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REGULATION OF PURINE BIOSYNTHESIS-
STUDIES IN THE CHICK AND FISH

by

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ABSTRACT

REGULATION OF PURINE BIOSYNTHESIS-
STUDIES IN THE CHICK AND FISH

Mary Margaret Welch

Purines are one of the major components of all living systems. Much work has been done on elucidating the structure and biosynthetic pathway of these compounds, but the regulation of their synthesis in biological systems is still not understood. Early research on purine biosynthesis emphasized the purification and characterization of individual enzymes and intermediates involved in the de novo biosynthesis of purines. More recently, study has been devoted to elucidating how these enzymes interact within the cell—the effects of substrate availability, inhibition by cellular metabolites, and the control of enzyme activity.

The purpose of this study has been to evaluate purine synthesis and regulation in the domestic chicken. Weber and co-workers (Prajda, N., Morris, H. P., and Weber, G. (1976) Cancer Research 36, 4639-4646) have shown that, in rat liver which is undergoing rapid proliferation, two enzymes—amidophosphoribosyltransferase and IMP dehydrogenase—show increased activity, representing a basic shift in metabolic flux under such conditions. These workers have also shown that the activity of these, as well as several other enzymes of purine biosynthesis, increases in malignant tissue. It was of interest, then, to see if the
uricotelic chicken reacts to an increase in the need for purines within the cell in the same manner as rat and tumor tissue.

By treating immature roosters with the hormone β-estradiol, it is possible to induce rapid liver growth. The strategy adopted by these liver cells to provide the purines needed for rapid proliferation differs from that seen in rat tissues. The activities of amidophosphoribosyltransferase, xanthine dehydrogenase, AMP-S synthetase and lyase, AMP deaminase, IMP dehydrogenase, and GMP synthetase do not increase under this stimulation. However, the rate of de novo purine synthesis triples and the intracellular level of PRPP doubles within 24 hours after β-estradiol treatment. Measurement of de novo synthesis of AMP and GMP shows that the guanine branch of purine biosynthesis is induced at 12 and 24 hours after stimulation, but the A:G ratio returns to control levels by 48 hours, even though the rate of de novo synthesis is still elevated. These data show that the regulation of purine biosynthesis in chickens differs from that exhibited in the rat tissues. The de novo pathway in these animals is regulated at the level of PRPP availability, not by the activity of amidophosphoribosyltransferase which is the first committed enzyme in the biosynthetic pathway. The induction of the GMP branch of the purine interconversion pathway is not regulated at the level of IMP dehydrogenase, as it is in rat cells, but by the increase in availability of IMP due to the induction of de novo synthesis. Therefore, it appears that the enzymes of purine biosynthesis are maintained at sufficient levels within the chicken liver to support rapid growth. The regulation of the rate of purine biosynthesis in chickens occurs at the level of substrate availability. It is
clear that the strategy developed by these cells to deal with such stress situations differs from that used in other systems.

The purine nucleotide cycle (punc cycle) is composed of three enzymes which interconvert IMP and AMP, with the release of ammonia. It has been postulated (Braunstein, A. E., (1957) Adv. Enz. 19, 335-377) that this cycle may be responsible for the ammonia excreted by the gill in teleost fish. By using Fundulus heteroclitus, a euryhaline fish, it was possible to show that the levels of AMP-$S$ lyase and AMP deaminase, enzymes of the punc cycle, are raised when the ammonia excretion of these fish is elevated by adaption to fresh water. Therefore, the purine nucleotide cycle may be involved in the formation of branchially excreted ammonia in these animals.
ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Dr. Frederick Rudolph who served as my advisor and mentor during the course of this research. Also, I am grateful to Dr. Kathleen Matthews for encouragement and for critical reading of the manuscript.

I also wish to thank Dr. Jean Vorhaben for providing samples of adenylophosphonopropionate, Wade Baugher for providing partially purified rat muscle adenylosuccinate synthetase, and Dr. Dan Louie for performing the partial hepatectomies on rats during the initial period of this research.

I would especially like to thank Wade Baugher, Steve Beissner, Vicky B. Schandle and Sandra Clark for their support and for many helpful and enjoyable discussions.
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ABBREVIATIONS

AMP, ADP, ATP—Adenine mono-, di-, and triphosphate nucleotide
GMP, GDP, GTP—Guanine mono-, di, and triphosphate nucleotide
IMP, IDP, ITP—Inosine mono-, di-, and triphosphate nucleotide
AMP-S—Adenylylsuccinate (5'-monophosphate)
XMP—Xanthine monophosphate nucleotide
OMP—Orotate monophosphate nucleotide
APP—Adenylylophosphonopropionate
NAD, NADH—Nicotinamide-adenine dinucleotide, reduced form
NADP, NADPH—Nicotinamide-adenine dinucleotide phosphate, reduced form
P_i—Phosphate ion
PP_i—Pyrophosphate ion
PRPP—5-phospho-α-D-ribosyl pyrophosphate
HGPRT—Hypoxanthine-guanine phosphoribosyltransferase
APRT—Adenine phosphoribosyltransferase
DNA—Deoxyribonucleic acid
RNA—Ribonucleic acid
punc cycle—Purine nucleotide cycle
DEAE—Diethylaminoethyl
EDTA—Ethylenediaminetetraacetic acid
HEPES—N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
PP0—2,5-diphenyloxazole
POPOP—1,4-bis(5-phenyloxazol-2-yl)-benzene
Tris--Tris(hydroxymethyl)aminomethane

HPLC--High pressure liquid chromatography

PEI-cellulose--Polyethyleneimine cellulose

Abbreviations of purine biosynthetic intermediates are listed in Table 1.
INTRODUCTION

Purines are one of the major components of all living systems. Much work has been done on elucidating the structure and biosynthetic pathway of these compounds, but the regulation of their synthesis in biological systems is still not understood. Early research on purine biosynthesis emphasized the purification and characterization of individual enzymes and intermediates involved in the de novo biosynthesis of purines. More recently, study has been devoted to elucidating how these enzymes interact within the cell--the effects of substrate availability, inhibition by cellular metabolites, and the control of enzyme activity (Henderson et al., 1976).

The purpose of this study has been to evaluate purine synthesis and regulation in the domestic chicken. Weber and co-workers (Weber et al., 1976) have shown that, in rat liver which is undergoing rapid proliferation, two enzymes--amidophosphoribosyltransferase and IMP dehydrogenase--show increased activity. These workers have also shown that the activities of these, as well as several other enzymes of purine synthesis de novo, increase in malignant tissue. It was of interest, then, to see if uricotelic animals such as the chicken react to increased growth rate and purine utilization in the same manner as rat and tumor tissue.

In the system chosen for study, immature roosters were treated with the hormone β-estradiol which induces a rapid increase in RNA, DNA,
and protein synthesis in the liver. This tissue is a model for a non-malignant fast-growing cell, analogous to the regenerating rat liver system. To gain an understanding of the regulation of purine biosynthesis in the chicken, several factors which have been shown to influence the rate of de novo synthesis in other tissues were compared in livers from normal and β-estradiol treated chickens. This included measuring the levels of several enzymes involved in purine biosynthesis, interconversion, and catabolism and determining the levels of PRPP and the adenine and guanine pools within the cell. In addition, the rate of purine biosynthesis de novo was measured in both control and β-estradiol treated chicks and the adenine:guanine ratio of newly-formed purines was determined. The results of these experiments have led to a better understanding of the regulation of purine biosynthesis in uricotelic animals.

The purine nucleotide cycle (punc cycle), which interconverts IMP and AMP, is thought to be responsible for ammonia production in several systems (Braunstein, 1957 and Schultz and Lowenstein, 1976). Certain teleost fish can be adapted to both fresh and salt water. These animals normally excrete large amounts of ammonia through the gill epithelium and upon adaptation to fresh water release higher levels of ammonia, possibly in exchange for Na⁺. The levels of several enzymes thought to be involved in ammonia production as well as the level of the punc cycle enzymes were determined in fish adapted to both environments. It was found that glutaminase and glutamate dehydrogenase levels do not change but that AMP deaminase and AMP-S lyase levels are elevated in the fish adapted to the fresh water environment, suggesting an involvement of the punc cycle in the production of branchially excreted ammonia.
LITERATURE REVIEW
INTRODUCTION

The study of purines is one of the oldest fields in biochemistry, beginning in 1776 with the discovery of uric acid by Scheele (Henderson and Paterson, 1973). Since that time many biological functions have been discovered for these heterocyclic compounds: adenine and guanine play a major cellular role as constituents of both RNA and DNA, and both adenine and guanine act as precursors for various coenzymes—NAD, S-adenosyl methionine, riboflavin, and folic acid; adenine nucleotides are the major energy carriers within the cell, and both 2',3'-cyclic adenine mononucleotide and 2',3'-cyclic GMP serve as mediators of cellular response to hormonal stimulation (Henderson, 1972).

The major purine components of the cell are the 9-(5-phosphopentosyl) derivatives of adenine, guanine, hypoxanthine, and xanthine. These molecules—termed nucleotides—are composed of a purine base, D-ribofuranose (or deoxy-D-ribofuranose in the case of DNA), and one to three phosphates esterified to the 5-carbon of the ribose (esterification can also occur at the 2 & 3-carbon position of the ribose). Nucleosides lack the phosphate while the term base refers to the heterocyclic purine ring system (Figure 1). Other common purines include succinyl adenine monophosphate ribonucleotide (AMP-S) (See Figure 1), which is an intermediate in AMP biosynthesis but never accumulates in normal cells, and uric acid, which is the endproduct of purine catabolism and is found extracellularly as the free base. Many other purine derivatives are found...
Figure 1a.

AMP

Adenosine

dAMP
Figure 1b

Guanine

Hypoxanthine

Succinyladenine

Xanthine

Uric Acid
in nature but only in specialized systems. For example, caffeine accumu-
lates in coffee beans while theophylline can be found in tea leaves.

Uric acid, the first purine discovered, was synthesized by Fischer
in 1882. The determination of its structure led to biosynthetic studies
using radioactive precursors, and by 1956 the biochemical source of each
atom of the purine ring was established (Figure 2). Most of the reac-
tions of the biosynthetic pathway were elucidated by J. M. Buchanan, G.
R. Greenberg, and co-workers between 1951 and 1959 using cell-free
pigeon liver homogenates. The key discoveries made during this time were:
(1) the identification if inosine 5'-monophosphate as the true end-
product of de novo synthesis (Greenberg, 1951),
(2) the isolation, in 1947, of aminoadazolecarboxamide ribonucleotide
(AICAR) which was shown to be incorporated into nucleic acid
purines in rats (Shive et al., 1947), and
(3) identification of glycineamide ribonucleotide (GAR) and formylglycine-
amide ribonucleotide (FGAR) by Golthwait and Greenberg (between 1954
and 1956) as intermediates located near the beginning of the biosyn-
thetic pathway (Golthwait et al., 1954 and 1955).

These discoveries made it clear that each biosynthetic intermediate was
a ribonucleoside monophosphate and the other steps of the pathway were
quickly elucidated (for review see Buckanan and Hartman, 1959 and

The pathway of purine biosynthesis de novo is shown in Figure 3.
The names of each intermediate are listed in Table 1 along with the
standard abbreviations. Table 2 lists the name of each enzyme involved
in purine synthesis. The purine biosynthetic scheme has several interest-
ing features:
Figure 2.
Precursors of the Purine Ring System
Figure 3.

 Biosynthesis of the Purine Ring. Encircled numbers refer to enzymes listed in Table 2.
<table>
<thead>
<tr>
<th>Old Nomenclature</th>
<th>Enzyme Commission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoribosyl amine (PRA)</td>
<td>Ribosylamine phosphate</td>
</tr>
<tr>
<td>Glycineamide ribonucleotide (GAR)</td>
<td>Phosphoribosyl glycineamide</td>
</tr>
<tr>
<td>Formylglycineamide ribonucleotide (FGAR)</td>
<td>Phosphoribosyl formylglycineamide</td>
</tr>
<tr>
<td>Formylglycineamidine ribonucleotide (FGAM)</td>
<td>Phosphoribosyl formyl-glycineamidine</td>
</tr>
<tr>
<td>Aminoimidazole ribonucleotide (AIR)</td>
<td>Phosphoribosyl aminimidazole</td>
</tr>
<tr>
<td>Aminoimidazolecarboxylate ribonucleotide (CAIR)</td>
<td>Phosphoribosyl aminimidazole carboxylate</td>
</tr>
<tr>
<td>Aminoimidazole succinocarboxamide ribonucleotide (SAICAR)</td>
<td>Phosphoribosyl aminimidazole succinocarboxamide</td>
</tr>
<tr>
<td>Aminoimidazolecarboxamide ribonucleotide (AlCAR)</td>
<td>Phosphoribosyl aminimidazole carboxamide</td>
</tr>
<tr>
<td>Formamidoinimidazolecarboxamide ribonucleotide (FAI CAR)</td>
<td>Phosphoribosyl formamidoimidazole carboxamide</td>
</tr>
<tr>
<td>Inosinate (IMP)</td>
<td>Inosine 5'-monophosphate</td>
</tr>
</tbody>
</table>
Table 2.
Enzymes of Purine Biosynthesis de novo

<table>
<thead>
<tr>
<th>Systematic Name</th>
<th>Trivial Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ribose phosphate pyrophosphokinase</td>
<td>PRPP synthetase</td>
</tr>
<tr>
<td>2. Ribosylamine-5-phosphate:pyrophosphate phosphoribosyl transferase (glutamate amidating)</td>
<td>Amidophosphoribosyltransferase</td>
</tr>
<tr>
<td>3. Ribosylamine-5-phosphate:glycine ligase (ADP)</td>
<td>Phosphoribosylglycineamide synthetase</td>
</tr>
<tr>
<td>4. 5'-Phosphoribosyl-N-formylglycineamide:tetrahydrofolate 5,10-formyltransferase</td>
<td>Phosphoribosylglycineamid formyltransferase</td>
</tr>
<tr>
<td>5. 5'-Phosphoribosyl-formylglycineamide:L-glutamine amido-ligase (ADP)</td>
<td>Phosphoribosylformylglycinemidide synthetase</td>
</tr>
<tr>
<td>6. 5'-Phosphoribosyl-formylglycineamide cyclo-ligase (ADP)</td>
<td>Phosphoribosylaminomidazole synthetase</td>
</tr>
<tr>
<td>7. 5'-Phosphoribosyl-5-amino-4-imidazole carboxylate carboxylyase</td>
<td>Phosphoribosylaminomidazole carboxylase</td>
</tr>
<tr>
<td>8. 5'-Phosphoribosyl-4-carboxy-5-amino imidazole:Laspartate ligase (ADP)</td>
<td>Phosphoribosyl-aminoimidazole succinocarboxamide synthetase</td>
</tr>
<tr>
<td>9. Adenylosuccinate AMP-lyase</td>
<td>Adenylosuccinate lyase</td>
</tr>
<tr>
<td>10. 5'-Phosphoribosyl-5-formamido-4-imidazolecarboxamide:tetrahydrofolate 10-formyltransferase</td>
<td>Phosphoribosyl-aminimidazole carboxamide formyltransferase</td>
</tr>
<tr>
<td>11. IMP 1,2-hydrolase (decyclizing)</td>
<td>Inosinate cyclohydrase</td>
</tr>
</tbody>
</table>
(1) The ribose-5-phosphate moiety is added in the first synthetic step and each biosynthetic intermediate is in the form of the monophosphate ester,

(2) phosphoribosylamine (PRA), the product of the first committed step in purine biosynthesis, is unstable in aqueous solutions and has never been isolated as the product of enzymatic synthesis,

(3) adenylosuccinate lyase, which cleaves fumarate from aminomimidazole succinocarboxamide ribonucleotide (SAICAR), is the same enzyme that cleaves fumarate from adenylosuccinate, the intermediate compound in de novo synthesis of AMP from IMP (Carter and Cohen, 1956),

(4) though all the enzymes are found in the soluble cytoplasmic fraction of cell extracts, there is evidence that the first four enzymes of de novo purine synthesis exist as a macromolecular complex (Rowe and Wyngaarden, 1968). Rowe et al. (1977) have shown that enzymes capable of synthesizing hypoxanthine from $^{14}C$-glycine chromatograph together on a controlled pore glass column equilibrated with propylene glycol. Though it is possible that other factors influence the chromatographic properties of the de novo purine biosynthetic enzymes, their existence as a multi-molecular complex would be advantageous in vivo due to the instability of several of the pathway's intermediates,

(5) this pathway appears to be universal and has been found in mammals, bacteria, fungi, plants, insects, and molluscs (Henderson and Paterson, 1973).

There are several small molecular weight compounds needed to support de novo synthesis of purines. These include the amino acids glutamine, glycine, and aspartate, the folate derivatives 5,10-methenyl tetrahydro-
folate and 10-formyl tetrahydrofolate, ribose-5-phosphate, and carbonate. The energy for synthesis is provided by the hydrolysis of one molecule of PRPP and four molecules of ATP.

Inosine monophosphate is considered to be the end-product of de novo synthesis, and it is from this compound that AMP and GMP are formed. This synthesis is termed the interconversion pathway and consists of two cycles, one involving AMP and the other GMP, as shown in Figure 4. Like the de novo pathway, the reactions involve only the monophosphate form and are found universally in nature (GMP reductase, however, is often very low in animals) (Henderson and Paterson, 1973).

Purine nucleotides can also be formed by adding the ribose-5-phosphate from PRPP to the free bases which are supplied by nucleic acid degradation and diet. This synthesis is termed the salvage pathway and is especially useful in cells which have lost the ability to synthesize purines de novo (such as mature erythrocytes). The salvage pathway is shown in Figure 5. Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and adenine phosphoribosyltransferase (APRT) are the major enzymes in this pathway. These enzymes compete with amidophosphoribosyltransferase, which catalyzes the first step in the de novo pathway, for the common substrate, PRPP (Henderson and Paterson, 1973).

Uric acid was the first purine discovered in biological systems and has since been recognized as the end-product of purine metabolism. All purines are funneled into the pathway to urate, as shown in Figure 5. In addition, this purine is the end-product of amino acid nitrogen metabolism (via de novo synthesis of purines) in animals such as reptiles and birds and its excretion in these animals is increased as dietary
The Purine Interconversion Pathway

Figure 4.
Figure 5a.

de novo Purine Synthesis and Salvage Pathways
Figure 5b.

Purine Catabolism
protein intake is raised (Katunuma et al., 1973). In all mammals, except the higher primates (including man), uric acid is further catabolized to ammonia and carbon dioxide via allantoin and urea, as shown in Figure 6.
Figure 6.

Degradation of Uric Acid
REGULATION OF DE NOVO PURINE SYNTHESIS

The study of the regulation of purine synthesis de novo has focused on control of the synthesis of phosphoribosylamine, the first compound in the biosynthetic pathway, since there appears to be no regulation of the pathway between the initial reaction and IMP. The amount of phosphoribosylamine (PRA) synthesized by a cell will determine the cell's rate of de novo purine production (Wyngaarden, 1976). PRA synthesis, in turn, is controlled by several factors. These include PRPP and glutamine levels within the cell, the amount of amidophosphoribosyltransferase present in the cell, and the presence or absence of inhibitors of amidophosphoribosyltransferase activity (Henderson, 1972). The influence of each of these factors will now be discussed in detail.

AMIDOPHOSPHORIBOSYLTRANSFERASE

Amidophosphoribosyltransferase catalyzes the first committed step in purine biosynthesis de novo and has been the focus of much research and speculation concerning its role in the regulation of purine synthesis (for review see Buchanan, 1973). This enzyme catalyzes the following reaction:

\[ \text{Mg-PRPP} + \text{glutamine} + \text{H}_2\text{O} \xrightarrow{\text{Mg}^{2+}} \text{PRA} + \text{glutamate} + \text{Mg-PP}_1. \]  

(rxn 1)

Amidophosphoribosyltransferase has been studied in pigeon liver (Hartman, 1956), rat liver (Caskey et al., 1964), mouse adenocarcinoma 755 (Hill and Bennett, 1969), human placenta (Holmes et al., 1973a), human
lymphoblasts (Wood and Seegmiller, 1973), Bacillus subtilis (Shiio and Ishii, 1969), Aerobacter aerogenes (Neirlich and Magasanik, 1965a), and Schizosaccharomyces pombe (Nagy, 1970). The first investigative work on this enzyme was exceedingly difficult due to the instability of enzymatic activity in tissue homogenates. Most amidophosphoribosyltransferase activity was lost after six hours at 4°C and the active enzyme would not survive overnight (Hill and Bennet, 1969). Caskey et al. (1964), using pigeon liver extracts, could keep the enzyme active for several days but its sensitivity to inhibitors would change spontaneously. Recent work using the enzyme isolated from human placenta has shown that amidophosphoribosyltransferase is stable at 4°C for four weeks if stored in β-mercaptoethanol and Mg²⁺ and is stable up to three months if frozen at -70°C in 60 mM β-mercaptoethanol. This has allowed more accurate studies of the physical properties of the enzyme (Holmes et al., 1973a and 1973b). Recently Itakura and Holmes (1979) have shown that amidophosphoribosyltransferase from human erythrocytes is inactivated upon exposure to oxygen. These researchers suggest that this enzyme contains an iron-sulfur center which is sensitive to oxygen and accounts for the in vitro instability of the catalytic activity. (The bacterial and avian liver enzymes are also unstable and have been shown to contain three iron atoms per subunit (Hartman, 1963, Rowe and Wyngaarden, 1968). The enzyme from B. subtilis, also, is inactivated upon exposure to oxygen (Wong et al., 1977)). Incubation of the protein with iron and sulfide restores the enzymatic activity. It has been suggested (Dixon et al., 1976) that iron-sulfur centers provide a site for ammonia binding in enzymes that utilize this small molecule.
Kinetic studies have shown that the $K_m$'s for PRPP vary between 0.02-0.48 mM and the $K_m$'s for glutamine between 0.5 and 5.5 mM (depending on the source of the enzyme) with maximal activity when $\text{Mg}^{2+}$ concentration is twice the PRPP concentration (Wyngaarden, 1976) (see Table 3). The enzyme isolated from pigeon liver (Rowe et al., 1970), adenocarcinoma 755 (Hill and Bennett, 1969), and $S$. pombe (Nagy, 1970) shows sigmoidal kinetics when PRPP concentration is plotted against reaction velocity. Human placental enzyme, however, shows hyperbolic kinetics (Holmes et al., 1973a) (the significance of this will be discussed later).

Amidophosphoribosyltransferase is inhibited by 5'-purine nucleotides (not nucleosides or bases) in a manner dependent on the source of the enzyme (Table 4). The adenine and guanine nucleotides can act additively, as is the case with amidophosphoribosyltransferase from human lymphoblasts (Wood and Seegmiller, 1973), adenocarcinoma 755 (Hill and Bennett, 1969), and $S$. pombe (Nagy, 1970), or in a synergistic fashion as exhibited by the enzyme from human placenta (Holmes et al., 1973a), pigeon liver (Wyngaarden, 1972), and $A$. aerogenes (Neirlich and Magasanik, 1965b). It is possible that this enzyme has two allosteric binding sites—one for 6-amino purine ribonucleotides and one for 6-hydroxy purines—that can exhibit co-operativity in binding the inhibitors (Wyngaarden, 1972). Recent work by Holmes et al. (1973b) using stabilized human placental enzyme has shown that amidophosphoribosyltransferase from this tissue dissociates into a small (133,000 daltons), active form when PRPP is added and reassociates to the large (220,000 daltons), inactive form when AMP or GMP is added. Activity is
Table 3.
Amidophosphoribosyltransferase
Michaelis Constants for PRPP and Glutamine

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Michaelis Constants</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRPP, mM</td>
<td>Glutamine, mM</td>
<td></td>
</tr>
<tr>
<td>Pigeon liver</td>
<td>0.23</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Pigeon liver</td>
<td>0.24</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Pigeon liver</td>
<td>0.06</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Rat liver</td>
<td>0.086</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Chicken liver</td>
<td>0.25-0.5</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma 755</td>
<td>0.47 (S0.5)</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Human lymphoblasts</td>
<td>0.25</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Aerobacter aerogenes</td>
<td>0.23-0.30</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>0.3</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>0.086</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>0.11</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>0.35 (S0.5)</td>
<td>5 (S0.5)</td>
<td></td>
</tr>
</tbody>
</table>

from Henderson (1972)
Table 4.

Inhibition of Amidophosphoribosyltransferase by Purine Nucleotides

from Henderson (1972)

<table>
<thead>
<tr>
<th>Enzyme Source Type of Inhibition</th>
<th>Parameter Measured</th>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
<th>GMP</th>
<th>GDP</th>
<th>GTP</th>
<th>IMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigeon liver synergistic</td>
<td>$K_1$ (mM)</td>
<td>0.09</td>
<td>0.09</td>
<td>0.037</td>
<td>0.086</td>
<td>0.38</td>
<td>--</td>
<td>0.18</td>
</tr>
<tr>
<td>Pigeon liver</td>
<td>$K_1$ (mM)</td>
<td>0.092-</td>
<td>0.038-</td>
<td>0.031-</td>
<td>0.086-</td>
<td>0.38-</td>
<td>--</td>
<td>0.18-</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>$K_1$ (mM)</td>
<td>2.5</td>
<td>0.64</td>
<td>1.1</td>
<td>0.35</td>
<td>5.4</td>
<td>--</td>
<td>3.5</td>
</tr>
<tr>
<td>Aerobacter aerogenes synergistic</td>
<td>$IC_{50}$ (mM)</td>
<td>ca. 0.7</td>
<td>1.3</td>
<td>--</td>
<td>0.4</td>
<td>--</td>
<td>0.65</td>
<td>1.8</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>$IC_{50}$ (mM)</td>
<td>0.2</td>
<td>0.27</td>
<td>not Inhib.</td>
<td>2</td>
<td>--</td>
<td>0.2-1</td>
<td>not Inhib.</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>$IC_{50}$ (mM)</td>
<td>&gt;5</td>
<td>--</td>
<td>--</td>
<td>0.5</td>
<td>--</td>
<td>--</td>
<td>0.5</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>% Inhibition at 5 mM</td>
<td>75</td>
<td>56</td>
<td>33</td>
<td>7</td>
<td>7</td>
<td>37</td>
<td>9</td>
</tr>
<tr>
<td>Brevibacterium ammonigenes</td>
<td>% Inhibition at 0.1 mM</td>
<td>2.7</td>
<td>40.4</td>
<td>38.3</td>
<td>55.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>at 1.0 mM</td>
<td>78</td>
<td>79.8</td>
<td>100</td>
<td>71.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Adenocarcinoma 755</td>
<td>% Inhibition at 2.33mM</td>
<td>30%</td>
<td>40%</td>
<td>8%</td>
<td>36%</td>
<td>42%</td>
<td>17%</td>
<td>55%</td>
</tr>
<tr>
<td>additive</td>
<td>at 3.33mM</td>
<td>2.0mM</td>
<td>3.33mM</td>
<td>3.33mM</td>
<td>2.33mM</td>
<td>6.67mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human lymphoblasts additive</td>
<td>$IC_{50}$ (mM)</td>
<td>2</td>
<td>--</td>
<td>--</td>
<td>1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Human placenta synergistic</td>
<td>% Inhibition at 5 mM</td>
<td>76%</td>
<td>--</td>
<td>--</td>
<td>70%</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
proportional to the per cent of the enzyme in the small form and, while the enzyme is allosteric in nature, nucleotide inhibition can be overcome by high PRPP concentrations. The pigeon liver enzyme appears to operate in the opposite manner—PRPP stabilizes the larger, 200,000 molecular weight active form, while AMP and GMP disassociates the enzyme to the smaller 100,000 dalton form (Itoh, Holmes and Wyngaarden, 1976). Thus, this enzyme exhibits a variety of characteristics, dependent on its source, and the interaction of these properties in vivo is far from being clearly understood.

As early as 1962 an alternate pathway for the formation of phosphoribosylamine was reported. The synthesis of PRA from ammonia, ribose-5-phosphate, and ATP (reaction 2) was described in wheat germ (Kapoor and Waygood, 1962), Ehrlich ascites cells (Hersovics and Johnstone, 1964), and *Escherichia coli* B (LeGal et al., 1967) extracts. In 1965, though, Neirlich and Magasanik reported that PRA could be formed in a non-enzymatic, pH dependent reaction, and this was taken as the explanation for the previous reports of ammonia-dependent PRA formation (Buchanan, 1973). It was also possible that two enzymatic steps were involved: PRPP synthesis from ribose-5-phosphate and ATP and then synthesis of PRA using ammonia as an alternative to glutamine (amidophosphoribosyltransferase will react with ammonia with a $K_m$ of 0.4 M at pH 8.0), possibly using only one subunit of the amidophosphoribosyltransferase (Reem, 1974).

In 1968 G. H. Reem purified (100-fold) 5-phosphoribosylamine synthetase (E. C. 6.3.4.7) from duck, chicken, and pigeon liver. This
enzyme will not accept PRPP or glutamine in place of ribose-5-phosphate
\( K_m = 1.8 \text{ mM} \) and ammonia \( K_m (\text{NH}_4\text{Cl}) = 6.3 \text{ mM} \) and is sensitive to inhibition by purine ribonucleotides. Continuing studies by Reem using Chinese hamster cell lines (Reem, 1977), though, have shown that cells which have the aminotransferase, but not the amidotransferase, activity cannot survive without exogenous, pre-formed purines. Thus, amidophosphoribosyltransferase appears to be necessary for de novo synthesis of purines (at least at levels that support life) and may be involved in the regulation of purine synthesis de novo.

**SUBSTRATE CONCENTRATION AND NUCLEOTIDE INHIBITION**

**PRPP**

5-phospho-α-D-ribosyl pyrophosphate (PRPP) is synthesized from ribose-5-phosphate and ATP with the hydrolysis of one high-energy phosphate bond (reaction 3). The enzyme which catalyzes this reaction is PRPP synthetase (E. C. 2.7.6.1) and is widely distributed (Wyngaarden, 1976). Because of the instability of the product, PRPP, the regulation of this enzyme has only recently come under direct study (Henderson, 1972). PRPP synthetase has been partially purified from *Salmonella typhimurium* (Switzer, 1969), human erythrocytes (Fox and Kelley, 1971b), and Ehrlich ascites tumor cells (Wong and Murray, 1969). The enzyme from each source has an absolute requirement for inorganic phosphate, uses MgATP\(^2\), instead of free ATP, as the true substrate \( K_m = 0.1-2.9 \text{ mM} \) for
S. typhimurium enzyme, 0.06 mM for Ehrlich ascites enzyme, and 0.014 mM for the human erythrocyte enzyme) and has an absolute requirement for Mg$^{2+}$ other than that necessary to form the ATP complex (Henderson, 1972). PRPP synthetase appears to be sensitive to three types of inhibition:

(1) competitive inhibition at the MgATP$^{2-}$ site by free ATP and ADP ($K_i$=0.01 mM) (Henderson, 1972, Wyngaarden, 1976),

(2) competitive inhibition at the ribose-5-phosphate site by 2,3-diphosphoglycerate ($K_i$=5 mM) and PRPP in human erythrocytes (Fox and Kelley, 1972, Hershko et al., 1969). The $K_i$ for PRPP (0.05 mM) is approximately ten times higher than the intracellular concentration of PRPP in these cells (Fox and Kelley, 1972), and

(3) non-competitive inhibition by purine nucleotides by a mechanism termed "heterogenous metabolic pool inhibition" (Fox and Kelley, 1972, Switzer, 1970). This includes inhibition by AMP, ADP, GDP, GTP, IDP, ITP, TDP, NADPH, NAD, and FAD with the di-and tri-phosphates being more potent than the monophosphates (nearly all nucleoside mono-, di-, and triphosphates inhibit the Ehrlich ascites enzyme (Wong and Murray, 1969)). The $K_i$ values for this inhibition are quite high and the inhibition depends on the total nucleotide concentration rather than the presence of any specific nucleotide (Wyngaarden, 1976).

In Ehrlich ascites cells it is known that the rate of PRPP synthesis in cells in vivo is only 10% of the maximal rate of the enzyme when measured in tumor extracts. Thus there appears to be at least some inhibition of PRPP synthesis in vivo (Henderson et al., 1975).
Recent work by Becker et al. (1977) on PRPP synthetase from human erythrocytes has shown that the active form of this enzyme is composed of an aggregate of 16 or 32 identical subunits. MgATP$^2-$, free Mg$^{2+}$, purine nucleotides, and the enzymatic reaction products, in the presence of inorganic phosphate, cause aggregation to the large, active form while 2,3-diphosphoglycerate, a potent inhibitor of activity, prevents this aggregation. The monomer, a polypeptide of approximately 33,000 daltons, shows less than 4% maximal activity in any aggregation containing less than 16 subunits. Within the erythrocyte, though, the effectors are present in high amounts so that only the active form of the enzyme should be present. Also, while 2,3-diphosphoglycerate inhibits PRPP synthetase from all sources, it is only in erythrocytes that concentrations are high enough to affect the activity of this enzyme. Thus it is important to realize that all in vitro studies may not be directly applicable to the in vivo regulation of purine synthesis (Meyer and Becker, 1977).

Kinetic studies of amidophosphoribosyltransferase show that the concentration of PRPP could well influence the rate of PRA synthesis. In mammalian tissues the intracellular PRPP concentration is approximately 10% of the $K_m$ values for the isolated enzyme, with the highest elevated value reported (mouse liver treated with 2-ethylamine-1,3,4-thiadiazole) approximately equal to the $K_m$ (LaLanne and Henderson, 1975). Amidophosphoribosyltransferase from adenocarcinoma 755 (Hill and Bennett, 1969) and S. pombe (Nagy, 1970) show sigmoidal kinetics below the $K_m$ and, in these systems at least, small increases in PRPP concentration could greatly influence the rate of PRA synthesis.
(Wyngaarden, 1976). The pigeon liver enzyme also shows sigmoidal kinetics below the $K_m$ (approximately 60 mM), but intracellular levels are 10-fold higher and the enzyme is probably near saturation in vivo (Rowe and Wyngaarden, 1968, Badenoch-Jones and Buttery, 1976, and Lipstein et al., 1978).

Cellular concentrations of PRPP can be influenced, not only by the rate of synthesis, but also by the rate of usage of PRPP (see Figure 7). Various compounds which have been shown to lower the concentration of PRPP in vivo (and decrease de novo purine synthesis) are:

1. the purine bases adenine, guanine, and hypoxanthine which form the corresponding nucleoside monophosphates through a phospho-ribosylpyrophosphate transfer reaction (Bagnara et al., 1974b),
2. orotic acid which forms OMP through a similar reaction (Kelley et al., 1970),
3. nicotinic acid, a precursor of NAD, which forms a 5-phosphoribosyl derivative using PRPP (Boyle et al., 1972).

Given the above facts about PRPP, its synthesis and its use, the question of its importance in purine biosynthesis arises. Study of de novo synthesis in patients suffering from Lesch-Nyhan syndrome leads to the conclusion that PRPP availability does indeed influence the rate of purine biosynthesis. The Lesch-Nyhan syndrome is a disease characterized by mental and developmental retardation, hyperuricemia, and no detectable activity of the enzyme hypoxanthine-guanine phosphoribosylpyrophosphate transferase (see Figure 5). (Lesch and Nyham, 1964, Seegmiller et al., 1967). The hyperuricemia is a result of increased de novo purine synthesis, in some cases a twenty-fold elevation (Henderson, 1972).
Figure 7.
Biosynthetic Pathways using PRPP
Studies on the mechanism of this increase have shown that (1) amidophosphoribosyltransferase purified from Lesch-Nyhan cells is as sensitive to feedback inhibition as the enzyme isolated from normal cells, (2) nucleotide concentrations are equal in normal and Lesch-Nyhan cells (Rosenbloom et al., 1968), and (3) normal levels of amidophosphoribosyltransferase are found in Lesch-Nyhan cells (Wood and Seegmiller, 1971). However, the concentration of PRPP in Lesch-Nyhan cells is elevated at least four-fold over normal cells. These data have led to the hypothesis that PRPP concentration is involved in the regulation of purine biosynthesis de novo, both in normal Lesch-Nyhan cells (Henderson, 1972).

Recent data on adenine phosphoribosylpyrophosphate transferase-deficient Chinese hamster ovary cells, however, reveal that these cells do not show the same increase in de novo synthesis as the Lesch-Nyhan cells, though the situation should be similar. Further study is needed to show that Chinese hamster ovary cells can be compared to erythrocytes obtained from Lesch-Nyhan patients and that the increase in de novo purine synthesis in Lesch-Nyhan patients is due only to an increase in PRPP and not, more specifically, to an increase in PRPP due to hypoxanthine-guanine phosphoribosylpyrophosphate transferase deficiency (Taylor et al., 1977).

Recently, PRPP levels in patients that exhibit the clinical symptom of hyperuricemia have been studied. Several of the experimental subjects have elevated PRPP levels (in erythrocytes) and increased purine biosynthesis de novo (Sperling et al., 1977). It has been shown that this increase is the result of a superactive PRPP synthetase which either (1) lacks sensitivity to feedback inhibition, (Sperling et al., 1977), (2) has an increased affinity for the substrate ribose-5-phosphate (Becker et al., 1974), or (3) has increased specific activity per enzyme
molecule (Becker et al., 1973). Though such mutations have been found in only a few individuals, these enzyme variants may lead to a better understanding of the regulation of purine synthesis in vivo.

PRPP Concentration versus Nucleotide Inhibition

Amidophosphoribosyltransferase in vitro is influenced both by PRPP and by the presence of purine ribonucleotides. A major effort in the study of the regulation of purine synthesis de novo has focused on the interaction of these two factors within the intact cell. The most definitive work in this area has been performed by J. Frank Henderson and co-workers (for review see Henderson et al., 1975). Using Ehrlich ascites tumor cells in vitro, study has been made of enzyme activities, substrate concentrations, and nucleotide influence on the de novo pathway. By incubating the ascites cells with adenine and guanine, intracellular levels of ATP and GTP can be raised--two times for ATP and three times for GTP (even though mono- and diphosphate levels are less than 10% of the triphosphate levels, it is possible that changes in these nucleotides are causing the effects discussed below. As previously mentioned, partially purified amidophosphoribosyltransferase is more sensitive to inhibition by monophosphates while PRPP synthetase is more sensitive to purine nucleotide triphosphates). Henderson and co-workers have shown that the activity of amidophosphoribosyltransferase in cells with elevated triphosphate levels does not change (compared to cells not incubated with bases and at the same concentration of PRPP). Previous incubation with adenine and guanine does influence the concentration of PRPP by lowering the rate of PRPP synthesis in cells with raised ATP and
GTP levels. In cells still in the presence of the purine bases, though, PRPP synthesis is increased to provide this substrate for the formation of AMP and GMP (Bagnara et al., 1974b). Purine biosynthesis de novo in the tumor cells is inhibited when these cells are incubated in the presence of purine bases (Henderson, 1962). It is now clear that, at least in Ehrlich ascites cells, this inhibition is not due to the increase in concentration of the newly formed ribonucleotides but to the lowering of the PRPP concentration by the conversion of the bases to the monophosphate derivatives and that this is sufficient to account for the decrease in de novo synthesis (Henderson et al., 1975). Barankiewicz and Henderson (1977) have now also shown that the lowering of intracellular ATP and GTP concentration does not influence the activity of amidophosphoribosyltransferase but does increase the activity of PRPP synthetase. These cells, though, show no increase in de novo synthesis and this suggests that some other mechanism may be involved in the regulation of purine biosynthesis. The possibility of other mechanisms of regulation has also been raised by Becker (1976) who found that incubation of cells with inosine caused a decrease in purine synthesis de novo in both normal and hypoxanthine-guanine phosphoribosylpyrophosphate transferase-deficient human fibroblasts. While this can be explained by the formation of IMP (inosine→hypoxanthine→IMP) and the subsequent lowering of PRPP concentrations in the normal cells, this mechanism is obviously not the cause of inhibition in the HGPRT-deficient cells. Herschfield et al., (1977) have shown that human lymphoblasts which are not capable of forming AMP from either adenine or adenosine are as sensitive to growth inhibition by these compounds as normal lymphoblasts.
This growth inhibition had previously been thought to be the result of the effects of the newly formed nucleotides on lymphoblast metabolism (Ishii and Green, 1973, Green and Chan, 1973). Since these cells cannot form adenine nucleotides, some other mechanism must be postulated. These three experiments suggest that some factor other than PRPP availability and nucleotide inhibition may influence the rate of purine biosynthesis de novo (Barankiewicz and Henderson, 1977, Becker, 1976, and Herschfield et al., 1977).

The regulation of purine biosynthesis in uricotelic animals is clearly different from the regulation exhibited by mammals. Chickens and other avians, which excrete amino acid nitrogen as uric acid, synthesize purines at a rate that is fifteen times higher than purine production in rats, even though the in vitro properties of such enzymes as amidophosphoribosyltransferase are very similar in both systems (Wyngaarden, 1959, Holmes et al., 1973a, Wood and Seegmiller, 1973, and Lipstein et al., 1978). Matsuda (1972) has shown that hypoxanthine and xanthine added to chicken liver homogenates do not inhibit PRA synthesis in this cell-free system. Rat liver subjected to the same conditions shows a definite inhibition of purine synthesis. A recent study by Lipstein et al., (1978) has shown that the levels of PRPP and ribose-5-phosphate are 200 and 6 times higher, respectively, in chicken liver than in rat liver. In contrast, the activity of HGPRT and APRT in chicken is only 2-5% the level found in rat liver (PRPP synthetase activity is approximately the same). Experimentally induced increases in the cellular levels of ribose-5-phosphate and PRPP cause increases in purine biosynthesis and show that the normal levels of these compounds do not saturate de novo purine synthesis in vivo. Katunuma et al., (1970 and
1973) have shown that amidophosphoribosyltransferase is ten times higher in chicken liver than in rat liver. Under conditions of elevated amino acid catabolism (high protein diet or fasting) both amidophosphoribosyltransferase and glutamine synthetase levels are raised in chicken but not in rat liver. This clearly indicates a difference in the regulation of purine synthesis de novo in uricotelic and ureotelic animals.

Glutamine Concentration

Glutamine is used in purine biosynthesis at the steps which form PRA and formylglycineamidine ribonucleotide (FGAM). Its concentration in some cells and tissues is known to be rate-limiting for purine biosynthesis de novo, for example, in Ehrlich ascites tumor cells (Henderson, 1962 and Fontenelle and Henderson, 1969). Added glutamine stimulates purine synthesis in Ehrlich ascites cells, liver extracts, and adrenalectomized rats but not in rabbit bone marrow slices or brain slices. It appears that at least some tissues have sufficient quantities of this amino acid to support normal levels of purine biosynthesis de novo (Henderson, 1972).

The glutamine concentration in rat liver varies between 1 and 7 mM. The enzyme amidophosphoribosyltransferase has a $K_m$ for glutamine which varies from 0.5 mM in rat liver (Caskey et al., 1964) to 1.6 mM for human placenta (Holmes et al., 1973a) and lymphoblasts (Wood and Seegmiller, 1973), to 2.0 mM for Ehrlich ascites cells (Bagnara et al., 1974) and 1.8 mM for mouse adenocarcinoma 755 (Hill and Bennett, 1969). Thus the intracellular concentration occupies a range near the $K_m$ values of amidophosphoribosyltransferase for glutamine in several mammalian systems (for review see Wyngaarden, 1976).
REGULATION OF ENZYME LEVEL

Mammalian cells do not exhibit the simple induction-repression mechanism for control of enzyme synthesis often found in bacterial systems. There are several examples, though, in which the rate of PRA synthesis may be influenced by the amount of enzyme present:

1. In human lymphocytes stimulated with phytohaemagglutinin or Concanavalin A (which initiates proliferation) it is known that cGMP levels and PRPP concentrations increase (Hovi et al., 1975) and that purine biosynthesis de novo is stimulated 100-fold. The rise in PRPP levels requires protein synthesis and appears to be due to the induction of a PRPP synthetase that is sensitive to cGMP (Chambers et al., 1974),

2. When mammalian hepatoma cells in culture are incubated with adenine, purine biosynthesis declines and cell growth is nearly stopped (see above). When the adenine is removed, cell growth resumes. Martin and Owen (1972) have shown that protein synthesis is required for this release from inhibition and that the levels of amidophosphoribosyltransferase, phosphoribisyl glycineamide synthetase and transformylase do not change. It is possible that the resynthesis of PRPP synthetase is necessary for the cells to resume growth,

3. In chickens maintained on high protein diets, the level of amidophosphoribosyltransferase is elevated. The resulting increase in purine biosynthesis de novo allows the excess amino acid nitrogen derived from the diet to be excreted as uric acid (Katunuma et al., 1973).
While these examples are far from definitive, it is possible that animal cells, in some cases, regulate purine biosynthesis by adjusting the levels of the necessary enzymes (also see Purine Synthesis in Cancer Cells, page 47).
REGULATION OF PURINE INTERCONVERSION AND CATABOLISM

IMP is considered to be the endproduct of de novo purine biosynthesis. However, this compound never accumulates within the cell since it is rapidly converted to adenine and guanine nucleotides, the major cellular purines. As shown in Figure 4, the synthesis of AMP and GMP operates in a cyclic manner, AMP and GMP being formed from IMP while each of these nucleotides can be reconverted to IMP. The enzymes involved in each branch of this interconversion pathway will be discussed in the following section.

Adenylosuccinate synthetase catalyzes the formation of adenylosuccinate (AMP-S) from IMP, aspartate, and MgATP$^{2-}$. This enzyme has been studied from various sources, including **Azotobacter vinelandii** (Markham and Reed, 1976), **E. coli** (Rudolph and Fromm, 1969 and Rudolph, 1971), rabbit muscle (Muirhead and Bishop, 1974), **B. subtilis** (Ishii and Shio, 1970), **S. pombe** (Nagy et al., 1973), rat liver, Novikoff ascites and Walker carcinoma 256 tumors (Clark and Rudolph, 1976a and 1976b) and human placenta (van der Weyden and Kelley, 1974). The $K_m$'s of these various enzymes are listed in Table 5. The chicken liver enzyme has a $K_m$ for aspartate that is ten-fold lower than that determined for the enzyme from any other source. This may reflect the use of purine synthesis as the pathway for excretion of amino acid nitrogen in these animals. Rudolph and Fromm (1969) have shown that the enzyme from **E. coli** exhibits a Random Ter Ter mechanism and it appears that the enzymes
Table 5.
Michaels Constants for Adenylosuccinase Synthetase from Various Sources
from Clark et al., 1977

<table>
<thead>
<tr>
<th>Varied Substrate</th>
<th>Rat liver</th>
<th>Fetal rat liver</th>
<th>Pig brain</th>
<th>Walker tumors</th>
<th>Novikoff ascites</th>
<th>Chicken muscle</th>
<th>Chicken liver</th>
<th>Rabbit muscle</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>360</td>
<td>440</td>
<td>230</td>
<td>381</td>
<td>840</td>
<td>30</td>
<td>20</td>
<td>290</td>
<td>350</td>
</tr>
<tr>
<td>IMP</td>
<td>30</td>
<td>21</td>
<td>47</td>
<td>30</td>
<td>12</td>
<td>171</td>
<td>20</td>
<td>107</td>
<td>20</td>
</tr>
<tr>
<td>GTP</td>
<td>15</td>
<td>10</td>
<td>11</td>
<td>16</td>
<td>12</td>
<td>12</td>
<td>17</td>
<td>12</td>
<td>10</td>
</tr>
</tbody>
</table>
from other sources follow similar kinetics. The inhibition of AMP-S synthetase by GDP ($k_i$=5-30 μM) and AMP ($K_i$=10-100 μM in normal cells, 50-190 μM in tumor cells) may be important to the regulation of the enzyme within the cell (Rudolph and Fromm, 1969, Markham and Reed, 1976, Ishii and Shio, 1970, Clark and Rudolph, 1976a and 1976b, Bishop et al., 1975, Wyngaarden and Greenland, 1963).

Adenylosuccinate lyase cleaves fumarate from AMP-S to form AMP. This same enzyme catalyzes an analogous reaction in the de novo pathway—the biosynthesis of AICAR from SAICAR. This has been shown experimentally by the constant ratio of the two enzymatic activities maintained during purification and by genetic analysis. AMP-S lyase has been partially purified from yeast (Carter and Cohen, 1956, Bridges and Cohen, 1968), chicken liver (Miller et al., 1959), Ehrlich ascites tumor cells (Brox, 1973), Neurospora crassa (Woodward and Braymer, 1966), and human erythrocytes (Barnes and Bishop, 1975). The $K_m$ for the substrate AMP-S varies between 1.0 and 10.0 μM depending on the source. This enzyme has been shown to be sensitive to heavy metal ions. AMP and inorganic phosphate inhibit the enzymatic reaction with $K_i$'s of 6-12 μM and 0.5 mM, respectively (Barnes and Bishop, 1975). Kinetic studies have shown that after AMP-S is cleaved, fumarate leaves the enzyme before AMP (Bridges and Cohen, 1968). The enzyme isolated from mammalian sources is notoriously unstable (Barnes and Bishop, 1975).

AMP deaminase is the enzyme responsible for the formation of IMP and ammonia from AMP. Its properties vary, depending on the source of the enzyme and the degree of purification. In all cases the potassium ion is required for maximal activity to a degree inversely proportional
to the AMP concentration and the presence of this metal also enhances hyperbolic, as opposed to sigmoidal, kinetics (Coffee and Solano, 1977). In rat muscle AMP deaminase is bound to the light meromyosin portion of the myosin particle (Shiraki et al., 1979a) (rabbit muscle AMP deaminase is bound to heavy meromyosin. It is interesting to note that muscle AMP-S synthetase, another enzyme in the purine nucleotide cycle, interacts with F-actin (Ogawa et al., 1978). Kinetic studies on the enzyme while it is still attached to myosin give different results than those performed using partially purified enzyme. Attachment to myosin doubles the $V_{\text{max}}$ of AMP deaminase and adding ATP again doubles the catalytic activity (GTP abolishes this activation). Only the muscle form of AMP deaminase, not the isozymes isolated from liver, kidney, or cardiac muscle, interacts with the myosin particle and shows activation (Shiraki et al., 1979b). In uncentrifuged rabbit muscle homogenates AMP deaminase is sensitive to inhibition by ATP and GTP when assayed at low (0.2 mM) AMP concentration, but both nucleotides activate the enzyme in the presence of high (5 mM) concentrations. The partially purified muscle enzyme is sensitive to GTP inhibition at all AMP concentrations while under similar conditions added ATP will increase the catalytic activity (Brady and Costello, 1974). It is a property of partially purified muscle enzymes from frog, pigeon, rabbit, rat and guinea pig that both ATP and GTP inhibit activity at levels which are considered to be physiological (Coffee and Solano, 1977, Brady and Costello, 1974, Ronca-Testoni, 1970). AMP deaminase isolated from other tissues such as brain, liver, spleen, lung, and kidney all show activation by ATP and inhibition by GTP. The liver
enzyme is activated approximately three-fold by ATP while the deaminase from brain is activated ten-fold (rat tissues, Setlow, Burger, and Lowenstein, 1966). It is possible that under normal cellular ATP concentrations (2-4 mM, Williamson and Brosnan, 1974) AMP deaminase is maximally activated (Moss and McGivan, 1975). Recent work by Ogasawara et al. (1975) has shown that muscle, kidney, and liver, and heart each contain separate isozymes with $K_m$'s for AMP of 24, 37, and 6 mM, respectively. Three isozymes have also been found in rat brain, only one of which is sensitive to ATP activation but these have not been correlated to the organ-specific forms (Ogasawara et al., 1974).

The formation of XMP from IMP is catalyzed by IMP dehydrogenase. This enzyme has been studied in _A. aerogenes_ (Brox and Hampton, 1988a), sarcoma 180 cells (Anderson and Sartorelli, 1968, Miller and Adamczyk, 1976), and human placenta (Holmes et al., 1974). In each case the apparent $K_m$ for IMP is 15 $\mu$M and the apparent $K_m$ for NAD is 45 $\mu$M. The enzyme requires the potassium ion for maximal activity, and the kinetic mechanism is strictly ordered with IMP binding as the first substrate. IMP dehydrogenase activity is inhibited by XMP and GMP which compete with IMP.

GMP synthetase, which catalyzes the formation of GMP from XMP and glutamine, has been purified from pigeon liver (Lagerkvist, 1958), calf thymus (Abrams and Bently, 1959), _A. Aerogenes_ (Moyed and Magasanik, 1957), and various _E. coli_ strains (Brevet et al., 1967, Udaka and Moyed, 1963, Patel et al., 1975 and 1977, and Spector et al., 1974). Glutamine serves as the nitrogen donor ($K_m$=25 $\mu$M in pigeon, 50 $\mu$M in calf thymus and in the mM range for bacterial) but ammonia can also be
used at high concentrations by the enzyme from most sources ($K_m = 39-50$ mM except in *E. coli* where it can be as low as 0.9 mM, Hartman and Prusiner, 1973). ATP provides the necessary energy and forms an AMP-XMP intermediate with the loss of pyrophosphate. The pigeon liver enzyme is saturated at 5 mM ATP but is inhibited at higher concentrations. The $K_m$ for XMP is 30 μM in this system (Lagerkvist, 1958).

GMP synthetase is inhibited by GMP, GDP, and GTP and also by adenosine and AMP (Hartman and Prusiner, 1973).

GMP reductase converts GMP into IMP and has been studied in *A. aerogenes* (Brox and Hampton, 1968b), *E. coli* (Mager and Magasanik, 1960), calf thymus (Stephens and Whittaker, 1973), human erythrocytes (Hershko et al., 1973), and *Artemia salina* (Renart and Sillero, 1974). There is a great deal of evidence that this enzyme has very low activity in most animal cells (Henderson and Paterson, 1973). Kinetic studies have shown that the $K_m$ for GMP varies between 1, 4 and 10 μM and the $K_m$ for NADPH varies between 13 and 31 μM. In bacterial systems this enzyme is inhibited by AMP, ADP, and ATP but in higher organisms these nucleotides have no effect. GMP reductase is found in large quantities in brine shrimp eggs (*Artemia salina*). This small animal stores GMP for its embryos in a di-GDP compound (Gp$_4$G). This compound is used to form AMP through the action of GMP reductase (Renart and Sillero, 1974).

The above discussion of the individual enzymes of the interconversion pathway shows that the activity of these enzymes is sensitive to nucleotide concentrations. Adenylosuccinate synthetase, which catalyzes the first enzymatic step in the formation of AMP is inhibited by GDP, AMP, and GMP (Wyngaarden and Greenland, 1963, Rudolph and Fromm, 1969). The formation of XMP, catalyzed by IMP dehydrogenase, is
inhibited by GMP, GDP, and the reaction product, XMP (Holmes et al., 1974). GMP reductase is inhibited by ATP, AMP, and IMP but only in bacterial systems (Mager and Magasanik, 1960) while AMP deaminase from liver is inhibited by GTP and GDP (Setlow et al., 1966). It is interesting to note that GTP is used in the synthesis of AMP, and ATP is used to make GMP, providing a possible mechanism for balancing the branches of the pathway.

As with the enzymes of de novo synthesis it is necessary to study the interconversion pathway enzymes in the living cell as well as in isolated, in vitro systems. Nucleotide concentrations, enzyme amounts, and substrate availability can all influence the in vivo activity of these enzymes. It is known that XMP and adenylosuccinate (AMP-S) are produced unequally from IMP and that adenine nucleotide synthesis is approximately three times that of guanine nucleotide synthesis (Snyder and Henderson, 1973a). Crabtree and Henderson (1971) have shown that in Ehrlich ascites tumor cells in vitro GMP synthesis is limited, first by the availability of glutamine and then by the concentration of IMP (there was no evidence that the level of IMP was ever high enough to make IMP dehydrogenase activity rate-limiting). AMP synthesis in these cells is limited by the availability of aspartate and, since AMP-S never accumulates, the activity of AMP-S synthetase. The interconversion of AMP to GMP is limited, first by the concentration of glutamine (used in the synthesis of GMP from XMP) and then by the activity of AMP deaminase. The formation of AMP from GMP is limited by the rate of IMP formation in the GMP reductase reaction.
Studies of the effect of nucleotide concentration on the interconversion pathway have been performed by Snyder and Henderson (1973b). Using the method discussed earlier to raise ATP and GTP concentration in Ehrlich ascites cells by incubation with adenine and guanine, these researchers have shown that AMP-S synthetase and lyase activity is not changed under these conditions, that AMP deaminase is inhibited no more than 16% at maximum GTP elevation and that IMP dehydrogenase activity is sensitive to GTP concentration and is inhibited up to 60%. Similarly, Barankiewicz and Henderson (1977) have shown that lowered concentrations of ATP and GTP have no effect on AMP deaminase, GMP reductase, and AMP-S synthetase but that IMP dehydrogenase activity is raised by 41%. These data show that nucleotide inhibition, as well as substrate availability and enzyme activity, plays a role in the regulation of the synthesis of AMP and GMP from IMP.

Very little work has been done on relating the rate of de novo purine synthesis to IMP branch point regulation. Herschfield and Seegmiller (1976) have analyzed the effect of added purine and purine analogs on the interconversion pathway by measuring the incorporation of radioactive formate into adenine and guanine-containing compounds in cultured human lymphoblasts. These workers found that added adenine or guanine diverts newly formed IMP to GMP or AMP, respectively. Added 6-methylmercaptopurine riboside (the riboside is used since it can be phosphorylated by adenosine kinase and thus the level of PRPP remains unchanged), an adenine analog, also channels the pathway to GMP production. In a slightly different experiment in which hypoxanthine, instead of formate, was used as the precursor to IMP, this same adenine analog
had no effect on the branch point, that is, relative AMP and GMP formation was the same as in untreated cells (incubation with adenine has the same effects as 6-methylmercaptourine on IMP from the two sources). One explanation for this phenomenon is that IMP formed by de novo purine synthesis enters a different pool than that formed by the salvage of hypoxanthine and that these two pools have different branch point regulation. It is also possible that the regulation of the IMP branch point is controlled by the level of IMP in the cells. IMP synthesis through the de novo pathway is inhibited when cells are incubated with purine bases. Under these circumstances IMP is limiting and if AMP can be formed by salvage the IMP formed by de novo synthesis is diverted to the synthesis of GMP. When hypoxanthine serves as the source of IMP, the intracellular levels are elevated and possibly, under these conditions, the synthesis of AMP and GMP does not need to be closely regulated. Two questions arise:

(1) is this phenomenon a characteristic of incubation with adenine or adenine analogs or does incubation with guanine cause similar results?

(2) do these data hold only for human lymphoblasts or is this pattern of inhibition also exhibited by other cell types?

Though the answers to these questions are not known, it is obvious that these cells have found a method to further adapt to their environment.

Recently, Brand and Lowenstein (1978) have shown that the levels of purine biosynthetic enzymes are differentially regulated in fasting rat and chicken. Under conditions which stimulate amino acid catabolism and gluconeogenesis from amino acids (e.g. starvation) AMP-S lyase activity in rat liver and spleen (but not in skeletal muscle, brain and
kidney) is lowered drastically (to 6.3% and 12% of controls, respectively), but AMP-S synthetase and AMP deaminase activities remain unchanged (the level amidophosphoribosyltransferase is not changed (Katunuma et al., 1970), but IMP dehydrogenase activity is elevated two-fold (Jackson et al., 1977)). In contrast, liver from starved chickens has elevated levels of AMP-S lyase (Brand and Lowenstein, 1978b), xanthine dehydrogenase (Freeland and Szapesi, 1971), and amidophosphoribosyltransferase (Katunuma et al., 1970), reflecting the involvement of these enzymes in the excretion of amino acid nitrogen in this system. In rats purine biosynthesis \textit{de novo} (as measured by formate incorporation into adenine, guanine, and hypoxanthine) is lowered during starvation, especially in the spleen. Formation of adenine is decreased to a much greater extent than the synthesis of guanine and hypoxanthine, a result consistent with the lowering of AMP-S lyase activity (Since this enzyme is also used in the \textit{de novo} synthesis of purines, it may also be involved in the lowering of overall purine synthesis). In chicken liver, AMP-S lyase, xanthine dehydrogenase, and amidophosphoribosyltransferase levels are raised during starvation, while urea cycle enzymes and amino acid degrading enzymes are raised in rat liver under similar conditions. This variance reflects the difference in methods of excretion of amino acid nitrogen (uric acid versus urea) in these two animals. These results suggest that AMP-S lyase has a regulatory role in purine biosynthesis in rats. The exceedingly low levels of this enzyme found in liver during starvation are inconsistent with the proposal that the purine nucleotide cycle is involved in the production of ammonia for urea synthesis in these animals (Moss and McGivan, 1975, Brand and Lowenstein, 1978).
Purines are catabolized by the formation and excretion of uric acid. In order to form this compound the monophosphate nucleotides must be converted first to the nucleoside and then to their respective base (see figure 5). Guanine is then converted to xanthine by the action of guanase while adenosine must be degraded to inosine by adenosine deaminase at the riboside level. Thus hypoxanthine and xanthine are formed and these compounds can be converted to uric acid by an enzyme known as xanthine oxidase. Xanthine oxidase is found in many species but most physical studies on this enzyme, which contains molybdenum, iron-sulfur centers, and flavin, have been performed on the enzyme isolated from cow's milk (Bray, 1975). Xanthine oxidase from chicken and rat liver also has a dehydrogenase activity which uses NAD as the oxidizing substrate in preference to molecular oxygen (which is used by the oxidase), and these forms can be interconverted by the use of reagents which affect sulfhydryl groups (the oxidase is formed in the presence of N-ethylmaleimide while dithiothreitol stabilizes the dehydrogenase form (Delia Corte and Stirpe, 1972)). The dehydrogenase can also be proteolytically cleaved to a form of the oxidase which has a lower molecular weight (Waud and Rajagopalan, 1976). In chicken, however, the dehydrogenase form is very stable and, in fact, NAD is the preferred oxidizing substrate in bony fish, amphibians, reptiles, and birds. The enzyme isolated from higher animals prefers NAD to molecular oxygen but the rate of reaction is only doubled—instead of increased more than ten-fold as in chickens—when NAD is used. The dehydrogenase form of the enzyme must be stabilized with dithiothreitol or similar compounds (Krenitsky et al., 1974).
PURINE BIOSYNTHESIS IN CANCER CELLS

Purines play a central role in the metabolism of all replicating cells. Because of their involvement in the synthesis of RNA and DNA, the biosynthesis of purines has been studied in tumors in an effort to find possible malignancy-linked changes in purine metabolism (many tumor drugs are purine analogs so that the study of purine regulation in these cells is important from a clinical, as well as a scientific, standpoint). Using Morris hepatomas with different growth rates, Weber and co-workers have attempted to correlate changes in purine metabolism and tumor growth rate (Weber et al., 1976). These investigators have shown that AMP-S synthetase (Jackson et al., 1975), AMP-S lyase (Jackson et al., 1976) and amidophosphoribosyltransferase (Katunuma and Weber, 1974) activities are elevated two to three times in hepatomas (as compared to normal rat liver) regardless of the growth rate of the tumors but that, of these enzymes, only amidophosphoribosyltransferase is elevated (approximately 1.5 times) in regenerating rat liver, an example of a normal, fast-growing tissue (Weber et al., 1976). Xanthine oxidase activity (which regulates purine catabolism in the rat) is decreased in all hepatomas but is not changed in regenerating liver (Prajda and Weber, 1975, Prajda et al., 1976). In contrast, IMP dehydrogenase is elevated in all tumors—from two to thirteen-fold—and the increase correlates with the growth rate of the hepatoma. This enzyme's activity also increases five-fold in regenerating rat liver (Jackson et al., 1975). Comparison of partially purified IMP dehydrogenase from rat liver and a
fast-growing hepatoma has shown no apparent kinetic or nucleotide inhibition differences between the enzymes form the two sources. This experimental evidence indicates that the increase in IMP dehydrogenase in hepatomas is not due to production of an altered enzyme (Jackson et al., 1977). Thus, from these studies and those by Henderson on the effects of changes in ATP and GTP concentrations, it appears that the GMP pathway is regulated in normal cells and that the capacity of this branch of the interconversion pathway must be increased to support a faster-than-normal growth rate.
THE EFFECT OF ESTROGEN TREATMENT ON IMMATURE CHICKENS

Production of egg proteins in mature female chickens is mediated by the hormone estradiol. These proteins, such as phosvitin, are synthesized in the liver and then released into the blood stream for transport to the oviduct. Synthesis of these same polypeptides can be induced in roosters and immature chicks by injection of large doses of β-estradiol. The plasma level of phosvitin is raised in these treated roosters since there is no oviduct to remove this egg protein from the bloodstream (McDonald and Riddle, 1945, Beuving and Gruber, 1971, Greengard et al., 1964). The synthesis of egg proteins can also be induced in other egg laying animals such as the lizard *Uta stansburiana* and the frog *Xenopus laevis* by treatment with β-estradiol (Hahn et al., 1969).

When immature chicks are first treated with β-estradiol, the appearance of phosvitin in the bloodstream is delayed by 20 hours but during this time the synthesis of protein, DNA, and RNA in the liver increases (Jost et al., 1973). (In *X. laevis* the delay is only 9-12 hours (Lanclos and Hamilton, 1975, and Wittliff and Kenney, 1972)). Forty-eight hours after treatment, the protein and RNA content and the weight of the liver has increased by 60% (see Figure 8). The amount of DNA also increases (1.3-fold) but this synthesis is not necessary for the stimulation of phosvitin production. RNA synthesis, though, is absolutely necessary if estrogen treatment is to affect the liver. If the same
Figure 8.
Total liver weight, DNA, RNA, and protein content of livers from 8-estradiol chicks. Solid lines represent control chicks and dashed lines represent experimental animals. from Jost et al., 1973
chicks are treated with 8-estradiol two weeks after the first injection
the appearance of phosvitin is delayed only six hours while protein,
RNA and DNA synthesis follows a pattern similar to that observed after
the primary stimulation with the hormone (Jost et al., 1973). The
livers of all chicks treated with 8-estradiol increase in weight and
fat content. This system can be used as an experimental model of a
normal, as opposed to malignant, fast-growing, rapidly replicating cell.
AMMONIA EXCRETION IN FISH

All animals can be classed as amminotelic, uricotelic, or ureotelic depending on their mechanism of excreting amino acid nitrogen (in the form of ammonia, uric acid, or urea, respectively). Lower orders, such as fish and amphibians, excrete large amounts of ammonia, mainly through their respiratory epithelium. In these animals the kidneys play only a minor role in nitrogen excretion. Certain species of fish (for example, Fundulus) can be adapted to live in salt or fresh water. When living in salt water these animals must rapidly excrete the Na⁺ and Cl⁻ that is ingested with food. Conversely, in fresh water these fish must sequester both ions against a concentration gradient. When marine animals such as the mudskipper (Periophthalmus sobrinus) and the blue crab (Callinectes sapidus) are adapted to fresh water, ammonia excretion is doubled (Gordon et al., 1965, Mangum et al., 1976). It has been postulated that this ion is exchanged with sodium in the environment in an attempt to increase uptake and maintain salt balance (Maetz, 1971 and Evans, 1975).

The source of ammonia excreted through the respiratory epithelium of fish and amphibians has been studied by many investigators. Goldstein and Forster (1961) have found in Myoxocephalus scorpio (the short-horn sculpin) that both glutamine and ammonia are formed in the liver and then transported to the gills by the bloodstream. Two molecules of ammonia can be formed from each molecule of glutamine using the enzymes glutaminase (glutamine→ glutamate and NH₃) and glutamate dehydrogenase
(glutamate → α-ketoglutarate and NH₃) and this, plus the free ammonia transported in the blood, can account for approximately 60% of the NH₃ excreted from the gills. In another experiment, though, these workers found that very little glutamine is removed from the blood during passage through the gill. They point out that, since amino acids taken up by the gill could be replaced by re-equilibration with the amino acids present in the red blood cells (amino acid concentration is approximately ten times higher than in plasma), the experimental numbers represent minimum values for the glutamine removed (Goldstein et al., 1964). Vellas and Serfaty (1974) also found that glutamine plays a major role in the transport and formation of NH₃ for excretion in the carp, Cyprinus carpio. In rainbow trout the gills remove glutamate and ammonia but very little glutamine from the blood. The glutamine level is low in these animals, coming mainly from dietary sources. Glutamine synthetase, which catalyzes the formation of glutamine from glutamate and NH₃, is present in significant amounts only in the brain, being responsible for ammonia detoxification in that tissue (Walton and Cowey, 1977). It has been proposed that the ammonia excreted by these teleost fish, which is not derived directly from plasma ammonia, is formed by the deamination of amino acids other than glutamate and glutamine, which are formed in the liver and then transported to the gills (Goldstein and Forster, 1961). Under some conditions, though, the liver may not be essential to ammonia excretion through deamination of amino acids, production of blood ammonia, or formation of blood amino acids. Kenyon (1967) found that ammonia excretion remains constant in hepatectamized eels suggesting that bronchial ammonia may be derived from other tissues and also from amino acid deamination at the gill.
In 1957, A. Braunstein proposed that the conversion of AMP to IMP could provide high levels of ammonia and that these nucleotides could operate cyclically, with aspartate providing the nitrogen which was to be released as ammonia. This was termed the purine nucleotide cycle (punc cycle—see Figure 9). This scheme has since been shown to be operational in other systems such as skeletal muscle homogenates (Lowenstein and Tornheim, 1970), rat brain homogenates (Schultz and Lowenstein, 1976) and in the kidneys (Bodusky et al., 1976). Marakewicz and Zydowo (1962) have measured AMP deaminase, which is responsible for the deamination of AMP, and glutaminase in the kidneys of 15 vertebrate species. In higher vertebrates such as rat and dogs, glutaminase is much more active than AMP deaminase but in fish and amphibians AMP deaminase is responsible for a greater amount of the ammonia produced. This was also true of carp gills. This observation, coupled with the fact that 40% of the glutamate in the trout liver is converted to aspartate (Walton and Cowey, 1977), supports the hypothesis that excretory nitrogen in teleost fish can be transported to the gill in the form of aspartate and that this amino acid is then deaminated via the purine nucleotide cycle (Walton and Cowey, 1977, Marakewicz and Zydowo, 1962, and Maetz, 1971).

Recently, Leray, Raffin, and Winninger (1979) have measured the presence of several enzymes involved in purine interconversion and catabolism in the gill of rainbow trout (the fresh water species, *Salmo gairdneri*). The levels of adenylate kinase, creatine kinase, AMP deaminase, adenosine deaminase and adenosine triphosphatase are very high in this tissue. 5'-Nucleotidase, purine nucleoside phosphorylase,
Figure 9.
The Purine Nucleotide Cycle
(punc cycle)
HGPRT and APRT, and adenosine kinase are present but at a lower level. AMP-S lyase and AMP-S synthetase, the enzymes which with AMP deaminase make up the purine nucleotide cycle, were present only in small amounts. The activity of the AMP-S synthetase present in this tissue was at the limit of detection with the spectral assay used. Since purine biosynthesis de novo in this tissue has not been demonstrated, these workers conclude that the purine enzymes present in trout gill play a dominant role in salvage and conservation of purines in this tissue (Leray et al., 1979).

Several comparative studies of fresh and salt water adapted euryhaline fish have been performed. Epstein et al. (1967) have shown that in Fundulus heteroclitus succinate-cytochrome c reductase, NADH-cytochrome c reductase, 8-hydroxybutyrate dehydrogenase, and glutaminase levels do not change (similar results were found in Onchorhynchus (Tripp, 1969)), Na⁺-K⁺ ATPase, though, which has been linked in many cells to the presence of a Na⁺ pump (Maetz, 1971) is raised in Fundulus as the fish is adapted to salt water. The flux of Na⁺ across the gill is known to be several fold higher in salt water (Epstein, 1967). Early cytochemical findings show that alkaline phosphatase and succinate dehydrogenase are increased upon adaption to fresh water. This may reflect the increased energy needs of the gill which are used to maintain the osmolarity of the blood (the difference between fresh water and the fish is greater than the difference between the fish and salt water). In the blue crab glutamine dehydrogenase activity in the gill increases with acclimation to low salinity (Silverthorn and Krall, 1975). Excretion of NH₃ in these animals also increases during fresh water
adaptation but the blood ammonia levels do not change. This species also shows a shift from ureotelism to amminotelism when placed in water of low salinity. This is consistent with the hypothesis that ammonia is the important counter ion in Na⁺ uptake in the blue crab (Mangum et al., 1976). The source of ammonia excreted in fresh water fish is still under question. Conflicting results such as those found when trout (Goldstein et al., 1964), carp (Vellas and Serfaty, 1974), and hepatectamized eel (Kenyon, 1967) are compared open the possibility that the answer to this question is species specific.
MATERIALS AND METHODS
MATERIALS

Chemicals

Glutamate dehydrogenase (50 units/mg in 50% glycerol and sodium phosphate, pH=7.3), AMP deaminase (61 units/mg in 66% glycerol and 0.33 M KCl), pyruvate kinase (500 units/mg, lyophylized solid), hexokinase (345 units/mg in 3.2 M ammonium sulfate) and glucose-6-phosphate dehydrogenase (230 units/mg, dry ammonium sulfate cake) were purchased from Sigma Chemical Company (St. Louis, Missouri). Lactate dehydrogenase was purchased from P-L Biochemicals (Milwaukee, Wisconsin). Partially purified AMP-S synthetase from rat muscle was kindly provided by Wade Baugher.

AMP $^{14}$C(U) 42 mCi/mmole (in 50% ethanol) was purchased from Schwarz/Mann (Orangeburg, N. Y.). Adenine $^{14}$C(U) 43.9 mCi/mmole was purchased from New England Nuclear (Boston, Massachusetts). Glutamine $^{14}$C(U) 45 mCi/mmole and formic acid, sodium salt $^{14}$C 46.2 mCi/mmole were purchased from ICN (Irvine, California).

PRPP was purchased from Sigma and P-L Biochemicals. The purity of the commercial compound was determined spectrophotometrically by use of orotidine phosphoribosyltransferase (P-L Biochemicals, lyophylized solid). The assay mixture contained 0.02 M Tris-HCl, pH=8.5, 6 mM MgCl$_2$, 0.2 mM 5-fluoro-orotate, and 0.1 ml of PRPP solution made of 3-4 mg PRPP dissolved in 4 ml cold, distilled water, in a total volume of 3.1 ml. The reaction was allowed to go to completion and the change in absorbance was used to calculate the amount of PRPP present ($\Delta e_{295}=3.8$ mM$^{-1}$cm$^{-1}$).
All other reagents purchased commercially were of highest purity available.

Materials

Cellulose and PEI-cellulose thin layer plates on plastic backing were purchased from EM Laboratories Inc. (Elmsford, N. Y.). The cellulose plates were washed overnight in isopropanol before use. PEI-cellulose plates were washed in 4 M sodium formate, pH=3.4, till the solvent front reached the top of the plate. The plates were dried and then washed overnight in methanol:water, 50:50 (v:v) with a wick of Whatman 3MM paper stapled on top (Crabtree and Henderson, 1971). Sheets of 3MM paper and 2.5 cm DE-81 cellulose disks were purchased from Whatman (Clifton, New Jersey).

Instrumentation

Radioactive compounds were detected using a Beckman LS-233 liquid scintillation system. This equipment has $^{14}$C, $^3$H, and $^{14}$C-$^3$H windows. The efficiency of counting was determined using the ratio between the $^{14}$C-$^3$H windows (see Figure 10). Counts per minute were converted to dpm's using an efficiency of 85-90% at ratios of 0.7-1.4. The $^{14}$C:$^3$H channel was used for all counting. 10 ml Nalgene Fimware system scintillation vials were used (Nalge Company, Rochester, N. Y.) to contain the scintillation fluid and radioactive compounds. Organic scintillation fluid containing 14.7 g PPO and 0.3 g POPOP in one gallon toluene was used for counting thin layer plates. The radioactivity of DEAE-cellulose disks was measured using scintillation fluid containing 2100 ml toluene, 1230 ml 100% ethanol, 12 g PPO, and 0.3 g POPOP. All compounds used were scintillation-grade reagents.
Efficiency of the Beckman LS-233 Liquid Scintillation Counter

Efficiency was calculated using the channels ratio method. Vials contained $^{14}$C-toluene and varying amounts of chloroform.
Centrifugation was carried out in a Beckman J-21 centrifuge (JA-20 and JA-21 rotors), a Beckman Model L2-65B ultracentrifuge (Type 35 rotor), and a Model L Beckman ultracentrifuge (Type 65Ti rotor). All centrifugations were carried out at 40 C.

All special assays were performed on a Cary 118 recording spectrometer using 1 cm path-length, 1 ml cuvettes at 300 C unless otherwise stated.

High pressure liquid chromatography was performed using a Glenco system (Glenco Scientific, Incorporated, Houston, Texas). A Partisil PXS 10/25 SAX column (25 cm/4.6 mm) was used to separate purine nucleotides using 5mM potassium phosphate at pH=4.7 and pH=3.1 (step-wise gradient). The flow rate was maintained at 1 ml per minute. Purine bases were separated on a Partisil PXS 10/25 SCX column (25 cm/4.6 mm) by isocratic elution using 10 mM ammonium phosphate at pH=3.2 with a flow rate of 1.33 ml per minute. Both columns were purchased from Whatman. The effluent from the columns was monitored at 254 nm using a Glenco ultraviolet absorbance monitor with a strip chart recorder. 1 ml fractions were collected and analyzed spectrally to determine the identity of the purine compounds.

SYNTHESIS

IMP (14C(U)) was synthesized from AMP (14C(U)) 42 mCi/mmole using AMP deaminase (61 units/mg protein). 100 µCi of radioactive AMP in 50% ethanol was lyophilized to dryness and dissolved in 1 ml buffer containing 0.01 M potassium citrate and 0.1 M potassium chloride, pH=6.5. 4 IU (µmoles/minute/mg protein) of AMP deaminase (in 66% glycerol and
0.33 M potassium chloride) was added and the reaction was monitored at 265 nm until there was no further decrease in absorbance. The reaction was stopped with 1.23 ml 95% ethanol and centrifuged at 46,000xg for 30 minutes. The reaction mixture was then dried to a volume of less than 1 ml and applied to a Whatman PXS 10/25 SAX high pressure liquid chromatography column. The nucleotides were eluted with 5 mM potassium phosphate at pH=4.7 and 3.1 (see Figure 11 for elution profile). AMP and IMP were well separated on this system. Fractions containing IMP were pooled, the test tubes rinsed with 95% ethanol which was added to the IMP, and the solution was dried to approximately 6 ml. Approximately 65 µCi was recovered and shown to be chemically and isotopically pure by HPLC and PEI-cellulose thin layer chromatography (see chromatographic separations described under in experimental results).

Adenylosuccinate lyase was purified from yeast and used to synthesize adenylosuccinate by the method of Carter and Cohen (1956). This enzyme was stored at -20°C. To synthesize AMP-S, 3 mmoles of AMP and 27 mmoles of fumarate were adjusted to pH=6 in 150 ml water. Five ml of yeast extract (AMP-S lyase) was added and the mixture was incubated overnight at 37°C. The reaction mixture was boiled for five minutes and then centrifuged at 31,300xg to remove the precipitate. The supernatant was diluted to 900 ml and placed on a DEAE cellulose column (Whatman DE-52) with water at a flow rate of 2.2 ml per minute. The column was rinsed with water until the absorbance of the effluent dropped to zero. The nucleotides were eluted with an 800 ml linear gradient of 0-0.5 M triethylammonium bicarbonate pH=8.2. Fractions containing AMP-S, as determined by optical spectra, were pooled and lyophilized.
Figure II.
The powder was taken up in a small amount of water and stored at \(-20^\circ\text{C}\). The final concentration was 16 mM. Purity was checked using HPLC (SAX column) and PEI-cellulose thin layer chromatography (Crabtree and Henderson, 1971), and no contaminating nucleotides, nucleosides, or bases were present.

Adenylophosphonopropionate (APP) was synthesized by the method of Brand and Lowenstein (1978) and was a gift from Dr. Jean Vorhaben.
EXPERIMENTAL ANIMALS

Newly hatched white leghorn roosters were obtained from Hendricks Grain Company and were given food (Purina Chick Startena--18% under protein) and water ad libitum. Chicks of two, three, and four weeks of age were used as experimental animals. The average weight of these chicks were 90, 160, and 250 gm, respectively. β-Estradiol was dissolved in propylene glycol at a concentration of 25 mg/ml and 0.2 ml/100 gm body weight was injected intramuscularly into the leg of each chick. Control chicks were matched by weight to the experimental animals and treated with propylene glycol (0.2 ml/100 gm weight) (Jost et al., 1973). 24 and 48 hours after treatment, the animals were killed by decapitation and the liver quickly excised and placed on ice. Livers from β-estradiol treated chicks were consistently larger and lighter in color than the matched controls.

The chicks used for the radioactive incorporation studies were treated with β-estradiol and at 24 and 48 hours were injected intraperitoneally with 20 μCi formate \( ^{14}C \)/100 gm body weight. These chicks were killed 30 minutes later and the livers removed and placed in 0.75 M perchloric acid (2 ml/lg liver).

Regenerating rat liver studies were performed using Sprague-Dawley rats. Partial heptatectomies (approximately 70% of the liver) were performed by the standard method (Higgins and Anderson, 1931) using 2 μl Ketaset/100 gm body weight as an anesthetic (ketamine hydrochloride, Bristol Laboratories, Syracuse, New York). Animals were sacrificed at
24 and 48 hours, the livers removed and homogenized in 1:10 (wt/vol.) ice cold 0.25 M sucrose, 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-'2-ethanesulfonic acid), pH=7.0, 1 mM EDTA (ethylenediamine tetraacetic acid, tetrasodium salt), and 1 mM β-mercaptoethanol, and centrifuged at 31,300xg for one hour. The supernatant was used for AMP-S lyase and AMP-S synthetase assays as described in Assays. Sham-operated animals were used as controls.

The fish used in this study were *Fundulus heteroclitus* (killifish, bait minnows) and were obtained locally. These fish were adapted to 33% or 10% salt water over a period of 14 days and were kept at 20±2°C and maintained on commercial fish food.

**PREPARATION OF TISSUES**

Livers removed from chickens were homogenized in four volumes of buffer containing 0.25 M sucrose, 50 mM HEPES, pH=7.0, 1 mM EDTA, and 2.5 mM β-mercaptoethanol. Homogenization was performed using a Potter-Elvehjen homogenizer. Each homogenate was centrifuged at 31,300xg for 20 minutes, filtered through glass wool, and then centrifuged at 113,700xg for one hour. The supernatant was filtered through glass wool to remove lipids. All centrifugation steps were performed at 4°C and at all other times the homogenates were kept at 0-4°C. Protein was measured using the biuret method. Bovine serum albumin was used as a standard (Gornall, et al., 1949).

The chicken livers which were used for metabolite assays were rapidly removed from decapitated chickens and clamped between aluminum tongs, which were precooled in liquid nitrogen, and then immersed in
liquid nitrogen. The time for freezing was less than ten seconds. The frozen tissue was pulverized in a stainless steel percussion mortar and the powder was used for the metabolite assays.

The gills removed from each fish were placed in 2 ml buffer containing 0.25 M sucrose, 50 mM HEPES, 1 mM EDTA, and 2.5 mM 8-mercaptoethanol and homogenized using a Tekmar tissumizer. This solution was then centrifuged at 46,000xg for 20 minutes, the supernatant removed and recentrifuged at 130,000xg for one hour. This solution was decanted and used for all purine enzyme assays. The solution was kept cold at all times. Protein was determined by the biuret method as described above. Gill homogenates which had been centrifuged at 500xg for ten minutes were used for the glutamate dehydrogenase and glutaminase assays.

METABOLITE ASSAYS

PRPP

The amount of PRPP in chicken liver was measured using the procedure of LaLanne and Henderson (1974). The adenine phosphoribosylpyrophosphate transferase used in this determination was prepared by the method of LaLanne and Henderson (1974) from Ehrlich ascites tumor cells and stored as a lyophilized powder at -20°C. For use in the assay, 30 mg of the powder was dissolved in 1.5 ml 26 mM Tris-HCl (tris (hydroxymethyl)amminomethane), pH=7.4. One ml of 100 μM Adenine with 100 μl of 37 mM 2,3-diphosphoglycerate (in 26 mM Tris-HCl, pH=7.4) added, was heated in a boiling water bath. Approximately 100 mg liver, taken directly from a percussion mortar kept in liquid nitrogen, was added to the buffer. The solution was mixed and placed in a boiling
water bath for 20 seconds to extract the PRPP from the cells, then transferred to an ice bath. This extract was centrifuged for 10 minutes at 8,000xg and the supernatant was used to measure the PRPP present.

To determine the amount of PRPP in the liver extract, 200 µl of the centrifuged extract was added to 25 µl of 22.5 mM CaCl₂ in 26 mM Tris-HCl, pH=7.4, and adenine (¹⁴C(U)) to a specific activity of 5.3 µCi/µmole. The reaction was started with 25 µl of APRT and stopped at 0 or 30 minutes with 5 µl of 5 N perchloric acid. The reaction mix was then neutralized with 5 µl of 4 M KOH in 2 M Tris base (Lowry and Passoneau, 1972). This solution was then cooled and centrifuged to precipitate the potassium perchlorate formed upon neutralization. 25 µl of the neutralized reaction mix was spotted on cellulose thin layer plates and chromatographed in n-propanol:ammonia:water (60:30:10) until the solvent front reached the top of the plate (or 50 µl was spotted on Whatman 3MM paper and chromatographed for 16 hours in the same solvent). The R_f for AMP is 0.16 and the R_f for adenine is 0.55. The recovery of known amounts of PRPP was 15%. This value was used to calculate nmole/mg protein.

To determine the standard curve for PRPP a reaction mixture containing 200 µl Tris buffer, 25 µl of APRT and 25 µl PRPP solution in 22.3 mM CaCl₂, 26 mM Tris-HCl, pH=7.4, to give 0-20 mM PRPP, was incubated at 37°C for 30 minutes. The percent conversion was determined by the chromatographic procedure described and used to construct a standard curve for the calculation of the amount of PRPP in the liver extracts. The protein in the PRPP level determinations was measured by dissolving the precipitate formed upon boiling in 2 ml 1 N NaOH,
adding 8 ml biuret reagent, and measuring the absorbance at 550 nm. The standard curve was constructed as described above.

**Adenine and Guanine Levels**

One gm of freeze-clamped liver powder was homogenized in 2 ml 0.75 N perchloric acid using a Tekmar tissumizer. This solution was centrifuged at 8,000xg for 10 minutes and the supernatant placed in a boiling water bath for one hour. 50 µl of this supernatant was placed on a Partisil PXS 10/25 SCX high pressure liquid chromatography column and fractions containing adenine and guanine was collected (see Figure 12). Ultraviolet spectra of the purine compounds were taken in 0.09 N HCl. The concentration of each base was determined by the absorbance at 249 nm for guanine (ε=11,100 M⁻¹cm⁻¹) and 263 nm for adenine (ε=13,000 M⁻¹cm⁻¹). This column gives virtually 100% recovery of adenine and guanine as determined by spectral measurements and radioactivity recovered using known concentrations of purine bases.

**Glutamate**

The amount of glutamate formed in the amidophosphoribosyltransferase and GMP synthetase reactions was determined by use of glutamate dehydrogenase. A reaction mixture containing 160 mM Tris-HCl, pH-9.0, 0.5 mM NAD, 4.4 units glutamate dehydrogenase in 50% glycerol, and 0-50 nmoles of glutamate in 1 ml was used to construct a standard curve for this reaction. Glutamate synthesized by the amidophosphoribosyltransferase or GMP synthetase reaction was determined by adding 100 µl of the reaction mixture for these enzymes to the glutamate dehydrogenase reaction mix and measuring the absorbance change at 340 nm (Δε=6.22 mM⁻¹cm⁻¹) (Bernt and Bergmeyer, 1974).
PURINE BIOSYNTHESIS DE NOVO

Livers removed from chickens injected with radioactive formate were placed in 0.75 M perchloric acid and homogenized with a Tekmar tissumizer. This solution was boiled for one hour to hydrolyze the RNA and DNA and solubilize the nucleotides formed, converting them to bases. The homogenate was then centrifuged and the supernatant treated according to the procedure of Chen et al. (1977). In this method the perchloric acid in the solution is removed by extraction with 0.5 M tricaprylyl tertiary amine in freon and then by further extraction with a freon wash. The radioactivity incorporated into purines was measured by chromatography of the water layer on Whatman 3MM paper. Adenine and guanine-hypoxanthine were separated according to Brand and Lowenstein (1978b) using water-saturated butanol:ammonia (100:1) for 18 hours ($R_f$ adenine=0.2, $R_f$ guanine and hypoxanthine=0.12). Guanine and hypoxanthine were separated using 95% ethanol:1 M ammonium acetate, pH=7.3, (7:3) (Pabst, 1961, $R_f$ adenine=0.1, $R_f$ guanine=0.44, and $R_f$ hypoxanthine=0.62). Purine bases were located under ultraviolet light, the spots cut out, and counted.

ENZYME ASSAYS

Adenylate Kinase

The adenylate kinase assay was as described by Schleitman and Greenwalt (1968). The assay mixture contained 1 mM NADP, 0.015 M glucose, 10 IU hexokinase. 0.8 IU glucose-6-phosphate dehydrogenase, 0.45 mM KCN, 3 mM ADP, 5 mM MgCl$_2$, and 57 mM glycyl-glycine buffer,
pH=8.0. 100 µl homogenate was used to start the reaction and NADPH formation was observed at 340 nm (Δε=6.22 mM⁻¹cm⁻¹).

**Glutamate Dehydrogenase**

Glutamate dehydrogenase activity in fish tissues was determined by the method of Schmidt (1974). The 500 xg supernatant was used in this assay which included 5 µl Triton-X 100, 0.2 mM NADH, 1 mM ADP, 50 mM triethanolamine, pH=8.0, 2.5 mM EDTA, 100 mM ammonium acetate, and 2 IU lactate dehydrogenase in 1 ml. The reaction was started by the addition of 7 mM α-ketoglutarate. The decrease in absorbance at 340 nm was recorded and used to calculate the activity of the enzyme (Δε=6.22 mM⁻¹cm⁻¹).

**Glutaminase**

Phosphate-dependent glutaminase activity was determined by the method of Curthoys and Weiss (1974). The assay mixture contained 0.15 M potassium phosphate, 20 mM glutamine, 50 mM Tris-HCl, pH=8.6, and 0.2 mM EDTA in 100 µl. The reaction was started by adding 20 µl of the 500xg supernatant from fish gill and incubated for 30 minutes at 37°C. The reaction was stopped by adding 10 µl 3 N HCl. To this reaction mixture was added 1 ml solution containing 2 mM NAD, 0.25 mM ADP, 0.03% hydrogen peroxide and 80 mM Tris-HCl, pH=9.4. 12 IU of glutamate dehydrogenase were added and the absorbance at 340 nm after 30 minutes was recorded. (The amount of glutamate formed in the glutaminase assay is determined by the formation of α-keotglutarate and NADH). The change in absorbance was compared to a standard curve and the amount of glutamate formed, and thus the activity of glutaminase in the homogenate, was determined.
EXPERIMENTAL RESULTS
DEVELOPMENT OF EXPERIMENTAL PROCEDURES

The enzymes of purine biosynthesis have been purified and studied from many sources (Henderson and Paterson, 1973). To facilitate the investigation of the regulation of purine synthesis in chicken liver, it was necessary to adapt the enzyme assay procedures used by other researchers to this system. The assay techniques developed satisfy several criteria:

(1) These assays can be used to measure enzyme activity in crude homogenates. Purine catabolism in avian livers is very rapid and the assays were developed to circumvent this problem. All samples were centrifuged at greater than 100,000xg to remove 5'-nucleotidase which is located in the microsomal fraction. Tests showed that no nucleotidase was present in the supernatant (see Table 6). The homogenates used were subjected to no other treatment to prevent loss of the enzymes by manipulation. In a number of previous studies by other investigators, the enzymes have been partially purified before their activity was measured.

(2) All assays can be performed rapidly and, except for analysis of AMP-S synthetase activity, the results can be determined within 24 hours. This condition was important because a large number of samples had to be analyzed within one day for several consecutive days.

(3) Each enzyme assay is adapted to the peculiarities of the chicken system. The levels of several enzymes found in chicken liver
Table 6.

Degredation of Purine Monophosphates

5'-Nucleotidase Levels

$^{14}\text{C}}\text{-AMP and }^{14}\text{C}}\text{-IMP were incubated with the liver homogenate for 30 minutes. The reaction was stopped by boiling, the reaction mixture was centrifuged, and the supernatant spotted on PEI-cellulose thin layer plates (25 μl). The plates were chromatographed in water with adenine, adenosine, and IMP (R_f's = 0.26, 0.39, 0.0, respectively) or inosine, hypoxanthine, and IMP (R_f's = 0.53, -0.35, 0.0, respectively) added as carriers.}

<table>
<thead>
<tr>
<th>% of counts remaining in the monophosphate</th>
<th>0 minutes</th>
<th>30 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP</td>
<td>97.3</td>
<td>96.7</td>
</tr>
<tr>
<td>AMP</td>
<td>97.1</td>
<td>96.9</td>
</tr>
</tbody>
</table>
interfere in certain assays, and the procedures developed eliminate this problem. Each of these assays can be used to measure purine biosynthesis in other systems with a minimum of revision. Due to the high rate of purine catabolism in the chicken, this system probably represents the most extreme case for analysis of purine enzymes and other systems should actually be easier to handle. Liver homogenates contain many enzymes involved in purine and amino acid metabolism. Because of this, several controls were run on the enzyme assays developed to insure that the absorbance or radioactivity change seen during the course of the assay reflected the activity of the desired enzyme.

To study the enzymes of the purine nucleotide cycle it was necessary to develop assays for AMP-S synthetase, AMP-S lyase, and AMP deaminase. The spectral assay used by Rudolph and Fromm (1969) to study AMP-S synthetase from *E. coli* could not be used due to the presence of high levels of AMP-S lyase in the chicken liver homogenate. This enzyme prevents the accumulation of AMP-S by forming AMP, a nucleotide which has an ultraviolet spectrum similar to that of IMP (Figure 13). Assays were monitored at varying wavelengths (e.g. 265 nm and 280 nm), and a GTP regenerating system was added to the reaction mixture in an attempt to find conditions under which the formation of AMP-S (or AMP) could be monitored spectrophotometrically (GDP inhibits the enzyme with a *K*<sub>i</sub> of 5-30 μM). None of these conditions gave satisfactory measurement of the enzyme's activity. Dialysis of rat liver homogenates will inactivate the AMP-S lyase present in that system but the enzyme from chicken liver is sufficiently stable to retain catalytic activity after similar treatment. Since AMP-S lyase activity is almost
Figure 13.

Spectra of AMP, IMP, and AMP-S
200-fold higher than AMP-S synthetase activity in chicken liver, inactivation of 95% of the AMP-S lyase present is not sufficient to allow measurement of AMP-S synthetase activity. Copper sulfate, which inhibits AMP-S lyase from rat liver (Ogawa et al., 1978) has very little effect on the enzyme from chicken liver, and this method could not be used to develop a spectral AMP-S synthetase assay. In an attempt to remove the interfering activity of AMP-S lyase, an inhibitor, adenylophosphonopropionate (APP) was tested. Brand and Lowenstein (1978) have found that this analogue inhibits partially purified chicken liver AMP-S lyase with a $K_i$ of 5.4 nM. This compound, however, is not a good inhibitor of AMP-S lyase activity in crude homogenates (Table 7). It is necessary to use 150 nM APP to inhibit the enzyme by 50% and 8-9% of the activity remained at 2 μM APP. The amount of enzyme used was sufficient to catalyze the formation of 5 nmoles of AMP per minute. (Using the radioactive assay developed for AMP-S synthetase, which utilizes endogenous AMP-S lyase, it was possible to show that the AMP-S formed by AMP-S synthetase is converted to adenine nucleotides even in the presence of 2 μM APP (see Table 8)). Higher concentrations of APP inhibited the activity of AMP-S synthetase from rat muscle (partially purified by Wade Baugher of this laboratory) (Table 9). Therefore, this approach could not be used to develop a spectral assay to measure the activity of AMP-S synthetase.

A radioactive assay developed by R. B. Hurlbert (personal communication) was used to measure the level of AMP-S synthetase in chicken liver homogenates. This method utilizes endogenous AMP-S lyase and adenylate kinase to trap the AMP-S synthesized in the form of adenine
### Table 7.

Inhibition of AMP-S Lyase by APP

% of enzyme activity remaining when assay included indicated concentration of inhibitor.

<table>
<thead>
<tr>
<th>Concentration of APP</th>
<th>% AMP-S Lyase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 nM</td>
<td>63</td>
</tr>
<tr>
<td>100 nM</td>
<td>59</td>
</tr>
<tr>
<td>150 nM</td>
<td>45</td>
</tr>
<tr>
<td>200 nM</td>
<td>38</td>
</tr>
<tr>
<td>400 nM</td>
<td>33</td>
</tr>
<tr>
<td>500 nM</td>
<td>28</td>
</tr>
<tr>
<td>700 nM</td>
<td>21</td>
</tr>
<tr>
<td>1,000 nM</td>
<td>15</td>
</tr>
<tr>
<td>2,000 nM</td>
<td>8.4</td>
</tr>
</tbody>
</table>
Table 8.

AMP-S Synthetase Assay

% of counts in products of AMP-S synthetase assay with and without APP. When APP is present, counts should remain with AMP-S--ADP due to the inhibition of AMP-S lyase.

<table>
<thead>
<tr>
<th>% of counts in</th>
<th>ATP</th>
<th>AMP-S--ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard Assay</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min.</td>
<td>0.44</td>
<td>0.24</td>
</tr>
<tr>
<td>15 min.</td>
<td>2.85</td>
<td>0.48</td>
</tr>
<tr>
<td>30 min.</td>
<td>5.3</td>
<td>0.92</td>
</tr>
<tr>
<td>45 min.</td>
<td>6.63</td>
<td>1.72</td>
</tr>
<tr>
<td><strong>+APP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min.</td>
<td>0.17</td>
<td>0.75</td>
</tr>
<tr>
<td>15 min.</td>
<td>2.28</td>
<td>1.06</td>
</tr>
<tr>
<td>30 min.</td>
<td>4.1</td>
<td>1.04</td>
</tr>
<tr>
<td>45 min.</td>
<td>6.66</td>
<td>1.89</td>
</tr>
</tbody>
</table>
Table 9.
Inhibition of AMP-S Synthetase Activity

Inhibition of AMP-S synthetase partially purified from rat muscle in the presence of APP. 100% activity=0.68 O. D./50 s.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>44% inhibition</td>
</tr>
<tr>
<td>50</td>
<td>56% inhibition</td>
</tr>
</tbody>
</table>
di- and tri-phosphate nucleotides (see Figure 14). These enzymes are present at levels 200 times and 110 times, respectively, the level of AMP-S synthetase activity in the chicken liver system. AMP-S formed by AMP-S synthetase was rapidly converted to AMP (by AMP-S lyase) and, in the presence of added ATP, to ADP (by adenylate kinase). Phosphoenolpyruvate and pyruvate kinase were added in excess to serve as both a GTP regenerating system and to convert the ADP formed to ATP. The radioactive nucleotides formed were separated in a chromatographic system (PEI-cellulose thin layer plates using 0.5 and 2 M sodium formate, pH=3.4, Crabtree and Henderson, 1971) in which the Rf's for AMP-S, ADP, and ATP were identical (see Figure 15). Radioactivity was never found to accumulate in AMP which is well separated in this system from IMP and the other adenine nucleotides. It was necessary to synthesize and purify radioactive IMP since the commercial preparations contained impurities which co-chromatographed with ATP. Chromatography of the commercial IMP on the SAX-HPLC column showed that 1.59% of the radioactivity was in purine bases and nucleosides, 95.4% was IMP, and 3.1% of the counts were present in compounds that required high concentrations of phosphate buffer to remove from the column. Chromatography on PEI-cellulose thin layer plates shows that 3.4% of the radioactivity stays with the ATP area. Since conversion in the AMP-S synthetase assay is usually 3-10%, the impurities present in the commercial IMP could mask the enzyme activity measured by this method (and could possibly inhibit the enzymatic activity). AMP deaminase was used to synthesize IMP from AMP and the reaction mixture was applied to the SAX-HPLC column. AMP and IMP are well separated in this chromatography system.
Figure 14.

AMP-S Synthetase Assay and possible fates of AMP

Heavy lines indicate reactions occurring in assay
Figure 15.

PEI-TLC separation of AMP-S Synthetase Assay Mixture
To determine the accuracy of the radioactive assay, the activity of AMP-S synthetase partially purified from rat muscle was measured using both the spectral and radioactive assays. Table 10 shows that these two methods give identical results. In analyzing these assays a chromatographic system was used in which AMP-S--ADP were well separated from ATP (Figure 15). Radioactivity was always found associated with AMP-S--ADP since the AMP-S lyase necessary to form AMP was not present in this preparation. When AMP-S synthetase activity was assayed in chicken liver homogenates using this method, radioactivity was always found associated with ATP, reflecting the equilibration of AMP-ADP-ATP by adenylate kinase (Figure 14). By trapping the radioactivity in ADP and ATP no IMP is formed due to the action of AMP deaminase. This prevents the cycling of the purine nucleotide cycle and eliminates the formation of IMP as a possible source of error.

The assay for AMP-S synthetase was done as suggested by R. B. Hurlbert (personnal communication). The assay solution contained 0.1 mM IMP (\(^{14}\text{IMP(U)}\)--specific activity=2.8 mCi/mmol), 2.5 mM aspartate, 0.125 mM ATP, 0.25 mM GTP, 50 mM HEPES, pH=7.0, 3 mM magnesium acetate, 2.5 mM phosphoenolpyruvate, and pyruvate kinase at 20 \(\mu\)g/ml (0.01 IU). 25 \(\mu\)l of the chicken liver homogenate was added to give a final volume of 250 \(\mu\)l. The mixture was incubated at 30\(^\circ\)C for 30 minutes and then stopped by boiling the reaction mixture for 5 minutes. The blanks contained no GTP or aspartate. The reaction mixture was centrifuged on a table-top centrifuge to remove the precipitated protein and 25 \(\mu\)l of the solution was spotted on PEI-cellulose thin layer plates. The plates were washed overnight with methanol:water (50:50) to remove purine bases
Table 10.

Activity of AMP-S Synthetase

Comparison of spectral and radioactive assays on the enzyme partially purified from rat muscle.

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Activity (nmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectral assay</td>
<td>0.56</td>
</tr>
<tr>
<td>Radioactive assay</td>
<td>1.24 for 2 min, 2.58 for 4 min, enzyme inactivated after 4 minutes due to dilution of protein.</td>
</tr>
</tbody>
</table>
and ribosides. The remaining nucleotides were separated using 0.5 M sodium formate, pH=3.4 (12 cm, approximately 35 minutes) and then to 18 cm with 2 M sodium formate, pH=3.4 (approximately 35 minutes) without drying between solvent changes. The Rf's were ATP-0.0, AMP-S-ADP-0.11, AMP-0.66, and IMP-0.5.

For the regenerating rat liver studies a spectral assay for AMP-S synthetase was used (Rudolph and Fromm, 1969). The rat liver homogenate was dialyzed overnight on 0.03 M potassium phosphate, pH=7.0, containing 1 mM EDTA and 1 mM dithiothreitol, to inactivate AMP-S lyase. The assay mixture contained 0.15 mM IMP, 0.06 mM GTP, 5 mM aspartate, 1 mM magnesium acetate, and 20 mM HEPES, Ph=7.0. The increase in absorbance at 280 nm was measured and the nmoles of AMP-S produced calculated by using the extinction coefficient of 11.7 mM$^{-1}$cm$^{-1}$. Other spectral assays used the above procedure with no dialysis.

It was possible to measure the activity of AMP-S lyase spectally using the conditions described by Carter and Cohen (1956). This enzyme is present in large quantities in avian livers, and the procedure required no modification. The activity of AMP-S lyase was measured spectrophotometrically in a 1 ml assay mixture containing 20 mM Tris-HCl, pH=7.0 (this was determined to be the pH optimum for the chicken liver enzyme) 1 mM EDTA, and 0.05 mM AMP-S. The decrease in absorbance at 280 nm was monitored and the nmoles formed calculated from a Δε of 10.7 mM$^{-1}$cm$^{-1}$ for AMP formation from AMP-S. When AMP deaminase activity was high, the change in absorbance was measured at 282 nm. AMP and IMP absorbance at this wavelength is identical (Δε$_{282}$=10.0 mM$^{-1}$cm$^{-1}$).
The AMP deaminase assay used was modeled after the procedure of Schultz and Lowenstein (1976). Since both AMP and ATP absorb at 265 nm, giving a high absorbance blank, it was necessary to use a 1 mm path-length cuvette to measure the activity of this enzyme. Addition of ATP to the reaction mixture demonstrated that AMP deaminase from chicken liver is activated by this nucleotide approximately three-fold (see Table 16). This increase in activity was the same in both control and β-estradiol treated chickens. By adding radioactive AMP to the assay mixture and separating the nucleotides present by use of an anion exchange high pressure liquid chromatography column (Figure 11) it was possible to show the accumulation of radioactivity in IMP (Table 11). The spectral assay containing ATP was used routinely to measure the activity of AMP deaminase in chicken liver homogenates. The IMP formed in the AMP deaminase reaction cannot be cycled into the purine nucleotide cycle nor diverted to the GMP pathway due to the absence of needed substrates (eg. aspartate and NAD) in the reaction mixture. The enzyme activity was monitored at 265 nm. The assay mixture contained 50 mM imidazole, pH=6.5, 0.10 M KCl, and 1 mM AMP. 0.5 mM ATP was added to the assay when chicken homogenates were used as the source of enzyme but not when the activity present in fish gill was assayed. The decrease in absorbance at 265 nm has a change in absorptivity of 8.86 mM⁻¹cm⁻¹.

The measurement of the activity of amidophosphoribosyltransferase is difficult due to the instability of both the substrate and product of this enzymatic reaction. The assay technique most frequently employed is the measurement of glutamate formed in the reaction by the use of glutamate dehydrogenase. This is both time consuming and difficult
**Table 11.**

Activity of AMP Deaminase

Standard AMP deaminase assays were incubated with $^{14}$C-AMP. The reaction was stopped by boiling and the supernatant applied to a SAX-HPLC column. The assay containing ATP was analyzed by chromatography on PEI-cellulose thin layer plates in 0.5 M formate, pH=3.4.

<table>
<thead>
<tr>
<th>% of counts in</th>
<th>AMP</th>
<th>IMP</th>
<th>ATP+ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard assay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min.</td>
<td>93.9</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>30 min.</td>
<td>91.4</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td><strong>Standard assay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ATP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min.</td>
<td>89.7</td>
<td>2.11</td>
<td>0</td>
</tr>
<tr>
<td>30 min.</td>
<td>43.5</td>
<td>7.7</td>
<td>45.5</td>
</tr>
</tbody>
</table>
since the glutamate dehydrogenase reaction does not go to completion. Thus, all results must be calculated by use of a standard curve. King and Holmes (1976) have developed an assay which utilizes the reaction of phosphoribosylamine with $^{35}$S-cysteine to form a stable compound. While this assay allows the determination of amidophosphoribosyltransferase activity using either glutamine or ammonia, the interaction of cysteine with other ribose-phosphates present in crude homogenates would raise the activity seen in the assay blank and perhaps obscure the amidophosphoribosyltransferase activity. An assay utilizing the differential chromatographic properties of glutamate and glutamine has been developed by Martin (1972). In this procedure the reaction mixture is spotted on disks of DEAE-cellulose which are then washed with distilled water. This removes the glutamine while the glutamate formed by the enzymatic reaction remains on the paper. By using radioactive glutamine it is possible to measure the activity of amidophosphoribosyltransferase. Several controls were run before this assay technique was used routinely. The amount of glutamate and glutamine which remains on the DEAE-cellulose disks after successive washings was measured by the use of radioactive glutamate and glutamine. Assay mixtures containing these compounds were placed on the disks and washed one to six times. As seen in Figure 16, very little glutamate is washed from the disks while most of the glutamine is removed. If the amount of radioactivity placed on each disks is known, it is possible to calculate the percentage of glutamine converted to glutamate by the homogenate and thus determine the activity of amidophosphoribosyltransferase.

The use of liver homogenates as the source of amidophosphoribosyltransferase introduces the possibility that compounds other than
Figure 16.
Glutamine and Glutamate Remaining on DEAE Disks after Washing

[Graph showing the remaining cpm (counts per minute) of glutamine and glutamate after different numbers of rinses.]
glutamate, but with similar chromatographic properties, give rise to the radioactivity which remains on the DEAE-cellulose disks after washing. To eliminate this possibility and to test the accuracy of this technique, the amount of glutamate formed during the enzymatic reaction was carefully measured by the glutamate dehydrogenase method. These two methods are in close agreement: 23.5 nmole of glutamate was formed in 60 minutes as measured by the DEAE-cellulose disk method and 22.6 nmole as determined by the glutamate dehydrogenase reaction (see Table 12). Thus the method of Martin (1972) using DEAE-cellulose to separate glutamine and glutamate is a valid technique for determining amidophosphoribosyltransferase activity.

5-phospho-α-D-ribosyl pyrophosphate (PRPP) is a high-energy compound which is unstable as a solid and in solution. Before the activity of amidophosphoribosyltransferase could be measured, the purity of the solid, frozen PRPP was determined by spectral assay. It was possible that the compounds formed by the breakdown of PRPP (pyrophosphate and ribose-5-phosphate, Pabst, 1961) could inhibit amidophosphoribosyltransferase. To eliminate this possibility, pyrophosphate and ribose-5-phosphate were added to the reaction mixture to simulate the breakdown of up to 50% of the PRPP (the commercial compound is approximately 80% PRPP). Measurement of the activity of amidophosphoribosyltransferase under these conditions shows no inhibition of enzymatic catalysis by these compounds (Table 13). Thus, commercial PRPP could be used in the amidophosphoribosyltransferase assay with no further purification.

Blanks for the amidophosphoribosyltransferase assay contained the complete reaction mixture without PRPP. The glutamate formed by other
### Table 12.

**Glutamate Formation**

Measurement of glutamate formed in the amidophosphoribosyltransferase and GMP synthetase assays using the glutamate dehydrogenase assay or the DEAE-cellulose disk method (Martin, 1972).

<table>
<thead>
<tr>
<th></th>
<th>nmoles formed in 60 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amidophosphoribosyltransferase</strong></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose disks</td>
<td>23.6</td>
</tr>
<tr>
<td>glutamate dehydrogenase</td>
<td>22.6</td>
</tr>
<tr>
<td><strong>GMP synthetase</strong></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose disks</td>
<td>0.68</td>
</tr>
<tr>
<td>glutamate dehydrogenase</td>
<td>0.72</td>
</tr>
</tbody>
</table>
Table 13.

Activity of Amidophosphoribosyltransferase

Activity of amidophosphoribosyltransferase in the presence of pyrophosphate and ribose-5-phosphate.

<table>
<thead>
<tr>
<th>Condition</th>
<th>nmoles/60 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard assay</td>
<td>24.2</td>
</tr>
<tr>
<td>+0.25 mM PP&lt;sub&gt;i&lt;/sub&gt; and R-5-P</td>
<td>26.1</td>
</tr>
<tr>
<td>+0.5 mM PP&lt;sub&gt;i&lt;/sub&gt; and R-5-P</td>
<td>26.7</td>
</tr>
<tr>
<td>+1 mM PP&lt;sub&gt;i&lt;/sub&gt; and R-5-P</td>
<td>24.2</td>
</tr>
</tbody>
</table>
enzymes in the homogenate could thus be measured and subtracted from the
amount of glutamate formed during the amidophosphoribosyltransferase
reaction. These blanks always showed very low activity. Vorhaben and
Campbell (1977) have found that glutaminase in chicken liver is located
in the nuclear and mitochondrial fractions of a tissue homogenate.
Thus, this enzyme would not interfere in the determination of the activ-
ity of amidophosphoribosyltransferase since the mitochondrial and nuclear
fractions are removed by high-speed centrifugation. Phosphate-dependent
glutaminase from rat muscle has been shown to be activated by PRPP so
that amidophosphoribosyltransferase activity measured in this tissue
gives falsely-high results (the glutaminase activity in the blank (no
PRPP) is lower than in the assay for amidophosphoribosyltransferase).
Hahn et al., (1969) have shown that rat liver glutaminase can be in-
activated by incubation at 50°C for 3 minutes. Thus the assay developed
for amidophosphoribosyltransferase from chicken liver can be used to
measure the activity of amidophosphoribosyltransferase from rat liver
with this modification.

The activity of amidophosphoribosyltransferase was measured by
the method of Martin (1972). The final concentration of substrates
in the assay was 1 mM PRPP, 3 mM MgCl₂, 12 mM β-mercaptoethanol, 50 mM
Tris-HCl, pH=7.0, and 2 mM glutamate [¹⁴C(U)---specific activity was
1 mCi/mmmole] in a final volume of 250 μl. 25 μl of the centrifuged
liver homogenate was used to start the reaction which was incubated in
a water bath at 37°C. The reaction was stopped by spotting 50 μl of
the reaction mixture on DEAE-cellulose disks at 0, 10, 20, and 30
minutes. The disks were dried under a heat lamp, rinsed four times in
2 liters of distilled water, dried again, and then counted. Blanks contained no PRPP. The reaction was linear with time and amount of enzyme added. Activity is expressed as nmoles/min/mg protein.

It was possible to adapt the assay procedure developed for the amidophosphoribosyltransferase reaction to the measurement of the activity of GMP synthetase. This enzyme, isolated from vertebrates, requires glutamine, XMP, and ATP. The large absorbance in the ultraviolet spectral region of the assay mixture for this enzyme plus the large number of possible fates of the substrates made it impossible to use spectral assays to measure the activity of this enzyme. Therefore, the method of measuring glutamate formation using DEAE-cellulose disks was adapted to the GMP synthetase reaction. The conditions to be used were chosen by referring to the study on the pigeon liver enzyme performed by Lagerkvist (1958). Using this data, the concentration of the substrates which optimize the enzyme's activity was determined. Thus, the assay was performed at pH=7.0, ATP concentration was less than 6 mM since the enzyme is inhibited above this concentration, and the concentration of XMP and glutamine was high enough to give maximal activity.

It was advantageous to keep the glutamine concentration as low as possible since the activity of the enzyme is calculated by the percent of glutamate formed. The conditions used coupled maximum enzyme activity with maximum sensitivity for the percentage of glutamine converted to glutamate. Once the assay was developed, this method of determining GMP synthetase activity was tested by using the glutamate dehydrogenase technique of determining glutamate formation. Though the amount of enzyme activity in chicken liver is very low, agreement between these
methods was good. The final concentrations in the assay mixture were 0.4 mM XMP, 0.2 mM glutamine \(^{14}\text{C}(\text{U})\)--the specific activity was 10 mCi/mmole}, 5 mM ATP, 100 mM Tris-HCl, pH=7.0, and 10 mM MgSO\(_4\). The reaction was started with 25 \(\mu\)l of the enzyme solution to give a final volume of 250 \(\mu\)l. The reaction was maintained at 37° C, then stopped by spotting 50 \(\mu\)l of the solution on DEAE-cellulose disks (numbered in pencil). The disks were then washed and counted as described for the amidophosphoribosyltransferase assay (Martin, 1972).

Xanthine oxidase from cow's milk converts xanthine to uric acid using molecular oxygen as the oxidizing substrate. In chickens, however, the enzyme is in the form of a dehydrogenase which uses NAD. The concentration of NAD used for the spectral assay of this enzyme was at least 10 times the \(K_m\) for this substrate (see Table 14). The activity of xanthine dehydrogenase was measured according to Massey et al. (1969) and Krenitsky et al. (1974). The final reaction mixture contained 0.1 mM xanthine, 0.1 M sodium pyrophosphate, pH=8.5, 0.3 mM EDTA, and 0.5 mM NAD in a volume of 1 ml. The decrease in absorbance was measured at 295 nm (\(\Delta\varepsilon=9.6 \text{ mM}^{-1}\text{cm}^{-1}\)).

The assay for IMP dehydrogenase activity was taken from Anderson and Sartorelli (1968). The concentration of IMP used in this assay is 10 times higher than that used in the assay for the rat liver enzyme by Jackson et al. (1977). The chicken homogenate does not show the interfering activity seen with the rat liver homogenates. The enzyme level was measured optically by monitoring the increase in absorbance at 290 nm. This change requires the presence of both NAD and IMP. It was necessary to show that the change in absorbance observed was due to the
Table 14.

Activity of Xanthine Dehydrogenase—Effects of NAD

<table>
<thead>
<tr>
<th>Concentration of NAD</th>
<th>ΔO.D. 290/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.002</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>0.76</td>
</tr>
<tr>
<td>0.4 mM</td>
<td>0.87</td>
</tr>
<tr>
<td>0.6 mM</td>
<td>0.83</td>
</tr>
<tr>
<td>0.8 mM</td>
<td>0.86</td>
</tr>
<tr>
<td>no xanthine</td>
<td>0.001</td>
</tr>
</tbody>
</table>

NADP gives approximately the same activity as NAD.

NAD and NADP together show only additive increases in activity.
formation of XMP and not to the synthesis of xanthine and uric acid resulting from the degradation of IMP (Figure 17). An IMP dehydrogenase assay containing radioactive IMP was analyzed using high pressure liquid chromatography (Figure 18). This procedure showed that XMP was indeed formed and that the amount of radioactivity in purine bases and ribosides did not increase during the incubation with the chicken liver homogenate (Table 15). XMP was the end-product of this reaction since the glutamine and ATP required for GMP formation were not present in the assay mixture. Thus, this spectral assay can be used to measure the activity of IMP dehydrogenase in the chicken liver system. The assay mixture used to measure IMP dehydrogenase activity contained 50 mM potassium phosphate, pH=7.4, 100 mM KCl, 1 mM EDTA, 2 mM IMP, and 0.2 mM NAD. The increase in absorbance due to XMP formation was measured at 290 nm ($\Delta \varepsilon = 5.4 \text{ mM}^{-1} \text{cm}^{-1}$). At this wavelength NAD reduction cannot be visualized and no absorbance change is observed when IMP is not included in the assay mixture.

An attempt was made to measure the level of GMP reductase in the chicken liver homogenates. The activity of this enzyme is exceedingly low in most higher organisms and its presence in vertebrate livers has never been demonstrated. It was not possible to detect this enzyme in the homogenates routinely used by the spectral assay employed. After these initial experiments, no further attempts were made to detect the presence of this enzyme. The final reaction mixture contained 40 mM Tris-HCl, pH=7.5, 0.2 mM GMP, 0.1 mM NADPH, and 4 mM 8-mercaptoethanol in a volume of 1 ml. The blank reaction contained no GMP (Brox and
5'-Nucleotidase

Nucleoside Phosphorylase

Xanthine Dehydrogenase

IMP → Inosine → Hypoxanthine → Uric Acid

+ NAD

GMP → XMP

+ NAD

Figure 17.
IMP Dehydrogenase Assay and IMP Degradation Pathway
FIGURE 18.

XMP

0.75 M PO₄

IMP

pH 4.7

NAD

4.7, 3.1

Urate

254 nm

Absorbance
Table 15.

Activity of IMP Dehydrogenase

$^{14}$C-IMP was incubated with the standard enzyme assay mixture. The reaction was stopped by boiling and the supernatant was applied to a SAX-HPLC column.

<table>
<thead>
<tr>
<th>Time</th>
<th>Urate and other bases</th>
<th>% of counts in</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min.</td>
<td>1.3</td>
<td>98.0</td>
</tr>
<tr>
<td>1 hour</td>
<td>1.5</td>
<td>78.1</td>
</tr>
<tr>
<td>2 hour</td>
<td>1.8</td>
<td>62.4</td>
</tr>
</tbody>
</table>
Hampton, 1968. The extinction change of NADPH to NADP at 340 nm is 6.22 mM$^{-1}$cm$^{-1}$. Various concentrations of GMP and NADPH were tried in this assay but the blank activity was always equal to that seen in the enzyme assay mixture.
REGULATION OF PURINE BIOSYNTHESIS DE NOVO

The liver from chickens treated with the hormone β-estradiol is a fast-growing, non-malignant tissue. In an analogous mammalian system, the regenerating rat liver, increases in the levels of several purine biosynthetic enzymes have been reported. (Experiments performed on this system confirm that the level of AMP-S synthetase and AMP-S lyase after partial hepatectomy is the same as in normal rat liver—Table 16.) The goal of this study was to determine if the avian system adapts to high growth rates in a manner similar to that of the fast-growing rat liver. In addition to determining the activities of purine biosynthetic and interconversion enzymes, the rate of purine biosynthesis, PRPP levels, and adenine and guanine pool sizes were determined in order to gain a better understanding of the regulation of purine biosynthesis de novo.

Eukaryotic tissues often respond to changes in their environment by changing the levels of proteins within the cell. The chicken liver, for example, produces large amounts of phosvitin when stimulated by the hormone β-estradiol (Jost et al., 1973). In rapidly growing, regenerating rat liver the levels of amidophosphoribosyltransferase and IMP dehydrogenase are raised (Katunuma and Weber, 1974, Jackson et al., 1977). The level of AMP-S lyase in the liver is lowered in starved rats but is raised in livers from chicks maintained under the same conditions (Brand and Lowenstein, 1978). Since AMP-S lyase is involved in de novo purine synthesis, the increased level of this enzyme may reflect the elevated rate of purine synthesis and uric acid excretion which accompanies increased amino acid degradation during starvation. The liver of avians
Table 16.

Activity of AMP-S Synthetase and AMP-S Lyase in Regenerating Rat Liver

"I" represents animals injected with anesthetic only. "S" represents animals which were anesthetized and cut open but from which no liver was removed. "O" represents animals from which approximately 70% of the liver was removed. Animals were sacrificed 24 and 48 hours after the operation was performed. Activity is in nmoles/min/mg protein.

<table>
<thead>
<tr>
<th></th>
<th>AMP-S Synthetase</th>
<th>AMP-S Lyase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.64±0.11</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>24 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.71</td>
<td>3.2</td>
</tr>
<tr>
<td>S</td>
<td>0.49</td>
<td>2.6</td>
</tr>
<tr>
<td>O</td>
<td>0.73</td>
<td>1.8</td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.66</td>
<td>2.5</td>
</tr>
<tr>
<td>S</td>
<td>0.39</td>
<td>1.8</td>
</tr>
<tr>
<td>O</td>
<td>0.72±0.11</td>
<td>2.1±0.1</td>
</tr>
</tbody>
</table>
is the main site of uric acid formation and excess amino acid nitrogen is converted to this compound through purine synthesis. Mammalian livers are also responsible for conversion of excess amino acid nitrogen to the excretory form, urea. Thus it was of interest to see if the rapid growth exhibited by the liver of chickens treated with β-estradiol is accompanied by a change in the level of purine biosynthesis and interconversion enzymes. Though purine biosynthesis de novo in uricotelic animals is higher than in mammals, the experimental results discussed in the literature review show that this system still has the capacity to increase the amount of purines synthesized. The levels of the enzymes involved in this biosynthesis can also increase as shown by (1) the higher activity of amidophosphoribosyltransferase in chickens fed a high protein diet (Katunuma et al., 1973) and (2) the rise in AMP-S lyase activity during starvation (Brand and Lowenstein, 1978b).

It is possible, though, that the activities of these enzymes are sufficient to support the rapid growth of the liver of β-estradiol treated chicks.

The activity of seven enzymes involved in purine biosynthesis, interconversion, and catabolism was measured in liver extracts obtained from normal and β-estradiol treated chickens using assays developed for the chicken system. The results obtained (Table 17) show that the activity present in the chicken homogenates does not change after hormonal treatment. This is in direct contrast to the regenerating rat liver system in which amidophosphoribosyltransferase activity is increased by 50% and IMP dehydrogenase activity is elevated five-fold.

The overall rate of purine biosynthesis was determined by the incorporation of formate into cellular purines (both acid soluble and
Table 17.  
Enzyme Levels in 17-estradiol-treated Chickens  

\[ p > 0.4 \]

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>24 hours n=6</th>
<th>48 hours n=6</th>
<th>Control n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amidophosphoribosyltransferase</td>
<td>4.7±1.2</td>
<td>5.9±0.4</td>
<td>5.6±2.1</td>
</tr>
<tr>
<td>AMP-S synthetase</td>
<td>0.21±0.01</td>
<td>0.17±0.02</td>
<td>0.17±0.03</td>
</tr>
<tr>
<td>GMP synthetase</td>
<td>0.062±0.03</td>
<td>0.042±0.002</td>
<td>0.044±0.027</td>
</tr>
<tr>
<td>AMP-S lyase</td>
<td>40.2±4.7</td>
<td>41.1±8.4</td>
<td>34.6±11.2</td>
</tr>
<tr>
<td>AMP deaminase +ATP</td>
<td>5.87±0.21</td>
<td>5.84±2.3</td>
<td>7.71±2.16</td>
</tr>
<tr>
<td>-ATP</td>
<td>2.93±0.85</td>
<td>2.4±0.99</td>
<td>2.52±0.62</td>
</tr>
<tr>
<td>IMP dehydrogenase</td>
<td>1.31±0.31</td>
<td>1.58±0.41</td>
<td>1.74±0.87</td>
</tr>
<tr>
<td>Xanthine dehydrogenase</td>
<td>12.9±0.3</td>
<td>14.1±2.1</td>
<td>14.8±5.9</td>
</tr>
</tbody>
</table>
those incorporated into RNA and DNA). As seen in Table 18, the rate of de novo purine synthesis is increased twelve hours after hormone treatment and remains at a high level for at least 48 hours. At 12 hours, though, much more of the newly synthesized IMP is being channelled into the GMP branch of the interconversion pathway, as shown by the adenine: guanine ratios in Table 19, than at any other time. By 48 hours the ratio of GMP and AMP synthesis has returned almost to normal, even though the overall rate of synthesis remains high. Thus, it appears that the rapid growth of the liver induced by the treatment with β-estradiol is supported by an increase in the rate of purine synthesis de novo. The branches of the purine interconversion pathways are more equally expressed during the initial 24 hours of this increase in de novo synthesis than under normal growth conditions.

5-Phospho-α-D-ribosyl pyrophosphate (PRPP), which is a substrate of the first committed step in purine biosynthesis, has often been implicated in the regulation of the rate of de novo synthesis (Henderson, 1972, Wyngaarden, 1976). Amidophosphoribosyltransferase, which forms phosphoribosylamine from PRPP, purified from pigeon liver shows sigmoidal kinetics at PRPP concentrations below the $K_m$ so that small changes in cellular levels of PRPP could greatly influence the rate of purine biosynthesis (Rowe and Wyngaarden, 1968, Wyngaarden, 1976). Determination of the level of PRPP in livers of chickens treated with β-estradiol shows that after 24 hours the amount of PRPP in the tissues has doubled (Table 20). The concentration of PRPP in both the 24 hour and control chickens (1.04 and 0.52 mM, respectively) is greater than the $K_m$ for the chicken livers enzyme (25-50 μM, Hartman, 1963).
**Table 18.**

**Purine Biosynthesis de novo**

Incorporation of $^{14}$C-formate into cellular purines ($p>0.001$)

<table>
<thead>
<tr>
<th></th>
<th>Adenine</th>
<th>Guanine-Hypoxanthine</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 hours n=6</td>
<td>20,000±6,500</td>
<td>57,500±13,000</td>
<td>77,500±19,500</td>
</tr>
<tr>
<td>24 hours n=6</td>
<td>35,500±11,000</td>
<td>40,000±15,500</td>
<td>75,000±26,500</td>
</tr>
<tr>
<td>48 hours n=6</td>
<td>43,500±13,000</td>
<td>50,000±9,000</td>
<td>90,000±22,000</td>
</tr>
<tr>
<td>controls n=7</td>
<td>15,500±6,500</td>
<td>16,500±9,000</td>
<td>32,000±15,500</td>
</tr>
</tbody>
</table>
Table 19.

Purine Biosynthesis—Adenine:Guanine Ratios

<table>
<thead>
<tr>
<th></th>
<th>A/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 hour</td>
<td>2.6</td>
</tr>
<tr>
<td>24 hour</td>
<td>3.7</td>
</tr>
<tr>
<td>48 hour</td>
<td>4.0</td>
</tr>
<tr>
<td>control</td>
<td>4.9</td>
</tr>
</tbody>
</table>
Table 20.

Cellular PRPP Levels

<table>
<thead>
<tr>
<th></th>
<th>nmoles/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>8.3±2.8 (p&gt;0.001)</td>
</tr>
<tr>
<td>n=6</td>
<td></td>
</tr>
<tr>
<td>48 hours</td>
<td>5.0±2.5</td>
</tr>
<tr>
<td>n=6</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>4.2±1.5</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
</tr>
</tbody>
</table>
The influence of purine nucleotides on de novo synthesis is not well understood. To determine if the concentration of these compounds changes during the increase in purine biosynthesis that follows β-estradiol treatment. Soluble pool sizes of adenine, guanine, and hypoxanthine were determined. The values listed in Table 21 show that, while the cellular pool sizes varied with each animal, there was no significant change in the levels of adenine, guanine, and hypoxanthine after stimulation with β-estradiol.

Purine biosynthesis in chickens is maintained at a high level, since these animals excrete amino acid nitrogen as uric acid. Katunuma et al. (1973) have shown that under conditions of high rates of amino acid catabolism, such as starvation or high protein diets, amidophosphoribosyltransferase levels increase while, under similar conditions, urea cycle enzymes are increased in ureotelic animals (Brand and Lowenstein, 1978b). Lipstein et al. (1978) have recently shown that activation of the oxidative pentose phosphate pathway by methylene blue results in an increase in the availability of PRPP and a rise in purine production. Thus, in chicken liver, purine synthesis is not saturated under normal conditions.

Amidophosphoribosyltransferase has often been implicated in the regulation of purine biosynthesis de novo (Henderson, 1972 and Wyngaarden, 1976). This enzyme in vitro is influenced by PRPP and glutamine concentration and inhibited by various nucleotides. Weber et al. (1976) have shown that under conditions of rapid growth, amidophosphoribosyltransferase and IMP dehydrogenase activity in rat liver increases. The result of these experiments show that in rapidly
<table>
<thead>
<tr>
<th></th>
<th>µmoles/gram wet weight</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>adenine</td>
<td>guanine</td>
<td>hypoxanthine</td>
</tr>
<tr>
<td>24 hours</td>
<td>1.34±0.25</td>
<td>0.55±0.11</td>
<td>1.52±0.21</td>
</tr>
<tr>
<td>n=6</td>
<td></td>
<td></td>
<td>24 hour control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.71±0.31</td>
</tr>
<tr>
<td>48 hours</td>
<td>1.73±0.26</td>
<td>0.75±0.14</td>
<td>0.96±0.15</td>
</tr>
<tr>
<td>n=6</td>
<td></td>
<td></td>
<td>48 hours control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.14±0.19</td>
</tr>
<tr>
<td>Control</td>
<td>1.61±0.54</td>
<td>0.64±0.34</td>
<td>1.35±0.50</td>
</tr>
<tr>
<td>n=12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 21. Purine Pool Sizes in β-estradiol-treated Chickens
growing chicken liver the activities of several purine biosynthetic enzymes (amidophosphoribosyltransferase, AMP-S synthetase, AMP-S lyase, AMP deaminase, IMP dehydrogenase, GMP synthetase, and xanthine dehydrogenase) do not change. In these livers, though, the rate of purine biosynthesis (as measured by formate incorporation) triples and the levels of PRPP within the cell is doubled. However, the soluble pools of adenine and guanine do not change in size during rapid growth.

The regulation of purine biosynthesis de novo is not understood. Much of the experimental data on this pathway indicates that the overall rate of synthesis is regulated by the rate of phosphoribosylamine formation. This may be controlled by the activity of PRPP synthetase or amidophosphoribosyltransferase and thus such factors as nucleotide inhibition and substrate availability may influence the rate of purine formation. The experimental results discussed above agree with this viewpoint and indicate that the amount of PRPP within the cell is an important factor in the regulation of de novo purine synthesis in chicken liver. The level of amidophosphoribosyltransferase in this system appears to be sufficient to support rapid growth. Similarly, IMP dehydrogenase activity does not change under conditions of rapid tissue growth and increased guanine formation (this increase is shown by the decrease in the adenine:guanine ratios observed in the formate incorporation study). This is in direct contrast to the results observed in an analogous mammalian system (regenerating rat liver) and may reflect the ability of uricotelic animals to synthesize large amounts of purines as a means of excreting amino acid nitrogen. Under conditions of rapid growth, the levels of all the enzymes measured in chicken liver are sufficient to provide the purine nucleotides needed for the synthesis of RNA and DNA, even though the overall rate of purine synthesis de novo is increased.
AMMONIA EXCRETION IN FUNDULUS HETEROCYLITIS

When euryhaline fish are adapted to fresh water, branchial ammonia excretion is increased (Gordon, et al., 1965, Mangum et al., 1976). There are three possible sources of this ammonia: (1) glutamate through glutamate dehydrogenase, (2) glutamine through glutaminase and glutamate dehydrogenase, and (3) AMP through AMP deaminase. Fundulus heterocylitis are a common salt water fish which can be adapted to fresh water. To evaluate the three possible sources of branchially excreted ammonia, these fish were maintained in both fresh and salt water and then the activity of glutamate dehydrogenase, glutaminase, AMP-S lyase, AMP-S synthetase, and AMP deaminase in gill homogenates was measured.

The results (see Table 22) show that neither glutamate dehydrogenase nor glutaminase activity changes when these fish are adapted to fresh water. However, the levels of AMP deaminase and AMP-S lyase are doubled in the gills of the fish which were maintained in a low salinity environment. Also, preliminary experiments showed that the levels of these enzymes in the liver did not change when F. heterocylitis were adapted to fresh water. This organ is thought to produce ammonia and glutamate which are transported, by the blood, to the gills for excretion (Goldstein and Forster, 1961, and Goldstein et al., 1964). AMP-S lyase and AMP deaminase, along with AMP-S synthetase, compose the purine nucleotide cycle (punc cycle) which has been implicated in ammonia production in rat skeletal muscle, brain, and kidney (Lowenstein and
Table 22.

Enzyme Levels in Gills from *Fundulus heteroclitus*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fresh Water (n=5)</th>
<th>Salt Water (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate Dehydrogenase</td>
<td>110±50</td>
<td>110±10</td>
</tr>
<tr>
<td>Glutaminase</td>
<td>2.9±1.7</td>
<td>3.9±1.8</td>
</tr>
<tr>
<td>AMP-S Lyase (p&gt;0.01)</td>
<td>3.2±0.3</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>AMP Deaminase (p&gt;0.01)</td>
<td>210±50</td>
<td>110±15</td>
</tr>
<tr>
<td>AMP-S Synthetase</td>
<td>---0.03-0.33 nmoles/min/mg protein</td>
<td>---</td>
</tr>
</tbody>
</table>

*Changes in this enzyme were impossible to detect due to contamination with 5' nucleotidase.*
Tornheim, 1970, Schultz and Lowenstein, 1976, Bodusky et al., 1976). The conversion of AMP to IMP may be responsible for the increased ammonia excreted by these fish when maintained in a fresh water environment. Since enzymes of the punc cycle are elevated, the cycling of this pathway may be involved in the formation of branchially excreted ammonia (AMP-S synthetase is hard to measure in this system since 5'-nucleotidase is present in the soluble fraction of the homogenate even after centrifugation at 130,000xg. The data in Table 22 represent low values for the activity of this enzyme). The systemic source of ammonia is unknown but it is possible that adenine, adenosine, or aspartate are transported via the bloodstream to provide the ammonia excreted by the gills.

Maetz (1971) has stated that teleost fish excrete 150-1,000 umoles NH$_3$/kg body weight/hour. In fresh water 1,000 umoles NH$_3$/kg body weight/hour can be formed by the action of AMP deaminase. It is definitely possible that AMP deaminase (and perhaps the complete punc cycle) is involved in the excretion of ammonia in euryhaline fish which have been adapted to a fresh water environment.
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