at 11:00 p.m. On the following day, the pre and post nephrectomy schedule was the same as in the first series of nephrectomy experiments. Injections at 12 and 24 hours after NEPHRX were not possible because most of the animals died in tetany within 12 hours after the surgery.

The results of this series of experiments are shown in Figure 15. The pre-PTX injection shows the usual response to RTCT. As expected, the increase in plasma phosphate resulting from PTX caused an increased hypocalcemic response to the hormone 10 hours after PTX. Nephrectomy in PTX animals did not result in a further increase in plasma phosphate at the time measured. In fact the plasma phosphate fell approximately 1 mg% in the first hour after nephrectomy. The same relationship to high plasma phosphate was seen in these experiments. The rise in plasma phosphate led to an increased sensitivity of the animals to the hypocalcemic effects of RTCT, but had no effect on the sensitivity of the hypophosphatemic response.

It is important to note the $\Delta$Ca:$\Delta$PO$_4$ ratio following RTCT administration to these treated animals changes greatly. The normal $\Delta$Ca:$\Delta$PO$_4$ ratio
in intact rats given RTCT is 0.67. This ratio varies to as high as 1.37 after 24 hours after nephrectomy. Table 6 shows the ΔCa:ΔPO₄ ratios for both series of experiments. The ΔCa:ΔPO₄ ratio is significantly higher in all of the treated groups than the intact animals ratio. The most important observation to be made from this data is that the ΔPO₄ values for all the groups are not significantly different. The differences in the ΔCa:ΔPO₄ ratios result from the changes in the ΔCa values. These results can be interpreted to mean that the function of the hormones is to lower plasma phosphate and the changes in plasma calcium are results of the change in phosphate, and influenced by the plasma levels of calcium and phosphate. This supports the postulate that the calcitonins affect phosphate movement in bone (Kennedy et al., 1969).

These experiments were subsequently confirmed by experiments comparing the pancreatic hormone glucagon to thyrocalcitonin. The experiments were designed the same way except that an additional group of rats was given glucagon. The responses to RTCT were exactly the same as those described here (Tanzer et al., 1970).

Experiments to determine the effect of adrenalectomy and cortisol on thyrocalcitonin function

The adrenal corticosteroids have been shown to significantly lower plasma phosphate in rats, causing a decreased sensitivity to the hypocalcemic action of thyrocalcitonin (Thompson et al., 1968). This observation is consistent with our postulate that the action of the calcitonins
is related to plasma phosphate levels (Kennedy et al., 1969). The next
group of experiments was designed to test the effect of adrenalectomy and
the glucocorticoid, cortisol, on bone and kidney, and to relate these ef-
ficts to thyrocaltcitonin function.

For the first series of experiments, animals prelabeled with \(^{45}\)Ca
and \(^{32}\)P (100 \(\mu\)C each) 14 days before the experiment were divided into
four groups: Group 1, controls; Group 2, thyrropectomized (TX); Group 3,
adrenalectomized (ADX); Group 4, adrenalectomized and thyroidectomized
(ADXTX). Adrenalectomy and thyroidectomy were performed simultaneously
48 hours prior to the experiment. After an initial blood sample was
obtained, 2.5 mg calcium \(\equiv\) calcium chloride/100 gm body weight was in-
jected intraperitoneally. A second injection was given 30 minutes later.
Blood samples were taken at one and two hours following the initial cal-
cium injection.

The plasma calcium values are shown in Figure 16. Adrenalectomy did
not alter plasma calcium in intact or thyroidectomized rats. In addition, at
both one and two hours after calcium administration, Groups 2 and 3
were significantly higher than their thyroid intact counterparts. These
results showed that adrenalectomy did not have any effect on the hypo-
calcemic action of thyrocaltcitonin.

The plasma phosphate values are summarized in Figure 17. Adrena-
lectomy itself had no effect on plasma phosphate in thyroid intact or TX
animals. Following calcium administration, the plasma phosphate rose in
all groups except the control group. At one hour after injection, the ADX animals were significantly higher than their adrenal intact counterparts. By two hours, the plasma phosphate in adrenal intact animals was returning to normal; whereas, the ADX groups were still rising. These results indicate that the thyroid intact animals maintained lower phosphate levels than the TX following calcium injection; but, that adrenalectomy negated this effect.

The normalized data for $^{45}$Ca and $^{32}$P are summarized in Tables 7 and 8. Table 7 shows the $^{45}$Ca values during the experiment. While adrenalectomy alone had no effect on total calcium, it significantly affected plasma $^{45}$Ca. 48 hours after adrenalectomy, plasma $^{45}$Ca was 30% higher in the ADX group. Removal of the adrenals did not affect the total calcium responses to calcium challenge in TX and TI animals; however, it abolished the suppressive effect of the hormone on the removal of $^{45}$Ca from stable bone. Table 8 shows the plasma $^{32}$P values for the same time period. While the $^{32}$P levels were not statistically different between ADX and adrenal intact groups at 0 time, the ADX animals were much higher. The difference between TX and control animals was similar to that seen in plasma total phosphorus, while the controls stayed the same, the TX animals rose after the injection of calcium. As in total phosphate, adrenalectomy abolished the difference between ADX and ADXTX $^{32}$P levels. Both groups were rising together at 2 hours after calcium injection. The radioactivity results indicated that adrenalectomy increased $^{45}$Ca and $^{32}$P removal from bone while abolishing the ability of thyrocalcitonin to suppress the removal of these isotopes. However, adrenalectomy did not affect the abil-
ity of thyrocalcitonin to lower plasma total calcium.

To test the specificity of the responses in adrenalectomized animals, it was necessary to prove the completeness of adrenalectomy. As stated, only animals that could not survive for 24 hours at 4°C were used as ADX rats. In addition, blood glucose levels were determined for all animals before and after adrenalectomy. Plasma glucose levels are shown in Table 9. Adrenalectomy caused a significant drop in plasma glucose levels 48 hours after surgery. Animals with normal plasma glucose were used in the adrenal intact group. Calcium injection had no significant effect on plasma glucose levels at one hour.

The next series of experiments were designed to test the effect of adrenalectomy on the renal clearance of calcium and phosphate. Because of the well documented effects of parathyroidectomy on urine calcium and phosphate (Talmage and Kraintz, 1954), the influence of adrenalectomy on these changes was studied. The following groups were used: a) controls, b) adrenalectomized only, c) parathyroidectomized only, d) adrenalectomized and parathyroidectomized concurrently, and e) parathyroidectomized five days after adrenalectomy. The animals were injected 14 days prior to the first surgical treatment with $^{45}$Ca. Blood samples were taken daily for nine days. The procedure on the day of parathyroidectomy differed as follows: blood samples were taken from all animals; a water load was given by stomach tube (5% of body weight). After a two hour urine collection the appropriate groups were parathyroidectomized. Blood samples were
taken at 1, 2, 4, and 6 hours after PTX. A second water load was given at 2 hours, and a urine collection was made between the second and sixth hours post-PTX. For most of the experiments the animals were maintained on low calcium diet beginning one day prior to the adrenalectomy. A duplicate series of experiments was run maintaining the animals on stock lab chow.

The plasma calcium and phosphate changes throughout experiments in which the animals were maintained on low calcium diet are shown in Figure 18. Again adrenalectomy alone did not significantly affect subsequent plasma calcium or phosphate concentrations. Also, in every case, parathyroidec- tomy was followed by a rapid fall in plasma calcium levels and a concomitant rise in plasma phosphate levels. While it is obvious that ADX did not prevent the plasma calcium fall after PTX, it did appear to slightly decrease the rate of fall. The drop in PTX vs. ADXPTX at 4 hours is significant a p < .05. Similar results were obtained in experiments in which the animals were maintained on stock diet.

Urine calcium and phosphate are given in Table 10. Typical changes for both urine calcium and phosphate concentrations following PTX can be noted in animals with functional adrenals. Immediately after PTX there was a rise in urinary calcium and a drop in urinary phosphate. These results are consistent with the reported values (Talmage and Kraintz, 1954). Adrenalectomy affected these results in two ways. It prevented the rise in calcium excretion following PTX, and significantly lowered
phosphate excretion in parathyroid and thyroid intact rats. Adrenalectomy appeared to increase the renal tubular reabsorption of both ions therefore negating the usual effect of PTX on calcium excretion but not on phosphate.

As in the series of experiments with ADXTX rats, adrenalectomy significantly increased the removal of $^{45}\text{Ca}$ from stable bone pools. This data is summarized in Table II. Five days after adrenalectomy, the adrenal intact animals were approximately 30% lower than their ADX counterparts. These results also showed that adrenalectomy did not affect the decrease in bone resorption, measured by $^{45}\text{Ca}$ removal from stable bone, that follows parathyroidectomy.

The results of these experiments led us to postulate that the effects on plasma phosphate that we had seen following calcium injection to ADXTX animals, were a result of the effects of adrenalectomy on the renal excretion of phosphate. In addition, adrenalectomy had no significant effect on the ability of the parathyroids or thyrocalcitonin to maintain eucalcemia and euphosphatemia (Talmage and Kennedy, 1970).

**Review of experiments studying the effect of cortisol on thyrocalcitonin function run concurrently in this laboratory**

The results of published experiments being run concurrently in this laboratory are included here to help develop the argument (Talmage et al., 1970). This series of experiments was designed to test the effect of cortisol on the function of endogenous thyrocalcitonin. The natural
cortocosteroid was given subcutaneously to half the rats for 5 days prior to the experiment. The dose of 5 mg/kg body wt/day resulted in a retardation of weight gain and a reduction in endochondrial bone formation (Jee et al., 1966); however, it had no effect on number or morphology of osteocytes, osteoblasts, or osteoclasts. Following cortisol treatment and 48 hours after thyroidectomy, the animals were subjected to a high calcium challenge administered by peritoneal lavage with fluid containing 12.0 mg% Ca. The animals were lavaged for 4 hours at half hour intervals.

The plasma and lavage calcium values are shown in Figure 19. Treatment with cortisol had no effect on the ability of TI animals to maintain a significantly lower plasma calcium level than TX rats. Also, it did not effect the transport of calcium from the lavage rinse into the animals. As had been reported before, the TX animals are not able to transport calcium as rapidly.

Plasma and lavage phosphate values are shown in Table 12. Although the TX animals tended to transfer more phosphate to the lavage fluid than TI, there were no significantly different points. This trend was not seen in the cortisol treated animals. Plasma phosphate levels were significantly higher in TX animals. This difference was also seen in cortisol treated rats but the differences were smaller and only border line significance was seen.

The only effect of cortisol seen in this series of experiments was in the rate of removal of $^{85}$Sr from stable bone. These results are shown
in Figure 20. Cortisol itself significantly suppressed the turnover from deep bone as shown by the lower plasma $^{85}$Sr in cortisol treated animals. Later experiments showed that cortisol at this dose also suppresses $^{32}$P turnover from bone. Administration of the steroid did not affect the usual differences in rates of removal in TX and TI animals. As in the total calcium results, the transfer of calcium from the lavage rinse into the animal was less in TX rats so the removal of $^{85}$Sr from bone was greater.

These three series of experiments showed that the absence of the adrenal corticosteroids resulted in an increased movement of calcium and phosphate from stable bone and conversely pharmacological levels of cortisol caused a marked suppression of the removal of these ions from bone as shown by $^{45}$Ca and $^{32}$P movement. It is also important to note the effect of adrenalectomy on the renal excretion of the two ions. There was a marked retention of calcium and phosphate by the kidney in adrenalectomized rats but no effects were seen on plasma total calcium and phosphate. Our results showed that there were only minor effects of adrenalectomy on parathyroid function, and while cortisol had no effect on thyrocalcitonin function, adrenalectomy significantly altered the thyroid intact animals ability to lower plasma phosphate following calcium challenge (Talmage and Kennedy, 1970; Talmage et al., 1970).

**Responses of thyroidectomized and thyroid intact rats to calcium injection**

The next group of experiments was designed to further study phosphate
changes following calcium injection to TX and TI rats. Previous experi-
ments had shown that calcium administration caused an unexpected rise in
plasma phosphate in TX animals while TI rats remain euphosphatemic.

The standard experimental procedure was as follows: Parathyroid
transplanted rats, weighing 180-220 gms, were thyroidectomized; radiocal-
cium and radiophosphorus were administered either 12 hours or 10 days
prior to the experiment. Forty-eight hours post thyroidectomy, after an
overnight fast, the animals were bled, injected intraperitoneally with
calcium (2.5 mg Ca as CaCl₂/100 gm body weight); then bled at 1, 2, and 4
hours after the injection.

The plasma calcium and phosphate results are shown in Figure 21. As
expected, calcium administration caused a marked hypercalcemia in TX rats.
The dose of calcium used caused only a slight rise in plasma calcium
levels in intact animals, followed by a significant fall due to thyro-
calcitonin (Talmage et al., 1964). As in the previous experiments, cal-
cium injection caused a significant rise in plasma phosphate levels in TX
rats; conversely, the intact group fell significantly. At both 1 and 2
hours after calcium administration, the calcium and phosphate levels of
the two groups were different with \( p < .001 \). This data reemphasizes that
the presence of the thyroid not only protects against hypercalcemia but
also against the increase in phosphate caused by calcium administration.

Plasma \(^{45}\)Ca and \(^{32}\)P levels for experiments in which the isotopes are
in stable bone (10 day label) are shown in Figure 22. There was a marked
suppression of removal of $^{45}$Ca from stable bone as a result of calcium injection to TI rats, but there was no change in the TX group. Similarly, there was a suppression of $^{32}$P removal from stable bone in TI animals. At both one and two hours after calcium injection, the plasma $^{32}$P in the TI group was significantly lower than the pre-injection level. The TX rats, on the other hand, showed a significant increase in plasma $^{32}$P at one hour after injection. The TI group is significantly lower than the TX at 1, 2, and 4 hours after injection. The suppression of stable $^{45}$Ca and $^{32}$P removal after calcium injection is caused by a decrease in bone resorption in response to the secretion of thyrocalcitonin. However, in thyroidectomized animals, there is an increase in removal of $^{32}$P but not $^{45}$Ca from deep bone.

The results of the experiments in which the isotopes were injected 12 hours prior to calcium injection are shown in Figure 23. In this figure the dotted line represents approximately a 30% fall in plasma radioactivity over the 4 hour experimental period in control animals not given calcium. There was no difference between the plasma $^{45}$Ca levels of TX and TI rats in the four hours after calcium injection. In addition, the calcium treated animals fell the same as controls over the 4 hour period.

For the first hour after calcium injection the fall in plasma $^{32}$P was the same in both groups. By two hours, there was a significant rise in plasma $^{32}$P in TX animals while the TI group continued to fall. At two
hours, the groups were significantly different. The TX and TI groups are the same and higher than the controls at four hours. The data from this series of experiments suggested that the thyroid (thyrocalcitonin) causes a suppression of removal of $^{32}$P from both stable and freely exchangeable bone. Also, the calcium and phosphate effects of the hormone on bone are not concomitant; i.e., thyrocalcitonin can effect phosphate without changing calcium (Kennedy and Talmage, 1970a).

In order to rule out renal retention of phosphate in TX animals as a cause for the observed differences, two types of experiments were conducted. Series I followed the standard protocol, except that all the animals were nephrectomized (NX) 10 hours before calcium injection. As a result of nephrectomy, the starting plasma phosphate levels in these experiments were very high (~18 mg%). Series II also used nephrectomized animals; but prior to calcium injection, both groups (TX and TI) were lavaged for 3 1/2 hours with fluid containing 7 mg% calcium and no phosphate. The lavage procedure was designed to lower plasma phosphate while maintaining plasma calcium (Talmage et al., 1957).

The results from Series I are shown in Figure 24 and Table 12. There is a significant difference between TX and TI plasma calcium levels after calcium administration. As expected, the TI animals were able to maintain lower calciuems even at these relatively low starting plasma levels. Thyroidectomy itself had no effect on plasma phosphate, but calcium injection caused a significant rise in TX animals, and TX and TI
were significantly different at 2 and 3 hours after injection.

The radioactivity results (12 hour label), shown in Table 13, are harder to interpret because of the abnormally high phosphate levels. Thyroidectomy had no effect on the preinjection $^{32}$P levels, but following calcium injection there was a significant suppression of removal from freely exchangeable bone in TI rats. Plasma $^{45}$Ca was not different in TX and TI rats after calcium injection.

The results of Series II verified that the kidney is not necessary for the observed response. These results are shown in Table 14. Since the animals had been nephrectomized for 18 hours, the prelavage plasma phosphate levels were higher than those for Series I rats. The lavage treatment resulted in a drop in plasma phosphate or about 3 mg%.

Following calcium injection, the TX animals showed higher plasma phosphates than the TI group at one and two hours. The plasma calcium values of the TI group were significantly higher than the TX levels at the prelavage bleed, further confirming our earlier results, but the two groups showed no differences after calcium injection. As expected, the $^{45}$Ca plasma levels were not different at any bleed but the plasma $^{32}$P levels tended to be higher in TX rats after calcium administration.

Both Series I and II experiments rule out the kidney and relate the observed responses to bone. These results, however, are more interesting when interpreted in light of earlier studies by Minkin and Talmage (1968). Their study showed that thyrocalcitonin was not secreted in animals with
plasma calcium levels below 8.5 mg%. In the Series I experiments, the plasma calcium levels never rose above 8.6 mg%. Therefore, there should have been little hormone secreted in the TI rats, and the subsequent phosphate differences did not appear to be due to thyrocalcitonin.

In order to study the effects of the hormone in rats with low plasma calciums and no parathyroid hormone induced bone resorption, the next group of experiments conducted used parathyroidectomized (PTX) lavaged rats. The animals were thyroidectomized 48 hours prior to lavage and then PTX 12 hours before the experiment. All rats were lavaged 3 1/2 hours using a rinse containing 7.0 mg% calcium and no phosphate prior to calcium injection.

The results of these experiments are shown in Table 15. The PTX treatment resulted in starting plasma calcium levels well below 7.0 mg%. The lavage rinse caused only a slight rise in plasma calcium, and following calcium administration, there were no significant differences between TX and TI groups. Conversely, lavage treatment caused a decrease in plasma phosphate in both groups so that the animals were euphosphatemic. After calcium administration there was a marked increase in plasma phosphate in both groups. The TX rats tended to be higher throughout the period after calcium injection, but there were no statistical differences because of great variability.

In order to study these responses more carefully, the lavage procedure was dropped out and the experiments run on PTX animals with high
phosphate levels. For these experiments, thyroidectomized and intact rats were PTX from the eye 12 hours prior to calcium adminstration, then the usual protocol followed. Both 10 day and 12 hour radioisotope labels were used.

The plasma calcium and phosphate values are shown in Figure 25. All animals had starting plasma calcium values of less than 7.0 mg%. The TI animals tended to have higher plasma calcium and these differences were significant at some time periods. However, there was no evidence of a hypocalcemic effect of thyrocalcitonin under these conditions. The plasma phosphate levels of the TX group were significantly higher in some experiments but overall the high phosphates were variable and not significantly different.

The plasma $^{32}$P and $^{45}$Ca (10 day label) levels are shown in Figure 26. This figure shows the results of two experiments separately. The dashed line shows the results of experiments in which the rats were TX for 48 hours. The solid line shows the results obtained when the animals were TX 4 hours prior to receiving calcium. Plasma $^{45}$Ca levels were not different following calcium injection. Forty-eight hours after TX and 12 hours after PTX (dashed line), the plasma $^{32}$P levels in the TX group were significantly higher; however, after calcium injection, the $^{32}$P in the TX rats showed a rise while the TI groups levels fell significantly. In the experiment using acutely TX animals, the TX and TI groups had the same $^{32}$P levels prior to injections. Following calcium administration,
the TX $^{32}$P levels stayed the same while the TI plasma $^{32}$P levels fell significantly. These results indicated a retention of stable bone phosphate ($^{32}$P) due to the presence of thyrocalcitonin.

The $^{45}$Ca and $^{32}$P values for experiments using 12 hour label are shown in Figure 27. Again, TX had no effect on the response to calcium injection. The $^{45}$Ca values were the same for both groups throughout the experiment. As in experiments using parathyroid intact animals, the short label $^{32}$P increased in TX animals while falling significantly in TI rats in the 3 hours after calcium injection. These experiments further support our concept that thyrocalcitonin causes a phosphate retention by all fractions of bone.

A final series of experiments was run in which parathyroid hormone secretion and function were almost totally suppressed by maintaining the animals on low phosphate diet for 3 days prior to the experiment. After the third day on low phosphate diet, the animals were PTX 12 hours, and TX 4 hours before calcium injection. The pretreatment resulted in PTX animals with normal plasma calcium and phosphate levels for the duration of the experiment. The results of these experiments are shown in Figure 28.

Calcium injection resulted in an increase in plasma calcium in both groups, but there was no difference between TX and TI values at any time period. The lower plasma calcium level in the TX group at 0 hour was not significantly different from TI. The plasma phosphate levels did not
change significantly in TX rats throughout the experiment. Following
calcium injection, however, the plasma phosphate in TI animals fell and
was not back to normal at three hours after the injection. In addition
to being another example of a phosphate response to thyrocalcitonin when
no calcium response is observed, these results show that at normal plasma
calcium and phosphate levels, a retention of phosphate by bone caused by
thyrocalcitonin does not cause hypocalemia (Kennedy and Talmage, 1970b).
CONCLUSIONS

It is frequently stated rather dogmatically in the literature that the calcitonins act in mammals by inhibiting bone resorption. There is no doubt that one of the results of these hormones is to decrease bone resorptive processes. However, the studies reported here give convincing evidence that this is a secondary, not the primary action of this family of hormones. This conclusion is based on the irrefutable evidence that the $\Delta\text{Ca:}\Delta\text{P}_i$ ratios following hormone administration are variable, and in all cases studied except for late nephrectomy, were always less than unity. The evidence for greater phosphate changes than calcium include studies on the administration of calcitonins from a variety of species. Other situations in which the $\Delta\text{Ca:}\Delta\text{P}_i$ varied were following thyroidectomy and nephrectomy. This is a rather important point as resorption of bone or inhibition of such processes should produce changes in calcium and phosphate removal in the ratio these ions exist in bone salts, which is 1.25. This type of effect on bone resorption was demonstrated by Talmage, et al., (1959) for parathyroid action on bone.

It is therefore suggested that the calcitonins act on bone cells in a way which affects calcium and phosphate removal from bone, but not specifically on bone resorption processes. Some of the factors that appear to be able to change the $\Delta\text{Ca:}\Delta\text{P}_i$ are such things as the diet, the type of calcitonin used (species), and particularly the phosphate homeostasis existing in the animal at the time the hormone titer rose (endogenous or exogenous).
Figure 29 shows a direct relationship between plasma phosphate and the $\Delta\text{Ca} : \Delta P_i$ resulting from the injection of RTCT or CUBE. The results from several experiments using equal, standardized doses of the hormone are included to show this relationship. The response is measured as $\Delta\text{Ca} : \Delta P_i$ to emphasize that in normal cases the $\Delta P_i$ is constant but the ratio varies from 0.1 to 1.0. It can be seen that if the initial plasma phosphate level is between 7 and 14 mg%, the $\Delta\text{Ca} : \Delta P_i$ is directly proportional. The maximum $\Delta\text{Ca} : \Delta P_i$ ($\sim 1.25$) is obtained at 15 mg% and higher phosphate levels have no further effect. It is interesting, though unexplained at this time, that the curve levels off at the $\Delta\text{Ca} : \Delta P_i$ equal to the Ca:P$_i$ in bone.

Having shown a variability in the changes in plasma calcium and phosphate resulting from the action of these hormones, attention was next turned toward directly relating the physiological function of the calcitonins to bone phosphate. In these studies, many situations arose where there were changes in phosphate without changes in calcium. Such observations would also support the postulate that the calcitonins do not work solely by inhibiting bone resorption.

In intact rats with low plasma phosphate, exogenous thyrocalcitonin causes a normal change in plasma phosphate but no concomitant change in plasma calcium. Using radioactive tracers in both stable and freely exchangeable bone, changes in plasma $^{32}$P are observed without changes in plasma $^{45}$Ca. Furthermore, in parathyroidectomized animals with plasma
calcium below the level at which thyrocalcitonin is thought to be secreted, the thyroid causes a suppression of removal of $^{32}$P from bone without affecting $^{45}$Ca movement. Plasma total phosphate changes without calcium changes are seen following calcium injection to parathyroidectomized animals fed low phosphate diet to maintain their normal plasma calcium and phosphate levels. This data indicates that either thyrocalcitonin acts on bone phosphate or there is a new hormone directly affecting phosphate transport.

In summary, we conclude that thyrocalcitonin directly affects phosphate transport in mammalian bone. Since we are not as yet in favor of a new hormone, there are two explanations of how thyrocalcitonin works. The first is that the hormone acts independently on calcium and phosphate transport in bone. The alternate explanation and the one which we prefer is that the calcitonins affect phosphate transport in all species and in mammals thyrocalcitonin acts primarily on phosphate transport producing, secondarily, a suppression of calcium transport from bone.
TABLE 1. Comparison of calcitonins from three species injected into rats.

<table>
<thead>
<tr>
<th></th>
<th>Mg Ca/100 ml plasma</th>
<th></th>
<th>Mg Pi/100 ml plasma</th>
<th></th>
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<td></td>
<td>Preinjection</td>
<td>1 Hr</td>
<td>2 Hr</td>
<td>Pre</td>
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<td>RTCT (10)</td>
<td>10.8 ± .05</td>
<td>8.5 ± .1</td>
<td>7.9 ± .09</td>
<td>10.1 ± .2</td>
</tr>
<tr>
<td>CUBE (10)</td>
<td>10.7 ± .09</td>
<td>8.4 ± .14</td>
<td>7.6 ± .19</td>
<td>10.2 ± .29</td>
</tr>
<tr>
<td>FUBE (6)</td>
<td>10.1 ± .08</td>
<td>9.4 ± .13</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Changes in calcium and phosphate following injection were significant with $p < .001$.

RTCT = Rat thyroid extract
CUBE = Chick ultimobranchial extract
FUBE = Frog ultimobranchial extract

Number of animals in parenthesis.
TABLE 2. The response of ultimobranchialectomized and control R. pipiens to calcium injection (5.0 mg/100 gm B.W.).

<table>
<thead>
<tr>
<th></th>
<th>Mg Ca/100 ml Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>8.0 ± .6</td>
</tr>
<tr>
<td>UBX</td>
<td>7.0 ± .6</td>
</tr>
</tbody>
</table>

All points are the average of at least 4 animals ± standard error.
UBX = ultimobranchialectomized
Calcium injected at 0 Time.
TABLE 3. Response of ultimobranchialectomized and intact *R. catesbeiana* to calcium challenge administered through peritoneal lavage.

<table>
<thead>
<tr>
<th></th>
<th>Plasma Calcium in mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9.0 mg % Ca Lavage For 6 Hours</td>
</tr>
<tr>
<td>INTACT</td>
<td>(4) 11.4 ± .58</td>
</tr>
<tr>
<td>UBX</td>
<td>(4) 11.2 ± .12</td>
</tr>
</tbody>
</table>

UBX = ultimobranchialectomized

Number of Animals in parenthesis.
TABLE 4. Effect of diet on response of rat to RTCT.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Plasma calcium (mg/100 ml)</th>
<th></th>
<th>Plasma PO4(mg P&lt;sub&gt;i&lt;/sub&gt;/100 ml)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
<td>1 hr</td>
<td>2 hr</td>
<td>0 hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Starved overnight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TI (15)</td>
<td>10.4 ± .1</td>
<td>9.6 ± .1</td>
<td>10.0 ± .1</td>
<td>9.9 ± .2</td>
</tr>
<tr>
<td>TX (15)</td>
<td>10.5 ± .1</td>
<td>8.7 ± .1</td>
<td>9.3 ± .1***</td>
<td>10.3 ± .3</td>
</tr>
<tr>
<td>2. Regular diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TI (10)</td>
<td>10.5 ± .1</td>
<td>10.1 ± .1</td>
<td>10.1 ± .1</td>
<td>10.1 ± .3</td>
</tr>
<tr>
<td>TX (10)</td>
<td>10.5 ± .1</td>
<td>9.0 ± .1</td>
<td>9.7 ± .1***</td>
<td>10.6 ± .3</td>
</tr>
<tr>
<td>3. Ca-Lactate supplement</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TI (5)</td>
<td>10.3 ± .2</td>
<td>8.5 ± .2</td>
<td>9.6 ± .2</td>
<td>10.1 ± .5</td>
</tr>
<tr>
<td>TX (5)</td>
<td>10.0 ± .1</td>
<td>7.7 ± .1</td>
<td>8.2 ± .1***</td>
<td>10.7 ± .4</td>
</tr>
<tr>
<td>4. Low PO4 diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TI (5)</td>
<td>12.2 ± .3</td>
<td>12.1 ± .2</td>
<td>12.3 ± .2</td>
<td>6.8 ± .3</td>
</tr>
<tr>
<td>TX (5)</td>
<td>12.6 ± .4</td>
<td>9.6 ± .1</td>
<td>11.0 ± .3***</td>
<td>7.1 ± .5</td>
</tr>
</tbody>
</table>

All rats received 10 μg of the rat thyrocalcitonin (RTCT) preparation.

* p < .01
** p < .005
*** p < .001

Number of animals in parenthesis.

(Talmage and Kennedy, 1969)
TABLE 5. The effect of thyroxine and long term thyroidectomy on the response of rats to RTCT.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Mg Ca/100 ml Plasma</th>
<th>Mg P_i/100 ml Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1 Hr</td>
</tr>
<tr>
<td>1. All animals 10 μg T4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TX (5)</td>
<td>10.4 ± .12</td>
<td>7.7 ± .11</td>
</tr>
<tr>
<td>TI (5)</td>
<td>10.5 ± .12</td>
<td>9.0 ± .3</td>
</tr>
<tr>
<td>2. No T4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TX (5)</td>
<td>10.2 ± .13</td>
<td>8.4 ± .19</td>
</tr>
<tr>
<td>TI (5)</td>
<td>10.0 ± .08</td>
<td>9.2 ± .15</td>
</tr>
<tr>
<td>3. 7 Days Post TX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TX + T4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TX (14)</td>
<td>9.9 ± .1</td>
<td>8.6 ± .1</td>
</tr>
<tr>
<td>TI (5)</td>
<td>9.9 ± .2</td>
<td>9.3 ± .4</td>
</tr>
</tbody>
</table>

* p < .05
** p < .005
*** p < .001
TX = thyroidectomy
TI = thyroid intact
T4 = thyroxine
Number of animals in parenthesis.
TABLE 6. Plasma calcium and phosphate changes one hour after the administration of 25 MRC mU rat TCT.

<table>
<thead>
<tr>
<th></th>
<th>Pre-nephx</th>
<th>Post nephx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact</td>
<td>PTX</td>
</tr>
<tr>
<td>No. animals*</td>
<td>13</td>
<td>38</td>
</tr>
<tr>
<td>ΔCa in mg/100 ml</td>
<td>-1.0 ± .05</td>
<td>-1.6 ± .11</td>
</tr>
<tr>
<td>ΔPO4 in mg P/100 ml</td>
<td>-1.5 ± .13</td>
<td>-1.4 ± .15</td>
</tr>
<tr>
<td>ΔCa/ΔP1</td>
<td>0.67</td>
<td>1.14</td>
</tr>
</tbody>
</table>

PTX

<table>
<thead>
<tr>
<th></th>
<th>Pre-nephx</th>
<th>Post nephx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>ΔCa in mg/100 ml</td>
<td>-0.7 ± .05</td>
<td>-1.7 ± .12</td>
</tr>
<tr>
<td>ΔPO4 in mg P/100 ml</td>
<td>-1.1 ± .10</td>
<td>-1.3 ± .14</td>
</tr>
<tr>
<td>ΔCa/ΔP1</td>
<td>0.64</td>
<td>1.31</td>
</tr>
</tbody>
</table>

All changes significant at p < .005 determined by Student's t-test.
PTX = parathyroidectomized;
nephx = nephrectomized.
*Control group (non-injected) same size.
(Kennedy et al; 1969)
TABLE 7. Plasma $^{45}$Ca values two days after thyroidectomy-adrenalectomy (Normalized to Pre-Adx values).

<table>
<thead>
<tr>
<th>Time Sequence</th>
<th>-48 Hrs</th>
<th>Time 0</th>
<th>+1 Hr</th>
<th>+2 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Controls</td>
<td>100 ± 7.5</td>
<td>88 ± 10.0</td>
<td>78 ± 7.4</td>
<td>64 ± 10.0</td>
</tr>
<tr>
<td>2. TX at -48 Hrs</td>
<td>100 ± 5.6</td>
<td>94 ± 2.5</td>
<td>107 ± 2.5</td>
<td>87 ± 7.7</td>
</tr>
<tr>
<td>3. Adx at -48 Hrs</td>
<td>100 ± 6.9</td>
<td>133 ± 3.5</td>
<td>125 ± 3.4</td>
<td>105 ± 2.2</td>
</tr>
<tr>
<td>4. Adx at -48 Hrs</td>
<td>100 ± 4.7</td>
<td>127 ± 5.2</td>
<td>124 ± 4.2</td>
<td>112 ± 4.5</td>
</tr>
</tbody>
</table>

All values represent the average of at least 4 animals ± standard error. Calcium injected at Time 0 to all animals.

* 1 vs. 2 significant at p < .001.
** 1 or 2 vs. 3 or 4 significant at p < .001.

Adx = Adrenalectomy.
TX = Thyroidectomy.

(Talmage & Kennedy, 1970)
TABLE 8. Plasma $^{32}$P values two days after thyroidectomy-adrenalectomy. (Normalized to PreAdx values)

<table>
<thead>
<tr>
<th>Time Sequence</th>
<th>-48 Hrs</th>
<th>0</th>
<th>+1 Hr</th>
<th>+2 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Controls</td>
<td>100 ± 6.3</td>
<td>97 ± 4.5</td>
<td>106 ± 7.9</td>
<td>96 ± 3.1</td>
</tr>
<tr>
<td>2. TX at -48 Hrs</td>
<td>100 ± 4.7</td>
<td>106 ± 4.4</td>
<td>114 ± 6.7</td>
<td>133 ± 16.2</td>
</tr>
<tr>
<td>3. Adx at -48 Hrs</td>
<td>100 ± 2.8</td>
<td>111 * 6.0</td>
<td>111 ± 6.3</td>
<td>131 ± 10.8</td>
</tr>
<tr>
<td>4. Adx at -48 Hrs</td>
<td>100 ± 5.2</td>
<td>128 ** 3.7</td>
<td>120 ± 6.7</td>
<td>119 ± 4.9</td>
</tr>
</tbody>
</table>

* 1 vs. 3 significant at p < .05.
** 4 vs. 2 significant at p < .005.
*** 1 vs. 3 significant at p < .01.
**** 1 vs. 2 significant at p < .001.

Adx = adrenalectomy
TX = thyroidectomy

All points are the averages of at least 4 animals ± standard error. Calcium injected to all animals at 0 Time.
TABLE 9. Plasma glucose changes following adrenalectomy and thyroidectomy.

<table>
<thead>
<tr>
<th>Time Sequence</th>
<th>PreAdx - 48 Hr</th>
<th>0 Hr</th>
<th>+1 Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>136 ± 9.9</td>
<td>157 ± 6.8</td>
<td>129 ± 4.2</td>
</tr>
<tr>
<td>2. TX at -48 Hr</td>
<td>143 ± 8.5</td>
<td>153 ± 12.5</td>
<td>149 ± 16.3</td>
</tr>
<tr>
<td>3. Adx at -48 Hr</td>
<td>161 ± 10.1</td>
<td>85 ± 7.4</td>
<td>63 ± 3.7</td>
</tr>
<tr>
<td>4. Adx at -48 Hr</td>
<td>TX at -48 Hr</td>
<td>157 ± 10.4</td>
<td>89 ± 4.9</td>
</tr>
</tbody>
</table>

All values are the average of 8 animals ± standard error.

Adx = adrenalectomy
TX = thyroidectomy

* 1 or 2 vs. 3 or 4 different with p < .001.
### TABLE 10. Urine Ca and PO₄ changes after parathyroidectomy.

<table>
<thead>
<tr>
<th></th>
<th>Mg/hr X 10²</th>
<th>Urine Collections</th>
<th>1-3 Hrs Pre Time 0</th>
<th>2-6 Hrs Post Time 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Controls</td>
<td>0.65 ± .02</td>
<td>*0.25 ± .02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Adx at -5 Days</td>
<td>0.60 ± .04</td>
<td>0.17 ± .02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. PTX at Time 0</td>
<td>0.85 ± .07</td>
<td>1.25 ± .04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Adx at -5 Days PTX at Time 0</td>
<td>0.55 ± .02</td>
<td>**0.15 ± .01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate (P₄)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Controls</td>
<td>9.6 ± .4</td>
<td>7.6 ± 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Adx at -5 Days</td>
<td>1.5 ± .2</td>
<td>1.1 ± .4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. PTX at Time 0</td>
<td>7.8 ± .5</td>
<td>&lt; 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Adx at -5 Days PTX at Time 0</td>
<td>2.3 ± .1</td>
<td>&lt; 0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values represent the average of at least 5 animals ± standard error.

* 1 vs. 3 significant at p < .001.
** 3 vs. 4 significant at p < .001.
*** 5 or 7 vs. 6 or 8 significant at p < .001.
**** 5 or 6 vs. 7 or 8 significant at p < .001.
Adx = Adrenalectomized.
PTX = Parathyroidectomized.

(Talmage & Kennedy, 1970)
TABLE II. Plasma $^{45}$Ca values in rats parathyroidectomized 5 days after adrenalectomy (Normalized to Pre-Adx values).

<table>
<thead>
<tr>
<th>Time Sequence</th>
<th>-5 Days</th>
<th>0 Time</th>
<th>+ 1-2 Hrs</th>
<th>+ 4-6 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Controls</td>
<td>100 ± 6.2</td>
<td>89 ± 8.0</td>
<td>101 ± 7.1</td>
<td>110 ± 6.9</td>
</tr>
<tr>
<td>2. PTX at Time 0</td>
<td>100 ± 3.6</td>
<td>91 ± 5.0</td>
<td>80 **</td>
<td>94 **</td>
</tr>
<tr>
<td>3. Adx at -5 Days</td>
<td>100 ± 5.5</td>
<td>129 *</td>
<td>130 ± 5.5</td>
<td>135 ± 4.4</td>
</tr>
<tr>
<td>4. Adx at -5 Days</td>
<td>100 ± 5.2</td>
<td>135 ± 4.4</td>
<td>116 **</td>
<td>118 ± 1.5</td>
</tr>
</tbody>
</table>

PTX at Time 0

All values represent the average of at least 6 animals ± standard error.

* 1 or 2 vs. 3 or 4 significant at p < .001.
** 1 vs. 2 and 3 vs. 4 significant at p < .005.
*** 1 vs. 2 and 3 vs. 4 significant at p < .05.

Adx = Adrenalectomized.
PTX = Parathyroidectomized.

(Talmage & Kennedy, 1970)
TABLE 12. Plasma and lavage phosphate levels in cortisol treated rats given a calcium challenge by peritoneal lavage.

<table>
<thead>
<tr>
<th>Lavage Period**</th>
<th>Pre</th>
<th>1</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lavage Fluid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (5)</td>
<td>-</td>
<td>4.2 ± .2</td>
<td>3.3 ± .3</td>
<td>3.0 ± .2</td>
<td>2.6 ± .2</td>
<td>2.5 ± .1</td>
<td>2.1 ± .2</td>
</tr>
<tr>
<td>TX (5)</td>
<td>-</td>
<td>4.5 ± .1</td>
<td>3.6 ± .2</td>
<td>3.6 ± .3</td>
<td>3.0 ± .2</td>
<td>2.6 ± .1</td>
<td>2.3 ± .1</td>
</tr>
<tr>
<td>Control-cortisol (4)</td>
<td>-</td>
<td>4.3 ± .4</td>
<td>3.1 ± .1</td>
<td>2.8 ± .2</td>
<td>2.6 ± .1</td>
<td>2.6 ± .2</td>
<td>2.2 ± .2</td>
</tr>
<tr>
<td>TX-cortisol (5)</td>
<td>-</td>
<td>4.0 ± .3</td>
<td>3.6 ± .2</td>
<td>2.7 ± .1</td>
<td>2.7 ± .2</td>
<td>2.4 ± .3</td>
<td>2.1 ± .2</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (5)</td>
<td>11.5 ± .14</td>
<td>-</td>
<td>-</td>
<td>7.2 ± .33</td>
<td>-</td>
<td>6.6 ± .28</td>
<td>-</td>
</tr>
<tr>
<td>TX (5)</td>
<td>10.3 ± .41</td>
<td>-</td>
<td>-</td>
<td>8.1 ± .33</td>
<td>-</td>
<td>7.8 ± .67</td>
<td>-</td>
</tr>
<tr>
<td>Control-cortisol (4)</td>
<td>10.7 ± .47</td>
<td>-</td>
<td>-</td>
<td>7.2 ± .23</td>
<td>-</td>
<td>6.1 ± .14</td>
<td>-</td>
</tr>
<tr>
<td>TX-cortisol (5)</td>
<td>9.4 ± .2</td>
<td>-</td>
<td>-</td>
<td>7.6 ± .30</td>
<td>-</td>
<td>7.2 ± .44</td>
<td>-</td>
</tr>
</tbody>
</table>

* p < .05
** Lavage Periods are 30 min each
TX = thyroidectomized
Number of animals in parenthesis.
TABLE 13. Plasma $^{45}$Ca and $^{32}$P changes (12 hr label) following calcium injection (2.5 mg/100 g BW) to thyroidectomized rats 10 hours after nephrectomy.

<table>
<thead>
<tr>
<th></th>
<th>Counts/min as % of Pre TX</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 Hrs Post TX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre Calcium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>$^{45}$Ca</td>
<td>TX-NX (7)</td>
<td>79.4 ± .8</td>
<td>64.9 ± 1.5</td>
<td>62.6 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>TI-NX (8)</td>
<td>73.3 ± 1.1</td>
<td>60.3 ± 0.8</td>
<td>58.9 ± 0.3</td>
</tr>
<tr>
<td>$^{32}$P</td>
<td>TX-NX (7)</td>
<td>95.9 ± 2.7</td>
<td>88.5 ± 4.1</td>
<td>96.2 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>TI-NX (8)</td>
<td>89.6 ± 2.4</td>
<td>79.8 ± 2.9</td>
<td>78.9 ± 3.2</td>
</tr>
</tbody>
</table>

NX = nephrectomy
TX = thyroidectomy
TI = intact thyroid

* p < .05
** p < .01
*** p < .001

Number of animals in parenthesis
TABLE 14. The response of nephrectomized-thyroidectomized rats to calcium injection (2.5 mg/100 g BW) following buffer lavage.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mg/100 ml plasma</th>
<th>Pre lavage</th>
<th>Pre calcium</th>
<th>1 Hr</th>
<th>2 Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 Hr NX</td>
<td>7.2 ± .3</td>
<td>7.8 ± .2</td>
<td>8.6 ± .2</td>
<td>8.0 ±  .3</td>
<td></td>
</tr>
<tr>
<td>48 Hr TX</td>
<td>**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 Hr NX</td>
<td>8.6 ± .3</td>
<td>7.9 ± .2</td>
<td>8.7 ± .2</td>
<td>7.7 ±  .3</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>18 Hr NX</td>
<td>17.5 ± .4</td>
<td>15.1 ± .2</td>
<td>16.3 ± .6</td>
<td>17.7 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>48 Hr TX</td>
<td>**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 Hr NX</td>
<td>18.2 ± .6</td>
<td>14.3 ± .4</td>
<td>14.7 ± .5</td>
<td>15.2 ±  .6</td>
<td></td>
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</tbody>
</table>

* p < .05
** p < .01

TX = thyroidectomized
NX = nephrectomized
Number of animals in parenthesis.
TABLE 15. The response of parathyroidectomized-thyroidectomized rats to calcium injection (2.5 mg/100 g BW) following buffer lavage.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mg/100 ml plasma</th>
<th>Pre lavage</th>
<th>Pre calcium</th>
<th>1 Hr</th>
<th>2 Hr</th>
<th>3 Hr</th>
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<tbody>
<tr>
<td>Calcium</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>12 Hr PTX (5)</td>
<td></td>
<td>5.5 ± .2</td>
<td>6.6 ± .1</td>
<td>7.7 ± .2</td>
<td>6.7 ± .1</td>
<td>6.0 ± .1</td>
</tr>
<tr>
<td>48 Hr TX</td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 Hr PTX (7)</td>
<td></td>
<td>6.3 ± .2</td>
<td>6.9 ± .1</td>
<td>7.6 ± .1</td>
<td>6.7 ± .2</td>
<td>6.0 ± .1</td>
</tr>
<tr>
<td>Phosphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 Hr PTX (5)</td>
<td></td>
<td>11.3 ± .6</td>
<td>10.9 ± .9</td>
<td>12.6 ± 1.1</td>
<td>13.7 ± 1.1</td>
<td>13.2 ± .7</td>
</tr>
<tr>
<td>48 Hr TX</td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTX (7)</td>
<td></td>
<td>12.3 ± .4</td>
<td>10.7 ± .5</td>
<td>12.1 ± .4</td>
<td>13.0 ± .7</td>
<td>12.8 ± .6</td>
</tr>
</tbody>
</table>

* p < .025  
TX = thyroidectomized  
PTX = parathyroidectomized  
Number of animals in parenthesis.
Figure 1. Dose response curve for chicken ultimobranchial extract (CUBE) and rat thyrocalcitonin (RTCT) administered to rats. ⚫️ = CUBE; ▲ = RTCT. (Talmage and Kennedy, 1969).
Figure 2. Elution pattern of rat thyrocalcitonin on Sephadex G-100.
Dashed line shows the approximate activity peak. Column size 1.5 x 100 cm,
eluant 0.1N formic acid; temperature 4°C; flow rate 9.0 ml per hour; fraction
size 3.0 ml.
Figure 3. Elution pattern of chicken ultimobranchial extract on Sephadex G-100. Dashed line shows the approximate activity peak. For column specifications see Figure 2.
Figure 4. Gel filtration of rat thyrocalcitonin on Sephadex G-100 for molecular weight estimation. For column specifications see Figure 2. Column void volume, determined by Blue Dextran 2000 (M.W. = 2 x 10^6), 49ml. a = bacitracin (1496); b = cytochrome c (12,400).
Figure 5. Chromatography of rat thyrocalcitonin on carboxymethyl cellulose (Sigma). Column size, 0.9 X 15 cm; eluant, ammonium acetate, pH 4.0; linear increase in ionic strength from 0.01M to 0.5M (shown by dashed line); temperature 4°C; flow rate 8 ml per hour; fraction size 2.0 ml. Dotted line shows the approximate activity peak. \( \Delta Ca \) = change in plasma calcium at 60' in Meq/ L.
Figure 6. The response of intact and ultimobranchialectomized frogs to high calcium administered through peritoneal lavage. □ = intact; ○ = ultimobranchialectomized (UBX). Numbers in parenthesis equal number of animals.
Figure 7. Removal of $^{85}$Sr by lavage with 9.0 mg% calcium in intact and ultimobranchialectomized frogs. All values are normalized to the average of the first three washes. For legend, see Figure 6.
Figure 8. Plasma calcium and phosphate responses of intact Rana pipiens to 500 MRC μU chicken ultimobranchial extract. ○ = plasma calcium; □ = plasma phosphate; ⬇️ - time of injection. All points represent the average of at least 12 animals. **p < .01 for 2 hours vs. 1 hour and 2 hours vs. 0 hours.
Figure 9. Response of thyroidectomized and thyroid-intact rats, all bearing parathyroid transplants, to 10 µg of RTCT. TX = thyroidectomized rats; TI = thyroid-intact rats; p values: * < .01; ** < .005; *** < .001; □ = TI; ○ = TX. (Talmage and Kennedy, 1969)
Figure 10. Response of thyroidectomized and thyroid-intact rats to 5 µg (25 MRC mU) of CUBE. See Figure 9 for legend. (Talmage and Kennedy, 1969).
Figure 11. Plasma calcium and phosphate responses of thyroidectomized and thyroid-intact rats to phosphate challenge administered through peritoneal lavage. See Figure 9 for legend.
Figure 12. Plasma calcium and phosphate changes in thyroidectomized and thyroid-intact rats following bilateral nephrectomy. See Figure 9 for legend. NX = nephrectomy. ▼ = time of NX.
Figure 13. Response of rats to consecutive phosphate and calcium challenge administered through peritoneal lavage. ♦ indicates time of switch in lavage rinse. See Figure 9 for legend.
Figure 14. Response of plasma calcium and phosphate to thyrocalcitonin in rats before and after nephrectomy. 

- a) ○ = non-injected;  
- b) ○ = injected;  
- c) ⇣ = time of TCT administration;  
- d) *p < .005 difference from control, **p < .001 based on sum drop of injected group plus the increase in control values;  
- e) all points represent at least 30 animals. Note: phosphate scale = 1/2 calcium scale. (Kennedy et al., 1969).
Figure 15. Plasma calcium and phosphate responses to thyrocalcitonin in intact, parathyroidectomized, and parathyroidectomized-nephrectomized rats. All points represent the average of 8 animals; PTX = parathyroidectomized. See Figure 14 for legend. (Kennedy et al., 1969).
Figure 16. Plasma calcium changes following calcium injection to adrenalec-tomized-thyroidectomized rats. ADX = adrenalectomy; TX = thyroidectomy; number of animals in parenthesis; *p < .001. (Talmage and Kennedy, 1970).
Figure 17. Plasma phosphate changes following calcium injection to adrenalectomized-thyroidectomized rats. *p < .025; ** p < .01; ***p < .005.

See Figure 16 for legend. (Talmage and Kennedy, 1970).
Figure 18. Plasma calcium and phosphate changes following parathyroidectomy in adrenalectomized rats. ADX = adrenalectomy; PTX = parathyroidectomy; ○ = controls (including sham operated); ★ = ADX only; □ = PTX only; ● = acute ADX-PTX (ADX-PTX I); ■ = ADX 5 days-PTX (ADX-PTX II); shaded area shows range of controls; all changes in calcium and phosphate are significant with at least p .005. All points represent the average of at least 10 animals. (Talmage and Kennedy, 1970).
Figure 19. Effect of cortisol (5 mg/Kg/day for 5 days) on thyrocalcitonin function. Plasma and lavage calcium values. TX = thyroidectomized; TI = thyroid intact. ⋄ = TX; ■ = TI; ○ = TX with cortisol; □ = TI with cortisol. For plasma values, TX is different from TI with p < .01. All points represent the average of at least 9 animals. (Talmage et al., 1970).
o, □ = Cortisol treated
•, ■ = Non-treated

Calcium - mg/100ml

Plasma

Lavage

Hours Of Lavage
Figure 20. Effect of cortisol (5 mg/kg/day for 5 days) on $^{85}$Sr removal by lavage. See Figure 19 for key. Statistical differences: TI vs. TX, $p < .01$; TI cortisol vs. TX cortisol, $p < .01$; TX-cortisol vs. TX, $p < .025$; TI vs. TI cortisol-individual values not significantly different, but lines are different with $p < .001$. All points represent the average of at least 9 animals. (Talmage et al., 1970).
Figure 21. Plasma calcium and phosphate changes following calcium injection (2.5 mg/100g B.W.) to thyroidectomized and thyroid-intact rats. TX = thyroidectomized; TI-thyroid-intact, ● = TX; ■ = TI; ◄ = time of calcium injection; ***, p < .001; number of animals in parenthesis.
Plasma Calcium And Phosphate Changes
After Calcium Injection (2.5 mg/100g B.W.)
Figure 22. Changes in plasma $^{45}$Ca and $^{32}$P (10 day label) after calcium injection (2.5 mg/100g B.W.) to thyroidectomized and thyroid-intact rats. See Figure 21 for legend. *, $p < .05$; ***, $p < .001$; number of animals in parenthesis.
Figure 23. Changes in plasma $^{45}$Ca and $^{32}$P (12 hr label) after calcium injection (2.5 mg/100g B.W.) to thyroidectomized and thyroid-intact rats. Dashed line shows fall in plasma levels in control animals not injected with calcium. For legend see Figure 21. ***, $p < .001$. Number of animals in parenthesis.
Figure 24. Plasma calcium and phosphate changes following calcium injection (2.5 mg/100g B.W.) to thyroidectomized -nephrectomized and thyroid-intact-nephrectomized rats. Nephrex=nephrectomized. For legend see Figure 21. *p < .05; **p < .01; ***p < .005. Number of animals in parenthesis.
Figure 25. Plasma calcium and phosphate changes following calcium injection (2.5 mg/100g B.W.) to thyroidectomized-parathyroidectomized and thyroid-intact-parathyroidectomized rats. PTX - parathyroidectomized. See Figure 21 for legend. Number of animals in parenthesis.
Figure 26. Plasma $^{45}\text{Ca}$ and $^{32}\text{P}$ (10 day label) changes following calcium injection ($2.5 \text{ mg/100g B.W.}$) to thyroidectomized-parathyroidectomized and thyroid-intact-parathyroidectomized rats. TX = thyroidectomized; PTX = parathyroidectomized; TI = thyroid-intact; ○ = TX 48 hours, PTX 12 hours; □ = TI, PTX 12 hour; ● = PTX 12 hour, TX ½ hours; ■ = PTX 12 hour, TI. Number of animals in parenthesis. *, p < .05; **, p < .025; ***, p < .01; all comparisons are made on TX versus corresponding TI.
Figure 27. Plasma $^{45}$Ca and $^{32}$p (12 hour label) changes following calcium injection (2.5 mg/100g B.W.) to thyroidectomized-parathyroidectomized and thyroid-intact-parathyroidectomized rats. See Figure 21 for legend. PTX = parathyroidectomized. Number of animals in parenthesis. ***,p < .001.
Figure 28. Response of thyroidectomized-parathyroidectomized and thyroid-intact-parathyroidectomized rats to calcium injection (2.5 mg/100g B.W.) after 3 days on low phosphate diet. PTX = parathyroidectomized; see Figure 21 for legend; number of animals in parenthesis. *p < .01; **p < .005.
Figure 29. Relationship of the effect of thyrocalcitonin to the starting plasma phosphate levels. a) PTX = parathyroidectomized; b) Nephx = nephrectomized; c) *low phosphate diet from Nutritional Biochemical Corporation, Cleveland, Ohio; d) **ΔCa/ΔPO₄ = change in plasma calcium at 1 hour after thyrocalcitonin divided by the change in plasma phosphate at 1 hour. (Kennedy et al., 1969).
\[
\frac{\Delta C_i}{\Delta \text{PO}_4}
\]

Plasma PO$_4$ as mg P/100ml

<table>
<thead>
<tr>
<th>Group</th>
<th>Starting Plasma C$_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Low Phosphate diet*</td>
<td>12.2</td>
</tr>
<tr>
<td>2 Normal 12 Hour fast</td>
<td>10.3</td>
</tr>
<tr>
<td>3 Normal 18 Hour fast</td>
<td>9.9</td>
</tr>
<tr>
<td>4 PTX-18 hr., 1 hr. Post Nephx</td>
<td>7.2</td>
</tr>
<tr>
<td>5 PTX-18hr.</td>
<td>7.4</td>
</tr>
<tr>
<td>6 12 Hours post Nephx</td>
<td>9.7</td>
</tr>
<tr>
<td>7 24 Hours post Nephx</td>
<td>9.1</td>
</tr>
<tr>
<td>8 1 Hour post Nephx</td>
<td>10.6</td>
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BIBLIOGRAPHY


APPENDIX I

Lavage Fluid Composition

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<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>6.75g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.0g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1225g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.19g</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>0.67ml</td>
</tr>
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</table>

Calcium added as CaCl₂
Phosphate added as Na₂HPO₄