INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeib Road
Ann Arbor, Michigan 48106
75-22,000

CARNES, David Lee, Jr., 1946-
SOME ASPECTS OF PURINE METABOLISM IN
ISOLATED BONE CELLS.

Rice University, Ph.D., 1975
Biology

Xerox University Microfilms, Ann Arbor, Michigan 48106
RICE UNIVERSITY

SOME ASPECTS OF PURINE METABOLISM IN ISOLATED BONE CELLS

by

DAVID L. CARNES, JR.

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Thesis Director's signature:

[Signature]

Houston, Texas

November, 1974
ACKNOWLEDGEMENTS

This work was carried out in the laboratory of Dr. J. W. Campbell to whom I express my sincere gratitude for guidance throughout the course of this investigation and for invaluable aid in the preparation of this manuscript.

I also wish to express my appreciation to Dr. F. B. Rudolph, Dr. S. Subtelny, and Dr. C. W. Philpott for their many helpful discussions and for their critical reading of the manuscript.

In addition my thanks go to Gwynfryn Hopkins for his invaluable technical assistance in carrying out the microscopy and to Ruth Parks, my typist, for her special efforts in the preparation of this manuscript.

Sincere thanks also go to Dr. Jean Vorhaben for many valuable discussions and for her unending encouragement.

Finally, I am especially grateful to my wife, Paula, for her trust, patience, and encouragement during this period of research.

This research was supported by a training grant from the National Institute of Dental Research, number 5-T01-DE-118-11. I gratefully acknowledge this support.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>i</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ii</td>
</tr>
<tr>
<td>List of Micrographs</td>
<td>ii</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Purine Metabolism</td>
<td>3</td>
</tr>
<tr>
<td>De Novo Synthesis</td>
<td>4</td>
</tr>
<tr>
<td>Purine Salvage and Interconversion</td>
<td>7</td>
</tr>
<tr>
<td>Purine Supply</td>
<td>12</td>
</tr>
<tr>
<td>Precursor Incorporation</td>
<td>14</td>
</tr>
<tr>
<td>Purines and Bone Metabolism</td>
<td>17</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td></td>
</tr>
<tr>
<td>Bone Cell Isolation</td>
<td>25</td>
</tr>
<tr>
<td>Incubation Procedures</td>
<td>33</td>
</tr>
<tr>
<td>RNA Extraction</td>
<td>34</td>
</tr>
<tr>
<td>Chromatographic Procedures</td>
<td>34</td>
</tr>
<tr>
<td>Measurement of Radioactivity</td>
<td>38</td>
</tr>
<tr>
<td>Microscopy</td>
<td>38</td>
</tr>
<tr>
<td>Materials</td>
<td>39</td>
</tr>
<tr>
<td>EXPERIMENTAL RESULTS</td>
<td></td>
</tr>
<tr>
<td>De Novo Purine Biosynthesis</td>
<td>43</td>
</tr>
<tr>
<td>Purine Salvage and Interconversion</td>
<td>47</td>
</tr>
<tr>
<td>Hormonal Effects on Purine Salvage and Interconversion</td>
<td>58</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>69</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>73</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1  $^{14}$C-Glucose Metabolism in Isolated Bone Cells ................................. 28
Table 2  De Novo Purine Biosynthesis in Isolated Bone Cells
          a) Acid Soluble Fraction ............... 45
          b) RNA Fraction .......................... 46
Table 3  Conversion of $^{14}$C-Adenine into other Purine Compounds .................. 52
Table 4  Conversion of $^{14}$C-Hypoxanthine into other Purine Compounds
          a) 15 minute incubation ................. 53
          b) 60 minute incubation .................. 54
Table 5  Conversion of $^{14}$C-Guanine into other Purine Compounds .................. 55
Table 6  Conversion of $^{14}$C-Adenosine into other Purine Compounds
          a) 30 minute incubation ................. 56
          b) 15 minute incubation .................. 57
Table 7  Conversion of $^{14}$C-Adenosine into other Purine Compounds in the Presence of Thyrocalcitonin ..................... 63
Table 8  Conversion of $^{14}$C-Hypoxanthine into other Purine Compounds in the Presence of Thyrocalcitonin ................. 64
Table 9  Conversion of $^{14}$C-Adenosine into other Purine Compounds in the Presence of Parathyroid Hormone .................. 65
Table 10 Conversion of $^{14}$C-Hypoxanthine into other Purine Compounds in the Presence of Parathyroid Hormone ................. 66
Table 11 Incorporation of $^{14}$C-Purine Precursors into RNA in the Presence of Hormone .... 67
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Purine Biosynthesis De Novo</td>
<td>5</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Pathways of Purine Salvage and Interconversion</td>
<td>8</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Precursors of the Purine Ring System</td>
<td>16</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Flow Diagram of $^{14}$C-Adenine Metabolism</td>
<td>52</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Flow Diagram of $^{14}$C-Hypoxanthine Metabolism</td>
<td>53</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Flow Diagram of $^{14}$C-Guanine Metabolism</td>
<td>55</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Flow Diagram of $^{14}$C-Adenosine Metabolism</td>
<td>56</td>
</tr>
</tbody>
</table>

## LIST OF MICROGRAPHS

<table>
<thead>
<tr>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minced Bone Before Collagenase Treatment</td>
<td>29</td>
</tr>
<tr>
<td>Minced Bone After Collagenase Treatment</td>
<td>30</td>
</tr>
<tr>
<td>Isolated Cell From Centrifuged Pellet</td>
<td>31, 32</td>
</tr>
</tbody>
</table>
LITERATURE REVIEW
INTRODUCTION

Realization of the important functions of purine nucleotides in a variety of cellular processes has stimulated interest in the measurement of these metabolites in cells and cell extracts. Purine nucleotides act as carriers and reservoirs of chemical energy; they participate as cofactors, activators, inhibitors and substrates in many enzymatic reactions; and they are precursors of nucleic acid formation. Most of these processes exhibit some type of dependence on the concentration of the participating nucleotide since, in many cases, the rates of reactions are controlled by nucleotide concentration. To establish the existence of such control in vivo, it is necessary to measure intracellular nucleotide concentrations. This is then a time when an increasing number of experiments are focused on the behavior of purine nucleotide pools and nucleotide transport, and knowledge of the factors which affect or control these parameters is beginning to accumulate for a great many tissues.

However, information about many of the basic features of purine ribonucleotide metabolism and its regulation in bone is lacking. Evidence that purine ribonucleotides can be synthesized from preformed purines and from non-purine precursors by bone is not available in the literature; and, although it is clear that bone must utilize purine bases,
little is known about the operation of the pathways of purine ribonucleotide metabolism as an integrated and regulated whole. This study was directed toward the identification of the pathways of synthesis, interconversion, and catabolism of purines as occurs in bone cells in vitro.

PURINE METABOLISM

Although a complete review of purine metabolism will not be attempted, it is necessary to establish a basic understanding of the pathways involved. The ensuing discussion will therefore be focused on the basic aspects of the metabolic pathways of purines as they operate in animal cells and tissues.

All cells need a balanced supply of purine nucleotides. Maintenance of balanced intracellular concentrations is essential for energy metabolism, protein and nucleic acid synthesis, and the various more specialized functions in metabolism of the purine nucleotides. In virtually all cells and organisms two fundamentally different pathways exist for synthesis and supply of purine nucleotides (Kornberg, 1957). One pathway involves the synthesis de novo from small molecule precursors. These are combined in successive reactions to form nucleotides directly. The second is the use of preformed purines from exogenous sources or produced by the breakdown of nucleic acids. To this second pathway the term
"salvage" is applied, although this should not be taken to mean that this is of secondary physiological importance to synthesis de novo (Roux, 1973).

Synthesis De Novo

A summary of the pathway for IMP synthesis in animal cells is given in Figure 1. Only certain key features of the pathway warrant discussion here.

Of primary importance is the fact that the initial purine compound formed through synthesis de novo is neither a free base nor nucleotide, but rather the nucleotide inosinic acid (Greenberg, 1951; Schulman and Buchanan, 1952). In addition, all intermediates of the pathway are 5'-phosphoribosyl derivatives. The metabolically functional nucleotide forms of adenine and guanine (AMP and GMP) are formed directly from inosinic acid (IMP) (Henderson and Khoo, 1965).

The agent contributing the phosphoribosyl unit in IMP synthesis is phosphoribosyl pyrophosphate (Hartman et al., 1955; Hartman et al., 1956). This compound also contributes the phosphoribosyl unit in the direct conversion of the free bases to their nucleotides in the salvage pathway. Phosphoribosyl pyrophosphate also participates in the formation of orotidylic acid in pyrimidine biosynthesis de novo and in nicotinamide coenzyme production. These facts have regulatory
Figure 1
Purine Biosynthesis De Novo

Guanine

5'-dGMP

Pi

Phosphoribosylformamide

Phosphoribosylglycinamide

5'-dAMP

Phosphoribosylaminomimidazole

Carboxamide

5'-dIMP

Phosphoribosylaminomimidazole

Carboxamide
significance since the levels of phosphoribosyl pyrophosphate are generally believed to be a rate limiting factor in the de novo purine pathway (Blakeley and Vitols, 1968; Rosenbloom, 1968; Murray, 1971). Depletion of phosphoribosyl pyrophosphate by exogenous purine (Henderson and Khoo, 1965) or excess orotic acid (Rajalakshmi and Handschumacher, 1968) can shut down de novo purine nucleotide synthesis.

The intermediate 5-amino-4-imidazole carboxamide (AIC) ribonucleotide is also of some importance. The inability of some cell types, namely the mature mammalian red cell, to synthesize purines de novo has been found to be due to a defective first portion of the pathway (Fontenelle and Henderson, 1969b). But these cells will incorporate AIC and AIC riboside into purines providing evidence for the existence of at least part of the complete synthetic pathway (Lowy and Williams, 1960).

Finally, the main site of purine synthesis in animals is the liver (Smellie et al., 1958) although several extrahepatic tissues have also been reported to possess the capability. Brain (Howard et al., 1970), adrenal cortex (McKerns and Ryschkewitsch, 1973), and corpora lutea (McKerns, 1973) all have been shown to possess the de novo pathway.
Purine Salvage and Interconversion

Some mammalian tissues such as red blood cells (Lowry et al., 1962) and platelets (Holmsen and Rozenberg, 1968) are incapable of forming purine nucleotides de novo; and bone marrow (Lajtha and Vane, 1958) and leukocytes (Scott, 1962) have a very limited capacity for such synthesis. These tissues must therefore rely on an exogenous supply of preformed purines. The utilization of preformed purines by all cells is mediated by the purine phosphoribosyl transferase enzymes which convert free purines to their monophosphate ribotides (Williams and Buchanan, 1958). This is the so-called "salvage" pathway (Kornberg, 1957), and while the de novo route of purine synthesis is the same in all normal cells, the salvage routes are far more diverse in their nature and distribution (Hauschka, 1973), and there are inconsistencies in the information available for the salvage pathways in animal cells.

The known pathways for purine salvage and interconversion in animal cells are summarized in Figure 2. IMP is a central compound since both triphosphate precursors of nucleic acids -- ATP and GTP -- are derived from it. The pathways leading to AMP and GMP involve more than one enzyme and are not freely reversible by the same route. In the absence of exogenous purine sources, all adenine nucleotides are formed from IMP by way of the intermediate adenylosuccinate (Hartman, 1970). This intermediate is a product of
Figure 2
Pathways of Purine Salvage and Interconversion

1. 5'-Nucleotidases
2. Guanine deaminase
3. Adenosine deaminase
4. AMP deaminase
5. Nucleoside phosphorylases
6. Adenine phosphoribosyltransferase
7. Hypoxanthine-guanine phosphoribosyltransferase
8. Adenosine kinase
9. Adenylosuccinate synthetase
10. Adenylosuccinate lyase
11. IMP dehydrogenase
12. XMP aminase
13. Xanthine oxidase
14. GMP reductase

De Novo Synthesis

AMP → Adenylosuccinate → IMP → XMP → Guo → GMP → Nucleotides
the condensation of L-aspartic acid and IMP, catalyzed by adenylosuccinate synthetase. The concentration of aspartate has been shown to be a rate limiting factor (Crabtree and Henderson, 1971), and the aspartate analogue hadacidin is a very strong reversible inhibitor of the reaction (Shigeura and Gordon, 1962; Hartman, 1970). Cleavage of the adenylosuccinate to form AMP is catalyzed by adenylosuccinate lyase—an enzyme which also participates in the de novo pathway (see Figure 1, reaction 6). All guanine nucleotides are normally derived from IMP by the intermediate formation of xanthosine ribotide (XMP). This dehydrogenation reaction is catalyzed by IMP dehydrogenase (McFall and Magasanik, 1960). The second step, amine transfer to XMP by the enzyme GMP synthetase, utilizes L-glutamine. The concentration of glutamine has been shown to be rate-limiting (Ravio and Seegmiller, 1973b; Fontenelle and Henderson, 1969a; Herschko et al., 1967; Crabtree and Henderson, 1971), and the glutamine analogue 6-diazo-5-oxo-L-norleucine (DON) inhibits the reaction (Sonne et al., 1956; Levenberg et al., 1956).

The interconversion of compounds in the adenine and guanine nucleotide pools must proceed through IMP, and since the synthetic routes are not reversible, other enzymes exist that make this interconversion possible. The enzyme AMP deaminase is essential for the conversion of adenine compounds to guanine compounds (Hauschka, 1973). Conversely, the conversion of guanine compounds to adenine compounds is
directly dependent on the enzyme GMP reductase (Lowy et al., 1962; Lowy et al., 1961; Mager and Magasanik, 1960; Guarino and Yüregir, 1959). AMP deaminase has been found in a wide variety of tissues (Lowenstein, 1972), but GMP reductase has limited distribution and generally low activity in animal cells. This accounts for the limited ability of guanine precursors to enter the adenine nucleotide pool (Hartman, 1970). However, in some mammalian cells such as reticulocytes and erythrocytes, both xanthine and guanine are effective precursors for adenine nucleotides (Cook and Vibert, 1966).

The purine bases are incorporated into nucleotides by way of phosphoribosylation utilizing phosphoribosyl pyrophosphate in the presence of their respective phosphoribosyl transferases (Raivio and Seegmiller, 1970). Two purine phosphoribosyl transferases have been found — one having a specificity for adenine and 5-amino-4-imidazole carboxamide, the other acting on hypoxanthine, guanine and to a limited extent xanthine (Hartman, 1970; Krenitsky et al., 1969a,b; Murray et al, 1970; Murray, 1971). As previously mentioned, the supply of phosphoribosyl pyrophosphate is a very important factor in determining the rate of conversion of free bases to their respective purine nucleotides.

Purine nucleoside monophosphates are hydrolyzed to their respective nucleosides by 5'-nucleotidases (Kit, 1970). Further degradation of nucleosides to free bases occurs by
phosphorolysis with the exception of adenosine. This nucleoside is deaminated to inosine by adenosine deaminase, an enzyme which has widespread distribution and is distinct from AMP deaminase (Hoagland and Fisher, 1967). The phosphorolytic cleavage of purine nucleosides yields pentose-1-phosphate and free purine base. This reaction is reversible, but it is not clear whether the equilibrium favors nucleoside formation or breakdown in all tissues (Hartman, 1970). In addition, the phosphorylases catalyze slow base exchange between free purines and their nucleosides (Paterson and Simpson, 1965; Reichard and Sköld, 1958; Murray et al., 1970).

Finally, it should be pointed out that adenosine kinase is the only purine ribonucleoside kinase generally found in animal cells (Green and Ishii, 1972). Of the naturally occurring purine ribosides, only adenosine serves as a substrate for the enzyme (Kit, 1970).

It is evident that purine salvage and interconversion is one of the more complex metabolic processes because of the existence of many alternative pathways for a given intermediate. Only intensive investigation on the particular tissue of interest can clarify the pathways peculiar to it. Extrapolation from known pathways in one tissue can only be used as a guide in determining potential experimental approaches in another tissue.
PURINE SUPPLY

From the foregoing discussion, it can be seen that nucleotides can be formed by a de novo pathway from simple metabolites, or alternatively from preformed purine bases or nucleosides. It should also be evident that differences in the dependence of certain cell types upon the synthesis de novo of purine nucleotides or the utilization of preformed purines must exist, since there are cell types and tissues that possess both the de novo pathway and the capacity to utilize preformed bases. The relative dependence upon one pathway or the other may be an important factor in the particular cell or tissue metabolism. In addition, there are those tissues which, even though they possess the de novo pathway, cannot make sufficient purine nucleotides for their requirements and which must therefore also depend upon a supply of purines formed in some other tissue.

The liver, with its great capacity for de novo purine synthesis, is of primary interest as a source of purines (Henderson and LePage, 1959a; Lajtha and Vane, 1958; Mager et al., 1967). The dependence of bone marrow cells on the liver for their purine supply was established by Lajtha and Vane (1958). Intact rabbits incorporated $^{14}$C-formate readily into adenine and thymine of marrow cell DNA. Hepatectomy or portal occlusion caused a 10-50 fold decrease in the ratio of adenine to thymine incorporation. Earlier
work (Smellie et al., 1956) showed that the addition of liver enzymes needed for synthesis de novo of purines increased the incorporation of $^{14}C$-formate into adenine and guanine of Ehrlich ascites tumor cell RNA in vitro, giving rates similar to those found in vivo. Marrow, neoplastic tumor cells, and other cell types gave similar results (Smellie et al., 1958). From this work it was concluded that the liver in mammals is the main source of exogenous purines for bone marrow cells and possibly for the cells of all other peripheral tissue (Lajtha and Vane, 1958). This conclusion was extended and reinforced by experiments which provided more direct and definitive evidence that the liver is the principal source of purines for other tissues. The basic experiment was to isolate the liver from the rest of the organism, perfuse and label it with radioactive purine precursors, then restore normal circulation and examine nonhepatic tissues for the presence of radioactivity in purines (Pritchard et al., 1970). The transfer of label was indeed demonstrated for many tissues and constituted direct evidence that the liver could act as a source of purine supply. Moreover, the chemical identity of the compounds supplied was limited to hypoxanthine, guanine, or a compound converted to either by the rat red cell.

The importance of the erythrocyte as the vehicle for purine transport had been evident since the dependence of bone marrow on the liver for purine supply was reported.
Early work suggested that the nucleotides in red cells were the transport form for purines utilized by other tissues (Henderson and LePage, 1959a). This work was later confirmed and the detailed biochemical mechanism involved clarified in studies of purine metabolism in the rabbit (Hershko et al., 1967) and human erythrocyte (Mager et al., 1966). The results of these studies confirmed that erythrocytes take up adenine, guanine, hypoxanthine and xanthine and convert them to nucleotides; however, hypoxanthine and xanthine appear to be the only purines released from red cells (Mager et al., 1967). Little or no free adenine or guanine is found in the plasma of rats (Henderson and LePage, 1959b). The major plasma purines are the 6-oxypurines hypoxanthine and xanthine (Bennett and Krueckel, 1955). Furthermore, consistent with these reports in plasma, it has been reported that xanthine is the main 6-oxypurine present in the liver (Reid and Stevens, 1958). Thus it seems reasonable to state that the 6-oxypurines are most important in purine supply to other tissues and that the liver and erythrocytes play a major role in this supply.

PRECURSOR INCORPORATION

The use of isotopic precursors is the method of choice for the study of purine ribonucleotide pathways, with special attention given to the probable fate of the various precursors.
The enzymatic activities in the system of interest, the accessibility of the precursor to these pertinent enzymatic activities, and any feedback effects of the precursor or related metabolites on the activities are primary factors to consider when determining the precursor fate (Hauschka, 1973). A knowledge of the expected metabolites which can be derived from a labeled precursor is also valuable in designing experiments and choosing analytical systems.

Figure 3 illustrates the metabolic origin of each of the carbon and nitrogen atoms of the purine ring. These assignments have been fully corroborated by subsequent studies on the synthetic pathway (for review see Hartman, 1970; Henderson, 1972). Observations from many laboratories have established that the incorporation of labeled precursors into adenine and guanine compounds in all cases occurs as anticipated from Figure 3. Thus the incorporation of $^{14}\text{C}$-glycine or $^{14}\text{C}$-formate into purines is the conventional method of assessing the synthesis de novo of these compounds.

The literature regarding the incorporation of preformed purine precursors has been extensively reviewed (Murray et al., 1970). $^{14}\text{C}$-hypoxanthine and $^{14}\text{C}$-adenine are the more commonly used purine precursors because their conversion to nucleotides is generally more efficient than for other purines. Guanine can be rapidly degraded without significant incorporation into nucleotide pools. $^{14}\text{C}$-hypoxanthine has been shown to enter the adenine and guanine nucleotide pools
Figure 3
Precursors of the Purine Ring System

- Glycine
- Formate
- Glutamine
- CO₂
- Aspartate
- Formate

N1, N2, N3, C4, C5, C6, C8, N9
in equivalent amounts in most tissues, while $^{14}$C-adenosine is incorporated into the adenine nucleotide pool rather selectively. Of the free guanine which is not degraded, most remains in the guanine nucleotide pool with little if any conversion to adenine compounds. With the exception of adenosine, labeling with purine nucleosides does not usually offer any distinct advantages. This is because no nucleoside kinases with a high affinity for inosine or guanosine have been reported, and rapid degradation of these nucleosides to their free bases occurs as a result of nucleoside phosphorylase activity. However, the presence of adenosine kinase usually allows for rapid incorporation of this compound and better than 90 per cent of the radioactivity of this precursor is generally found in adenine nucleotides. Thus $^{14}$C-precursors and the proper analytical systems make it possible to investigate which specific salvage and interconversion pathways are operational in a given tissue.

PURINES AND BONE METABOLISM

Although the complex hormonal environment required to support physiological bone growth is partially understood, the precise metabolic responses of bone-forming cells to specific hormones and hormone combinations are largely unknown. Reports of the effects of specific hormones on the metabolism of isolated bone cells in vitro suggest that
an understanding of purine pathways in these cells may shed some new light on the problem. For example, glucocorticoids are known to alter bone cell nucleoside and RNA metabolism (Peck et al., 1967; Peck et al., 1969) by inhibiting the incorporation of radioactive nucleosides into the intracellular free nucleotide pool -- an effect which appears to result from decreased nucleoside phosphorylation. Glucocorticoids also appeared to deplete bone cells of ribosomal RNA by inhibiting RNA synthesis and accelerating its breakdown.

Other reports indicate that insulin stimulates the transport or phosphorylation or both of pyrimidine nucleosides in isolated bone cells (Peck and Messinger, 1970; Peck et al., 1971). Although the physiological significance of this is unknown, it has been suggested that the phosphorylation of pyrimidine nucleosides may contribute significantly to cellular nucleotide pools during periods of tissue growth and proliferation.

The large number of morphological and metabolic changes in bone after parathyroid hormone treatment, and the inference that at least one of the biological effects of the hormone, the hypercalcaemic response, is dependent upon the formation of new RNA has prompted several investigators to examine RNA synthesis in bone (Steinberg and Nichols, 1967; Steinberg and Nichols, 1971; Wermeskerken, 1968). Osteoclast response to parathyroid hormone has also been studied using
a variety of radioactive precursors administered in vivo. The hormone has been reported to increase the rate of \(^3\text{H}\)-uridine incorporation into cellular RNA (Bingham and Owen, 1968; Bingham et al., 1969). Furthermore, it has been reported, without reference to cell type, that both RNA and DNA synthesis are increased in bone cells in vivo following parathyroid administration (Park and Talmage, 1968).

In view of these hormonal effects on RNA metabolism and the implied effects on cellular nucleotide pools, it seems critical to understand the basic features of purine nucleotide metabolism in bone cells, especially in light of recent reports indicating hormonal effects on purine nucleotide biosynthesis in other tissues. ACTH is now known to stimulate purine nucleotide biosynthesis in adrenal cortex (McKerns and Ryschkewitsch, 1973). Gonadotropins have a similar effect in corpora lutea (McKerns, 1973).

Furthermore, one of the best characterized effects of parathyroid hormone upon bone cells is an increase in cellular cyclic-AMP levels. This has been convincingly demonstrated by Chase and co-workers (Chase and Aurbach, 1970; Chase et al., 1969; Aurbach and Chase, 1970; Aurbach et al., 1972). It is also known that thyrocalcitonin acts to increase cyclic-AMP concentration by affecting a separate, independent site (Rodan and Rodan, 1974). There
is mounting evidence in the literature that purine nucleotides may be of importance in modulating the response of adenyl cyclase to hormone activation. It has been shown that GTP is required for glucagon stimulation of adenyl cyclase in plasma membranes from rat liver (Rodbell et al., 1971; Rodbell et al., 1974), for prostaglandin E₁ activation of platelet membranes (Krishna et al., 1972), for glucagon stimulation of β-cell tumors of the golden hamster (Goldfine et al., 1972) and for β-andrenergic stimulation in turkey erythrocytes (Bilezikian and Aurbach, 1973). These reports suggest that the responsiveness to nucleotides may be a wide-spread, if not universal, characteristic of adenyl cyclase and may provide a cellular means for modulation of the enzyme in response to hormonal stimulation (Bell, 1973).

Similarly, it has been demonstrated that exogenous adenosine rapidly increases cyclic-AMP levels in guinea pig cerebral cortex slices (Sattin and Rall, 1970). Other experimental evidence exists which suggests that adenosine may be a physiological regulator of cyclic-AMP metabolism in brain (Rall and Sattin, 1970), platelets (Mills and Smith, 1971), and adipose tissue (Pain et al., 1972) and, very recently, a similar effect on bone cells by adenosine has been suggested (Peck et al., 1974). In view of the pattern of modulation and interrelationship which is emerging between purine nucleotides and related compounds and
cyclic-AMP, and since cyclic-AMP levels are closely related to the effects of two of the more important hormones which act on bone, there is obviously a need to elucidate the operation of purine nucleotide pathways in bone.

In addition to their indirect effects in bone cells, purine nucleotides have also been directly implicated in the mineralization process. Leonard et al. (1971) have postulated a cyclic process for the mineralization of skeletal tissue in which ATP present at the mineralizing site reacts with calcium ion to form a Ca-ATP complex. This complex would then be hydrolyzed, first to pyrophosphate and later to hydroxyapatite. During the process, adenosine and orthophosphate would be formed and would combine to regenerate ATP. The process was tested using embryonic chick femur with regard to adenosine formation and phosphorylation, and it was shown that ATP could be hydrolyzed to adenosine and regenerated. Further testing of this hypothesis has not been done. There also appears to be a relationship between alkaline phosphatase and 5'-nucleotidase in human bone (Goldberg and Belfield, 1974). The presence of alkaline phosphatase in bone has been known for many years (Robison, 1923; Robison and Soames, 1924) although its role in bone growth and mineralization is controversial (Robison, 1923; Jibril, 1967; Kuhlman, 1965; Woltgens et al., 1971; Strates et al., 1971).
5'-Nucleotidase has also been known to occur in bone and teeth for several years (Gibson and Fullmer, 1967; Mäkinen and Paunio, 1970). However, in spite of the fact that it is more active in bone at physiological pH's than alkaline phosphatase, it has not been studied extensively (Reis, 1951) although it has been suggested that the alkaline phosphatase/5'-nucleotidase ratio may be a mechanism for suppressing bone growth or turnover in long bones. Since changes in the 5'nucleotidase activity would be expected to alter the availability of nucleic acid precursors, this is another example where an integrated knowledge of purine ribonucleotide pathways might be of value.

Finally, the problem of outstanding interest in bone research today is the role played by the osteocyte in bone metabolism and calcium homeostasis (Vaughn, 1968). Any understanding of the metabolic pathways which operate in this cell type should be a welcome addition to the body of knowledge available to investigators as they attempt to elucidate this unknown role and its biochemical and cellular mechanisms of action.

Thus in spite of the obvious need for maintenance of precursor pools for RNA synthesis, the several known effects of purine nucleotides and adenosine on cyclic-AMP levels, and the implication of ATP in calcification, there have been no systematic investigations of the pathways of purine
metabolism in bone cells. The de novo synthesis rates are unknown and no investigations of the alternative mechanism for maintenance of purine nucleotide pools -- salvage of purine bases and nucleosides -- have been undertaken. Indeed, there have been no studies comparing the pathways of nucleotide synthesis from a number of purine precursors. In short, little is known about the operation of the pathways of purine metabolism in bone as an integrated whole. It was therefore the objective of this study to determine if bone cells are capable of synthesizing purines de novo, and if so, to what extent this synthesis contributes to the purine metabolism of the cell. A further objective was to investigate the patterns of incorporation of preformed purine bases into the cellular nucleotide pool and the interconversions of the purine nucleotides in this pool. The final objective was to ascertain the effects, if any, of parathyroid hormone and thyrocalcitonin on these pathways of incorporation and interconversion.
MATERIALS AND METHODS
BONE CELL ISOLATION

The main experimental system used was the isolated bone cell from newborn rat calvaria. This system was chosen because of its simplicity and because it permits a consideration of bone metabolism without cartilage contamination. In addition, the rat is the classical animal of endocrine research and much of the current research thrust is in the area of the influence of hormones on bone metabolism.

Collagen preparations have been used to digest tissues and disperse cells effectively for many years (Lasfargues, 1957; Hinz and Syverton, 1959; Cavanaugh et al., 1963; Hifner and Brown, 1971; Coon, 1966; Wiebkin and Muir, 1973) without altering their function (Rodbell, 1964). This method was extended to the dispersion of viable cells from rat bone by Peck et al. (1964). Cells were isolated from the frontal and parietal bones obtained from the calvaria of 17-21 day old rat fetuses and newborn rats. The isolated cells were characterized by studies of their glucose metabolism and the impression gained upon histological examination. The cells isolated by collagenous treatment appear to be representative of the entire cell population in the parent bone. This has been reinforced by the similarity in the metabolism of glucose between whole bone segments and isolated cells (Cohn and Forscher, 1962). Further studies (Birge and Peck, 1966) have shown that freshly dispersed
cells could synthesize collagen in suspension and it is generally agreed that the technique permits the direct investigation of bone cell metabolism. Many studies utilizing the technique (Peck et al., 1967; Peck et al., 1974) or slight modifications thereof (Hekkelman and Moskalewski, 1969; Rodan and Rodan, 1974) have appeared providing further evidence that isolated cells do in fact provide a suitable model for the study of the regulation of bone metabolism. One disadvantage of the technique is that the isolated cells are made up of the several different types of bone cells. However, the sophisticated methodology required for the isolation of bone cells separated as to type has not been developed although attempts have been made (Smith et al., 1973; Park and Talmage, 1967). In these attempts to isolate specific cell types, the cell yields are too low for biochemical studies.

To prepare the isolated cells used here, the frontal and parietal bones were dissected as aseptically as possible from the calvaria of newborn rats (less than 24 hours old). Twelve to fifteen newborns were used per isolation. The bones were cleaned of superficial periosteum, dura and cartilage along major suture lines by scraping with a scalpel. As each calvarium was dissected and cleaned, it was rinsed with a jet of isolation buffer from a syringe and placed in a petri dish containing the isolation buffer. The total time necessary to accumulate sufficient calvaria was usually
30 minutes. The isolation buffer was a Hank's balanced saline solution (Hanks and Wallace, 1949), calcium- and magnesium-free, containing 100 units/ml each of potassium penicillin G (U.S.P.) and streptomycin sulphate (U.S.P.).

The calvaria were then minced with scissors and placed in a siliconized Erlenmeyer flask containing 4 ml of the isolation buffer and collagenase (3 mg/ml). The flask was placed in a metabolic bath and incubated for 90 minutes at 37°C with mild shaking.

At the end of the incubation period, the flask was supported at an angle and the debris allowed to settle for 10 minutes. The supernatant fluid was then transferred to a 12 ml siliconized conical test tube and centrifuged 6 minutes at 1000 rpm's in a clinical centrifuge. The resulting supernatant fluid was discarded and the cellular pellet was resuspended in isolation buffer without collagenase and recentrifuged. This wash procedure was repeated two more times. The cells were finally suspended in the incubation medium and standardized to $10^6$ cells by counting in a hemacytometer.

Histologically, the collagenase treated bone showed areas of complete cell removal (micrographs on page 30), suggesting that in those areas affected by the enzyme there was no selective removal of cell type. Furthermore, no definite areas of hematopoietic marrow cells were recognized in sections of undigested bone. An electron microscopic
examination of the centrifuged pellet showed that intact mononuclear cells were present with the morphology of normal bone cells (electron micrographs on pages 31-32) (Cameron, 1972; Jande, 1973).

The viability of the cells was evaluated by vital dye uptake (Peck et al., 1964), dye exclusion (McLimans, 1957), and glucose metabolism. Over 90 per cent of the isolated cells appeared to be viable as evidenced by cytoplasmic staining or dye exclusion after incubation for 30-60 minutes. Studies of glucose metabolism, presented in Table 1, were in agreement with published values of Peck et al. (1964) who reported that inocula of $10^6$ cells converted approximately 0.2 per cent of the glucose present in the medium to carbon dioxide within two hours.

### TABLE 1

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>dpm's</th>
<th>% Conversion $10^6$ cells/hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6,578</td>
<td>0.011</td>
</tr>
<tr>
<td>2</td>
<td>29,809</td>
<td>0.046</td>
</tr>
<tr>
<td>3</td>
<td>35,452</td>
<td>0.061</td>
</tr>
<tr>
<td>4</td>
<td>34,438</td>
<td>0.060</td>
</tr>
</tbody>
</table>

Cells incubated for one hour in Krebs-Ringer phosphate buffer, pH 7.4, 5mM glucose, containing 25μC 14C-glucose(u). Evolved CO₂ trapped in hyamine hydroxide and counted by scintillation spectrometry.
Minced Bone before Collagenase Treatment

Stain -- Methylene Blue/Azure II
Minced Bone after Collagenase Treatment

Stain -- Methylene Blue/Azure II
Isolated Cell from Centrifuged Pellet

(28,000 X)
Isolated Cell from Centrifuged Pellet

(28,000 X)
INCUBATION PROCEDURES

The basic incubation system was identical for all experiments reported in this study. The only variable was the final incubation volume which was 1 ml for the studies on the de nova pathway and 0.5 ml for all performed precursor studies. One million cells were suspended in Krebs-Ringer phosphate buffer (Umbreit et al., 1957) pH 7.4 containing 10mM D-glucose and preincubated for 15 minutes at 37°C with mild shaking. The desired ¹⁴C-labeled precursor was then added and incubation was continued for the required time. The reactions were stopped by the addition of 25 µl of ice-cold 4.2M perchloric acid. The sample was kept cold for 10 minutes before centrifuging at 2000 rpm's in a clinical centrifuge. The resulting acid soluble supernatant fluid was removed and neutralized with 25 µl of 4.42M potassium hydroxide and kept in the cold for 15 minutes to precipitate potassium perchlorate. The acid insoluble pellet was set aside for RNA extraction. The cold, neutralized acid-soluble extract was then centrifuged to remove the precipitated perchlorate and the resulting sample frozen until the desired chromatographic procedures were performed. These were usually begun on the next day.

This procedure was modified slightly when investigating the effects of parathyroid hormone and thyrocalcitonin. After the 15 minute preincubation period, there was a one
minute exposure to 0.1 µg of the desired hormone (5.7 x 10^-2 and 1.1 x 10^-2 units, respectively). The \(^{14}\)C-labeled precursor was then added and the cells incubated for 15 minutes. All other procedures were as described above.

**RNA EXTRACTION**

RNA was extracted by the method of Schmidt and Tannhauser (1945) as modified by Peck et al. (1969). The pellet remaining after perchloric acid precipitation was resuspended in 0.9 ml of 0.3N potassium hydroxide and heated at 37°C in a water bath for a minimum of 18 hours. This served to hydrolyze RNA to its component nucleotides (Munro and Fleck, 1966). DNA and protein were re-precipitated by the addition of 0.1 ml 70 per cent (v/v) perchloric acid followed by standing at 4°C for 30 minutes. It was then centrifuged and the resulting supernatant fluid removed and kept frozen until analyzed for \(^{14}\)C-adenine and \(^{14}\)C-guanine by chromatography.

**CHROMATOGRAPHIC PROCEDURES**

Adenine, guanine and AIC were separated by the method of Wong et al. (in press). One half ml of N hydrochloric acid was added to the neutralized acid-soluble extract along
with 0.5 μmole of each of the unlabeled compounds to act as carrier. The mixture was then heated for 30 minutes in a boiling-water bath to hydrolyze all purine compounds to their free bases. The mixture was cooled and centrifuged to remove any precipitated material. The sample was placed on a 4 x 12 mm column of Dowex 50W-X8 which had been washed to remove chromagenic material. The column was converted to the acid form by washing with 5 ml 3N hydrochloric acid and then water until chloride-free. $^{14}C$-glycine or $^{14}C$-formate and unidentified impurities in the $^{14}C$-AIC precursor were eluted from the column with 4 ml of N hydrochloric acid. Guanine and AIC were eluted with an additional 8 ml N hydrochloric acid. The presence of the desired compounds in their respective fractions was verified spectrophotometrically by scanning from 320 μm to 240 μm on a Beckman DB spectrophotometer. The respective fractions were evaporated to dryness in a Buchler test-tube evaporator or under an infrared heat lamp. The residue was redissolved in 20-50 ul N hydrochloric acid and the entire sample spotted on thin layers of cellulose. The sheets were developed with N-butanol, iso-butyric acid, water and 28 per cent aqueous ammonia (w/v) (37.5 : 18.25 : 12.5 : 1.25, by volume). Adenine and guanine were located under U.V. light. The amount of radioactivity incorporated into these bases was determined by cutting the thin layer sheets into equivalent sections along their entire length and subjecting them to
scintillation spectrometry.

Adenine and guanine from the RNA fractions were also separated as described above. The only modification was adjusting the RNA sample with 5N potassium hydroxide to a pH of 9 to precipitate the perchlorate after heating in the water bath. The sample was kept cold for 30 minutes and then centrifuged and the resulting supernatant fluid made acidic with the addition of 0.5 ml N hydrochloric acid before applying to the column.

The purine bases and ribosides were separated by the two-dimensional method of Pataki (1967). Twenty by twenty cm cellulose thin layer sheets were predeveloped in n-propanol and dried overnight. A standard solution containing, in μg/5μl, uric acid(2), xanthine(2), adenine(2), guanine(2) and hypoxanthine(2) was applied and dried in a stream of cold air. A second standard solution containing, in μg/5μl, adenosine(2.5), guanosine(2.5), inosine(2.5) and xanthosine(2.5) was then applied and dried in the same manner. Twenty-five μl of the acid-soluble fraction from the preformed precursor experiments was applied and allowed to dry. The sheets were developed in the first direction with n-propanol, 15 per cent aqueous ammonia (w/v) and water (6 : 3 : 1, by volume). After drying, the chromatograms were turned 90° and developed in the second dimension with iso-propanol, saturated ammonium sulphate (room temperature) and water (2 : 79 : 19, by volume). The separated compounds
were located under U.V. light and identified by reference to a standard chromatogram. The amount of radioactivity incorporated into each compound was determined by scintillation spectrometry.

The purine ribotides were separated according to the method of Crabtree and Henderson (1971). Polyethyleneimine (PEI) cellulose thin layers were first developed with 4M sodium formate buffer, pH 3.4, dried and then developed overnight with methanol and water (1:1 v/v). Twenty-five μl of acid-soluble extract from the preformed precursor experiments was applied plus a standard solution containing, in μg/5μl, AMP(3), IMP(3), GMP(3), ADP(3), GDP(3), ATP (3) and GTP(3). A wick of Whatman No. 3mm paper was stapled to the top of the sheet, and it was developed overnight with methanol and water (1:1 v/v) to wash salts, bases and ribosides onto the paper wick which was then discarded. For separation of the ribonucleotides, the sheets were developed with increasing concentrations of sodium formate buffers, pH 3.4, as follows: 0.5M formate buffer to a line 2.5 cm above the origin, then 2M formate buffer to a line 7.0 cm above the origin, and finally 4M formate buffer to the top of the plate. After drying, the nucleotide-containing areas were visualized under U.V. light and the radioactivity in each determined by cutting the strips along their entire length and subjecting them to scintillation spectrometry.
MEASUREMENT OF RADIOACTIVITY

Radioactivity was measured by liquid scintillation spectrometry using either a Beckman LS-133 or Packard Tri-Carb model 2002 instrument. Quench curves for both instruments were determined using a set of $^{14}$C standards of known radioactivity and quench. The Beckman instrument was equipped with internal standardization to determine counting efficiency, while this was determined by the channels ratio technique for the Packard instrument.

The scintillation fluid used for counting consisted of 5.5 grams Permablend per liter toluene. Ten ml per counting vial was used. Occasionally, Bray's solution (Bray, 1960) was used to count aqueous based samples when determining the radioactivity of the precursor compounds.

MICROSCOPY

Fragments of minced calvaria from before and after collagenase treatment and the cell pellet remaining after the final wash with isolation buffer were prepared for microscopy in the following manner. The tissue was placed in paraformaldehyde-glutaraldehyde fixative (Karnovsky, 1965) for 180 minutes. The fixative was then decanted and the tissue washed four times with 0.2M cacodylate buffer, pH 7.4, and then left in cold buffer overnight. The tissue was then post-fixed in 0.1 per cent osmium tetroxide (w/v)
in Millonig's buffer, pH 7.4 (Millonig, 1961), for 90 minutes. Post-fixation was omitted in some cases. The post-fixed tissue was rinsed twice in tap-water and dehydrated in ethanol. Ninety-five per cent ethanol was added dropwise to the tissue in tap-water until the volume doubled, and then half the mixture was decanted. This procedure was repeated twice. The tissue was then rinsed in three changes of absolute ethanol of 15 minutes each. This was followed with two rinses in propylene oxide. The tissue was then placed in a 1:1 mixture of propylene oxide and Epon (Luft, 1961) and allowed to set uncovered on top of a 60°C oven overnight. The tissue was then placed in fresh Epon (Luft, 1961) for final embedding. Polymerization took place at 60°C for 36 hours. The tissue was then sectioned for both light and electron microscopy.

MATERIALS

D-glucose $\left[^{14}\text{C} \right]$ 234 mCi/mmole, glycine $\left[^{14}\text{C} \right]$ 102 mCi/mmole, and formic acid, sodium salt $\left[^{14}\text{C} \right]$ 54.3 mCi/mmole were purchased from New England Nuclear, Boston, Massachusetts. Adenine $\left[8^{-14}\text{C} \right]$ 54.2 mCi/mmole, guanine sulphate $\left[8^{-14}\text{C} \right]$ 56 mCi/mmole, hypoxanthine $\left[8^{-14}\text{C} \right]$ 62 mCi/mmole, and adenosine $\left[8^{-14}\text{C} \right]$ 47 mCi/mmole were purchased from Amersham-Searle, Arlington Heights, Illinois.
Collagenase (3.4.4.19) CLS II, 300 units/mg was purchased from Worthington Biochemical, Freehold, New Jersey. Porcine thyrocalcitonin, 116 units/mg and bovine parathyroid hormone, 570 units/mg were generously provided by Dr. Cary Cooper of The University of North Carolina, School of Medicine, Chapel Hill, North Carolina.

All other reagents were purchased from commercial sources and were of the highest available purity.

Eastman Chromagram Sheet 6065 cellulose thin layers with fluorescent indicator were purchased from Scientific Products, Houston, Texas. Baker-flex Cellulose PEI polyethyleneimine thin layers with fluorescent indicator were purchased from J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Permablen™ I (PPO, 91%, Dimethyl POPOP, 9%) was purchased from the Packard Instrument Co., Downers Grove, Illinois.

In order to insure a readily available supply of newborn rats a breeding colony was established. Adult rats were obtained from Texas Inbred Rat Co., Houston, Texas and the Holtzman Company, Madison, Wisconsin. All breeders were between 100-120 days of age on the first mating and were used for subsequent matings until the litter size became too small to be useful. Three to four females were placed in a cage with two males and allowed to remain for twenty days. On the twenty-first day the
females were removed and each placed in a separate maternity cage until a litter was produced or she was found to be non-pregnant. All females were rested for three weeks between birth and the next mating.

In addition to newborns produced within the colony, term pregnant females were purchased from the Holtzman Co. and placed in maternity cages immediately upon arrival until they gave birth. These females were then utilized within the colony. Litter size ranged from ten to eighteen animals per litter.

All animals were maintained on a standard laboratory diet and tap water in a constant temperature room.
EXPERIMENTAL RESULTS
DE NOVO PURINE BIOSYNTHESIS

$^{14}$C-Formate and $^{14}$C-glycine were used to determine the presence of purine biosynthesis de novo. No incorporation of these precursors into adenine and guanine of either the acid-soluble fraction (Table 2a) or the acid-insoluble RNA fraction (Table 2b) was detected in any system employed. This was true in Krebs-Ringer phosphate buffer alone or with aspartic acid, glycine, sodium formate or L-glutamine added. It is possible that the availability of 5-phosphoribosyl-1-pyrophosphate may have been limiting in this experiment, but this seems unlikely since significant hexose monophosphate shunt activity has been demonstrated in bone slices in vitro (Cohn and Forscher, 1962).

While there was no evidence of the complete de novo pathway in the isolated bone cells with either precursor, the existence of a portion of the pathway was considered since other cells, such as the mature mammalian erythrocyte (Lowy and Williams, 1960) and erythrocytes of other vertebrates (Wong et al., in press), although incapable of synthesizing purines de novo, do possess the final steps of the pathway and can carry out the synthesis of the purine ring from sodium formate and 5-amino-1-ribosyl-4-imidazole carboxamide (AIC riboside). In order to determine whether the final reactions leading to the formation of purine nucleotides may occur in bone cells, the cells were incubated with sodium formate and AIC riboside in Krebs-Ringer
phosphate buffer. Labeling of adenine and guanine from both the acid-soluble and RNA fractions resulted as shown in Tables 2a and 2b. It then appears that bone cells possess the enzymatic mechanisms for the formation of 10-formyl-tetra-hydrofolic acid and the final steps of de novo purine synthesis. The inability of the cells to synthesize purines de novo then must result from an absence of one or more reactions prior to the formation of 5-amino-1-ribosyl-4-imidazole-carboxamide-5'-phosphate. It remains to be determined whether this partial pathway is of any significance in the maintenance of purine nucleotides in the cells -- a condition which would depend on the availability of the AIC ribosyl compound to the cell. The only available evidence to this effect has been reports of AIC in human urine (Braunstein and Vilenkina, 1958; McGeer et al., 1961) indicating that it may be a constituent of plasma.

It is concluded from these results that bone cells must have an absolute requirement for externally supplied preformed purine compounds.
TABLE 2a

*De Novo* Purine Biosynthesis in Isolated Bone Cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Adenine</th>
<th>Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Formate-$^{14}$C</td>
<td>0.74</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Adenine</th>
<th>Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Formate-$^{14}$C</td>
<td>0.74</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glycine</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Adenine</th>
<th>Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine-$^{14}$C</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Adenine</th>
<th>Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine-$^{14}$C</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium Formate</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Adenine</th>
<th>Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Formate-$^{14}$C</td>
<td>0.74</td>
<td>2.6</td>
<td>48.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-amino-1-ribosyl-4-imidazole-carboxamide</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$10^6$ cells isolated from newborn rat calvaria incubated in 1 ml of Krebs-Ringer phosphate buffer, pH 7.4, for one hour at 37°C.
**TABLE 2b**

**De Novo** Purine Biosynthesis in Isolated Bone Cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Incubated</th>
<th>Conc.</th>
<th>Isolated RNA Fraction</th>
<th>Adenine</th>
<th>Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μmoles/ml</td>
<td></td>
<td>pmoles</td>
<td>pmoles</td>
</tr>
<tr>
<td>A. Sodium Formate-&lt;sup&gt;14&lt;/sup&gt;C</td>
<td></td>
<td>.74</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose (40μc)</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Sodium Formate-&lt;sup&gt;14&lt;/sup&gt;C</td>
<td></td>
<td>.74</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose (40μc)</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td></td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td></td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Glycine-&lt;sup&gt;14&lt;/sup&gt;C</td>
<td></td>
<td>.5</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose (50μc)</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. Glycine-&lt;sup&gt;14&lt;/sup&gt;C</td>
<td></td>
<td>.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (50μc)</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td></td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td></td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium Formate</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. Sodium Formate-&lt;sup&gt;14&lt;/sup&gt;C</td>
<td></td>
<td>.74</td>
<td></td>
<td>1.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Glucose (40μc)</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-amo-1-ribosyl-4-imidazole-carboxamide</td>
<td></td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

10<sup>6</sup> cells isolated from newborn rat calvaria incubated in 1 ml of Krebs-Ringer phosphate buffer, pH 7.4, for one hour at 37°C.
PURINE SALVAGE AND INTERCONVERSION

The data obtained from the incubation of isolated bone cells with several of the naturally-occurring purines indicated that these compounds could serve, to varying degrees, as precursors of adenine or guanine nucleotides or both. $^{14}$C-Adenine was extensively metabolized to nucleotides and approximately 85 per cent of the label incorporated remained in the adenine nucleotide fraction. $^{14}$C-Guanine was utilized primarily for the formation of guanine nucleotides while $^{14}$C-hypoxanthine served as an effective precursor for both adenine and guanine nucleotides; the ratio of incorporated label from $^{14}$C-hypoxanthine approached 1:1 for these two nucleotide pools. The purine bases were presumably incorporated into nucleotides by way of phosphoribosylation utilizing phosphoribosyl pyrophosphate in the presence of their respective phosphoribosyl transferases. The utilization of the preformed bases for nucleotide formation provides evidence that 5-phosphoribosyl pyrophosphate is, in fact, formed by these cells.

In the one case where data were available, there was one-half as much GMP formed as AMP; even in those cases where no comparison was possible, there was still substantial GMP formed when $^{14}$C-adenine served as the precursor (Table 3). This conversion of adenine compounds to guanine compounds indicated the presence of an active adenylate
deaminase enzyme. When \(^{14}\)C-hypoxanthine was the precursor, incorporation into AMP and IMP was approximately equal but incorporation into GMP was much lower (Table 4a). However, after a longer incubation period (60 minutes as opposed to 15 minutes), the level of GMP was equal to or greater than that of AMP or IMP (Table 4b). Also, after a 60 minute incubation at an increased precursor concentration, incorporation into GMP exceeded that of either AMP or IMP (Table 4b). This represents additional evidence for the presence of adenylate deaminase.

The formation of IMP was also indicated when \(^{14}\)C-guanine served as the radioactive precursor (Table 5). Label also appeared in the adenine nucleotide fraction (not shown), which indicates that the conversion of guanine compounds to adenine compounds takes place in these cells to some extent, although most certainly at a level much lower than the reverse reaction above. The presence of GMP reductase is thus indicated, although its activity may be low. The pathway from IMP to GMP is not freely reversible in mammalian cells (Green and Ishii, 1972), and the presence of the reductase is required to carry out this conversion. Similar enzymes have been reported only in erythrocytes (Herschko et al., 1963 and reticulocytes (Cook and Vibert, 1966) although their activity is low. It is interesting to consider whether this conversion occurs at all in vivo at normal guanine nucleotide levels.
In contrast to the metabolism of the other purine precursors, especially $^{14}$C-adenine and $^{14}$C-hypoxanthine, $^{14}$C-adenosine did not serve as a very effective precursor for nucleotide formation (Tables 6a and 6b). There was substantial incorporation into inosine and hypoxanthine indicating a rapid deamination of adenosine. The low incorporation of label into the nucleotide fraction and especially the failure to detect label in the guanine pool make this data difficult to interpret. The ultimate result of adenosine deamination is the formation of significant levels of hypoxanthine; yet there was no incorporation of label into IMP, and subsequently GMP, by way of the phosphoribosyl transferase reaction. Apparently there was an inhibition of this pathway, although the cause of this is not known. Since the levels of product formed in this study referred only to the radioactive substrates, it is possible that a high endogenous level of IMP inhibited the pathway (Hartman, 1970) and was responsible for diluting any labeled guanine compounds formed to below a level that could be detected. Furthermore, if endogenous aspartate and glutamine concentrations were low, there would be little conversion of IMP to either nucleotide pool. The label which appeared in the adenine nucleotide pool was probably the result of the phosphorylation of the precursor by adenosine kinase, and thus no phosphoribosyl transferase activity was required.
Another complication in interpreting the isotope incorporation data was that the metabolism of the precursor may influence the incorporation-concentration relationship. Such an effect was especially likely for adenosine because the extent of deamination of this nucleoside differs at different concentration levels in other tissues (Namm, 1973; Lomax and Henderson, 1973). Phosphorylation should have a competitive advantage over deamination, provided the concentration of adenosine remains low. The extensive deamination of adenosine in this study probably resulted from the fact that the adenosine concentration was high. The experiments with $^{14}$C-adenosine then gave no indication that it was metabolized to any intermediate before it was incorporated into the adenine nucleotide pool; rather they indicated that the adenosine was utilized for nucleotide synthesis exclusively through a nucleoside kinase reaction. This conclusion was similar to that from studies in the heart (Goldthwait, 1957; Lu and Feinberg, 1971; Wiedmeier et al., 1972) and Ehrlich ascites cells (Lomax and Henderson, 1973). It is also reinforced by data from the other precursor studies. When $^{14}$C-adenine served as the precursor, there was significant adenosine formed while the level of inosine was low. Furthermore, label appeared in IMP. When $^{14}$C-hypoxanthine served as the precursor, no adenosine was formed. Following the formation of AMP from adenine or hypoxanthine, there were two routes by which it could be
converted to inosine: 1) dephosphorylation to adenosine followed by deamination and 2) deamination to IMP followed by dephosphorylation. The data indicate that the second route was probably preferred. Since no adenosine was detected from hypoxanthine, the first route was probably not an important route of AMP breakdown in these cells. The label in inosine and hypoxanthine in the adenine experiment probably resulted from 5'-nucleotidase activity on IMP. The fact that adenosine levels remained high after a one hour incubation period indicated that deaminase activity was not high. Rather adenosine was probably being formed from AMP and continually reprophosphorylated. This has been suggested to occur in Ehrlich ascites cells (Lomax and Henderson, 1973) and may be a normal occurrence in cellular metabolism. In view of studies which report an effect of adenosine on cyclic-AMP levels in several tissues, including bone (see Literature Review), further studies into adenosine formation and the roles of adenosine kinase and adenosine deaminase in these cells should prove interesting and informative.
TABLE 3
Conversion of $^{14}$C-Adenine into other Purine Compounds

<table>
<thead>
<tr>
<th>Product</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Expt. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>47.5</td>
<td>31.8</td>
<td>23.8</td>
<td>18.5</td>
</tr>
<tr>
<td>Guanine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Xanthine</td>
<td>11.7</td>
<td>10.4</td>
<td>8.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Total Base</td>
<td>59.2</td>
<td>42.2</td>
<td>32.0</td>
<td>23.4</td>
</tr>
<tr>
<td>Adenosine</td>
<td>28.4</td>
<td>17.2</td>
<td>16.8</td>
<td>6.5</td>
</tr>
<tr>
<td>Inosine</td>
<td>9.5</td>
<td>9.3</td>
<td>8.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total Nucleoside</td>
<td>37.9</td>
<td>26.5</td>
<td>25.3</td>
<td>12.7</td>
</tr>
<tr>
<td>AMP</td>
<td>30.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IMP</td>
<td>16.3</td>
<td>7.3</td>
<td>3.4</td>
<td>3.3</td>
</tr>
<tr>
<td>GMP</td>
<td>18.1</td>
<td>12.8</td>
<td>11.3</td>
<td>8.9</td>
</tr>
<tr>
<td>Total Nucleotide</td>
<td>280.4</td>
<td>205.7</td>
<td>155.5</td>
<td>95.3</td>
</tr>
</tbody>
</table>

Cells incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing 5mM glucose and 55.2 nmoles adenine-8-$^{14}$C.

Figure 4
Flow Diagram of $^{14}$C-Adenine Metabolism

Diagram illustrating the metabolism of $^{14}$C-Adenine with pathways for Guanine Nucleotides and Adenine Nucleotides, including Guanosine, Inosine, Adenosine, Hypoxanthine, and Xanthine.
TABLE 4a
Conversion of $^{14}$C-Hypoxanthine into other Purine Compounds

<table>
<thead>
<tr>
<th>Product</th>
<th>pmoles/$10^6$ cells/15 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>Adenine</td>
<td>(26.6)</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>-</td>
</tr>
<tr>
<td>Guanine</td>
<td>42.2</td>
</tr>
<tr>
<td>Xanthine</td>
<td>12.9</td>
</tr>
<tr>
<td>Total Base</td>
<td>55.1</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0</td>
</tr>
<tr>
<td>Inosine</td>
<td>31.5</td>
</tr>
<tr>
<td>Guanosine</td>
<td>10.1</td>
</tr>
<tr>
<td>Total Nucleoside</td>
<td>41.6</td>
</tr>
<tr>
<td>AMP</td>
<td>16.6</td>
</tr>
<tr>
<td>IMP</td>
<td>17.9</td>
</tr>
<tr>
<td>GMP</td>
<td>9.5</td>
</tr>
<tr>
<td>Total Nucleotide</td>
<td>46.5</td>
</tr>
</tbody>
</table>

Cells incubated in Krebs-Ringer phosphate buffer containing glucose and 48.3 nmoles hypoxanthine-8-$^{14}$C.

Figure 5
Flow Diagram of $^{14}$C-Hypoxanthine Metabolism
**TABLE 4b**

Conversion of $^{14}$C-Hypoxanthine into other Purine Compounds

<table>
<thead>
<tr>
<th>Product</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Expt. 4</th>
<th>Expt. 5</th>
<th>Expt. 6</th>
<th>Expt. 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Guanine</td>
<td>147.5</td>
<td>106.4</td>
<td>93.5</td>
<td>163.1</td>
<td>123.0</td>
<td>65.2</td>
<td>55.5</td>
</tr>
<tr>
<td>Xanthine</td>
<td>27.1</td>
<td>22.6</td>
<td>22.1</td>
<td>16.6</td>
<td>13.2</td>
<td>3.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Total Base</td>
<td>169.6</td>
<td>129.0</td>
<td>115.6</td>
<td>179.7</td>
<td>136.2</td>
<td>68.6</td>
<td>56.9</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Inosine</td>
<td>93.9</td>
<td>88.4</td>
<td>80.7</td>
<td>169.0</td>
<td>119.1</td>
<td>108.3</td>
<td>63.8</td>
</tr>
<tr>
<td>Guanosine</td>
<td>13.5</td>
<td>12.4</td>
<td>2.1</td>
<td>93.3</td>
<td>38.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total Nucleoside</td>
<td>107.4</td>
<td>100.8</td>
<td>82.8</td>
<td>262.3</td>
<td>157.2</td>
<td>108.3</td>
<td>63.8</td>
</tr>
<tr>
<td>AMP</td>
<td>46.9</td>
<td>44.1</td>
<td>35.5</td>
<td>20.3</td>
<td>14.8</td>
<td>9.5</td>
<td>6.0</td>
</tr>
<tr>
<td>IMP</td>
<td>8.0</td>
<td>5.1</td>
<td>1.5</td>
<td>18.7</td>
<td>16.1</td>
<td>14.5</td>
<td>6.7</td>
</tr>
<tr>
<td>GMP</td>
<td>51.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>26.5</td>
<td>13.9</td>
<td>11.1</td>
</tr>
<tr>
<td>Total Nucleotide</td>
<td>173.3</td>
<td>107.2</td>
<td>88.9</td>
<td>83.0</td>
<td>145.1</td>
<td>43.1</td>
<td>67.2</td>
</tr>
</tbody>
</table>

Cells incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing 10mM glucose and 48.3 nmoles hypoxanthine-8-$^{14}$C (68.4 nmoles, Expts. 4-7).
TABLE 5
Conversion of $^{14}$C-Guanine into other Purine Compounds

<table>
<thead>
<tr>
<th>Product</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Expt. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Guanine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xanthine</td>
<td>19.4</td>
<td>16.9</td>
<td>16.9</td>
<td>16.8</td>
</tr>
<tr>
<td>Total Base</td>
<td>19.4</td>
<td>16.9</td>
<td>16.9</td>
<td>16.8</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Inosine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Guanosine</td>
<td>21.6</td>
<td>11.9</td>
<td>52.2</td>
<td>38.2</td>
</tr>
<tr>
<td>Total Nucleoside</td>
<td>21.6</td>
<td>11.9</td>
<td>52.2</td>
<td>38.2</td>
</tr>
<tr>
<td>AMP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IMP</td>
<td>13.9</td>
<td>13.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GMP</td>
<td>3.0</td>
<td>1.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total Nucleotide</td>
<td>44.2</td>
<td>38.8</td>
<td>38.4</td>
<td>34.5</td>
</tr>
</tbody>
</table>

Cells incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing 5mM glucose and 51.0 nmoles guanine-$8-{^{14}}C$ (Expts. 1-2) or 68.0 nmoles guanine-$8-{^{14}}C$ (Expts. 3-4).

Figure 6
Flow Diagram of $^{14}$C-Guanine Metabolism
TABLE 6a
Conversion of $^{14}$C-Adenosine into other Purine Compounds

<table>
<thead>
<tr>
<th>Product</th>
<th>pmoles/$10^6$ cells/30 minutes</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td></td>
<td>393.3</td>
<td>327.0</td>
<td>204.1</td>
</tr>
<tr>
<td>Guanine</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Xanthine</td>
<td></td>
<td>9.1</td>
<td>4.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Total Base</td>
<td></td>
<td>402.4</td>
<td>331.9</td>
<td>205.3</td>
</tr>
<tr>
<td>Adenosine</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inosine</td>
<td></td>
<td>536.9</td>
<td>404.7</td>
<td>359.9</td>
</tr>
<tr>
<td>Guanosine</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total Nucleoside</td>
<td></td>
<td>536.9</td>
<td>404.7</td>
<td>359.9</td>
</tr>
<tr>
<td>AMP</td>
<td></td>
<td>7.6</td>
<td>5.5</td>
<td>5.7</td>
</tr>
<tr>
<td>IMP</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GMP</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total Nucleotide</td>
<td></td>
<td>101.7</td>
<td>96.0</td>
<td>71.9</td>
</tr>
</tbody>
</table>

Cells incubated in Krebs-Ringer phosphate buffer containing glucose and 25.0 nmoles adenosine-8-$^{14}$C.

Figure 7
Flow Diagram of $^{14}$C-Adenosine Metabolism
TABLE 6b

Conversion of $^{14}\text{C}$-Adenosine into other Purine Compounds

<table>
<thead>
<tr>
<th>Product</th>
<th>pmoles/$10^6$ cells/15 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 4</td>
</tr>
<tr>
<td>Adenine</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>344.3</td>
</tr>
<tr>
<td>Guanine</td>
<td>0</td>
</tr>
<tr>
<td>Xanthine</td>
<td>6.4</td>
</tr>
<tr>
<td>Total Base</td>
<td>350.7</td>
</tr>
<tr>
<td>Adenosine</td>
<td>-</td>
</tr>
<tr>
<td>Inosine</td>
<td>541.0</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0</td>
</tr>
<tr>
<td>Total Nucleoside</td>
<td>541.0</td>
</tr>
<tr>
<td>AMP</td>
<td>14.9</td>
</tr>
<tr>
<td>IMP</td>
<td>-</td>
</tr>
<tr>
<td>GMP</td>
<td>0</td>
</tr>
<tr>
<td>Total Nucleotide</td>
<td>50.3</td>
</tr>
</tbody>
</table>

Cells incubated in Krebs-Ringer phosphate buffer containing glucose and 25.0 nmoles adenosine-8-$^{14}\text{C}$. 
HORMONAL EFFECTS ON
PURINE SALVAGE AND INTERCONVERSION

Incorporation of $^{14}$C-adenosine and $^{14}$C-hypoxanthine was also investigated in the presence of two hormones known to exert an influence on bone cell metabolism -- thyrocalcitonin and parathyroid hormone (Tables 7-11). When $^{14}$C-adenosine served as the precursor in the presence of thyrocalcitonin, the most significant effect observed was the appearance of label in the guanine pool: in the presence of the hormone, incorporation into guanosine and GMP took place. There was also an increase in the amount of labeled nucleotide formed, especially AMP, over control values. This changed pattern of incorporation in the presence of the hormone could have resulted from an increase in hypoxanthine/guanine phosphoribosyl transferase activity acting to convert hypoxanthine formed from the deamination of adenosine. It could also have resulted from a stimulation of adenosine kinase. It was possible to differentiate between these two possibilities by using $^{14}$C-hypoxanthine as a precursor in the presence of the hormone. In this case, there was no increase in the incorporation of label in guanine compounds as well as only a small increase in AMP and other nucleotides. The adenosine level remained unchanged. Therefore no stimulation of phosphoribosyl transferase activity by the hormone is evident. It is
likely that the observed increase in incorporation when $^{14}$C-adenosine served as the precursor was the result of increased kinase activity with the formation of AMP and its subsequent metabolism. In view of the relationship emerging between nucleotide modulators and cyclic-AMP, the apparent stimulation of the conversion of adenine to guanine compounds is most interesting since the guanine nucleotides, especially GTP, have been reported to be the most effective modulators of adenylyl cyclase (Bell, 1973).

Analysis of the label in the adenine and guanine of the acid-insoluble RNA fraction indicated that thyrocalcitonin decreased incorporation of label into the adenine and guanine of RNA. These data are, however, difficult to interpret without knowledge of the intracellular concentration of nucleotides and therefore the actual specific radioactivities of the RNA fractions. Therefore no comment can be made on whether or not this represents a true decrease in RNA synthesis or reflects decreased incorporation into the acid-soluble precursor pool. When $^{14}$C-adenosine was the precursor, there was an increase in incorporation in the acid-soluble pool, thus a decrease in RNA synthesis seems apparent. However $^{14}$C-hypoxanthine was not incorporated into the acid-soluble pool to any greater extent in the presence of the hormone and therefore no statement concerning RNA synthesis can be made. No reports in the literature indicate any effect of the hormone on RNA synthesis.
Furthermore, the increase in the uptake of adenosine may be related to reports of adenosine modulation of adenyl cyclase (see Literature Review).

The data were equally difficult to interpret when the cells were incubated in the presence of parathyroid hormone. Changes in the incorporation of label into the acid-soluble purine compounds were apparent. When $^{14}$C-hypoxanthine served as the precursor, there was an increased incorporation into guanine, inosine and IMP. The increased incorporation into these compounds probably occurs from an increase in phosphoribosyl transferase activity resulting in the formation of more IMP and ultimately in the products of its metabolism. When $^{14}$C-adenosine was the precursor, increased incorporation into guanine, guanosine, hypoxanthine, inosine, AMP and GMP was observed. The same difficulty occurs when interpreting these data as occurred when interpreting the data in the hormone's absence. It is impossible to determine from these data whether the increased incorporation is the result of an increase in adenosine deaminase activity, with subsequent conversion of the hypoxanthine formed to IMP by phosphoribosyl transferase activity; or if it is the result of increased adenosine kinase activity and conversion of the AMP formed. It is suggested that at the high concentration of adenosine significant deaminase activity is responsible for the increased incorporation into inosine and hypoxanthine. However, increased incorporation
into AMP, and thus other nucleotides, is the result of increased adenosine kinase activity. Considerable evidence implicates nucleoside kinases in the uptake of nucleosides by animal cells (Hauschka, 1973), facilitated diffusion being the mechanism which probably applies. The portion of intracellular nucleoside that is not phosphorylated is known to increase as the contribution of simple diffusional transport increases at high nucleoside concentrations (Lindberg et al., 1969). Although the data suggest such an occurrence in this experiment, further study is needed to elucidate the transport mechanisms of these cells to verify such a conclusion. Regardless of the mechanism, it can be concluded that the hormone stimulates the incorporation of label into the acid-soluble purine pool. A similar effect of the hormone on the pyrimidine pool has been reported (Steinberg and Nichols, 1968). The physiological significance of this action has not been determined although it may contribute significantly to cellular nucleotide pools during periods of increased RNA synthesis.

Further consideration of the data indicates that incorporation of labeled precursor into bone cell RNA was stimulated by parathyroid treatment (Table 11). This increase in labeling may be entirely secondary to increased formation of RNA precursor rather than an increase in DNA-directed RNA synthesis. In order to evaluate this consideration, knowledge of the specific activities of the acid-
soluble precursor pool and the acid-insoluble RNA pool is needed. Such experiments have been done using an in vivo pulse labeling technique (Steinberg and Nichols, 1968) and it has been suggested that there is a clearly demonstrable increase in the true rate of RNA synthesis after one hour independent of simultaneous change in labeling of the acid-soluble precursor pool. The results of this study indicate an immediate effect of parathyroid hormone on bone cells and it should be possible through further experiments on isolated cells to determine whether this immediate effect extends to the stimulation of RNA synthesis. The relation of the change in RNA synthesis to other hormonally induced events within the cell such as ion translocation, phosphate entry, calcium transport as well as later induction of cellular differentiation is at present undetermined. Studies with isolated cells should be able to distinguish between those effects which are immediately apparent as a result of hormonal stimulation and those which require a lag time before becoming evident and to evaluate their interdependence in determining the final expression of the hormone on the bone cell.
TABLE 7

Conversion of $^{14}$C-Adenosine into other Purine Compounds in the presence of Thyrocalcitonin

<table>
<thead>
<tr>
<th>Product</th>
<th>pmoles/10$^6$ cells/15 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-hormone</td>
</tr>
<tr>
<td>Acenine</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>351.9</td>
</tr>
<tr>
<td>Guanine</td>
<td>0</td>
</tr>
<tr>
<td>Xanthine</td>
<td>1.7</td>
</tr>
<tr>
<td>Adenosine</td>
<td>-</td>
</tr>
<tr>
<td>Inosine</td>
<td>521.2</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0</td>
</tr>
<tr>
<td>AMP</td>
<td>5.7</td>
</tr>
<tr>
<td>IMP</td>
<td>1.0</td>
</tr>
<tr>
<td>GMP</td>
<td>0</td>
</tr>
</tbody>
</table>

Cells incubated in Krebs-Ringer phosphate buffer, pH 7.4, 10mM glucose containing 25.0 nmoles adenosine-$^{14}$C and 0.1µg salmon calcitonin.
TABLE 8

Conversion of $^{14}$C-Hypoxanthine into other Purine Compounds in the presence of Thyrocalcitonin

<table>
<thead>
<tr>
<th>Product</th>
<th>pmoles/10$^6$ cells/15 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-hormone</td>
</tr>
<tr>
<td>Adenine</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>-</td>
</tr>
<tr>
<td>Guanine</td>
<td>14.1</td>
</tr>
<tr>
<td>Xanthine</td>
<td>20.0</td>
</tr>
<tr>
<td>Adenosine</td>
<td>2.5</td>
</tr>
<tr>
<td>Inosine</td>
<td>15.8</td>
</tr>
<tr>
<td>Guanosine</td>
<td>20.1</td>
</tr>
<tr>
<td>AMP</td>
<td>9.0</td>
</tr>
<tr>
<td>IMP</td>
<td>7.1</td>
</tr>
<tr>
<td>GMP</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Cells incubated in Krebs-Ringer phosphate buffer, pH 7.4, 10mM glucose, containing 48.3 nmoles hypoxanthine-8-$^{14}$C and 0.1μg salmon thyrocalcitonin.
TABLE 9

Conversion of $^{14}$C-Adenosine into other Purine Compounds in the presence of Parathyroid Hormone

<table>
<thead>
<tr>
<th>Product</th>
<th>pmoles/$10^6$ cells/15 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-hormone</td>
</tr>
<tr>
<td>Adenine</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>484.6</td>
</tr>
<tr>
<td>Guanine</td>
<td>0</td>
</tr>
<tr>
<td>Xanthine</td>
<td>4.4</td>
</tr>
<tr>
<td>Adenosine</td>
<td>-</td>
</tr>
<tr>
<td>Inosine</td>
<td>1084.0</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.2</td>
</tr>
<tr>
<td>AMP</td>
<td>30.4</td>
</tr>
<tr>
<td>IMP</td>
<td>7.0</td>
</tr>
<tr>
<td>GMP</td>
<td>0</td>
</tr>
</tbody>
</table>

Cells incubated in Krebs-Ringer phosphate buffer, pH 7.4, 10mM glucose, containing 25.0 nmoles adenosine-8-$^{14}$C and 0.1μg porcine parathyroid hormone.
TABLE 10

Conversion of $^{14}$C-Hypoxanthine into other Purine Compounds in the presence of Parathyroid Hormone

<table>
<thead>
<tr>
<th>Product</th>
<th>pmoles/10^6 cells/15 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-hormone</td>
</tr>
<tr>
<td>Adenine</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>-</td>
</tr>
<tr>
<td>Guanine</td>
<td>19.1</td>
</tr>
<tr>
<td>Xanthine</td>
<td>3.5</td>
</tr>
<tr>
<td>Adenosine</td>
<td>2.2</td>
</tr>
<tr>
<td>Inosine</td>
<td>6.1</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0</td>
</tr>
<tr>
<td>AMP</td>
<td>11.7</td>
</tr>
<tr>
<td>IMP</td>
<td>4.6</td>
</tr>
<tr>
<td>GMP</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Cells incubated in Krebs-Ringer phosphate buffer, pH 7.4, 10mM glucose, containing 48.3 nmoles hypoxanthine-$^{14}$C and 0.1μg porcine parathyroid hormone.
TABLE 11

Incorporation of $^{14}$C-Purine Precursors into RNA in the Presence of Hormone

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Incorporation pmoles/$10^6$ cells/15 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Adenine</td>
</tr>
<tr>
<td>$^{14}$C-hypoxanthine</td>
<td>5.5</td>
</tr>
<tr>
<td>$^{14}$C-adenosine</td>
<td>76.1</td>
</tr>
</tbody>
</table>

Incubation conditions per Tables 7-10.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Incorporation pmoles/$10^6$ cells/15 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Adenine</td>
</tr>
<tr>
<td>$^{14}$C-hypoxanthine</td>
<td>5.5</td>
</tr>
<tr>
<td>$^{14}$C-adenosine</td>
<td>76.1</td>
</tr>
</tbody>
</table>

Incubation conditions per Tables 7-10.
In spite of the complexities in interpreting isotope incorporation data, especially in terms of purine salvage, some conclusions can be drawn from this study. Synthesis of cellular nucleotides by the salvage pathways takes place in these cells. Although the initial nucleotide formed differs according to the precursor, the process of phosphorylation represents the first step in utilization of preformed purines. Allowing for the fact that some of the present nucleotide synthesized from radioactive precursor may be underestimated, it is nevertheless possible to conclude that the salvage of preformed purines exceeds their synthesis de novo. Indeed no de novo synthesis is detectable at all. Thus, the salvage process must be more important for the maintenance of bone cell nucleotide pools, and bone is a tissue with an absolute requirement for preformed purine from some exogenous source.

It is also concluded from this study that these cells are able to convert compounds in the adenine nucleotide pool into the guanine pool. This conversion could have important implications to these cells, since any adenine converted may be lost to the cell. There are only two possible means of resupplying the adenine pool: 1) through additional exogenous purine supply or 2) the presence of GMP reductase to convert GMP back to IMP. It is suggested that the reductase may be present in these cells and further study is needed to
verify its existence and ascertain its role. Thus the interrelationship of the adenine and guanine nucleotide pools needs further investigation.

Additional factors exist which suggest some interesting aspects for further study. First, adenosine appears to be a very poor precursor of nucleotides in these cells, in contrast to its effectiveness in other tissues. Studies should be done with regard to pool sizes and specific activities in order to determine the true effectiveness of this precursor. Secondly, the roles of adenosine deaminase and adenosine kinase should be studied further in view of the effect of adenosine on cyclic-AMP levels reported for many tissues. The intracellular formation of this metabolite may have a significant regulatory function.

Finally, the preliminary studies into hormonal effects on the salvage pathways, though inconclusive, did raise interesting aspects for further study. Thyrocalcitonin seems to stimulate formation of guanine compounds, and GMP has been reported to be an effective modulator of adenyl cyclase activity in many cell types. It is interesting to speculate, then, that this stimulation may be one of the biochemical mechanisms through which the hormone acts, and this deserves additional investigation. Also, parathyroid hormone was shown to have an immediate effect on the incorporation of label into the acid-soluble pool as well as RNA. Further study is needed to determine if an actual increase in DNA-
directed RNA synthesis occurs which should shed new light on the relation of other hormonally induced events and changes in RNA synthesis.


