# "THE BIOSYNTHESIS OF PARATHYROID HORMONE AND ITS BINDING TO THE CELLS OF BONE"

Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Dentistry, University of Sydney.

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## PREFACE.

This thesis contains a report on experiments designed and carried out by the author to fulfill the requirements for the degree of Doctor of Philosophy in the Faculty of Dentistry, University of Sydney. The author was a research student, supported by the National Health and Medical Research Council of Australia, in the Department of Pathology, University of Sydney. Without the support of the Council and the permission of Professor T. Magarey (Head of Department), this work would not have been done.

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R.L. O'GRADY. B.Sc. B.D.S. (Hon).

# CONTENTS.

## INTRODUCTION.

The metabolism of bone cells is controlled, in part at least, by the levels of various hormones in the circulation. Parathyroid hormone (PTH) is one of these hormones, and its relationship to bone cell metabolism is the subject of this thesis. It is a low molecular weight, basic protein, frequently referred to as a polypeptide, that is synthesised and secreted by the parathyroid glands. This hormone is also involved with the kidney (Albright and Ellsworth, 1929) and the small intestine (Rasmussen and De Luca 1963) but the major interest for the present work is its involvement with bone.

The existence of the parathyroid glands was first noted by Sandstrom (1880) who described the external parathyroids in man and later Kohn (1895) discovered the internal parathyroids. In 1908, MacCallum and Voegtlin showed that parathyroidectomy had an effect on the concentration of calcium in serum. The first physiologically active extracts from parathyroid glands were isolated independently by Hanson (1924) and Collip

(1925).

Once these crude hormone preparations became available, experimental work designed to look at the effects of this parathyroid substance began to absorb the efforts of a number of investigators. By 1929, Albright and Ellsworth had demonstrated that an injection of parathyroid gland extract was followed by :

(a) a rise in serum calcium

(b) a rise in urinary excretion of calcium

(c) a fall in serum phosphorus

(d) a rise in urinary excretion of phosphorus

Also, they demonstrated that parathyroidectomy was followed by :

(a) a fall in serum calcium

(b) a fall in urinary excretion of calcium

(c) a rise in serum phosphorus

(d) a fall in urinary excretion of phosphorus

From that time until the present, there has been a good deal of discussion as to whether the kidney or bone is the major target organ for parathyroid hormone

(PTH) in the control of serum calcium, but this argument is irrelevant to the present discussion which concerns PTH and bone. What was important concerning these early results was the fact that it was possible to relate the parathyroid glands to the bony skeleton. Products of these glands were capable of increasing the amount of calcium in both blood and urine, so where did this calcium come from? Bone was recognised as the major calcium containing tissue in the body and was the logical choice for further investigation.

At this stage, on the assumption that the parathyroids stimulated loss of calcium from bone, people began to look for evidence of bone destruction after the administration of parathyroid extract. In these investigations, the osteoclasts received much attention as these were the cells considered to be involved in the process of bone resorption. In 1931, Jaffe et. al. stated:

"Decalcification is the only established specific effect of parathyroid hormone on bone, and the resorption is generally evidenced by the presence of enlarged haversian canals or by lacunae

containing osteoclasts on the periosteal and endosteal surfaces of bone".

Also Jaffe et. al. were able to induce bone lesions in experimental animals which presented the essential features of osteitis fibrosa cystica (von Recklinghausen's disease) in varying degrees of severity, depending on the relation of the parathormone dose to the calcium intake and duration of treatment with parathormone (Jaffe et. al. 1931, 1932; Bodansky and Jaffe, 1932).

By histological methods, Selye (1942) and Ingalls et. al. (1943) showed increased osteoclastic resorption after treatment with parathyroid hormone. Perhaps of more significance in the present context, these experiments demonstrated that similar changes occurred in nephrectomised animals, suggesting that the hormone might affect bone directly. One of the most convincing demonstrations that the parathyroids secreted something that had a direct action on bone was reported by Barnicot (1948). He transplanted parathyroid glands of ten day old mice to parietal bone from the same animal and

grafted them to the cerebral hemisphere of a litter mate. After 10-14 days, very active bone resorption, accompanied by osteoclasts, was occurring on the surface of the bone graft under the attached parathyroid. In some specimens, new bone deposition was occurring on the opposite surface of the bone graft, while in others perforation of the bone had occurred. He carried out similar experiments replacing the parathyroids with pituitary, thyroid, adrenal or cartilage tissue but in none of these did local bone resorption occur. Barnicot also made the observation that, since the central region of the parietal bone of a ten day old mouse contained few osteoclasts, and since all of these cells appeared to be absent in his two day old grafts, the numerous osteoclasts at the site of bone resorption in later stages must have been newly differentiated. In other words, he suggested that the parathyroids stimulated the differentiation of multi-nuclear osteoclasts.

The work of Chang (1951) supported these results and there was reasonable evidence that the number of osteoclasts was increased by the action of parathyroid hormone. An extension of this view was that the

hormone stimulated release of calcium to the circulation via osteoclasia. However, doubt has been expressed that these cells are important in the minute to minute regulation of serum calcium, (Belanger, 1965; Talmage et. al., 1965).

In 1950, Heller et. al. reported changes in bone cells of experimental animals that had received toxic doses of parathyroid extract. In the rat there was considerable destruction of osteocytes, marked increase in osteoclasts, but relatively little destruction of osteoblasts. In puppies there was practically no destruction of osteocytes, only a moderate increase in osteoclasts and death of most of the osteoblasts. They stated that there was no microscopically visible evidence of phagocytosis in osteoclasts and that their role in bone resorption was based on presumptive evidence.

From the middle of the 1950's Gallard has been much involved in the study of bone and parathyroid hormone in organ culture. In his early work he used parietal bone and in 1959 he reported that osteoclasts were not

found in these explants except when parathyroid hormone has been present in the culture medium.

From this work he concluded :

1) "No significant lysis of bone matrix has ever been found without giant osteoclasts in close contact with, or in the immediate vicinity of, the "dissolving areas"."

2) "Parathormone is indispensable for creating the conditions favouring the survival, the formation and the functioning of osteoclasts."

In 1961 he reported results of experiments in which he had cultured radii from mouse embryos. As with the parietal bone cultures, he noted in these that parathyroid extract (PTE) induced the formation of multinuclear osteoclasts that were involved in the dissolution of bone matrix.

It is fairly well accepted then that osteoclast numbers rise after stimulation by parathyroid hormone, but where do these cells come from, and are they the result of a direct effect of the hormone? They might

arise by division of pre-existing osteoclasts or from the undifferentiated cell pool either via coalescence of mononuclear cells or via successive nuclear divisions without cytoplasmic division. The autoradiographic evidence of Young (1962) suggested that they arose by the coalescence of "osteoprogenitor" cells. The studies of Owen and Bingham (1967) indicated that the injection of parathyroid extract into rabbits resulted in increased RNA synthesis in both osteoclasts and preosteoclasts. These workers classified osteoprogenitor cells as either preosteoblasts, on the periosteal surface of the developing femur, or preosteoclasts if they were on the endosteal surface. The increased nucleic acid synthesis was apparent only in the cells on the endosteal surface, suggesting a direct stimulatory effect on the cells destined to become osteoclasts. It is probable then that osteoclasts arise by coalescence of mononuclear cells.

Also, they described an opposite effect on those cells responsible for bone matrix formation - that is decreased uptake of radioactive RNA precursors in osteoblasts and in preosteoblasts. Further, they demonstrated

that these cells had undergone deleterious morphological changes and they attributed these changes to the effect of parathyroid extract. Some years earlier, evidence that PTE influenced osteoblasts was presented by Gaillard (1959, 1961) when he noted the disappearance of typical osteoblasts in cultured parietal bone and radius when the medium contained PTE. Also, from his work on the incorporation of $^{14}C$ - glycine (1961), he suggested that PTE impaired the ability of osteoblasts to synthesise collagen long before histological changes in these cells became evident. Other experiments (Talmage et. al. 1965) using $^{3}H$-proline as a collagen precursor showed that parathyroid hormone suppressed the ability of the osteoblasts to lay down bone matrix. The effects of intraperitoneal injections of PTE into rats on the fine structure of bone cells in the tibia were reported (Cameron et. al., 1967). The osteoblasts showed the greatest alteration. Their mitochondria were swollen and exhibited dense granules. Ribosomes were separated from the membranes and both rough and smooth endoplasmic reticulum systems were distended. They noted no change in the osteoclasts in the material studied. These results were in line with earlier work in vitro (Gaillard 1965,

unpublished electron micrographs of Scherft) when radii which had been incubated *in vitro* with one U $ml^{-1}$ of PTE, showed a loss of ribosomes and a reduction in the amount of endoplasmic reticulum in the osteoblasts as compared to control bone incubated without PTE.

At this stage of the discussion, it appears that parathyroid hormone may be involved in the regulation of osteoclasts, osteoblasts and of undifferentiated bone cells, the latter also being referred to as osteo-progenitor cells (Young, 1962) or preosteoblasts or preosteoclasts (Owen and Bingham, 1967).

The osteocyte is the only bone cell type that does not appear in this list, but it has not been disregarded. An attempt has been made to separate osteocytes into more than one group of cells (Belanger *et. al.*, 1963). In chicks, rats and dogs, small osteocytes were predominant near formative bone surfaces. These cells were surrounded by organic matrix of high density as demonstrated by alpharadiography. Larger, more mature osteocytes, located further from formative surfaces, were surrounded by bone matrix of much lower density. Also, toluidine

blue staining of sections indicated areas of meta-chromasia surrounding the larger, more mature osteo-cytes. (Belanger, 1965). This was interpreted as indicating areas of bone containing accessible sulphated mucopolysaccharides or, expressed another way, areas where bone mineral had been removed. These areas coincided with areas of rarefaction on alpharadiography. By histochemical methods he demonstrated alkaline phosphatase and protease activity over these larger osteocytes rather than over the smaller cells. From these results, he suggested that mature osteocytes were engaged in bone resorption in normal tissue and that this mechanism was enhanced by a variety of factors including parathyroid hormone. Belanger called this mechanism "osteolysis" and he suggested that the osteocyte was the important cell in the minute to minute regulation of serum calcium.

These concepts received further support (Talmage et. al., 1965) from experiments that showed the failure of PTH to affect $^{45}Ca$ or $^{32}P$ recently deposited in bone, whereas radioactivity administered two weeks before the experiment was released by the action of PTH. Those

results were interpreted to mean that parathyroid hormone-induced calcium mobilisation occurred in mature bone rather than in recently formed bone; that is away from sites of osteoclastic activity. Organ culture of mouse radii, done by Talmage in Gaillard's laboratory (Talmage et. al., 1965), showed that osteocytic lacunae were larger in bones that had been incubated with PTH than in the contralateral bones used as controls. At the ultrastructural level changes were found in the osteocytes of the tibia similar to those mentioned above for the osteoblasts. (Cameron et. al., 1967). These changes were far less marked than in the osteoblasts.

The simplest way to summarise at this point would be to say that parathyroid hormone has been implicated in the regulation of all bone cell types, that it apparently stimulates different responses in different cell types, and that very little is known about its subcellular mechanism of action.

From the time that an active extract was recovered from parathyroid glands in the 1920's until the present, a great deal of effort has gone into preparing a pure

sample of parathyroid hormone and characterising its composition and structure. The original method of Collip (1925) for preparing crude extracts of the parathyroid glands involved the use of hot hydrochloric acid. More recently Rasmussen and Craig (1961) demonstrated that the use of hot hydrochloric acid or hot, 80% acetic acid for the initial dissociation of PTH from other constituents in bovine parathyroid glands resulted in partial hydrolysis of the hormone. Aurbach (1959) introduced an extraction procedure employing phenol instead of hot acid, and Rasmussen et. al. (1964) used a cold HCL/urea/cysteine solution. Both of these techniques apparently overcame the hydrolysis problem. Rasmussen and Craig (1962) introduced gel filtration on Sephadex G50 into the purification procedure. Aurbach and Potts (1964) also used G50 but they found that Sephadex G100 was more efficient for fractionation of parathyroid gland extracts. Many other procedures were used in the characterisation of the hormone including counter current distribution, ion exchange chromatography, paper chromatography, starch gel electrophoresis and polyacrylamide gel electrophoresis.

There is general agreement now that at least the bovine parathyroid hormone has been completely characterised. Brewer and Ronan (1970) reported the amino acid sequence of parathyroid hormone recovered from extracts of bovine parathyroid glands. It is a single chain polypeptide containing 84 amino acids, giving it a molecular weight around 10,000. The molecule has an N-terminal alanine and a C-terminal glutamine; it contains two methionine residues but no cysteine. It is a basic protein with nine lysine residues and five arginines; five of the eleven glutamic residues and three of the nine aspartics are amides.

Porcine PTH has also been isolated and partially characterised, (Littledike and Hawker, 1966), and is similar in size to the bovine material.

Once the amino acid sequence of bovine parathyroid hormone was established, Potts et. al. (1971b) began making fragments of the molecule by the technique of peptide synthesis. They synthesised a thirty four amino acid peptide that corresponded to the N-terminal 34 amino acids of bovine PTH. This fragment was biologically

active. A 1-29 N-terminal fragment produced by dilute acid treatment of bovine PTH had activity comparable to the 1-34 synthetic molecule, (Potts et. al. 1971a). The N-terminal alanine was essential for activity and they suggested that the minimum fragment required for biological activity occupied a continuous sequence from residue 1 (ala) to a point between residue 20 (arg.) and 29 (glu). The activity of these fragments was greater in vitro than in vivo, so it is possible that the carboxy terminal end of PTH may provide some sort of protective function in the circulation. It may be of interest to note that the active portion of the PTH molecule is similar in size to calcitonin.

Synthetic peptide synthesis is a very powerful technique with tremendous potential in the field of molecular biology, but it has only recently been applied to parathyroid hormone (Potts, et. al., 1971b). The older and more frequently reported methods for synthesising parathyroid hormone have utilised the in vitro incubation techniques of organ and tissue culture. (Gallard, 1961; Raisz, 1963; Roth and Raisz, 1964, 1966; Raisz et. al., 1965; Hamilton and Cohn, 1969; Au et. al.

1970; Sherwood et. al., 1970, 1971; Martin et. al., 1971).

It was shown that both the secretory activity of the parathyroid glands and the effect of PTH on bone could be demonstrated in tissue culture (Gaillard 1961). The effect of varying concentrations of calcium in the culture media then received a great deal of attention. Raisz (1963), using chick and rat parathyroid glands, showed that decreasing the ($Ca^{2+}$) caused an increase in the cytoplasmic/nuclear volume while it had no effect on thyroid, kidney or lung. Also, using his *in vitro* assay technique, he demonstrated that the amount of PTH secreted in culture varied inversely with the ($Ca^{2+}$) of the medium. Roth and Raisz (1964, 1966) published ultrastructural studies of rat parathyroid glands exposed to varying concentrations of calcium over varying time periods up to a maximum of 96 hours in culture. Briefly, they showed an inverse relationship between the ($Ca^{2+}$) of the medium and the degree of organisation of the protein-synthetic and Golgi complexes in the parathyroid cells. Further evidence (Raisz *et. al.* 1965) indicated that the rate of uptake of radioactive RNA and protein

precursors, and the rate of secretion of radioactive protein into the medium was inversely related to the ($Ca^{2+}$) of the medium. Uptake of radioactive amino acids by bovine parathyroid glands slices in culture was reported (Hamilton and Cohn, 1969) but they were unable to demonstrate the presence of PTH in the medium. This was not surprising as their method of tissue preparation was far more disruptive than other methods employing whole glands. Sherwood et. al. (1970, 1971) suggested that parathyroid hormone in the circulation, or secreted in culture, was of lower molecular weight than that extracted from bovine glands or from glands in tissue culture. They suggested that a prohormone was synthesised in the cell and that this was modified before secretion. Potts et. al. (1971 a) disputed this and suggested that the smaller molecule found in the circulation or in culture medium was the result of hydrolysis that had occurred after secretion. This objection was based on immunological techniques and the question remained open at the time of Endocrinology 1971.

All these organ culture media have included serum

products in some form or other. While more protein secretion probably occurs under these conditions, the serum content poses a formidable problem in the subsequent purification of any PTH from the culture medium. Au et. al. (1970) had been using antibodies to bovine PTH as a final purification step in the recovery of rat parathyroid hormone from fractions of culture medium that had been subjected to chromatographic procedures. It may yet prove possible to recover pure PTH by this method.

From the material referred to above, it is obviously possible to make radioactive parathyroid hormone by an organ culture technique (Raisz et. al., 1965; Au et. al. 1970). It must also be possible to recover a pure, labelled, molecular species which would then be very useful in the search for where parathyroid hormone binds to its target cells. This knowledge regarding binding sites is vital to a full understanding of parathyroid hormone function.

## MATERIALS AND METHODS.

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## I. PREPARATION OF PARATHYROID GLANDS FOR CULTURE.

Parathyroid glands were obtained from adult albino rats. Animals were killed by a blow on the head and the thyroid lobes were removed aseptically and placed in culture medium. With stainless steel razor blades, thyroid tissue was dissected away from the parathyroid glands under a dissecting microscope. After gently washing in culture medium, 4-5 parathyroid glands were placed on a Millipore filter (0.45 micron, 11 mm. diam.) supported on stainless steel gauze in a Falcon organ culture dish (Cat. No. 3010). Approximately 0.9 ml. of medium was sufficient to keep the tissue wet without submerging it.

## II. CULTURE MEDIA

a) Normal calcium medium.

The medium used was BME, Basal Medium (Eagle) based on Earle's salts prepared from dry powder (Gibco). The calcium concentration of this medium was 7.2 mg% (1.8 mM).

b) Low calcium culture medium.

This was based on Earle's balanced salt solution

except that the calcium concentration was only 1.8 mg% (0.45 mM). Amino acids, cofactors and other metabolites were added so that the final culture medium approximated a low calcium version of BME.

c) Radioactive medium

Low calcium medium was made up but the normal complement of amino acids was omitted. Instead, amino acids, uniformly labelled with $^{14}C$ (Amersham : Protein hydrolysate $^{14}C$ (U) CFB-25), were added to this medium in amounts varying from 1-5 uC $ml^{-1}$. The specific activity of these amino acids was 52 mC per m Atom of carbon. Cold tryptophan was added to replace that destroyed by acid hydrolysis of the radioactive protein from which the $^{14}C$ amino acids were obtained. Cold glutamine was added to conform with the composition of Eagle's medium.

d) Concentrated medium

BME, was made up at four times the normal concentration. This medium was used, after recovery of parathyroid hormone from glass ampoules by dilute acid, to provide a vehicle for the hormone which approximated normal Eagle's medium.

All culture media were sterilised by Millipore filtration and stored at $4^{o}C$ in glass bottles. All media contained sodium bicarbonate at 2 g. $l^{-1}$ to give a physiological pH when equilibrated with 5% $CO_2$. All culture media contained penicillin at 100 units $ml^{-1}$. This was the only antibiotic used. Some cultures were supplemented with 20% horse serum (Commonwealth Serum Laboratories, Melbourne, Aust.,) or with bovine serum albumin (1 mg $ml^{-1}$). (Sigma Chemical Co., St. Louis, U.S.A.).

## III. CONDITIONS OF CULTURE.

Organ cultures were maintained at $37^{o}C$ in an oven saturated with water vapour, and flushed continuously with a gas mixture containing 70% air, 25% oxygen, 5% carbon dioxide. Parathyroid glands were incubated for periods ranging from one to fourteen days. Culture medium was replaced every one, two or three days during this time. Medium recovered from the cultures was fractionated by gel filtration either immediately after removal or after storage at $-70^{o}C$ for a short period of time. When the cultures were terminated, the glands

were fixed for histological examination.

## IV. EXAMINATION OF CULTURE MEDIA

a) Gel filtration :

Medium from gland cultures was chromatographed on Sephadex G50 medium in a column 1.5 x 100 cm. long. The maximum sample load was 1.5 ml. The buffer used to dvelop the column was 0.2M ammonium acetate/acetic a cid (NH4Ac/HAc), pH 4.6. The flow rate through the c olumn was 20 ml. $hr.^{-1}$. Fractions were collected on an LKB Ultrorac at 4.9 ml. intervals.

Material recovered from G50 was rechromatographed on Sephadex G25 fine in a column 1.5 x 30 cm. The developing buffer was either 0.2M ammonium acetate/ acetic acid pH 4.6, or 0.1% acetic acid. The flow rate t hrough the column was 12 ml. $hr.^{-1}$ and the fraction size was 2.3 ml. per tube.

All gel filtration was carried out at $4^{o}c$.

The effluent from Sephadex columns was monitored at 280 nm in a dual beam Isco A-2 ultraviolet analyser (path length 0.5 cm) and the resulting graph plotted by a recorder.

Aliquots of column fractions were examined for radio-activity in a Philips liquid scintillation analyser (LSA). Samples were prepared for counting by one of two methods :

1) 0.4 ml. aliquots of fractions were dried on glass filter paper and placed in a scintillant solution containing 0.4% PPO, 0.002% POPOP in toluene.

2) 0.1 ml. aliquots of fractions were suspended in one part Triton X100 to two parts of the above toluene scintillant (Patterson and Greene, 1965).

The external standard channels ratio facility of the Philips LSA was used to compute decompositions per minute (DPM's) automatically.

Fractions recovered from column chromatography

were placed in 20 ml glass ampoules and lyophilised. Buffer salt was removed from these samples by redissolving them in distilled water and further freeze drying. This procedure was repeated until all the buffer salt was removed, and required a total volume of water about three times the volume of the original buffer.

b) Paper chromatography

Material recovered from column chromatography was examined by ascending paper chromatography using Whatman No. 4 paper and N-butanol:acetic acid:water (25:6:25) as the solvent system. Samples, dissolved in dilute acetic acid, were applied to the paper in volumes ranging from five to twenty five microlitres, the diameter of any one spot being restricted to 5 mm. After the samples were dried, the paper was pinned in the form of a cylinder and placed vertically in a large jar. Twenty five ml. solvent was then added carefully to the bottom of the jar, wetting the base of the paper as evenly as possible. When the solvent front had travelled about fifteen cm., the paper was removed from the jar and dried in a fume cupboard. Once dry, the paper was sprayed with Ninhydrin (Nynhydrin spray - Sigma Chemical Co.), or it was cut into small pieces and examined for radio-

activity by liquid scintillation analysis.

c) Ion exchange chromatography

The composition of fractions recovered from gel filtration was examined by both anion and cation exchange chromatography.

Anion exchange on Sephadex DEAE, A25 was carried out in small columns (0.6 x 8 cm), set up in Pasteur pipettes, and equilibrated with 0.1 N HCl. Samples, dissolved in 0.1 N HCl, were loaded onto the columns, and ten column volumes of 0.1 N HCl were washed through and collected. The solution used to wash these columns was then changed to 0.2M Tris - HCl, pH 5.0, and a further ten column volumes collected. The content of radioactivity in the fractions was then measured by adding 0.1 ml. aliquots to the Triton X 100/toluene scintillant and counting these in the liquid scintillation analyser.

Cation exchange on Dowex 50 (Sulphonic acid groups) was also employed. The same procedure was used as for anion exchange chromatography except that the initial solvent was 0.05 M acetic acid and the final buffer

was 0.2 M ammonium acetate/acetic acid (pH 4.6).

d) Disc electrophoresis on 15% polyacrylamide gel.

The composition of fractions recovered from gel filtration was examined by disc electrophoresis on 15% polyacrylamide gel. This technique was based on the work of Reisfeld et. al. (1962).

Stock Solutions.

| | | |
|---|---|---|
| A. | N KOH | 48.0 ml. |
| | N Ac (Glacial) | 17.2 ml. |
| | Tetramethylethylenediamine (teemed) | 4.0 ml. |
| | $H_2O$ to make 100 ml. | |
| B. | N KOH | 48.00 ml. |
| | HAc (glacial) | 2.87 ml. |
| | Teemed | 0.46 ml. |
| | $H_2O$ to make 100 ml. | |
| C. | Acrylamide | 60.0 g. |
| | methylene bisacrylamide | 0.4 g. |
| | $H_2O$ to make 100 ml. | |

D. Acrylamide 10.0 g

methylene bisacrylamide 2.5 g

$H_2O$ to make 100 ml.

E. Riboflavin 4.0 mg.

$H_2O$ to make 100 ml.

Tray Buffer (pH 4.5)

$\beta$ -alanine 31.2 g.

HAc (glacial) 8.0 ml.

$H_2O$ to make 1 litre

The following working solution were prepared from the stock solutions:

Small Pore Solution :(pH 4.3)

1 part A

2 parts C

1 part $H_2O$

This solution was mixed before use with an equal volume of a freshly prepared solution of ammonium persulphate (0.28 g./100 ml.). This resulted in a solution containing 15% acrylamide.

Large Pore Solution (pH 6.8)

1 part B

2 parts D

1 part E

4 parts $H_2O$

The gels were set up in glass tubes (7.0 cm. long by 0.5 cm. internal diameter) stood vertically on rubber stoppers to close the ends. Small pore solution (0.85 ml) was pipetted in, overlaid with 0.1 ml. distilled water to give a flat surface and polymerised for thirty minutes under an infra-red lamp. After polymerisation, the water was removed and then large pore solution (0.15 ml) was added above the 15% gel. This was overlaid with 0.1 ml water and polymerised for thirty minutes of ultraviolet light. Following removal of the water and the rubber stopper, the glass tubes containing the gels were set vertically in the upper electrophoresis tray such that the upper ends just protruded above the base of the upper tray while the lower portion of the gels just dipped into the beta-alanine buffer in the lower tray. Samples (10-40 microlitres) dissolved in beta-alanine buffer containing 5% sucrose and pyronin-gamma were placed on

top of the large pore gel. Pyronin-gamma was included as a tracker dye. The sucrose was present to prevent loss of sample when buffer was added to the upper tray to cover the gels. Electrophoresis was carried out at 6-8 m-amp per tube with the upper electrode the anode and the lower one the cathode. When the tracker dye neared the lower end of the gel, current was disconnected and the gels were removed with 7% acetic acid in a syringe with a long needle. There was insufficient protein in the sample to stain by normal methods so the gels were sliced in cross section at 1.5 mm. intervals, solubilised in 30% hydrogen peroxide at 50°C overnight (Young & Fulhorst, 1965) and the radioactivity measured by liquid scintillation in the Triton X 100/toluene scintillant.

## V. DETECTION OF BIOLOGICAL ACTIVITY.

Both *in vivo* and *in vitro* techniques were used to detect parathyroid hormone activity.

a) *In Vivo* :

Albino rats weighing about 100 g. were used. They were thyroparathyroidectomised and then maintained on

a standard diet. After three days, blood was removed by cardiac puncture, the test solution (0.5 ml.) injected intraperitoneally, and a further blood sample removed six hours later. Blood samples, normally about 0.3 ml., were centrifuged to remove the cells, and the plasma calcium was measured by atomic absorption spectrophotometry using a Zeiss PMQ II spectrophotometer. Heparin was used to prevent coagulation. A rise in the plasma calcium concentration over the six hours was taken as evidence of parathyroid hormone activity.

b) <u>In Vitro</u>

In this system the measure of biological activity was the ability of a sample to stimulate removal of calcium from the shaft of a two day old rat radius or ulna during twenty four hours incubation <u>in vitro</u>. The contralateral bone of the same animal in an equal volume of the same medium, minus the test sample, was used as the control.

Bone for these tests was obtained aseptically from animals anaesthetised with a lethal dose (5 mg.) of Nembutal injected intraperitoneally. Stainless steel razor blades were used to trim away muscle and connective

tissue, and to obtain equal sized pieces of tissue from the mid-shafts of the bones. The tissues were kept moist in Eagle's medium and tissue preparation was carried out with the aid of a binocular dissecting microscope.

Incubation medium for control cultures consisted of three parts $10^{-3}$N HCL to one part of the concentrated medium referred to in part II (d). Freeze-dried samples from column chromatography were redissolved in $10^{-3}$N HCL and mixed with concentrated medium for test cultures. For some of these incubations bovine serum albumin was incorporated in the medium at one mg. $ml.^{-1}$. Cultures were terminated after 24 hr., the bone fixed for histological examination and themedia kept for calcium determinations. Anti-bovine PTH gamma globulin (supplied by W. Britton, Dept. of Pathology, University of Sydney) was used in some of these cultures to block the hormone activity. In these latter cultures, the samples were incubated for 60 mins. with the antibodies before addition of the bone shaft. Conditions of incubation were the same as for parathyroid glands.

In a small number of experiments bone shafts were incubated with parathyroid glands or in whole medium in which parathyroid glands had been cultured previously.

Plasma and medium calcium concentrations were measured by atomic absorption spectrophotometry at 423 nm in an air/acetylene flame.

Stock Solutions

(a) Calcium standard

| | |
|---|---|
| $CaCO_3$ (dehydrated) | 0.2500 g. |
| NaCl | 8.3900 g. |
| KCl | 0.2860 g. |
| $H_2O$ to a final volume of | 1 litre |

(b) Calcium blank

| | |
|---|---|
| NaCl | 8.390 g. |
| KCl | 0.286 g. |

(c) Oxine solution

4 g. oxine $l^{-1}$ in 1% acetic acid with wetting agent.

All water used to make these solutions was deionised, distilled water. The oxine solution was used as the

diluent for preparing the set of calcium standards. All test solutions were diluted one in twenty with oxine solution. This dilution brought the test solutions within the range of the standard curve (0.0 to 0.5 mg.% w.r.t. calcium). The standard curve was linear over this range.

The results of these tests were arranged into two groups, calcium concentration of control ($x_1$) and test ($x_2$) media. These groups were treated as two populations and subjected to a t-test with the aid of a computer. The Null Hypothesis was that there was no significant difference between the two groups.

## VI. BINDING OF RAT PARATHYROID HORMONE TO RAT BONE

Parathyroid hormone, produced in organ culture from radioactive amino acids, purified and reconstituted in Eagle's medium, as described above, was incubated with bone for periods of time from ten to ninety minutes. Conditions of incubation were the same as for parathyroid gland cultures. Bone specimens were recovered from two day old rats. Parietal was most frequently the bone of choice and was prepared in pieces about 2-4 mm. square.

In a smaller number of experiments long-bone pieces were employed.

After the period of incubation, the bone was removed and fixed for autoradiography, and the incubation medium was subsequently examined for hormone activity by the *in vitro* detection system described above.

Gamma globulin referred to above was used in one experiment to block the binding of rat PTH to bone.

Bone was also incubated with $^{14}C$ amino acids to see how rapidly they reached the interior of bone cells.

## VII. HISTOLOGICAL TECHNIQUES.

### a) Fixation.

All tissues were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer for a minimum period of one hour. Tissues were then washed in a few changes of buffer and post-fixed in osmium tetroxide for one hour. Excess osmium tetroxide was removed by three changes of cacodylate buffer.

b) Dehydration

Tissues were submerged for ten minutes in each of 30%, 50%, 70% and 90% ethanol. This was followed by 4 x 15 min. in 100% A.R. ethanol followed by 4 x 15 min. in 100% A.R. acetone.

c) Infiltration and embedding

After the last wash in acetone, the tissues were placed in a 50% acetone'50% araldite mixture for 12 hours on a rotor to assist infiltration of the resin.

Araldite is an epoxy resin prepared as follows:

| | |
|---|---|
| Araldite base (CY 230) | 96 g. |
| DDSA (HY 964) | 32.8 g. |
| MNA (HY 906) | 17.8 g. |
| DMP 30 (HY 960) | 1.92 g. |

These chemicals were mixed for 1½-2 hours and stored in a freezer until required.

After 12 hours in acetone/araldite, tissues were transferred to 100% araldite and kept overnight at 37°C in a vacuum oven at 100 mm Hg. pressure, to remove any acetone retained by the tissues. Following this,

tissues were transferred to fresh araldite in rectangular embedding wells, and the resin polymerized for 24 hours at 45°C followed by 48 hours at 60°C.

d) Sectioning and staining of tissue for light microscopy.

Thick sections (0.5 micron) were cut with glass knives on a Huxley, a Porter-Blum MT 1 or an LKB ultratome. Sections were transferred to a drop of distilled water on a clean glass slide, dried down and then stained with 1% toluidine blue in 1% sodium carbonate. Photomicrographs were taken with a Zeiss Photomicroscope.

e) Sectioning and staining of tissue for electron microscopy.

Thin sections (500-1000Å) for electron microscopy were cut on a diamond knife on a Huxley microtome. Sections were picked up unsupported, on acid cleaned, 300 mesh copper grids. They were stained with uranyl acetate (2 min.) and lead citrate (2 min.). Electron micrographs were taken with a Siemen's Elmiskop 1.

## VIII. AUTORADIOGRAPHY.

This technique was used to demonstrate where $^{14}$C-PTH

was localised in bone. Sections of undecalcified bone (0.5 micron thick) were dried down as flat as possible onto specially cleaned glass slides. Ilford K5 emulsion, diluted 1:3 with distilled water was used to coat these slides. Slides were dipped into the emulsion and allowed to dry in a vertical position, thus allowing a fairly thin layer of emulsion over the sections. These preparations were then exposed to a wet oxidising environment for three hours to minimise background (Caro et. al. 1962) This was done in light-tight McIntosh and Fildes jars, and at the end of three hours, the environment was changed to a dry, reducing atmosphere (silica gel, dry nitrogen). Slides remained in these jars under $N_2$ for the duration of the exposure time. The period of exposure, i.e. from dipping the slides to developing the emulsion, was within the range 2-4 weeks. The development time was 6 min. using Kodak D19B developer at 17°C. This was followed by an acid stop bath (1% HAc) for 30 sec. and then 5 min. in Ilford Hypam rapid fixer. The slides were then washed for 10 min. in tap water, the emulsion toughened in 10% formalin for 15 min., followed by a further 10 min. washing in water. The sections were then stained with 1% toluidine blue

containing 1% sodium carbonate.

## IX. GRAIN COUNTS.

Developed silver grains in the autoradiographs were counted over the different types of bone cells and over areas of bone matrix. Background grain counts were made over areas or araldite adjacent to the tissues. These background counts were used as controls and they were made over areas of araldite equivalent in size to the areas covered by the tissues. Grain counts were done directly, with the aid of microscope rather than from photographic prints, and represented total grains over complete sections. The ability to recognise silver grains was aided by the use of a Nomarski phase interference optical system (Nomarski, 1955). By this system it was possible to ensure that the silver grains were lying in a plane above the plane of the tissue section.

# RESULTS.

## I. PARATHYROID GLAND CULTURES.

## II. COLUMN CHROMATOGRAPHY

## III. HOMOGENEITY OF THE G50 SECOND PEAK

a) Sephadex G25

b) Ion exchange chromatography

c) Paper chromatography

d) Polyacrylamide gel electrophoresis

## IV. DETECTION OF BIOLOGICAL ACTIVITY.

a) In vivo

b) In vitro

## V. ESTIMATION OF THE AMOUNT OF RECOVERED PTH

## VI. AUTORADIOGRAPHIC LOCALISATION OF RAT PARATHYROID HORMONE IN RAT BONE.

## I. PARATHYROID GLAND CULTURES

Rat parathyroid glands were maintained in culture for time periods ranging from one to fourteen days. The majority of cultures were terminated between two and five days. At the end of the culture period, glands were fixed for histological examination and the medium was analysed for molecules that had been secreted during the period of incubation. Some six hundred parathyroid glands were used in these experiments - a few were incubated in media containing horse serum, or bovine serum albumin but the majority were exposed to media from which serum additives were specifically excluded. A total of three hundred and seventy glands were incubated in low calcium medium.

The presence or absence of serum in the media did not appear to alter the histological appearance of the parathyroid cells. However, from the aspect of PTH secretion, glands incubated without serum did not secrete very much after the first week. Au et. al. (1970), who used serum in their cultures of rat parathyroid glands reported high levels of secretion up to the end of the

second week of incubation.

At the level of the light microscope, the majority of cultured glands appeared to be very healthy. In most of them it was difficult to find evidence of necrosis of parathyroid cells. However, some glands showed gross necrosis at the periphery which was most probably caused by careless handling during removal from the animal and preparation for organ culture. In these latter cases, the fractionation pattern obtained from gel filtration of the culture medium was different to that from healthy cultures, and this material was not used in subsequent experiments.

Figures 1 - 3 are photomicrographs of parathyroid glands which had been cultured for one, five and nine days respectively. Figs 1 and 2 are of glands cultured in normal Eagle's medium while the third gland (Fig. 3) was cultured in low calcium medium. Each of these tissues was obviously healthy and gave some indication of the success of the organ culture system.

By electron microscopy, cultured parathyroid glands

were similar to tissue which had been fixed immediately after removal from the animal. The nuclei were irregular in shape, and the bulk of the heterochromatin was arranged around the inner leaflet of the nuclear envelope, with a smaller quantity dispersed throughout the nucleus. Nucleoli were frequently observed. The cytoplasm of these cells was rich in mitochondria and many cells had a well developed Golgi complex. Another prominent feature of the cytoplasm was the presence of large numbers of ribosomes. These were arranged mainly in clumps which did not appear to be membrane bound. Endoplasmic reticulum was present only to a limited extent and ribosomes were always attached to it. This rough endoplasmic reticulum was somewhat more developed in cells that had been cultured in the low calcium media than in those from normal Eagle's medium, but there were always far more ribosomes that appeared to be unattached to membranes.

Figures 4 and 5 depict glands that had been cultured for 5 days in Eagle's medium without serum. There is little rough endoplasmic reticulum, mitochondria are common and secondary lysosomes are prominent (Fig. 4.).

Those lysosomal bodies are more common in cultured tissue, and their numbers appear to increase as the period of incubation is increased. After eight days incubation (Fig. 6) in a similar medium, the cells appear little different. The rough endoplasmic reticulum is possibly less prominent but it is noted that lipid droplets are increased in number. Accumulation of lipid is a feature of most of the glands, particularly the longer term cultures (Figs. 6, 13-18), and is an indication of some change in the metabolic pattern. Cells in organ culture are probably hypoxic as oxygen can reach them only by diffusion in the absence of circulating erythrocytes, and this could account for the increased lipid accumulation.

Figures 7 and 8 show cells from three day cultures where the medium was normal Eagle's, supplemented with 20% horse serum. There are no gross morphological differences between the cells in these pictures and those in all the others of this series. Even though serum in the culture medium may aid the secretion of PTH in organ culture (Au et. al. 1970) it does not appear to be necessary to maintain the integrity of parathyroid cells, at least

in these relatively short term cultures.

The remaining electron micrographs (Figs 9-18) are of parathyroid tissue that had been cultured in low calcium (1.8 mg%) medium without any serum additives. Although most of the ribosomes still appear to be unattached to membranes, the rough endoplasmic reticulum is more highly developed that in cells cultured in media containing more calcium (compare Figs 9-18 with Figs 4-8). A more marked difference between these groups is the greater prominence of the Golgi apparatus in cells in the low calcium cultures (Figs 15, 16, 18). Both of these observations are consistent with the greater secretion of PTH by parathyroid glands in culture with low calcium medium (Raisz et. al, 1965). The plasma membranes of adjoining cells showed a marked degree of interdigitation. This feature was more marked in cells from lower calcium cultures, as reported earlier by Roth and Raisz (1966). However, the highest calcium concentration in the present experiments was less than 2 mM which meant that all these cultures lay in the low calcium range as outlined by these workers. Because of this, the variation in this feature of the plasma

membranes was less marked than in the other experiments referred to. Another feature of the cell contacts was the presence of desmosomes, which were noted in all tissue, irrespective of the culture medium.

These results indicate that rat parathyroid glands may be maintained in organ culture without grave cytological effects, and that, by morphological criteria, the cells are capable of synthesizing and secreting protein or polypeptide molecules.

## II. COLUMN CHROMATOGRAPHY

Fractionation of parathyroid gland culture medium on Sephadex G50 produced the following results :

(a) Only one UV - absorbing peak was found consistently. The material in this peak was a mixture of small molecules below the fractionation range of G50. It contained amino acids, niacinamide, riboflavin and other low molecular weight substances from the original medium, plus any small molecules that may have been released by the parathyroid glands. In the column system used, this peak eluted at about 150 ml. effluent volume. Even when the most sensitive scale of the Isco analyser was employed, no definite peak could be found within the

fractionation range of this column when the sample was parathyroid gland culture medium.

A second UV - absorbing peak was found in those few runs where the sample of medium contained either horse serum or bovine serum albumin. This peak eluted in the void volume of the column, corresponding to about 60 ml. effluent volume.

Further characterisation of this column system was achieved by employing bovine pancreatic ribonuclease (Sigma) as a marker protein. Like parathyroid hormone, this molecule is a basic protein, and with a molecular weight around 13,500, it should elute just ahead of the hormone. When ribonuclease was run on G50, two peaks were found. The first, at around 90 ml. was within the fractionation range of the Sephadex. A smaller peak followed at about 150 ml. corresponding to the low molecular weight substances referred to above. This latter peak probably contained breakdown products of ribonuclease. From these results it was predicted that parathyroid hormone would probably elute at a volume of some 90-100 ml.

(b) Fractions from parathyroid gland culture medium that contained Carbon - 14 were then examined by liquid scintillation analysis. Three consistent, radioactive peaks were found (Fig. 19). The first and smallest one appeared in the void volume at about 60 ml effluent volume. A second, larger peak at about 100 ml was within the fractionation range of G50. The third and largest radioactive peak appeared at 150 ml, coinciding with the UV - absorbing material mentioned above. The radioactivity in this last peak was associated with unused amino acids in the medium and possibly small peptides. These three peaks consistently appeared in exactly the same positions for any one particular column. Because of the low temperature and pH involved, it was possible to use the same column for months at a time.

The second radioactive peak, eluting in a position just after that for ribonuclease, was subsequently shown to contain parathyroid hormone activity. In support of these results, highly purified bovine parathyroid hormone (Wilson Lab.) was run on the same column. Although the sample contained insufficient protein to monitor at 280 nm, biological activity was

demonstrated at an effluent volume equivalent to the second radioactive peak from parathyroid gland culture medium.

## III. HOMOGENEITY OF THE G50 SECOND PEAK.

The second radioactive peak from G50 was examined further to assess its purity.

(a) Sephadex G25

First it was run on Sephadex G25 fine (1.5 x 30 cm) at 12 ml $hr^{-1}$. When the eluting buffer was 0.2M $NH_4$ AC/H Ac (pH 4.6), all the radioactivity eluted in the void volume (23 ml) (Fig. 20). This demonstrated that the G50 peak was not contaminated by small molecules and that radiolysis was not a problem, at least with material up to two weeks old.

Dilute acetic acid was also used in this system, because other workers in the field have done so, (Au. et. al. 1970). However, further fractionation of the second radioactive peak was not achieved.

(b) Ion exchange chromatography

Secondly, it was examined by ion exchange chromatography. Separation of different molecular species

in the G50 peak was not achieved by anion exchange on Sephadex DEAE. All the radioactivity of the sample was eluted with the HCL. The subsequent treatment of these columns with Tris buffer produced no radioactive material (fig. 21). Liquid scintillation analysis of the Sephadex at the completion of these runs indicated that no radioactivity remained bound to it.

When a cation exchanger (Dowex 50) was used, radioactive molecules from the second G50 peak were bound to the resin and no radioactivity eluted with the acetic acid wash. Development of these columns with the 0.2 M $NH_4$ Ac/HAc buffer resulted in the displacement of all the radioactivity from the resin.(Fig. 22).

(c) Paper chromatography.

Thirdly, the G50 material was examined by ascending paper chromatography, and compared with highly purified bovine. - PTH (Wilson Lab.). The bovine preparation was inhomogeneous. Some of the sample remained at the origin, while smaller molecules migrated with an Rf value of 0.55. The latter probably consisted of breakdown products of the hormone. The sample of rat protein from the G50 second peak remained at the origin. None of the radioactivity migrated up the paper.

(d) Polyacrylamide gel electrophoresis

Fourthly, it was examined by disc electrophoresis in 15% polyacrylamide gel.

The results available from this technique (fig. 23) suggested that the G50, second radioactive peak was probably homogeneous. Great difficulty was encountered in preparing samples for electrophoresis because of the minute quantities of PTH that were available and the necessity to apply them in small volumes to the gels. The samples used in this series contained only picogram quantities of radioactive PTH and only one set of results was obtained. In this instance, the radioactivity was restricted to one section (1.5 mm slice) of the gel.

## IV. DETECTION OF BIOLOGICAL ACTIVITY

### (a) In Vivo

This technique was discarded after some fifty animals were used because the results were at best inconsistent and at worst confusing. The in vivo method is useful for microgram quantities of hormone, but does not appear to be sensitive enough for the nanogram quantities available in these experiments.

(b) <u>In vitro</u>

This method was developed to yield consistent results with the small quantities of PTH that were recovered from parathyroid gland culture media containing no serum additives. In this system the measure of biological activity was the ability of a sample to stimulate removal of calcium from the shaft of a two day old rat radius or ulna during twenty four hours incubation <u>in vitro</u> with 0.9 ml of medium. The contralateral bone of the same animal in an equal volume of medium, minus the test sample, was used as the control. It must be emphasised here that these experiments were carried out merely to detect calcium mobilising ability and not as an assay related to units of activity of bovine PTH or of any other standard.

The first few experiments in this series were exploratory in nature. Bone shafts were incubated in the presence of parathyroid glands, and also in media with which glands had been cultured previously. These tests indicated that the glands were secreting some calcium mobilizing substance and that this effect was greater when the glands had been cultured in low calcium medium. This second effect supported the results of

Raisz et. al., (1965) who reported that low calcium media stimulated rat parathyroid glands in culture. Because of these results, low calcium media were used for all subsequent cultures of parathyroid glands.

Once the in vitro testing system was established, it was employed in a detailed examination of reconstituted fractions recovered from column chromatography of parathyroid gland culture medium. The only area of the G50 eluate that exhibited calcium mobilizing ability was that associated with the second radioactive peak. This activity was still demonstrable after the material had been rerun on G25 and recovered from the void volume fraction. The results of thirty-seven tests are shown in Table 1. The calcium concentrations of the control media were pooled (mean 4.67 mg%, standard deviation 1.04) as were those of the test media (mean 5.66 mg%, standard deviation 1.11). These two populations were subjected to a t test and the value of t was found to be -3.9301. On these results, the probability that the Null Hypothesis (i.e. that the test values were not significantly greater than the control values) was correct was $< 0.001$. Therefore, the calcium concen-

trations of test media were significantly above those of control media.

Highly purified bovine PTH (Wilson Lab.) was tested in the <u>in vitro</u> system after the hormone had been chromatographed on G50 and recovered from the same position of the elution pattern as the rat material. Similar statistical treatment of these results (Table 11) indicated a mean calcium concentration of 5.55 mg% for control media (standard deviation 0.1732) and 6.55 mg% for test media (standard deviation 0.2646) with a t value of -6.3245, and a $p < 0.001$. These results supported the hypothesis that the G50 second radioactive peak referred to above was rat parathyroid hormone.

To enhance this claim, gamma globulin from a rabbit that had been stimulated by highly purified bovine PTH was incubated with the rat hormone for an hour before the addition of the bone shaft, no variation in the calcium concentrations of control and test media was demonstrable after the subsequent 24 hour incubation. From this, it was concluded that antibodies to bovine PTH cross react with rat PTH.

The G50, second radioactive peak, recovered from rat parathyroid gland culture medium, with the ability to mobilize calcium from bone, will now be referred to as rat parathyroid hormone.

## V. ESTIMATION OF THE AMOUNT OF RECOVERED PTH

It was estimated that the PTH recovered from column fractions was in nanogram quantities. Unfortunately, there was insufficient pure protein for it to be weighed, but an estimation was made, based on the probable degree of incorporation of radioactivity. Obviously, when the parathyroid glands were put into organ culture, they contained cold amino acids. The culture medium contained cold glutamine and tryptophan, but the rest of the added amino acids available to the cells were uniformly labelled with $^{14}C$. Therefore, PTH synthesised and secreted in culture, particularly after the first day, was probably heavily labelled with radioactivity. Further, rat parathyroid glands do not appear to store PTH (Cameron 1968), so that secreted in culture probably represented newly synthesised protein. The activity of the $^{14}C$ protein hydrolysate added to culture media

was 52 m C per m-Atom of Carbon. Carbon - 14 was added to culture media at 1-5 u C $ml^{-1}$. If every carbon atom in PTH was $^{14}C$ from this source, then the amount of PTH with an activity of $10^5$ DPM would be approximately 17 nanograms. In the experimental system, the yield of radioactive PTH from 1.5 ml. of parathyroid gland culture medium, fractionated on G50, varied from $10^4$-$10^5$ DPM. If this amount of recovered PTH was labelled totally with carbon - 14, this amount of radioactivity would represent approximately 1.7-17 ng. PTH. However, culture media contained cold glutamine and tryptophan as well as the $^{14}C$ amino acids; also, the glands added to culture contained cold amino acids. Therefore, the amount of rat PTH containing $10^5$ DPM would be greater than 17 ng, but it would still be in nanogram rather than microgram amounts.

## VI. AUTORADIOGRAPHIC LOCALISATION OF RAT PARATHYROID HORMONE IN RAT BONE.

In developed autoradiographs grains were present over osteoblasts (Figs. 24, 25, 26, 28) and osteo-progenitor cells (Figs. 25-28) as classified by Young (1962). A smaller number of grains was also found over

osteocytes (Fig. 25), calcified matrix and superficially placed macrophages (Fig. 27). The distribution of grains is shown in Tables. 111, 1V.

Autoradiographs of bone which had been incubated with PTH for ten minutes were technically very poor, the background being very high. Despite this, grain counts were attempted and it was decided that little radioactive material had bound to the tissue in this time. However, no conclusions were drawn from these experiments.

After incubation times of thirty and sixty minutes, grain counts over tissue were considerably above background, and it was from these results that the conclusions presented in this thesis were drawn.

The location of silver grains over tissue was similar for both the 30' and 60' incubations (Tables 111, 1V). Radioactive PTH (total activity 50,000 DPM) used in the 60' incubation was recovered from the second radioactive peak of G50/ This material was dissolved in 0.9 ml. medium and the tissue incubated

with it was taken from the contral part of a parietal bone of a two day old rat. The PTH used for the 30' incubation (a similar amount of radioactivity) was initially fractionated on G50 and then rechromatographed on G25. Part of this material was incubated in a separate dish with gammaglobulin, raised against bovine - PTH, before addition of parietal bone. The bone for these incubations was taken from an area of two day old rat parietal bone close to the lateral suture line and it contained a much larger percentage of osteoprogenitor cells than that used for the 60' incubation.

The PTH remaining in solution at the end of the 60' incubation was tested for calcium mobilising ability. At the end of this second incubation the calcium concentration of the test medium (8.3 mg%) was 24% higher than that in the control medium (6.7 mg%); ie the parietal bone had been exposed to biologically active, radioactive parathyroid hormone.

In autoradiographs from the 60' incubation, Table V, 1552 grains were counted over 1,012 osteoblasts out of a possible 1960 osteoblasts; i.e. 52% of the osteoblasts

were labelled with an average of 1.52 grains per cell. 1869 grains were counted over 1350 osteoprogenitor cells out of a possible 2800 such cells; i.e. 48.3% of the osteoprogenitor cells were labelled with an average of 1.38 grains per cell. 188 grains were counted over 97 osteocytes out of a possible 420 cells; i.e. 23% of the osteocytes were labelled with an average of 1.22 grains per cell. Also in these autoradiographs, the number of grains per unit area of bone matrix was approximately double that of the background.

Of the grains counted over osteoblasts and osteoprogenitor cells, 75% of those over the former and over 90% of those over the latter were located very close to the peripheries of these cells.

In the experiments mentioned above where parietal bone was incubated for 30' with radioactive PTH plus gamma globulin, the grain count over cells did not appear to be above background but the autoradiographs were not as good technically and little weight was put on them.

TABLE 1.

Calcium mobilizing tests for rat PTH

$(Ca^{2+})$ (mg%)

| Control ($x_1$) | Test ($x_2$) | Control ($x_1$) | Test ($x_2$) |
|---|---|---|---|
| 3.7 | 4.3 | 5.0 | 6.1 |
| 3.6 | 4.5 | 5.0 | 5.8 |
| 3.6 | 4.3 | 5.2 | 6.4 |
| 3.6 | 4.1 | 5.8 | 6.4 |
| 5.8 | 6.6 | 5.8 | 6.3 |
| 3.2 | 3.7 | 3.4 | 4.7 |
| 3.3 | 3.7 | 3.5 | 5.3 |
| 6.7 | 8.3 | 3.5 | 4.8 |
| 6.1 | 7.1 | 3.5 | 5.2 |
| 5.8 | 6.6 | 3.4 | 4.9 |
| 5.2 | 6.6 | 3.0 | 4.0 |
| 5.0 | 6.0 | 3.1 | 4.2 |
| 5.8 | 6.4 | 5.3 | 7.2 |
| 5.2 | 6.0 | 5.3 | 7.5 |
| 5.4 | 6.0 | 4.4 | 5.8 |
| 5.4 | 6.0 | 4.9 | 5.2 |
| 5.0 | 6.5 | 5.1 | 5.9 |
| 5.2 | 6.2 | 5.2 | 5.6 |
| 4.1 | 5.1 | | |

mean of $x_1$ = 4.6730 mean of $x_2$ = 5.6568

standard deviation of $x_1$ = 1.0415 standard deviation of $x_2$ = 1.1107

$t$ = -3.9301 degrees of freedom 72 $p < 0.001$

## TABLE 11.

### Calcium mobilizing tests for bovine PTH

($Ca^{2+}$) (mg%)

| control ($x_1$) | test ($x_2$) |
|---|---|
| 5.5 | 6.4 |
| 5.4 | 6.6 |
| 5.8 | 6.9 |
| 5.5 | 6.3 |

mean of $x_1$ 5.5500 mean of $x_2$ 6.5500

standard deviation of $x_1$ 0.1732 standard deviation of $x_2$ - 0.2646

t 6.3245 degrees of freedom - 6.0000 $p < 0.001$

TABLE 111

GRAIN COUNTS — RAT PARIETAL BONE

60 MIN. $^{14}$C-PTH — 14 SECTIONS

| Osteo-blasts | Osteo-cytes | Osteo-progenitor cells | Macro-phages | Matrix | Back-ground |
|---|---|---|---|---|---|
| NUMBER OF GRAINS | | | | | |
| 1552 | 118 | 1869 | 176 | 267 | 249 |
| % OF TOTAL COUNTS | | | | | |
| 37 | 3 | 44 | 4 | 6 | 6 |

TABLE IV.

GRAIN COUNTS — RAT PARIETAL BONE

30 MIN. $^{14}$C-PTH — 12 SECTIONS

| Osteo-blasts | Osteo-cytes | Osteo-progenitor cells | Matrix | Back-ground |
|---|---|---|---|---|
| NUMBER OF GRAINS | | | | |
| 618 | 63 | 1192 | 126 | 314 |
| % OF TOTAL COUNTS | | | | |
| 25 | 3 | 52 | 6 | 14 |

## TABLE V.

## Autoradiography (60 min. incubation)

| total no of cells | | | total no of labelled c cells | | |
|---|---|---|---|---|---|
| osteo-blasts | osteo-cytes | osteo-progenitor cells | osteo-blasts | osteo-cytes | osteo-progenitor cells |
| 1960 | 420 | 2800 | 1012 | 97 | 1350 |

| % of cells labelled | | | no. of grains/labelled cell | | |
|---|---|---|---|---|---|
| osteo-blasts | osteo-cytes | osteo-progenitor cells | osteo-blasts | osteo-cytes | osteo-progenitor cells |
| 52 | 23.0 | 48.3 | 1.52 | 1.22 | 1.38 |