PARATHYROID FUNCTION IN THE BOX TURTLE, *TERRAPENE CAROLINA TRIUNGUIS*

A Dissertation

Presented to

the Faculty of the Graduate School

University of Missouri

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

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by

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May, 1973

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ACKNOWLEDGEMENTS

I wish to express my appreciation to Professor Robert P. Breitenbach for his guidance and encouragement during this study and in the preparation of this dissertation. I also wish to thank Professor Roger M. deRoos for his suggestions and critical reading of the manuscript.

I am indebted to many of my friends and colleagues for their assistance at various stages of this study.

This study was largely made possible by a USPH Environmental Physiology Traineeship.

Lastly, I wish to express my deep gratitude to my mother, Mrs. Ethel Magliola, and my future wife, Naomi, who have encouraged and supported me throughout the course of this study.

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CHAPTER I

INTRODUCTION

A fundamental component of the concept of homeostasis is that the functional integrity of a cell is dependent upon maintaining the relative constancy of its internal environment. Since all cell membranes are more or less permeable to a variety of organic and inorganic constituents, the cells are, not surprisingly, closely dependent upon the properties of the solution which surrounds them. Two of these properties, the relative and the absolute concentration of ions in solution, are of special significance in mineral metabolism. Seen from the perspective of evolution, extracellular mineral regulation was achieved by a modification and amplification of more primitive processes involved in intracellular homeostasis.

Biologists are in general agreement that life began in the sea, a solution so vast in its bulk that its ionic composition changes very slowly. Because of the relative stability of its ionic composition, the life forms which evolved and remained in the sea had no need for complex osmotic and ionic regulatory mechanisms. Most marine invertebrates, in fact, are ion conformers, i.e., their internal environment closely approximates seawater in ionic composition (Baldwin, 1964). Even these osmotically "labile" animals are capable of limited osmo- and ion regulation to insure normal cellular function. Most marine invertebrates are capable of maintaining certain electrolytes at concentrations above or below

their surroundings (Prosser, 1961a, b).

In the vertebrates, however, every ion is regulated. The complicated regulatory mechanisms found in vertebrates are a reflection of their early evolutionary history. Physiological and paleontological evidence provides considerable support for the hypothesis that vertebrates originated in freshwater, although an alternative saltwater theory of vertebrate origin is supported by a voluminous fossil record (Robertson, 1957). The relative proportions of the different ions in freshwater are different from what they are in the internal medium of the cell, and more importantly, the total ion content is much reduced from that of seawater. The protovertebrates which moved from the oceans and estuaries into freshwater were confronted with a new and harsh environment in which to survive "they had to rid themselves of excess water while at the same time conserve ions which were in poor supply in the environment" (Smith, 1961). The solution to this problem was largely achieved through the evolution of a glomerular kidney capable of excreting a hypotonic urine and possession of an almost impermeable integument to reduce the rate of entry of water. The integument is made relatively waterproof largely by impregnation with mucous secreted by epidermal glands (Baldwin, 1964).

The subsequent movement of vertebrates to land required both salt and water conservation. Salt conservation was not new to vertebrates colonizing land since their freshwater antecedents had been faced with a similar difficulty. How-

ever, the terrestrial vertebrate now was separated from the abundant water supply of his previous habitat and exposure to the dry atmosphere posed a special and additional problem of desiccation. For the first time, the conservation of water became an overriding consideration, a problem which teleosts reinvading the hyperosmotic sea also had to face (Baldwin, 1964). Conservation of water was achieved in two ways. First, keratinization of the epidermis provided a completely waterproof covering for the animal as protection against cutaneous water loss. Second, reduction in the size of the glomerulus in marine teleosts and terrestrial reptiles, and the capacity to form a concentrated urine, by addition of a water-absorbing segment, the loop of Henle, to the kidney tubule in birds and mammals, provided protection against urinary water loss (Baldwin, 1964).

Basic vertebrate form was largely determined by the special osmoregulatory challenges of the environment. The pattern of osmo- and ion regulation in the vertebrates involves locally restricted groups of cells, situated between the circulation of the animal and the exterior, which are specialized for ionic exchange. Several routes of ionic regulation evolved in the vertebrates, each incorporating groups of these specialized transport cells in configurations consistent with their functions. Thus, the kidney is composed, in part, of cells organized into tubules which collect an isosmotic blood filtrate from which either essential ions can be reabsorbed or into which unwanted ions can be secreted

from the blood and excreted in the urine (Smith, 1961). Specialized transport cells lining the gill epithelium of freshwater fishes are capable of active salt absorption while their marine counterparts extrude salts in the opposite direction (Prosser, 1961b; Smith, 1961; Baldwin, 1964). In those reptiles and birds which because of habitat limitations found it advantageous to drink seawater to supply their water needs, the kidney was inadequate for ionic regulation and thru selection pressure extra-renal routes of excretion evolved, i.e., nasal salt glands (Prosser, 1961b; Smith, 1961).

The maintenance of calcium in the body fluids at a remarkably constant level attests to its important functional role in the life processes of the organism. The very integrity of the membranes which control the influx and efflux of calcium in the organism is dependent on the presence of calcium in the proper concentration. Membranes deprived of calcium ion cannot retain their selective permeability properties (Kleeman, Massry, and Coburn, 1971). In elasmobranchs and marine cyclostomes, which have a high calcium ion concentration in the blood, one of the specific functions of calcium is concerned with reducing the permeability of the cell membranes to water (Urist, 1961, 1963). Calcium is also an essential coupling factor or "biologic transducer" in both excitation-contraction coupling and stimulus-secretion coupling. Calcium also plays an important role in the activation of many intracellular enzyme systems (Rasmussen, 1971).

The fine regulation of phosphorus (as phosphate) can

also be attributed to its involvement in a number of essential cellular processes. This anion is a structural component of bone crystal hydroxyapatite; an integral part of nucleotides and, as such, is necessary for nucleic acid formation; and phosphate is important in membrane biodynamics as a component of phospholipids (Frieden and Lipner, 1971).

Although the regulation of magnesium in the body fluids is essentially unknown, its requirement as a cofactor for many intracellular enzymatic reactions is well documented (Guyton, 1966).

A discussion of the evolution of mineral homeostatic mechanisms with regard to calcium, phosphorus, and magnesium logically should begin with the most rudimentary control system and progress to the most advanced. With this in mind, the cyclostomes will be reviewed first.

The hagfishes are marine cyclostomes which are unusual in the vertebrate hierarchy for two reasons: (1) They are the only vertebrates which are isosmotic with their environment. (2) They have a non-mineralized endoskeleton. The fact that hagfishes are in osmotic equilibrium with their environment does not imply a lack of ion regulation. The body fluids of hagfishes resemble seawater qualitatively not quantitatively, and each ion can be maintained separately against an osmotic gradient. In hagfishes both calcium and magnesium are maintained at concentrations below the environmental level, albeit at high levels relative to vertebrates with mineralized endoskeletons, while phosphate is maintained

at a concentration greater than seawater, but at essentially the same levels found in all vertebrates. With regard to biological solubility, the blood of hagfishes contains calcium phosphate ion products which greatly exceed the minimum ion product necessary for calcification of bone or cartilage in mammals. Consequently, the blood of hagfishes is supersaturated with respect to calcium and phosphate, and should precipitate out in the blood. The reasons that it does not is due to two factors: (1) Because of the high ionic strength of hagfish serum. (2) Hagfishes do not have nucleation centers for precipitation of apatite in cartilage matrix, and hence calcification does not occur.

Unlike the hagfishes, the freshwater-adapted lamprey has a blood ionic composition and total electrolyte concentration which resembles that of freshwater teleosts. Thus, the blood of the lamprey is hypertonic with respect to freshwater, although the concentration gradient for calcium is less than any other ion. Lampreys have calcium phosphate ion products favorable for calcification, but like the hagfishes, the cartilage matrix is apparently non-calcifiable (Urist, 1963).

Cyclostomes, therefore, have no requirement for or ability to store calcium and phosphate in the endoskeleton. They rely on ion storage in body fluids and extra-skeletal organs, or obtain calcium from the external environment. Cyclostomes, then, regulate calcium through an "open cycle system" involving skin, gill epithelium, gut, and kidneys,

by circulating tremendous volumes of water across the gill membranes and maintaining their plasma ionic concentration with mechanisms that resist passive ion movement toward equilibrium, by concentrating ions from freshwater (lampreys) and eliminating ions into seawater (hagfishes). Although this is an "archaic" type of mineral regulation, it is sufficient as long as the ionic composition of the medium is reasonably stable, and probably represents the situation in the ancestral vertebrate. Thus, the organism is "ionically and continuously dependent upon the external environment" (Urist, 1963).

While the gills, kidney, and to a lesser extent, the gut, control mineral balance or turnover, fine regulation of calcium and phosphate in the plasma of vertebrates could not be accomplished until the evolution of a mineralized endoskeleton (Urist, 1961, 1963).

The ancestors of the elasmobranchs evolved jaws, crania, and vertebrae of calcified cartilage. By their ability to store calcium in the skeleton to protect the body fluids against changes in the external environment, they represented the beginnings of ionic independence. The modern elasmobranchs, like the cyclostomes, are adapted to high total cation concentrations and high calcium concentrations in their blood; calcium, phosphorus, and magnesium levels also characteristically vary within wide ranges. Thus, although the elasmobranchs evolved a calcified endoskeleton and with it achieved some degree of ionic independence, they have not

succeeded in precisely regulating ionically like vertebrates with bony endoskeletons, and probably regulate calcium largely through an "open cycle system" much like cyclostomes (Urist, 1961).

The paleontological evidence suggests that bone evolved in the primitive freshwater ostracoderms, whose plasma must have resembled a teleost rather than a cyclostome or an elasmobranch. "Bone as a tissue seems to have been evolved by the vertebrates as an integral part of their achievement of osmotic independence" (Urist, 1961). "Hormonal control of mineral metabolism does not appear until the evolution of bone as the storage unit for a closed system for regulating the turnover of phosphate and calcium ions" (Urist, 1963).

The first group in which such a system operates is the teleosts. All teleosts have the ability to absorb calcium and phosphorus directly from their environment at the level of the gills and surface epithelium (Simmons, 1971). Both ions are stored in bone and in considerable quantities in soft tissues (Fleming, 1967). Urist (1966) insists that teleostean bone by being in physico-chemical continuity with blood, can serve as a labile resevoir for minerals which can be withdrawn upon demand to maintain homeostasis. Bone, thus, behaves as part of a closed cycle system. Moss (1962) concurs that osteocytes of cellular teleostean bone are capable of exercizing some inherent degree of homeostatic control of calcium and phosphate metabolism. On the other hand, Moss (1963) argues that while acellular bone (which most teleosts

possess) acts as a repository for calcium and phosphate, it is not involved in mineral homeostasis, and thus, like the cyclostomes which lack apatite, these teleosts must rely upon a constant continuity with the external environment. If the rate of withdrawal is not enough to maintain equilibrium in the absence of an extra-skeletal source of these minerals, then bone in these fishes cannot function in mineral homeostasis (Fleming, 1967). Fleming (1967) suggests that the deposition of acellular bone may be for purposes of conserving and recycling phosphate which is limiting in the environment, and not be involved in calcium homeostasis, since environmental calcium is physiologically unlimited. The question as to whether an open or a closed cycle system operates in teleosts with acellular bone remains unanswered, although it is known that they can maintain plasma levels of calcium and phosphate within narrow limits.

The endocrine control of mineral homeostasis is not well understood in teleosts. The following structures have all been implicated: hypophysis (Pang, Griffith, and Pickford, 1971), adrenals (Simmons, 1971), gonads (Simmons, 1971), Corpuscles of Stannius (Pang, 1971a), ultimobranchial bodies (Chan, Jones, and Smith, 1968; Pang, 1971b; Pang, Clark, and Thomson, 1971), and thyroid (Simmons, 1971). Because resorptive bone changes rarely have been demonstrated after mammalian parathyroid extract and estrogen administration to teleosts with cellular and acellular bone, the major influence of the endocrine organs seems to focus on regulation of ionic

fluxes across the gills and other membranes (Simmons, 1971). On the other hand, marked changes in the levels of plasma and urinary calcium and phosphate following administration of estrogens, adrenocorticotropin, and growth hormone have been reported (Fleming, 1967; Simmons, 1971). However, these changes are, in large part, dependent upon the availability of calcium ions in the environmental medium (Simmons, 1971).

As is obvious from above, the endocrine control of mineral homeostasis evolved before the appearance of the parathyroid glands. Skin, gut, gill, kidney, adrenal glands, and many other organs are capable of affecting the level of calcium and phosphorus in the body fluids.

The evolution of the parathyroid glands appears concomitantly with the transition from an aquatic to a terrestrial existence. The cells of the parathyroid gland evolved from the specialized cells of the gill epithelium as regulators of calcium and phosphate homeostasis when the gills disappeared. This development was accompanied by migration of these specialized cells to a new position as parathyroid parenchyma, a process which can be followed ontogenetically in all terrestrial vertebrates.

The requirement for a parathyroid hormone may be related to the specialization of the bony skeleton as a compact structure for support in the terrestrial vertebrates. For if, as a consequence of the additional role of support for bone, the physico-chemical equilibrium between blood and bone is not sufficiently high to maintain calcium at levels compat-

ible with normal functioning, in the absence of parathyroid glands, then a secretion of parathyroid hormone is necessary. By mobilizing calcium from bone against a concentration gradient, parathyroid hormone permits terrestrial vertebrates to maintain the higher calcium levels necessary for normal physiological performance. The extent of a parathyroid hormone requirement would depend not only on the slope of the concentration gradient between blood and bone, but on the ease of communication between bone and fluid phases. The fact that teleosts do not possess parathyroids and yet are apparently capable of maintaining calcium at levels compatible with normal functioning, suggests that either, (1) the physico-chemical relationship between blood and bone is of such a nature as to allow maintenance of calcium levels sufficiently high for normal physiological functioning, at equilibrium, (2) the availability of calcium in bone may be so limited that homeostasis is achieved by extra-skeletal mechanisms or, (3) in an environment physiologically unlimited in calcium, the problem facing teleosts may be one of preventing hypercalcemia instead of hypocalcemia, hence there would be no requirement for a parathyroid hormone (Talmage, 1967).

Parathyroid regulation of mineral metabolism in reptiles virtually has not been elucidated. The limited research to date has been concerned with the effects of parathyroidectomy and parathyroid extract administration on calcium and phosphate levels in plasma and urine, effects which are qualitat-

ively similar in snakes and lizards to those of mammals. One unusual facet of mineral metabolism in reptiles is the absence of a calcium response to parathyroidectomy and parathyroid extract administration in turtles (Clark, 1964, 1965). In addition, bone chips from turtles incubated in plasma taken from the same animal do not deplete the plasma of calcium and phosphate, suggesting that a secretion of parathyroid hormone is not required for turtles to maintain normal calcium levels (Talmage, 1967). Turtles are unique in possessing an unusually large amount of mineralized bone tissue, mostly associated with their protective shells. Such a large mass could provide exceptional area for ionic exchange between blood and bone and might explain why plasma calcium and phosphate can remain at normal levels even in the absence of parathyroid glands. The only work to date on the physiological responses to parathyroidectomy and parathyroid extract administration was performed on several aquatic species of pond turtles (Clark, 1964, 1965). Several hypotheses can be proffered to try to account for the unresponsiveness to parathyroid gland ablation and parathyroid extract injection. It may be that aquatic turtles can maintain their calcium levels even in the absence of parathyroid glands either by drinking water containing calcium or by absorbing calcium directly from the environmental water through their mucous membranes. In this regard, Dunson and Weymouth (1965) have shown that softshell turtles, Trionyx spinifer can actively transport sodium into the body, presumeably across pharyngeal

and anal membranes. The apparent refractoriness of bone to parathyroid extract may indicate that the large reserves of bone mineral are unavailable for homeostatic purposes. It is also conceivable that calcium homeostasis in these reptiles is not under the control of the parathyroid glands; instead, a role for these glands in phosphate metabolism may be revealed. The purpose of the experiments was to assess the mineral responses to parathyroid extract in the three-toed box turtle, Terrapene carolina triunguis. Plasma calcium, phosphate, and magnesium levels were taken as indices of hormone activity. Shifts in the concentration of one or more of these ions were interpreted as target organ responsiveness, with the hope of elucidating the functional status of parathyroid glands in a reptile where a role for a parathyroid hormone has not been defined with certainty. It was thought revealing to repeat the study of Clark (1965) using a terrestrial species which can be separated from an environmental source of calcium which might otherwise interfere with a response to a calcium altering hormone.

CHAPTER II

LITERATURE REVIEW

Literature on the reptilian parathyroids is devoted almost exclusively to descriptive studies of the embryology, location, and structure. Only recently have studies been concerned with attempts to define the physiological role of the parathyroids in the regulation of mineral metabolism.

Anatomy, Embryology, and Histology of the Parathyroids

The parathyroid glands are permanent features of all reptiles studied and presumeably are present in all reptiles. As in other vertebrates, the parathyroids are derived from the branchial pouches (usually pouches III and IV) which arise as evaginations of the pharyngeal epithelium. In all reptiles, the adult parathyroids retain a close vascular relationship with the "great vessels" of the heart. This feature is traceable to the parallel development of the branchial pouches forming the parathyroids and the aortic arches forming the definitive arteries. Two pairs of parathyroids (designated parathyroids III and IV) persist in the adult, with certain notable exceptions as will be discussed later.

The basic pattern in adult snakes is two pairs of parathyroid glands, a cranial pair at the bifurcation of the carotid arteries, and a caudal pair between the anterior and posterior lobes of the thymus (Greep, 1963; Clark, 1967a). The parathyroids in snakes develop from outpocketings of the

pharyngeal pouches as in other reptiles but their precise embryological origin is much in question. Five visceral pouches appear as evaginations from the pharyngeal wall which give rise to epithelial structures in all species studied. The fate of the epithelial structures, thereafter, seems to differ according to the species. Verdun (1898) is the only author to observe more than two pairs of persisting parathyroids. He described five pairs of parathyroids in Coluber but some may represent accessory parathyroid tissue. Harrison and Denning (1929) observed five pairs of "epithelial bodies" in embryos of Thamnophis radix; those derived from pouches I-III atrophy before "full term", while the "epithelial bodies" derived from the ventral portions of pouches IV and V persisted into the adult as definitive parathyroids. In snakes of the genus Coluber and Tropidonotus, however, the third and fourth pouches developed persistent parathyroids, while the epithelial anlage of pouch V existed as only a transitory structure (Johnson, 1922). Recently, Oguro (1970) noted the presence of two pairs of parathyroids in Elaphe quadrivirgata. Characteristic of snakes, much variation occurs in the location of the cranial pair of parathyroids, but the caudal pair always remain associated with the thymus.

Lizards generally, but not invariably, possess only one pair of parathyroids (derived from pouch III) that occupy the concavity of the carotid arch on either side in more or less intimate contact with the adventitia of the internal

carotid artery. This relationship has been observed in the genus Varanus (Adams, 1952), in the genera of Lialis and Aprasia of the family Pygopodidae (Underwood, 1957), and in the following species: Cnemidophorus gularis, Crotaphytus collaris, Sceloporus undulatus, Hemidactylus frenatus, and H. flaviviridis (Adams, 1953). The early work of van Bemmelen (1888a) and Wettstein (1931) established that the primitive lizard-like reptile, Sphenodon, regularly possessed two pairs of parathyroids corresponding to parathyroids III and IV. Nevertheless, it was commonly stated that all lizards lost parathyroid IV before birth (Adams, 1939). However, it is now known that several other lizards possess two pairs of parathyroids as adults. Verdun (1898) and Adams (1939) reported parathyroid IV in Lacerta. Adams (1953) noted a second pair in the concavity of the aortic arches of Phrynosoma cornutum as did Underwood (1957) in Delma fraseri, Xiphocercus valenciennii, and a number of anoline lizards (personal communication, cited by Greep, 1963). The existence of two pairs of parathyroids is reminiscent of the situation seen in other reptiles with two pairs of parathyroids originating from pouches III and IV.

A very thorough study on the parathyroids of the Crocodilia was performed by van Bemmelen (1888b), who described only one pair of glands (Greep, 1963). By virtue of their location near the carotid fork they have been designated parathyroid III by later authors. In young Alligator mississippiensis, Reese (1931) described a small body em-

bedded within the connective tissue capsule of the thyroid as well as "several small, more or less spheroidal bodies situated on each side of the neck, near, or even imbedded in the thymus glands", but due to their unusual cytology their parathyroid nature is questionable (Greep, 1963).

Two pairs of parathyroids have been found in all species of turtles studied since 1910. The freshwater turtles Chrysemys picta picta (Shaner, 1921a; Johnson, 1922; and Clark, 1964, 1965), Chrysemys picta marginata (Shaner, 1921b), Pseudemys scripta and Graptemys pseudogeographica (Clark, 1964, 1965), and genera of Chelydra and Trionyx (Johnson, 1922) each possess four parathyroids as persisting adult structures. Parathyroid III is embedded within the thymus, and parathyroid IV is in close association, wholly or partly embedded, within the ultimobranchial body (the ultimobranchial body attains striking dimensions on the left side while remaining wholly or partially absent on the right side). The association between the parathyroids and the ultimobranchials has been described in detail by Shaner (1921a, b) and Johnson (1922). The thymic-parathyroid gland associations simply reflect a common origin since the thymus and third parathyroid develop respectively from dorsal and ventral buds of the same visceral pouch and parathyroid IV and the ultimobranchial body develop as a closely coordinated complex between arches IV and V (Johnson, 1922).

The parathyroid glands of reptiles are small, spherical to ovoid bodies, white or yellow in color. They range in

size from 0.2 mm in diameter in small lizards, viz., Chalcides ocellatus (Sidky, 1965) and Anolis carolinensis (Clark, 1968a) to 1.0 mm in diameter in snakes and turtles, viz., Notechis (Greep, 1963), Chrysemys picta and Pseudemys scripta (Clark, 1964, 1965), Natrix sipedon and Coluber constrictor (Neudeck, 1969), Elaphe quadrivirgata (Oguro, 1970) and Thamnophis sirtalis (Clark, 1971a).

Individual parathyroid glands of reptiles consist of compact, anastomosing cellular cords as well as cells arranged in follicular fashion around a central lumen. The cell cords are separated by connective tissue trabeculae which extend into the parenchyma from the outer connective tissue capsule (Adams, 1952; Rogers, 1963; Clark, 1967a; Clark and Khairallah, 1969; Oguro, 1970; Clark, 1971a).

The parathyroid glands of most mammals are composed of two cell types: the principal or chief cell and the less common oxyphil cells. The chief cells, which synthesize and secrete parathyroid hormone are polygonal with a round or ovoid centrally placed nucleus. The cytoplasm contains considerable amounts of glycogen but only faint granulation to indicate a secretory product. Oxyphil cells are larger, contain less glycogen and have unusually large concentrations of closely packed mitochondria (Gorbman and Bern, 1962; Clark, 1967a; Bloom and Fawcett, 1968). "Water-clear" variants in which the cytoplasm takes on a vacuolated appearance, as well as a host of other cell types have been described, but to what extent they represent fixation artifacts is not

known (Bloom and Fawcett, 1968).

In most reptiles studied only one cell type has been found, presumeably corresponding to the mammalian chief cell. This condition exists in the lizard, Lacerta (Peters, 1941), in the snake, Elaphe quadrivirgata (Oguro, 1970), and in the freshwater turtles, Graptemys pseudogeographica, Chrysemys picta and Pseudemys scripta (Clark, 1965). Adams (1952) described in Varanus varius a highly vacuolated cell with a clear cytoplasm which he saw in addition to the chief cell. However, he did not regard it as being homologous with the mammalian "water-clear" cell. Rogers (1963) distinguished three cell types in the Australian lizards, Trachysaurus rugosus and Tiliqua occipitalis: the clear cell, the dark cell, and the epithelial cell. Sidky (1965) was the first to report a seasonal variation in the histology of the parathyroid glands of the lizards, Scincus scincus and Chalcides ocellatus, but attributed the changes in cellular appearance to the level of secretory activity and not as evidence of more than one cell type.

The epithelial cells of the reptilian parathyroid have essentially the same cytological characteristics as the mammalian chief cell. In those reptilian species in which the cells are arranged in follicles about a central lumen, both the cytoplasm and the luminal contents stain with periodic acid-Schiff reagent (Rogers, 1963; Sidky, 1965; Clark, 1967a). Occasionally "empty" cells which have lost their PAS-positive stainability are seen lining the follicles.

As suggested by some workers (Rogers, 1963; Clark, 1965; Sidky, 1965), the luminal contents may represent stored parathyroid hormone in the form of colloid. At least in mammals, the paucity of secretory granules in the cytoplasm of the chief cells and the correspondingly low gland content of parathyroid hormone suggest that the chief cells do not have mechanisms for storage of preformed hormone (Capen, 1971). In reptiles, this may not be the case, in view of the conspicuous number of follicular structures in the reptilian parathyroid gland and the widespread distribution of membrane-bound, electron dense cytoplasmic granules measuring about 3000-5000 A^{o} (Clark and Khairallah, 1969). It has been suggested that the appearance of colloid-filled follicles represents glandular degeneration since it is known that in man, follicles are observed in older individuals. The presence of follicles containing colloid-like material in young individuals and even embryos of reptiles, however, indicates that follicle formation is not solely a function of age (Clark, 1965; Sidky, 1965).

Reptilian Parathyroid Physiology

Both parathyroidectomy and parathyroid extract administration have been performed on several species of lizards in an attempt to elucidate the role of the parathyroid glands in mineral metabolism.

Parathyroidectomy invariably results in the animals exhibiting hyperexcitability and tremors, although the on-

set of tetany, its duration, and its severity differ in each species studied. In the first investigation of its kind, Peters (1941) removed the parathyroids of Lacerta viridis. One-third of the animals parathyroidectomized in the fall developed tetany after a latent period of 3-17 days; symptoms of tetany developed only after seven weeks in animals parathyroidectomized in the spring. Nevertheless, all parathyroidectomized animals died within three months. More recently, Sidky (1966) compared the effect of parathyroidectomy on two species of lizards, Chalcides ocellatus and Varanus griseus and found that the animals responded with classic symptoms of tetany; however, the onset of spasms was sooner (21 hours to six days) in the larger V. griseus than in the smaller C. ocellatus (six to 75 days). Interestingly, the smaller animals reacted more severely to parathyroid ablation since young V. griseus and the smaller C. ocellatus died in tetany after a maximum period of three months while the larger V. griseus rarely manifested any signs of hyperexcitability. Also, the severity of the tetanic seizures increased and the time of their commencement decreased with increasing environmental temperature. Sidky (1966) was the first to measure changes in mineral levels in response to parathyroidectomy; total plasma calcium concentrations in V. griseus fell from 11.0-12.8 mg/100 ml in both the sham-operated and intact control animals to 5.8-8.0 mg/100 ml in the parathyroidectomized group. The intramuscular administration of a ten per cent calcium glu-

conate solution to parathyroprivic animals was accompanied by relief from the symptoms of tetany, presumeably due to a return to normal of the plasma calcium concentration. In the only study of a lizard with four parathyroid glands, Clark (1968a) reported that parathyroidectomy of Anolis carolinensis resulted in tetanic convulsions within one to two days and death in two to nine weeks. Although death was attributed to starvation, tetany was believed indirectly to be involved, since tetany developed whenever an animal attempted to eat. Subsequently, Clark, Pang, and Dix (1969), reported that the behavioral changes that were associated with tetany in parathyroidectomized individuals could be correlated with shifts in the blood calcium and phosphate values. Total plasma calcium and phosphate concentrations in sham-operated A. carolinensis were 10.45 ± 0.43 mg/100 ml and 6.03 ± 0.39 mg/100 ml respectively. Following parathyroidectomy, the plasma calcium levels fell significantly to 4.58 ± 0.42 mg/100 ml while the plasma inorganic phosphorus levels rose significantly to 10.37 ± 0.90 mg/100 ml.

Administration of 97 IU of mammalian parathyroid extract (PTE) per animal (route not given) produced no significant change in serum calcium or phosphorus concentrations, when compared with saline-injected controls (11.99 ± 1.78 mg/100 ml vs. 8.74 ± 0.68 mg/100 ml and 7.52 ± 0.32 mg/100 ml vs. 7.13 ± 0.73 mg/100 ml, respectively), although there was a trend toward hypercalcemia. On the other hand, PTE administered intramuscularly to parathyroidectomized lizards produced both a significant hypercalcemia and hypophosphatemia

when compared to the controls. Serum calcium values for controls were 5.01 ± 0.28 mg/100 ml and rose to 8.70 ± 0.54 mg/100 ml in experimentals while serum inorganic phosphorus values fell from 11.93 ± 0.68 mg/100 ml in the controls to 7.69 ± 1.16 mg/100 ml in the PTE-injected group. McWhinnie and Cortelyou (1968) investigated the effects of PTE administration in intact iguanid lizards, Dipsosaurus dorsalis and Sceloporus grammicus; the former received a cumulative dose of 105 USP units PTE (15 USP units daily for one week) and the latter received a cumulative dose of 70 USP units PTE (ten USP units daily for one week), both given intraperitoneally. In contrast to the lack of an effect of PTE on serum calcium levels in sham-operated A. carolinensis, plasma calcium levels in D. dorsalis rose significantly from control values of 15.14 ± 0.44 mg/100 ml to 18.17 ± 0.52 mg/100 ml in PTE-injected animals. In agreement with the data in sham-operated A. carolinensis was the lack of a shift in plasma inorganic phosphorus after PTE administration from 8.12 ± 0.61 mg/100 ml in the controls to 8.64 ± 0.67 mg/100 ml in experimental animals.

McWhinnie and Cortelyou were the only workers to measure urinary levels of calcium and phosphorus in lizards. Control D. dorsalis had calcium values of 0.134 ± 0.021 mg/100 mg urine pellet and phosphorus values of 0.048 ± 0.016 mg/100 mg urine pellet. Pellets produced during seven days of PTE treatment had calcium contents which were 8.1-fold greater than controls (1.084 ± 0.203 mg/100 mg urine

pellet) while the phosphorus contents were elevated 19.8-fold above the corresponding control animals (0.952 ± 0.366 mg/100 mg urine pellet). Similar changes in response to PTE treatment were observed in S. grammicus. Significant hypercalciuria and hyperphosphaturia resulted following PTE administration; control urine pellets contained 0.059 ± 0.008 mg/100 mg calcium and 0.153 ± 0.011 mg/100 mg phosphorus while urine pellets from experimental animals contained 0.145 ± 0.016 mg/100 mg and 0.431 ± 0.064 mg/100 mg calcium and phosphorus, respectively.

In an effort to determine if bone was the source of the additional calcium in the blood of parathyroidectomized D. dorsalis and S. grammicus following PTE treatment, citric acid content of hindlimb bones was determined. The citric acid content in the PTE-injected group and in the corresponding control group was not significantly different. Umanski and Kudokotzev (1951) studied the effect of PTE administration on the limb regenerative capacity of Lacerta agilis and observed an increased number of osteoclasts in bone.

Parathyroid function has been investigated in three species of snakes: Elaphe quadrivirgata (Oguro, 1970), Thamnophis sirtalis (Clark and Srivastava, 1970; Clark, 1971a), and Rhabdophis tigrinus tigrinus (Oguro, 1972). Parathyroidectomy in E. quadrivirgata was followed by a significant decline in serum calcium values to 60, 67, and 62 per cent of control levels respectively at seven, ten, and 20 days post-operation. Serum calcium concentrations were 14.0

mg/100 ml in controls and 5.6 mg/100 ml in parathyroidectomized T. sirtalis. The decline in plasma calcium was significant at ten days and remained at this low level throughout the six week experimental period. At the same time, serum inorganic phosphorus rose from 4.96 mg/100 ml in controls to near 12.4 mg/100 ml in parathyroidectomized animals after six weeks. These values were significantly different from controls at 18 days. In the most recent study by Oguro (1972), serum calcium levels were measured in parathyroidectomized and sham-operated male and female R. tigrinus tigrinus. The males and females were compared separately. Males exhibited statistically significant hypocalcemia at ten and 20 days post-operation while females were not significantly hypocalcemic until after 20 days. Parathyroidectomized T. sirtalis exhibited tetany about ten days after the operation, but in E. quadrivirgata and R. tigrinus tigrinus, the majority of parathyroidectomized animals did not manifest the neuromuscular disturbances associated with tetany. Since parathyroidectomy in T. sirtalis was accompanied by the most severe hypocalcemia of those species examined, perhaps calcium was not lowered sufficiently in the other species to elicit tetany.

Intact T. sirtalis (Clark and Srivastava, 1970; Clark, 1971a) were injected intraperitoneally with distilled water and bled 20 hours later to obtain control readings and then injected with either 1.18 IU PTE/100 g body weight or 2.35 IU PTE/100 g body weight and bled 24 hours later. Serum

calcium concentrations during the control period averaged 12.16 ± 0.56 mg/100 ml and rose to 14.24 ± 0.92 mg/100 ml at the lower dose and 15.92 ± 1.04 mg/100 ml at the higher dose of PTE. Serum phosphate values were depressed to 2.60 ± 0.62 mg/100 ml at the lower dose and 3.97 ± 0.40 mg/100 ml at the higher dose of PTE from control values of 4.81 ± 0.53 mg/100 ml. Serum calcium levels were significantly raised at the higher dose administered; however, only the lower dose caused a significant hypophosphatemia, which is difficult to explain.

Doyon and Kareff (1904) were the first investigators to parathyroidectomize a turtle. They removed only one pair of parathyroids from an "African tortoise" which led to paralysis and death; no measurements of either serum calcium or phosphorus were made. In the only other study of chelonians, Clark (1964, 1965) parathyroidectomized two species of aquatic turtles, Chrysemys picta and Pseudemys scripta and made simultaneous measurements of both serum and urine calcium and phosphorus in sham-operated and parathyroidectomized individuals up to eight weeks after gland removal. Complete parathyroidectomy did not provoke behavioral changes symptomatic of tetany, nor did it significantly alter serum calcium, serum phosphorus, urinary calcium, or urinary phosphorus in relation to sham-operated controls, even eight weeks after gland ablation. Within one week of the operation, only urine phosphorus showed significant change from controls. By three weeks the phosphorus levels had returned to about

control values. Despite the lack of responsiveness to parathyroidectomy, injection of sodium citrate was capable of inducing tetany and lowering calcium in the serum to a level that abolished blood clotting.

Clark administered PTE to both intact and parathyroidectomized C. picta and P. scripta as single injections of either 50 or 100 units per animal or as single injections of either 100 or 200 units per kilogram body weight. Controls received equivalent volumes of distilled water. The animals were bled and urine was collected before the operation and for up to ten days thereafter. Calcium and phosphorus were measured. While 50 units of PTE did not produce significant changes, 100 units of PTE resulted in a six-fold rise in urinary phosphorus in both intact and parathyroidectomized animals as compared with controls. The serum calcium was also elevated 20 per cent above control levels after three days in intact and parathyroidectomized animals although the change was not significant. In contrast, serum phosphorus and urinary calcium in both intact and parathyroidectomized groups did not vary appreciably from control values.

PTE administration did not produce any significant change in either osteoclast number or calcium content in bones of either intact or parathyroidectomized turtles. Mammalian PTE-injected hatchlings of Graptemys pseudogeographica (Clark, 1965) had 33 per cent more osteoclasts than their distilled water-injected controls. Osteoclasts of adult P. scripta also increased in number in response to PTE but due

to the small sample size, statistical comparisons could not be performed. Fibulae of C. picta and P. scripta were wet-ashed for determinations of calcium content (Clark, 1965). Fibulae from normal animals receiving the placebo contained 0.122 ± 0.03 mg Ca/mg bone while fibulae from animals which received 189-378 units of PTE contained 0.124 ± 0.010 mg Ca/mg bone. Likewise, parathyroidectomized controls contained 0.111 ± 0.010 mg Ca/mg bone and parathyroidectomized animals injected with PTE had a similar value of 0.129 ± 0.010 mg Ca/mg bone.

Overview of Actions of Parathyroid Hormone and Calcitonin in Mammals

The primary regulation of calcium and phosphorus metabolism is under the dual endocrine control of parathyroid hormone (PTH) and (thyro)calcitonin (TCT, CT). Their complementary and antagonistic actions are responsible for maintaining the concentration of ionic calcium in the plasma within physiological limits.

All actions of PTH tend to promote an increased movement of calcium ion into the extracellular fluid. Parathyroid hormone does so principally through its direct effects on bone by increasing the calcium mobilization from the skeleton. To a lesser extent, PTH also conserves calcium by stimulating absorption from the gut and reabsorption by the renal tubule (Munson, Hirsch, and Tashjian, 1963; Arnaud, Tenenhouse, and Rasmussen, 1967). The renal response

is characterized by a rapid onset which is limited in magnitude and a high sensitivity to slight fluctuations in hormone concentration. The response of bone on the other hand is sluggish in onset, relatively insensitive, but of nearly unlimited capacity. Another effect of the hormone, that of stimulating urinary phosphate excretion, produces hypophosphatemia and, thereby, secondarily causes plasma calcium to rise (Kleeman, Massry, and Coburn, 1971).

Calcitonin opposes the action of PTH by directly inhibiting PTH-induced skeletal calcium release (Hirsch and Munson, 1969).

The parathyroids are of unquestionable importance in calcium homeostasis, since parathyroidectomy frequently results in hypocalcemic tetany, paralysis and eventually death. The significance of calcitonin as a normal functioning component in homeostasis is not well established, particularly since removal of the thyroid gland, which secretes calcitonin in mammals, does not appear to alter blood calcium levels (Cooper, Hirsch, and Munson, 1970). However, calcitonin has been shown to effectively counteract hypercalcemia induced by PTH (Hirsch and Munson, 1966), vitamin D (Hirsch and Munson, 1969), and calcium infusion since the ability to resist a calcium challenge is significantly impaired by removal of the thyroid glands (Care, Duncan, and Webster, 1967, cited in Copp, 1969). These kinds of experiments are of doubtful value in assessing the physiological function of calcitonin, since hypercalcemic stress of this

magnitude is never encountered in the normal situations to which an organism is exposed. The data, then, seem to indicate that in the moment-to-moment control of calcium homeostasis, the primary effector is PTH. Evidence suggests that calcitonin may be involved in combating brief episodes of postprandial hypercalcemia (Hirsch, 1971). Kennedy and Talmage (1971) offer another explanation consistent with the data, namely that the effects of calcitonin on calcium fluxes are secondary to its more basic function of control of phosphate transport. In any event, the primary endocrine role in calcium homeostasis, under normal conditions, is played by PTH with calcitonin acting in a subsidiary capacity, but serving to control acutely imposed hypercalcemia.

Actions of Parathyroid Hormone on Plasma Calcium and Phosphate Concentrations

Single administrations of PTE or PTH in dogs and human subjects results in a rise in plasma calcium and a concomitant fall in plasma phosphate. At the same time PTH is exerting its vascular effects, the concentration of phosphate in the urine is increasing and the concentration of calcium in the urine briefly falls, but is followed by a sustained rise. The time course of the hormone's effects depends, of course, on the species, but generally the hyperphosphaturia culminates in two to three hours and is relatively short-lived while the hypercalcemia reaches a maximum in about eight hours but persists for 24-36 hours. The hypercalcemia

is attributable to the dissolution of hydroxyapatite in bone, releasing calcium and phosphate at a rate greater than the normal bone-blood physico-chemical equilibrium allows. The hypophosphatemia is caused by an increase in the rate of excretion of phosphate ions by the kidney (Arnaud, Tenenhouse, and Rasmussen, 1967).

Parathyroidectomy, on the other hand, produces a fall in the concentration of calcium in the plasma and a rise in the concentration of phosphate in the plasma. In addition, parathyroidectomy produces a fall in the urinary excretion of both electrolytes, although calcium initially rises (Talmage, 1956). Shifts in the plasma and urinary levels of calcium and phosphate can be attributed to a removal of the principal effects of PTH on its target organs, bone and kidney.

Actions of Parathyroid Hormone on Bone

Parathyroid hormone stimulates bone resorption *in vitro* (Gaillard, 1965; Raisz, 1965) and *in vivo* (Munson, Hirsch, and Tashjian, 1963) and inhibits bone formation (Gaillard, 1961; Bronner, 1962; Vaes and Nichols, 1962). Until recently, the former process was believed to occur primarily through an increase in the number and activity of osteoclasts (Toft and Talmage, 1960). Recently investigators, led by Talmage, have implicated the osteoblast-osteocyte complex as the responsive targets in PTH action on bone, at least as PTH relates to mineral homeostasis, thus relegating the

osteoclast to the specialized role of controlling large-scale bone remodeling (Talmage, 1969, 1970). Parathyroid hormone does not affect the removal of recently deposited surface calcium (labile fraction of bone mineral), but instead mobilizes calcium from the deep stores of stable bone (Munson, Hirsch, and Tashjian, 1963; Talmage and Elliott, 1958, cited in Copp, 1969).

Bone resorption is a complex process involving both the dissolution of bone mineral and the destruction of organic matrix. Mineral removal appears to precede matrix destruction, since early release of calcium by PTH reflects a change in ionic flux across the membrane while matrix breakdown requires RNA and protein synthesis (Raisz *et al.*, 1969).

The breakdown of organic matrix is accomplished by enzymatic degradation. The data suggest that PTH may accomplish this either through *de novo* synthesis of enzymes or by activation of latent enzymes. Actinomycin D and puromycin, which inhibit RNA and protein synthesis, respectively, prevent the calcium-mobilizing effect of PTH both *in vivo* (Rasmussen, Arnaud, and Hawker, 1964; Tashjian, Levine, and Munson, 1964) and *in vitro* (Tashjian, Ontjes, and Goodfriend, 1964; Raisz, 1965; Raisz and Niemann, 1967). However, when Raisz and Niemann (1967) studied this phenomenon, the hormone-induced release of calcium from bone grown in tissue culture was biphasic: an early release of calcium which was actinomycin D-independent and which therefore did not require enzyme synthesis, and a later release of bone mineral which

was prevented by the actinomycin D and was therefore presumeably dependent on enzyme synthesis.

The activation of latent enzymes by PTH is presumed to occur through a hormone-induced release of whole lysosomal contents into the lacunar spaces surrounding bone. These lysosomes contain hydrolytic enzymes which when released into the local acid environment of bone, hydrolyze the organic constituents of bone matrix. PTH-induced synthesis and release of lysosomal enzymes has been demonstrated both in vitro (Vaes, 1968a) and in vivo (Orimo, Ohata, and Fujita, 1970). Lysosomes contain all the enzymes required for the breakdown of the ground substance, but lack an enzyme for collagen hydrolysis. Walker, Lapiere, and Gross (1964) demonstrated the appearance of a collagenolytic factor in the culture media of bones treated with PTH. Such a factor would explain the increase in hydroxyproline excretion observed after PTH administration, presumeably due to collagen degradation in resorbing bone (Bates, McGowan, and Talmage, 1962).

The dissolution of bone mineral is largely dependent on alterations in carbohydrate metabolism which lead to the formation of products which can produce demineralization (Schartum and Nichols, 1962). Parathyroid hormone, by controlling the rate of utilization of metabolites derived from glucose, stimulates the production of organic acids (citrate and lactate) and CO_2 by bone cells both in vitro (Cohn and Forscher, 1961, 1962; Mecca, Martin, and Goldhaber, 1963;

Cohn, 1964; Cohn et al., 1970; Hekkelman, 1971) and in vivo (Firschein et al., 1958). This local accumulation of organic acids creates a low pH environment at resorptive sites which is responsible for dissolving the hydroxyapatite (Neuman and Dowse, 1961; Schartum and Nichols, 1962).

Actions of Parathyroid Hormone on Kidney

Perhaps the earliest manifestation of action is the enhanced excretion of phosphate ions in the urine. The exact mode and mechanism of action of PTH, and the site of its phosphaturic action, are still subjects of controversy in the literature.

Earlier investigators working with crude extracts of parathyroid glands could not dissociate possible direct effects of the hormone on the renal tubule from changes in glomerular filtration rate (GFR) which affects the filtered load of phosphate ions (Handler and Cohn, 1952). A direct effect on the renal tubule without alterations in GFR subsequently has been demonstrated using purified hormone preparations (Pullman et al., 1960; Samiy et al., 1960; Rasmussen, 1961; Hirsch and Munson, 1964).

Actinomycin D and puromycin are incapable of blocking the action of PTH on the kidney (Rasmussen, Arnaud, and Hawker, 1964). Unlike bone where PTH-induced hypercalcemia can be abolished by metabolic inhibitors, the mechanism whereby PTH regulates phosphate excretion apparently does not require RNA or protein synthesis.

There have been a number of studies to determine if the major renal action of PTH is to reduce proximal tubular reabsorption of phosphate or to stimulate tubular phosphate secretion. Most investigators have concluded that PTH directly inhibits renal tubular reabsorption (Pitts et al., 1958; Carone, 1964; Samiy, Hirsch, and Ramsay, 1965). Bartter (1961) has shown a constant rate of phosphate reabsorption by the kidney of the dog over a wide range of filtered loads. The simplest way to interpret these data is to postulate the existence of a renal threshold maximum for the reabsorption of phosphate rather than a variable rate of phosphate secretion into the kidney tubules. Comparative studies on the chicken (Levinsky and Davidson, 1957), American alligator (Hernandez and Coulson, 1956), and the American goosefish (Marshall and Grafflin, 1928) provide compelling evidence in favor of phosphate secretion into the kidney tubules.

Almost all the changes in final urinary phosphate excretion can be ascribed to changes in proximal tubular phosphate reabsorption, suggesting also that relatively little of the filtered load of phosphate is transported in the distal portions of the nephron (Strickler et al., 1964).

The renal handling of phosphate is apparently complicated by an interrelation with sodium ion. Acute saline loading (Massry, Coburn, and Kleeman, 1969), extracellular volume expansion, and PTH administration in dogs (Suki et al., 1969; Goldberg et al., 1972) results in parallel and insep-

arable inhibition of sodium and phosphate reabsorption in the proximal tubule. The proximally unreabsorbed sodium is transported in the distal segment, while the phosphate which has only a proximal transport site is excreted in the urine.

Parathyroid hormone also affects the renal handling of calcium by promoting its reabsorption by the renal tubules and therefore decreasing its clearance (Talmage, 1956; Kleeman, Rockney, and Maxwell, 1958; Buchanan, 1961; Kleeman et al., 1961; Biddulph et al., 1970). Since a PTH-induced rise in plasma calcium also produces a rise in the filtered load, the renal effects of PTH on urinary calcium are usually masked. Data from stop-flow experiments (Wesson and Lauler, 1959; Widrow and Levinsky, 1962) and micropuncture studies on rats and hamsters (Lassiter, Gottschalk, and Mylle, 1963), have established that calcium is actively reabsorbed along the entire length of the nephron. Parathyroid hormone enhances the transport of only a small fraction of the filtered calcium in the distal tubule as well as the late segment of the proximal tubule and the ascending limb of the loop of Henle. A linear correlation between calcium and sodium clearances in the dog (Walser, 1961; Massry et al., 1968) has indicated some type of relationship between factors modulating sodium excretion and those modulating calcium excretion.

Regulation of Synthesis and Secretion of Parathyroid Hormone and Calcitonin

Because of the development of sensitive radioimmunoassays for both PTH and TCT, direct evidence for ionic calcium regulation of PTH and TCT secretion has been provided. Reduction in the circulating level of ionic calcium stimulates the secretion of PTH by the parathyroid gland and suppresses the release of TCT by the thyroid gland. Elevation of plasma ionic calcium levels suppresses PTH secretion and stimulates TCT secretion (Potts *et al.*, 1968; Lee, Deftos, and Potts, 1969; Oldham *et al.*, 1971). Thus, by utilizing a negative-feedback control system, these two hormones effectively regulate plasma calcium concentration over a narrow physiologic range. While the concentration of calcium to which the parathyroid glands are exposed is inversely related to the amount of PTH which is released, the phosphate concentration in the plasma has no direct regulatory influence on the secretion of PTH (Sherwood *et al.*, 1968). The circulating level of magnesium is just as effective as calcium in controlling PTH secretion (Sherwood *et al.*, 1972), except at very low magnesium concentrations where secretion is markedly depressed (Sherwood *et al.*, 1972). Furthermore, the divalent cation concentration (calcium plus magnesium) and hormone release vary in a first-order relationship (Sherwood *et al.*, 1971). *In vitro* organ culture systems and slice incubation systems designed to measure the biosynthesis and secretion of PTH have established

that while calcium ion regulates both the release and synthesis of PTH, magnesium ion regulates only its secretion (Hamilton et al., 1971; Sherwood et al., 1971; Sherwood et al., 1972).

Parathyroid Hormone and Intracellular Calcium Activity

"Extracellular calcium homeostasis is achieved by an adaptation and extension of those more ancient mechanisms involved in intracellular calcium homeostasis" (Rasmussen, 1971). Thus, in order to gain an understanding of those mechanisms involved in calcium homeostasis at the organismal level demands an understanding of the mechanisms involved in intracellular calcium homeostasis.

The concentration of ionic calcium in the cytoplasm of typical mammalian cells is 10^{-2} to 10^{-6} times the concentration of calcium in the extracellular fluid. However, if the total calcium content of the cell were in an ionized form and evenly distributed within the cell, it would exist at a concentration equal to or greater than that of the extracellular fluid (Borle, 1967). It is apparent then that calcium is unequally distributed within the cell among various subcellular compartments, and that within each compartment, calcium may exist in either ionic, bound or colloidal form (Rasmussen, 1966, 1971). Three intracellular calcium compartments have been identified: a mitochondrial pool, a microsomal pool, and a cytosolic pool (Ebashi and Endo, 1968; Borle, 1972; Rasmussen et al., 1972). Calcium ex-

change among the intracellular compartments is continuous and rapid, and calcium within the cell cytoplasm communicates freely with the extracellular fluids. Movement of calcium across the plasma membrane and the subcellular membranes involves energy-linked transport in one direction and passive influx in the opposite direction. In all cases, passive influx raises the level of calcium in the cytosol, while ATP-dependent calcium "pumps" operate to reduce the level of calcium in the cytosol either by expulsion across the cell membrane into the extracellular fluid or by calcium uptake and sequestration by mitochondria and endoplasmic reticulum (Rasmussen, 1966, 1971; Borle, 1967; Ebashi and Endo, 1968). The mitochondrial and microsomal compartments act as calcium traps by deposition of calcium phosphate and as buffers by maintaining the ionic calcium concentration in the cytoplasm at very low levels.

In a series of experiments directed at analyzing calcium movements in kidney and HeLa cells in monolayer cultures, Borle has surprisingly concluded that regulation of intracellular calcium concentration is not governed by the exchange of calcium across the cell membrane. Rather, the dominant control factor in intracellular calcium activity and transport is the exchange of calcium between the subcellular compartments and the cytoplasm (Borle, 1967).

Parathyroid hormone stimulates calcium uptake in kidney cells (Borle, 1968). This is a consequence of both increased influx across the cell membrane and expansion of the

intracellular calcium pool (Borle, 1970a). It is most probable that PTH stimulates calcium influx through changes in membrane permeability such that the rate of diffusion for calcium or a calcium-carrier complex would be accelerated (Friedmann and Park, 1968; Borle, 1970a). While PTH stimulates a two-fold increase in the size of the cytoplasmic pool, it enlarges the mitochondrial pool 22-fold and stimulates calcium release from mitochondria 500 per cent. The effect of having greater calcium exchange between mitochondrial and cytoplasmic pools than between extracellular fluids and the cell, is for PTH to raise the intracellular level of ionized calcium primarily by mobilizing it from mitochondrial stores rather than by influx of calcium across the cell membrane.

This increased intracellular calcium could explain many reported effects of PTH:

1. Expansion of the intracellular exchangeable cytoplasmic pool by PTH secondarily stimulates calcium efflux (Borle, 1972). One could easily adopt the system of calcium transport in HeLa or kidney cells with one modification to explain the action of PTH on calcium transfer in bone, kidney and gut (Borle, 1968; Talmage, 1969, 1970): in bone, from the extracellular fluid peculiar to bone across the cellular layer of osteoblastic "lining" cells, and into the extracellular fluid proper; in kidney, from the renal tubule across the renal cortical cells into the interstices of the peritubular capillary network; and in gut, from the lu-

men of the intestine across the epithelial cells of the mucosa and into the extracellular fluid of the portal circulation. Transcellular transport, then, would represent a particular type of calcium exchange between the cell and the extracellular fluid, but due to the polarized nature of the cells involved, transport would become directional.

2. Parathyroid hormone inhibits citrate-decarboxylation activity in mouse calvaria in tissue culture (Cohn et al., 1970). This block in citrate metabolism may be due to a PTH-induced elevation of bone intracellular ionic calcium levels which then might inhibit the rate of citrate conversion in the cell either through inhibition of the citrate-handling enzymes themselves or through competition with the enzymes for citrate. Underutilization and hence accumulation of citrate in PTH-stimulated bone cells might lead to mineral resorption via chelation of calcium by citrate.

3. One of the effects of PTH in renal tissue is to stimulate gluconeogenesis (Nagata and Rasmussen, 1968, 1970). An increase in the extracellular calcium concentration also leads to an enhanced rate of gluconeogenesis. A striking similarity in the metabolite profiles of various glycolytic and Krebs cycle intermediates was seen following calcium infusion and PTH administration. This led to the possibility that both PTH and extracellular calcium act by increasing the intracellular level of ionic calcium, which in turn leads to changes in the rate of gluconeogenesis. A PTH-induced increase in the intracellular calcium pool was in-

dicated by the striking inhibition of isocitrate dehydrogenase, an enzyme known to be extremely sensitive to calcium.

Parathyroid Hormone and Adenosine 3', 5'-Monophosphate

Sutherland et al. (1968) have developed a concept to account for the diverse actions of adenosine 3', 5'-monophosphate (cyclic AMP) in different cells. In this two messenger concept, the first messenger, either external stimuli or hormone, reacts with the target cell and stimulates the formation therein of a second messenger (cyclic AMP). This first messenger is believed to interact with a membrane-bound adenyl cyclase which functions both as a "discriminator for environmental signals and as a signal generator" (Bradham, Holt, and Sims, 1970). The increased concentration of cyclic AMP within the cell, acting as a second messenger, activates one or more processes within the cell.

Since its initial discovery, cyclic AMP has been credited with mediating a major part of the endocrine control system (Robison, Butcher, and Sutherland, 1968; Sutherland et al., 1969; Sutherland, Robison, and Butcher, 1968).

The role of the adenyl cyclase-cyclic AMP system in the mediation of PTH action has been amply demonstrated. Purified PTH markedly stimulates adenyl cyclase in rat kidney in vitro (Dousa and Rychlik, 1968). Similar results were obtained on the renal cortical adenyl cyclase of the rabbit (Streeto, 1969).

To account for the hormonal specificity of adenyl cyclase, a specific receptor on the membrane must serve as recognition site for the hormone, existing either as a subunit of the adenyl cyclase enzyme (Robison, Butcher, and Sutherland, 1967) or as a separate component of the adenyl cyclase system (Schwartz and Hechter, 1966).

Chase and Aurbach (1968) reported that while PTH and vasopressin activate adenyl cyclase in plasma membrane fractions of rat kidney, the receptor sites for each hormone are located in anatomically separable areas. Specifically, PTH-sensitive adenyl cyclase is localized in cortical membrane fractions whereas vasopressin-sensitive adenyl cyclase is restricted to the medullary membrane fractions. These observations are consistent with the known physiological actions of both PTH and vasopressin on kidney, since PTH acts primarily on the proximal tubule of the nephron and vasopressin primarily on the distal convoluted tubule and collecting ducts.

Activation of adenyl cyclase from fetal rat calvaria by PTH in vitro has been shown (Chase, Fedak, and Aurbach, 1969) thus establishing cyclic AMP as a common intermediate in the action of PTH on its principal target tissues, bone and kidney. More recently, workers from the same laboratory measured increases in the concentration of cyclic AMP in bone fragments incubated with PTH (Chase and Aurbach, 1970).

If the mechanism of action of PTH is mediated by an increase in the intracellular concentration of cyclic AMP

in kidney and bone, then either the natural cyclic nucleotide or its synthetic analogue, dibutyryl adenosine 3', 5'-monophosphate (DBcAMP) should be able to mimic the actions of PTH.

Rasmussen, Pechet and Fast (1968) present data which indicate that infusion of DBcAMP in thyroparathyroidectomized rats produces changes in the urinary excretion of calcium, phosphate and hydroxyproline which mimic those changes produced after PTH administration. Similarly, DBcAMP mimicked the actions of PTH on kidneys of intact mongrel dogs. Infusion of DBcAMP either systemically or directly into the renal artery suppressed proximal sodium and phosphate reabsorption (Agus et al., 1971).

Addition of cyclic AMP to embryonic bone in organ culture had little effect on calcium release but DBcAMP, which is more active than the natural nucleotide, significantly enhanced calcium mobilization from bone (Raisz et al., 1969). When DBcAMP is cultivated with calvaria from 18-day-old mouse embryos and compared with PTH-cultivated calvaria, the production of lactate and the release of calcium and phosphate are qualitatively similar (Hermann-Erlee, Van Zuylen, and Hekkelman, 1972). Vaes (1968b) has also shown that DBcAMP stimulates synthesis of lysosomal enzymes in bone in vitro thus resembling the effects of PTH.

The adenyl cyclase-cyclic AMP system functions effectively in translocation of the hormonal signal because the concentration of cyclic AMP within the cell can be precisely

regulated. In order to faithfully reproduce the hormonal signal, both quantitatively and temporally, requires the rapid metabolism of cyclic AMP. Highly specific phosphodiesterases exist in several tissues and inactivate the cyclic nucleotide by hydrolyzing it to 5' AMP and pyrophosphate (Robison, Butcher, and Sutherland, 1968).

Wells and Lloyd (1967) have demonstrated that large doses of theophylline produce an increase in the level of plasma calcium in parathyroidectomized rats. This effect is presumeably due to a potentiation of cyclic AMP activity since it is known that theophylline is a potent inhibitor of phosphodiesterase. In yet another study, theophylline potentiated the response of thyroparathyroidectomized rats to small doses of DBcAMP, as judged by the changes in urinary excretion of calcium, phosphate, and hydroxyproline and by alteration in plasma calcium and phosphate levels (Rasmussen, Pechet, and Fast, 1968).

Another consequence of PTH-induced activation of the adenyl cyclase-cyclic AMP system is an enhanced excretion of cyclic AMP in the urine (Chase and Aurbach, 1967). The phosphaturia normally exhibited following PTH administration is preceded by the rise in urinary cyclic AMP. This raises the possibility that the rise in phosphate excretion represents the secondary effect of the intermediate cyclic AMP within the cell of the renal tubule rather than the direct effect of phosphate transport out of the cell.

Cyclic AMP has also been implicated in the mediation

of the action of PTH on calcium transport across the gut. Pretreatment of everted intestinal loops with DBcAMP increased the transport of calcium in preparations from vitamin D-treated but not vitamin D-deficient rats (Harrison and Harrison, 1970).

Abe and Sherwood (1972) have presented first evidence for the involvement of the adenyl cyclase-cyclic AMP system in the mediation of the effects of calcium ion on PTH secretion. In an _in vitro_ culture system, parathyroid glands incubated with DBcAMP and theophylline caused significant stimulation of PTH secretion.

Since in the regulation of glycogenolysis a cyclic AMP-dependent protein kinase has been identified as the initial link in a cascading series of protein phosphorylation reactions, the likelihood that protein phosphorylations lie behind the various metabolic manifestations of cyclic AMP has been established (DeLange _et al._, 1968; Walsh, Perkins, and Krebs, 1968; Johnson, Maeno, and Greengard, 1971).

In this regard, Winickoff and Aurbach (1970) have succeeded in identifying a cyclic AMP-sensitive histone kinase from bovine renal cortex. As far as can be ascertained to date, it appears as if this histone kinase remains attached to a binding protein in the resting state. Increased levels of endogenous cyclic AMP react with the binding protein which then uncouples from the kinase. Thus activated, the histone kinase may participate in additional phosphorylation reactions leading to the ultimate physiological response

(Aurbach et al., 1972).

Role of Calcium in the Two Messenger System

The widespread occurrence of both ionic calcium and cyclic AMP as mediators of hormone action has led Rasmussen to propose a role for calcium as a second element in the two messenger control system (Rasmussen and Tenenhouse, 1968; Rasmussen, 1970). The number of systems in which both calcium and cyclic AMP have been implicated as possible second messengers is considerable (Rasmussen, 1970).

The relationship between cyclic AMP and ionic calcium in the intermediation of PTH action is characterized by a number of responses:

1. In isolated renal tubules, the rate of gluconeogenesis is increased by PTH, cyclic AMP, and by raising the calcium content of the medium (Nagata and Rasmussen, 1970).

2. Calcium is not required for PTH to increase cyclic AMP within the cell, but an increase in the rate of gluconeogenesis is observed only in the presence of calcium (Nagata and Rasmussen, 1970).

3. The rise in cyclic AMP following administration of PTH to kidney cell cultures is accompanied by an increased intracellular concentration of ionic calcium (Borle, 1970b). Unlike PTH, cyclic AMP is not capable of enhancing calcium influx from the extracellular fluid, rather it expands the cytoplasmic pool by mobilizing calcium from subcellular compartments (Borle, 1972).

In addition, several laboratories have confirmed the existence of an enzyme which acts on the product of a cyclic AMP-dependent protein kinase reaction and which requires calcium for its expression (Corbin and Krebs, 1968; Walsh et al., 1968; Kuo and Greengard, 1969)

In retrospect, Rasmussen et al. (1972) have formulated an hypothesis which can presently account for the interrelation of cyclic AMP and ionic calcium in the evocation of PTH action on kidney cells and possibly on bone cells.

Excitation of the cell by PTH leads to activation of adenyl cyclase. This enzyme, in turn, acts on ATP to form cyclic AMP and pyrophosphate, raising the concentration of cyclic AMP within the cell. At the same time, an increase in the concentration of ionic calcium in the cytoplasm is brought about by a stimulation of calcium uptake from outside the cell by PTH and by mobilization of calcium from intracellular pools (mitochondria and endoplasmic reticulum) by cyclic AMP. The increase in cyclic AMP also leads to an activation of a cyclic AMP-dependent protein kinase, a phosphorylating enzyme which activates other enzyme substrates. The increased calcium within the cell serves two functions:

1. The products of the phosphorylation reaction catalyzed by cyclic AMP-sensitive protein kinases are now sensitive to calcium. Activation of the calcium-dependent enzyme may lead directly, or through a number of subsequent phosphorylating reactions, indirectly, to final cell acti-

vation. In the case of PTH, this may be a change in cell metabolism or an activation of a transcellular transport of calcium or phosphorus.

2. The increase in calcium concentration within the cell may act as a feedback inhibitor of further adenyl cyclase activation, since it is known that high calcium concentrations inhibit the formation of cyclic AMP (Bradham, Holt, and Sims, 1970).

CHAPTER III

MATERIALS AND METHODS

Adult male and female three-toed box turtles, Terrapene carolina triunguis, which weighed 270-615 g were used in this study. The animals were road collected in Boone and several other counties comprising the Ozark plateau of Missouri during the spring months of 1970 and 1971. All animals were maintained in shallow holding tanks containing continuously flowing tap water and equipped with dry platforms. The animals were kept indoors at room temperature which did not vary appreciably during the year, and on a 12:12 photoperiod. All animals were fed a diet consisting of dog food, lettuce, fruits, and earthworms ad libitum every third day. For each experiment, animals were randomly selected from the holding tanks and individually housed in stainless steel aquaria. They were maintained in the aquaria without food for one week prior to experimentation and then were fasted during the course of experimentation. All animals had free access to shallow vessels of distilled water during the pre-experimental and experimental periods. The animals were sexed on the basis of a number of secondary sexual characteristics such as plastron shape, eye color, hindlimb claw length, and epidermal coloration according to Ditmars (1936) and Carr (1952). Females which showed elevated calcium, magnesium, or phosphorus values, presumeably due to an estrous condition were not used.

Chemical Determinations

Plasma calcium and magnesium concentrations were analyzed on a Perkin-Elmer Model 303 atomic absorption spectrophotometer. One-tenth ml aliquots were diluted and plasma proteins precipitated by the addition of 2.0 ml trichloroacetic acid-lanthanum oxide (LaTCA) solution (315 ppm LaO in 10 per cent TCA). Samples were centrifuged at 2500 rpm for 30 minutes and the supernatants collected into tubes for reading. The samples were compared with calcium and magnesium reference standards. Standards of 0.25, 0.5, 0.75, and 1.0 mg/100 ml calcium were prepared from a reference standard (Fisher) containing 1000 ppm calcium. Likewise, standards of 0.025, 0.05, 0.10, and 0.20 mg/100 ml magnesium were prepared from a reference standard (Fisher) containing 1000 ppm magnesium. Calcium and magnesium were read simultaneously and paired aliquots were prepared for each bleeding time.

Plasma inorganic phosphorus was determined by a micromodification of the method of Dryer, Tammes, and Routh (1957). One-tenth ml aliquots were added to 1.9 ml of 10 per cent TCA in a centrifuge tube. After separation of the clear supernatant from the precipitate by centrifugation, 0.5 ml was placed in a cuvette to which was added 0.1 ml molybdate solution (1:3 dilution of 0.008 M ammonium molybdate) and 1.0 ml of the reducing agent, N-phenyl-p-phenylenediamine. The samples were allowed to stand for precisely ten minutes and read at 770 mμ on a Beckman DU spectrophotometer. Standards

of 0.1, 0.2, 0.5, and 1.0 mg/100 ml inorganic phosphorus were run by taking 1.0 ml of a KH_2PO_4 TCA solution of different dilutions through the entire procedure and then comparing the unknowns with the standard values. Duplicate determinations were made for each bleeding time.

Operative Procedure

Cannulation

Animals in experiments I and IV were cannulated for infusion of chemical agents and blood collection. This permitted experimental manipulation with a minimum of disturbance to the animal. The turtles were anesthetized with Fluothane (Halothane) by placing them in a desiccator containing air saturated with the inhalant. After 45-60 minutes, the anesthetized animals were removed and secured in an upright position to a flat, wooden board.

An artery was sought for cannulation which was both large enough to cannulate easily yet superficial to the peritoneum so that infection would be minimized. A blood vessel which supplies the carapace lying just anterior to the junction of the epigastric, marginocostal, and pelvic arteries was selected (Ashley, 1955).

In order to expose the blood vessel for catheterization, a roughly square segment of carapace, 2.5 cm on a side, was removed with a fine-toothed jewelers saw. The area from which the piece of carapace was excised corresponded to the posterior half of the third costal shield and the anterior

half of the fourth costal shield just above the marginal scutes. Care was taken when removing the shell fragment not to sever blood vessels in the underlying fascia. Thrombin was topically applied to small bleeders. The carapace fragment was kept moist in Ringer's solution for extracellular fluid replacement (Na^+ 146 mEq/l, K^+ 4 mEq/l, Ca^{++} 5.4 mEq/l, and Cl^- 155.4 mEq/l) until replaced. After carefully teasing apart the overlying connective tissue and fat bodies to expose the blood vessel, three loose ligatures were placed around it. The distal ligature (farthest from heart) was tied securely, occluding the artery completely. The ligature nearest to the heart was tied at the free ends only, creating a permanent loop. The middle ligature was tied in a very loose knot which could be drawn tight when the cannula was inserted in the artery. A polyethylene cannula, size PE 10 or PE 50 (Intramedic) six inches in length (0.1 ml) was inserted into an incision made in the wall of the artery with fine surgical forceps. The tip of the cannula was beveled and then blunted for ease of entry and as a precaution against puncturing the arterial wall while *in situ*.

The cannulae were graphite-coated by immersing the cannulae in hot (*not* boiling) water and forcing a graphite-alcohol suspension through the tubing with a syringe. After coating, a cationic surface active agent, benzalkonium chloride, which binds to the graphite-coated plastic was applied by soaking overnight. This provided a surface on

which heparin could be adsorbed and thus a surface superior to silicone in preventing thrombus formation (Whiffen and Gott, 1964).

After securing the cannula in place with two or three ligatures, the operative area was sprinkled with Terramycin and Neomycin and packed with thrombin-soaked Gelfoam, The cannula was laid along the carapace and taped in position and the carapace "window" was replaced. The border around the "window" was also packed with Gelfoam and finally sealed with bone wax. The cannula was filled with heparinized saline (200 U/cc) and a small volume of about 0.5 ml was flushed through the cannula. The collecting end of the cannula was sealed with Seal-ease after each bleeding maneuver.

Although the straight polyethylene cannulae proved satisfactory for the majority of animals used, some still developed clots just above the tip of the cannula before the scheduled termination of the experiment. To eliminate the problem of clotting, presumeably due to the pooling of blood in the occluded artery, a "T" shaped cannula was devised which would allow for an uninterrupted flow of blood through the vessel. Sampling could be achieved by tapping the flow through the vertical extension of the "T". The cannulae were constructed from polyethylene tubing, size PE 90 and PE 160 (Intramedic). PE 160 was heated slowly near the outer cone of a Bunsen burner flame until it became malleable at which time it was drawn out until the diameter of the ends approximated the outside diameter of

PE 10 tubing. Midway between the tapered ends, the thickness was equal to the original thickness of the PE 160 tubing. At this point along its length, a hole was made in the wall with the tip of a hot syringe needle large enough for PE 90 to be inserted. The PE 90 was pushed through the opening as little as possible to present the minimum amount of interference to blood flow. The junction was sealed with melted polyethylene smeared around and cooled rapidly. The cannulae were not graphite-coated and the tapered ends were beveled as little as possible. The procedure for inserting the cannulae was identical with that above except two incisions were required in the vessel wall for the tapered ends.

Cardiac Puncture

Animals in experiments II and III were bled by serial cardiac puncture. After anesthetization they were placed ventral side up on a hollowed-out Styrofoam block. A square "window" approximately 2.5 cm on a side was cut out of the plastron along the midline and immediately posterior to the hinge. This revealed the heart and aortic arches. Blood was withdrawn using a 24 gauge needle. Following each sampling maneuver, the exposed area was sprinkled with Terramycin and Neomycin, the plastron plug was replaced, and its border sealed with bone wax.

Experimental Protocol

A total of 55 animals were used in this study. Four separate experiments were conducted to determine the effect of injection of parathyroid extract (PTE, Eli Lilly and Co.) and turtle parathyroid gland extract on intact animals. Total plasma calcium, magnesium, and inorganic phosphorus concentrations were measured. Controls received equivalent volumes of physiological saline adjusted to the same pH as PTE. Hematocrits were determined for each blood sample obtained since low hematocrits might be symptomatic of hemorrhage at the site of cannulation or cardiac puncture. All data were analyzed for statistical significance at the 0.05 probability level using Student's t-test.

Experiment I

Twenty turtles were randomly divided into three groups. In all cases, the experimental period commenced three days after cannulation. A straight polyethylene cannula was inserted. Blood samples were taken and hematocrit determinations were made at the time of cannulation, zero time, three, four, six, eight, nine, 12, 24, 36, and 48 hours, and three, four, five, six, and seven days after treatment. Blood samples of 0.4 ml were taken and replaced with equivalent volumes of physiological saline. Immediately after the zero time sample, turtles in one group of seven animals received *via* the cannula 200 USP units/kg body weight PTE. One ml possessed a potency of 100 USP units. Turtles in

a second group of seven animals received 100 USP units/kg body weight PTE. Six control animals received equivalent volumes of physiological saline. All blood samples were centrifuged at 2500 rpm for 30 minutes in Wintrobe tubes for hematocrit determination. The plasma samples were drawn off and frozen until analyzed.

Experiment II

Eleven turtles were randomly divided into two groups. The experimental period began three days after removal of the plastron plug. Blood samples were taken and hematocrit determinations were made at the time of the operation, zero time, four, six, eight, 12, 24, 36, and 48 hours, and three, four, five, six, and seven days after treatment. Blood samples of 0.4 ml were withdrawn by heart puncture. Immediately after the zero time sample, seven turtles in one group were injected intramuscularly in the pectoral region with 200 USP units/kg body weight PTE. The control group of four animals was similarly injected with an equivalent volume of physiological saline. The blood samples were centrifuged and the plasmas harvested as in Experiment I.

Experiment III

Thirteen turtles were randomly divided into two groups. The experimental period began three days after removal of the plastron plug. Blood samples were taken and hematocrit determinations were made at the time of the operation, zero time, one, two, three, four, five, six, seven, and eight

days after treatment began. Blood samples of 0.4 ml were withdrawn by cardiac puncture. One group of seven turtles was injected intramuscularly in the pectoral region from day one through day seven with 100 USP unit/kg body weight PTE.(total dosage 700 USP units/kg body weight PTE). The six control animals received an equivalent volume of physiological saline administered likewise from day one through day seven. The blood samples were handled as mentioned above.

Experiment IV

Eleven turtles were randomly divided into two groups. The experimental period was initiated three days after cannulation. All animals were provided with a "T" shaped cannula for hormone infusion and blood collection. Blood samples were drawn and hematocrit determinations were made at the time of cannulation, zero time, four, eight, 12, 24, 36, and 48 hours, three, four, five, six, and seven days after treatment. Blood samples of 0.4 ml were taken and replaced with equivalent volumes of physiological saline. One group of six turtles received *via* the cannula 1.0 ml/kg body weight of an acid extract of turtle parathyroid glands. Parathyroid glands were removed from freshly killed turtles, *Chrysemys picta* and *Pseudemys scripta*, weighed and homogenized in cold 0.1 N HCl (10 ml HCl/mg organ weight). The homogenates were centrifuged at 27,000 x G for 30 minutes and the precipitates discarded. The preparations were frozen until time of use (Clark, 1968b). Before adminis-

tration, the gland extract was adjusted to pH 6.90 with 0.1 N KOH. The five control animals received an equivalent volume of physiological saline, pH 6.90.

CHAPTER IV

RESULTS

Normal total plasma calcium levels in 55 intact T. c. triunguis averaged 9.72 ± 0.60 mg/100 ml.

The average plasma inorganic phosphate concentration in 49 intact T. c. triunguis was 3.41 ± 0.25 mg/100 ml.

Total plasma magnesium levels in 47 intact T. c. triunguis averaged 4.30 ± 0.47 mg/100 ml.

Experiment I

Initial values for total plasma calcium levels were 9.86 ± 0.47 mg/100 ml and 9.77 ± 0.29 mg/100 ml for experimentals and controls, respectively. Intra-arterial injection of 200 USP units of mammalian PTE/kg body weight produced significant elevations in total plasma calcium concentration when compared with saline-injected controls (see Figure I). Significant differences were observed at eight, 12, and 24 hours, and at three, five, and six days after hormone administration. Immediately following PTE injection there was a transient fall in the plasma calcium level which returned to normal by four hours, and continued its upswing, peaking at six hours with a mean value of 12.98 ± 0.07 mg/100 ml. At the end of 48 hours the calcium concentration had returned to pre-treatment levels during which time it fluctuated widely, but then it began to increase steadily apparently beyond the seventh day at which time the experiment was terminated. On the other hand,

Figure I. Experiment I. Effect of commercial bovine parathyroid extract (PTE) at a dose of 200 USP units/kg body weight given as a single intra-arterial injection on calcium, phosphorus, and magnesium levels (mg/100 ml) in plasma of intact Terrapene carolina triunguis. The vertical bars are one-half the standard error of the means. Numbers beside points indicate number of animals. Points of significant difference ($p < 0.05$) between the experimental group and the control (saline-injected) group are indicated by an asterisk (*).

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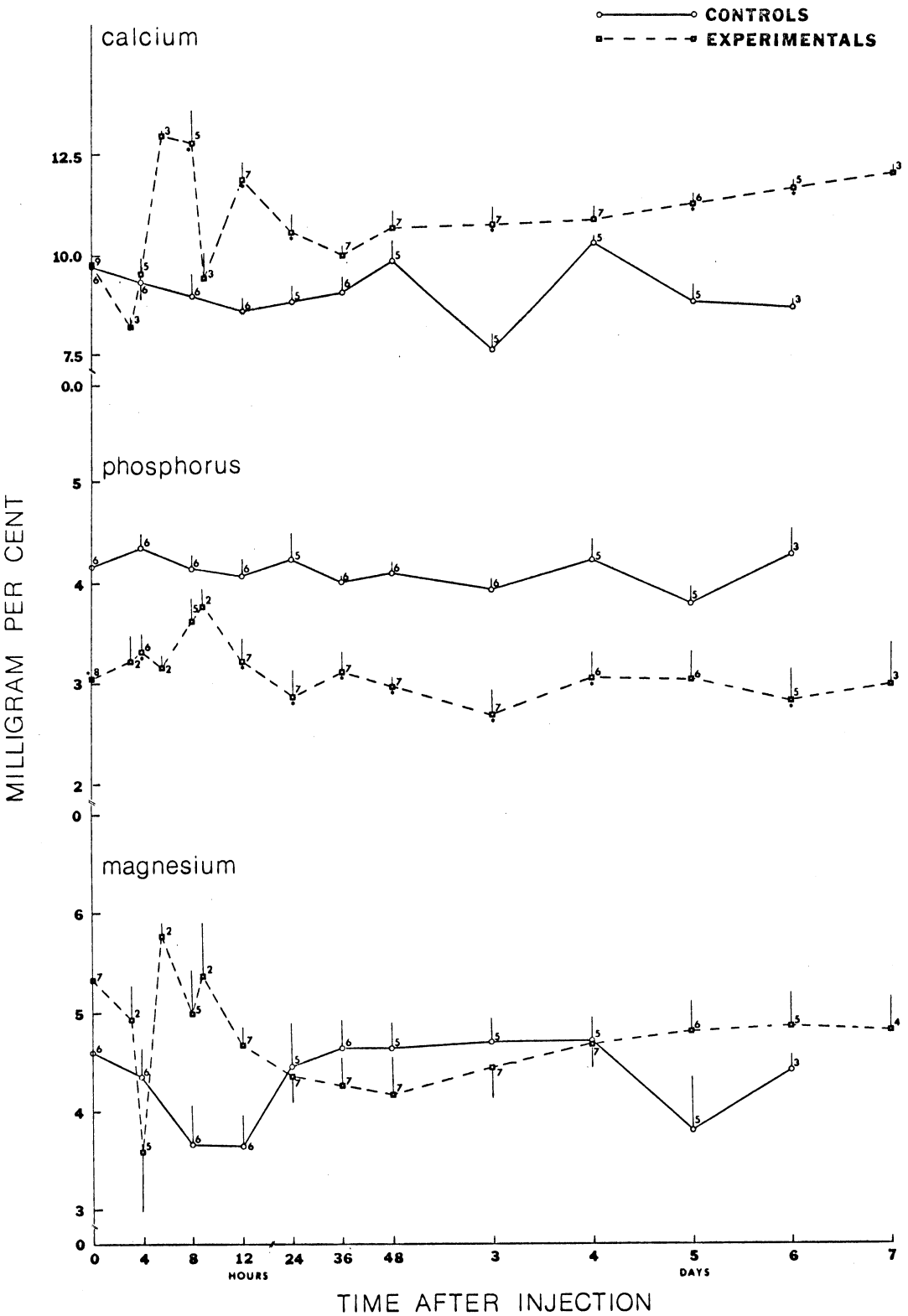

controls increased less than 1.0 mg/100 ml above the mean zero time value through six days.

When the values were plotted as a per cent change from the average zero time value, the same points of significance between control and experimental groups persisted (Figure II).

Initial plasma inorganic phosphate levels in the control group were significantly higher than experimental levels. Zero time plasma phosphate levels averaged 4.16 ± 0.16 mg/100 ml for controls and 3.06 ± 0.18 mg/100 ml for experimentals. Thus, although levels of this parameter remained remarkably stable throughout the experimental term, phosphate values of the PTE-injected group were significantly depressed at four, 12, 24, 36, and 48 hours, and at three, four, and six days after treatment when compared with controls (see Figure I).

When the same values were expressed as a per cent change from zero time, an entirely different pattern is seen (see Figure II). At no point in the experiment was there a significant difference between PTE-injected animals and the saline-injected controls. A brief, non-significant rise in phosphate concentration in the experimental group at eight and nine hours after PTE injection was the only change in an otherwise horizontal concentration profile.

The average values of plasma magnesium concentrations of controls and experimentals, respectively, were 4.59 ± 0.23 mg/100 ml and 5.36 ± 0.32 mg/100 ml. Changes in plasma magnesium concentration of PTE-injected animals paralleled those of controls (see Figure I). There were no significant

Figure II. Experiment I. Effect of commercial bovine parathyroid extract (PTE) at a dose of 200 USP units/kg body weight given as a single intra-arterial injection, on calcium, phosphate, and magnesium levels (per cent change from average zero time value) in plasma of intact Terrapene carolina triunguis. The vertical bars are one-half the standard error of the means. Numbers beside points indicate number of animals. Points of significant difference ($p < 0.05$) between the experimental group and the control (saline-injected) group are indicated by an asterisk (*).

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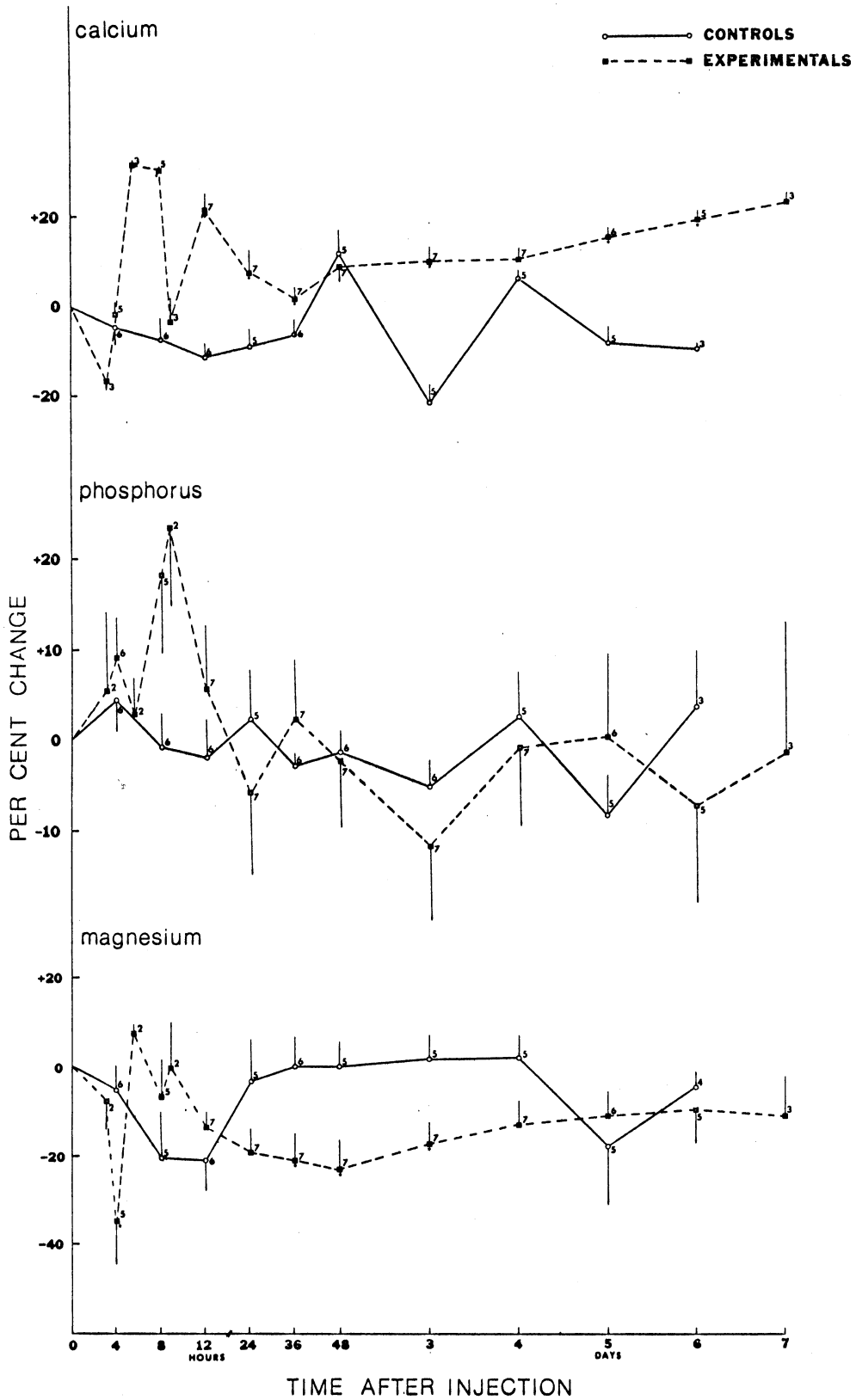

differences between groups at any sampling period.

When expressed as a per cent change from zero time, the PTE-injected group was significantly hypomagnesemic at four, 36, and 48 hours, and at three days following hormone administration (see Figure II). Initially there was considerable fluctuation in the plasma magnesium levels of experimentals. At four hours, the plasma magnesium level fell significantly below the control value, then rose above the control value at six, eight, nine, and 12 hours, only to again fall below the control level at 24 hours. After 24 hours, however, experimentals consistently remained below controls except at five days post-injection when the control value deflected below the experimental value.

Animals were similarly injected with 100 USP units of PTE/kg body weight. Zero time control values averaged 9.77 ± 0.29 mg/100 ml which was significantly higher than controls which averaged only 7.60 ± 0.20 mg/100 ml. Accordingly, experimental values were significantly reduced at 24, 36, and 48 hours, and at four days when compared with saline-injected controls (see Figure III).

When the discrepancy between zero time values was negated by expressing them as a per cent change, plasma calcium concentrations of the PTE-injected group were significantly higher than control values at 12 hours and at three days (see Figure IV). The response elicited by 100 USP units of PTE was qualitatively similar to the higher dose response. The experimentals initially became hypo-

Figures III and IV. Experiment I. Effect of commercial bovine parathyroid extract (PTE) at a dose of 100 USP units/kg body weight given as a single intra-arterial injection, on calcium, phosphorus, and magnesium levels (mg/100 ml in Figure III above; per cent change from average zero time value in Figure IV below) in plasma of intact Terrapene carolina triunguis. The vertical bars are one-half the standard error of the means. Numbers beside points indicate number of animals. Points of significant difference ($p < 0.05$) between the experimental group and the control (saline-injected) group are indicated by an asterisk (*).

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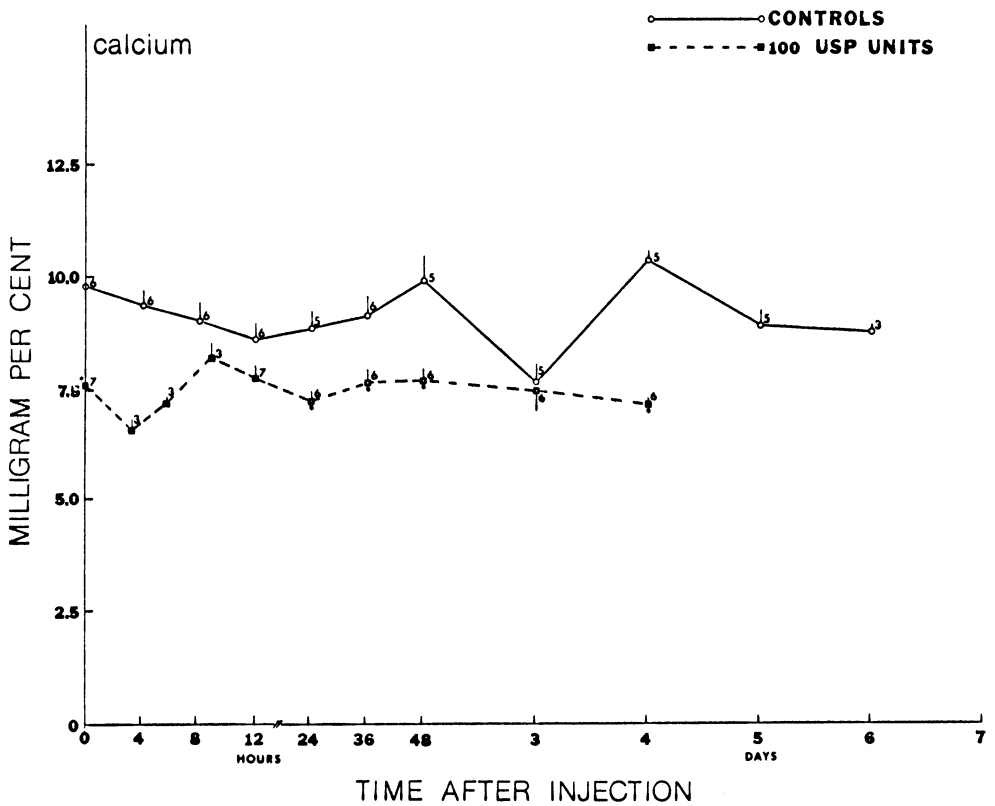
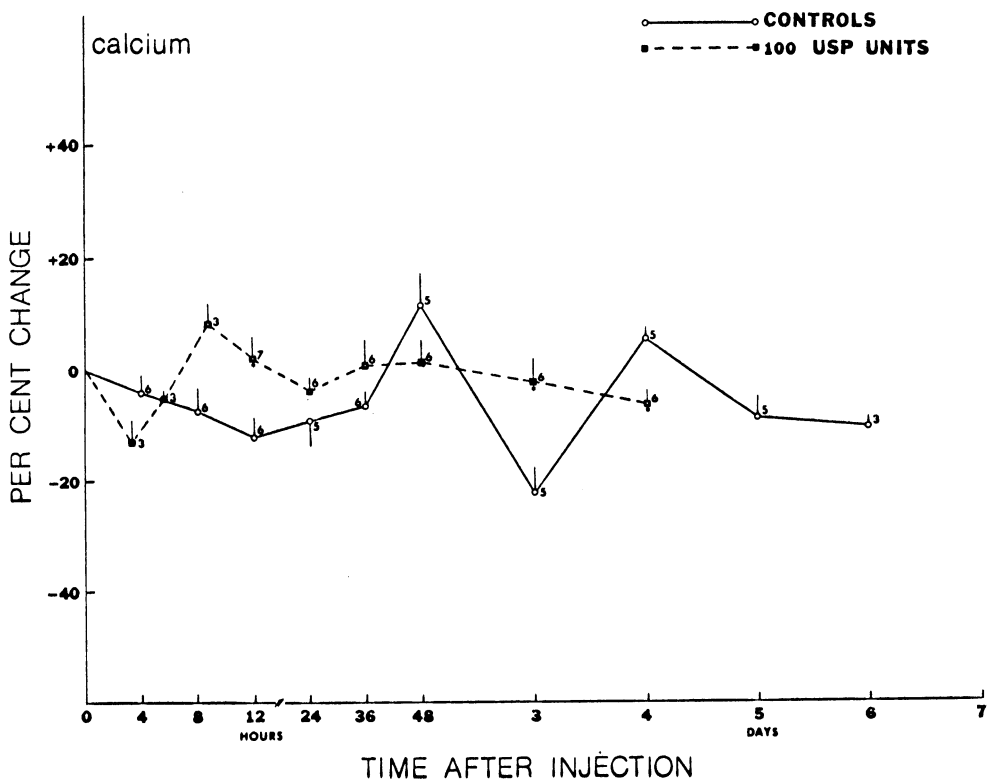




calcemic, then abruptly hypercalcemic, followed by a gradual return to normal. Control values were approximately ten per cent below zero time levels by six days.

PTE-induced changes in groups receiving 100 and 200 USP units of the hormone were compared (see Figures V and VI). Plasma calcium concentrations in the high dosage group were significantly elevated above the values of the low dosage group at all times except at eight hours.

When the values were expressed as a per cent change from zero time the differences were significant only at six and 12 hours and at four days.

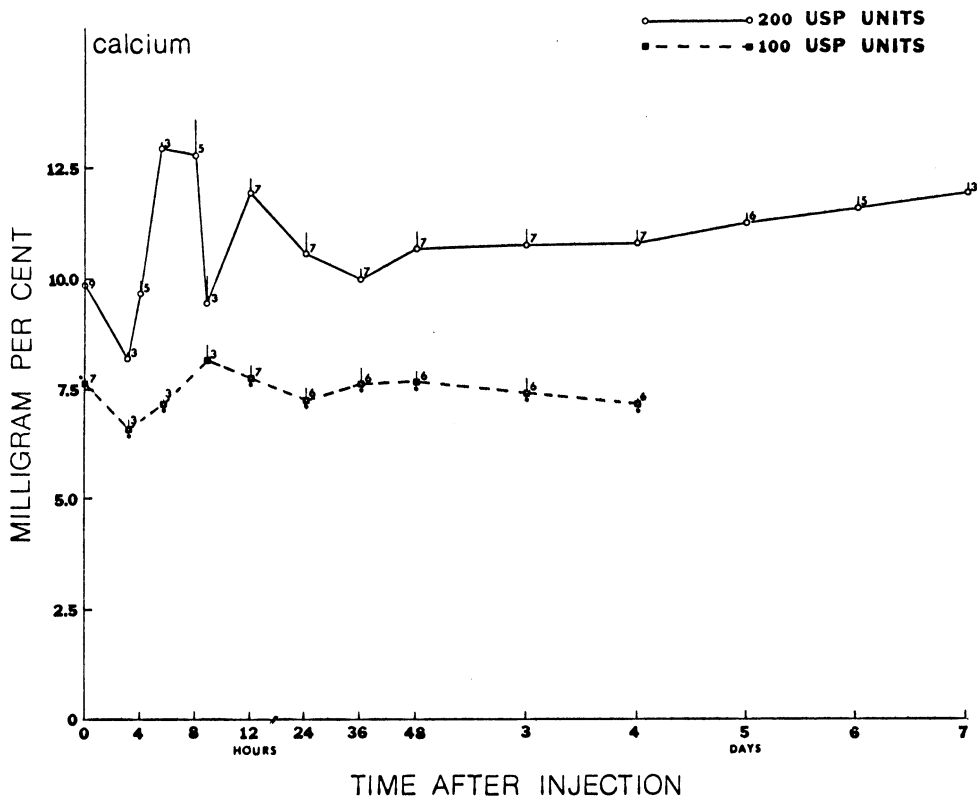
Experiment II

Experimental animals received 200 USP units of PTE/kg body weight by an intramuscular route of injection. Controls were similarly injected with physiological saline. Pre-treatment levels of total plasma calcium averaged 9.28 ± 0.17 mg/100 ml and 9.61 ± 0.46 mg/100 ml for controls and experimentals, respectively. Parathyroid extract produced an abrupt rise in plasma calcium concentration after which the calcium level remained quite stable through the seven day study. Controls were somewhat less stable, but exhibited no appreciable net change in calcium concentration after seven days. The PTE-injected group had significantly elevated plasma calcium levels at four, 12, and 36 hours, and at five days when compared with controls (see Figure VII).

When plotted as a per cent change from zero time, sig-

Figures V and VI. Experiment I. Effect of commercial bovine parathyroid extract (PTE) at two different doses (200 USP units/kg body weight and 100 USP units/kg body weight) given as a single intra-arterial injection, on calcium, phosphorus, and magnesium levels (mg/100 ml in Figure V above; per cent change from average zero time value in Figure VI below) in plasma of intact Terrapene carolina triunguis. The vertical bars are one-half the standard error of the means. Points of significant difference between experimental groups ($p < 0.05$) are indicated by an asterisk (*).

PLASMA MINERAL LEVELS



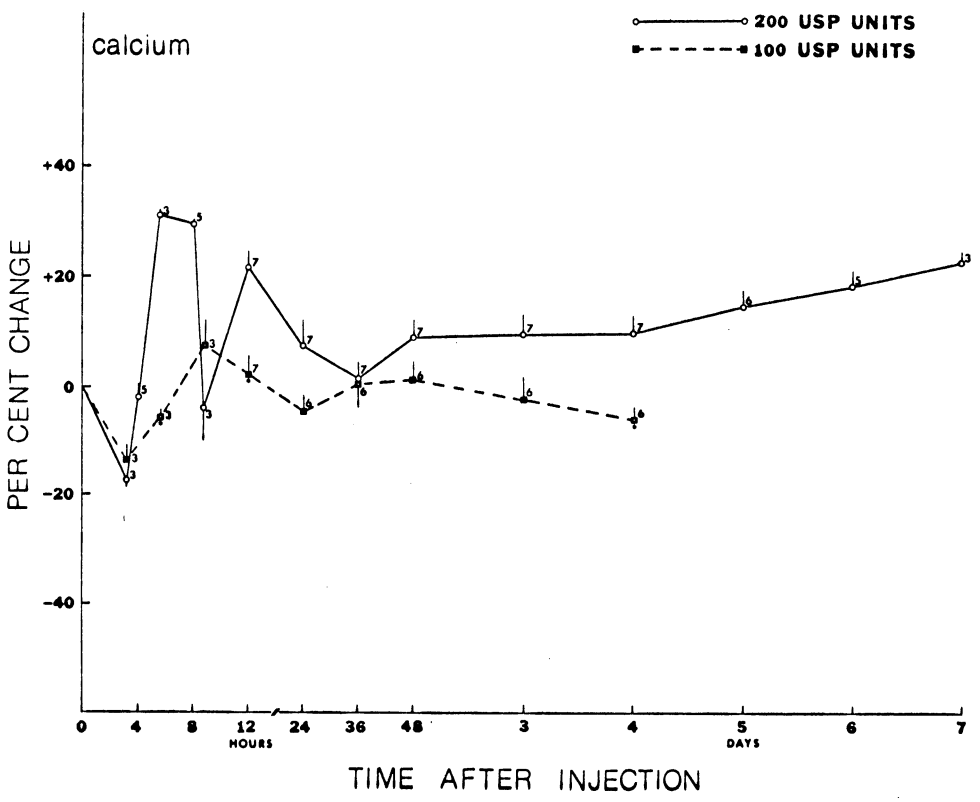
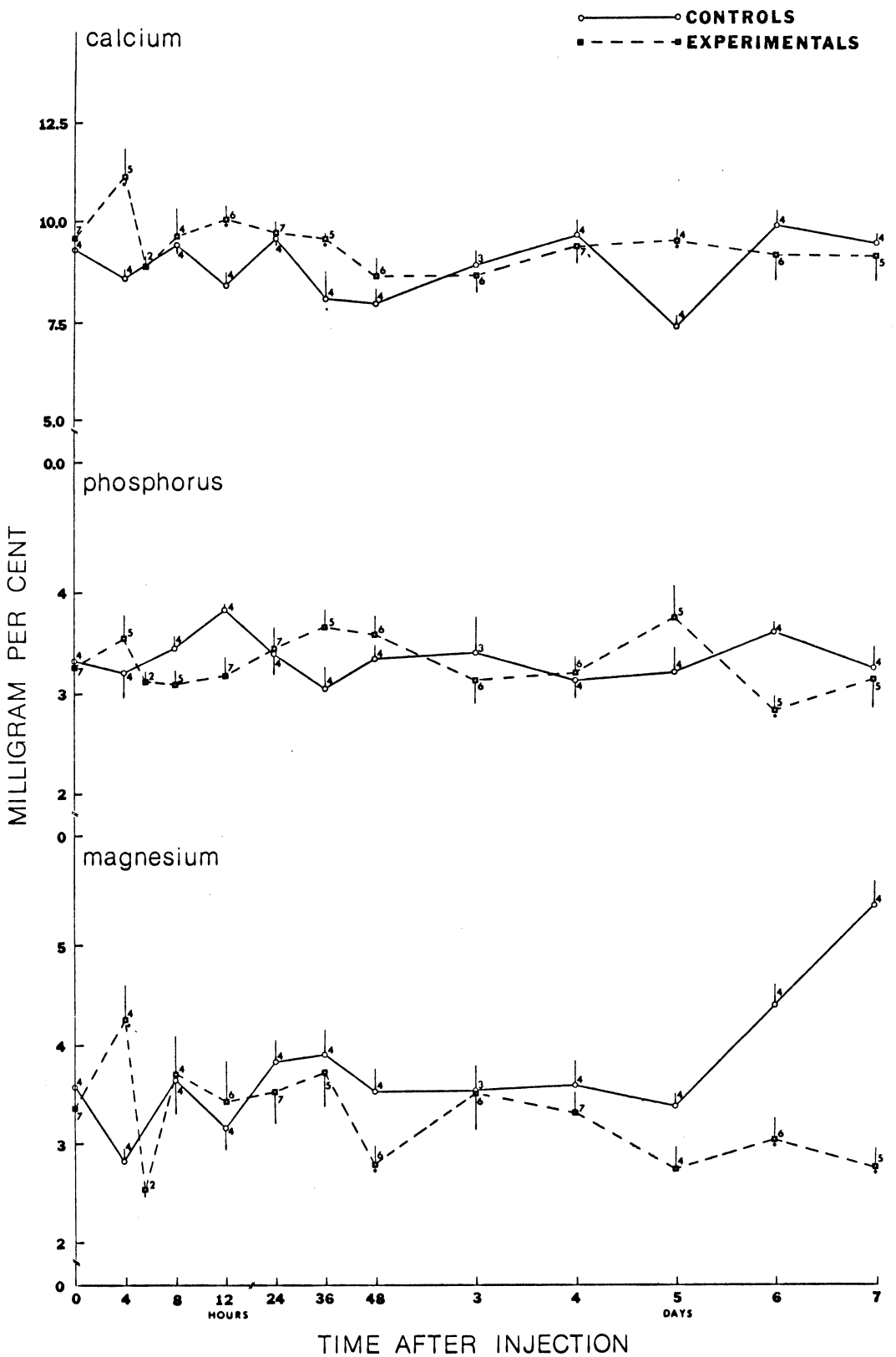


Figure VII. Experiment II. Effect of commercial bovine parathyroid extract (PTE) at a dose of 200 USP units/kg body weight given as a single intramuscular injection on calcium, phosphorus, and magnesium levels (mg/100 ml) in plasma of intact Terrapene carolina triunguis. The vertical bars are one-half the standard error of the means. Points of significant difference between the experimental group and the control (saline-injected) group ($p < 0.05$) are indicated by an asterisk (*).

PLASMA MINERAL LEVELS



nificant points of difference at 12 and 36 hours were abolished (see Figure VIII).

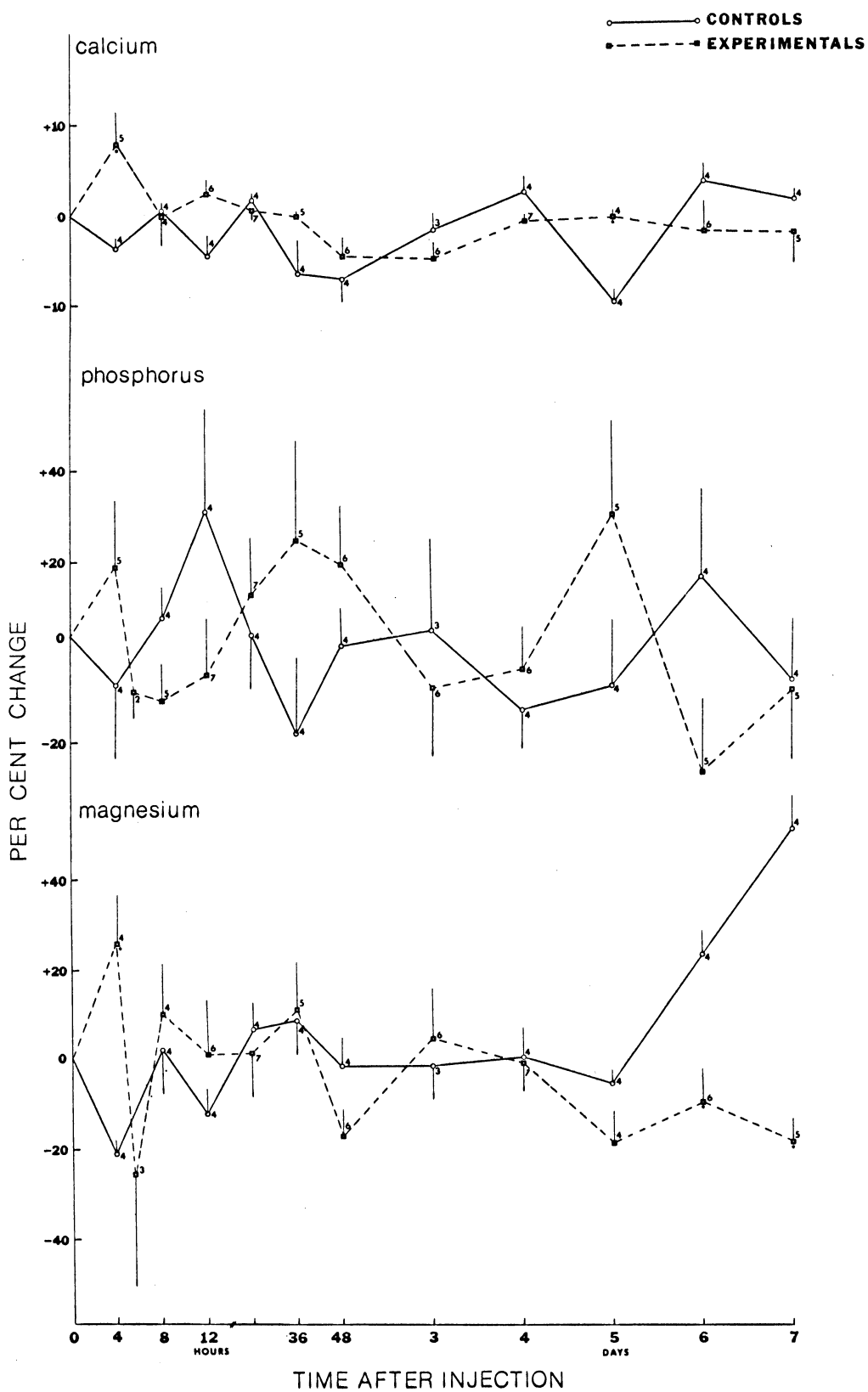
Pre-treatment levels of plasma inorganic phosphate of control and experimental groups respectively averaged 3.32 ± 0.32 mg/100 ml and 3.25 ± 0.16 mg/100 ml. Concentration profiles of both groups intersected frequently and after seven days of treatment, control and experimental values differed by less than five per cent from pre-treatment levels (see Figure VII). Only at six days was there a significant difference between groups, the controls having a greater phosphate concentration than the experimentals.

When plasma phosphate concentration was expressed as a per cent change from zero time, there were no points of significant difference between groups (see Figure VIII).

Initial values for the magnesium concentration in plasma averaged 3.57 ± 0.21 mg/100 ml and 3.36 ± 0.32 mg/100 ml for controls and experimentals, respectively. Experimentals responded rapidly to PTE administration. Plasma magnesium levels were increased significantly over controls at four hours (see Figure VII). Thereafter, the plasma magnesium levels of the hormone-injected group gradually declined to 20 per cent below pre-treatment levels. Meanwhile, levels in control turtles were maintained near the zero time value until day five, when the control group became hypermagnesemic. Apparently the values were still rising at the time the experiment was terminated. Signif-

Figure VIII. Experiment II. Effect of commercial bovine parathyroid extract (PTE) at a dose of 200 USP units/kg body weight given as a single intramuscular injection on calcium, phosphorus, and magnesium levels (per cent change from average zero time value) in plasma of intact Terrapene carolina triunguis. The vertical bars are one-half the standard error of the means. Points of significant difference ($p < 0.05$) between the experimental group and the control (saline-injected) group are indicated by an asterisk (*).

PLASMA MINERAL LEVELS



icant differences between groups were distinguished on days six and seven.

When the values were plotted as a per cent change from zero time as in Figure VIII the same disparities between experimentals and controls were evident. Experimentals, however, were not significantly depressed at 48 hours as above.

Experiment III

Experimentals received several small doses of PTE (100 USP units/kg body weight/daily) spaced over a one week period and controls received physiological saline on an identical timetable. The control group had plasma calcium levels which averaged 10.30 ± 0.44 mg/100 ml while the experimental group averaged only 8.40 ± 0.66 mg/100 ml for the same parameter. Hence, although control levels remained stable and experimental levels progressively rose, significant differences between groups were largely obscured. Indeed, only on day four were calcium levels of the PTE-injected group higher than control levels (see Figure IX).

When the data were treated as a per cent change from zero time, the hypercalcemia elicited by PTE administration was statistically borne out. Plasma calcium concentration of the experimental group was significantly elevated over the control group at four, seven, and eight days (see Figure X). Moreover, levels in the experimental group were consistently higher than controls on days one, three, five, and six, but the levels were not statistically significant.

Figure IX. Experiment III. Effect of commercial bovine parathyroid extract (PTE) at a dose of 100 USP units/kg body weight given daily for one week by intramuscular route of injection on calcium, phosphorus, and magnesium levels (mg/100 ml) in plasma of intact Terrapene carolina triunguis. The vertical bars are one-half the standard error of the means. Points of significant difference ($p < 0.05$) between the experimental group and the control (saline-injected) group are indicated by an asterisk (*).

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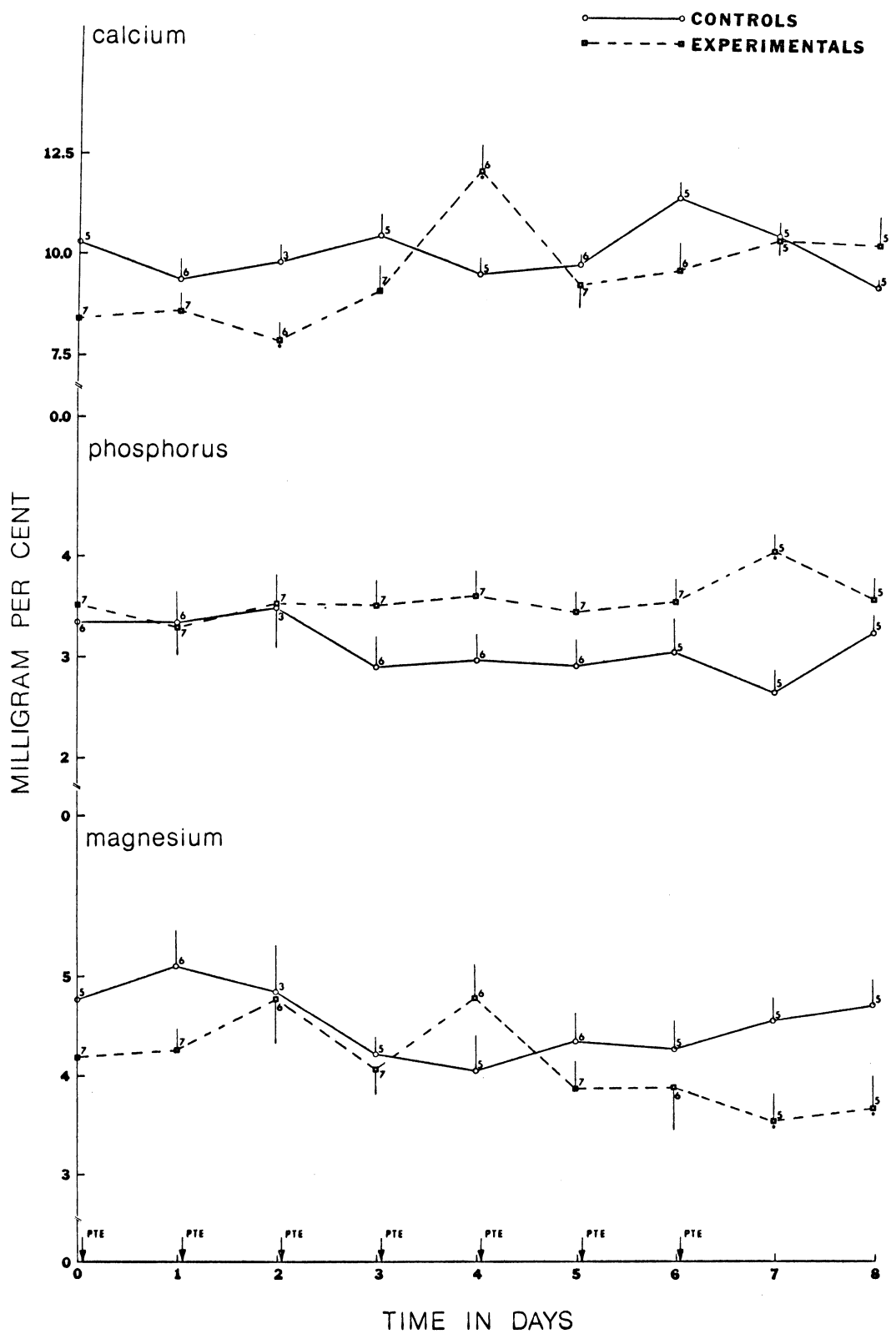
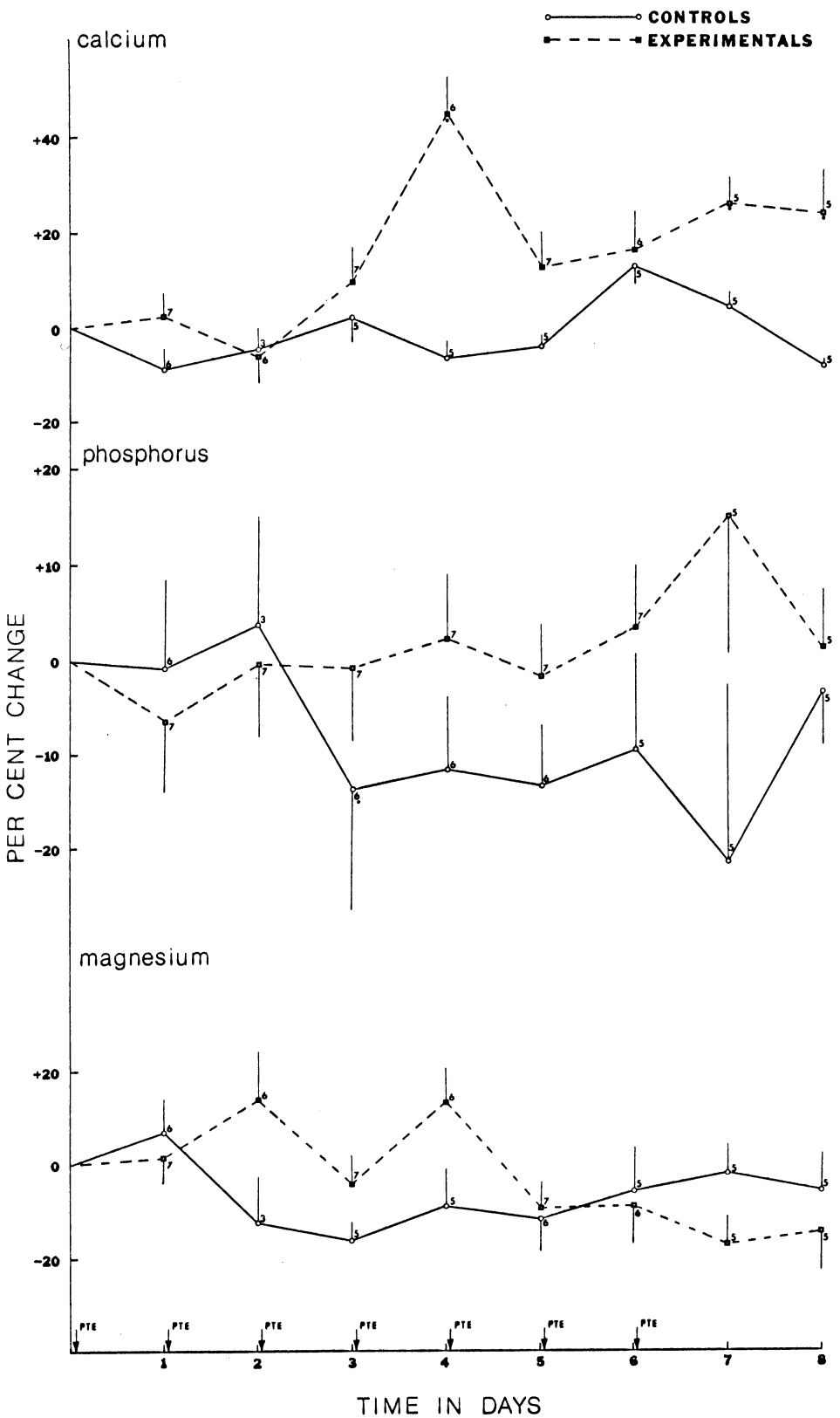


Figure X. Experiment III. Effect of commercial bovine parathyroid extract (PTE) at a dose of 100 USP units/kg body weight given daily for one week by intramuscular route of injection on calcium, phosphorus, and magnesium levels (per cent change from average zero time value) in plasma of intact Terrapene carolina triunguis. The vertical bars are one-half the standard error of the means. Points of significant difference ($p < 0.05$) between the experimental group and the control (saline-injected) group are indicated by an asterisk (*).

PLASMA MINERAL LEVELS



Initial phosphate concentrations of plasma of controls and experimentals, respectively, averaged 3.35 ± 0.37 mg/100 ml and 3.52 ± 0.30 mg/100 ml. Small, multiple doses of PTE had no effect on plasma phosphate levels. Phosphate levels in controls were depressed at seven days but there were no other points of significant difference between groups (see Figure IX).

When plotted as a per cent change from zero time, no significant differences between groups were observed (see Figure X).

Initial magnesium concentrations in plasma of controls and experimentals, respectively, averaged 4.78 ± 1.55 mg/100 ml and 4.20 ± 0.19 mg/100 ml. Parathyroid extract had no effect on plasma magnesium levels. After six days of treatment, controls and experimentals were separated by only 0.38 mg/100 ml plasma magnesium, however, at seven and eight days magnesium levels in controls were significantly higher (see Figure IX). These differences at seven and eight days of treatment were abolished when the values were plotted as a per cent change from zero time (see Figure X).

Experiment IV

Preparations of acid extracts of turtle (Pseudemys scripta and Chrysemys picta) parathyroid glands were administered intra-arterially into experimental subjects and assayed for hypercalcemic activity. Pre-treatment calcium levels in plasma of control and experimental groups, respect-

ively, were 12.98 ± 1.57 mg/100 ml and 9.67 ± 1.18 mg/100 ml. Gland preparations were apparently ineffective in altering plasma calcium concentrations. There were no significant differences between groups (see Figure XI).

When the values were expressed as a per cent change from zero time, an entirely different pattern is seen. When plotted in this manner, the experimental group displayed distinct elevations in plasma calcium levels (see Figure XII). At 36 hours the PTE-injected group had values more than 55 per cent above pre-treatment levels. Significant differences between groups were observed at 24, 36, and 48 hours. Moreover, during the seven day period calcium levels in the experimental group never returned to pre-treatment levels, while controls fell as much as 25 per cent below zero time values.

Control animals had zero time inorganic phosphate concentrations of 3.34 ± 0.29 mg/100 ml and experimentals had plasma phosphate levels of 3.25 ± 0.19 mg/100 ml. Parathyroid extract was unable to alter plasma phosphate concentrations. Levels of this parameter were consistently lower in the experimental group but never reached significant levels when expressed either as absolute values or as per cent change from zero time values (see Figures XI and XII).

Prior to hormone injection, magnesium concentrations in plasma averaged 4.41 ± 0.52 mg/100 ml and 4.15 ± 0.43 mg/100 ml for control and experimental groups, respectively. Plasma magnesium concentrations remained stable throughout

Figure XI. Experiment IV. Effect of an acid extract of turtle (Pseudemys scripta and Chrysemys picta) parathyroid glands (10 ml HCl/100 mg gland weight) given as a single intra-arterial injection on calcium, phosphorus, and magnesium levels (mg/100 ml) in plasma of intact Terrapene carolina triunguis. The vertical bars are one-half the standard error of the means. Points of significant difference ($p < 0.05$) between the experimental group and the control (saline-injected) group are indicated by an asterisk (*).

PLASMA MINERAL LEVELS

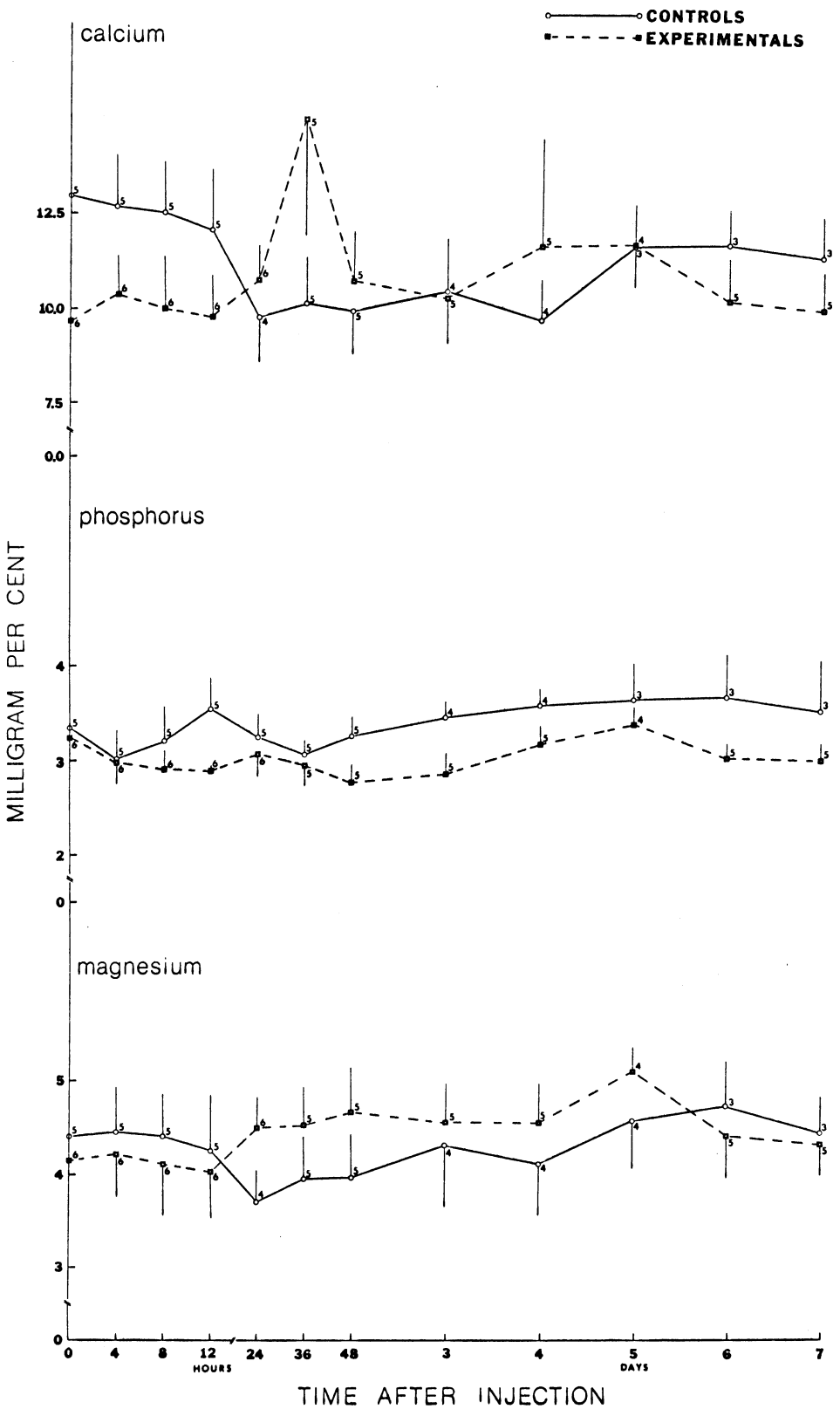
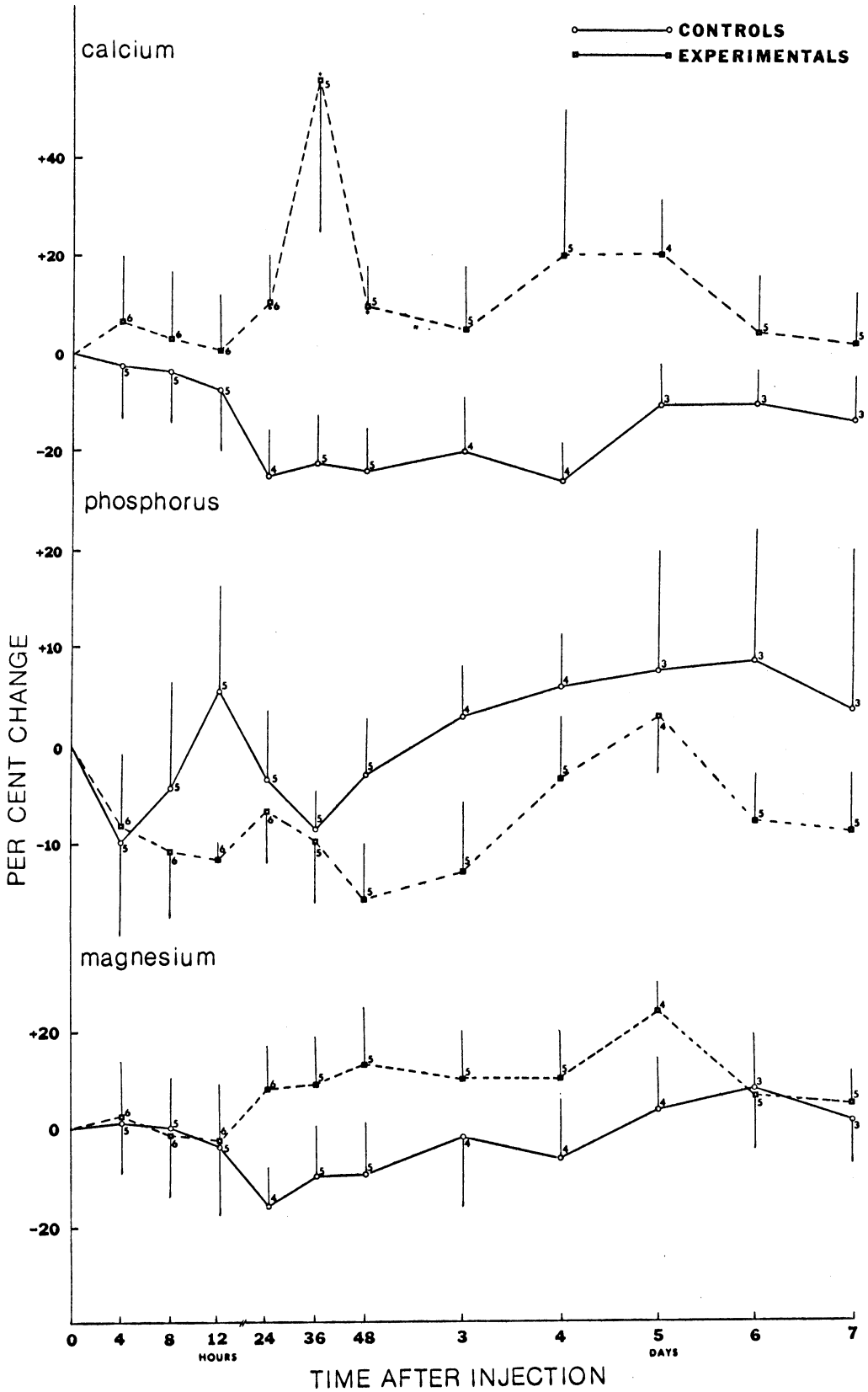


Figure XII. Experiment IV. Effect of an acid extract of turtle (Pseudemys scripta and Chrysemys picta) parathyroid glands (10 ml HCl/100 mg gland weight) given as a single intra-arterial injection on calcium, phosphorus, and magnesium levels (per cent change from average zero time value) in plasma of intact Terrapene carolina triunguis. The vertical bars are one-half the standard error of the means. Points of significant difference ($p < 0.05$) between the experimental group and the control (saline-injected) group are indicated by an asterisk (*).

PLASMA MINERAL LEVELS



the duration of the experiment and no significant changes were observed between PTE-injected and control groups (see Figures XI and XII).

CHAPTER V

DISCUSSION

The major objective of this research has been to assess mineral responses to parathyroid extract in a species of reptile which possesses exceptional stores of bone mineral and which is well-adapted to a terrestrial existence. The objective was pursued through four separate experiments in order to explore any differences in mineral responses due to experimental protocol.

Resting Plasma Mineral Levels

Plasma calcium concentrations in 55 untreated Terrapene carolina triunguis averaged 9.72 ± 0.60 mg/100 ml which is in good agreement with the resting levels in Pseudemys scripta and Chrysemys picta measured by Clark (1965). Pooled calcium values for 61 animals used in her study averaged 9.60 ± 0.24 mg/100 ml. Hutton and Goodnight (1957) are the only investigators to measure plasma electrolytes in the Terrapene genus. Unfortunately, they were able to detect only 1.4 mg/100 ml of calcium in the plasma, a value which must be regarded cautiously since it is at variance with all recently published measurements of plasma calcium in reptiles and all other vertebrates. The equally low value of 1.60 mg/100 ml plasma calcium in an unspecified species of the genus Pseudemys reported by the same authors is also an anomaly in the literature. The only other investigator to make direct resting measurements of electrolyte levels in turtles was Smith (1929).

He analyzed the serum, perivisceral and pericardial fluids, and the bile of several freshwater and marine turtles. The sera contained from 12.40 mg/100 ml of calcium in Caretta caretta to 22.0 mg/100 ml of calcium in Chrysemys marginata belli and Chelydra serpentina. However, in view of the fact that only one or two determinations were made on each species, the representative nature of these values must be questioned. The plasma calcium levels of T. c. triunguis found in these experiments also compare favorably with previously reported values from several species of snakes and lizards, and from the American alligator.

Plasma inorganic phosphorus concentrations in 49 untreated T. c. triunguis averaged 3.41 ± 0.25 mg/100 ml, a value which is in good accord with other published values in other turtles. Normal serum phosphate of 61 C. picta and P. scripta had a mean value of 2.88 ± 0.12 mg/100 ml (Clark, 1965). Normal phosphate levels in Pseudemys and Terrapene, measured by Hutton and Goodnight (1957), averaged 3.58 and 2.78 mg/100 ml, respectively. The values reported by Smith (1929) in chelonians also resemble those reported here. The concentration of phosphate in the plasma of turtles, as a group, is considerably below the plasma phosphate levels reported in other reptiles. Plasma phosphate levels in the Eastern garter snake, Thamnophis sirtalis, had a mean value of 4.81 ± 0.53 mg/100 ml (Clark, 1971a). And, McWhinnie and Cortelyou (1968) reported mean plasma phosphate values of 8.12 ± 0.61 and 6.98 ± 1.32 mg/100 ml, respectively, in the

iguanid lizards, Dipsosaurus dorsalis and Sceloporus grammicus.

Total plasma magnesium concentrations in 47 untreated T. c. triunguis averaged 4.30 ± 0.47 mg/100 ml. The values resemble magnesium levels in Terrapene and Pseudemys which measured 4.54 and 3.08 mg/100 ml, respectively (Hutton and Goodnight, 1957). Smith's measurements (1929) of plasma magnesium in chelonians range from a low value of 1.20 mg/100 ml in Graptemys geographica to a high value of 11.52 mg/100 ml in Chrysemys marginata belli, but again his small sample size prevents serious consideration of these values. Magnesium concentrations in the plasmas of ten species of Natrix and 14 species of Thamnophis measured 6.24 and 6.08 mg/100 ml, respectively, and represent the only published values for non-chelonian reptiles (Dessauer, 1956).

The normal concentration of calcium and phosphate in the plasma of human subjects ranges from 9.0 to 11.0 mg/100 ml and from 2.50 to 4.50 mg/100 ml, respectively, which are in good agreement with the published values in reptiles, generally, and in T. c. triunguis, in particular. Plasma magnesium concentrations in man are substantially lower than the values calculated for T. c. triunguis or any other reptile.

Variability in Plasma Mineral Levels

There was considerable variation in the concentration of plasma electrolytes both before and during the experiments. And, a notable lack of uniformity in responsiveness to ident-

ical hormonal treatment was a consistent feature of the turtles in the present study. Several factors might have contributed to the observed variability.

An annual cycle in the level of secretory activity of the parathyroids has been observed in two species of lizards, Scincus scincus and Chalcides ocellatus (Sidky, 1965). However in turtles, Clark (1965) was unable to detect any seasonal variation in secretory activity. The data in this thesis are in agreement with Clark. Plasma mineral levels in untreated, male T. c. triunguis were similar throughout the year, suggesting an absence of seasonal parathyroid and ultimobranchial secretory cycles.

Increased secretion of estrogens during the breeding season in egg-laying reptiles produced hypercalcemia, hyperphosphatemia, and hypermagnesemia (Dessauer, Fox, and Gilbert, 1956; Clark, 1967b). The imposition of an estrogenized condition, with its associated disturbance of plasma mineral levels, on mineral homeostasis could certainly interfere with a mineral response to PTE. Since parts of these studies were conducted in the spring and in the fall, at which time or times, the box turtle breeds (Carr, 1952), females with elevated plasma mineral levels, presumeably due to reproductive activity, were not used. Interestingly enough, most females had plasma mineral levels within the normal range at the times when they were expected to be in estrous. It is possible that the stress associated with captivity upset the box turtle's normal reproductive pattern and they became

acyclic. Autopsies were not performed to assess reproductive condition.

All animals used in the study were fasted for one week prior to the administration of hormone or placebo, but they were allowed free access to deionized water at all times. By eliminating the dietary source of minerals, plasma mineral levels would be expected to be relatively stable, in contrast to the situation in which the animals would be fed. Yet in spite of these precautions, the variability was striking, a fact which suggests that the absence of stability in plasma mineral levels is an inherent characteristic of the turtle's physiology. Clark (1965) made a similar observation with regard to variability in plasma mineral levels and attributed the instability to a lack of precise control of mineral homeostasis. The source of variability certainly was not due to the methodology, since the sensitivity limits and reproducibility of the techniques used in mineral determinations were extremely high.

In view of the great variability in mineral levels, zero time values of PTE-injected and control groups were equated and all subsequent determinations of plasma mineral concentrations were expressed as a per cent change from the zero time value, in order to make comparisons between experimental animals and their controls more meaningful.

Plasma Calcium

Parathyroid extract (PTE) elicited the most conspicuous calcium response when introduced directly into the bloodstream via the indwelling cannula. When 200 units of a commercial bovine extract were given by this route as a single injection, plasma calcium rose over 30 per cent in just six hours, fluctuated widely for the next 30 hours, and then steadily increased to and perhaps beyond the seven day period of the experiment. Controls exhibited no comparable fluctuations in either direction, through the seven days of experimentation.

Parathyroid extract was also administered through a cannula at a dose of 100 units/kg body weight. This lower dose produced a calcium response qualitatively similar to a response to 200 units PTE/kg body weight, although the magnitude of the response was proportionately reduced. Both doses of PTE produced an initial fall in plasma calcium before causing it to rise. Intravenous injections of PTH in rats (Tashjian, Ontjes, and Munson, 1964) and dogs (Parsons, Neer, and Potts, 1971) produce the same transient fall in plasma calcium before causing it to increase. This effect has usually been attributed to calcitonin contamination of PTH. However, evidence obtained with calcitonin-free purified PTH shows that the transient hypocalcemia is due to PTH itself and results from a calcium shift into bone (Parsons and Robinson, 1971). These results strengthen current suggestions that entry of calcium into bone cells occurs very early in the response to PTH and may represent its primary action.

Acid extracts of turtle parathyroid glands given as a single administration did not produce significant hypercalcemia until 24 hours post-injection. The response was maximal at 36 hours at which time the plasma calcium concentration was almost 50 per cent above pre-injection levels, but by the seventh day, in contrast to the response to 200 units/kg body weight of mammalian extract, levels were almost back to normal. The lack of coincidence in the appearance of the hypercalcemic peaks might be attributable to a species specificity of the hormonal preparations. Judging from the delayed responsiveness of the turtle to reptilian PTE, but not the mammalian PTE, it would appear that the latter is more potent. Since in mammals, PTH synthesized and stored in the gland is higher in molecular weight than the circulating form (Potts et al., 1972), it is quite plausible that in the reptile the circulating form represents a biologically active fragment of a larger less active precursor molecule from which it is cleaved during secretion. Extracts of parathyroid glands, then, may contain the hormone predominantly in the storage or precursor form and may not manifest the metabolic heterogeneity expected of hormones from different animal sources. One might predict that the reptilian extract, on the basis of presumed greater chemical similarity to the recipients' native hormone, would have more of an impact on the calcium response in the turtle than the mammalian extract. On the other hand, the mammalian extract which contains an active principle more unlike the native hormone than the reptilian

extract, would be more resistant to degradation, possess a longer circulating half-life, and thus evoke a greater mineral response. In any event, since the potency of the reptilian extract was not determined by bioassay, it is impossible to make meaningful assessments of relative kinetics and potencies. Finally, it should be recognized that making assessments of relative potencies of hormonal preparations based on expected responses, extrapolated from evidence gained in mammals, may not be particularly meaningful in infra-mammalian classes.

When 200 units of mammalian PTE were administered by an intramuscular route, a degree of hypercalcemia comparable to that in Experiment I was not observed. The onset of hypercalcemia was just as sudden, but it was of lesser magnitude, and the response was not nearly as protracted, since calcium values returned to normal by six hours. It is well-established in mammals that PTH exerts an immediate effect on bone resorption which involves a membrane permeability change, and a later effect which requires a change in enzyme synthesis (Raisz and Niemann, 1967). It is reasonable to assume that the threshold for responding may be different for each process. Since both processes contribute to the hypercalcemia, a high dose, by activating both processes, might produce a prolonged hypercalcemia, while a smaller dose, by activating the early response, with the presumeably lower threshold for activation, might produce only a transient rise in calcium. If the same amount of PTE is administered, a smaller fraction per unit of time would be expected to reach the skeleton and

produce a calcium response if injected intramuscularly, than if injected directly into the bloodstream. This may account for the discrepancy seen between groups of turtles receiving identical quantities of extract but by different routes.

The dichotomy in the response may also reflect differences in the site of injection. Parathyroid extract administered through the cannula arrives at the vascular bed of the carapace, an exceptional area of mineral reserve, before entering the general circulation. Parathyroid extract injected into the pectoral region circulates systemically and does not reach the carapace until significantly diluted.

Although the effects of PTE, given *via* an indwelling cannula, have not been investigated in reptiles prior to this study, PTE has been administered in reptiles both intramuscularly and intraperitoneally as single injections. Clark (1965) injected two species of freshwater turtles, *P. scripta* and *C. picta* with 200 units of mammalian PTE intramuscularly in the pectoral region. Since one experiment in the present study employed an identical experimental procedure, direct comparisons can be made. After three days of hormone treatment, serum calcium concentrations rose 20 per cent above control values in intact and parathyroidectomized turtles. Assuming that the 20 per cent elevation in plasma calcium after three days is maximal over the ten day study (Clark has no accompanying figures or tables), then both *P. scripta* and *C. picta* exhibit a delayed reaction to PTE since a comparable degree of hypercalcemia was demon-

strated as early as four hours in T. c. triunguis. However, Clark did not make her initial determination until 24 hours after PTE injection and any earlier response was missed. If, as it appears, a larger dose, or a more effective avenue of injection is required to extend the hypercalcemia to several days, then both P. scripta and C. picta give the appearance of heightened sensitivity to PTE. The disparity in responsiveness, however, may be dose-related. While T. c. triunguis received only 200 units of PTE/kg body weight, both P. scripta and C. picta received 200 units of PTE/animal or approximately 400 units of PTE/kg body weight, since animals in both studies were of comparable size.

Intact Eastern garter snakes, T. sirtalis, responded to intraperitoneal injection of mammalian PTE at a dose of 2.35 IU PTE/100 g body weight with increased serum calcium values 20 hours after injection, in agreement with the previous studies (Clark, 1971a).

Although it has not been directly demonstrated that bone is the principal target of PTE in reptiles and therefore the source of the elevated calcium, several indirect parameters have been identified which indicate enhanced bone resorption.

Umanski and Kudokotzev (1951) noted that unsuccessful limb regeneration in the lizard, Lacerta agilis, may be due to rapid muscle redifferentiation without concomitant bone remodeling. They induced bone remodeling with daily injections of PTE to a cumulative dose of 96 USP units. Deossifi-

cation was so severe in many lizards receiving PTE that the cranium was deformed, and the limb and jaw bones softened to the extent that movement could not be supported, nor food eaten. Microscopic studies showed that bone resorption had occurred, and that the number of osteoclasts was elevated.

More recently, Clark (1965) detected increased numbers of osteoclasts in PTE-injected P. scripta and hatchling G. pseudogeographica, than in controls, although the small sample sizes did not permit statistical comparison. In a collateral experiment, calcium contents of fibulae of normal and parathyroidectomized C. picta and P. scripta treated with PTE or distilled water were not significantly different. Parathyroidectomized turtles receiving PTE showed slightly higher calcium content than did water-injected parathyroprivic turtles.

In light of the possibility that demineralization and elevated osteoclast counts in bones of PTE-treated reptiles may be accompanied by metabolic changes, citric acid levels of limb bones from D. dorsalis were measured by McWhinnie and Cortelyou (1968). The citric acid content of limb bones from PTE-injected lizards (cumulative doses of 70 units) was elevated 14 per cent, but was not significantly increased.

In the present study, daily injections of PTE were given intramuscularly at a subliminal level (100 units/kg body weight) for a period of one week. Between day two and day four the plasma calcium increased over 50 per cent. Over the last four days of the experiment, the slope of the line

describing the concentration curve paralleled the curve reported for Experiment I. The absence of an early calcium response suggests that the quantities of hormone administered were insufficient to elicit a response. But, by the third day the circulating levels of hormone must have been adequate to evoke a response, bolstered by repetitive injections of PTE. If this is the explanation for the appearance of the response, one has to assume that either the half-life of circulating PTH in the turtle is considerably longer than in the mammal, (perhaps due to the low metabolic rate of this reptile) or that the metabolic manifestations of a sub-threshold dose are difficult to detect, and subsequent administrations of hormone are necessary to provide a detectable response. Both factors may be responsible for the results.

Other investigators have used multiple injections of PTE in other reptiles. McWhinnie and Cortelyou (1968) injected the iguanid lizard, D. dorsalis intraperitoneally with mammalian PTE at 24 hour intervals for one week, plasma calcium levels increased 20 per cent above controls by day seven. Their results compare favorably with those reported here for T. c. triunguis, but since their samples were taken only at day seven, the time course of the calcium responses cannot be analogized. Since, on a relative weight basis, D. dorsalis received the equivalent of 2100-4200 units of PTE, compared with only 700 units delivered to T. c. triunguis, the capacity to respond to PTE seems to be at least as well-developed in the terrestrial turtle as in other reptiles.

Clark, Pang, and Dix (1969), studied the effect of PTE in A. carolinensis. Intact A. carolinensis were sacrificed two hours after receiving four injections of commercial bovine PTE totaling 97 units in a 24 hour period. The animals responded variably to PTE. Half of the group remained in the normal range and half showed elevated calcium levels. On the whole, however, the group was consistently hypercalcemic (37 per cent above controls) in agreement with data presented herein.

The capacity of intact turtles to respond to PTE quite possibly was moderated by antagonistic actions of calcitonin. The rapid recovery from the PTE-induced hypercalcemia may be attributable to stimulation of endogenous calcitonin release by elevated calcium levels in the plasma. While a role for calcitonin in reptiles has not been established, acid extracts of ultimobranchial bodies of skinks (Moseley et al., 1968) and turtles (Clark, 1968b) contained hypocalcemic activity when assayed in rats. Unfortunately, reptilian ultimobranchial extracts have also been assayed in turtles, lizards, and snakes without significant effects on serum calcium values (Dix, Pang, and Clark, 1970; Clark, 1971b). The main cells of the ultimobranchial gland of the turtle (Khairallah and Clark, 1971) and lizard (Sehe, 1965) ultrastructurally and histochemically resemble the thyroid "C" cell of mammals (Ekholm and Ericson, 1968). The most consistent and conspicuous feature of these cells is the presence of numerous membrane-bound granules of moderate to

intense electron density measuring approximately 150-200 mμ. They are found in proximity to the outer face of the Golgi apparatus extending to the plasma membrane, suggestive of a secretory product, perhaps calcitonin, being transported to the cell periphery for release.

Plasma Inorganic Phosphorus

PTE produced no significant changes in plasma phosphate levels in contrast to the typical hypophosphatemia produced in mammals. Mammalian PTE at a dose of 200 units/kg body weight, given either intramuscularly or via an indwelling arterial cannula, as either single or multiple injections, and acid extracts of turtle PTE given as single injections via an indwelling arterial cannula failed to elicit any changes in plasma phosphate levels.

The lack of a shift in plasma phosphate concentrations in response to PTE has also been reported in other reptiles. Plasma phosphate levels remained stable in intact A. carolinensis given 97 IU PTE (Clark, Pang, and Dix, 1969); in intact D. dorsalis and S. grammicus administered cumulative doses of 105 and 70 units of PTE, respectively (McWhinnie and Cortelyou, 1968); and in intact and parathyroidectomized P. scripta and C. picta given 50, 100, or 200 units of PTE (Clark, 1965).

The lack of a shift in plasma phosphate in response to PTE in turtles and lizards is difficult to explain in view of the known phosphaturic action of PTE in reptiles (Clark, 1965; McWhinnie and Cortelyou, 1968). Since turtles are

unusual in possessing such large amounts of bone, mobilization of this exceptional area of mineral reserve by PTE may be adequate to maintain the level of phosphate in the plasma in the face of increased elimination by the kidneys.

In other experiments, parathyroidectomized A. carolinensis developed significant hypophosphatemia 26 hours after receiving the first of four injections of PTE (Clark, Pang, and Dix, 1969). Single intraperitoneal injection of 1.18 IU PTE/100 g body weight to intact Eastern garter snakes, T. sirtalis significantly depressed plasma phosphate values to 54 per cent of normal at 24 hours (Clark, 1971a).

Plasma Magnesium

Plasma magnesium levels were only altered in the first experiment by PTE administration. The role of PTE in magnesium homeostasis has not been previously studied in a reptile. However, regulation of this ion has been examined in mammals in recent years, with the discovery that magnesium deficiency may have profound influences on the ability of the parathyroid glands to function in calcium homeostasis (MacIntyre, Boss, and Troughton, 1963; Heaton, 1965).

The regulation of magnesium metabolism by the parathyroid gland appears to be very similar to the actions of parathyroid hormone on calcium metabolism. PTH increases the plasma magnesium levels presumeably by mobilizing this ion from skeletal reserves and by promoting its reabsorption by the renal tubule (MacIntyre, Boss, and Troughton, 1963; Heaton, 1965).

Hypermagnesemia is not an invariably observed conse-

quence of PTE administration. In agreement with the absence of an effect of PTE in T. c. triunguis, Heaton (1965) reported no increase in the plasma magnesium concentrations in intact rats on normal magnesium diets after PTE treatment. Since the calcium to magnesium ratio in bone is extremely low, changes in plasma magnesium levels comparable with calcium changes in plasma would presumeably require higher doses of hormone extract. Since the changes associated with magnesium are smaller than those associated with calcium, perhaps, the hormonal control of magnesium is not as important to its homeostasis as is calcium.

Turtles in Experiment I given 200 units of mammalian PTE/kg body weight by an intra-arterial route of injection became significantly hypomagnesemic within 24 hours and remained hypomagnesemic until day five. This is irreconcilable with the magnesium responses in other experimental groups. Since this experiment consistently also produced the greatest impact on mineral responses, one might anticipate that higher doses of PTE would produce similar effects in the other groups. However, a common reabsorptive mechanism in the kidney for calcium and magnesium could explain the hypomagnesemia observed, since increased filtration of both ions, associated with PTE-induced skeletal demineralization, might increase the clearance of both ions if they are competing for the same reabsorptive site.

Conclusions

Parathyroid extract injection produced significant elevations in plasma calcium levels in experiments in which it was used. Turtle parathyroid gland extract also elevated plasma calcium levels. The time courses and magnitudes of the calcium responses were not the same in each group. There were generally no significant changes in the plasma concentrations of phosphate and magnesium. The effect of PTE on plasma calcium is qualitatively similar to that which occurs in mammals although larger doses may be required in reptiles to produce a comparable effect and the period of response is more protracted. The general inability of PTE to effect changes in plasma phosphate and magnesium levels may reflect a relative insensitivity of the mammalian extract in reptiles.

These data indicate that box turtles, T. c. triunguis, can respond to PTE with increased plasma titers of calcium, presumeably through mobilization of calcium from bone. The doses administered to produce these responses cannot be considered supernormal since they are well within the range tested in other reptiles, birds, and mammals. Clark (1965) did not observe a change in plasma calcium following PTE injection in freshwater turtles, which were kept in water containing calcium. In the present study, the hypercalcemic responses to PTE could not be influenced by an external source of calcium. This discrepancy in calcium responses between aquatic and terrestrial turtles to PTE, may lend credence to the hypothesis that active uptake of calcium from the environ-

mental water can interfere with a response to PTE. In view of the general inability of PTE to effect a response in phosphate and magnesium and in view of the consistent response in calcium, some involvement of the parathyroid glands of the box turtle in calcium metabolism seems likely. Although the data do not exclude a role of the parathyroid glands in either phosphate or magnesium metabolism, they do suggest that in this species the primary role of the parathyroid glands is calcium homeostasis.

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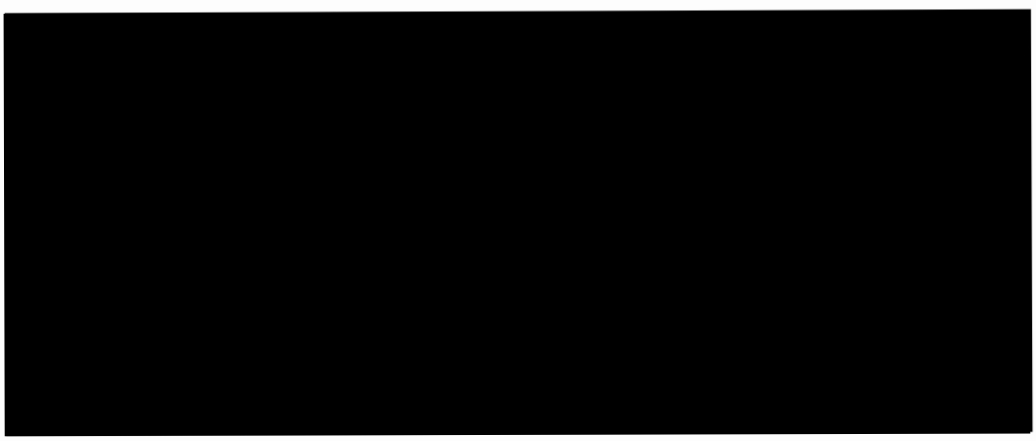
The undersigned, appointed by the Dean of the Graduate Faculty, have examined a thesis entitled

Parathyroid Function in the Box Turtle, Terrapene carolina triunguis.

presented by Lawrence Magliola

a candidate for the degree of Master of Arts

and hereby certify that in their opinion it is worthy of acceptance.



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