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PARATHYROID HORMONE INFLUENCES ON RNA SYNTHESIS IN BONE CELLS

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STATEMENT OF THE PROBLEM
A dual function of parathyroid hormone (PTH) in its target tissue, bone, has been well established (Talmage, 1962). One function is to increase the rate of bone remodelling by increasing the number of active osteoclasts, and the second is to control calcium homeostasis by hormonal action on existing bone cells to stimulate transport of calcium ion to the fluid phase of the extracellular spaces.

Recent investigation of metabolism associated with PTH function indicates that there is an involvement of ribonucleic acid (RNA) synthesis in the action of the hormone in producing its effects in bone (Talmage et al., 1965; Martin et al., 1965).

The following problems have been studied:

A. The effect of increased endogenous PTH secretion on RNA synthesis in different cell types of bone.

B. The effect of increased endogenous PTH secretion on deoxyribonucleic acid (DNA) synthesis in mesenchyme cell population of bone.

C. The effect of increased extracellular calcium ion concentration of bone cell RNA synthesis, which may exemplify the close relationship between calcium ion transport across cell boundary, stimulation of RNA synthesis, and PTH function.

D. The effect of thyrocalcitonin and cortisone on RNA synthesis in bone and a possible relationship of the steroid to parathyroid hormone action.
LITERATURE REVIEW
A. **Immediate effects of PTH on bone cell metabolism**

The metabolic phenomena underlying bone resorption and the action of parathyroid hormone on bone cell metabolism are still poorly understood.

Modification of bone metabolism after treatment of animals with parathyroid extract (PTE) has been reported, but in most of these studies (Gaillard, 1955; Johnston et al, 1961) animals were treated for several days with PTE. In such experiments, the observations are the sum of primary and secondary effects of hormone on target tissues, with little delineation between the two events.

One way to avoid this complication is to study the sequence of events following a single dose of PTE. This approach may reveal the more basic effects of parathyroid hormone and its role in bone resorption (Heller et al, 1950).

The effect of parathyroid hormone on such factors controlling cell metabolism such as nucleic acid synthesis, protein synthesis, enzyme induction, enzyme alteration through molecular conversion, generation of cofactors, and cell permeability will most likely be translated ultimately into a fairly specific and direct stimulation of calcium and phosphate transport.

However, efforts have failed to uncover any specific mechanism of action of this hormone on calcium transport. One of the difficulties was the lack of availability of pure hormone and most of the studies were carried out using PTE which was composed of many non-specific peptides besides PTH (Hawker et al, 1966). This may have contributed to the confusion in the biochemical and physiological analyses of the effect.
Pure hormone is now available and there are excellent ways of inducing
dogenous PTH secretion in vivo; therefore, most of the studies have to
be repeated using these physiological means rather than administering
pharmacological doses of mixtures of various polypeptides.

1. Calcium homeostasis

It was first demonstrated by Talmage and Elliott (1958) that PTH
mobilizes calcium from the areas of bone that are not in immediate con-
tact with surrounding vascular fluid media to the fluid spaces. This
was effected through metabolic processes in bone cells which enable calcium
to be dissolved in greater concentration than the solubility of apatite
crystal, although the apatite crystal does maintain a constant low con-
centration of calcium in the fluid space by a physicochemical equilibrium.
Using the enteroperitoneal lavage technique, the difference in removal
of Ca$^{45}$, Ca$^{40}$, P$^{32}$, and inorganic phosphate in PTH-stimulated and para-
thyroidectomized (PTX) rats was shown within one hour. The rats were
labeled with radioisotope for 24 hours or 2 to 3 weeks before the lavage
procedure. These data clearly demonstrated that PTH acts on the stable
portion of bone, while the reactive, exchangeable, or more recently formed
bone is lost by continuous, physicochemical exchange processes.

Jeffrey and Bayne (1964) confirmed the experiments by observing the
increase of radioactivity in serum within six hours after PTE injection.
The results showed that the increase in serum calcium levels was due to
an effect on the slower exchanging fractions of bone. Further, Talmage
et al (1965), demonstrated direct evidence of this effect by in vitro
incubation of bone fragments in pooled serum. Raisz (1963) has observed
that in embryonic rudiment organ cultures of bone, the release of Ca$^{45}$ is stimulated by PTH within one half hour to three hours after addition of the hormone to the culture medium.

Parsons and Robinson (1968) also demonstrated evidence for the rapid hypercalcemic action of PTH. They measured the mobilization of calcium from perfused cat tibia 20 minutes after an intravenous injection of PTH and observed an increase of about 15%.

2. **Phosphate transport**

Egawa and Neuman (1963) examined PTH action on phosphate transport at the cellular level using P$^{32}$ incorporation as the criterion. The acid soluble organic phosphate fraction of calvaria was greater one hour after PTH injection compared to control animals.

3. **Carbohydrate metabolism**

*In vitro* studies of carbohydrate metabolism using glucose-$\mu$-C$^{14}$ indicate that PTE treated animals produced 10-20% more lactic acid within 6 hours after PTE administration (Vaes and Nichols, 1962). The specific activity of lactate was the same in treated and control animals, indicating that the excess lactate produced under the influence of PTE was due to an increased catabolism of glucose. Dowse et al (1963) were not able to observe any increase in citric acid levels *in vitro* as a result of PTH treatment. The only significant finding was an accumulation of lactate in the aerobic condition after 2 hours of PTE treatment.

Yates and Talmage (1965) found that PTX had a different effect on the accumulation of citrate and lactate in femurs incubated *in vitro*. Both citric acid and lactic acid produced by bone cells decreased four hours after PTX. Citrate production was greater in the diaphysis, and
lactate production was greater in the metaphysis. They postulated that
the type and ratio of bone cells in any one region might be associated
with the predominant metabolic end-product. Hekkelman and Hermann-Erlee
(1968) demonstrated the rapidity of the lactate accumulation in vitro
after the addition of PTE to embryonic cultures of bone. A significant
difference was noted within 30 minutes.

Since Dickens (1941) first reported a high content of bone citrate,
emphasis has been given to a strong correlation of the two organic acids,
citrate and lactate, and the response of their levels to PTH administra-
tion. However, no clear cut mechanism for the relation has been determined.

Neuman et al (1956) compared dog spongiosal blood, marrow blood, and
arterial blood after PTE administration, and observed that the citrate
output from bone increased immediately after PTE injection. They also
observed an increase in arterio-venous difference in citric acid, pyruvic
acid, and lactic acid in leg bone five hours after PTE injection (Martin
et al, 1958). Schooley and Otero (1960) confirmed this observation by
studying an isolated perfused rat hind limb one hour after PTE. They
found that the total plasma citrate was two to three times greater than
that found in a control perfusion, but the lactate increased only slightly.
Vaes and Nichols (1962) and Schartum and Nichols (1961a, 1961b) observed
that the rate of production of lactate was about 100 times that of citrate.
They also noted that there was no direct correlation between the amount
of lactate produced in vitro by bone and the transfer of calcium and
phosphate into surrounding fluid. Also, using iodoacetate which blocks
the triose-phosphate dehydrogenase reaction, there was a decreased mobili-
zation of calcium and phosphate in vitro suggesting that glycolysis is
important in some way and that the end-product of glycolysis, lactic acid, is not, as such, the critical factor in calcium and phosphate mobilization from bone.

Goldhaber (1961) demonstrated that malonate inhibited bone resorption presumably by inhibition of succinic dehydrogenase (SDH). Since then, many reports on the long term effects of PTH on either increased or decreased SDH have been made. It appears that this difference of PTH effect could be accounted for by the length of time elapsing after hormone injection. These results were clarified by the study of Mills and Bavetta (1965) which showed that administration of hormone to young rats resulted in an early stimulation of SDH activity (within two hours) in metaphyses, diaphyses and calvaria. This was followed by a return to normal activity in 12 to 24 hours. Hermann-Erlee (1965) reported an effect of PTH on lactic dehydrogenase. Two hours after administration, there was an inhibitory effect on the enzymatic activity in contrast to stimulatory effects on the activity of malic dehydrogenase and glutamic dehydrogenase.

An increased activity of acid phosphatase and a decreased activity of magnesium-dependent adenosine triphosphatase (ATPase) was observed six hours after PTH administration in a decalcified bone slice of femoral metaphysis (Doty and Robinson, 1968). However, endogenous stimulation of PTH resulted in stimulation of ATPase activity (two times) and a decrease in acid phosphatase activity in bone (Doty, 1968).

Hermann-Erlee (1966) reported a decrease in nicotinamide adenine dinucleotide phosphate (NADP) content of in vitro cultures of embryonic mouse calvaria six hours after the addition of hormone, although the level of nicotinamide adenine dinucleotide (NAD) was increased significantly
by four hours. This confirmed the hypothesis of Hekkelman (1965) who stated that the effect of PTH on carbohydrate metabolism is partly due to the NADP level.

4. Synthesis of bone matrix

Heller et al. (1950), Kroon (1958), and Young (1963c) reported that PTE administration led to a disappearance of the typical osteoblast, and in vitro biochemical studies have indicated that PTH inhibits bone collagen synthesis.

Gaillard (1961, 1962) found in radioautographs of radii from six hour organ cultures, a decreased content of labeled glycine in the osteoblasts if PTE was added. De Vogd van der Straaten (1962a, b) found similar evidence of an influence of PTE on the glycine-C\textsuperscript{14} binding capacity using quantitative radioautographic analyses of the bony shaft of cultivated mouse radius rudiments. Johnston et al. (1962), using rat calvaria in tissue culture, reported a decreased rate of hydroxyproline labeling with proline-C\textsuperscript{14} three hours after the last dose of PTE was administered (75 units every two hours for three days). Vaes and Nichols (1962), using femoral metaphyses, observed a slight decrease (10\%) in the in vitro incorporation of glycine-C\textsuperscript{14} six hours after a subcutaneous injection of a massive dose of PTE (100 units) into mice. Flanagan and Nichols (1964) subsequently reported that the in vitro incorporation of proline-C\textsuperscript{14} into bone collagen was reduced after a single dose of PTE in young rats. Depression of proline-C\textsuperscript{14} uptake into collagen proline and hydroxyproline was observed over intervals ranging from six hours to 18 hours after addition of PTE. Nichols et al. (1965) reported further studies on the effect of PTE on bone biosynthetic mechanisms and indicated that the site of PTE effect may be the assembly of the collagen molecule on the ribosomes.
Heersche and De Vogd van der Straaten (1965) extended the previous work on autoradiographic analysis with radioactive proline. Flash labeling revealed that two hours of exposure of PTE was sufficient to inhibit the incorporation of proline.

Analyzing the percent of labeling of osteoblasts following H3-proline injection and the extraction of bone collagen for measuring relative incorporation of radioactive proline during in vitro incubations of bone fragments, Cooper and Talmage (1965) could not demonstrate any change in osteoblastic function in response to stimulation of endogenous PTH secretion brought about by several hours of peritoneal lavage. However, collagen synthesis was clearly inhibited three hours after the last dose of PTE when the animals was injected with commercial PTE.

Much evidence indicates that the ground substance of bone matrix is involved in the resorptive process of PTH. However, these processes are slow in response (Bradford et al, 1959; Bronner, 1961; Johnston et al, 1961). Johnston et al (1962) was the only group to report an early response. They observed that the rate of incorporation of glucose-\(^{14}\)C, measured as specific activity of hexoseamine, was above the control level within three hours after the last injection of PTE (three doses every two hours).

B. Bone cell modulation

The modulation or cellular transformation of bone in response to PTH administration is a dramatic and rapid process. Jaffe and Bodansky (1930a, 1930b) described the general histological changes which characteristically follow the administration of large doses of PTE. They observed varying degrees of osteitis fibrosa cystica in dogs after giving
varying amounts of hormone at different time intervals. In the production of osteitis fibrosa cystica, they found resorption in both cortical and trabecular bone, a wide distribution of osteoclasts, and an invasion of fibrous tissue. Similar results were also obtained in guinea pigs (Jaffe et al, 1930). Using intact, parathyroidectomized (PTX), and hormone treated rats, Bulbring (1931) found that the bone of the animals treated with hormone showed resorption at first which later gave way to a fibrous condition and still later to bone apposition. These last observations were confirmed by Selye (1932). He demonstrated that an early response of osteitis fibrosa gave way to increased numbers of osteoblasts and hypercalcification, producing the condition referred to as marble bone disease. Pugsley and Selye (1933) studied this in greater detail. Johnson (1932a, 1932b) was able to produce a condition of osteitis fibrosa similar to that described by Selye in both rats and puppies. McLean and Bloom (1937) also published a short note describing the cellular changes of osteitis fibrosa. Burrow (1938), using Hanson and Collip's PTE, studied in great detail the transformation of bone cells in the hyper and hypoparathyroidal state in the albino rat. Histological changes were examined in proximal tibia, distal femur, lumbar vertebrae, metatarsus and rib. Numbers of osteoclasts, osteoblasts, fibrous tissue, hemorrhage, cysts, resorption of trabecular and cortical bone were all carefully examined in response to dosage and time after PTE administration. The first histological evidence of the beginning of osteitis fibrosa was described. Osteoblasts changed from their normal shape to a spindle shape and eventually formed a loose syncytium-like mesenchyme. A large dose of PTE completed the change within 12 hours. The resorption of nearly all trabecular as well as some cortical bone occurred within
24 hours. Osteoclasts did not reach their maximal number until about 48 hours after the first injection of PTE. This demonstrated that osteoclasts cannot be the effector of the decalcification, because they reached their maximum a day after most of the resorption had taken place.

Most of the information about cellular transformation in bone has been obtained from studying either long term changes or the relatively slower changes induced by PTE, therefore Heller et al (1950) gave emphasis to the rapidity of the cellular changes following toxic doses of PTE. The earliest response examined was two hours after treatment. The effect of a single dose of PTE in the young rat, puppy, kitten, and guinea pig was examined in detail for histological changes in the metaphysis of distal femur and proximal tibia.

Gaillard (1955), Raisz (1963), and Goldhaber (1962) confirmed these cellular transformation phenomena in an in vitro explanted tissue culture system with the introduction of PTE into the medium.

The functional specialization of an individual bone cell is largely determined by the microenvironment in which it is situated (Basset, 1962; Young, 1963a, 1963b), and this microenvironment represents the sum of the chemical and physical factors which impinge on the individual cells. Young (1963b), applying the concept of Jacob and Monod (1961), proposed that, in the presence of an increased level of PTH, stimuli in the microenvironment initiate or induce a change in cell specialization. A stimulus in the microenvironment exerts a profound effect on the organization of the cell by inactivating the repressor molecule and activating the structural gene. The specific factor which may have induced this cell specialization has been the subject of extensive research. For further elucidation,
Gaillard (1965) applied actinomycin D (AMD), a potent inhibitor of DNA dependent RNA synthesis, to study the mechanism of PTH action in tissue culture. The results indicated that AMD reduced the histological changes induced by PTE. Talmage et al (1965) examined this hypothesis in vivo by stimulating endogenous PTH instead of injecting pharmacological doses of PTE which contain polypeptides other than PTH. They observed that in their response to endogenous stimulation, animals pretreated with AMD showed no increase in osteoclast number in the distal metaphysis of the femur, but those treated while the rat was being stimulated showed no effects on cellular transformation. This indicated that the rapidity of induction, or trigger mechanism of osteoclast formation by PTH was a process sensitive to AMD; i.e., AMD was able to block stimuli in the microenvironment which were produced in response to PTH. This may in part support the proposal of Young. Using tritiated thymidine as a marker for the modulation of cell types, Young (1962, 1963c) studied bone cell changes in the rat following PTE administration. His results indicated that osteoblasts revert to the osteoprogenitor state as indicated by labeled thymidine. From these studies, he proposed that the various types of bone cells represent different functional states of the same cell. Specialized cells like the osteoblast and their incorporation into osteoclasts arise by modulation of the osteoprogenitor cells. Histological studies indicating this transformation of osteoprogenitor cells into other bone cells were reported by Kroon (1958) and Young (1961).

The fact that treatment with PTH is followed by an increase in the number of osteoclasts in bone has been demonstrated many times. This was described in detail by Heller et al (1950) and noted in tissue
culture (Gaillard, 1955; Goldhaber, 1962), and reported as a concomitant of stimulation of endogenous hormone (Toft and Talmage, 1960; Talmage and Doty, 1962).

The controversy concerning the cell type directly involved in osteoclast formation has not been settled, and the source actually may be heterogeneous. Experiments designed to discern the precursor of the osteoclast have been carried out by many investigators. They indicated either (1) that the precursor cell(s) do not undergo mitosis or (2) that all precursor cells do not go through a DNA synthesis period because only one or two out of many nuclei in the osteoclast were labeled with tritiated thymidine. The suggested precursor includes the osteoblast (Tonna, 1960; Tonna and Cronkite, 1961a, 1961b), osteocyte (Messier and Leblond, 1960), monocyte (Fishman and Hay, 1962), and phagocyte (Jee and Nolan, 1963).

Talmage et al (1965), using tritiated thymidine as a marker for the bone cell transformation which occurs during induction of increased endogenous parathyroid activity, made a recent contribution to the question concerning osteoclast formation. They attempted to pinpoint the possible cell type(s) involved in osteoclastic proliferation by examining the turnover of tritiated thymidine labeled cells during a time of increased secretion of endogenous PTH. The results indicated that osteoclasts are formed by fusion of undifferentiated mesenchyme cells, and osteoblasts and osteocytes are eliminated as the normal precursor during rapid proliferation after PTH stimulation.

The questions still remaining concerning the cellular transformations in bone are numerous: what is the inductive factor for the transformation of osteoblast into osteocyte, the fate of the osteocyte, and mesenchymal
cell to osteoblast, osteoblast to mesenchymal cell, and possible change of osteocyte to osteoblast? Owing to technical problems, the change of generation time consequent to hormonal stimulation observed in other tissue has not been studied.

C. Parathyroid hormone and RNA synthesis

With the introduction of the hypothesis that a hormone may act as a gene activator and stimulate the production of RNA and protein synthesis (Karlson, 1963), actinomycin D (AMD) was used to demonstrate the involvement of RNA synthesis in PTH function in calcium and phosphate metabolism. AMD not only prevents messenger-RNA (m-RNA) synthesis, it is also a potent inhibitor of ribosomal-RNA (r-RNA) formation (Girard et al, 1964). Furthermore, conclusions drawn from the use of this inhibitor do not allow one to be specific about the site of action of the hormone (Girard et al, 1964).

Eisenstein and Passavoy (1964) administered PTE and AMD simultaneously. Eighteen hours later, the hypercalcemia and bone lesions usually induced by this large dose of PTE had been inhibited in the AMD treated animals. Milhoud et al (1964) reported similar observations. Noting changes in the serum calcium levels of PTX'd rats maintained on high calcium diet and treated with PTE and AMD simultaneously, Rasmussen et al (1964) concluded that there were two different periods of response to AMD. In the initial three hour period, the animals are insensitive to AMD, and calcium mobilization by PTE is not associated with RNA synthesis. In the later period, up to 28 hours, the animals are sensitive to the inhibitory action of AMD, indicating a requirement for RNA synthesis in mobilizing calcium from target tissue. The phosphaturic response was the same in
control and AMD treated animals. The possibility of absorption from the
gut as a contributor to an increased level of calcium was not considered
in this experiment. The insensitivity of the first period may not be
entirely due to the failure of AMD to inhibit bone cells. Rasmussen
failed to examine the effect of AMD on PTX'd rats which could have served
as a control in this experiment. Tashjian et al (1964) observed a similar
phosphaturic response and calcium mobilization. From this, it was con-
cluded that the action of exogenous PTH required an intact genetic
mechanism and the concurrent synthesis of RNA. However, the AMD did not
seem to interfere with the ability of endogenous hormone to maintain a
normal calcium level for at least 7 hours. Khoo and Kowalewski (1965)
extended the above type of experiment and confirmed that endogenous hor-
mode action on the serum calcium level was not disrupted until 10 to 15
hours after AMD treatment. This effect is observed in the absence of
kidney, adrenal, and thyroid glands.

Tashjian (1965) transplanted parathyroid glands from AMD animals and
measured the maintenance of serum calcium levels, and noted that the
synthesis of PTH in the gland was disturbed by AMD. In intact AMD treated
rats, he suggested that an increased secretion of endogenous PTH overcame
the inhibitory action of AMD up to 12 hours; that time when the gland
became exhausted. He also proposed that calcium mobilization promoted
by PTH depended not only on the quantity of hormone at the effective site,
but also on the optimum quantity of mediator(s) of hormone action. These
mediators may be short lived products of gene activity and therefore,
inhibited by AMD.

Gaillard (1965) found that AMD reduced cellular proliferation
ordinarily induced by PTH. Raisz (1965) confirmed this observation.
He also reported the competitive action of PTH induction and AMD inhibition of bone resorption. AMD inhibition of the release of Ca\(^{45}\) into the medium can be overcome by increasing the dose of PTH in the incubating medium.

Belanger (1968) observed the effect of AMD on the synthesis of activity of proteolytic enzymes. Twenty-four hours after AMD administration, proteolytic activity decreased by two thirds. From this, he proposed that AMD sensitive RNA which is involved in the protein synthesizing scheme is inhibited by the drug.

Talmage et al. (1965), using an endogenous PTH stimulation system, confirmed the observations of Gaillard and Raisz that AMD inhibited the expected increase in osteoclasts in bone and reduced the osteoclast numbers to the level of PTX'd animals. This may indicate that an AMD sensitive process is necessary even for a PTX'd rat to maintain its calcium level on the other hand, it does not clearly show whether or not AMD is an inhibitor of other requisites in calcium mobilization. Parathyroidectomy prior to, at the same time, or subsequent to the administration of AMD always resulted in the expected change in serum calcium, lavage fluid calcium and urine calcium and phosphate levels. They concluded that PTH does not affect the same site in the cell which is affected by AMD. Endogenous PTH required a continuous production of enzymes ultimately responsible for its action on bone and kidney, but this action was not necessarily related directly to the production of AMD sensitive RNA synthesis.

Early works on the demonstration of changes in RNA of bone cells have been extensively reviewed by Young (1963a).
Based on Casperson's technique of localizing nucleic acids by their absorption of ultraviolet light at 2600 A, Hamberger and Hyden (1947) found that, as the osteoblast transformed from the mesenchyme cell, it accumulated an intensely ultraviolet absorbing nucleolus, and material of a similar nature accumulated in the cytoplasm. Using the methyl green pyronin staining technique and enzyme extraction of RNA with RNase, Cappellin (1949) confirmed Hamberger and Hyden's observations. Other workers verified many of these findings in bones of several animals (Calvert, 1950; Follis, 1951; Prichard, 1952; Schajowicz and Cabrini, 1954; Monesi and Bettini, 1958).

The osteoclast has not received much attention. Bhaskar et al (1956) stated that these cells showed cytoplasmic basophilia and that this could be removed by RNase; therefore, it was due to RNA. Morse and Greep (1960) reported that cytoplasmic RNA tended to accumulate in the vicinity of the nuclei. Owen (1967) extensively investigated RNA synthesis and decay in bone cells, particularly its pattern in different cell types and different stages of cell differentiation. Steinberg and Nichols (1968a) described the normal pattern of RNA synthesis in surviving bone fragments in vitro, the characteristics of labeling, and subcellular localization of its components. Bulk RNA preparations were resolved by sucrose density gradients into components with sedimentation coefficients 28S, 18S, and 4S. Rapidly labeled RNA demonstrated the polydisperse sedimentation pattern. The highest specific activity was found in the 4-18S fraction which was insoluble in 10% sodium chloride, and which is therefore presumed to be m-RNA with a half life of 4.3 hours.

Burckard and Mandel (1958) extracted RNA and DNA from bones and found a relatively low DNA content. The elevated ratio of RNA to DNA
suggested that bone had a relatively high content of RNA; this was ascribed to the storage of RNA in the osteoblast. Vaes and Nichols (1962) measured the ratio of RNA and DNA in bone, and found the ratio to be relatively low and constant in response to the hormone.

Burckard et al (1959) studied the turnover of bone RNA. After injection of P^{32} the specific activity of RNA was measured in bone of adult animals sacrificed between 12 hours and 8 days. The maximum specific activity was observed about 24 hours after injection; thereafter the specific activity decreased gradually, but was still measurable at the end of 8 days.

During the last several years, investigations of the effect of PTH on RNA synthesis in the target organ have been started in several laboratories.

Egawa and Neuman (1964) observed an increased P^{32} incorporation in the kidney nucleic acid fraction in response to PTE administration. The relative specific activity was increased by 128% within 75 minutes after PTE administration. This same trend was observed up to 4 1/2 hours.

Talmage et al (1965), by quantitative autoradiography, were the first to report an effect of PTH on bone cells. Labeling by H^{3}-cytidine showed that a 100% increase in the mesenchymal cell population occurred in the metaphysis of the femur, a few hours after stimulation of endogenous PTH secretion.

Martin et al (1965), on the other hand, reported a decrease of 17% in the incorporation of H^{3}-uridine after four days of treatment with PTE in cultures of calvaria.

Using a biochemical cell-fractionation technique, Talmage (1966) extended his observation on the stimulation of RNA synthesis in femoral
metaphyses. An increase was shown within one hour after stimulation of secretion of PTH.

Raisz and Nieman (1967) observed a biphasic effect of PTH on uridine-5-T incorporation into RNA of embryonic ulnae and tibia in organ culture. The earliest effect was seen one hour after treatment. The rate of incorporation was depressed by PTH up to 5 hours, sharply increased from 6 to 9 hours, and then returned to normal.

Steinberg and Nichols (1966, 1968a, 1968b) also found a similar mode of stimulation of RNA synthesis by PTH. A single injection of the hormone in vivo markedly stimulated the rate of synthesis in two different modes. With in in vitro incubation of metaphyseal chips, there was a lag for several hours in the synthesis which reached a peak at 12 hours, then gradually returned toward the normal level. On the other hand, flash labeling in vivo showed a much earlier stimulation of RNA synthesis. The differences observed in these two systems can probably be reconciled by a consideration of the dramatic dissimilarities which exist between environmental conditions in vivo and in vitro.

By quantitative autoradiographic analysis of uridine-5-T in the periosteum of the rabbit, Owen and Bingham (1968) studied the diverse effects of RNA on the different cell types, osteoblasts and osteoclasts. Both nuclear and cytoplasmic RNA showed a similar effect on a particular cell type. The osteoblast which is responsible for bone growth was markedly inhibited and the osteoclast which resorbs bone was greatly stimulated. The early effect was observed in one half to one and one half hours, and this effect was sustained for 24 hours.

Van Wermeskerken (1968) also reported an effect of PTE on calvaria of the embryonic rat. His observations confirmed earlier evidence for the biphasis effect observed by Raisz and Nieman (1967).
D. **Hormonal effects on RNA synthesis**

When Karlson (1963) proposed an explanation for hormone action on RNA metabolism based on the simple Jacob and Monod (1961) model for microbial enzyme induction and the ecdysone-induced chromosomal "puffing" in dipteran salivary glands (Clever and Karlson, 1960), he suggested that the hormones act as inducers which, by combining with appropriate repressors, control messenger RNA (m-RNA) synthesis and thus regulate synthesis. There is no evidence for the direct interaction of hormone and repressor or genes for those enzymes whose synthesis may be controlled by hormone. There are two important considerations resulting from recent work which must be taken into account and necessitate a re-evaluation of some oversimplified concepts (Tata, 1967). Messenger RNA may be transported from the nucleus to the cytoplasm as a complex with a smaller ribosomal unit and one or more of the mechanisms regulating ribosomes could determine the type and rate of response to the hormone operating via control of m-RNA synthesis. There is also the possibility that the hormone could regulate the type and rate of protein synthesis at the level of translation of m-RNA by ribosomes.

Extensive reviews on the hormonal control of RNA metabolism have been written during the last few years by Karlson, 1965; Karlson and Skeries, 1966; Tata, 1966, 1967; Tsuda, 1966a, 1966b; Zalokar, 1967; and Korner, 1968.

1. **Growth hormone (STH)**

The effect of growth hormone on the control of RNA biosynthesis was reviewed by Korner (1965). STH treatment stimulated the incorporation of labeled precursor into all types of RNA, including m-RNA (Talwar
et al, 1963, 1965; Korner, 1964, 1965, 1966; Dawson et al, 1966; Jackson and Sells, 1967; Sells and Takahashi, 1967; and Venugopalan, 1967), and increased the activity of RNA polymerase (Pegg and Korner, 1965). Synthesis of ribosomal RNA (r-RNA) and rapidly labeled 45S and 55S nuclear RNA, which is presumed to be the precursor of r-RNA, were stimulated, and soluble RNA (s-RNA) was affected to a lesser extent (Earl and Korner, 1966; Jackson and Sells, 1967). DNA-RNA hybridization studies indicated that STH does not stimulate a new species of m-RNA (Drews and Brawerman, 1967). Although the stimulating effect of STH on RNA synthesis was reported in the case of in vivo administration of the hormone, use of the hormone in vitro had no effect on RNA synthesis of diaphragm muscle (Kostyo, 1966), and liver slices (Jackson and Sells, 1967).

2. Follicle stimulating hormone (FSH)

FSH administered to immature hypophysectomized rats significantly increased the total RNA and the ratio of RNA to DNA in the ovary (Callantine et al, 1965). FSH and concomitant luteinizing hormone stimulated nuclear RNA synthesis in testicular nuclei in vitro. The hormonal influence was quantitative rather than qualitative in the type of RNA synthesized (Goswami et al, 1968).

3. Adrenocorticotropic hormone (ACTH)

The in vivo response of adrenal cortex to ACTH administration included both a prompt increase in precursor incorporation into cytoplasmic and nuclear RNA (Logan et al, 1955; Bransome and Reddy, 1963; Bransome and Chargaff, 1964; Farese, 1966), and a net gain in adreno-cortical cytoplasmic RNA (Bransome and Reddy, 1961; Farese and Reddy,
1963). Despite this seemingly straightforward indication that ACTH enhanced adrenal RNA synthesis (Farese and Schnure, 1967), it has been suggested that ACTH may increase adrenal RNA by decreasing RNA degradation (Imrie and Hutchinson, 1965). Contrary to a stimulatory effect, there is indication that a transient decrease of precursor incorporation into RNA can also be observed (Ferguson, 1963; Ferguson and Morita, 1964). Ferguson et al. (1967) analyzed this and interpreted that this inhibitory effect was mediated by the corticosteroid it produced and was not a direct effect of ACTH. These conflicting reports reside in the fact that most of these observations were made after successive injections of ACTH, except for two reports (Fiala et al., 1956; Bransome and Cadwgan, 1968) which followed the observation after a single injection.

The conflicting status of this hormonal effect was clarified by Bransome (1967) who observed that there was a biphasic response to the hormonal stimuli. One injection of ACTH stimulated the synthesis of RNA within 15 minutes, but following the initial stimulation, 24 hours after the injection, a secondary inhibition of RNA synthesis was observed. Therefore, the interpretation of the hormonal stimuli will vary due the time after the first injection and the dose of the hormone.

4. Thyroid stimulating hormone (TSH)

Begg and Munro (1965) and Tata (1966) reported that TSH stimulated the uptake of precursor in rapidly labeled RNA fractions, and the first effect of TSH on nuclear RNA metabolism was AMD sensitive and may have involved synthesis of m-RNA for a specific nuclear protein. Creek (1965) studied the sequence of this stimulation by giving a single dose of TSH. The uridine-H³ incorporation into the RNA and TCA soluble fractions of
thyroid was increased by 2 hours, and this corresponded to a decrease in dry weight and total thyroidal protein. The total RNA content of the thyroid was not increased until 12 hours after hormonal treatment.

5. **Prolactin**

Sherry and Nicoll (1967) reported that AMD injected into the crop sac of pigeons inhibited the thickening response of the tissue to prolactin stimulation. RNA extracted from the stimulated tissue was highly effective in stimulating the thickening of the crop sac of a second pigeon. RNase completely eliminated this response. This suggested that prolactin-induced tissue response is mediated through RNA synthesis.

6. **Thyroid hormone**

Administration of thyroxin to thyroidectomized rats or to the early stage of tadpoles rapidly accelerated the synthesis and degradation of RNA by liver and muscle, and total RNA synthesis in the rat salivary gland (Moore and Hamilton, 1964; Tata, 1965, 1966; Strohm et al., 1964; Kidson and Kirby, 1965; Nakagawa et al., 1967; Ackerman et al., 1968; Eaton, 1968). Whereas the synthesis and turnover of all types of nuclear RNA were affected, the effect of hormone was most marked on r-RNA turnover as revealed by the analysis of rapidly labeled nuclear RNA *in vivo*, the product of RNA polymerase formed *in vitro*, and by hybridization of RNA and DNA (Leader and Barry, 1967).

7. **Insulin**

Attempts have been made to correlate the inhibitory effect of AMD on RNA synthesis and the fundamental mechanism of action of insulin. It was demonstrated that the action of insulin involved the induction of enzyme
synthesis by stimulating the renewal of cellular RNA (Manchester and Young, 1961; Benjamin and Gellhorn, 1964; Wool and Moyer, 1964; Weber et al, 1965; Gutman et al, 1966; Singh and Chaikoff, 1967). Insulin in vivo as well as in vitro produced a stimulation of various types of RNA synthesis such as rapidly labeled RNA, 6-12S nuclear RNA, and cytoplasmic RNA (Wool, 1963, 1965; Wool and Munro, 1963; Kidson and Kirby, 1965; Mayne and Barry, 1966). Salmon et al (1967) reported that the administration of insulin enhanced the incorporation of tritiated uridine into RNA as early as 1 hour. A similar observation was made by Wool et al (1968) who showed in heart muscle that insulin reversed the decrease of RNA synthesis of the diabetic condition within 1 hour. The rates of r-RNA, 18S and 28S RNA synthesis were affected more than that of 4S RNA.

8. Glucocorticoids

Hydrocortisone and cortisol were shown to cause in different tissues either an increase or a decrease in the synthesis of RNA and the activity of enzymes. Nuclear RNA polymerase activity in rat and duck thymus decreased acutely within 30 minutes in response to cortisol injection (Nakagawa and White, 1966, 1967, 1968; Fox and Gabourel, 1967; Bottoms et al, 1968). The early decrease in RNA polymerase activity in thymic nuclei of cortisol treated rats was in harmony with reports of diminished RNA metabolism in lymphoid tissue exposed to thymolytic corticoids both in vivo (Gabourel and Fox, 1965; Brinck-Johnson and Dougherty, 1965; Feigelson and Feigelson, 1966; Makman et al, 1966) and in vitro (Kidson, 1965; Makman et al, 1966; Young and Klurfeld, 1968). A decrease in RNA metabolism was also observed in duck spleen treated with cortisol and prednisolone (Bottoms et al, 1968). In contradistinction, RNA polymerase
activity in liver was increased within 10 minutes following the injection of corticosteroid (Lang and Sekeris, 1964a, 1964b; Barnebei and Sereni, 1964; Barnebei et al, 1965; Lukacs and Sekeris, 1967, 1968), and the template activity of nuclear RNA was increased (Barnebei et al, 1964; DeLoecker, 1964; Garren et al, 1964; Lang and Sekeris, 1964a; Dukes and Sekeris, 1965; Greenman et al, 1965; Shereshevska, 1965; Drews and Bondy, 1966; Drews and Brawerman, 1967; Jakubovic et al, 1967; Leader and Barry, 1967; Lukacs and Sekeris, 1967; Schmid et al, 1967; Venkov et al, 1967). There is also an increased template activity of nuclear RNA in prostate (William-Ashman et al, 1964) and in uterus (Gorski, 1964). A direct action of corticosteroids on genes and on enhancement of m-RNA synthesis has been suggested (Barnebei and Sereni, 1964; Lang and Sekeris, 1964; Venkov et al, 1967). The corticosteroids induced the formation of a new species of m-RNA without influencing the relative quantity of newly formed m-RNA. In contrast, other experiments indicated that corticosteroids provoked a non-specific stimulation of RNA synthesis (Garren et al, 1964; Greenman et al, 1965). Kidson (1965) observed that the initial events involved in the regulation of RNA and protein synthesis by cortisol were very rapid, and suggested that this steroid hormone acts as an effector molecule in induction and repression.

9. Aldosterone

Castles and Williamson, 1967; Fimognari et al, 1967; De Weer and Crabbe, 1968). Stimulation of RNA synthesis was observed within 90 minutes after treatment of rat kidney (Castles and Williamson, 1967). Uptake of radioactivity in rapidly labeled RNA fractions from isolated toad bladder were stimulated (Rousseau and Crabbe, 1968). The RNA synthesis in isolated toad bladder and rat kidney was also found to be stimulated 30 minutes prior to any change in sodium transport (Porter et al, 1964; Castles and Williamson, 1967). Corticosteroids elevated the level of intracellular RNA in isolated toad bladder (Edelman et al, 1963), and in rat kidney (Castles and Williamson, 1965). In both preparations, the stimulation, of sodium transport and the increased levels of RNA were blocked by AMD (Edelman et al, 1963; Castles and Williamson, 1965).

10. Adrenaline

The effect of adrenaline was studied on mouse liver RNA. It was found that adrenaline increased the incorporation of a precursor within 13 minutes and this was soon followed by inhibition within 60 minutes. A marked effect on 4-16S RNA presumed to be m-RNA was observed (Decallonne et al, 1967).

11. Testosterone

One of the early effects of gonadohormones on the target tissue of hormone deficient animals is the enhancement of the RNA synthesizing capacity (Hancock et al, 1962; Wicks and Kenney, 1964; Greenman et al, 1965; Hancock, 1965; William-Ashman, 1965; Kockakian and Hill, 1966). AMD in vivo and in vitro selectively obviated the androgen-dependent enhancement of nuclear RNA polymerase activity (Liao et al, 1966). RNA,
rich in guanine and cytosine, was synthesized as the chromatin section where testosterone has its greatest effect (Liao et al, 1966; Liao and Lin, 1967), and nuclear RNA, rich in template activity which indicated that the level of m-RNA in the target tissue, such as prostate, is under the control of testicular hormone (Liao, 1965).

12. Estrogen

The early response of the ovariectomized rat uterus to estradiol is characterized by a rapid increase in RNA synthesis followed by an increase in protein synthesis (Mueller et al, 1961; Noteboom and Gorski, 1963; Ui and Mueller, 1963; Hamilton, 1964; Gorski et al, 1965; Hamilton et al, 1965; Miller and Emmens, 1967). Nuclear RNA synthesis, assayed by pulse labeling with uridine-\textsuperscript{H\textsmaller{3}}, increased as early as 2 minutes after administration, and it was followed by a decrease in the rate of nuclear and cytoplasmic protein synthesis (Means and Hamilton, 1966b). This indicated that the earliest effect of this hormone occurs at synthesis of nuclear RNA of the uterine cell. Available information suggests that this effect may involve RNA polymerase (Gorski, 1964), inactivation of repressors in the regulation of RNA polymerase (Talwar et al, 1964), nuclear membrane permeability (Szego, 1965; Means and Hamilton, 1966a), and chromatin template activity (Barker and Warren, 1966).

RNA synthesized by uterine cell nuclei of the estrogen treated rat had greater in the guanosine + cytosine over adenosine + uridine ratio than that of the noninjected uterine cell nuclei (Trachewsky and Segal, 1967). Hamilton et al (1965) reported that the uterine nuclear RNA synthesized as a concomitant of 20 minutes of estrogen stimulation exhibited a sedimentation profile characteristic of r-RNA. Greenman and Kenney (1964)
observed that there was an increase in number of ribosomes due to estrogen administration in uterine cells. Similar reports were also made by Moore and Hamilton (1964) and Greenman et al (1965). The stimulation of RNA synthesis was abolished by AMD (Trachowsky and Segal, 1967). These results are compatible with the observation that AMD blocks various aspects of the early estrogen response (Talwar and Segal, 1963; Ui and Mueller, 1963; Nicolette and Gorski, 1964; Hamilton, 1964; Means and Hamilton, 1966a).

E. Effects of PTH on DNA synthesis and mitosis of cells

Although a few investigators have studied the effect of PTH on cellular modulation in bone using tritiated thymidine as a marker, no reports are available on the effect of PTH on changes in the DNA synthesis. Few studies have been published in which PTH was reported to have influenced the mitotic rate.

Borle and Neuman (1965) observed that the administration of PTE in HeLa cell cultures induced an increase in the rate of mitosis within 1 day. Martin et al (1965) observed that triated thymidine incorporation in explanted calvaria increased after 4 days of treatment with PTE. Perris and Whitfield (1967) reported a stimulatory effect of PTH on mitoses in bone marrow and thymus cells. PTH secretion was stimulated by injecting a chelating agent, ethylenediaminetetraacetic acid (EDTA) or sodium phosphate. Differences in the mitotic rate of bone marrow cells and thymic cells were observed within 2 hours after the treatment.
F. Methods of cell isolation for metabolic studies

A logical development in the study of tissue or organ function in vitro is the use of isolated cell suspensions. In contrast to the in vivo situation where circulation is intact, in vitro preparations, such as tissue slices or parts of an organ, which have been widely used present serious difficulties related to transport of diffusion of nutrients and metabolites. Furthermore, the tissue is composed of a heterogeneous cell population, and therefore the responses of a tissue to various stimuli may be the sum of the responses of each cell type. Thus, the need for a preparation consisting of a disaggregated homogeneous cell population is obvious.

Various methods have been described for the preparation of dispersed cells. The extracellular coat or intercellular cement is removed mechanically or with suitable solvents so that only a final limiting surface remains on the uninjured cell. The physical properties and chemical composition of the intercellular components vary widely among animal species, as well as with the developmental stage of an organism, but in general they are composed of protein, polysaccharides, and cations. Most of the methods for dispersion have utilized (1) mechanical treatment, (2) use of chelating agents, or (3) enzymes (Rinaldini, 1958).

Early work on this subject has been reviewed extensively by Rinaldini (1958), Mäteyko and Kopac (1963), and, with special regard to embryonic and invertebrate tissues, by Mascona (1962). In the following discussion, methods innovated since these reviews will be listed.

The view that mild mechanical treatment of the tissue is best for preserving cell metabolism was reinforced by the detailed morphological
studies of Mateyko and Kopac (1963). Howard and Green (1965) separated liver cells by repeatedly drawing the finely chopped tissue into and out of a wide mouth pasteur pipette and then filtering through nylon mesh or stainless steel mesh. Next, Bannister and Morton (1957), Berry (1962), and Berry and Simpson (1962) perfused liver with 0.4 M sucrose and dispersed part of the liver into single cells. High yields of cells were obtained by perfusion of the liver with a calcium-free solution containing a chelating agent such as EDTA or citrate and subsequent dispersion by mechanical means (Jacob and Bhargava, 1962; Tye and Bhargava, 1963, 1965; Castagna and Chaveau, 1963; Takeda et al., 1964; Ichihara et al., 1965; Ontko, 1967). Sodium tetraphenylboron (TPB), a specific agent for complexing with potassium, has been found to dissociate adult mouse tissue in vitro. Liver, kidney, and brain cell suspensions were obtained by this method (Rappaport and Howze, 1966a).

After discovering that the aggregation of cells may involve monovalent cations, Rappaport and Howze (1966b) extended the application of other possible sodium and potassium chelating agents such as malic acid, pyruvic acid, citric acid, EDTA, ethanol, picric acid and perchloric acid. It was found that connective tissue was dispersed by 4 M ethanol at pH 8.3 through complex formation with sodium. Epstein (1967) also used TPB for dispersing liver cells and studied cell size distribution and polyploidy of mammalian liver cells. The above methods produced a poor yield of cells (Jacob and Bhargava, 1962); probably because the mechanical treatment damaged the cells by rupture of the plasma membrane.

Since Rous and Jones (1916) dispersed avian and mammalian tissues with trypsin, other proteases, singly or in combination with other
enzymes, have also been used. However, for many tissues trypsinization remains the standard method (Mascona, 1962). The use of trypsin has many drawbacks: digestion is slow and incomplete, the cells tend to remain in clumps, and there is a viscous residue (Gwatkin and Thomson, 1964), nevertheless, epithelial cells of the small intestine (Harrer et al, 1964) and thyroid cells (Tong et al, 1962) have been effectively isolated.

Collagenase has been used in the preparation of cells from adipose tissue (Rodbell, 1964), from embryonic heart (Cavanaugh et al, 1963), and from the Islets of Langerhans (Clark and Steiner, 1968).

Pronase was used to dissociate mouse lung, kidney, and other tissue (Gwatkin and Thomson, 1964; Houba, 1967). Lysozyme dissociated rabbit epithelial cells (Huang, 1965), and hyaluronidase dissociated rat intestine epithelial cells (Perris, 1966). Howard et al (1967) observed that the use of a single enzyme in the dispersion of liver cells was due more to mechanical treatment than to enzyme action. However, hyaluronidase used in combination with collagenase was very effective in the preparation of isolated Ehrlich ascites carcinoma cells (Yamada and Ambrose, 1966). The nature of this synergistic effect is not clear. Yamada and Ambrose (1966) suggested that hyaluronidase assisted the permeation of collagenase, and then collagenase attacked the interstitial fibers, resulting in a disintegration of tissue structure with little influence on the cells themselves. A similar effect of hyaluronidase in combination with trypsin was also reported by Mateyko and Kopac (1963). These workers suggested that one enzyme aided the permeation of the other by unmasking reactive groups. Bernfield and Fell (1967) applied a combination of
of pancreatin and trypsin for the separation of a proliferating and differentiating cell population from pancreatic epithelium. Sequential treatment of tissue with more than two enzymes is also very effective for this purpose.

Adrenal sections were preincubated with trypsin, DNase and RNase. After being washed with soybean trypsin inhibitor, the sections were finally dissociated by indubation with collagenase and hyaluronidase (Halerston and Feinstein, 1968). Smith (1965) liberated chondrocytes from adult cartilage by sequential treatment of the tissue with papain, collagenase and finally with pronase.

G. Isolation of bone cells and biochemical studies of isolated bone cells

The study of bone fragments in vitro has provided evidence for a distinct pattern of bone cell metabolism. They have observed the composite of metabolic activity of many cell types within bone, including osteoprogenitor cells, osteoblasts, osteocytes, and osteoclasts. Yates and Talmage (1965) proposed that factors modifying bone metabolism exert their influence through discrete effects on different cell types in various stages of activity or differentiation. Although a homogeneous cell population has not yet been obtained, bone cells have been dispersed from bone by both enzymatic and/or mechanical means (Flanagan and Nichols, 1963; Peck et al, 1964). De Vogd van der Straaten (1966) offered the consideration that information concerning the metabolism of the individual cells will be fundamentally insufficient to explain the formation of bone as a tissue, and represents the metabolism of cells in transition. He pointed out the fact that there had been bone formation and that dedifferentiation and/or overgrowth by "fibroblasts" could not be prevented.
Calvaria from rat fetuses at term or from new born rats have been cleaned, minced, and shaken in a buffer containing collagenase with the result that the isolated cells were found to be histologically intact, and growing and multiplying in culture. In contrast with the enzymatic method, the mechanical method was applied to older, more heavily calcified bones, and the cells were liberated by shearing force. However, cells harvested enzymatically or mechanically may still be damaged (Takeda et al, 1964). By using a cytochemical alkaline phosphatase reaction, it was shown that the harvested cells contained osteoblasts and osteocytes. Moreover, a radioassay of CO₂ and lactate after cultivation with labeled glucose under aerobic conditions revealed metabolic traits reminiscent of intact bone tissue. Glucose metabolism and amino acid transport have been studied extensively in mechanically dispersed bone cells. Nichols (1965) compared the metabolism of isolated cells with cells still attached to their connective tissue framework. Isolated cells incubated in suspension utilized less oxygen and glucose, and formed less lactic acid than cells in bone. However, oxygen consumption increased as the density of the cell suspension increased, suggesting the importance of an intercellular relationship in vitro. A kinetic study of the entry of 3-O-methyl glucose into the cells supported the idea that the bone cell membrane was passively permeable to this glucose analog and therefore probably to glucose. It was also demonstrated that thyrocalcitonin or PTE had no apparent effect on sugar uptake, suggesting that the mode of action of the hormones on glucose metabolism is somewhere along its metabolic pathway. Active transport of alpha-amino iso-butyric acid (AIB) into bone cells was demonstrated. This process was temperature, oxygen, and energy dependent.
Evidence for two different mechanisms was revealed; one independent of and the other dependent on the ambient sodium and potassium concentration in the medium. Variation of the external calcium, magnesium and phosphate concentration had no effect on this system. A biphasic response of transport of AIB to the injection of PTH in vivo was observed. In the first phase (8 to 12 hours after administration), PTH stimulated the uptake by 30%, while in the second phase (48 to 72 hours) the transport activity was depressed by 45%.

Similar to the intact bone, the glucose metabolism of enzymatically isolated cells is anaerobic, and the hexose-monophosphate shunt is active. After enzymatic isolation, bone cells continue to accumulate amino acid rapidly by an active transport mechanism. Comparison of amino acid uptake by isolated cells and whole bone segments of the same age revealed a similar degree of uptake. Cells mechanically isolated from older bone do not synthesize collagen under similar in vitro conditions (Planagan and Nichols, 1963). However, in PTE treated cells, a suppression of the bound form of proline was observed despite the fact that the free proline pool was increased intracellularly. An examination of the subcellular fraction showed that only the "microsome" fraction was suppressed by 30%. This indicated that the locus of action of PTH was at some step in the assembly of collagen molecules, and from data related to the concentration of free amino acid precursors in the cell, can be said to be independent of the general protein synthetic mechanisms (Nichols et al, 1965). Collagen synthesis by enzymatically isolated cells in a simple medium proceeded slowly for 6 hours, but was not longer detectable after 12 hours, although non-collagen protein synthesis continued. However,
dispersed cells have been found to synthesize collagen when cultured on a flat surface (Peck and Dirksen, 1966). After an initial latent period of three days, the cells began to proliferate, and organic matrix was demonstrated in an area of the culture where cells piled up on one another. A response to ascorbic acid was shown after 10 days of cultivation. Whereas freshly dispersed cells showed no response to stimulation of collagen synthesis, non-proliferating cells in the primary culture were able to produce a complex organic matrix containing collagen and chondroitin sulfate (Peck et al, 1967a). Isolated bone cells were extremely sensitive to Vitamin C. Vitamin C promoted collagen synthesis by directly stimulating the hydroxylation of the proline-rich peptide.

Lipid synthesis was studied using a primary culture of collagenase dispersed cells. The incorporation of $^{14}$C-glycerol into a lipid was examined by Birge and Peck (1966). Lecithins and triglycerides were the two most heavily labeled fractions, and accounted for over 70% of the total lipid radioactivity. Monoglyceride, diglyceride, phosphatidic acid, phosphatidyl ethanolamine, lysophosphatidyl ethanolamine, lysolecithin, and phosphatidyl inositol were the other products formed.

RNA synthesis of mechanically isolated cells was studied by Steinberg and Nichols (1966, 1968a). The turnover of both nuclear RNA and cytoplasmic RNA was examined with respect to the influence of PTE.

Peck et al (1967b) examined the direct effect of glucocorticoids on the protein and nucleic acid metabolism of cells dispersed from rat calvaria and maintained in primary culture, and which were capable of forming collagen and sulfated acid mucopolysaccharides. Hydrocortisone in physiologic concentration was found to inhibit collagen, non-collagen,
and RNA synthesis, and to deplete the cells of RNA within five hours. These effects were not associated with a significant alteration in DNA metabolism.
MATERIALS AND METHODS
A. General procedures

All rats used in these studies were adult males (180-230 grams) of the Holtzman strain. The animals were maintained on a normal diet (Holtzman) and tap water. Twenty-four hours before they were used in experiments, the rats were placed on a calcium-free diet (Klein, 1968) and de-ionized water.

All surgery was conducted with the rats under light ether anesthesia. Parathyroidectomy and thyroidectomy were accomplished using fine jeweler's forceps under a dissecting microscope. Thyroidectomy was performed three days prior to use in experiments and each rat received 10 micrograms of thyroxine daily.

B. Stimulation of endogenous parathyroid hormone secretion

This was accomplished by peritoneal lavage with a calcium-free rinse (Talmage and Elliott, 1958). Basically this procedure consisted of the intraperitoneal administration of 30 ml of isotonic fluid at 37°C through a stainless steel plug sewn into the ventral abdominal wall. After a one hour equilibration period this fluid was withdrawn and replaced with fresh rinse. This hourly procedure of continuous calcium depletion was continued up to eight hours.

C. Live bone incubation in vitro

At timed intervals during the peritoneal lavage procedure, the animals were sacrificed and the femurs were cleaned of muscle and cartilage tissue. The femurs were then separated into metaphysis and diaphysis. For bone fragment preparation, they were further split into numerous bone
slices. Each piece of bone was washed with isotonic saline using a Pasteur pipette to remove blood and bone marrow. Pieces representing the two regions of bone were incubated in separate flasks containing 1.5 ml of pooled rat serum. Pooled rat serum was obtained from large (300-400 gm) male rats by cardiac puncture, centrifuged at 300X G for 20 minutes, and stored at -18°C until used. All samples were incubated in a water-bath shaker (Eberbach Corporation) apparatus at 37°C with constant shaking at approximately 100 oscillations/minute. The atmosphere used consisted of a mixture of 95% oxygen and 5% CO₂. All flasks containing the media were equilibrated with this gas mixture for 30 minutes to adjust the pH 7.4. The atmospheric condition was controlled by gassing the individually stoppered vessels every 15 minutes. One hour incubations were terminated by immediately pouring off the medium. Bones to be analyzed were washed 3 times in saline and immediately frozen in a -70°C dry ice-ethanol bath and stored at -20°C until analyzed.

For incubation, pooled rat serum was mixed with tritiated nucleoside(s), cytidine (specific activity: 23.8-25.5 c/mM cytidine-5-T), uridine (specific activity: 12.8-16.2 c/mM uridine-5-T), and thymidine (specific activity: 14.8-17.0 c/mM thymidine-6-T). The radioactivity of nucleoside ranged from 5µc to 10µc in 1.5 ml of serum. Radioactive phosphorus, 32P, was used for the flash labeling of bone. Thirty microcuries of 32P were added to each flask. Procedures for continuous perfusion of bone in vitro are described in Section IV.
D. Chemical methods of analysis

1. Homogenization of bone fragments

For better decalcification during extraction of nucleic acids, the bones were homogenized in a bone mill (spex model 5000, Spex Corporation). The chamber was specially designed and had an internal diameter of 12 mm and was 4 cm high. Leakage of the homogenate which occurred in a commercial chamber was prevented by placing a rubber O-ring between the cap and the cylinder. A solution of 95% ethanol with 2% potassium acetate or polyvinyl sulfate (2 or 5 mg/ml) was added to the bones: 0.5 ml for metaphyseal bone, and 1.0 ml for diaphyseal bone. For homogenization, three to four small stainless steel ball bearings were added to the chamber. The temperature was maintained below 2°C during homogenization by precooling the chamber and its content in a -70°C bath. The metaphyses and diaphyses were homogenized for approximately 70 and 80 seconds respectively.

2. Extraction and determination of RNA

RNA from both the metaphysis and diaphysis was determined by a modification of the method of Schmidt and Thannhäuser (1945). The outline is shown in Figure 1. The homogenate, 100-200 mg of metaphyseal bone or 200-300 mg of diaphyseal bone, was transferred to a centrifuge tube to which was added 5.0 N perchloric acid (PCA). After allowing 20-30 minutes for precipitation and decalcification at 2°C, the precipitate was spun down in a refrigerated centrifuge. The acid soluble and lipid soluble fractions were extracted with cold 0.1 N PCA in ethanol, then with ethanol: ether (3:1), first cold (4°C), then at 50°C. After storing in the freezer
Figure 1

Extraction procedure of "RNA fraction".
Modified from Schmidt and Thannhauser (1945).
Bone homogenate in 0.2 - 0.25N PCA

2X 0.1N PCA in ETOH

Acid soluble fraction

1X cold ether
1X ether 50° 15'
2X chloroform: methanol (1:2)
      50° 15'

lipid, mucopolysaccharide fraction

Digestion in 0.3N KOH
2 hr.
followed by acidification,
2X 0.5N PCA

Acid soluble (RNA) fraction

Precipitate of DNA and Protein

RNA by orcinol reaction

DNA by diphenylamine reaction
at -20°C for at least 24 hours, the last supernatant was discarded and then extracted with chloroform:methanol (1:2) at 50°C. The residue was washed twice in cold 0.5 N PCA to remove all of the calcium matrix from the bone.

The RNA from the metaphysis and diaphysis was hydrolyzed with 0.3 N KOH for at least 2 hours at 37°C. The solution was neutralized, then acidified to a pH of 2.0 by the addition of PCA. After 30 minutes of precipitation at 2°C the precipitate was centrifuged and washed twice with cold 0.5 N PCA. The supernatants were pooled to form the RNA fraction. During the early phases of this research, the contamination contributed by other components in the diaphysis was not eliminated, and RNA was extracted by the method of Ogur and Rosen (1950). After the tissue homogenate was free of acid soluble and lipid components, the residue was extracted with 1.0 N PCA at 4°C, then washed twice with cold 1.0 N PCA. The combined supernatants formed the RNA.

The RNA concentration was determined by Drury's modification of the orcinol procedure (Drury, 1948). An aliquot of RNA hydrolysate was mixed with a reagent consisting of purified orcinol, hydrochloric acid, and ferric ammonium sulfate, then heated in a water bath for 45 minutes to develop the color reaction. After cooling the test tubes in running tap water, the optical density (O.D.) was measured at 660 m\(\mu\) using yeast RNA (Sigma Chemical) as the standard. The RNA hydrolysate was also measured at 260 m\(\mu\) and 280 m\(\mu\) and the concentration of RNA was determined according to Fleck and Monroe (1962).

RNA was also extracted from homogenates of bone by the sodium dodecyl sulfate-phenol method of Perry (1958). The homogenate was further homogenized
in a Teflon homogenizer for 1 minute with 0.3% sodium dodecyl sulfate in 0.01 M acetate buffer (pH 5.1). Phenol (10 ml) was added to this homogenate, which was then heated for 10 minutes at 60°C while being shaken in a water bath. It was then shaken for 20 minutes at room temperature, centrifuged (10 minutes at 1000X G), and the top aqueous layer was obtained. The aqueous layer was further extracted with phenol twice and the phenol layer was discarded. The aqueous layer which contained RNA was precipitated by 95% ethanol with 2% potassium acetate. The precipitate was incubated for 20 minutes with DNase (Worthington, 300 µg/ml) to remove contaminating DNA. The RNA was dissolved in isotonic saline and its concentration was determined by measuring the O.D. at 260 mµ. One mg/ml of RNA was equivalent to an O.D. reading of 20.

3. Extraction and determination of DNA

After RNA extraction, the residue was extracted twice with 0.5 N PCA at 80°C and once with cold 0.5 N PCA. The supernatants were pooled to form the DNA fraction. DNA was determined by the method of Burton (1956). Fifteen gm of diphenylamine was dissolved in 1 liter of glacial acetic acid and 15 ml of concentrated sulfuric acid, and acetaldehyde (10 mg/l) was added immediately before use. One ml of sample was mixed with 2 ml of reagent and the reaction was carried out at room temperature for 24 hours. The optical density was measured at 600 mµ using calf thymus DNA (Sigma Chemical) as a standard.

4. Sedimentation profile analysis of RNA

The intact RNA was dissolved either in water or saline to give a final concentration of 2-4 mg/ml. An aliquot was carefully layered on a
28 ml linear sucrose gradient of 5-40% sucrose (12:18) containing 0.1 M NaCl, 1.0 mM EDTA and 0.01 M sodium acetate, pH 5.0-5.1 (Steele and Busch, 1966), and centrifuged at 25,000 rpm for 15 hours in a Spinco SW-25.1 rotor at 4-6°C. The sucrose gradients were fractionated by an ISCO density gradient fractionator with modified turntable system for automatic collection of samples for measurement of radioactivity (Steele and Park, unpublished). Rat liver nucleolar RNA was extracted (Steele et al, 1965) and used as a reference for the sedimentation coefficient of the RNA fraction.

5. RNA nucleotide analysis

Since the RNA hydrolysate contained peptides and other contaminants which interfered with the assay of its concentration during earlier studies, the nucleotides were separated by chromatography on ion exchange resins (Dowex-1,X-8, formate). The specific activity of this pyrimidine nucleotide was determined and served as evidence for the incorporation of radioactive precursor into RNA.

For determination of total RNA and its specific activity, the hydrolysate was neutralized with 5 N PCA. The precipitate, potassium perchlorate, was removed by centrifugation. The resulting supernatant fraction was applied to a column of Dowex-1-formate, X8, 200-400 mesh (1 x 11 cm). The substance which passed through the resin column was collected and water washed (20 ml), and both optical density at 250 mμ and radioactivity were measured. The cytidine nucleotide was eluted with 0.05 M formic acid (Herbert et al, 1957). Four nucleotide fractions were also eluted with a linear gradient of formic acid (0-3.75 M) from a smaller column (0.5 x 18 cm). The nucleotide fraction was desiccated and resuspended in 0.1 N HCl and the specific activity was determined (Hurlbert et al, 1954).
6. Measurement of radioactivity

Radioactivity was measured by liquid scintillation spectrophotometry (Model 720, Nuclear Chicago Corporation). The concentration of the primary and secondary fluors of Bray's mixture (Bray, 1960) was doubled in order to increase the counting efficiency for the sample quenched by PCA. Radioactivity measurements were corrected for quenching by using the quenching curve of a tritiated nucleoside standard. The RNA fraction from the sedimentation profile analysis was collected in a scintillation vial and acidified with PCA to bring the pH to approximately 2.0. It was then hydrolyzed at 80°C for 15 minutes and counted in the same manner, after the fluor was added. Radioactive phosphorus was also counted in the same manner as the tritiated compound, except that it was corrected by its own characteristic quenching curve.

7. Autoradiography

Autoradiograms were prepared as previously described by Cooper (1965). Bones labeled with tritiated nucleoside or proline were fixed for 24-48 hours in Bouin-Holland fixative (Hartz, 1947), decalcified for 3 days in 18.5% EDTA (approximate pH 7.0), and double embedded in nitrocellulose and paraffin. Sections, 5 μ thick, were treated with periodic acid-Schiff reagent (Hotchkiss, 1948), warmed, dipped for 15 seconds in Kodak NTB-2 nuclear track liquid emulsion (45°C) and dried for 1 hour. They were then exposed in light tight black bakelite slide boxes at 4°C under low humidity for suitable periods of time (2-4 weeks). Subsequent to development at 17°C with Kodak Dektol developer, the sections were usually counter-stained with hematoxylin and examined.
EXPERIMENTAL RESULTS
The experimental data presented in this section have been reported in part in the following publications:


A. Modification of RNA extraction technique for bone

Employing the extraction technique of Schmidt and Thannhauser (1945), it was found that the RNA fraction contained certain chemical components which caused a brownish precipitate in the orcinol reaction and this interfered with the O.D. measurement of the ribose content in the RNA fractions. Diaphyseal bone contained more of this component than metaphyseal sections of bone. Therefore the differential of optical density in the ultra violet light range between 260 m$\mu$ and 280 m$\mu$ was used according to Fleck and Munro (1962). The ratio of 280 m$\mu$ to 260 m$\mu$ was much higher than that of a hydrolysate of standard RNA, and was therefore not suitable for quantitation. Thus, other extraction techniques were applied to solve this problem. Schneider's (1945) extraction technique using trichloroacetic acid in place of PCA was tried and a worse result was obtained. The desium dodecyl sulfate-phenol extraction method (Perry, 1958) produced cleaner RNA fractions with more variation in yield than other techniques,
but recovery of the DNA fraction was more difficult. Ogur and Rosen's (1950) extraction with cold 1.0 N PCA resulted in an RNA fraction similar to yeast RNA, and the absorption in the ultraviolet range was also similar to standard RNA. Complete extraction was difficult within 24 hours, and extraction for longer than this period produced an interfering substance. Removal of this interfering substance by charcoal absorption, and ion exchange resin (Dowex-2, X-8) was attempted, but these failed to eliminate the interfering component. Because PCA does not cause the complete precipitation of protein at a concentration lower than 0.5 N, during removal of the acid soluble fraction the concentration of PCA was 0.25 N. In addition, chloroform:methanol (1:2) at 50°C was added, presumably to extract mucopolysaccharide, and the time for hydrolysis with 0.3 N KOH was reduced to 2 hours. The procedure eliminated the brown precipitate in the orcinol reaction in both the diaphysis and metaphysis (Figure 1).

B. Development of a continuous perfusion system for live bone in vitro

Use of biochemical techniques to study bone fragments in vitro has produced a wealth of data on the processes of bone formation and resorption. However, the unphysiologic nature of these experiments has raised serious questions about the significance of much of this information. Diffusion of nutrients and waste products was retarded, metabolites are selectively bound to bone mineral, and metabolically active marrow cannot be completely removed by conventional methods (Borle, Nichols, and Nichols, 1960). Washing the bone preparations with a stream of isotonic saline did not remove all of the marrow, especially in the case of those cells deep within vascular channels or in spongiosa. Removal of marrow cells and blood from vascular channels was achieved by attaching either
the distal or proximal end of the femur to thick walled rubber tubing connected to a vacuum pump with a trap reservoir or aspirator connected to a tap water supply. The bone was immersed in either isotonic saline or buffered Krebs-Ringer salt solution. Closing the open end of the bone while washing was very effective and 1 minute of washing under a negative pressure of 10 to 20 pressure square inch was sufficient to remove marrows. The time between cutting and the beginning of washing was critical for the removal of marrow. Delay of washing caused coagulation in portions of the spongiosa and imperfect removal of this area. In case of delay, washing with a mild stream of saline by syringe before it was attached to the pumping manifold helped to remove coagulant. The piece of bone was immediately attached to the end of the perfusion manifold and immersed in the incubation medium (Figure 2). The continuous perfusion system was consisted of a peristaltic pump (Technicon, Model 1), manifold tubing connected to the tube (Technicon, type AO), and various sizes of snap cap vials. The detail is shown in Figure 2. The flow rate was 0.2 ml/minute, and bubbling in the incubation medium was prevented by attaching a glass capillary U-tube (Scientific Products). Excess gass pressure was released by a syringe needle on the vial cap. Maintenance of the pH was achieved by gassing with a 90% O₂ + 10% CO₂ (or 95% O₂ + 5% CO₂) gas mixture for 30 minutes prior to the beginning of the bone incubation. A control vial containing the incubation medium without bone was set up and the pH was constantly monitored by a combination electrode (Corning, 476050). The pH was maintained at 7.6. The gas mixture was continuously fed into the manifold and mixed with the same volume of incubation medium before it was returned to the vial. This was accomplished by using tubing of the same
Figure 2

Continuous recirculating perfusion system of femoral bone in vitro. Details appear in the legend on the Figure.
Continuous Recirculating Perfusion System

A. Gas mixture, 95% O2:5% CO2
B. Humidifying chamber
C. Silt valve
D. Peristaltic pump, TECHNICON
E. T-tube, 0.0 T.ECHNICON
F. Bone Vital
G. Bone and connector
H. Debubbling
I. Water bath
J. Pumping tube, 0.194, 0.021 D.

Technical Information:

- Flow rate: 2 ml/min
- Pumping tube: 0.194, 0.021 D.
- Gas mixture: 95% O2:5% CO2
- Humidifying chamber
- Silt valve
- Peristaltic pump, TECHNICON
- T-tube
- Water bath
- Bone and connector
- Debubbling
- Pumping tube
- Flow rate: 2 ml/min
specifications and a peristaltic pump. Evaporation of the incubation medium was prevented by bubbling the gas mixture through a reservoir of saline before it was mixed with medium. Any excess of pressure was released by a slit valve. The production of organic acids and the examination of bone by histology after incubation indicated normal maintenance of cell morphology and metabolites. There was a 15 fold incorporation of $^3$H-uridine in the metaphysis and a 3 fold incorporation in the diaphysis of the bone fragments incubated in flasks under the same conditions as continuous perfusion. The incubation medium was examined for cells which have been released during perfusion, and some marrow cells were being released during perfusion when washing had not been complete.

C. Bone cell isolation technique for metabolic study

Two groups of bone researchers have successfully established an isolated bone cell system for bone metabolism studies (Flanagan and Nichols, 1963; Peck et al, 1964). The composition of cell types in their systems was not well defined. Peck et al (1964) showed that the system contained osteoblasts and osteocytes. However, multinucleated and very fragile osteoclasts were not found. No attempts to isolate the osteoclast have been published.

Since the mononucleated cell types are easily isolated by the above methods, efforts were made to isolate and conserve the integrity of the multinucleated osteoclasts, as well as the mononucleated bone cells from mature adult bone in vitro.

The application of sonification using an ultrasonic apparatus (Bromwell Scientific), streptolysin O, a lysing agent of blood cells, saponin, lysozyme, papain, pepsin, panprotease, trypsin, pronase, or
tetraphenylboron, a chelating agent for potassium ions in appropriate
buffered media for various lengths of time and under various conditions
all resulted in either necrotic or disintegrated osteoclasts. Some intact
osteoclasts were obtained by slicing a piece of the bone and chipping it
with a sharp needle. After the marrow cells were removed by isotonic
saline, the metaphysis was sliced with a sharp razor blade or scalpel.

Crude collagenase (Worthington or Sigma) and bovine testicular
hyaluronidase (Worthington or Sigma) were applied individually for dif-
f erent lengths of time, different degrees of shaking or swirling, and
different buffer mediums as listed in Table 1. No bone cells were
separated from the calcified matrix by any of the methods employed.
Subsequently, two of these enzymes, mixed in different proportions, were
used and the medium was checked periodically for the removal of cells
whose morphology was quickly checked by toluidine blue. Various degrees
of shaking were also applied. A combination of 2 mg/ml of collagenase
and 10 mg/ml of hyaluronidase in calcium and magnesium free buffered
physiological saline yielded the best osteoclasts. The specific activity
of collagenase in commercial preparations varied as well as the activity
of other peptidases. It appeared that this variation in peptidase affected
the morphology of the isolated osteoclasts, therefore, fractionation of
collagenase was carried out by gel filtration and 3 fractions were obtained
following the method of Keller and Mandl (1963). The details of this
fractionation are shown in Figure 3. The elimination of "Fraction 3" aided
in obtaining a better shaped osteoclast. The coincidence of "Fraction 3"
and the brown color in the crude fraction suggested that commercial prepa-
rations with a lighter color may contain less of this harsh component.
<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>Medium</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Saponin</td>
<td>0.010%</td>
<td>0.9% NaCl</td>
<td>30, 60, 90 min.</td>
</tr>
<tr>
<td></td>
<td>0.025%</td>
<td>0.9% NaCl</td>
<td>30, 60, 90 min.</td>
</tr>
<tr>
<td></td>
<td>0.050%</td>
<td>0.9% NaCl</td>
<td>30, 60, 90 min.</td>
</tr>
<tr>
<td></td>
<td>0.075%</td>
<td>0.9% NaCl</td>
<td>30, 60, 90 min.</td>
</tr>
<tr>
<td>B. Streptolysin 0</td>
<td></td>
<td></td>
<td>2, 5 min.</td>
</tr>
<tr>
<td>C. Trypsin, Panprotease,</td>
<td>0.1, 0.25%</td>
<td>Calcium magnesium-free Krebs-Ringer, phosphate buffered</td>
<td>45, 60, 90, 120 min.</td>
</tr>
<tr>
<td>Pronase</td>
<td>0.1, 0.25%</td>
<td></td>
<td>45, 60, 90, 120 min.</td>
</tr>
<tr>
<td></td>
<td>0.1, 0.25%</td>
<td></td>
<td>45, 60, 90, 120 min.</td>
</tr>
<tr>
<td>D. Papsin</td>
<td>22, 55, 110, 220, and 330 units</td>
<td>Phosphate buffered saline, pH 5.3</td>
<td>30, 60 min.</td>
</tr>
<tr>
<td>E. Lysozyme</td>
<td>2.0, 5.0 mg</td>
<td>Phosphate buffered, citrate buffered, and oxalate buffered saline pH 7.4</td>
<td>30, 60, 90 min.</td>
</tr>
<tr>
<td>F. Ultrasonicator (Bronwill)</td>
<td>Setting 10, 20, 40, 60</td>
<td>Krebs-Ringer, phosphate buffered. 4°C</td>
<td>5, 20, 40, 60, 120 and 180 seconds</td>
</tr>
</tbody>
</table>
Table 2
PROCEDURES FOR BONE CELL FRACTIONATION

Rat Distal Femur

Perfuse with salt solution —> Cells collected: Fraction A (Osteoclasts and Myeloid cells)

Incubate in Pooled Rat Serum with Uridine-5-T for 2 hours, at 37 C and pH 7.4 using recirculation system.

Extracted as "Total bone" RNA (Fractions B and C)

Perfuse with hyaluronidase and collagenase (1% and 0.5% in calcium-free Krebs-Ringer) 60 to 90 minutes using recirculation system.

Perfuse with salt solution —> Cells collected on membrane filter (Fraction B)

Remaining bone (Fraction C)
Figure 3

Collagenase purification. An eluent pattern from Sephadex G-200 gel filtration chromatographic column.

Temperature: 25°C

Developing Buffer: 0.33 M Calcium acetate in 0.025 M Tris, pH 7.2

Flow rate: 3 ml/hr

Column size: 6.0 x 180 cm

Load: 10 mg of crude collagenase

Volume per tube: 3.0 ml
Collagenase with lighter color gave better results. The osteoclasts that were removed are shown in Figure 4A. The isolated osteoclasts were resuspended in 5% sucrose and examined under a phase contrast microscope at 100 X magnification. The intact cells extended their cytoplasmic processes in the medium and remained intact through the experimental period of 6 hours. The other cell types were also separated and the integrity of their morphology was observed (Figure 4B).

The separation of a homogeneous cell population of osteoclasts, osteoblasts and mesenchyme cells was not achieved owing to (1) that the reduced number of osteoclasts in this fraction, and (2) that the cells were removed from the bone surface as a strand. For a metabolic study of isolated bone cells, a partial separation of bone cell types was attempted. Separation of the mixture of osteoblasts and mesenchymal cells from osteocytes buried in the calcified bone matrix was accomplished. An outline of the procedure is illustrated in Table 2. After indubation in a continuous flow system with 5 mg/ml of collagenase and 10-20 mg/ml of hyaluronidase, the bone was washed with buffered isotonic saline to remove cells trapped in the bone. These dissociated cells were collected in siliconized glassware and collected on a membrane filter (Ga-1, Gelman). Cells collected on the membrane consisted of mesenchymal cells and osteoblasts (Figure 5). The osteocyte fraction remained in the bone matrix. Oxygen consumption of these dissociated cells was compared with that of intact bone, and appeared slightly stimulated. Lactate production of the osteocyte fraction was about 1/2 of the total produced by whole bone. Removal of bone collagen and a check on the integrity of osteocytes after this treatment were carried out. Intact bones were labeled with tritiated proline for 1 hour. After the
Figure 4

Isolated Osteoclasts

A. Osteoclasts isolated by enzyme treatment. Three osteoclasts are shown among marrow cells. Stained with 0.1% of toluidine blue. Magnification: X 100.

B. An osteoclast resuspended in 5% sucrose after enzyme treatment. Examined under phase contrast microscope. Magnification: X 100.
Figure 5

A strand of isolated osteoblasts and mesenchymal cells from metaphysis. Stained with 0.1% toluidine blue. Magnification: X 100.
1 hour labeling period, some of the bones were incubated in buffered saline to serve as controls and other pieces were treated with enzyme mixture. Autoradiographs were prepared and the degree of labeling was examined (Figure 6A, B). The results indicated that the enzyme mixture did not remove all of the collagen or that fraction labeled with tritiated proline for 1 hour, and also showed that the osteocyte was capable of incorporation of tritiated proline during isolation.

D. Effects of endogenous parathyroid hormone on RNA metabolism

1. RNA metabolism in femoral metaphysis and diaphysis

Table 3 contains pertinent data concerning recovery values and the relative specific activity values of RNA extracted from trabecular bone of metaphysis and compact bone of the diaphysis. The specific activity of cytidine in RNA was 2.5 times higher and the RNA content 10 times higher in metaphysis than in the diaphysis. The total RNA content did not change significantly throughout the period of stimulation of parathyroid hormone secretion.

In response to the stimulation of PTH secretion, the specific activity or incorporation of $^3$H-cytidine or $^3$H-uridine into RNA followed quite a different pattern in the two areas of bone. There was an immediate increase in the metaphysis and a biphasic change in the diaphysis which showed an immediate decrease followed by an increase. An increase (17%) in the metaphysis was observed after 20 minutes of stimulation and there was a consistent increase afterwards (up to 50% by 8 hours). A decrease of 20% in the incorporation was also shown in the diaphysis and it persisted up until 4 hours. After 4 hours of lavage, this effect was reversed, and
Figure 6

Radioautograph of Osteocyte Fraction

A. Longitudinal section of metaphysis of a femur. Intact bone was labeled with 10 μc of tritiated proline for 1 hr. and treated with enzyme mixture. Magnification: X 50.

B. Longitudinal section of diaphysis of a femur. Labeled with 10 μc of 3H-proline for 1 hr. and treated with enzyme mixture. Magnification: X 100.
until the 8th hour, the rate of increased nucleoside incorporation paralleled that of the metaphysis. In the metaphysis, PTH alone produced a small but significant increase (10%) in the incorporation rate, which was not further affected by peritoneal lavage (Figure 7).

Twenty four hours after nephrectomy, the incorporation of labeled nucleoside increased by 38% in diaphysis, but there was no change in metaphysis (Table 6B). Nephrectomy stimulated the secretion of endogenous PTH through chelation of calcium ions by phosphate which accumulated in extracellular fluid in the absence of the kidney (Talmage et al., 1960). The experiment described above demonstrated the effects of endogenous PTH on the incorporation of radioactive nucleosides into RNA in specific areas of bone. In order to characterize the reaction, the pattern of incorporation of labeled precursors was examined by autoradiography, sedimentation profile analysis of phenol extracted RNA, and ion exchange chromatography for the analysis of nucleotides in hydrolysates.

The distribution of tritiated cytidine after its uptake by bone cells during the bone-serum incubation is illustrated in Figure 8. These autoradiographs are of bone sections taken from animals in which endogenous parathyroid secretion had been stimulated by five hours of peritoneal lavage. Figures 8A and 8B demonstrate that, at the one hour incubation period, the $^3$H-cytidine was incorporated into all types of bone cells and concentrated primarily in the nuclei of bone cells. No attempts were made to analyze the quantitative differences among cell types or between control and stimulated animals. This result demonstrated that all cell types of bone take up the nucleoside precursor.
Specific Activity of $^3$H-cytidine-RNA Extracted from Femurs of the Peritoneal Lavage-Serum Incubation.

Specific activity for nonlavaged control animals was set at 100. Represented by dotted line on graphs. All closed figures are statistically different from control value with $p < 0.01$.

○,●: Parathyroid-intact lavaged animals
□,■: Parathyroidectomized lavaged animals
Figure 8

Autoradiographs of metaphysis and diaphysis of femur after 1 hr. of incubation with $^3$H-cytidine. Bones were taken from the parathyroid-intact rat after 5 hr. of peritoneal lavage. PAS and hematoxylin stained. Magnification: X 400.

A. Metaphysis
B. Diaphysis
Figure 9A shows the sedimentation profile of the phenol extracted RNA from the metaphysis of a non-lavaged control rat and a profile of rat liver nucleolar RNA which was used as a reference marker for the sedimentation coefficient (Steele and Busch, 1966). The metaphyseal RNA was composed mainly of RNA fractions with sedimentation coefficients of approximately 6S, 18S, and 28S (Perry, 1958; Steele and Busch, 1966). All fractions of RNA, as well as those having a coefficient of 35S and higher, incorporated tritiated nucleoside. The RNA extracted from the diaphysis of the control non-lavaged rats (Figure 9C) showed a similar profile and incorporation pattern; but the radioactivity per unit optical density was lower than that of metaphysis and paralleled the results obtained with the acid-extracted RNA (Table 2). Figure 9B is the profile of RNA from the metaphysis of a rat lavaged with calcium-free rinse for 8 hours. There was a decrease in the relative amount of the 6S region, and an increase in the 18S and 28S regions compared to the non-lavaged controls. Regions 22S and 35S are present in the RNA from the lavaged animals, whereas a distinct peak was absent in the profile of control rats. Lavage increased the incorporation of precursor in all RNA fractions over that of the controls. The diaphyseal RNA exhibited an increase in the incorporation of tritiated cytidine in all fractions similar to metaphyseal RNA. No changes in sedimentation profile were observed (Figure 9D). This demonstrated that the increased incorporation of precursor observed with acid extracted RNA was due to the increase in 6S, transfer RNA, 18S and 28S, ribosomal RNA as well as in other fractions of RNA. The relative increase of 18S and 28S fractions over 6S RNA indicated that there was an increase in ribosomal RNA synthesis. This was
Table 3

Yield and Specific Activity of Extracted RNA and DNA Fraction

<table>
<thead>
<tr>
<th></th>
<th>Yield µg/mg bone</th>
<th>Specific Activity cpm/µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metaphysis</td>
<td>2.58 ± 0.20</td>
<td>46.4 ± 1.5</td>
</tr>
<tr>
<td>Diaphysis</td>
<td>0.24 ± 0.07</td>
<td>18.4 ± 0.7</td>
</tr>
<tr>
<td>DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metaphysis</td>
<td>0.66 ± 0.04</td>
<td>10.2 ± 0.7</td>
</tr>
<tr>
<td>Diaphysis</td>
<td>0.07 ± 0.01</td>
<td>3.4 ± 0.2</td>
</tr>
</tbody>
</table>

The values are for femurs from parathyroid-intact non-lavaged rats.

Incubating conditions: 5 µc of $^3$H-cytidine (23.8 c/mM) of 5 µc of $^3$H-thymidine (14.8 c/mM) in 1.5 ml of pooled rat serum for 1 hr. at 37°C.
also demonstrated in the appearance of the 35S fraction which is a type of precursor for ribosomal RNA (Scherrer et al., 1963).

An analysis of hydrolyzed RNA by ion exchange chromatography was carried out to give an indication of the specificity of cytidine incorporation and the degree of incorporation of radioactivity from 3H-cytidine into other nucleosides. The data are presented in Table 4. The total recovery of radioactivity in nucleotide fractions averaged 98%, and the loss of a small quantity of UV absorbing material indicated that there was some contamination which was negligible in the specific activity value of RNA presented in Table 2. The 8 hour lavage caused an increase of approximately 60% in the incorporation of nucleosides into metapyseal RNA over the non-lavaged controls which was in close agreement with the data presented in Figure 7. The distribution of radioactivity of the 3H-cytidine precursor was found to occur at a ratio of approximately 55% in cytidine and 45% in other nucleosides, primarily in uridine and DNA fractions.

2. RNA metabolism of "mesenchyme-osteoblast" and "osteocyte" fractions separated by enzymic digestion

(a) Fraction B: "mesenchyme-osteoblast" fraction

This is the fraction that contains essentially all of the mesenchyme and osteoblast cell populations and a remnant of the myeloid population, but few or no osteoclasts (Figure 5). The only osteoclasts which might be found in this fraction would be partially trapped or newly trapped cells released by the enzymatic degradation of matrix. This is the fraction which, according to a previous report describing
Table 4

Specific Activity of Cytidine Nucleotide
of Metaphysis of Femur

<table>
<thead>
<tr>
<th></th>
<th>2', 3'-CMP cpm/μg</th>
<th>Fraction of CMP/total nucleotide</th>
<th>Fraction of radioactivity of CMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-lavaged</td>
<td>111.3 ± 3.3</td>
<td>.185</td>
<td>.547</td>
</tr>
<tr>
<td>8 hr. lavaged</td>
<td>180.5 ± 7.5</td>
<td>.190</td>
<td>.510</td>
</tr>
</tbody>
</table>

CMP was determined by an absorbance at 275 mλ.

Recovery of total radioactivity ranged from 97.3 to 98.5% of sample loaded on column.

Quantity of material which passed through resin column was less than 3% of total optical density at 260 mλ.
parathyroid effects on tritiated cytidine incorporation into the RNA of bone cells (Talmage et al., 1965), should show the major stimulatory effects of PTH. Figure 10 shows the effect of peritoneal lavage on fraction B and the total metaphysis. The incorporation of tritiated uridine into metaphyseal RNA using a recirculating perfusion system was considerably higher than bone fragment incubation as described in Section III, 1. After 8 hours of lavage, \(^3\)H-uridine increased by 150% compared to a 50% increase by bone fragment incubation. Fraction B consisted of 80% of the total RNA content. The effect in fraction B was very dramatic and reached 204% after 8 hours of parathyroid stimulation (Figure 10). Similarly, but to a lesser extent, effects were also demonstrated in the shaft incubated by the perfusion method. The early inhibitory effect was no longer observed when only fraction B was analyzed (Figure 11).

(b) Fraction C: osteocyte fraction

This consisted almost entirely of osteocytes still located in their individual lacunae (Figure 6). Preliminary tests with oxygen consumption, lactic acid production, and \(^3\)H-proline uptake indicated that these cells were still viable following the separation procedure as described in Section IV, C. The data are summarized in Table 5. There was an immediate suppression of tritiated uridine incorporation into osteocytes by parathyroid hormone. This suppression was reversed after 8 hours of lavage when it returned close to the normal level.

These studies of fractions B and C indicated that it was the difference in the ratio of total RNA of fractions B and C between the metaphysis and shaft which probably accounted for the difference in the net RNA
Figure 9

Sedimentation profiles for RNA of metaphysis and diaphysis from rat femur after 1 hr. of incubation with $^3$H-cytidine (50 $\mu$C/1.5 ml serum).

A. RNA of metaphysis, non-lavaged and liver nucleolar RNA. Figures on each peak shows S values.

B. RNA of metaphysis, 8 hr. lavaged.

C. RNA of diaphysis, non-lavaged.

D. RNA of diaphysis, 8 hr. lavaged.

---: Optical density at 254 m$\mu$ of bone RNA

-----: Radioactivity of bone RNA

-----: Optical density of liver nucleolar RNA
Comparison of calcium ion and endogenous PTH on the effect of incorporation of $^3$H-uridine into RNA fraction of total bone and Fraction B. Bones were taken from parathyroid-intact rat after 2 hr. and 8 hr. of peritoneal lavage with buffered saline or with 10 mg% of calcium in buffered saline.

-■-■- : Fraction B, buffer lavaged.
-●-●- : Fraction B, calcium lavaged.
-□-□- : Total bone, buffer lavaged.
Comparison of calcium ion and endogenous PTH on the effect of incorporation of $^3$H-uridine into RNA fraction of total bone and fraction B. Bones were taken from parathyroid-intact rat after 2 hr. and 8 hr. of peritoneal lavage with buffered saline or with 10 mg% of calcium in buffered saline.

- - - : Fraction B, buffer lavaged.
- - - : Fraction B, calcium lavaged.
- - - : Total bone, buffer lavaged.
- - - : Total bone, calcium lavaged.
Figure 12

Specific activity of $^3$H-thymidine-DNA extracted from femurs of the peritoneal lavage-serum incubation. Specific activity for non-lavaged control animal was set at 100. Represented by dotted line on graphs. All closed figures are statistically different from control value with $p < 0.01$.

○, ○: Parathyroid-intact lavaged animals.

☐: Parathyroidectomized lavaged animals.
Figure 13

Autoradiograph of metaphysis of femur after 1 hr. of incubation with $^3$H-thymidine (10 µc/1.5 ml serum). Bone was taken from the parathyroid-intact rat after 5 hr. of peritoneal lavage.
synthesis rate seen when the whole metaphysis or shaft was extracted (Figure 7 and Table 4). In the shaft, fraction C contained 65% of the extracted RNA, whereas only 15% of the extracted RNA was found in the metaphysis fraction.

E. Effects of endogenous PTH and calcium ions on DNA synthesis of femoral metaphysis and diaphysis

The specific activity of DNA extracted from the metaphysis and diaphysis of femurs from control animals is given in Table 3. The rate of incorporation of thymidine into DNA of trabecular bone was approximately three times the rate for the compact bone. There was no change in the total DNA in bone during 8 hours of lavage. Figure 12 contains data demonstrating the effects of parathyroid stimulation and parathyroidectomy on the rate of incorporation of $^{3}H$-thymidine into DNA extracted from these two types of bone. An effect of endogenous parathyroid stimulation was observed after 4 hours of lavage. Both the metaphysis and diaphysis showed similar responses except the degree of stimulation was much less in the diaphysis. Since parathyroidectomy negated this stimulation, it can be assumed that it was caused by increased endogenous PTH secretion. Autoradiographs (Figure 13) indicated that $^{3}H$-thymidine uptake in vitro for 1 hour was essentially restricted to the nuclei of the mesenchyme population of bone cells (Tonna and Cronkite, 1961a; Young, 1962).

The effect of calcium ions was examined by lavage with a rinse containing calcium at a concentration of 5.8 mg to 12.0 mg per 100 ml of lavage fluid. There was neither a stimulation nor inhibition of DNA synthesis up to 8 hours of lavage in the metaphysis or shaft. In nephrectomized rats, 24 hours after surgery, the rate of incorporation of
tritiated thymidine was increased to 146% in the shaft and decreased to 80% in the metaphysis.

F. Effect of calcium ions on RNA metabolism in femoral metaphysis and diaphysis

This experimental approach was originally designed to stabilize the rate of endogenous PTH secretion to a minimum level. Since the feedback system of parathyroid hormone secretion is controlled by the blood serum calcium level (Talmage et al., 1960), challenging the animal with high calcium will increase the calcium level in serum and inhibit parathyroid secretion. However, a rapid stimulation of RNA synthesis by this method was observed (Figure 7), rather than a maintenance of the control level. This effect tapered off and remained constant through the lavage. The transitionary effect of this effect of calcium ions was also observed. The rats were given a peritoneal lavage with a high calcium rinse for two hours, and after removal of the fluid the animals were allowed to recover for one hour before the femurs were removed for use in the bone-serum incubation procedure. The $^3$H-cytidine incorporation rate into the RNA of metaphyseal bone returned to the starting level. In contrast, the one hour given to the animals with stimulated endogenous PTH secretion did not reduce RNA synthesis in the metaphysis. In the diaphysis, similar effects were obtained except that the inhibitory effect observed during early hours of lavage was not demonstrable in rats subjected to high calcium challenge (Figure 11). Figure 14 shows the sedimentation profile of phenol extracted RNA from rats challenged with a rinse containing 10 mg Ca per 100 ml for 2 hours. The patterns of optical density and
Comparison of calcium-free and high calcium lavage on sedimentation profile for RNA of metaphysis, and pattern of incorporation of $^3$H-uridine.

Bones were incubated with $^3$H-uridine (50 μc/1.5 ml serum) for 1 hr. in vitro.

A. RNA of metaphysis, non-lavaged and liver nucleolar RNA. Figures on each peak shows S value.

B. RNA of metaphysis, 2 hr. calcium-free lavaged, intact rat.

C. RNA of metaphysis, 2 hr. 10 mg% calcium lavaged, intact rat.

D. RNA of metaphysis, 2 hr. 10 mg% calcium lavaged, PTX rat.

--- : Optical density of bone RNA at 254 mμ.

--------- : Radioactivity.

--------- : Optical density of liver nucleolar RNA.
Comparison Of Ca-Free And High Calcium Lavage On Sedimentation Profiles For RNA Of Metaphysis

A. Intact, Non-lavaged Rat Liver Nucleolar RNA

B. Intact, 2 hr. Ca**-free

C. Intact, 2 hr. 10 mg% Ca**

D. PTX, 2 hr. 10 mg% Ca**
radioactivity were similar to those of animals lavaged with buffer and no difference was indicated between intact and PTH'd rats.

Fraction B of metaphysis as well as diaphysis was stimulated in a manner similar to that observed as a result of the calcium-free lavage, but it remained at the same level whereas it was increased by endogenous parathyroid stimulation (Figure 10). Fraction C was inhibited by the high calcium challenge and remained inhibited up to 8 hours of lavage (Table 5).

The effect of various concentrations of calcium on metaphyseal RNA synthesis was examined to test the specificity of this stimulatory effect. These studies are summarized in Figure 15. The two intermediate levels (5.8 and 6.2 mg%) of calcium concentration failed to increase RNA synthesis when compared to experiments in which a higher calcium concentration (6.9 to 12.0 mg%) was used. This intermediate concentration was used in an attempt to bracket that concentration which produced a situation of no net transfer of calcium between peritoneal fluid and the animal during an equilibration period. In turn, this would prevent any increase in endogenous parathyroid secretion. Because of the duplication of a PTH-like effect with high calcium on RNA synthesis, the specificity was also tested in PTH'd and thyroparathyroidectomized rats. Animals were subjected either to a lavage containing no calcium or to one with a calcium content adjusted to 12 mg%. The removal of the parathyroids or the whole thyroid-parathyroid complex negated the effect produced by the calcium-free lavage in parathyroid intact rats. However, removal of these glands in no way affected the results produced by a high calcium lavage (Figure 16).
Table 5

COMPARISON OF Ca-FREE AND HIGH CALCIUM LAVAGE CONTENT ON BONE RNA SYNTHESIS IN FRACTION C

<table>
<thead>
<tr>
<th></th>
<th>Calcium-Free</th>
<th>12 mg% Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 Hour</td>
<td>8 Hour</td>
</tr>
<tr>
<td>Metaphysis</td>
<td>80.4±2.5</td>
<td>104.5±4.6</td>
</tr>
<tr>
<td>Diaphysis</td>
<td>81.4±2.8</td>
<td>93.2±3.4</td>
</tr>
</tbody>
</table>

Non-lavaged bone expressed as 100.
Comparison of various concentrations of calcium ion on the effect of incorporation of $^3$H-uridine into RNA fraction of metaphysis of femur.

Bones were taken from the animals lavaged 2 to 3 hr. Incubated in pooled rat serum (1.5 ml) with 10 µc of $^3$H-uridine.
Effect of Intermediate Lavage Calcium Concentration on Bone RNA Synthesis (2-3 HPhenol/Lavage)

Metaphysis
Figure 16

Effect of parathyroidectomy and thyro-parathyroidectomy on the incorporation of $^3$H-uridine into RNA fraction of metaphysis of femur.

Bones were taken from animals lavaged for 8 hr. with either calcium-free or 10 mg% calcium rinse. Incubated with 10 $\mu$C of $^3$H-uridine per 1.5 ml of pooled rat serum for 1 hr.

Values from parathyroidectomized rat were set as control.
Effect Of Parathyroidectomy And Thyroparathyroidectomy
On Bone RNA Synthesis (8 Hour Peritoneal Lavage)
G. Preliminary experiments on thyrocalcitonin and cortisone effects on RNA synthesis

Thyrocalcitonin prepared by Klein (1968) from hog thyroid was injected at a dose of 2-4 units every two hours and the effect of this hormone on metaphyseal and diaphyseal bone RNA synthesis was examined by $^{32}$P flash labeling in bone-serum incubations. The results indicated that no significant change was induced by thyrocalcitonin although the serum calcium level showed the characteristic reduction (Table 6C). Injection of thyrocalcitonin while the animal was being challenged with calcium-free buffered lavage fluid did not significantly reduce the increase in RNA synthesis which was observed during a calcium-free buffered lavage without thyrocalcitonin administration (Table 6A, 6B). This may indicate that stimulation of RNA synthesis may be related to movement of calcium ions across bone cells. Rats treated with cortisone for 6 days with 5 mg/day of cortisone were challenged with 10 mg% of high calcium for 8 hours. The stimulatory effect on RNA synthesis caused by high calcium was not observed in cortisone treated rats (Table 7). This indicated that the effect of calcium on RNA synthesis may be mediated through a cortisone sensitive process.
Table 6

Effect of TCT Incorporation of $^3$H-cytidine, $^3$H-uridine, and $^{32}$P into RNA Fraction of Femoral Bone

A. Buffer lavaged for 8 hr. Thyrocalcitonin (TCT) injected at 0, 2, and 5 hr. 8 units per injection.

<table>
<thead>
<tr>
<th>Group</th>
<th>Metaphysis</th>
<th>Diaphysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact, non-lavaged</td>
<td>100.0 ± 3.3</td>
<td>100.1 ± 2.4</td>
</tr>
<tr>
<td>Intact, lavaged</td>
<td>158.3 ± 6.2</td>
<td>125.1 ± 4.6</td>
</tr>
<tr>
<td>Intact, lavaged with TCT</td>
<td>146.1 ± 8.5</td>
<td>131.9 ± 8.9</td>
</tr>
<tr>
<td>PTX, lavaged</td>
<td>115.8 ± 2.5</td>
<td>103.3 ± 3.1</td>
</tr>
<tr>
<td>PTX, lavaged with TCT</td>
<td>109.0 ± 6.2</td>
<td>94.5 ± 5.6</td>
</tr>
</tbody>
</table>

B. Injected with TCT for 24 hr. 12 doses of 8 units every 2 hr.

<table>
<thead>
<tr>
<th>Group</th>
<th>Metaphysis</th>
<th>Diaphysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact, with TCT</td>
<td>98.3 ± 3.8</td>
<td>110.3 ± 3.1</td>
</tr>
<tr>
<td>Nephrectomized</td>
<td>101.5 ± 2.4</td>
<td>138.1 ± 3.1</td>
</tr>
<tr>
<td>Nephrectomized, with TCT</td>
<td>92.8 ± 5.8</td>
<td>130.9 ± 8.0</td>
</tr>
</tbody>
</table>

C. Injected with TCT (2 units) for 1 hr. Incubated with $^{32}$P for 30 min.

<table>
<thead>
<tr>
<th>Group</th>
<th>Metaphysis</th>
<th>Diaphysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact, with TCT</td>
<td>90.7 ± 4.7</td>
<td>108.7 ± 4.3</td>
</tr>
</tbody>
</table>

Incubating condition: 10 µc of $^3$H-cytidine or $^3$H-uridine per 1.5 ml of pooled rat serum, 1 hr. at 37°C, or 30 µc of $^{32}$P was added to each flask.
Table 7

Effect of Cortisone on Incorporation of
$^{3}H$-uridine into RNA Fraction of Bone

<table>
<thead>
<tr>
<th>Group</th>
<th>Metaphysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>100.0 ± 2.9</td>
</tr>
<tr>
<td>Intact, lavaged</td>
<td>127.4 ± 3.6</td>
</tr>
<tr>
<td>Intact, injected, lavaged</td>
<td>107.7 ± 5.4</td>
</tr>
</tbody>
</table>

Injected with cortisone acetate (5 mg) for 6 days. Lavaged with 10 mg% calcium for 8 hr. Bones were incubated with 10 μc of $^{3}H$-uridine/1.5 ml rat serum for 1 hr. at 37°C.
DISCUSSION
A. Technical innovations for the study of bone cell metabolism

1. Evaluation of perfusion technique

Employment of preexisting vascular channels for the perfusion of bone in vitro for the study of metabolism without the interference of contaminating bone marrow cells was established. Parsons and Robinson (1968) devised a simple perfusion method for the study of calcium equilibrium in isolated bone. Their flow rate of blood through bone, which was claimed to be physiological, was identical to our system, but recirculation of the blood was not possible in their system. Since PTH functions through the production of intermediates which enhance bone resorption (Johnston et al, 1962), if the medium is not recirculated to bone, then the factor may be washed out and the endocrine effect lost. In this system, the medium can be recirculated or even mixed with other factors. The dessication or evaporation which accompanied the flask bone fragment incubation was eliminated by humidifying the gas before it was mixed with the circulating medium. The penetration of circulating medium was checked by toluidine blue and the calcium ion stain, Glyoxal BIS(2-hydroxyanil) (GBHA) (Kashiwa, 1966) as well as by autoradiography of proline and uridine. This showed that penetration was uniform and very rapid. The possibility that removal of bone cells occurred was eliminated by failure to find any osteoclasts in the medium.

The isolation of an organ or tissue from the body always gives rise to the basic question of whether or not the isolated counterpart represents the in vivo physiological process. How much this factor influences
the metabolism of bone in vitro cannot be completely assessed, however, it is probable that this system represents an improvement over preexisting systems for the in vitro study of bone.

2. **Evaluation of cell separation methods**

The synergistic action of hyaluronidase and collagenase observed in dispersion of bone cells was also reported in the dispersion of liver cells (Howard et al, 1967) and in the preparation of Ehrlich ascites carcinoma cells (Yamada and Ambrose, 1966). According to Yamada and Ambrose (1966), this combination of enzymes was superior to other enzymes such as trypsin for the preservation of cell morphology and cell wall components. The possible damage due to the enzymes was examined. The lactic acid production, oxygen consumption, and uptake of tritiated proline indicated that the isolated cells were close to the intact cell populations examined. The test with GBHA showed that the isolated osteoblasts and osteocytes reversed their staining property with this stain. This may indicate that some change in the ionized calcium binding capacity of the cells was effected.

Fraction B is still a mixture of mesenchymal cells and osteoblasts and needs further separation into homogeneous populations. The difficulty of differentiating the osteoblast from the mesenchyme cell in its isolated condition has prevented further attempts at separation despite several proper flotation techniques available at this moment (Mell, 1964; Mateyko and Kopac, 1963).
B. Evaluation of modification of extraction of RNA

The application of Schmidt and Thannhauser's extraction procedure for RNA resulted in some component(s) other than nucleotides in the RNA fraction. It is generally understood that this particular procedure also yields peptides which do not interfere with the orcinol reaction for ribose determination (Munro, 1965). However, some component in bone causes a brown precipitate and this was not eliminated by a decrease in hydrolysis time, Dowex-2 ion exchange chromatography, or charcoal absorption. Attempts to extract RNA by the methods of Ogur and Rosen (1950), Schneider (1945), or phenol-SDS (Perry, 1958) were not completely successful either because of the incomplete removal of contaminants or the failure of quantitative recovery. Addition of the chloroform:methanol step in the procedure eliminated this particular component which existed in a larger quantity in shaft than in metaphyseal bone. The decreased time of hydrolysis in 0.3 N KOH to 2 hours reduced the peptide component and the ratio of UV absorption at 260 m\(\mu\) and 280 m\(\mu\) was very close to the value of Fleck and Munro (1962).

C. RNA synthesis and parathyroid hormone

1. Demonstration of RNA synthesis in bone

Following several attempts to relate RNA synthesis and parathyroid function by the use of AMD, a nonspecific RNA synthesis inhibitor, Talmage et al (1965), by autoradiography, first observed the increased incorporation of tritiated cytidine in an undifferentiated mesenchymal cell population after 8 to 16 hours of stimulation of endogenous PTH. This apparently supports a functional transfer toward an increased formation of osteoclasts.
In extending this observation, the rapid response of RNA synthesis, measured by the uptake of $^3$H-cytidine and chemical extraction method, to stimulation of endogenous PTH secretion was observed. Twenty minutes of stimulation caused an increase in the uptake of precursor in metaphyseal trabecular bone and an inhibition in compact bone of diaphysis. A similar observation was reported by Steinberg and Nichols (1968b) in the uptake of $^{32}$P in the RNA fraction of metaphyseal bone and by Egawa and Neuman (1964) in the uptake of $^{32}$P in the RNA fraction after PTE administration. Owen and Bingham (1968) also reported the rapidity of the response. They observed a stimulation in osteoclasts and a depression in osteoblasts within one half to one and one half hours after PTE treatment. The rapidity with which hormones may stimulate or depress the rate of RNA synthesis was reported (Tata, 1966, 1967). Raisz and Niemann (1967) and Van Wermeskerken (1968) confirmed the immediate suppression of RNA synthesis by PTE in embryonic bone cultures. The opposite effect of hormone on different parts of the same tissue was the first case in which the same hormone evoked different responses in cells of a tissue (Tata, 1967). Karlson (1963) proposed that the hormone may act as a gene activator and stimulate DNA-dependent RNA synthesis, and Tata (1967) extended this hypothesis to include the ribosomal translational level as well as the genetic transcriptional level. The sedimentation profile analysis indicated that, after two hours of stimulation, the incorporation of radioactivity in the region between 6S and 18S, presumed to be m-RNA synthesis, was increased. An indication of the stimulation of synthesis in the m-RNA fraction was also reported by Steinberg and Nichols (1968b) by flash labeling and sedimentation profile analysis. This
indicated the involvement of all types of RNA including t-RNA, r-RNA, and high molecular weight preribosomal RNA. After 8 hours of stimulation, an increased incorporation was indicated in all fractions analyzed. The relative quantity of low molecular weight RNA and high molecular weight RNA was changed. In the metaphysis a peak of 22S and 35S RNA was noticed after 8 hours of lavage.

These data still do not indicate the precise site and mechanism of action of parathyroid hormone. However, the investigation of two early hormone-dependent cellular responses, namely subcellular ion translocations and RNA synthesis, is relevant for an evaluation of their interdependence and their relation to parathyroid hormone action in the bone cell.

2. Demonstration of different responses in bone cell fractions

The cellular composition of bone tissue is very unique in that the morphological or positional characteristics can be associated with the function of a particular bone cell type. The osteoclast is multinucleated and active in reabsorption of bone structure. Osteocytes are buried in bone matrix. The osteoblast lines the bone surface, and the osteoprogenitor or mesenchymal cell is located behind the osteoblast and is the only type of cell which can synthesize DNA and go through mitosis. These two types of cells are difficult to identify when they are isolated because of the similarity of their morphology.

Owen and Bingham (1968) analyzed the pattern of RNA synthesis in response to PTE. By quantitative radioautography, they found that there are immediate and quite opposite effects on the cells responsible for
bone resorption and bone growth. There is a significant stimulation of RNA synthesis in the osteoclast and a depression of RNA synthesis in the osteoblast.

Although there are many indications that the same hormone can act on different cells in tissue (Tata, 1967), as mentioned before, this is the first example of a hormone acting in opposing directions on RNA synthesis in different cell types of the same tissue. This differential effect on separate cell types and the relative quantity or mass of RNA may account for the biphasic response of diaphyseal bone and the immediate increase observed in metaphyseal bone. The biphasic response of bone RNA synthesis induced by PTE was observed by Steinberg and Nichols (1966, 1968b), Raisz and Niemann (1967), and Van Wermeskerken (1968). The report of Martin et al (1965) on the inhibitory response to PTE may be the result of observing the response at a time later than 40 hour which is the usual period of effectiveness (Owen and Bingham, 1968). In previous work, Talmage et al (1965) observed a 100% stimulation of RNA synthesis is an undifferentiated mesenchymal cell population.

In this work, the separation of mesenchyme cells and osteoblasts was not feasible; therefore, it was not possible to estimate the separate contribution of these cell types to the sum of the stimulation of RNA synthesis by PTH. Presumably either the quantity of mesenchyme cells or the extent of the stimulatory effect on mesenchyme cells was larger than that of the osteoblasts. Combining Talmage et al (1965), Owen and Bingham (1968) and this work, the diverse response of all bone cells to PTH is clearly demonstrated. Osteoclasts and mesenchyme cells are stimulated, osteoblasts are depressed, and osteocytes are immediately depressed, but return to normal within 8 hours.
3. **Comparison of parathyroid hormone and calcium ion effects on RNA synthesis**

High calcium content in lavage fluid stimulated incorporation of tritiated precursor into RNA of bone cells, and its effect was similar to the effect of endogenous PTH on bone cells. It was demonstrated clearly that this hypercalcemic effect was induced by the calcium ion itself and not by surgical effects or secretion of thyrocalcitonin evoked by hypercalcemia. This effect was observed in fraction B in which there was a net increase in RNA synthesis, and also in fraction C in which there was a net suppression in incorporation of tritiated precursor into RNA fraction. A difference between endogenous PTH and high calcium was observed, however, after 8 hours of lavage for RNA stimulation produced by calcium reached a maximum early and leveled off while the PTH stimulated RNA synthesis continuously increased. Another influence was transient effect of calcium on the stimulation of RNA synthesis by high calcium while endogenous PTH continued to increase even after the stimulation of secretion of RNA was terminated. Differences were also observed in the DNA synthesis in fraction B, which was stimulated only following increased PTH secretion. This will be discussed later.

The similarity of effect of calcium ion to that of PTH has been observed in another aspect of metabolism related to calcium and bone. Isolated perfused rat hearts exposed to increased calcium concentration demonstrated a marked activation of phosphorylase within 15 seconds of treatment (Friesen et al., 1966). This activation of phosphorylase is normally caused by cyclic AMP. The possibility of PTH action through cyclic AMP has also been suggested by Wells and Lloyd (1968) and by
Chase and Aurbach (1968) in studies on kidney tissue. Metabolism of citric acid, an organic acid often considered to be important in PTH-induced resorption of bone, also was inhibited by an increased concentration of calcium ion (Simpson, 1967). Delong and L'Heureux (1968) observed that the metabolism of pyruvate-2-\(^{14}\)C, measured by the evolution of \(^{14}\)CO\(_2\) in bone homogenates, was stimulated under the hypercalcemic conditions of incubation media containing \(14\, \text{mg}\%\) calcium; the last production was observed in media containing \(6\, \text{mg}\%\) calcium. The same metabolic effect was also induced by PTE.

A transient effect of calcium was reported by Costello and Wynn (1968) who observed that calcium ion stimulated a temporary increase in mitochondrial respiration in the absence of phosphate \textit{in vitro}. Johnston \textit{et al} (1965), who measured the effect of calcium infusion on bone matrix metabolism, reported similar transient results. Rats were killed 3 hours after the injection of calcium, and aspects of bone metabolism were measured \textit{in vitro}. The results indicated that the effects of calcium ions were dissipated by this time.

The known effects of calcium ions on physiological processes are numerous and many undiscovered influences undoubtedly exist. Calcium has been shown to inhibit Na-K activated ATPase (Epstein and Whitman, 1966) and stimulate the release of epinephrine (Greenberg and Kolen, 1966). The possibility that the calcium effect is due to the secretion of thyrocalcitonin is excluded from the study of the effect of calcium in thyro-parathyroidectomized rats. Thyrocalcitonin injection, itself, did not change the rate of nucleoside incorporation and the calcium effect was observed in rats without thyroid-parathyroid complex. The possibility
that the calcium effects were produced through secretion or release of other endocrine compounds of through physiological processes which only occur in vivo may be eliminated by the observation of Cooper and Talmage (1965) where increased concentration of calcium ions in the medium in vitro, as well as PTH administration in vivo, inhibited collagen biosynthesis. The reports of Simpson (1967) and Delong and L'Heureux (1968) also lend support to these observations.

The real basis for the similarity of effects between calcium ions and PTH may remain obscure until other aspects of these two processes and the mechanism of PTH function itself are more completely defined. One possible explanation is that the hormone induce lysis or resorption of bone. In turn this may produce a local hypercalcemia around bone cells, a situation which could be mimiced by an increased concentration (Belanger et al, 1963). Another possibility is that both calcium and PTH may lead to increase in intracellular calcium. This postulate is favored both by Borle and Talmage. Intracellular increases in calcium ion content due both to increasing calcium concentration of the medium and by addition of PTH were reported by Borle (1968). If calcium ion transport is passive (Borle, 1968) and is influenced by the extracellular calcium level, it may be that PTH primarily affects cell membranes by permitting passage of calcium inward and thereby inducing intracellular increase in calcium. Talmage (1968) has recently explained Borle's findings and the results reported in this thesis by such a process, and suggests that all other actions of PTH result from the production of increased intracellular concentration of calcium. This would explain not only RNA studies, but all the studies of Belanger (Belanger et al, 1963) on osteolysis.
around osteocytes. It also accounts for the findings of Johnston et al (1962) who identified intermediates produced by PTE stimulated bone fragments, which were active in inducing metabolic changes previously attributed to the action of PTE itself. In a similar type of system, Douglas and Poisner (1961) reported that calcium ions were an immediate stimulus for the release of certain catecholamine in vitro and suggested that same factor was involved in action on the cell membrane leading to an increased influx of calcium ions. Also McCann (1963) has recently proposed the theory on the action of the releasing factors of the hypothalamus on anterior pituitary cells. The releasing factors act first on the cell membranes to increase the passage of calcium into the cells, and in turn release hormone from cells.

It is of interest to note that, for several years now, Talmage (1962) has been proposing the theory that parathyroid hormone has a dual role in bone, one to cause the transformation of bone cells and thereby increase the rate of bone remodelling and the other to transport calcium from bone to extracellular fluids against a concentration gradient. The similarity of effects of calcium ions and PTH on RNA synthesis would be a basis for changes in cellular activities, the movement of calcium ions into the cell; these could also be involved in transport of calcium between body compartments.

The studies reported here also demonstrated that the effect of high calcium challenge on RNA synthesis in bone cells was not seen in animals administered pharmacological doses of cortisone. This would agree with the studies of Peck et al (1967) in which it was reported that, in a system utilizing isolated bone cells, hydrocortisone inhibited the cells
of RNA synthesis within 5 hours. A further extension of our studies which must be undertaken, is the study of the effect of cortisone on the increase in RNA synthesis produced by parathyroid stimulation. Concurrent studies in our laboratory have demonstrated that cortisol, a similar adrenal steroid, does not inhibit parathyroid influence on calcium transfer from bone to extracellular fluids. If adrenal corticoids inhibit the RNA effect of both high calcium and PTH, but not the calcium transfer effect of the hormone, it would suggest that the action of the hormone at the cell membrane was a primary effect.

D. Parathyroid hormone and DNA synthesis

Parathyroid hormone increases the rate of mitosis in HeLa cell cultures (Borle and Neuman, 1965) after 1 day of treatment, and $^3H$-thymidine incorporation in calvaria was stimulated after 4 days of culture with PTE in vitro (Martin et al, 1965). In our studies the response in stimulation of DNA synthesis, measured by $^3H$-thymidine uptake, was shown in metaphysis as well as in diaphysis of long bone after only 4 hours of stimulation of endogenous PTH. The experiment was terminated after 8 hours of stimulation and it is not known whether or not this stimulation lasted longer than this period. However, 24 hours after nephrectomy, a condition which markedly stimulates the secretion of PTH, metaphyseal bone incorporation of $^3H$-thymidine decreased to below the control level while that of the shaft remained higher than the normal level. It is known, however, that the pathological effects of nephrectomy are first seen in the metaphyseal area of bone. Perris and Whitfield (1967) have claimed an even more rapid response in metabolic activity
following PTH administration. The tissues examined were bone marrow and thymus, and the response was noted in two hours.

The question asked in our work is "Why did not calcium administration also cause an increase in the stimulation of $^3$H-thymidine incorporation into bone cells?" If we base the stimulation of RNA synthesis as increased intracellular calcium caused either by increasing extracellular calcium or by the opening of the membrane to calcium by PTH, one would expect a calcium load to mimic the effect of PTH in stimulation of DNA synthesis as well as of RNA. There are reports of such DNA synthesis stimulation by calcium ion in other cell types. For thymus cells and fibroblast cells of the mouse, Perris and Whitfield (1967) reported a marked stimulation of DNA synthesis following increased calcium ion concentration in the extracellular fluid. However, no change in the rate of DNA synthesis could be observed in certain strains of HeLa cells in an in vitro system (Sinclair, 1968).

The only conclusion that can be reached is that different cell types display permeabilities to the calcium ion. Those cells which are stimulated by PTH to undergo mitosis are essentially unresponsive to calcium except under hormonal stimulation. In the absence of hormone, increase in extracellular calcium produce little if any effect. Such a postulate, obviously, needs further confirmation.
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