THYROCALCITONIN[1,2]

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Until recently the major hormonal regulation of calcium and phosphate metabolism was considered to be brought about by the actions of parathyroid hormone (PTH) (1–9), vitamin D (2, 10–15), and to a lesser degree, several of the more classical hormones, i.e. thyroxine (16–18), corticosteroids (16, 19, 20), and growth hormone (21, 22). However, an exciting new perspective has been added to this field of inquiry by the discovery and isolation of a new hormone, thyrocalcitonin (TCT), which has a unique and dramatic effect upon calcium and phosphate metabolism. Although the actual discovery of this substance was presaged by older findings and suggestions (23), the experimental observations establishing its existence have all been made in the past six to seven years. This review is an attempt to describe as completely as possible the history of this hormone's discovery and isolation, and of studies concerned with its mechanism of action. The period covered is from 1960 to July 1967.

Discovery

Early work had shown that the concentration of calcium in blood plasma remains constant in spite of wide fluctuations in intake and excretion of this ion. This led McLean & Hastings (24) to conclude that "the level of calcium in blood is one of nature's physiological constants." Exploration of the nature of this homeostatic system has concentrated upon a negative feedback relationship between the plasma calcium concentration and the rate of secretion of PTH (25, 26). In this scheme, a fall in plasma calcium stimulates PTH secretion which, in turn, acts upon several organs to restore plasma calcium concentration which then inhibits further hormone secretion. The rapidity and fine degree of control exerted by this system has been well illustrated by

[1] The work from the authors' laboratories was supported by grants from the National Institutes of Health (AM 09650), (AM 09494), and the Atomic Energy Commission (AT(30-1)3489). We wish to thank Miss Sharon Rockey for typing and assistance with the manuscript.

[2] The following abbreviations have been used: TCT (thyrocalcitonin); EDTA (ethylenediaminetetra-acetic acid); PTH (parathyroid hormone); PTE (parathyroid extract); TCA (trichloroacetic acid); PTX (parathyroidectomized); TPTX (thyroparathyroidectomized); EGTA (sodium ethylenebis-oxyethylenenitrilotetra-acetic acid); TAR (peptide with TCT activity first isolated by Tenenhouse et al. (47)).

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the work of Copp et al. (25), and more recently by direct measurements of PTH concentrations in plasma by radioimmunoassay (27–30). A tacit assumption has been that when plasma calcium concentrations increased above normal, the only means of controlling this rise was the complete or nearly complete cessation of PTH secretion. However, a consideration of the differences in the time course of the responses of different organs to the injection of PTH in a parathyroidectomized (PTX) animal, led Rasmussen to point out in 1961 that wide oscillations of plasma calcium concentration would be expected if the system operated in this fashion (9). In late 1960, Sanderson and co-workers published the first definitive study bearing out this prediction (31) and clearly suggested there was a positive control exerted to lower plasma calcium when it was raised by artificial means. In their study, they infused either EDTA or calcium into normal or TPTX dogs, and measured the rate of change of plasma calcium following cessation of the infusion. In normal animals, the plasma calcium rose or fell, respectively, when calcium or EDTA was infused, but returned to the control value within three to four hours after the infusion was stopped. However, in the TPTX animals, the plasma calcium change induced by either agent was greater initially, and the plasma calcium had not returned to control values 24 hours after cessation of infusion. The altered response to EDTA infusion was readily explicable in terms of the control system involving PTH, but the failure of plasma calcium to fall after calcium infusion in the TPTX animals could not be accounted for by this mechanism. Unfortunately, neither the authors nor other workers in the field recognized the full significance of this finding until a year later when Copp and co-workers showed that perfusion of the dog thyroid and parathyroid glands with hypercalcemic blood led to the release of a substance which caused hypocalcemia (32). The substance was called calcitonin because of its postulated role in regulating the level or 'tone' of plasma calcium in extracellular fluids. In further work aimed at distinguishing between a thyroid or parathyroid origin of this substance, Copp et al. employed sheep because it was possible to perfuse these two glands separately in this animal (33). From these studies, they concluded that the parathyroids were the source of this agent. However, in spite of initial hopeful reports, it has not been possible to isolate calcitonin from parathyroid tissue.

In the meantime, MacIntyre and colleagues (34–36) in England carried out similar perfusion studies and confirmed the data of Copp et al. (32). In later work this group chose the goat to carry out separate perfusions of thyroid and parathyroids. These studies clearly indicated that the thyroid was the source of this substance and not the parathyroids (37). This finding was soon confirmed by others (38–40). Simultaneously, in the United States, Hirsch, Gauthier & Munson came to a similar conclusion on somewhat different evidence (41), and shortly thereafter, prepared extracts of porcine thyroid tissue which caused both hypocalcemia and hypophosphatemia within one hour after injection into rats (42). Because of its thyroid origin, Hirsch,

Voelkel & Munson designated this agent thyrocalcitonin (42), to distinguish it from the possible calcitonin from parathyroid tissue.

Several groups soon reported that the substance was readily extracted with hydrochloric acid from fresh thyroid tissue (41–43). This has been confirmed by numerous other workers and hypocalcemic activity has now been detected in the thyroid tissue of all species tested including rat, goat, pig, guinea pig, dog, cow, monkey, and man (37, 42–46).

Chemistry

Hypocalcemic activity, presumably thyrocalcitonin, can be extracted from the thyroid glands of a number of mammals by treatment with dilute hydrochloric acid. Preliminary studies of these crude extracts by Hirsch, Voelkel & Munson (42), Foster et al. (43), and MacIntyre, Foster & Kumar (36) indicated that the active component was protein in nature. The activity was destroyed by treatment with pepsin (36, 42), or trypsin (36, 42), as well as by boiling in excess acid or alkali (36). These initial observations were soon followed by reports from three laboratories of methods for the preparation and partial purification of porcine thyrocalcitonin (42, 47, 48).

The procedure developed by Hirsch, Voelkel & Munson (42) used fresh frozen porcine thyroid glands as a starting material. The glands were homogenized in 0.1 *N* HCl at 4° C. The residue was removed by centrifugation, and the supernatant further purified by column chromatography on Sephadex G-50. The active material, which was eluted as part of a large protein peak, could be further purified on a carboxymethylcellulose column, and still appeared as part of a large protein peak. A 500-fold purification of the initial extract was achieved.

A second isolation procedure was that developed by Baghdiantz et al. (43) and Gudmundsson, MacIntyre & Soliman (48). Acetone dried thyroid powder was mixed with 0.2 *N* HCl at 60° to 70° C and allowed to stand at room temperature for one hour. Dialysis and salt fractionation of the extract was followed by column chromatography on Sephadex G-100 and G-50 using 0.1 *N* formic acid as the eluant. Final purification was carried out on Sephadex G-50 and Bio Gel P-10, or by recycling at least twice through Sephadex G-50. The material obtained after two cycles through Sephadex G-50 was found, by starch gel electrophoresis, to consist of at least two peptides, both of which possessed biological activity. To explain this observation, Gudmundsson et al. (48) suggested that there might be two thyrocalcitonin peptides. Although this suggestion must be considered, a more realistic interpretation is that the two active peptides represent overlapping fragments of a parent molecule which has been partially hydrolyzed by the hot acid used in the extraction procedure. Such treatment would almost certainly hydrolyze some of the weaker peptide bonds of a number of proteins, including thyrocalcitonin, and so result in a wide spectrum of peptides, some of which retain at least partial biological activity, a situation analogous to that

seen with PTH (6). Partial characterization of one of these peptides was accomplished. The molecular weight was estimated to be approximately 3000, and preliminary amino acid analysis revealed no cystine (48). The second biologically active peptide was not characterized. An approximately 20,000-fold purification was achieved.

The third method which was used in the isolation of thyrocalcitonin was that of Tenenhouse, Arnaud & Rasmussen (47), which was a modification of the method developed by Hawker, Glass & Rasmussen (49) for the isolation and purification of PTH. Porcine thyroid glands were dehydrated and defatted by successive extractions with acetone and chloroform. The resulting powder was treated initially with 8 *M* urea–0.2 *N* HCl–0.1 *M* cysteine at 4° C for one hour, followed by solvent and salt fractionation, precipitation of the active material with trichloroacetic acid (TCA), and recovery of this precipitate as a lyophilized powder. A single passage of this TCA powder through Sephadex G-75 yielded a preparation which was judged homogeneous by the following criteria: a single band was obtained on both starch gel and polyacrylamide disc electrophoresis, and threonine was found to be a single N-terminal residue. The empirical formula derived from amino acid analysis was, Lys_8, His, Arg_4, Asp_7, Thr_4, Ser_4, Glu_{10}, Pro_4, Gly_5, Ala_8, Val_4, Met, 1/2 Cys, $Ileu_3$, Leu_9, Tyr_2, Phe_2, Try, $(\text{-CONH}_2)_7$. The molecular weight of 8666 calculated from the amino acid composition was in excellent agreement with the value of 9700 (8700 in the presence of mercaptoethanol) obtained by sedimentation analysis. This peptide has become known as the TAR peptide.

A radioimmunoassay was developed for this peptide by Arnaud & Littledike (50). By this assay, it was possible to demonstrate that this TAR peptide was released from the thyroid when pigs were infused with calcium. However, work by Hawker, Rasmussen & Glass (51), Potts et al. (52), and Littledike & Arnaud (57) soon demonstrated that the TAR peptide, isolated by Tenenhouse, Arnaud & Rasmussen (47) was not the active hormone. The preparation of the TAR peptide contained a contaminant of some 3 to 5 per cent of the total protein which contained all of the biological activity. It was also found by Hawker, Rasmussen & Glass (53) that the TAR peptide was present in parathyroid tissue.

Within the past year, several groups have reported the isolation of highly purified thyrocalcitonin peptides which upon partial characterization are remarkably similar in their amino acid content. Both Hawker, Rasmussen & Glass (51), and Potts, Reisfeld, Hirsch & Munson (52) employed the initial extraction procedure described by Tenenhouse, Arnaud & Rasmussen (47) to prepare a TCA-precipitate. Hawker et al. (51) then purified the TCA precipitate by solvent fractionation, gel filtration upon Bio Gel P-6, partition chromatography on Sephadex G-25, and finally, ion exchange chromatography upon carboxymethylcellulose. The product appeared as a single band upon polyacrylamide gel electrophoresis, with the biological activity residing within this band. After initial extraction and gel filtration on Sephadex G-75 as described by Tenenhouse et al. (47), Potts et al. (52) employed preparative

disc gel electrophoresis to obtain an apparently homogeneous peptide. MacIntyre (54) has described a different procedure which employed initial extraction with either hot dilute HCl, phenol, or a mixture of *n*-butanol: pyridine:acetic acid:water (32:24:12:30), followed by precipitations with acetone and TCA, partition chromatography on Bio Gel P-60, and finally, gel filtration on Sephadex G-50. This material was also subjected to gel filtration on Sephadex G-75 in order to estimate its molecular weight. The value obtained by this procedure was 3900 (54). However, by sucrose density centrifugation, O'Riordan et al. (56) had previously estimated the molecular weight of the biological activity to be 5000 to 6000.

All three groups have reported preliminary amino acid compositions of their respective peptides. These are summarized in Table I. Hawker et al. (51) and Potts et al. (52) attempted to obtain the best integral values for their data without assuming any molecular weight. The values from the two groups are quite similar but not identical. The discrepancies may be due to remaining heterogeneity, as well as to the fact that the amounts of purified material available are very limited, and extensive characterization remains to be done.

TABLE I

COMPARISON OF AMINO ACID COMPOSITIONS OF THYROCALCITONIN PREPARATIONS OF THREE DIFFERENT LABORATORIES

Amino Acid	*Hawker*[a]	*Potts*[b]	*MacIntyre*[c]
Aspartic acid	4	2	5
Threonine	3	3	2
Serine	7	6	4
Glutamic acid	3	2	2
Proline	3–4	4	2
Glycine	6	5	4
Alanine	2	3	2
Valine	2	2	1
Cystine	0	0	0
Methionine	1	1	1
Isoleucine	0–1	0	0
Leucine	3	4	3
Tyrosine	1	1	1
Phenylalanine	4	4	3
Lysine	1	1	0–1
Histidine	2	2	1
Arginine	3	3	2
Tryptophan	1	–	1
	46–48	43	34-35

Values are residues per mole: [a]Taken from Hawker, Rasmussen & Glass (53). [b]Taken from Potts & Munson (52). [c]Taken from MacIntyre (54).

MacIntyre (54) approached his data in a somewhat different fashion. He calculated the amino acid analysis data, assuming a mol wt of 3900 as determined by gel filtration on Sephadex G-75. If the data of Hawker et al. (51) and Potts et al. (52) are recalculated in this manner, the compositions of the three peptides are almost identical. However, the molecular weight estimate is open to question, because it is approximately the lower limit of molecular weights that can be estimated on G-75 (55). Furthermore, it conflicts with the value of 5000 to 6000 obtained by O'Riordan (56).

The isolation of a nearly identical thyocalcitonin peptide has also been reported by Littledike & Arnaud (57). Isolation was achieved by gel filtration on Sephadex G-75 followed by partition chromatography on G-25. Two other groups have reported preliminary work on the isolation of thyrocalcitonin. White, Allan & Anast (58) have employed a phenol extraction and gel filtrations on Bio Gels P-30 and P-2 to obtain a partially purified preparation. Tashjian & Voelkel (59) have used batch chromatography on CM-Sephadex and gel filtration on Bio Gel P-2 to also obtain a partially purified preparation.

In summary, it would appear that porcine thyrocalcitonin has been isolated simultaneously by at least four groups of workers, that it is a polypeptide with a molecular weight in the range of 5000, and that it has not been completely characterized. It should be noted that none of the isolation procedures are ideal. The recovery of total biological activity in each is very low, and the reasons for these low yields of activity are not yet clear. Nor has it yet been completely settled that thyrocalcitonin is a simple polypeptide. The possibility remains that there are other constituents of the hormone molecule. Likewise, the biological activity of these preparations vary somewhat, but, here, the difficulty lies in differences in assay methods, subspecies of rat employed, and the basis upon which the data are reported. For example, based upon weight, 0.2 μg of the preparation of Hawker et al. (51) produce a half-maximal response; but if based upon Lowry protein (60), using bovine serum albumin as a standard, then 0.03 to 0.05 μg produce a half-maximal response. All preparations represent purifications of at least 400,000-fold.

The problem of hypocalcemic principles from other tissues has also received some attention. Kraintz has reported (61) that salivary glands protect rats from the hypercalcemic effects of parathyroid extract (PTE)–a possible calcitonin-like effect–but has not reported the extraction of activity from this gland. However, Japanese workers have extracted a substance from parotid glands, parotin, which produces hypocalcemia (62). Furthermore, several pituitary polypeptides cause hypocalcemia but only in rabbits (63). It has also been reported that ACTH induces hypocalcemia in the rabbit, but not in other species (64). All the pituitary peptides which induce hypocalcemia also lead to fatty acid mobilization. Freisen (63) has suggested that the hypocalcemia may be secondary to this hyperlipemia, through the formation of insoluble calcium salts of the fatty acids. Chausmer et al. (65) and Gud-

mundsson et al. (48) found no calcitonin-like activity in extracts of other non-endocrine tissues.

Biological Assay

The rat and the mouse have been used to assay hypocalcemic activity of thyrocalcitonin preparations. Hirsch, Voelkel & Munson (42) described an assay procedure which depends upon the decrease in the concentration of calcium in the plasma of 150 to 180 g Holtzman rats, maintained on a low calcium diet for four days, one hour after the subcutaneous administration of the test substance. Their standard preparation was the 100,000 g supernatant fraction of a cold 0.1 *N* HCl extract of fresh hog thyroid tissue. They defined a unit as that amount of this standard preparation (10 μg of nitrogen) required to lower the plasma calcium "significantly (about 1 mg/100 ml)." The index of precision of ten consecutive assays was 0.23 ± 0.025. Kumar et al. (66) reported the details of an assay procedure previously employed by Baghdiantz et al. (43) for the purification of porcine TCT. Piebald (hooded) 135 to 145 g rats were used. Biological activity of a test preparation was measured in an assay system in which the degree of hypocalcemia produced 50 minutes after its intravenous injection or infusion (via the tail vein) was compared with the level of plasma calcium in rats given an injection vehicle alone. The index of precision of six consecutive assays varied between 0.12 and 0.37. Comparison of the subcutaneous or intraperitoneal routes of administration with the intravenous route showed that the latter yielded greater assay sensitivity. An extract of porcine thyroid glands carried through the salt fractionation procedure of Baghdiantz et al. (42) was used as a standard preparation. This has been adopted by the Medical Research Council (MRC) Department of Standards[4] as a standard preparation of TCT. Approximately 0.01 unit of this material given intravenously in the Kumar assay system produces a 10 per cent fall in the plasma calcium. Each ampule of the MRC standard preparation contains freeze-dried material equivalent to approximately 250 μg protein/mg and 0.25 unit. It is considered to be stable and is available on request for assay standardization.

The present authors have had experience with the subcutaneous injection, and intravenous injection and infusion techniques, and find, as did Kumar et al. (66), that the infusion method is most sensitive, but that the single intravenous injection appears to combine both convenience and sensitivity. We inject the test substance into the external jugular vein of 80 to 90 g Wistar or Holtzman rats which have been on a low calcium diet for two to four days and measure the plasma calcium 1.5 hrs later. The assay response depends upon diet, age, sex, and strain of animal employed (67–69). Differences in response are probably due, in part, to the differences in rates of bone

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resorption. Thus, young animals on a low calcium diet are quite sensitive, but old animals on a low phosphate intake are highly insensitive. Sprague-Dawley and Holtzman strains are more sensitive than Charles River or Blue Spruce rats. The reasons for these differences are not apparent.

There have been several attempts to assay hypocalcemic material in plasma. White & Ahmann (70) have reported detectable changes in the plasma calcium 30 minutes after rats were injected intraperitoneally with as little as 1.5 ml of whole plasma obtained from normal young human females. Markedly increased diurnal variations in plasma hypocalcemic activity were found in an osteopetrotic subject. Arnaud, Littledike & Tsao (50) have used gel filtration to partially resolve the protein components of lyophilized plasma on Sephadex G-75. By this means, they were able to concentrate TCT-like activity from the plasma of normocalcemic young pigs. This material caused hypocalcemic and hypophosphatemic responses in rats which were identical in time of onset, duration, and degree to those produced by highly purified porcine TCT. There was no significant difference between the slopes of the log dose responses of the two preparations. The concentation of TCT-like material in the plasma was at least 0.5 to 1.0 M.R.C. units/liter. Although further work is needed before precise quantitation is possible, it would appear that a substance which is biologically similar to the TCT isolated from the porcine thyroid gland circulates in the plasma of normal animals, and that this material may have physiologic importance in the normal as well as the hypercalcemic organism.

No standardized *in vitro* assay has been reported, but effects of TCT preparations on bone culture systems have been described [Friedman & Raisz (71) and Aliapoulios, Goldhaber & Munson (72)], and this approach may eventually yield a workable assay.

Origin

It is now generally agreed that the source of thyrocalcitonin is the thyroid gland. Initial immunofluorescence studies using an antibody to the TAR peptide led Hargis, Williams, Tenenhouse & Arnaud (73) to the conclusion that all thyroid cells contain thyrocalcitonin. However, more recent studies, particularly those of Pearse and co-workers (74–76), have led to the conclusion that the mitochondrial rich "C" cells of the thyroid are the source of this hormone. These cells were first described by Nonidez as argyrophil parafollicular cells (77). These cells change morphologically when changes are induced in plasma calcium concentration. They respond to an acute increase in plasma calcium concentration by discharging their content. Furthermore, Pearse has shown by immunofluorescent techniques (76) that TCT is localized in these cells. A particularly interesting feature of these cells is their ability to concentrate injected 5-hydroxytryptophan, and to convert it to 5-hydroxytryptamine (78). A similar property has been ascribed to adrenal medullary cells, enterochromaffin cells, mast cells, pituitary corticotrophs, pituitary melanotrophs, and pancreatic islet β-cells (74, 76). Pearse

has suggested that cells producing polypeptide hormones may have common cytochemical and cytological properties. In this regard, most of these organs also have high levels of α-glycerophosphate dehydrogenase (74), a mitochondrial enzyme believed to be important either in shuttling reducing power between cellular compartments, or in the synthesis of phospholipids. The latter is of interest because polypeptide-producing cells contain lipid-bound vesicles averaging 2000 A° in diameter (79–81). These vesicles disappear during periods of high secretion, and it is assumed that a high rate of turnover of phospholipids would be a part of the secretory process. In keeping with this hypothesis, is the fact that TCT has been found to be associated with particulate fractions of thyroid homogenates (82, 83). Bauer & Teitelbaum (82) have partially purified these particles, have shown that they release TCT upon sonic disruption, and that they appear to be similar, in electron micrographs, to those seen in the parafollicular or "C" cells of the intact thyroid. The embryologic origin of the "C" cells has also been investigated and present evidence suggests that they originate in the ultimobranchial gland (68, 76). This organ disappears in mammalian development, but persists in fish and lower forms. An association of this gland with calcium metabolism had been suggested in previous studies in fish (84, 85), although the thinking then was that these glands might possess a parathyroid-like function. Clearly, in the light of recent evidence, their functional properties need to be reinvestigated. The older data could well be explained if these glands were the source of a calcitonin-like activity in fish.

Control of Secretion

Very few studies have been carried out concerning the factors regulating TCT secretion. This is due primarily to the fact that no immunoassay or other sensitive bioassay is available to detect this substance in blood. However, the physiological studies of Sanderson et al. (31), Copp et al. (8), Anast et al. (86), Tenenhouse et al. (87), Talmage et al. (88), Pechet (89), and Williams et al. (90) imply that the hormone is secreted in response to a rise in plasma calcium concentration. A peculiar feature of this response is the fact that there appears to be an inverse relationship between the thyroidal content of TCT and the plasma calcium (91). Thus, in patients with chronic hypocalcemia due to pseudohypoparathyroidism, the thyroidal content has been found to be extremely high, whereas in subjects with hyperparathyroidism, the content is quite low (91). This would imply that the rate of synthesis is unable to keep pace with the rate of secretion, and that there is no close coupling between the two processes. However, this particular question requires a more thorough investigation using sensitive assay techniques and direct measurements of secretion rates.

The relationship between the classical thyroid hormones, thyroxine and tri-iodothyronine, and TCT is not clear. They are apparently liberated by different cells in the thyroid. Nevertheless, Morii & Talmage (92) have found that rats on an iodine-deficient diet are less able to dispose of a calcium load.

From this, they conclude that these animals are less able to produce TCT than normal controls. However, other interpretations are possible.

Shortly after the discovery of the thyroidal origin of TCT, Gittes, Wells & Irvin proposed that the parathyroids were the source of a TCT-releasing factor (93, 94). However, subsequent investigations leave this conclusion in doubt, and the present concensus is that such is not the case (46).

Biologic Effects

Thyrocalcitonin causes a decrease in plasma calcium and phosphate (42, 48, 86, 95, 96). This effect has been demonstrated using crude acid extracts from the thyroid glands of rat, rabbit, dog, hog, ox, monkey (42), and goat (37), as well as highly purified preparations from the pig [Gudmundsson et al. (48); Tenenhouse et al. (87); Pechet (89)]. All species so far tested, including man, have responded in a similar manner, but the effects in man have not been dramatic (44, 97–100). The effect is rapid, the calcium reaching its lowest level one to two hours after TCT administration. The rate at which plasma calcium returns to its control level depends upon a variety of factors, including route of administration, duration of infusion, and endocrine status of the animal. Changes in plasma phosphate usually parallel the changes in plasma calcium (42, 48, 86, 95), and Gudmundsson et al. (48) have demonstrated that magnesium levels are unchanged by TCT administration.

The induced hypocalcemia is not due to an increase in renal calcium excretion as evidenced by the effectiveness of the hormone in the nephrectomized rat (42). Nor is it due to an increased loss of calcium in the gut, since TCT has been shown to produce hypocalcemia after removal of the gastrointestinal tract (101). This evidence eliminates both the gastrointestinal tract and the kidney as sole sites of action of TCT, although it does not exclude them as possible sites of hormone action. Under some circumstances, TCT causes phosphaturia [Rasmussen et al. (95); Robinson et al. (102)] and a decrease in calcium and magnesium excretion (86, 89, 95). This hypocalcuria and hypomagnesuria may be secondary to the hypocalcemia. However, the increase in urinary phosphate is more difficult to explain. Robinson et al. (102) has suggested that it is attributable to a direct effect of TCT on the kidney. An alternate explanation suggested by Rasmussen et al. (95) is that the phosphaturia is secondary to the hypocalcemia. This is supported by their observation that infusion of EGTA induces hypocalcemia which is also associated with increased urinary phosphate excretion.

Hirsch et al. (42) and Tashjian (105) reported that TCT was effective in PTX animals. However, Gudmundsson et al. (48) found that TCT produced hypocalcemia in PTX rats only if the plasma calcium was maintained near normal with a high calcium diet. If the plasma calcium was not maintained post-operatively, TCT treatment produced a fall only in plasma phosphate. Stahl et al. (112), on the other hand, found that TCT injection in the PTX dog leads to a fall only in serum calcium. All groups have concluded that TCT is active in the absence of PTH, and that the hypocalcemia is not the

result of an inhibition of PTH secretion. The data of Gudmundsson et al. and Stahl et al. further demonstrate that the fall in phosphate and calcium induced by TCT are not mutually dependent.

The current working hypothesis, subscribed to by most investigators in the field, is that the major effect of TCT is that of inhibiting bone resorption (48, 71, 72, 86, 89, 103–111). The evidence in support of this is substantial. Thyrocalcitonin inhibits the urinary excretion of hydroxyproline in PTX animals [Pechet (89); Martin (104)]. The rate of excretion of hydroxyproline is considered to be an index of bone resorption (115, 116). Prockop & Kivirikko (116) have shown that this is probably correct for adult animals, but might not be valid in growing animals where there is a significant pool of soluble collagen with a rapid turnover. However, the possibility that only a soluble pool of collagen in bone is influenced by TCT is unlikely since PTH-induced osteolysis and increased urinary excretion of hydroxyproline is abolished by TCT (69, 86, 114). In addition, Rasmussen & Belanger (117) have shown by histochemical means that TCT inhibits the osteocytic osteolysis induced by PTH infusion in the TPTX rat. Johnston & Deiss have also reported the results of a detailed study of the *in vivo* action of TCT on several parameters of bone metabolism (106). Calvaria from animals treated with TCT utilized less glucose and produced less lactic acid than controls when incubated *in vitro*. These calvaria also incorporated less proline into hydroxyproline and less glucose into glucosamine. These effects, with one exception, are opposite to those seen in bones from animals treated with PTE, the exception being the decrease in proline incorporation. Johnson & Deiss (106) explain this as being due to a negative feedback on collagen synthesis initiated by the decreased bone resorption. On the other hand, Wase et al. (109, 110) and Foster et al. (107, 118) have determined the effect of the chronic administration of TCT on growing rats. Their data indicate that these animals retain more calcium and have denser bones than those of appropriate controls. The unanswered question is whether this is due solely to an inhibition of bone resorption by TCT, or whether this hormone has additional effects upon bone formation. Foster et al. (118) have interpreted their data as leading to the conclusion that formation is enhanced, but other interpretations of their data are possible.

Gudmundsson et al. (48) have reported a decrease in the effectiveness of TCT in vitamin D-deficient rats. They suggest that the failure of TCT to produce a further decrease in plasma calcium in the PTX animal maintained on a normal diet, and in the vitamin D-deficient animal is due to the inability of TCT to lower plasma calcium below the level observed under these conditions. An alternative explanation is that these animals have lower rates of bone resorption, hence are less responsive to TCT.

The effect of actinomycin D on TCT action has been studied by Tashjian (105), Gudmundsson et al. (48), and Anast et al. (86). This agent which modifies the effect of PTH on bone (113) does not alter the hypocalcemic effect of TCT. However, Tashjian (105) found that the recovery from the

TCT-induced hypocalcemia in actinomycin D-treated rats was delayed at least three hours, and resembled the pattern seen in PTX animals treated with TCT. He concluded that the action of parathyroid hormone was responsible for the recovery from TCT-induced hypocalcemia.

An *in vitro* effect of TCT has been reported by Friedman & Raisz (71, 118) and Aliapoulios et al. (72). Both groups have demonstrated that TCT inhibits the PTH-induced resorption of bone in tissue culture. In the system of Freidman & Raisz, PTH-induced ^{45}Ca release can be divided into two phases, a slow initial phase which is insensitive to actinomycin D and a more rapid late phase which is inhibited by actinomycin D (120).

In addition, PTH has a dual effect upon uridine incorporation, an initial inhibition during the first five to six hours, and a late stimulation. TCT inhibits both phases of calcium release without affecting the early PTH-induced inhibition of uridine incorporation, but does block the later stimulation of uridine incorporation (121).

These *in vitro* observations lead to the same conclusion as the *in vivo* studies: the major physiologic effect of TCT is that of inhibiting bone resorption. The major function of the hormone appears to be that of protecting the animal from the dangerous effects of hypercalcemia. It may also function in some capacity as a bone growth substance. However, this possibility remains to be established.

There is one interesting aspect of TCT action which requires further study. Both *in vivo* (86) and *in vitro* (119), when both hormones (PTH and TCT) are present, TCT initially blocks the effects of PTH. However, the effect of PTH is eventually expressed even in the continued presence of TCT. The mechanism and significance of this "escape" is not known.

Biochemical Mode of Action

Very little is known concerning the mode of action of TCT at the biochemical level. The only lead comes from the work of Tenenhouse & Rasmussen (69, 122) using isolated Ehrlich ascites tumor cells. These cells release pyrophosphatase activity into the incubation medium. The addition of PTH to this system leads to an inhibition of enzyme activity, while TCT alone increases enzyme activity slightly. When added together, TCT completely blocks the effects of PTH. These effects are probably brought about by changes in calcium, magnesium, and phosphate transport, induced by these hormones, across the plasma membranes of these cells. The relationship of these effects upon isolated ascites cells to the action of these hormones upon bone cells is not known. However, much recent evidence suggests that pyrophosphate and pyrophosphatase are intimately related to the processes of bone formation and bone resorption (123, 124).

Clinical Aspects

There has as yet been no clinical disease state which has been clearly related to over- or under-production of thyrocalcitonin. However, there is one

report (125) which attributes persistent hypocalcemia, intermittent tetany, and goiter to overproduction of TCT. Both plasma calcium and plasma phosphate rose after thyroidectomy.

TCT also may be related in an important way to the clinical manifestations of hyperparathyroidism. Two clinically-defined entities have been noted in hyperparathyroidism: the renal form and the bone form. The first is characterized by predominantly renal signs and symptoms with little or no evidence of altered bone metabolism, and the second by striking changes in bone morphology and metabolism (126). These two forms of hyperparathyroidism were considered so different that they were thought to result from overproduction of two different parathyroid hormones (127). However, they may represent the situations in which a compensatory increase in TCT production is able (renal form) or unable (bone form) to suppress the effects of excess PTH upon bone resorption without altering its effects upon renal function.

Much hope has been expressed that TCT will be a useful therapeutic agent in the treatment of osteoporosis and other bone disorders. Foster et al. have reported a response to TCT in adult humans with the hypercalcemia of malignancy (98), and Milhaud & Job have reported that TCT was efficacious in an infant with idiopathic hypercalcemia (100). However, normal human adults respond poorly to TCT, and it is not yet known whether this hormone will have widespread use in these disorders (89, 114). The responsiveness to TCT is dependent on the age of the animal, in that the younger the animal, the more sensitive it is to the hormone (68, 128). This might explain the poor effects observed, thus far, in man, since most trials have been done in elderly subjects. In this same vein, Copp (68) has recently demonstrated that pretreatment of rats with either thyroxine or growth hormone increases their sensitivity to TCT. This data has been interpreted to mean that TCT is most effective in states where bone resorption is most active. This property of TCT is most important when considering its therapeutic potential. It suggests that in a condition such as Paget's disease, characterized by very active rates of bone turnover, TCT may be most efficacious.

Note added in proof:

Since this review was completed, several reports of further purification and characterization of this hormone have appeared. Putter et al. (129), using a combination of countercurrent distribution and column chromatography, have obtained what they believe to be pure thyrocalcitonin. The material is a peptide with the following amino acid composition: His, Arg_2, Asp_4, Glu, Thr_2, Ser_4, Pro_2, Gly_3, Ala, $Cyst_2$, Val, Meth, Leu_3, Tyr, Phe_3, Try. They also presented evidence suggesting that the peptide possesses no free terminal amino group and that five of the six potential carboxyl groups are present as amides.

Potts (130) has recently revised the amino acid composition of his product. The significant changes are the total absence of lysine and the presence

of two cysteine residues, as well as the absence of a free terminal amino group. The total number of residues was reduced from 43 to 33.

Parkes & Copp (131) have reported the isolation of hypocalcemic polypeptides which they believe to be calcitonin, from the ultimobranchial glands of chickens and dogfish. This evidence supports the proposal that these glands play a role in the control of calcium homeostasis in lower animals, and that these glands and the "C" cells of mammals are functionally as well as embryologically identical.

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