COUPLED NUCLEOSIDE PHOSPHORYLASE
REACTIONS IN ESCHERICHIA COLI

by

John Lewis Ott

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Physiological Bacteriology

Approved:

In Charge of Major Work

Head of Major Department

Dean of Graduate College

Iowa State College
1956
INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI Microform DP12892
Copyright 2005 by ProQuest Information and Learning Company.
All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>4</td>
</tr>
<tr>
<td>Deaminases Active on Adenine or Adenosine</td>
<td>4</td>
</tr>
<tr>
<td>Nucleosidases</td>
<td>14</td>
</tr>
<tr>
<td>Nucleoside phosphorylase</td>
<td>14</td>
</tr>
<tr>
<td>Hydrolytic nucleosidases</td>
<td>21</td>
</tr>
<tr>
<td>Nucleoside-N-transglycosidase</td>
<td>22</td>
</tr>
<tr>
<td>Transaminases Active with Adenine</td>
<td>25</td>
</tr>
<tr>
<td>METHODS</td>
<td>27</td>
</tr>
<tr>
<td>Organism and Medium</td>
<td>27</td>
</tr>
<tr>
<td>Enzyme Preparations</td>
<td>28</td>
</tr>
<tr>
<td>Cell-free extracts</td>
<td>28</td>
</tr>
<tr>
<td>Dialyzed preparations</td>
<td>28</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation</td>
<td>29</td>
</tr>
<tr>
<td>Analytical Procedures</td>
<td>29</td>
</tr>
<tr>
<td>Ammonia</td>
<td>30</td>
</tr>
<tr>
<td>Protein</td>
<td>30</td>
</tr>
<tr>
<td>Orthophosphate</td>
<td>30</td>
</tr>
<tr>
<td>Ribose</td>
<td>31</td>
</tr>
<tr>
<td>Spectrophotometry</td>
<td>31</td>
</tr>
<tr>
<td>Paper chromatography</td>
<td>33</td>
</tr>
<tr>
<td>Assay of radioactivity</td>
<td>37</td>
</tr>
<tr>
<td>EXPERIMENTARY</td>
<td>40</td>
</tr>
<tr>
<td>Adenosine Deaminase</td>
<td>40</td>
</tr>
<tr>
<td>Method of analysis</td>
<td>40</td>
</tr>
<tr>
<td>Effect of concentration of enzyme</td>
<td>41</td>
</tr>
<tr>
<td>pH optimum</td>
<td>44</td>
</tr>
<tr>
<td>Effect of phosphate ions</td>
<td>44</td>
</tr>
<tr>
<td>Effect of aerobic and anaerobic conditions</td>
<td>47</td>
</tr>
<tr>
<td>Deamination of Adenine</td>
<td>49</td>
</tr>
</tbody>
</table>

T12089
<table>
<thead>
<tr>
<th>Method of analysis</th>
<th>49</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of inosine on deamination of adenine</td>
<td>51</td>
</tr>
<tr>
<td>Effect of pH on production of ammonia from adenine and inosine</td>
<td>52</td>
</tr>
<tr>
<td>Formation of Adenosine from Adenine and Inosine</td>
<td>53</td>
</tr>
<tr>
<td>Method of analysis</td>
<td>53</td>
</tr>
<tr>
<td>Effect of phosphate and aerobic conditions on the formation of adenosine</td>
<td>54</td>
</tr>
<tr>
<td>pH optimum</td>
<td>56</td>
</tr>
<tr>
<td>Enzymatic test system and unit of activity</td>
<td>58</td>
</tr>
<tr>
<td>Effect of concentration of inosine</td>
<td>63</td>
</tr>
<tr>
<td>Rate of reaction</td>
<td>63</td>
</tr>
<tr>
<td>Effect of dialysis</td>
<td>63</td>
</tr>
<tr>
<td>Effect of various ions on activity</td>
<td>70</td>
</tr>
<tr>
<td>Quantitative analysis of adenosine, adenine, hypoxanthine, and inosine in a mixture</td>
<td>73</td>
</tr>
<tr>
<td>Spectrophotometric test system</td>
<td>79</td>
</tr>
<tr>
<td>Stoichiometry</td>
<td>80</td>
</tr>
<tr>
<td>Effect of divalent cations on stoichiometry</td>
<td>80</td>
</tr>
<tr>
<td>Precipitation of phosphate by divalent cations</td>
<td>82</td>
</tr>
<tr>
<td>Phosphorolysis of inosine and adenosine</td>
<td>83</td>
</tr>
<tr>
<td>Chromatographic test system</td>
<td>84</td>
</tr>
<tr>
<td>Equilibrium constant</td>
<td>88</td>
</tr>
<tr>
<td>Kinetics</td>
<td>88</td>
</tr>
<tr>
<td>Phosphorolysis of adenosine and inosine</td>
<td>93</td>
</tr>
<tr>
<td>Formation of purine bases from adenosine and inosine</td>
<td>96</td>
</tr>
<tr>
<td>Formation of ribose-1-phosphate</td>
<td>98</td>
</tr>
<tr>
<td>Reaction of adenine and hypoxanthine with ribose-1-phosphate</td>
<td>102</td>
</tr>
<tr>
<td>Stoichiometry and equilibrium constants for the phosphorolytic reactions</td>
<td>105</td>
</tr>
<tr>
<td>Elimination of transamination as a reaction mechanism</td>
<td>107</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>114</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>126</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>128</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>139</td>
</tr>
</tbody>
</table>
INTRODUCTION

Advances in the knowledge of nucleic acid metabolism have been rapid in the last fifteen years. The importance of nucleic acids has been known for a long period of time both for the part they play in the cell nucleus and for their role in the form of nucleotides as co-factors in many enzymatic reactions. The introduction of tracer techniques involving both heavy isotopes of nitrogen, $^{15}\text{N}$, and carbon, $^{13}\text{C}$, and particularly the radioactive isotopes of carbon, $^{14}\text{C}$, and phosphorus, $^{32}\text{P}$, has to a large degree made the rapid progress in this field possible. Many of the one, two, and three carbon units, carbon and nitrogen, and nitrogen compounds that serve as precursors of the purine and pyrimidine bases have been found. Progress is being made in elucidating the individual steps involved in joining these precursors to form the bases. This phase of the study of nucleic acid metabolism starting from the simple and working toward the more complex compounds, however, is far from complete.

Many of the enzymes involved in the various steps in degradation and synthesis of nucleic acids have been isolated, although few have actually been purified in the crystalline state. These isolated enzyme studies have added much to our knowledge of nucleic acid metabolism. Many of the comparable enzymes from animals and from microorganisms have been shown
to have similar action and properties.

A considerable gap in our knowledge exists regarding the steps between the precursors and the highly polymerized nucleic acids of living cells. The use of tracer techniques in labeling complex compounds such as nucleosides and nucleotides, and the determination of their incorporation into the nucleic acids of cells have given some indication of the role of these compounds in synthesis. Results in this phase of the problem are quite variable and seem to show species differences much more than are shown by the isolated enzyme studies.

The problem has also been attacked by a study of nucleic acid structure starting with the complexity of the highly polymerized nucleic acids. Progress is being made on the step-wise degradation of polymerized nucleic acid as it exists in the cell. The tetranucleotide hypothesis that was popular for a period has been shown to be erroneous. Some periodicity has been found, however, and further work should demonstrate both the sequence of the nucleotides and the spatial arrangement of nucleic acids.

Much of the work from the different approaches used in the study of nucleic acid metabolism has indicated a common pathway for synthesis of many of the purine nucleosides or nucleotides. The study reported here is mainly concerned with an isolated enzyme system that catalyzes the interconversion of the purine nucleosides inosine and adenosine. This
riboyl transfer mechanism could be a required step in the actual synthesis of nucleic acids by providing the adenosine moiety found in nucleic acid from inosine and the purine base adenine. It could be considered as a reaction somewhat analogous to transamination in amino acid and protein synthesis. Should it be demonstrated that only one nucleoside can be formed from the precursors of the base and ribose, the ribosyl transfer mechanism would provide a means of synthesis for the other nucleosides. It further provides another link in the formation of more complex compounds, nucleosides, from smaller precursors, the purine bases.
REVIEW OF LITERATURE

The present discussion will deal with the literature on the metabolism of adenine and adenosine and their deamination products, hypoxanthine and inosine. For information of the general field of nucleic acids reference is made to the very extensive two volume work published in 1955 (Chargaff and Davidson, 1955). The early work on nucleic acids was reviewed by Jones (1920) and Levene and Bass (1931). Literature on purine and pyrimidine metabolism in microorganisms was reviewed by Friedkin (1953). Recent investigations on many of the enzymes involved in nucleic acid metabolism were reviewed by Kalckar and Klenow (1954). Biosynthesis of the purines was reviewed by Greenberg (1953).

Deaminases Active on Adenine or Adenosine

The presence of an adenosine deaminase was described by Jones in 1911. This early work was carried out with very crude enzyme preparations and substrates. The actual presence of adenosine deaminase was by inference from the products of the reaction of pig pancreas on thymus nucleic acid. Jones (1920) has summarized much of this early work. Both guanine and adenine could be deaminated by certain of these crude extracts. The production of hypoxanthine from adenine and xanthine from guanine was determined by isolation of the compounds. The two enzymes were designated guanase and adenase.
and a difference in distribution of the enzymes in various tissues was found. Dog liver appeared to have an adenosine deaminase since free adenine was not converted into hypoxanthine but hypoxanthine was formed in large amounts from nucleic acid.

Probably the first convincing demonstration of adenosine deaminase was reported by György and Röther (1927). They demonstrated the production of ammonia from adenosine and from sodium nucleinate by various tissues and tissue extracts. Schmidt (1928) purified the tissue extracts and found that two enzymes could be separated. One was active on adenosine, the other on muscle adenylic acid (adenosine-5'-phosphate). Neither enzyme was active on adenine, nor on guanine, guanosine, guanylic acid, or yeast adenylic acid (adenosine-3'-phosphate). Another direct demonstration of adenosine deaminase was reported by Dixon and Lemberg (1934). Crude xanthine oxidase prepared from milk contained an enzyme that deaminated adenosine. Adenine was oxidized by the preparation but not deaminated.

The first report on deaminase action of microorganisms on adenine compounds was that by Lutwak-Mann in 1936 although it was known much earlier that putrefaction of nucleic acid solutions would result in ammonia production (Jones, 1920). Lutwak-Mann (1936) described the dephosphorylation and deamination of various adenine containing compounds. This study was made with washed suspensions of Bacterium coli (Escheri-
The compounds decomposed were: adenosinetriphosphate (ATP), adenosine-5'-phosphate, adenosine-3'-phosphate, adenosine, and adenine. The phosphorylated compounds were dephosphorylated as well as deaminated and dephosphorylation appeared to precede deamination. Adenosine was most rapidly deaminated. The deamination was stimulated by the addition of phosphate or arsenate. The deamination of adenine depended on the presence of phosphate ions for appreciable production of ammonia. The end product in all cases was hypoxanthine. No free ribose could be detected after microbial action on the ribose containing compounds. *Bacterium dispar*, *Bacterium cloacae*, and *Bacterium lactis-aerogenes* showed similar activities, *Streptococcus faecalis* showed only slight activity, and *Pseudomonas pyocyanea* showed little activity on the phosphorylated compounds but was very active in the deamination of adenine and adenosine.

These observations were extended in 1938 by Stephenson and Trim. They also worked with washed cell suspensions of *Bact. coli*. With adenosine-5'-phosphate as substrate, they found the percentage increase in phosphate release greater than the percentage of ammonia formed during the early stages of the reaction. They concluded, as had Lutwak-Mann (1936), that dephosphorylation preceded deamination. They further demonstrated that 1 per cent phenol completely inactivated the deaminase activity but had no effect on dephosphorylation. The deaminase activity was greatest with adenosine as the
substrate. The addition of catalytic amounts of adenosine or inosine to adenine resulted in a great increase in the deamination of adenine. Adenylic acid also showed this effect but to a lesser extent. Ribose when added in amounts equal to the adenine present, doubled the rate of deamination of adenine. The catalytic effect of adenosine decreased with time, but the rate was restored with fresh adenosine. All of the ribose containing compounds were attack by the cell suspensions and gave hypoxanthine as the end product. No free ribose could be detected. Manometric studies indicated that CO₂ or H₂ was formed from free ribose and to a greater extent from ribose combined with adenine or hypoxanthine. This fermentation of adenosine and inosine could well explain the decreased catalytic effect of adenosine with time. In regard to the catalytic effect of adenosine Stephenson and Trim (1938, p. 1747) state,

> It is premature to discuss the nature of the catalytic effect of adenosine on the deamination of adenine. It is conceivable that the NH₂ group of adenine may be transferred to inosine which is then rapidly deaminated. The maximum rate of deamination of adenine in the presence of adenosine is still less than that of the adenosine, so evidence thus afforded is not inconsistent with this view.

Stephenson and Trim do not present any direct data concerning the effect of phosphate on the deamination of adenine or adenosine. Most of their experiments were conducted in phosphate buffer so that the effect of phosphate could not be determined. In most other respects the data reported confirm the findings of Lutwak-Mann (1936).
In 1938 Gale reported that cells of _Escherichia coli_ would deaminate adenosine. The main problem under study was the deamination of amino acids. He found that adenosine, adenylic acid, and inosine enhanced the deamination of aspartate. These results were also obtained with cell-free preparations. One possible explanation of the catalytic effect of adenosine and inosine would involve transamination from aspartate to inosine to form adenosine. The adenosine would then be deaminated to reform the inosine required as the amino acceptor. On the basis of the rate of deamination of adenosine as compared to the rate of deamination of aspartate, Gale concluded that adenosine was not active in the aspartate system through deamination but for some other unknown reason. Nothing more was done to establish the possibility of the transamination reaction between aspartate and inosine.

Borsook and Dubnoff (1939 a, b) prepared enzyme systems from _Aspergillus wentii_ that deaminate free adenylic acid or adenylic acid when combined with nucleic acid. Free adenine was not attacked. The enzyme system was a complex mixture and the possibility of dephosphorylation of adenylic acid before deamination was not ruled out. The distribution of the deaminases for adenosine and adenylic acid in the tissues and blood of the rabbit was described by Conway and Cooke (1939). They could not demonstrate an effect of phosphate on adenosine deaminase. Phosphate, however, did have an inhibitory effect on the adenylic acid deaminase. McElroy
and Mitchell (1946) found adenosine deaminase in certain temperature sensitive strains of Neurospora. Adenine deaminase was not present.

The problem of the existence of an enzyme that would deaminate adenine was at least partially clarified by the work of Florkin and Frappez (1940). They found an adenine deaminase in tissues of invertebrates but no adenosine deaminase. In vertebrates the reverse was true. Adenine was not deaminated but many tissues were shown to contain an adenosine deaminase. At the present time it is generally accepted that tissues of higher animals do not contain enzymes capable of deaminating free adenine (Schmidt, 1955). The presence or absence of adenine deaminase in microorganisms is not established. Adenine deamination by bacterial cells can be explained by a combined action of several enzymes. The inosine activation of the deamination of adenine as described by Stephenson and Trim (1938) could be one mechanism for adenase action without involving an enzyme that would deaminate free adenine. Nevertheless, adenine deaminase as a distinct enzyme may be present in some bacteria (Chargaff and Kream, 1943).

Adenosine deaminase from animal tissue has been purified to a considerable extent. Methods for this purification have been reported by Brady (1942), Zittle (1946) and Kalckar (1947 c). These enzyme preparations have been shown to be active only on adenosine and adenine deoxyriboside. The specificity of the adenosine deaminase from animal tissue has
also been demonstrated by Schaedel, et al. (1947).

In 1946 Mitchell and McElroy found that amylase preparations (takadiastase) from Aspergillus oryzae contained an adenosine deaminase. In contrast to the adenosine deaminase of animal tissue (Schaedel, et al., 1947), the Aspergillus oryzae enzyme was quite unspecific in its deaminase activity. The takadiastase preparation was fractionated with acetone and alcohol and with ammonium sulfate (Kaplan, et al., 1952). This purified enzyme would deaminate adenosine-5'-phosphate, adenosine-3'-phosphate, DPN, ATP, and ADP as well as adenosine. Adenine, TPN, and adenosine-2'-phosphate were resistant to deamination.

Although most bacterial adenosine deaminase preparations appear to be active on both adenosine and adenine deoxyriboside, Kalckar, MacNutt, and Hoff-Jørgensen (1952) working with a dialyzed enzyme from Lactobacillus helveticus found a deaminase for adenosine only. Adenine deoxyriboside was not deaminated by their preparation.

 Chargaff and Kream (1948) indicated that adenine could be deaminated by cells of E. coli. The reaction was carried out on filter paper incubated in a moist atmosphere. The reaction was stopped by heating the paper to 100° C. The products were then determined by chromatography of the paper used for the reaction. The main enzyme reported in this paper was cytosine deaminase from a cell-free preparation. The deamination of adenine was mentioned as also occurring but no
data were given. Further work on this adenine deaminase was published in 1952 (Kream and Chargaff, 1952). The reaction was studied both on paper and in vitro in the usual manner. The production of ammonia indicated 59 to 67 per cent of the adenine had been deaminated. A cell-free juice prepared from E. coli would deaminate cytosine. An extract from rabbit liver would also deaminate guanine and adenosine. The cell-free extracts and liver extracts, however, were inactive on adenine.

It would appear that adenosine deaminase was present in the rat liver homogenates used by Richert and Westerfeld (1950) in their study on purine metabolism. They studied the increased oxygen uptake produced by the xanthine oxidase in the liver homogenates. Xanthine oxidase catalyzes the following reactions:

\[
\text{Hypoxanthine} \quad \frac{1}{2} \text{O}_2 \rightarrow \text{Xanthine} \quad \frac{1}{2} \text{O}_2 \rightarrow \text{Uric acid}
\]

Adenosine, adenosine-5'-phosphate, guanine and guanosine showed an increase in oxygen uptake and formation of uric acid. Xanthine oxidase is not active on nucleosides or nucleotides, therefore, deamination and hydrolysis (or phosphorolysis) of the amino-containing nucleosides must have occurred. Adenine gave no increase in oxygen uptake. This was due to the absence of adenase from rat liver. Although no adenine deaminase has been demonstrated in tissues of higher animals (Schmidt, 1955), the absence of oxygen uptake with adenine as substrate is somewhat surprising. Xanthine oxidase catalyzes the oxidation of adenine to 2,8 dihydroxyadenine
(Nicolaier, 1902; Dixon and Thurlow, 1924; Bendich, et al., 1950). The rate of adenine oxidation is much less than the rate of hypoxanthine or xanthine oxidation by xanthine oxidase (Klenow, 1952) which may account for the results reported for adenine by Richert and Westerfeld (1950).

Wang and Lampen (1951) indicated that whole cells of *E. coli* could deaminate adenosine. When the cells were ground with alumina to form a cell-free extract, the deaminase was inactivated. Cell-free extracts from *Lactobacillus pentosus* contained nucleosidases active on adenosine but no adenosine deaminase was present. With similar preparations from *E. coli* the split of adenosine to adenine and ribose could not be demonstrated. Instead, hypoxanthine and ribose phosphate were formed. The preparation was free of adenine deaminase. They gave no explanation for these results with the extract of *E. coli* on adenosine. All of the results with the *E. coli* extracts were given only in a footnote in the article. Although cells of *E. coli* ground with alumina have adenosine deaminase destroyed, they stated that the extracts of *E. coli* were prepared in a similar manner to the extracts from *L. pentosus*, e.g., by grinding with powdered glass (Kalnitsky et al., 1945). Under these conditions, adenosine deaminase may not have been destroyed and the reaction may have involved adenosine deaminase and inosine nucleosidase to form hypoxanthine and ribose phosphate (or ribose).

Marmur (1951) demonstrated the deamination of adenosine
and adenosine-5'-phosphate by whole cells of *E. coli* and cell-free extracts from *Proteus morganii*. The cell-free extracts were inactive on adenine even in the presence of inosine.

Sutton (1951) demonstrated deamination of adenine by cell suspensions of *Aerobacter aerogenes* and *E. coli*. Hypoxanthine was found as the end product of the action of *E. coli* on adenine.

Friedman and Gots (1952) reported that resting cell suspensions of *E. coli* exhibited deaminase activity for adenine, adenosine, adenosine-3'-phosphate, isoguanine, 2,6-diaminopurine, cytosine, cytidine, and cytidylic acid.

*Salmonella breslau*, *E. coli*, and mutants from these parent strains were reported by Rashba and Krochko (1954) to deaminate adenine, guanine, and ribonucleic acid. The vibro organism (Agarwala, et al., 1954) has been demonstrated to deaminate adenine, adenosine, adenylic acid, and ATP.

Williams and McIntyre (1955) have found an adenosine deaminase in cell-free extracts of *Bacterium cadaveris*. By electromphoretic separation they found a fraction that was devoid of adenosine deaminase activity, but contained aspartate deaminase. They concluded that adenosine deaminase was not necessary for aspartate deaminase activity. This work confirmed Gale's conclusions (1938) that deamination of adenosine does not play a role in aspartase activity. Williams and McIntyre (1955) were unable to obtain adenosine deaminase free from aspartate deaminase. They did not deter-
mined the effect of their enzyme preparations on adenine.

**Nucleosidases**

Purine nucleosidase was discovered by Levene and Medigreceanu (1911 a, b) in various tissue extracts of the dog. They also proposed the term "nucleosidase" for the enzyme catalyzing the cleavage of the N-glycosidic bond (Levene and Medigreceanu, 1911 c). The preparations used were active on purine nucleosides but had very little activity on pyrimidine nucleosides. Deutsch and Laser (1929) found a pyrimidine nucleosidase in bone marrow of cattle. Klein (1935) made the next important observations on nucleosidases. He found that extracts from spleen, lung, liver and heart muscle were activated by arsenate ions and to a lesser extent by phosphate ions. He further demonstrated a specificity for purine nucleosides, although both ribose and deoxyribose nucleosides were split by the same enzyme. A specific pyrimidine nucleosidase was also found. An effect of phosphate on nucleosidase activity was found by Dische (1938). Hemolyzed human erythrocytes caused phosphorylation of the D-ribose present in adenosine. The phosphorylated ribose was decomposed by the enzyme system and so could not be characterized.

**Nucleoside phosphorylase**

The stimulatory effect of phosphate or arsenate on nucleosidase and the phosphorylation of ribose bound in adenosine
was explained by Kalckar in a series of papers in 1945 and 1947 (Kalckar 1945 a, b; 1947 a, b, c, d). Using a nucleosidase prepared from rat liver, Kalckar was able to show the phosphorolysis of inosine, the formation of a new acid-labile phosphate ester, and the synthesis of inosine from hypoxanthine and this new ester (Kalckar 1945 b). More complete data were reported in the papers published in 1947 (Kalckar 1947 c, d). Phosphorolysis of guanosine was demonstrated. A decrease in inorganic phosphate was shown which equaled the amount of guanine liberated. The phosphate was found in an extremely acid-labile organic compound. Analysis and properties of this phosphate compound indicated that it was ribose-1-phosphate. The effect of arsenate was somewhat similar to the effect of phosphate. A dialyzed enzyme preparation did not split inosine. The addition of arsenate resulted in the formation of hypoxanthine from the inosine. The reaction was slower with arsenate than with phosphate. Free ribose was formed by arsenolysis and the spontaneous breakdown of the ribose-arsenate compound. Ribose-1-phosphate was prepared from inosine in the presence of inorganic phosphate and the rat liver nucleosidase. The ribose-1-phosphate was isolated as the barium salt. When an excess of the sodium salt of ribose-1-phosphate was allowed to react with hypoxanthine in the presence of nucleosidase, inosine was formed and an equivalent amount of hypoxanthine disappeared. Similar results were obtained with guanine and ribose-1-phosphate.
These results indicated that the following reactions were catalyzed by the enzyme system:

\[
\text{Inosine} + \text{Orthophosphate} \rightleftharpoons \text{Hypoxanthine} + \text{Ribose-1-phosphate}
\]

\[
\text{Guanosine} + \text{Orthophosphate} \rightleftharpoons \text{Guanine} + \text{Ribose-1-phosphate}
\]

The enzyme was specific for inosine and guanosine; there was no phosphorolysis of adenosine or xanthosine. Although the action on deoxyribosides was not determined, Kalckar (1947 d) suggested that the enzyme would be active on guanine and hypoxanthine deoxyribosides. This was later demonstrated to be true (Friedkin, et al., 1949).

The formation of deoxyribose-1-phosphate by the action of rat liver nucleoside phosphorylase on guanine deoxyriboside was demonstrated in 1950 (Friedkin and Kalckar, 1950; Friedkin, 1950). Deoxyribose-1-phosphate proved to be even more acid-labile than ribose-1-phosphate. It was stable at alkaline pH values and was isolated as the barium or cyclohexylamine salt. The cyclohexylamine salt of deoxyribose-1-phosphate reacted with hypoxanthine in the presence of the nucleoside phosphorylase to form hypoxanthine deoxyriboside. The formation of the hypoxanthine deoxyriboside was shown spectrophotometrically by methods developed by Kalckar (1947 a, b) and by microbiological assay (Hoff-Jørgensen, et al., 1950). With the deoxyribose-1-phosphate, as with ribose-1-phosphate, no synthesis occurred with adenine or with pyrimidines.

The presence of phosphoribomutase in crude rat liver preparations of nucleoside phosphorylase complicated the
isolation of ribose-1-phosphate (Abrams and Klenow, 1951). Phosphoribomutase catalyzed the following reaction:

Ribose-1-phosphate $\rightarrow$ Ribose-5-phosphate

The enzyme was destroyed by surface denaturation which did not effect nucleoside phosphorylase.

Rat liver and calf thymus preparations contained an enzyme that formed deoxyribose-5-phosphate from deoxyribose-1-phosphate. This enzyme was named phosphodesoxyribomutase (Manson and Lampen, 1951 a). The nucleoside phosphorylase in the preparations cleaved hypoxanthine deoxyriboside and also catalyzed the arsenolysis of hypoxanthine deoxyriboside.

The nucleoside phosphorylase from rat liver was shown to catalyze the formation of guanine deoxyriboside and xanthine deoxyriboside from the bases and deoxyribose-1-phosphate (Friedkin, 1952 a). Xanthosine was formed from xanthine and ribose-1-phosphate. The enzyme preparation contained more guanine deoxyriboside phosphorylase activity than xanthine deoxyribose phosphorylase activity.

Use was made of nucleoside phosphorylase and ribose-1-phosphate or deoxyribose-1-phosphate to form nucleosides with various substituted purine and pyrimidine bases. Friedkin (1952 b) found that the following bases would react with ribose-1-phosphate or deoxyribose-1-phosphate in the presence of horse liver nucleoside phosphorylase: 8-azaguanine (also Friedkin, 1954), 2-thiouracil, 5-amino uracil and other substituted uracils. He also found evidence for the formation
of the ribosidic analogue of thymidine. Korn, et al. (1953) found the riboside of 4-amino-5-imidazolecarboxamide (carboxamide) following the action of beef liver nucleoside phosphorylase on the carboxamide and ribose-1-phosphate. They presented evidence that adenine reacted with ribose-1-phosphate to form adenosine. The enzyme preparation converted adenosine completely to inosine, or to inosine and hypoxanthine in the presence of inorganic phosphate. The following reactions were postulated:

\[
\text{Adenine} + \text{Ribose-1-phosphate} \xrightarrow{\text{nucleoside phosphorylase}} \text{Adenosine} + \text{Orthophosphate}
\]

\[
\text{Adenosine} \xrightarrow{\text{adenosine deaminase}} \text{Inosine} + \text{Ammonia}
\]

\[
\text{Inosine} + \text{Orthophosphate} \xrightarrow{\text{nucleoside phosphorylase}} \text{Hypoxanthine} + \text{Ribose-1-phosphate}
\]

Hypoxanthine was not formed from adenine in the absence of ribose-1-phosphate. The beef liver nucleoside phosphorylase, therefore, had a different specificity than the enzyme prepared by Kalckar (1947 c) from rat liver.

Nicotinamide reacted with ribose-1-phosphate in the presence of an enzyme from hog liver to form nicotinamide riboside (Rowen and Kornberg, 1951).

Nucleoside phosphorylases have also been found in bacterial cells. Manson and Lampen (1950) reported resting cells of \(E. \text{ coli}\) metabolized hypoxanthine deoxyriboside and thymidine.
The deoxyribose disappeared from the medium. In the presence of arsenate the deoxyribose and free base accumulated in the medium. Cell-free extracts contained nucleosidases active on the same substrates. With the cell-free extracts a deoxyribose-phosphate compound was formed. They extended these observations on cell-free extracts in 1951 (Manson and Lampen, 1951 b). They found that guanine deoxyriboside was split by the extract as was uracil deoxyriboside. Adenine deoxyribo­side was not attacked. The following reactions were shown to take place by spectrophotometric analysis and by chromatographic methods:

\[
\text{Hypoxanthine Deoxyriboside} + \text{Thymine} \rightarrow \text{Hypoxanthine} + \text{Thymidine}
\]

\[
\text{Hypoxanthine Deoxyriboside} + \text{Uracil} \rightarrow \text{Hypoxanthine} + \text{Uracil Deoxyriboside}
\]

These reactions were also reported by Hoffmann (1952).

Lampen (1952) reported that the extracts of \textit{E. coli} used by Manson and Lampen (1950, 1951 b) produced arsenolysis of adenine deoxyriboside. The inability to demonstrate phosphorolysis (Manson and Lampen, 1951 b) was the result of technical factors in the analysis and the inhibitory effect of adenine on the nucleosidase. Adenine was identified as a cleavage product from adenosine and from adenine deoxyriboside. Adenine nucleosides have been obtained by condensation of adenine and ribose-1-phosphate or deoxyribose-1-phosphate.
Nucleosidase activity in cells of *E. coli* was reported by Friedman and Gots (1952). Inosine, guanosine, uridine, and thymidine were cleaved by the cells. A nucleoside phosphorylase active on inosine was reported in cell-free extracts of *Bacterium cadaveria* (Williams and McIntyre, 1955). Phosphate was required for the splitting of inosine.

In many of the preceding papers nucleosidase activity was demonstrated for both purine and pyrimidine nucleosides. Although the enzymes were not separated, most workers indicated that there are two nucleoside phosphorylases, one active on purine ribosides and deoxyribosides, and another active on pyrimidine ribosides and deoxyribosides. Specific pyrimidine nucleoside phosphorylases have been found in dog bone marrow and calf kidney (Manson and Lampen, 1949; Friedkin and Roberts, 1951), rabbit liver and intestinal mucosa, and horse liver (Friedkin and Roberts, 1954a, b).

Paege and Schlenk (1950) reported that cytidine and uridine were split by enzymes in whole cells of *E. coli* and *A. aerogenes*. Cytidine was deaminated by the cells and the uridine split to uracil and ribose-1-phosphate. A purified cell-free extract from *E. coli* was prepared that was free of cytidine deaminase (Paege, 1951). The enzyme formed uracil and ribose-1-phosphate from uridine but was inactive on cytidine and on purine nucleosides. A synthesis of uridine was demonstrated with uracil and ribose-1-phosphate.
Hydrolytic nucleosidases

The discovery of the phosphorolytic cleavage of nucleosides (Kalckar, 1945) suggested that hydrolysis, as such, played no role in nucleosidase activity. The first exception to this was found by Carter in 1951. An enzyme was partially purified from yeast that split uridine to uracil and free ribose in the absence of phosphate or arsenate. The enzyme did not attack other ribosides.

Purine and pyrimidine nucleosidases were demonstrated in cell-free extracts of _L. pentosus_ by Wang (1950). Uridine, cytidine, inosine, adenosine, guanosine, and xanthosine were attacked and showed the formation of base and reducing sugar. No ribose phosphate ester was isolated. The cleavage was much faster in the presence of arsenate or phosphate, so it was suggested that the process was phosphorolytic rather than hydrolytic. An extension of this work was reported by Wang and Lampen in 1951. Attempts to isolate the phosphorylated ribose compound were unsuccessful. A somewhat different specificity for the enzymes was found also. The deoxyribo-sides of hypoxanthine, cytosine and uracil were attacked slowly, if at all, while the ribosides were very susceptible to attack. Most previously reported nucleosidases were equally active on both ribose and deoxyribose nucleosides (Klein, 1935; Friedkin and Kalckar, 1950). An explanation of the effects of phosphate and arsenate on the nucleosidases from _L. pentosus_ was given later (Lampen and Wang, 1952).
The pyrimidine nucleosidases were stable in phosphate, arsenate, succinate or sulfate buffer and showed the same activation in all these buffers. The purine nucleosidases were stable in tris(hydroxymethyl)-aminomethane-HCl (TRIS) buffer, but were inactivated in phosphate or arsenate buffer. The failure to detect phosphorylated ribose during the cleavage of nucleosides with the enzymes of _L. pentosus_ and the effects observed with the ions on the stability of the enzymes lead Lampen (1952) to conclude that the action was hydrolytic rather than phosphorolytic.

Heppel (1952) found hydrolytic nucleosidases in yeast active on purine ribosides. He separated two enzyme system from baker's yeast by ammonium sulfate fractionation and adsorption on calcium phosphate gel. One fraction catalyzed the phosphorolysis of inosine, guanosine, and nicotinamide riboside. The other fraction was a hydrolytic system active against inosine, guanosine, nicotinamide riboside, adenosine, xanthosine, and a number of synthetic nucleosides. The nucleoside phosphorylase required phosphate or arsenate and was reversible. The hydrolytic enzyme catalyzed complete splitting without phosphate or arsenate and no reversibility could be demonstrated with ribose-1-phosphate.

**Nucleoside-N-transglycosidase**

The transfer of the deoxyribosyl group from one purine or pyrimidine to another was first demonstrated by MacNutt
Dialyzed enzyme preparations of *Lactobacillus helveticus* were used in this work. Hypoxanthine deoxyriboside reacted with the corresponding pyrimidine bases to form uracil deoxyriboside, thymine deoxyriboside, 5-methylcytosine deoxyriboside, and cytosine deoxyriboside. Hypoxanthine deoxyriboside also reacted with adenine to form adenine deoxyriboside. Thymine deoxyriboside reacted enzymatically with adenine, guanine, hypoxanthine, xanthine, and 4-amino-5-imidazolecarboxamide to form the corresponding deoxyribosides. Various other purine deoxyribosides reacted with other purine bases to form the new deoxyribosides. Pyrimidine bases also reacted with pyrimidine deoxyribosides. These reactions were catalyzed in phosphate-free buffers by enzyme preparations that had been dialyzed for several days. Deoxyribose and deoxyribose-1-phosphate were inactive in the transfer reaction. The enzyme was considered to be a trans-N-glycosidase. The transfer reaction was also found in enzyme preparations from *Lactobacillus delbrueckii* and *Thermobacterium acidophilus*.

The transfer between hypoxanthine deoxyriboside and adenine as described by MacNutt (1952) may result from a transamination rather than by trans-N-glycosidase action. The possibility was ruled out by experiments reported by Kalckar, *et al.* (1952) using radioactive adenine. If the transfer were a transamination, then the hypoxanthine formed would be radioactive; if it were a transfer of the deoxyribosyl group then the adenine deoxyriboside formed would be radioactive. The
two possibilities are given below.

Transamination:

\[
\text{Adenine-3-}^{14}_C + \text{Hypoxanthine Deoxyriboside} \rightleftharpoons \text{Hypoxanthine-3-}^{14}_C + \text{Adenine Deoxyriboside}
\]

Trans-N-glycosidase action:

\[
\text{Adenine-8-}^{14}_C + \text{Hypoxanthine Deoxyriboside} \rightleftharpoons \text{Hypoxanthine + Adenine-8-}^{14}_C \text{Deoxyriboside}
\]

When the reaction was carried out with adenine-\(3-^{14}_C\), only adenine deoxyriboside was radioactive. The specific activity of the adenine deoxyriboside was essentially the same as the substrate adenine-\(8-^{14}_C\). No radioactivity was found in hypoxanthine or its deoxyriboside. The reaction was a transfer of the deoxyribosyl group and not a transamination as had been proposed by Stephenson and Trim (1938) for the corresponding ribose compounds. Attempts to carry out this reaction with adenosine were unsuccessful because of a powerful adenosine deaminase and riboside phosphorylase active on the ribose nucleosides.

Some observations by Kritskiy (1950, 1952) and Kriskiy and Melik-Sarkisyan (1953) on a nucleoside phosphorylase from rat liver can be interpreted as a transfer of the ribosyl group from one purine to another. These authors indicated, however, that a special mechanism was acting in which phosphate ions were bound to the enzyme itself and acted by phosphorolysis with the bound labile-phosphate taking the role of inorganic phosphate.
Hoffmann (1952) reported a transfer of deoxyribosyl groups from purine to purine or pyrimidine to pyrimidine catalyzed by cell-free extracts of *E. coli*. Phosphate or deoxyribose-1-phosphate was not required for the transfer. To obtain a transfer from a purine deoxyriboside to pyrimidine base or from a pyrimidine deoxyriboside to a purine base, phosphate was required. Apparently deoxyribose-1-phosphate was the intermediate in this transfer between purines and pyrimidines.

Transaminases Active with Adenine

Only a few reports have appeared on transamination with adenine or adenine compounds. This potentially important system for the formation of amino compounds has been largely neglected. Kalckar and Rittenberg (1947) have indicated a rapid and reversible deamination occurs in skeletal muscle of the rat involving adenylic acid. They suggested the amide nitrogen of glutamine as the source of nitrogen for the regeneration of the amino nitrogen of adenylic acid. Weil-Malherbe (1953) concluded that the amide group of glutamine was transferred to inosine triphosphate (ITP) forming ATP in rat brain homogenates. In a later publication Weil-Malherbe and Green (1955) suggested that the increased ammonia formation in the presence of ITP and glutamine was not due to deaminases present in the brain but was linked with proteolysis. Tsukada and Tukaguki (1954 a, b) found no evidence for amination of
inosinic acid or ITF in guinea pig brain slices or homogenates.

Gunsalus and Tonzetich (1952) found cell-free extracts of *E. coli* formed glutamate from $\alpha$-ketoglutarate and pyridoxal phosphate with adenine as the amino donor. Reamination of hypoxanthine and xanthine by the extracts of *E. coli* was demonstrated by transamination from glutamate (Gunsalus, 1952). Pyridoxal phosphate was a necessary co-factor. The reaction was found in various strains of *E. coli*.

Mardashev and Pavlova (1955) found glycine formation in rat liver slices from glyoxylic or glycolic acid with adenosine, guanosine, adenylic acid or guanine as the amino donor. No indication was given of the reversibility of the reaction.
METHODS

Organism and Medium

The organism employed in this investigation was *Escherichia coli*, strain E-26, Iowa State College Laboratory culture. The cells were grown in a medium of the following composition: 1.5 per cent deionized milk (Bacto), 0.75 per cent $K_2HPO_4$, 10 per cent tap water, made to volume with distilled water. The medium was sterilized for 15 minutes at 15 pounds pressure when prepared in quantities of 1 liter or less. When 9 liter quantities were used, sterilization was for 45 minutes at 15 pounds pressure.

*E. coli* was inoculated into 250 ml. of medium and incubated at 37° C. for 24 hours. The entire culture was inoculated into 1 liter of medium. After 24 hours' incubation at 37° C., 9 liters of medium were inoculated. The culture was incubated for 8 hours in a 37° C. incubator. The medium was vigorously aerated with sterile air passing through a fritted glass tube. Aeration cooled the medium so that the actual temperature of the culture was 31° to 32° C. The cell crop, averaging 4 to 5.5 grams of packed cells per liter of medium, was harvested in a Sharples centrifuge, washed with approximately 2 liters of cold distilled water and recentrifuged in the Sharples centrifuge. The washed cell paste was suspended in 0.5 ml. of cold distilled water for every gram of cell paste. The suspension was used immediately for the preparation
of cell-free extracts or kept in the frozen state until the extract was prepared. Storage of the cells at -10° C. before preparation of the extract had no appreciable effect on enzyme activity.

Enzyme Preparations

Cell-free extracts

Cell-free extracts were prepared by sonic disintegration of the cell suspension in a 9 kilocycle Raytheon oscillator for 30 minutes. Twenty to forty ml. of suspension were treated at one time. After sonic treatment the material was diluted with an equal volume of cold water. Debris was removed by centrifugation at approximately 15,000 rpm for 30 minutes in an International Refrigerated centrifuge equipped with a high speed head. Extracts may be stored at -10° C. for at least a year with no appreciable decrease in activity.

Dialyzed preparations

Dialysis of the cell-free extracts was carried out by two methods. In one, 5.0 ml. of extract was placed in dialysis tubing having a diameter of approximately 6.4 mm. The tubes were attached to a stirring motor that revolved at slow speed and placed in a 1 liter cylinder. Dialysis was carried out at 4° C. against distilled water. The water was replaced with fresh cold water every hour. After dialysis for various periods of time, the tubes were removed and the extract frozen until tested.
The second type of dialysis was carried out in an E-C electrophoresis-convection instrument. The cell-free extract was placed in a double dialysis sac in the cell holder of the instrument. The buffer jar was filled with 3 liters of cold distilled water. The water was kept at 1° C. by circulation through a refrigerated constant temperature bath kept at -3° C. The instrument was operated at 6 volts per centimeter for 5 hours. The water was replaced with fresh cold distilled water after 1 hour and again after 2 hours. The extract was removed from both compartments, mixed and frozen until tested.

**Ammonium sulfate fractionation**

In some cases inactive protein was removed by fractionation of the cell-free extract with ammonium sulfate. Cell-free extract was taken to 33 percent saturation by the addition of neutralized, saturated ammonium sulfate solution. All operations were carried out at 4° C. The precipitated protein was removed by centrifugation and discarded. The supernate was taken to 66 percent saturation with ammonium sulfate solution and the precipitated protein removed by centrifugation. The precipitate was dissolved in water and dialyzed against distilled water for 6 hours. The preparation was frozen until tested.

**Analytical Procedures**

Determinations of the products were carried out on aliquots of the reaction mixture after removal of the precipitated pro-
tein by filtration or centrifugation. Several methods of deproteinization were used depending on the product to be analyzed. Reference to these methods will be made with each determination discussed.

Standard procedures were used for the determination of many of the products. Consequently these methods will not be presented in detail. Modifications of the published procedures will be fully discussed.

**Ammonia**

Ammonia was determined by distillation with a Parnas-Wagner apparatus into acid followed by nesslerization. The color developed was read in a Klett colorimeter with a 520 filter. Known standards of ammonium sulfate solutions were run with each determination. Reaction mixtures used for ammonia determinations were deproteinized with 20 per cent (w/v) perchloric acid.

**Protein**

Protein content of the cell-free extracts was determined by the biuret method of Weichselbaum (1946). A standard curve was prepared with Armour's Bovine Plasma Albumin, Crystallized. The color was read in a Klett colorimeter with a 540 filter.

**Orthophosphate**

Orthophosphate was determined by the method of Lowry and Lopez (1946). The color developed was read after 10 minutes in a Klett colorimeter with a 660 filter. Reaction mixtures were deproteinized with 1/4 volumes of a saturated solution of
ammonium sulfate in 0.1 M acetate buffer at pH 4.

Ribose

Ribose was determined by the orcinol method of Mejbaum (1939). The period of heating was 40 minutes. A Klett colorimeter with a 660 filter was used to read the color developed. A commercial D-ribose product was used as the standard.

Spectrophotometry

A Beckman spectrophotometer, Model DU, was used for the determination of the absorption of various purine compounds in the ultraviolet. Cuvettes having a light path of 1.0 cm. were used for all determinations.

The purine and pyrimidine bases have characteristic absorption spectra in the ultraviolet. The absorption maxima for the various purine and pyrimidine compounds and their molar extinction values have been tabulated by Beaven, et al. (1955). The absorption maxima and molar extinction values obtained with the compounds used in this investigation are tabulated in Table 1 along with the values determined by Johnson (1955). Quite close agreement is found both in the wavelength of the maximum and in the molar extinction values. Since only small amounts of the compounds were taken for the determination of extinction values and since the errors introduced in weighing small quantities have a relatively large influence on the molar extinction values, the values found by Johnson (1955) were used to determine the concentration of
Table 1. Absorption maximum and molar extinction values for various purine bases and nucleosides in 1.0 N HCl

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molar extinction(^1) Wavelength of maximum m(\mu)</th>
<th>Molar extinction(^2) Wavelength of maximum m(\mu)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value at maximum</td>
<td>Value at maximum</td>
</tr>
<tr>
<td>Adenine</td>
<td>262</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td>13,150</td>
<td>12,900</td>
</tr>
<tr>
<td>Adenosine</td>
<td>257</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>14,600</td>
<td>14,500</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>248</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>10,800</td>
<td>10,700</td>
</tr>
<tr>
<td>Inosine</td>
<td>251</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>10,900</td>
<td>11,000</td>
</tr>
</tbody>
</table>

\(^1\) Determined by Johnson (1955).

\(^2\) Present investigation.

The solutions used for the absorption curves.

Solutions of adenine, adenosine, hypoxanthine, and inosine that were approximately 5 x 10\(^{-4}\) M were prepared in 1.0 N hydrochloric acid. Four aliquots were taken from each and diluted 1:10 with 1 N hydrochloric acid. The optical density of each solution was determined at various wavelengths. These values were averaged to give an average optical density reading for each wavelength. The actual concentration of each solution was determined at its maximum density by the following equation (Beaven, et al., 1955):

\[
A = \text{absorbance (optical density)} = \varepsilon_m c d = \log \frac{I_0}{I}
\]

where \(\varepsilon_m\) = molar absorptivity = molar extinction value

\(c\) = molar concentration

\(d\) = internal cell length in centimeters
$I_o$ = intensity of incident radiation
$I$ = intensity of transmitted radiation

The molar extinction values used were those given by Johnson (1955). The cell length was 1.0 cm. The actual concentrations of the solutions as determined at their maximum absorption were then used to determine the $E_m$ values at wavelengths other than the maximum. The concentrations of the solutions and the $E_m$ values found for the compounds are given in Table 2.

The absorption curves for these compounds are shown in Figure 1. The curves for adenine and adenosine are quite similar as are the curves for hypoxanthine and inosine.

Paper chromatography

The various purine compounds were separated by ascending paper chromatography (Block, et al., 1955). The reaction mixtures were deprotenized by the addition of 0.1 volume of 1.5 N sulfuric acid and the protein removed by centrifugation. Aliquots were placed along a line 1 inch from the edge of Whatman No. 1 filter paper (7½ by 11 inches). Aliquots of 5 microliters to 25 microliters were placed as spots. Aliquots of 0.05 ml. to 0.2 ml. were placed along a line 1½ inches long rather than on a single spot. The spot or band was neutralized with concentrated ammonium hydroxide applied to the chromatogram with a micropipet before the chromatogram was developed.

The chromatograms were developed in water adjusted to pH
Figure 1. Ultraviolet absorption spectra of purine compounds

Experimental conditions: Pure compounds dissolved in 1.0 N HCl. Concentration determined at absorption maximum from molar extinction values given by Johnson (1955). Concentration of solutions used: adenine, $4.81 \times 10^{-5}$ M; adenosine $5.24 \times 10^{-5}$ M; hypoxanthine, $4.95 \times 10^{-5}$ M; inosine, $5.23 \times 10^{-5}$ M.

Curves: (○) adenine; (●) adenosine; (□) hypoxanthine; (△) inosine.
Table 2. Molar extinction values at various wavelengths for adenine, adenosine, hypoxanthine, and inosine in 1.0 N HCl\(^a\)

<table>
<thead>
<tr>
<th>Molar extinction values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (\text{m} \mu)</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>230</td>
</tr>
<tr>
<td>235</td>
</tr>
<tr>
<td>240</td>
</tr>
<tr>
<td>245</td>
</tr>
<tr>
<td>248</td>
</tr>
<tr>
<td>250</td>
</tr>
<tr>
<td>255</td>
</tr>
<tr>
<td>257</td>
</tr>
<tr>
<td>260</td>
</tr>
<tr>
<td>262</td>
</tr>
<tr>
<td>265</td>
</tr>
<tr>
<td>270</td>
</tr>
<tr>
<td>280</td>
</tr>
<tr>
<td>290</td>
</tr>
<tr>
<td>300</td>
</tr>
</tbody>
</table>

\(^a\)Concentrations of solutions used: adenine, \(4.81 \times 10^{-5}\) M; adenosine \(5.24 \times 10^{-5}\) M; hypoxanthine \(4.95 \times 10^{-5}\) M; and inosine \(5.23 \times 10^{-5}\) M.

10 with concentrated ammonium hydroxide (Levenbook, 1953) and run at 7° C. The lower temperature gave better separation than 30° C. After drying at 100° C. for 10 minutes the position of the purine compounds was determined by fluorescence quenching under ultraviolet light from a Model SL Minervalight equipped with a 2537 filter (Wyatt, 1955). The separated compounds were recovered from the chromatogram by cutting out the spot and elution according to Vischer and Chargaff
All compounds were eluted in 4.0 ml. of 1.0 N hydrochloric acid overnight at room temperature.

**Assay of radioactivity**

Determinations of radioactivity were made on the compounds separated by paper chromatography. The spots visible under ultraviolet light were cut out and counted with a 1.4 mg./cm.\(^2\) end window (mica) Geiger-Müller tube.

A rough estimate of the efficiency of the counter and the effect of counting radioactivity from paper chromatograms rather than on glass is shown in Table 3. Adenine-8-C\(^{14}\) (1.09 microcuries/mg.) was dissolved in 0.1 M pyrophosphate buffer, pH 6.5. Aliquots from this solution, 5 microliters, were placed on a glass planchet and on Whatman No. 1 filter paper. One sample on paper was subjected to chromatography as given in that section. The spot visible under ultraviolet light was cut out after development of the chromatogram. The other spot placed on paper was cut out and counted, with no further treatment, both on the front side, i.e. the side on which the sample was placed, and on the back side, i.e. the same paper on the reverse side. From the amount of adenine-8-C\(^{14}\) used and its known specific activity, it was calculated that the 5 microliter aliquots contained 6530 disintegrations/minute.

The counter efficiency as shown by the counts on glass was 10.8 per cent (704 cpm divided by 6530 dpm). Very little difference was shown between the counts on paper, either front
or back or after chromatography. The counts on paper were only 38 per cent as large as those obtained with the same amount of solution on glass. This agrees quite well with the findings of Fink and Fink (1948) who found counts of BaC\textsuperscript{14}O\textsubscript{3} on paper one-third less than samples counted on glass.

All compounds from reaction mixtures were counted on paper. A comparison between these compounds is valid even though they have a lower count than if they had been counted on glass.

In some cases radioautography was used to detect radioactivity (Fink and Fink, 1948). Chromatograms developed as described in the section on paper chromatography were exposed to Kodak No-Screen X-ray Safety Film for 3 days. The exposed film was developed according to the recommendations of the manufacturer.
Table 3. Counter efficiency and absorption of radioactivity by filter paper

<table>
<thead>
<tr>
<th>Adenine-3-C(^{14}) placed on:</th>
<th>Counts/minute(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>704 ± 6</td>
</tr>
<tr>
<td>Paper--front(^c)</td>
<td>277 ± 4</td>
</tr>
<tr>
<td>Paper--back(^d)</td>
<td>265 ± 4</td>
</tr>
<tr>
<td>Paper--after chromatography(^e)</td>
<td>264 ± 3</td>
</tr>
</tbody>
</table>

\(^a\) Adenine-3-C\(^{14}\) containing 6530 disintegrations/minute/5 microliters placed in 5 microliter aliquots on material listed.

\(^b\) All counts corrected for background.

\(^c\) Front refers to the side of paper on which the solution was applied.

\(^d\) Back refers to the other side of the same paper spot.

\(^e\) Chromatography as given in methods.
EXPERIMENTAL

Adenosine Deaminase

It was found that a cell-free extract of *E. coli* would deaminate adenosine as indicated by the earlier work of Lutwak-Mann (1936), Stephenson and Trim (1938), and Gale (1938) on whole cells of *E. coli*.

**Method of analysis**

Reactions were carried out in Warburg flasks of conventional design. In almost all cases experiments were run in an atmosphere of nitrogen. The center compartment contained the cell-free extract and buffer. In all cases the cell-free extract was diluted with the buffer used for that particular experiment. The substrate contained in the buffer was placed in one side arm. It was tipped into the main compartment to start the reaction after temperature equilibration. All experiments were carried out at 30.4°C. The reaction was stopped by the addition of 0.5 ml. of 20 per cent perchloric acid contained in the second side arm. The total volume was 2.0 ml. For controls, the acid was tipped into the main compartment at the same time as the substrate. After incubation for the desired time and addition of acid, the contents of the flasks were placed in test tubes and the flasks washed with 3.0 ml. of water acidified with a small amount of perchloric acid. The mixture was filtered through retentive filter paper and aliquots taken for the determination of
ammonia content as given in the section on methods.

Table 4 shows the rate of production of ammonia from adenosine. The reaction was carried out in 0.1 M phosphate buffer, pH 7.7. The cell-free extract contained 135 mg. of protein. Substrate was 10 micromoles of adenosine. No gas production was found manometrically during the incubation of adenosine and cell-free extract. Deamination had reached completion (98 per cent) in the 30 minute incubation.

**Effect of concentration of enzyme**

The production of ammonia from adenosine is proportional to the enzyme concentration within limits (Figure 2). The reaction was conducted in 0.1 M glycine-phosphate (0.1 M in glycine and 0.1 M in phosphate) buffer, pH 7.9 with 5 micromoles of adenosine as substrate. The cell-free extract contained 31.5 mg. of protein per ml.

Table 4. Production of ammonia from adenosine

<table>
<thead>
<tr>
<th>Time minutes</th>
<th>Ammonia formed μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>5.4</td>
</tr>
<tr>
<td>30</td>
<td>9.8</td>
</tr>
</tbody>
</table>

\[a\] Each Warburg flask contained: phosphate buffer, pH 7.7, 75 micromoles; adenine, 10 micromoles; cell-free extract, 135 mg. protein; 0.5 ml. of 20 per cent HClO₄; total volume, 2.0 ml.; gas, N₂; temperature, 30.4°C; time, as indicated.
Figure 2. Formation of ammonia from adenosine as function of enzyme concentration

Warburg flasks contained: glycine-phosphate buffer, pH 7.9, 200 micromoles; adenosine, 5 micromoles; cell-free extract, as indicated; 0.5 ml. of 20 per cent HClO₄; total volume, 2.0 ml.; gas, N₂; temperature, 30.4° C.; time, 5 minutes. Ammonia determined as given in methods.
**pH optimum**

The pH optimum for the production of ammonia from adenosine was close to 8 (Figure 3). The usual test system was employed with 5 micromoles of adenosine as substrate and with 0.1 M glycine-phosphate buffer. The cell-free extract contained 19 mg. of protein. Stephenson and Trim (1933) found the optimum pH of adenosine deaminase was fairly sharp at pH 7.75. Lutwak-Mann (1936) found pH 8.8 as the optimum for deamination activity with adenosine as the substrate.

**Effect of phosphate ions**

Lutwak-Mann (1936) found that adenosine deaminase was activated by phosphate ions. The effect of various amounts of phosphate on the production of ammonia from adenosine is presented in Table 5. The usual test system was employed with 20 micromoles of adenosine as substrate and with 0.1 M tris(hydroxymethyl)-aminomethane (TRIS), pH 8.0, as the buffer.

Phosphate had no appreciable effect on the deamination of adenosine. The difference in activity between various enzyme preparations is also shown by the table. The production of ammonia per mg. of protein shows that the extract containing 18.1 mg. of protein was considerably less active than the extract containing 20.0 mg. of protein. The reason for the difference found in various extracts was not determined. The lack of an effect of phosphate on adenosine deaminase is in contrast to the results found by Lutwak-Mann (1936)
Figure 3. pH-activity curve for adenosine deaminase

Warburg flasks contained: glycine-phosphate buffer, of indicated pH, 200 micromoles; adenosine, 5 micromoles; cell-free extract, 19 mg. of protein; 0.5 ml. of 20 per cent HClO₄; total volume, 2.0 ml.; gas, N₂; temperature, 30.4° C.; time, 5 minutes. Ammonia determined as given in methods.
Table 5. Effect of phosphate on adenosine deaminase

<table>
<thead>
<tr>
<th>Phosphate added</th>
<th>Enzyme mg.</th>
<th>Ammonia formed</th>
<th>Specific activity ( \mu \text{M} ) ammonia/ ( \mu \text{M} ) protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu \text{M} )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>20.0</td>
<td>17.3</td>
<td>0.87</td>
</tr>
<tr>
<td>25</td>
<td>20.0</td>
<td>16.8</td>
<td>0.84</td>
</tr>
<tr>
<td>50</td>
<td>20.0</td>
<td>17.3</td>
<td>0.87</td>
</tr>
<tr>
<td>100</td>
<td>20.0</td>
<td>16.6</td>
<td>0.83</td>
</tr>
<tr>
<td>200</td>
<td>20.0</td>
<td>17.7</td>
<td>0.88</td>
</tr>
<tr>
<td>0</td>
<td>18.1</td>
<td>10.3</td>
<td>0.57</td>
</tr>
<tr>
<td>100</td>
<td>18.1</td>
<td>10.6</td>
<td>0.59</td>
</tr>
<tr>
<td>0</td>
<td>18.1</td>
<td>10.4</td>
<td>0.58</td>
</tr>
<tr>
<td>500</td>
<td>18.1</td>
<td>9.8</td>
<td>0.54</td>
</tr>
<tr>
<td>0</td>
<td>18.1</td>
<td>10.0</td>
<td>0.55</td>
</tr>
<tr>
<td>20</td>
<td>18.1</td>
<td>9.3</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Reactions carried out in Warburg flasks containing: TRIS buffer, pH 8.0, 150 micromoles; phosphate and cell-free extract, as indicated; adenosine, 20 micromoles; 0.5 ml. of 20 per cent \( \text{HClO}_4 \); total volume, 2.0 ml.; gas, \( \text{N}_2 \); temperature, 30.4° C.; time, 30 minutes. Ammonia determined as given in methods.

with whole cells. The reason for this difference is not known.

Effect of aerobic and anaerobic conditions

The production of ammonia from adenosine was carried out under anaerobic conditions with nitrogen as the gaseous atmosphere as a continuation of another problem not reported here. No gas production could be detected above the small amount of endogenous change when the cell-free extracts were allowed to act on adenosine. The effect of carrying out the reaction in air was determined in duplicate, one set with air as the gas atmosphere, the other with an atmosphere of nitrogen. Flasks with air contained in addition 0.3 ml. of 10 per
Table 6. Effect of aerobic conditions on adenosine deaminase

<table>
<thead>
<tr>
<th>Gas phase</th>
<th>$\text{O}_2$ $\mu$M</th>
<th>$\text{CO}_2$ $\mu$M</th>
<th>$\text{NH}_3$ $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>$\text{N}_2$</td>
<td>1.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Reaction</td>
<td>$\text{N}_2$</td>
<td>2.0</td>
<td>11.9</td>
</tr>
<tr>
<td>Difference</td>
<td>$\text{N}_2$</td>
<td>+0.2</td>
<td>+8.7</td>
</tr>
<tr>
<td>Endogenous</td>
<td>air $^b$</td>
<td>2.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Reaction</td>
<td>air $^b$</td>
<td>1.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Difference</td>
<td>air $^b$</td>
<td>+0.4</td>
<td>-0.3</td>
</tr>
</tbody>
</table>

$^a$Warburg flasks contained: phosphate buffer, pH 7.7, 150 micromoles; adenosine, 10 micromoles; cell-free extract, 17 mg. of protein; 0.5 ml. of 20 per cent $\text{HClO}_4$; total volume, 2.0 ml.; gas, as indicated; temperature, 30.4° C.; time, 100 minutes.

$^b$Flasks with air atmosphere had in addition 0.3 ml. of 10 per cent KOH or water in center well.

cent KOH or water in the center well. In all other respects the method of analysis was similar to the reactions previously reported. The results are given in Table 6.

The data presented on the manometric changes are typical for many experiments. The changes are quite small and of no significance. The difference in the amount of ammonia produced in air and in the absence of air is quite small also, 0.8 micromoles. This amounts to a decrease of about 9 per cent under the value found in the absence of air. A change of this magnitude has little, if any, significance.

The results indicate that the product of the deamination
of adenosine, inosine, is not broken down to the extent of the formation of any gaseous products. The possible cleavage of inosine to hypoxanthine and ribose, or ribose-1-phosphate, was not determined in this experiment. In contrast to the results obtained by Lutwak-Mann (1936) and Stephenson and Trim (1938), ribose was not fermented by the cell-free extract when combined with either adenine or hypoxanthine. Free ribose was not attacked by the cell-free extract.

Kinetic studies were not carried out with the adenosine deaminase.

Deamination of Adenine

Lutwak-Mann (1936) and Stephenson and Trim (1938) found that cells of *E. coli* would deaminate adenine. The deamination of adenine was much less than with adenosine as the substrate.

**Method of analysis**

Deamination of adenine by the cell-free extract was demonstrated in the same manner as was the deamination of adenosine. The reactions were carried out in conventional Warburg vessels under an atmosphere of nitrogen. The center compartment contained buffer and cell-free extract. One side arm contained the substrate in buffer. Substrate was tipped into the main compartment after temperature equilibration. The reaction was stopped by the addition of 0.5 ml. of 20 per cent perchloric acid from the second side arm. Incubation
was carried out at $30.4^\circ$ C. In the few experiments in which gas exchange was determined the total volume of fluid in the flask was 2.0 ml. In experiments in which no gas exchange was determined the total volume was 4.0 ml. Protein was removed by filtration and the ammonia determined.

Production of ammonia from adenine and from adenosine is given in Table 7. Substrates were present in 5 micromole quantities. The buffer was 0.1 M phosphate, pH 7.6. The cell-free extract contained 19 mg. of protein. No evolution of CO$_2$ occurred. A slight decrease in gas volume was noted which was calculated by use of the constants employed for CO$_2$ production. The changes were quite small and of no significance. Results of the manometric studies are included only to show that essentially no gas exchange occurred with either adenine or adenosine.

Table 7. Deamination of adenine and adenosine$^a$

<table>
<thead>
<tr>
<th>Substrate</th>
<th>NH$_3$$^\mu$M</th>
<th>CO$_2$$^\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>2.7</td>
<td>-0.7</td>
</tr>
<tr>
<td>Adenosine</td>
<td>4.6</td>
<td>-0.5</td>
</tr>
</tbody>
</table>

$^a$Warburg flasks contained: phosphate buffer, pH 7.6, 150 micromoles; substrate, 5 micromoles as indicated; cell-free extract, 19 mg. of protein; 0.5 ml. of 20 per cent HClO$_4$; total volume, 2.0 ml.; gas, N$_2$; temperature, 30.4$^\circ$ C.; time, 30 minutes. Carbon dioxide determined manometrically, ammonia determined as given in methods.
Table 8. Production of ammonia from adenine in the presence of inosine

<table>
<thead>
<tr>
<th>Inosine added $\mu$M</th>
<th>Adenosine added $\mu$M</th>
<th>Ammonia formed $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>0&lt;sup&gt;b&lt;/sup&gt; 20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.3</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Warburg flasks contained: TRIS buffer, pH 8.0, 350 micromoles; adenine, 20 micromoles; inosine, as indicated; cell-free extract, 20 mg. of protein; 0.5 ml. of 20 per cent HClO₄; total volume, 4.0 ml.; temperature, 30.4°C; time, 30 minutes.

<sup>b</sup> Same except adenine and inosine replaced by 20 micromoles of adenosine.

Appreciable production of ammonia occurred with adenine and it calculated to be only 59 per cent of that formed from adenosine. At other pH values and with other cell-free extracts that did not contain phosphate, the formation of ammonia from adenine was much less.

Effect of inosine on deamination of adenine

Stephenson and Trim (1938) found that adenosine or inosine in catalytic amounts increased the deamination of adenine. The effect of inosine on the formation of ammonia from adenine is given in Table 8. Cell-free extract contained 20 mg. of protein; the buffer was 0.1 M TRIS, pH 8.0; and the substrate was 20 micromoles of adenine. For comparison with adenosine
deaminase the activity shown by the same extract on 20 micro-
moles of adenosine is also given in the table. Inosine showed
a stimulatory effect on the formation of ammonia from adenine.
The deamination was considerably less than that from a similar
concentration of adenosine.

Effect of pH on production of ammonia from adenine and inosine

The effect of various pH values on the deamination of
adenine and adenine and inosine is shown in Table 9. The sub­
strate was 20 micromoles of adenine. The TRIS buffer contained
120 micromoles of phosphate.

The results of this preliminary study showed an optimum
formation of ammonia from adenine and inosine at pH 7, and
pH 8.0 for adenosine deaminase. The hypothesis proposed by
Stephenson and Trim (1938) for the effect of inosine on the
deamination of adenine may be expressed by the following
equations.

\[
\text{Adenine} + \text{Inosine} \rightarrow \text{Adenosine} + \text{Hypoxanthine}
\]

\[
\text{Adenosine} + \text{H}_2\text{O} \rightarrow \text{Inosine} + \text{Ammonia}
\]

The increased production of ammonia at pH 7 may be interpreted
that the reaction that forms adenosine has an optimum pH
below 8, possibly at pH 7. The formation of a small amount
of ammonia at pH 6 offers at least two explanations: (a) the
reaction that produces adenosine from adenine and inosine
does not take place to any appreciable extent at pH 6, or
(b) adenosine is formed at pH 6, but because the adenosine
deaminase present in the cell-free extract has little activity
Table 9. Effect of pH on the production of ammonia from adenine and from adenine and inosine

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Inosine added</th>
<th>Protein in extract</th>
<th>NH₃ formed</th>
<th>Specific activity (μM NH₃/ mg. protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS</td>
<td>8.0</td>
<td>0</td>
<td>20.0</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Phosphate</td>
<td>7.0</td>
<td>0</td>
<td>15.8</td>
<td>0.8</td>
<td>0.05</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.0</td>
<td>0</td>
<td>17.8</td>
<td>0.5</td>
<td>0.03</td>
</tr>
<tr>
<td>TRIS</td>
<td>8.0</td>
<td>5</td>
<td>20.0</td>
<td>1.6</td>
<td>0.09</td>
</tr>
<tr>
<td>Phosphate</td>
<td>7.0</td>
<td>5</td>
<td>15.8</td>
<td>4.6</td>
<td>0.33</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.0</td>
<td>5</td>
<td>17.8</td>
<td>1.1</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Warburg flasks contained: buffer, 350 micromoles, inosine and cell-free extract, as indicated; adenine, 20 micromoles; 0.5 ml. of 20 per cent HClO₄; total volume, 4.0 ml.; gas, N₂; temperature, 30.4°C; time, 30 minutes.

At this pH (see Figure 3) very little ammonia is formed from the adenosine. To test these possibilities a method was required that allowed for the quantitative determination of adenosine. The use of such a method is described in the next section.

Formation of Adenosine from Adenine and Inosine

Method of analysis

The formation of adenosine was determined by an enzymatic method using an adenosine deaminase prepared from calf intestinal mucosa. The method of Brady (1942) was followed.
in the preparation of the mammalian enzyme.

The presence of adenosine in a reaction mixture was determined by deproteinization with 5 per cent (w/v) trichloroacetic (TCA) acid. The protein was removed by filtration. An aliquot of the filtrate, 6.0 ml., was adjusted to pH 6.5 by the addition of 1.0 ml. of 0.12 N NaOH. Adenosine deaminase (10 mg. dry weight in 2.0 ml. buffer, pH 6.5) was added. The mixture was incubated for 1 hour in a 37° C. water bath. One ml. of 20 per cent perchloric acid was added and the precipitated protein removed by filtration. The ammonia present in an aliquot of the filtrate was determined as described under methods.

Adenosine deaminase gave a quantitative deamination of adenosine, and was inactive on adenine, inosine, and histidine as shown in Table 10. The buffer used was 0.075 M phosphate, pH 6.5. Amounts of adenosine varying between 2 and 10 micromoles were tested. Adenine and inosine were tested at 10 micromole concentrations only. Histidine buffer (3.0 ml. of 0.075 M histidine, pH 6.5) was used in place of phosphate buffer to test for any deamination of histidine. Incubation was in air at 37° for 60 minutes.

Effect of phosphate and aerobic conditions on the formation of adenosine

The possible role of phosphate in the formation of adenosine was suggested by the results obtained by Lutwak-Mann (1936). He found that the deamination of adenine required
Table 10. Activity of intestinal adenosine deaminase on adenosine, inosine, adenine, and histidine

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Quantity present</th>
<th>NH₃ determined</th>
<th>NH₃ produced (substrate-endogenous)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine</td>
<td>2.0</td>
<td>3.3</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>5.4</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5.0</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>9.5</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>11.8</td>
<td>10.6</td>
</tr>
<tr>
<td>Adenine</td>
<td>10.0</td>
<td>0.5</td>
<td>-0.7</td>
</tr>
<tr>
<td>Inosine</td>
<td>10.0</td>
<td>0.8</td>
<td>-0.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>225.0</td>
<td>1.1</td>
<td>-0.1</td>
</tr>
</tbody>
</table>

*aExperimental conditions: phosphate buffer, pH 6.5, 225 micromoles; substrates, as indicated; intestinal deaminase, 10 mg. dry weight; total volume, 7.0 ml.; gas, air; temperature, 37° C.; time, 60 minutes. Deproteinization by 1.0 ml. of 20 per cent HClO₄.*

*bSame except phosphate buffer replaced by histidine buffer, pH 6.5, 225 micromoles.
the presence of phosphate ions for appreciable activity.

The effect of phosphate was determined as a part of an experiment to evaluate the influence of aerobic conditions on the formation of adenosine. Reactions were carried out in Warburg vessels in duplicate, one set with an atmosphere of air, the other with an atmosphere of nitrogen. Substrate was 20 micromoles of adenine and 10 micromoles of inosine. The buffers used were 0.075 M histidine, pH 6.5, and 0.075 M phosphate, pH 6.5. The bacterial enzyme contained 2.6 mg of protein. Incubation was for 30 minutes at 30.4°C. The deproteinized filtrates were tested for the presence of adenosine with the mammalian adenosine deaminase. The results are presented in Table 11.

The average value for the production of adenosine was 5.2 micromoles with a standard deviation of 0.66 micromoles. This magnitude of error is not uncommon particularly when two different biological reactions are used. The error in the determination indicated that there was no significant difference in the production of adenosine under aerobic and under anaerobic conditions. Subsequent experiments were conducted in air. The reaction took place to the same extent in either phosphate or histidine buffer. With the crude extract phosphate was not required for the formation of adenosine from adenine and inosine.

pH optimum

The cell-free extracts contain enzymes that form adeno-
Table 11. Effect of phosphate and aerobic conditions on the formation of adenosine

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Gas phase</th>
<th>Adenosine $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>air</td>
<td>6.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>$N_2$</td>
<td>4.2</td>
</tr>
<tr>
<td>Average in histidine</td>
<td></td>
<td>5.2</td>
</tr>
<tr>
<td>Phosphate</td>
<td>air</td>
<td>4.9</td>
</tr>
<tr>
<td>Phosphate</td>
<td>$N_2$</td>
<td>5.5</td>
</tr>
<tr>
<td>Average in phosphate</td>
<td></td>
<td>5.2</td>
</tr>
<tr>
<td>Average in air</td>
<td></td>
<td>5.5</td>
</tr>
<tr>
<td>Average in $N_2$</td>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td>Overall average</td>
<td></td>
<td>$5.2 \pm 0.66$</td>
</tr>
</tbody>
</table>

Warburg flasks contained: buffer, pH 6.5, 350 micromoles as indicated; adenine, 20 micromoles; inosine, 10 micromoles; cell-free extract, 2.6 mg. of protein; total volume 5.0 ml.; gas, as indicated; temperature, 30.4°C.; time, 30 minutes. Adenosine determined by enzymatic method.
sine from adenine and inosine and also deaminate adenosine. The optimal pH was different for the two systems (Figure 4). The test system consisted of histidine buffer of indicated pH, 150 micromoles; adenine, 17.5 micromoles; inosine, 10 micromoles; cell-free extract, 1.3 mg. of protein; and water to make 5.0 ml. Incubation was at 30°C for 30 minutes. The difference between initial and final pH was not over 0.1 unit in any experiment. The test system for adenosine was similar in all respects except that 20 micromoles of adenosine was used instead of adenine and inosine. The results are presented as relative activity with an activity of 100 equal to 4 micromoles of adenosine produced. In the case of the adenosine deaminase the activity is in terms of micromoles of ammonia formed by the bacterial adenosine deaminase.

pH 6.5 was optimum for the formation of adenosine whereas that for adenosine deamination was 8.0. The relative activity of the adenosine deaminase was much less, even at its optimum, than the activity of the adenosine synthesizing system.

**Enzymatic test system and unit of activity**

The formation of adenosine from adenine and inosine was proportional to enzyme concentration within limits (Figure 5). The test system for the formation of adenosine as determined by the enzymatic method consisted of histidine buffer pH 6.5, 225 micromoles; adenine, 10 micromoles; inosine, 10 micromoles; cell-free extract; and water to 5.0 ml. Incubation was at 37°C for 1 hour. A unit of activity is arbitrarily
Figure 4. pH-activity curve for the formation of adenosine and for the deamination of adenosine

Reactions carried out in Warburg flasks containing: histidine buffer of indicated pH, 150 micromoles; adenine, 17.5 micromoles and inosine, 10 micromoles, or adenosine, 20 micromoles; cell-free extract, 1.3 mg. of protein; 0.5 ml. of 5 per cent TCA; total volume, 5.0 ml; temperature, 30.4°C.; time, 30 minutes. Adenosine formed determined by enzymatic method. Ammonia determined as given in methods. Curve (○): adenosine formed from adenine and inosine. Curve (●): ammonia formed from adenosine.
Figure 5. Formation of adenosine as a function of the concentration of enzyme

Experimental conditions: histidine buffer, pH 6.5, 225 micromoles; adenine, 10 micromoles; inosine, 10 micromoles; cell-free extract, as indicated; total volume, 5.0 ml.; temperature, 37° C.; time, 60 minutes. Adenosine determined by enzymatic method.
defined as the amount of extract catalyzing the formation of 1 micromole of adenosine. The specific activity of the enzyme system is expressed as units per mg. of protein.

Effect of concentration of inosine

The activity of the enzyme system as a function of the concentration of inosine is shown in Figure 6. The test system was changed for this experiment to contain 20 micro-moles of adenine. The extract contained 7.1 mg. of protein. A complete lack of activity without added inosine was not obtained, so the kinetics of the reaction cannot be determined from these data.

Rate of reaction

The rate of reaction decreases with time (Figure 7). The production of adenosine had essentially reached its maximal value after 15 minutes. In no case has the reaction been found to go to completion. When intestinal adenosine deaminase was added to a reaction mixture containing 5 micro-moles of adenine, 10 micromoles of inosine, 325 micromoles of histidine, pH 6.5, and E. coli extract containing 6.6 mg. of protein, essentially all of the adenine was converted to adenosine. The adenosine was deaminated by the intestinal deaminase present. From the 5 micromoles of adenine present, 4.7 micromoles of ammonia were formed. This represents 94 per cent of the adenine present.

Effect of dialysis

The effect of dialysis against distilled water on the
Figure 6. Formation of adenosine as a function of the concentration of inosine

Experimental conditions: histidine buffer, pH 6.5, 225 micromoles; adenine, 20 micromoles; inosine, as indicated; cell-free extract, 7.1 mg. of protein; total volume, 5.0 ml.; temperature, 37° C.; time, 60 minutes. Adenosine determined by enzymatic method. Specific activity equals the micromoles of adenosine formed per mg. of protein.
Figure 7. Rate of formation of adenosine

Experimental conditions: histidine buffer, pH 6.5, 225 micromoles; adenine, 10 micromoles; inosine, 10 micromoles; cell-free extract, 6.6 mg. of protein; total volume, 5.0 ml.; temperature, 37°C.; time, as indicated. Adenosine determined by enzymatic method. Specific activity equals the micromoles of adenosine formed per mg. of protein.
Table 12. Effect of dialysis on enzyme activity

<table>
<thead>
<tr>
<th>Time of dialysis hours</th>
<th>Protein in extract mg.</th>
<th>Adenosine formed μM</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.1</td>
<td>5.7</td>
<td>0.86</td>
</tr>
<tr>
<td>6</td>
<td>6.1</td>
<td>3.5</td>
<td>0.57</td>
</tr>
<tr>
<td>12</td>
<td>5.6</td>
<td>2.1</td>
<td>0.45</td>
</tr>
<tr>
<td>18</td>
<td>3.9</td>
<td>3.2</td>
<td>0.80</td>
</tr>
<tr>
<td>24</td>
<td>4.1</td>
<td>2.6</td>
<td>0.63</td>
</tr>
<tr>
<td>5^b</td>
<td>5.0</td>
<td>5.7</td>
<td>1.06</td>
</tr>
</tbody>
</table>

^ Experimental conditions: histidine buffer, pH 6.5, 225 micromoles; adenine, 10 micromoles; inosine, 10 micromoles; cell-free extract, as indicated; total volume, 5.0 ml.; temperature, 37° C.; time, 60 minutes. Adenosine determined by enzymatic method.

^b In electrophoresis-convection apparatus.

activity of the extract is shown in Table 12. The same initial cell-free extract was used for all experiments. The results show a decrease in activity after dialysis for 6 and 12 hours, and then an increase after 18 hours' dialysis. The decrease in activity after dialysis for 24 hours was too small to be of significance; i.e. activities of the 18 and 24 hours' dialysis are essentially equal. The dialysis in the electrophoresis-convection apparatus shows a slight increase in activity over the non-dialyzed extract. The possible presence of a dialyzable co-factor is indicated by the decrease in
activity after 6 and 12 hours of dialysis and/or the presence of an inhibitor that is more slowly dialyzable than the co-factor is indicated by the increase after dialysis for 18 and 24 hours.

The activity of dialyzed preparations can be restored to predialysis levels with boiled or charred extract. Extract that had not been subjected to dialysis was diluted with buffer so that it contained the same amount of protein used in earlier experiments (7.6 mg. per 1.0 ml. of fluid). The extract was placed in a boiling water bath for 5 minutes. Precipitated protein was removed by filtration. Another aliquot of untreated extract was placed in an evaporating dish and heated over a flame until only ash remained. Sufficient buffer was added to give a solution that contained the same amount of extract as used in the reaction. After thoroughly mixing the insoluble material was removed by filtration. The results of the addition of these preparations to a dialyzed extract are shown in Table 13. The charred extract had practically no activity when tested alone. The activity of the dialyzed extract was increased by the addition of both boiled and charred extract. The effect of boiled extract is uncertain. The activity was not destroyed by the 5 minute period of heating. The increase in activity with the addition of boiled extract to the dialyzed preparation may be due to the addition of enzyme that had not been inactivated or to some other factors in the extract.
Table 13. Restoration of activity in dialyzed extracts

<table>
<thead>
<tr>
<th>Additions</th>
<th>Adenosine produced μM</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.5</td>
<td>0.90</td>
</tr>
<tr>
<td>Boiled extract /²</td>
<td>4.3</td>
<td>1.13</td>
</tr>
<tr>
<td>Charred extract /² c</td>
<td>4.3</td>
<td>1.13</td>
</tr>
<tr>
<td>Boiled and charred extract /² c</td>
<td>4.7</td>
<td>1.20</td>
</tr>
<tr>
<td>Boiled extract only /² b</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Charred extract only /² c</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

²Experimental conditions: histidine buffer, pH 6.5, 225 micromoles; adenine, 10 micromoles; inosine, 10 micromoles; cell-free extract, 3.9 mg. of protein dialyzed for 18 hours; additions, as indicated; total volume, 5.0 ml.; temperature, 37°C; time, 60 minutes. Adenosine determined by enzymatic method.

²Boiled extract contained filtrate from 7.1 mg. of cell-free extract placed in boiling water bath for 5 minutes.

²Charred extract contained soluble ash from 7.1 mg. of cell-free extract.

Effect of various ions on activity

The results obtained with the charred extract indicated a possible effect of inorganic ions. The effect of various inorganic ions on the production of adenosine is shown in Table 14. Small concentrations of phosphate ions show considerable stimulation of activity on all dialyzed preparations. Phosphate had very little effect on undialyzed preparations (Table 11).
Table I. Effect of various ions on enzyme activity

<table>
<thead>
<tr>
<th>Ions added</th>
<th>Specific activity</th>
<th>Relative effect in per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>$2 \mu M \text{PO}_4^{3-}$</td>
<td>0.90</td>
<td>158</td>
</tr>
<tr>
<td>$20 \mu M \text{Mg}^{++}$</td>
<td>0.25</td>
<td>43</td>
</tr>
<tr>
<td>$20 \mu M \text{Mn}^{++}$</td>
<td>0.31</td>
<td>54</td>
</tr>
<tr>
<td>$20 \mu M \text{Ca}^{++}$</td>
<td>0.39</td>
<td>68</td>
</tr>
<tr>
<td>$20 \mu M \text{Ba}^{++}$</td>
<td>0.52</td>
<td>91</td>
</tr>
<tr>
<td>$20 \mu M \text{K}^+$</td>
<td>0.57</td>
<td>100</td>
</tr>
<tr>
<td>$20 \mu M \text{Na}^+$</td>
<td>0.52</td>
<td>91</td>
</tr>
<tr>
<td>$20 \mu M \text{SO}_4^{2-}$</td>
<td>0.53</td>
<td>93</td>
</tr>
<tr>
<td>$20 \mu M \text{Cl}^-$</td>
<td>0.54</td>
<td>95</td>
</tr>
<tr>
<td>$300 \mu M \text{F}^-$</td>
<td>0.56</td>
<td>98</td>
</tr>
<tr>
<td>$2 \mu M \text{PO}_4^{3-} + 20 \mu M \text{Mg}^{++}$</td>
<td>0.80</td>
<td>140</td>
</tr>
<tr>
<td>$2 \mu M \text{PO}_4^{3-} + 20 \mu M \text{Mn}^{++}$</td>
<td>0.84</td>
<td>147</td>
</tr>
<tr>
<td>$2 \mu M \text{PO}_4^{3-} + 20 \mu M \text{Ca}^{++}$</td>
<td>0.87</td>
<td>153</td>
</tr>
<tr>
<td>$2 \mu M \text{PO}_4^{3-} + 20 \mu M \text{Ba}^{++}$</td>
<td>0.80</td>
<td>140</td>
</tr>
</tbody>
</table>

*Experimental conditions: histidine buffer, pH 6.5, 225 micromoles; adenine, 10 micromoles; inosine, 10 micromoles; cell-free extract, 6.1 mg. of protein dialyzed for 6 hours; ion solutions, as indicated; total volume, 5.0 ml.; temperature, 37° C.; time, 60 minutes. Adenosine determined by enzymatic method.*
The divalent cations listed show an inhibitory effect on the reaction with the exception of those of barium. The inhibition shown with barium is too small to be of significance. The inhibition by the cations can be overcome by the addition of phosphate ions. The increase in activity, however, only restored the activity of the extract to that of undialyzed preparations. Phosphate alone showed no stimulation above undialyzed extract which had a specific activity of 0.86. The monovalent cations show no inhibitory action. Fluoride, chloride and sulfate ions have no effect on the enzyme activity.

These data indicate that there is no readily dialyzable organic co-factor required for the reaction. The restoration of dialyzed preparations to predialysis activity with small amounts of phosphate indicates that phosphate does not enter into the reaction in stoichiometric amounts. The data do not exclude the possibility that phosphate may cycle in the mechanism of the reaction. The role of phosphate may involve removing inhibitory divalent cations by forming relatively insoluble salts or complexes. Conversely, the inhibitory effect of the cations may be in removing the phosphate required for the reaction. Another hypothesis to explain the mechanism of inhibition by divalent cations involves the ions as co-factors for enzymes that shunt inosine, adenosine, or adenine into other reactions. A quantitative analysis of the reaction mixture was required to test this hypothesis.
Quantitative analysis of adenosine, adenine, hypoxanthine, and inosine in a mixture

The ultraviolet absorption spectra for adenine, adenosine, hypoxanthine, and inosine are shown in Figure 1. The absorption curves for adenine and adenosine are quite similar as are the curves for hypoxanthine and inosine. There are sufficient differences in the adenine and hypoxanthine curves so that the concentration of each compound in a mixture can be determined spectrophotometrically. The same holds true for adenosine and inosine.

Quantitative determination of two compounds in a mixture. The determination of two compounds having different absorption spectra has been described by Kerr et al. (1949) and Loring et al. (1952) for an adenine and guanine mixture and by Loring and Ploeser (1949) for a uridine and cytidine mixture. The analysis is based on the assumption that the total optical density, D, at each of two wavelengths, \( \lambda_1 \) and \( \lambda_2 \), is the sum of the densities due to each component at each wavelength. From the Beer-Lambert law two simultaneous equations can be written in which the concentration of the two components, \( A \) and \( B \), are expressed as functions of their molar extinction coefficient and optical densities at the respective wavelengths. These equations are as follows:

\[
D_{\lambda_1} = C_A E_A \lambda_1 \quad C_B E_B \lambda_1
\]

and

\[
D_{\lambda_2} = C_A E_A \lambda_2 \quad C_B E_B \lambda_2
\]
where

\( C_A \) and \( C_B \) = concentration of A and B in moles per liter

\( \lambda_1 \) and \( \lambda_2 \) = two different wavelengths

\( E_{A\lambda_1} \) and \( E_{A\lambda_2} \) = molar extinction coefficient of A at \( \lambda_1 \) and \( \lambda_2 \)

\( E_{B\lambda_1} \) and \( E_{B\lambda_2} \) = molar extinction coefficient of B at \( \lambda_1 \) and \( \lambda_2 \)

\( D_{\lambda_1} \) and \( D_{\lambda_2} \) = optical density at the two wavelengths

Solving these equations for \( C_A \) and \( C_B \) gave the following results:

\[
C_A = \frac{E_{B\lambda_2}D_{\lambda_1} - E_{B\lambda_1}D_{\lambda_2}}{E_{B\lambda_2}E_{A\lambda_1} - E_{B\lambda_1}E_{A\lambda_2}}
\]

and

\[
C_B = \frac{E_{B\lambda_2}D_{\lambda_1} - E_{B\lambda_1}D_{\lambda_2}}{E_{B\lambda_2}E_{A\lambda_1} - E_{B\lambda_1}E_{A\lambda_2}}
\]

This type of analysis was applied to mixtures of adenine and hypoxanthine and of adenosine and inosine. The wavelengths used were 240 millimicrons and 265 millimicrons. With the extinction values given in Table 2, the following equations were calculated:

\[
\text{Adenine} = \frac{9340 \ D_{265} - 5180 \ D_{240}}{9.11 \times 10^7}
\]

\[
\text{Hypoxanthine} = \frac{12300 \ D_{240} - 5500 \ D_{265}}{9.11 \times 10^7}
\]

\[
\text{Adenosine} = \frac{3550 \ D_{265} - 6060 \ D_{240}}{6.96 \times 10^7}
\]

\[
\text{Inosine} = \frac{12650 \ D_{240} - 6360 \ D_{265}}{6.96 \times 10^7}
\]

where \( D_{240} \) and \( D_{265} \) = optical density determined in the Beckman DU spectrophotometer at 240 millimicrons and 265 millimicrons, respectively
compound = concentration of that compound in moles per liter

Data obtained on mixtures of adenine and hypoxanthine were applied to these equations. The results of these calculations are given in Table 15. The recovery was quite satisfactory in all cases. The first eight solutions listed were run on the same original solutions. When the solutions are all calculated to the same original volume and the average taken, the average recovery for adenine was 96 per cent and the average recovery for hypoxanthine was 95 per cent.

Mixtures of solutions of known concentration of adenosine and inosine were also prepared. The formulae for determination of the concentration of adenosine and inosine were applied to the optical density data obtained on these solutions. Results are presented in Table 16. The concentrations calculated were in excellent agreement with the known concentrations. The first eight mixtures listed were prepared from the same original solutions. When the concentrations were calculated to the same volume, the average for adenosine was 102 per cent of the known concentration, and the average for inosine was 95 per cent of the known concentration.

Separation of purine bases and nucleosides. Spectrophotometric measurements on mixtures containing all four of the compounds involved in this study are not satisfactory to determine the concentration of each compound. The purine bases were separated from the nucleosides by a method used by
Table 15. Application of adenine-hypoxanthine concentration formulae to known mixtures

<table>
<thead>
<tr>
<th>Conc. found 10^{-5} M</th>
<th>Actual conc. 10^{-5} M</th>
<th>Recovery per cent</th>
<th>Conc. found 10^{-5} M</th>
<th>Actual conc. 10^{-5} M</th>
<th>Recovery per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.63 10^{-5} M</td>
<td>1.68 10^{-5} M</td>
<td>100</td>
<td>1.96 10^{-5} M</td>
<td>1.95 10^{-5} M</td>
<td>101</td>
</tr>
<tr>
<td>3.46 10^{-5} M</td>
<td>3.37 10^{-5} M</td>
<td>103</td>
<td>1.97 10^{-5} M</td>
<td>1.95 10^{-5} M</td>
<td>101</td>
</tr>
<tr>
<td>1.61 10^{-5} M</td>
<td>1.68 10^{-5} M</td>
<td>96</td>
<td>3.90 10^{-5} M</td>
<td>3.90 10^{-5} M</td>
<td>100</td>
</tr>
<tr>
<td>3.37 10^{-5} M</td>
<td>3.37 10^{-5} M</td>
<td>100</td>
<td>3.76 10^{-5} M</td>
<td>3.90 10^{-5} M</td>
<td>100</td>
</tr>
<tr>
<td>1.73 10^{-5} M</td>
<td>1.68 10^{-5} M</td>
<td>103</td>
<td>1.86 10^{-5} M</td>
<td>1.95 10^{-5} M</td>
<td>95</td>
</tr>
<tr>
<td>3.50 10^{-5} M</td>
<td>3.37 10^{-5} M</td>
<td>104</td>
<td>1.92 10^{-5} M</td>
<td>1.95 10^{-5} M</td>
<td>98</td>
</tr>
<tr>
<td>1.62 10^{-5} M</td>
<td>1.68 10^{-5} M</td>
<td>97</td>
<td>3.84 10^{-5} M</td>
<td>3.90 10^{-5} M</td>
<td>99</td>
</tr>
<tr>
<td>3.38 10^{-5} M</td>
<td>3.37 10^{-5} M</td>
<td>100</td>
<td>3.82 10^{-5} M</td>
<td>3.90 10^{-5} M</td>
<td>98</td>
</tr>
<tr>
<td>1.39 10^{-5} M</td>
<td>1.45 10^{-5} M</td>
<td>96</td>
<td>1.45 10^{-5} M</td>
<td>1.46 10^{-5} M</td>
<td>99</td>
</tr>
</tbody>
</table>

^Actual concentration determined from optical density and known molar extinction values on solution of the single compound. The same solutions were then combined in various proportions to give the mixtures tested.
Table 16. Application of adenosine-inosine concentration formulae to known mixtures

<table>
<thead>
<tr>
<th>Conc. found $10^{-5}M$</th>
<th>Actual conc. $10^{-5}M$</th>
<th>Per cent recovery</th>
<th>Conc. found $10^{-5}M$</th>
<th>Actual conc. $10^{-5}M$</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.66</td>
<td>1.72</td>
<td>97</td>
<td>1.71</td>
<td>1.63</td>
<td>102</td>
</tr>
<tr>
<td>1.67</td>
<td>1.72</td>
<td>97</td>
<td>3.30</td>
<td>3.35</td>
<td>98</td>
</tr>
<tr>
<td>3.65</td>
<td>3.45</td>
<td>106</td>
<td>1.42</td>
<td>1.68</td>
<td>95</td>
</tr>
<tr>
<td>3.46</td>
<td>3.45</td>
<td>100</td>
<td>3.28</td>
<td>3.35</td>
<td>98</td>
</tr>
<tr>
<td>1.35</td>
<td>1.72</td>
<td>108</td>
<td>1.54</td>
<td>1.68</td>
<td>92</td>
</tr>
<tr>
<td>1.77</td>
<td>1.72</td>
<td>103</td>
<td>3.26</td>
<td>3.35</td>
<td>97</td>
</tr>
<tr>
<td>3.54</td>
<td>3.45</td>
<td>103</td>
<td>1.46</td>
<td>1.68</td>
<td>87</td>
</tr>
<tr>
<td>3.46</td>
<td>3.45</td>
<td>100</td>
<td>3.28</td>
<td>3.35</td>
<td>98</td>
</tr>
<tr>
<td>1.36</td>
<td>1.34</td>
<td>102</td>
<td>1.57</td>
<td>1.59</td>
<td>99</td>
</tr>
<tr>
<td>2.60</td>
<td>2.56</td>
<td>102</td>
<td>2.68</td>
<td>2.68</td>
<td>98</td>
</tr>
</tbody>
</table>

*Actual concentration determined from optical density and known molar extinction values on the solution of the single compound. These solutions were then combined in various proportions to give the mixtures tested.*
Kerr and Seraldarian (1945). According to this method a solution of purine nucleosides and bases is made 0.05 N with H$_2$SO$_4$ and 0.02 volumes of 1 M AgNO$_3$ is added. The purine bases are quantitatively precipitated from solution and the nucleosides remain in the filtrate.

Applicability of this method was determined on known mixtures containing the buffer and the cell-free extract used in the experiments. A known mixture of adenine, hypoxanthine, adenosine and inosine was prepared. The concentration of each compound was determined from the optical density of a solution that contained a concentration of the compound similar to that used in the precipitation procedure. Aliquots of the mixture of purines and nucleosides were combined with 1.0 ml. of 1 N HClO$_4$, 1.0 ml. of cell-free extract of E. coli, 2.0 ml. of 0.075 M histidine buffer, pH 6.5, 1.0 ml. of 0.5 N H$_2$SO$_4$ and water to make 12.0 ml. After filtration to remove the precipitated protein, duplicate aliquots (5.0 ml.) were placed in flasks and 1.0 ml. of 0.2 M AgNO$_3$ solution added to one flask and 1.0 ml. of water to the other. The flasks containing the AgNO$_3$ solution were allowed to stand at room temperature for 30 minutes before filtration to remove the silver salts of the purine bases. Optical densities at 240 millimicrons and 265 millimicrons were determined on aliquots of each filtrate. The optical density of the filtrate that had not been treated with Ag$^+$ ions was considered to be due to all four compounds. By subtraction of the
Table 17. Recovery of added purines and nucleosides after precipitation with silver ions^* 

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quantity added</th>
<th>Recovery</th>
<th>Average recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
<td>Exp. 1</td>
</tr>
<tr>
<td>Adenine</td>
<td>2.02</td>
<td>2.12</td>
<td>2.05</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>2.59</td>
<td>2.76</td>
<td>2.51</td>
</tr>
<tr>
<td>Adenosine</td>
<td>2.06</td>
<td>1.90</td>
<td>2.12</td>
</tr>
<tr>
<td>Inosine</td>
<td>1.92</td>
<td>1.83</td>
<td>2.01</td>
</tr>
</tbody>
</table>

^Actual concentration determined spectrophotometrically on the single compounds. Each experiment contained in addition: 1.0 ml., 1 N HClO4; 1.0 ml. cell-free extract; 1.0 ml. 0.5 M H2SO4; 2.0 ml. 0.075 M histidine buffer, pH 6.5; water to 12.0 ml. Aliquots treated with 0.2 N AgNO3 as described in text.

Optical density of the flask that had been treated with Ag ions, the optical density of a solution of the bases was determined. The formulae derived in the preceding section for the determination of the concentrations of binary mixtures were applied to the optical density data, i.e. the density actually determined in case of the nucleosides or calculated, by difference, in the case of the bases. The results of this analysis are given in Table 17. The recovery of all compounds was satisfactory to show any fairly large changes occurring during a reaction.

Spectrophotometric test system

Reactions were carried out in Erlenmeyer flasks in a
37° C. water bath. Reaction mixtures contained 300 micro-
moles of buffer, pH 6.5, approximately 4 micromoles each of
adenine and inosine, cell-free extract, and water to make
4.0 ml. Incubation was for varying time intervals. Depro-
teinization was with 1.0 ml. of 1.5 N H$_2$SO$_4$. The solution
was diluted to 12.0 ml. with water and aliquots taken for
the spectrophotometric determination of the bases and the
nucleosides as indicated in the previous section.

**Stoichiometry**

Essentially a 1:1 ratio exists between reactants disap-
pearing and products formed during the course of the reac-
tions as shown by Table 18.

**Effect of divalent cations on stoichiometry**

One hypothesis to explain the mechanism of inhibition of
the reaction by divalent cations involves the ions as co-
factors for reactions that shunt adenine, adenosine, or
inosine into other reactions. If this were the case, the
ratio of reactants disappearing to products formed would not
be unity as found for the reaction in the absence of divalent
ions. The ratio of adenine and inosine disappearing to
adenosine and hypoxanthine formed was essentially the same
in the presence or absence of the divalent cations (Table 19).
The stimulation with phosphate ions also gave the same ratio
of reactants and products. These data indicate that the
divalent cations do not act as co-factors for side reactions.
Table 13. Stoichiometry of adenine-inosine reaction

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial</th>
<th>After 10 min.</th>
<th>Change</th>
<th>After 20 min.</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu M )</td>
<td>( \mu M )</td>
<td>( \mu M )</td>
<td>( \mu M )</td>
<td>( \mu M )</td>
</tr>
<tr>
<td>Adenine</td>
<td>4.3</td>
<td>3.3</td>
<td>1.0</td>
<td>3.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Inosine</td>
<td>4.0</td>
<td>3.1</td>
<td>0.9</td>
<td>2.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0</td>
<td>0.9</td>
<td>0.9</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0</td>
<td>0.8</td>
<td>0.8</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Average change</td>
<td>0.9</td>
<td></td>
<td></td>
<td>1.25</td>
<td></td>
</tr>
</tbody>
</table>

Experimental conditions: pyrophosphate buffer, \( \mathrm{pH} \ 6.5 \), 300 micromoles; substrates, as indicated; cell-free extract, 1.2 mg. of protein; total volume, 4.0 ml.; temperature, 37\(^\circ\) C.; time, as indicated. Compounds determined by spectrophotometric method.

Table 19. Effect of divalent cations on stoichiometry

<table>
<thead>
<tr>
<th>Ions added</th>
<th>Adenine</th>
<th>Adenosine</th>
<th>Inosine</th>
<th>Hypoxanthine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>- 1.2</td>
<td>+ 1.1</td>
<td>- 1.0</td>
<td>+ 1.3</td>
</tr>
<tr>
<td>50 ( \mu M ) Mn(^{++})</td>
<td>- 0.6</td>
<td>+ 0.5</td>
<td>- 0.6</td>
<td>+ 0.7</td>
</tr>
<tr>
<td>100 ( \mu M ) Mg(^{++})</td>
<td>- 1.0</td>
<td>+ 1.0</td>
<td>- 0.9</td>
<td>+ 1.0</td>
</tr>
<tr>
<td>100 ( \mu M ) Ca(^{++})</td>
<td>- 0.7</td>
<td>+ 0.8</td>
<td>- 0.6</td>
<td>+ 0.6</td>
</tr>
<tr>
<td>100 ( \mu M ) Ba(^{++})</td>
<td>- 0.8</td>
<td>+ 0.7</td>
<td>- 0.9</td>
<td>+ 0.8</td>
</tr>
<tr>
<td>8 ( \mu M ) PO(_4)(^{--})</td>
<td>- 1.7</td>
<td>+ 1.5</td>
<td>- 1.5</td>
<td>+ 1.9</td>
</tr>
</tbody>
</table>

Experimental conditions: histidine buffer, \( \mathrm{pH} \ 6.5 \), 300 micromoles; adenine, 4 \( \mu M \) micromoles; inosine, 4 \( \mu M \) micromoles; cell-free extract, 1.2 mg. protein, dialyzed 5 hours in electrophoresis-convection apparatus; water to 4.0 ml.; temperature, 37\(^\circ\) C.; time, 20 minutes. Compounds determined by spectrophotometric method.
Precipitation of phosphate by divalent cations

Another mechanism for inhibition by the cations would involve the removal of phosphate required for the reaction. The removal of orthophosphate from solution by these ions was determined by the Lowry and Lopez (1946) orthophosphate test on filtrates of solutions of inorganic phosphate treated with the cations. Results of this test are presented in Table 20. The Mg$^{2+}$ ions removed the least phosphate and were the least active in the inhibition of the formation of adenosine. The ions other than Mn$^{2+}$ may actually prevent more phosphate from participating in the enzyme reaction than is indicated by the results reported in Table 20. When the ions in 50 micromole quantities or less were tested for the removal of inorganic phosphate, no visible precipitate was formed and the phosphate test indicated that very little phosphate had been removed. The possibility exists that complexes are formed between the divalent cations and phosphate that are effective in removing the phosphate from participation in the enzyme reaction, but which are soluble and dissociate when the filtrate is diluted in the test for inorganic phosphate. From a quantitative viewpoint, the results presented in Tables 19 and 20 may not be strictly comparable. Nevertheless, the results do indicate that phosphate is probably involved in the formation of adenosine and hypoxanthine from adenine and hypoxanthine.
Table 20. Removal of phosphate ions by divalent cations

<table>
<thead>
<tr>
<th>Solution added</th>
<th>Phosphate found $\mu M$</th>
<th>Phosphate removed per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.4</td>
<td>0</td>
</tr>
<tr>
<td>50 $\mu M$ Mn$^{++}$</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>100 $\mu M$ Mg$^{++}$</td>
<td>6.1</td>
<td>27</td>
</tr>
<tr>
<td>100 $\mu M$ Ca$^{++}$</td>
<td>0.7</td>
<td>92</td>
</tr>
<tr>
<td>100 $\mu M$ Ba$^{++}$</td>
<td>1.9</td>
<td>77</td>
</tr>
</tbody>
</table>

*Experimental conditions: phosphate, 3.4 micromoles; salt solutions, as indicated; histidine buffer, pH 6.5, 300 micromoles; total volume, 4.0 ml. Aliquots of filtrate tested for inorganic phosphate by method of Lowry and Lopez (1946).

Phosphorolysis of inosine and adenosine

The results of the experiments with dialyzed preparations and data of the effect of the divalent cations and phosphate ions suggested that phosphate ions were involved in the reaction. The possibility of a phosphorolysis of the nucleosides was considered. When inosine or adenosine was incubated with cell-free extract and 100 micromoles of phosphate, a considerable disappearance of the nucleoside occurred (Table 21). The appearance of adenine and hypoxanthine was not determined in this experiment. Qualitative chromatographic analysis showed the appearance of adenine when adenosine was incubated with cell-free extract and phosphate. Hypoxanthine
Table 21. Phosphorolysis of adenosine and inosine

<table>
<thead>
<tr>
<th>Additions</th>
<th>Adenosine disappearing $\mu M$</th>
<th>Inosine disappearing $\mu M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>100 $\mu M$ phosphate</td>
<td>1.4</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Experimental conditions: histidine buffer, pH 6.5, 300 micromoles; adenosine, 4.47 micromoles, or inosine, 4.36 micromoles; phosphate, as indicated; cell-free extract, 2.6 mg. of protein; water to 4.0 ml.; temperature, 37° C.; time, 60 minutes. Compounds determined by spectrophotometric method.*

was formed from inosine under the same conditions. The phosphorolysis of the nucleosides did not occur to any appreciable extent when small amounts of phosphate were added. No attempt was made to detect the appearance of organic phosphate possibly formed during the reaction. It is to be expected that only 1.4 to 1.5 micromoles of such a compound would be formed when 100 micromoles of phosphate were incubated with the extract and the nucleoside. The disappearance of 1.5 micromoles of inorganic phosphate from 100 micromoles of phosphate is too small to detect with the methods available. An error of only 2 per cent in the analysis would be sufficient to prevent the detection of an organic phosphate.

Chromatographic test system

The spectrophotometric method for the determination of
the nucleosides and bases after precipitation as the silver
salts showed satisfactory recoveries (Table 17) when all
compounds were present in considerable quantity. When only
small amounts of a base or nucleoside are formed in a reac-
tion, the inherent errors in the determination make the error
in the concentration of that compound of considerable magni-
tude. This is illustrated in Table 22. If optical density
readings of 0.430 and 0.320 are assumed at 265 millimicrons
and 240 millimicrons respectively, then the optical density
of solutions with only 0.1 the concentration would have
optical densities of 0.048 and 0.032. If for some reason the
optical density of both solutions read 0.010 units low at
each wavelength, (not uncommon) then the concentrations cal-
culated for the two solutions will change. As shown in Table
22 this condition has very little effect on the concentra-
tions when the readings are in the range of 0.300 to 0.500.
When only small amounts are present, however, readings would
be quite low, and then a change of 0.010 optical density units
due to errors in the spectrophotometer or manipulations would
introduce errors of 12 to 60 per cent in the concentrations
calculated.

The quantitative determination of the compounds when
present in small amounts was accomplished by a chromatographic
separation using water adjusted to pH 10 as the solvent. The
chromatographic test system consisted of 90 micromoles of
pyrophosphate buffer, pH 6.5, substrate, usually 1.6 micro-
Table 22. Errors introduced into calculated concentrations by small errors in spectrophotometer readings\(^a\)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wavelength m(\mu)</th>
<th>(D^b) x 10(^{-5})M</th>
<th>(D^c) x 10(^{-5})M</th>
<th>Per cent error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>265</td>
<td>0.480</td>
<td>0.470</td>
<td>3.09</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>0.320</td>
<td>0.310</td>
<td>3.05</td>
</tr>
<tr>
<td></td>
<td>265</td>
<td>0.048</td>
<td>0.038</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>0.032</td>
<td>0.022</td>
<td>0.25</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>265</td>
<td>0.480</td>
<td>0.470</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>0.320</td>
<td>0.310</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>265</td>
<td>0.048</td>
<td>0.038</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>0.032</td>
<td>0.022</td>
<td>0.08</td>
</tr>
<tr>
<td>Adenosine</td>
<td>265</td>
<td>0.480</td>
<td>0.470</td>
<td>3.10</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>0.320</td>
<td>0.310</td>
<td>3.07</td>
</tr>
<tr>
<td></td>
<td>265</td>
<td>0.048</td>
<td>0.038</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>0.032</td>
<td>0.022</td>
<td>0.27</td>
</tr>
<tr>
<td>Inosine</td>
<td>265</td>
<td>0.480</td>
<td>0.470</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>0.320</td>
<td>0.310</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td>265</td>
<td>0.048</td>
<td>0.038</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>0.032</td>
<td>0.022</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\(^a\)All concentrations calculated from assumed optical density readings and errors introduced as indicated in text.

\(^b\)Assumed optical density reading.

\(^c\)Optical density reading with error of -0.010 units.
moles each of adenine and inosine, and cell-free extract in a total volume of 0.9 ml. Incubation was in a Dubnoff metabolic shaking incubator at 37° C. The reaction was stopped by the addition of 0.1 ml. of 1.5 N H₂SO₄. Protein was removed by centrifugation. Chromatography and elution of the spots were carried out as given in methods. The concentration of the individual compounds was determined in the spectrophotometer. To compensate for possible ultraviolet absorbing materials in the filter paper, the concentration was determined from the density at the absorption maximum less the density at 290 millimicrons as recommended by Vischer and Chargaff (1948). The data in Table 23 give the values used for these calculations.

Table 23. Molar extinction values used in calculation of concentrations of purine compounds separated by paper chromatography

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wavelength of maximum (m), Eₘₐₓ at 1 x 10⁻⁵ M sol.</th>
<th>Eₘ at 290 (m), Eₘₐₓ 290 - Eₘ₂₉₀ (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>257, 14,600</td>
<td>325, 0.138</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>268, 10,800</td>
<td>320, 0.105</td>
</tr>
<tr>
<td>Inosine</td>
<td>250, 10,900</td>
<td>500, 0.104</td>
</tr>
<tr>
<td>Adenine</td>
<td>262, 13,150</td>
<td>54.0, 0.126</td>
</tr>
</tbody>
</table>

¹Values are those given by Johnson (1955).

²Molar extinction values (Eₘ) determined on pure compounds.
When only small amounts of a compound were formed, larger aliquots of the reaction mixture were placed on the chromatogram so that the optical density readings were large enough that errors in reading did not introduce large errors in the concentrations calculated. In some cases the use of large aliquots on the chromatograms did not give good separation of the compounds. Under these conditions smaller aliquots were placed on several chromatograms. Spots from these chromatograms were combined and eluted with 4.0 ml. of 1.0 N HCl so that the concentration of the solution was higher than could be obtained from a single spot.

Equilibrium constant

The conversion of adenine and inosine to adenosine and hypoxanthine was demonstrated to be reversible.

\[
\text{Adenine + Inosine} \rightleftharpoons \text{Adenosine + Hypoxanthine}
\]

The equilibrium for the reaction is shown in Figure 8. The compounds were determined by the chromatographic method. Equilibrium is attained from both directions in approximately the same time; i.e., about 90 minutes. The equilibrium constant at 37° C. for the forward reaction was 2.6. The constant for the reverse reaction was 0.38.

Kinetics

The formation of adenosine as a function of the concentration of substrate is shown in Figure 9. Curve (O) shows the effect of increasing adenine in the presence of 0.76 micromoles of inosine; curve (□) shows the effect of in-
Figure 8. Equilibrium in the conversion of adenine and inosine to adenosine and hypoxanthine

Experimental conditions: pyrophosphate buffer, pH 6.5, 90 micromoles; cell-free extract, 1.3 mg. of protein; curve (O), adenosine, 1.62 micromoles and hypoxanthine, 1.49 micromoles; curve (●) adenine, 1.59 micromoles and inosine, 1.59 micromoles; total volume, 0.9 ml.; temperature, 37° C.; time, as indicated. Compounds determined by chromatographic method.
Figure 9. Formation of adenosine as a function of the concentration of substrate

Experimental conditions: pyrophosphate buffer, pH 6.5, 90 micromoles; cell-free extract, 0.8 mg. of protein; curve (○) inosine, 0.76 micromoles and adenine as indicated; curve (□) adenine, 0.76 micromoles and inosine as indicated; curve (△) adenine and inosine, each at amount indicated; total volume, 0.9 ml.; temperature, 37°C; time, 30 minutes. Compounds determined by chromatographic method.
μM ADENOSINE PRODUCED

μM SUBSTRATE
creasing inosine concentration in the presence of 0.76 micro-
moles of adenine; and curve (Δ) shows the effect of increas-
ing the substrates when both are present in equimolar concen-
trations.

The concentration of adenine required for half-maximum
rate was $2.3 \times 10^{-4}$ M; the concentration of inosine required
for half-maximum rate was $5.5 \times 10^{-4}$ M; and when both were
present in equimolar quantities, the concentration of each
required for half-maximum rate was $1.2 \times 10^{-3}$ M. The concen-
trations were determined by the method of Lineweaver and
Burk (1934) using the least squares method rather than the
graphic method.

The effect of the concentration of phosphate on the for-
mation of adenosine is shown in Figure 10. The cell-free
extract was fractionated with ammonium sulfate and dialyzed
as indicated in methods. Even under these conditions, appre-
ciable adenosine was formed without added phosphate. These
data do not permit a calculation of a constant for the concen-
tration of phosphate required for half-maximum rate. The
data do indicate that phosphate has a stimulatory effect on
the reaction.

**Phosphorolysis of adenosine and inosine**

The phosphorolysis reaction was carried out using the
chromatographic test system to make certain that the phos-
phorolysis obtained by the use of the spectrophotometric
system was not due, at least in part, to experimental error.
Figure 10. Formation of adenosine as a function of the concentration of phosphate

Experimental conditions: pyrophosphate buffer, pH 6.5, 90 micromoles; adenine, 1.59 micromoles; inosine, 1.59 micromoles; phosphate, as indicated; cell-free extract, 1.2 mg. of protein from extract precipitated between 33 and 66 per cent saturation with ammonium sulfate; total volume, 0.9 ml.; temperature, 37° C.; time, 10 minutes. Compounds determined by chromatographic method.
Table 24. Phosphorolysis of inosine and adenosine

<table>
<thead>
<tr>
<th>Phosphate added (μM)</th>
<th>Inosine split (μM)</th>
<th>Adenosine split (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.10</td>
<td>0.11</td>
</tr>
<tr>
<td>1</td>
<td>0.18</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.17</td>
</tr>
<tr>
<td>4</td>
<td>0.24</td>
<td>0.20</td>
</tr>
</tbody>
</table>

aExperimental conditions: pyrophosphate buffer, pH 6.5, 90 micromoles; inosine, 1.54 micromoles, or adenosine, 1.66 micromoles; cell-free extract, 0.8 mg. of protein; phosphate, as indicated; total volume, 0.9 ml.; temperature, 37 C.; time, 30 minutes. Adenosine and inosine determined by chromatographic method.

The results of this study are presented in Table 24. Both adenosine and inosine were split by the enzyme. The formation of the purine base from the nucleoside was increased by the addition of orthophosphate. Even without the addition of orthophosphate, considerable base was formed. The splitting could be due to the presence of orthophosphate in the extract or to hydrolysis rather than phosphorolysis.

Formation of purine bases from adenosine and inosine

The formation of adenine from adenosine and hypoxanthine from inosine was catalyzed by a cell-free extract that had been fractionated by ammonium sulfate and dialyzed against distilled water (Table 25). The rate of reaction is also indicated. The splitting of inosine appeared to be quite rapid (maximum value in 10 minutes) and was comparable to the
Table 25. The formation of purine bases from nucleosides catalyzed by a fractionated extract

<table>
<thead>
<tr>
<th>Time of reaction</th>
<th>Inosine split</th>
<th>Adenosine split</th>
</tr>
</thead>
<tbody>
<tr>
<td>minutes</td>
<td>(μM)</td>
<td>(μM)</td>
</tr>
<tr>
<td>10</td>
<td>0.09</td>
<td>0.06</td>
</tr>
<tr>
<td>30</td>
<td>0.09</td>
<td>0.08</td>
</tr>
<tr>
<td>60</td>
<td>0.09</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Experimental conditions: pyrophosphate buffer, pH 6.5, 90 micromoles; inosine, 1.54 micromoles, or adenosine, 1.66 micromoles; cell-free extract, 0.6 mg. of protein from extract precipitated between 33 and 66 per cent saturation with ammonium sulfate; total volume, 0.9 ml.; temperature, 37°C; time, as indicated. Adenosine and inosine determined by chromatographic method.

reaction with the crude extract (Table 24). The splitting of adenosine was somewhat slower, but after 60 minutes had reached essentially the same extent as with the crude extract in 10 minutes. These results indicate either the incomplete removal of orthophosphate by the fractionation procedure and dialysis or the hydrolysis of the nucleosides rather than a phosphorolysis.

The presence of pyrophosphate as the buffer used for these experiments inhibits the color formation by orthophosphate in the Lowry and Lopez (1946) method used for the determination of orthophosphate. A possible uptake of orthophosphate, therefore, could not be determined. The effect of orthophosphate on the splitting of the nucleosides (Table 24) indicated that phosphorolysis probably occurred, but the
possibility of a hydrolytic cleavage was not eliminated by these data.

Formation of ribose-1-phosphate

The phosphorolysis of inosine and adenosine (Tables 21 and 24) suggested that the reaction under study could be explained as the sum of two separate reactions.

Inosine + Orthophosphate $\rightleftharpoons$ Hypoxanthine + Ribose-1-phosphate
Adenine + Ribose-1-phosphate $\rightleftharpoons$ Adenosine + Orthophosphate
Sum: Adenine + Inosine $\rightleftharpoons$ Adenosine + Hypoxanthine

Ribose-1-phosphate was prepared according to the first reaction by a modification of the procedures used by Kalckar (1947 a) for the preparation of ribose-1-phosphate from inosine and by Friedkin (1950) for the preparation of deoxyribose-1-phosphate. Inosine (14.9 millimoles) in 1.0 ml. of 1.0 M phosphate buffer, pH 6.5, was incubated with 0.5 ml. of xanthine oxidase (Nutritional Biochemicals Corporation) and 0.5 ml. of cell-free extract of E. coli that contained 13.1 mg. of protein. Incubation was in a Warburg vessel at 30.6° C. for 3 hours. Oxygen uptake amounted to 34 micromoles and indicated that 17 micromoles of hypoxanthine had been oxidized to uric acid. The reactions taking place during incubation were probably:

Inosine + Orthophosphate $\rightleftharpoons$ Hypoxanthine + Ribose-1-phosphate
Hypoxanthine + $O_2 \xrightarrow{\text{xanthine oxidase}}$ Xanthine + $H_2O_2$ and
Xanthine + \( \text{O}_2 \xrightarrow{\text{Xanthine oxidase}} \text{Uric acid} + \text{H}_2\text{O}_2 \)

In the absence of catalase, one mole of hypoxanthine requires 2 moles of \( \text{O}_2 \) for oxidation by xanthine oxidase to uric acid.

After incubation the reaction mixture was placed in a test tube. The flask was washed with 3.4 ml. of 0.5 \( \text{M MgCl}_2 - 5.0 \text{M NH}_4 \text{Cl} \) solution, the washings transferred to the test tube and 0.2 ml. of 15 \( \text{N NH}_4 \text{OH} \) added. The test tube was placed in the refrigerator for 4 hours. The 0.5 \( \text{M MgCl}_2 - 5.0 \text{M NH}_4 \text{Cl} \) reagent was added in the proportion of 1.7 times as many millimoles of magnesium as millimoles of inorganic phosphate present in the reaction mixture. The precipitated magnesium ammonium phosphate was removed by centrifugation. The supernate was treated with 0.2 ml. of ammonical barium acetate and allowed to stand in the refrigerator overnight. The ammonical barium acetate (0.3875 \( \text{M barium acetate and 0.44 N NH}_4 \text{OH} \)) solution was added in the proportion of 1.3 times as many micromoles of barium as micromoles of ribose-1-phosphate expected. The precipitate that formed overnight was removed by centrifugation and discarded. The supernate was slowly added to 4 volumes of cold ethanol containing 0.1 volume of concentrated \( \text{NH}_4 \text{OH} \) and placed in the refrigerator for 10 hours. The precipitate was centrifuged and washed with ethanol. The supernate and washings were combined. The precipitate was washed with ether and dried at room temperature. A test for ribose showed its absence. A test with \( \text{Na}_2\text{SO}_4 \) for \( \text{Ba}^{++} \) indicated its absence. The precipitate was
probably water soluble, alcohol insoluble protein. The alcoholic supernate and washings were treated with 0.2 ml. of ammonical barium acetate and evaporated to approximately 5 ml. at room temperature under a stream of air. The precipitate present dissolved upon addition of 3.0 ml. of water. The solution was treated with 0.2 ml. of ammonical barium acetate and allowed to stand overnight in the refrigerator. The precipitate was removed by centrifugation and discarded. Cold ammonical ethanol was added (4 volumes) to the supernate and allowed to stand in the refrigerator for 4 hours. The precipitate was removed by centrifugation, washed with ethanol and with ether, and dried on the filter at room temperature. The yield was 7.6 mg. The theoretical yield of barium ribose-1-phosphate from 17 micromoles of inosine is 6.2 mg.

The orcinol test (Mejbaum, 1939) for pentose content on a solution of the barium salt indicated 50 per cent of the theoretical amount of pentose present. The barium salt was converted to the sodium salt by treatment with Na₂SO₄. The precipitated Ba₂SO₄ was removed by centrifugation. Aliquots of this solution were tested for orthophosphate by the Lowry and Lopez (1946) method before and after hydrolysis in 0.5 N HCl at room temperature for 30 minutes (Table 26). The results of the test for pentose and the presence of an equivalent amount of phosphate that was labile in 0.5 N acid at room temperature indicated that the compound formed was ribose-1-phosphate. The purity was 50 per cent and the small
Table 26. Inorganic phosphate content of ribose-1-phosphate

<table>
<thead>
<tr>
<th>Phosphate before hydrolysis</th>
<th>Phosphate after hydrolysis</th>
<th>Labile phosphate found</th>
<th>Theoretical labile phosphate</th>
<th>Ribose-1-phosphate per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>9.3</td>
<td>8.4</td>
<td>16.6</td>
<td>50</td>
</tr>
</tbody>
</table>

*Phosphate content by method of Lowry and Lopez (1946). Ribose-1-phosphate (Na+), 0.54 micromoles. Hydrolysis with 0.5 N HCl at room temperature for 30 minutes.

Table 27. Reaction of adenine and hypoxanthine with ribose-1-phosphate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial μM</th>
<th>Final μM</th>
<th>Change μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>1.55</td>
<td>1.01</td>
<td>- 0.54</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0.0</td>
<td>0.58</td>
<td>+ 0.58</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>1.52</td>
<td>0.99</td>
<td>- 0.53</td>
</tr>
<tr>
<td>Inosine</td>
<td>0.0</td>
<td>0.59</td>
<td>+ 0.59</td>
</tr>
</tbody>
</table>

*Experimental conditions: pyrophosphate buffer, pH 6.5, 90 micromoles; ribose-1-phosphate (Na+ salt), 0.77 micromoles; adenine or hypoxanthine, as indicated; cell-free extract, 0.8 mg. of protein; total volume, 0.9 ml.; temperature, 37° C.; time, 30 minutes. Compounds determined by chromatographic method.
quantity of the salt obtained prevented further purification.

Reaction of adenine and hypoxanthine with ribose-l-phosphate

When adenine was allowed to react with the ribose-l-phosphate preparation in the presence of cell-free extract, a disappearance of adenine and an equivalent appearance of adenosine was found. The results of this experiment are presented in Table 27. The results were obtained by chromatographic separation of the compounds and quantitative spectrophotometric determination of the eluted spots. The data indicate that the compound formed from inosine and the cell-free extract was ribose-l-phosphate and that the reaction was reversible. The rate of formation of adenosine and inosine from the purine bases and ribose-l-phosphate is shown in Figure 11. The rate of formation of adenosine appears to be somewhat greater than that of the formation of inosine. The difference is small and may be of little significance. The equilibrium constant for the reactions cannot be determined from the data presented. It would appear that the synthesis of adenosine was slightly more favored than that of inosine.

The data indicate that the mechanism of the reaction that forms adenosine and hypoxanthine from adenine and inosine involves a transfer of the ribosyl group from inosine to adenine with ribose-l-phosphate as the intermediate.
Figure 11. Rate of formation of adenosine and inosine from the purine bases and ribose-1-phosphate

Experimental conditions: pyrophosphate buffer, pH 6.5, 90 micromoles; ribose-1-phosphate, 0.77 micromoles; cell-free extract, 0.3 mg. of protein; curve (□) inosine formed from ribose-1-phosphate and hypoxanthine, 1.56 micromoles; curve (○) adenosine formed from ribose-1-phosphate and adenine, 1.52 micromoles; total volume, 0.9 ml.; temperature, 37° C.; time, as indicated. Compounds determined by chromatographic method.
Stoichiometry and equilibrium constants for the phosphorolytic reactions

The data presented in the preceding sections indicate that the following reactions are catalyzed by the cell-free extract.

\[
\text{Adenosine + Orthophosphate } \rightleftharpoons \text{ Adenine + Ribose-1-phosphate} \\
\text{Inosine + Orthophosphate } \rightleftharpoons \text{ Hypoxanthine + Ribose-1-phosphate}
\]

Quantitative data are necessary to show the stoichiometry of the reaction. Equivalent amounts of base are formed from the nucleosides and orthophosphate in the forward reaction and equivalent amounts of nucleosides are formed from the bases and ribose-1-phosphate. These data are presented in Table 28.

As indicated previously, orthophosphate cannot be determined in the presence of pyrophosphate. Changes in orthophosphate could not be determined in this experiment.

Data presented in Tables 21 and 25 indicate that cleavage of the nucleosides occurred in the absence of added orthophosphate. Determination of the amount of orthophosphate in the cell-free extracts by the method of Lowry and Lopez (1946) indicated that 0.16 micromole of orthophosphate was added with 0.8 mg. of the crude extract (as used in Table 21) and 0.02 micromole of orthophosphate with 0.6 mg. of the fractionated extract (as used in Table 25). Sufficient orthophosphate was present in the extract used in the experiments reported in Table 21 to account for the amount of nucleosides split. In
Table 28. Stoichiometry of bases and nucleosides involved in the phosphorolysis reactions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial $\mu$M</th>
<th>Final $\mu$M</th>
<th>Change $\mu$M</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>1.55</td>
<td>1.01</td>
<td>-0.54</td>
<td>Adenine + 0.77 $\mu$M</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0.00</td>
<td>0.58</td>
<td>+0.58</td>
<td>Ribose-1-phosphate</td>
</tr>
<tr>
<td>Inosine</td>
<td>0.00</td>
<td>0.59</td>
<td>+0.59</td>
<td>Hypoxanthine + 0.77 $\mu$M</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>1.52</td>
<td>0.99</td>
<td>-0.53</td>
<td>Ribose-1-phosphate</td>
</tr>
<tr>
<td>Adenine</td>
<td>0.00</td>
<td>0.39</td>
<td>+0.39</td>
<td>Adenosine + 4 $\mu$M</td>
</tr>
<tr>
<td>Adenosine</td>
<td>1.67</td>
<td>1.32</td>
<td>-0.35</td>
<td>Orthophosphate</td>
</tr>
<tr>
<td>Inosine</td>
<td>1.57</td>
<td>1.21</td>
<td>-0.36</td>
<td>Inosine + 4 $\mu$M</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0.00</td>
<td>0.34</td>
<td>+0.34</td>
<td>Orthophosphate</td>
</tr>
</tbody>
</table>

*aExperimental conditions: pyrophosphate buffer, pH 6.5, 90 micromoles; substrates, as indicated; cell-free extract, 0.8 mg. of protein; temperature, 37°C; time, 30 minutes. Compounds determined by chromatographic method.

The experiment reported in Table 25, however, the extract contained only 0.02 micromole of orthophosphate, yet 0.09 micromole of inosine and 0.10 micromole of adenosine were split. The reaction was either hydrolytic, there was another source of orthophosphate in the cell-free extract, or orthophosphate cycled in the phosphorolytic reaction.

The possibility of orthophosphate cycling in the reaction is presented in the following equations:

Nucleoside + Orthophosphate $\rightleftharpoons$ Base + Ribose-1-phosphate and
Ribose-1-phosphate + Water $\rightarrow$ Ribose + Orthophosphate

The enzymatic hydrolysis of ribose-1-phosphate was demon-
Table 29. Enzymatic hydrolysis of ribose-l-phosphate

<table>
<thead>
<tr>
<th>Extract</th>
<th>Initial μM</th>
<th>Final μM</th>
<th>Change μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>0.17</td>
<td>0.29</td>
<td>+0.12</td>
</tr>
<tr>
<td>Fractionated</td>
<td>0.03</td>
<td>0.07</td>
<td>+0.04</td>
</tr>
</tbody>
</table>

\(^a\)Experimental conditions: histidine buffer, pH 6.5, 75 micromoles; ribose-l-phosphate (Na\(^+\) salt), 0.16 micromoles; crude extract, 0.3 mg. of protein; fractionated extract, 0.6 mg. of protein; total volume, 1.0 ml.; temperature, 37\(^\circ\) C.; time, 30 minutes. Deproteinization with 4.0 ml. of acetate buffer, pH 4, saturated with ammonium sulfate.

The hydrolysis of ribose-l-phosphate and the apparent cycling of the orthophosphate released during hydrolysis make it impossible to determine the stoichiometry or equilibrium constants for the phosphorolytic reactions. Enzyme preparations devoid of orthophosphate and of hydrolytic activity on ribose-l-phosphate will be required before the stoichiometry and equilibrium constants of the phosphorolytic reactions in E. coli can be determined.

Elimination of transamination as a reaction mechanism

The phosphorolysis of inosine to form ribose-l-phosphate and the reaction of the latter with adenine to form adenosine can be used to explain the mechanism of the conversion of
adenine and inosine to adenosine and hypoxanthine. The possibility still exists that transamination from adenine to inosine is involved in the reaction. Whether this is the case can be demonstrated with radioactive adenine as one substrate in the reaction.

The two possibilities may be represented by the following equations.

Transamination:

\[ \text{Adenine-}^{14}\text{C} + \text{Inosine} \rightleftharpoons \text{Hypoxanthine-}^{14}\text{C} + \text{Adenosine} \]

Trans-N-ribosidation:

\[ \text{Adenine-}^{14}\text{C} + \text{Inosine} \rightleftharpoons \text{Adenosine-}^{14}\text{C} + \text{Hypoxanthine} \]

The formation of adenosine and hypoxanthine from adenine and inosine was carried out with adenine-\( ^{14}\text{C} \) as one substrate. The results are presented in Table 30. The radioactivity of the compounds isolated by paper chromatography was then determined. All counts are corrected for background. Flasks 1 through 6 served as control flasks for the concentration of substrate at zero time. Acid was added to these flasks before the enzyme preparation. No exchange of radioactivity occurred between adenine and inosine (flask 5) or between adenine and adenosine (flask 6) in the presence of acid inactivated enzyme. The incubated flask containing adenine (flask 8) showed no significant decrease in concentration after incubation with the enzyme. Some decrease in concentration of inosine (flask 9) and adenosine (flask 10) occurred.
Table 30. Formation of radioactive adenosine from adenine-3-C¹⁴ and inosine

<table>
<thead>
<tr>
<th>Flask</th>
<th>Substrate</th>
<th>Time of reaction min.</th>
<th>Compound found</th>
<th>Radioactivity c./min.</th>
<th>Quantity found µM</th>
<th>Change in quantity µM</th>
<th>Specific activity c./min./µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>0</td>
<td>None</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Adenine</td>
<td>0</td>
<td>Adenine</td>
<td>20,500</td>
<td>1.41</td>
<td></td>
<td>14,500</td>
</tr>
<tr>
<td>3</td>
<td>Inosine</td>
<td>0</td>
<td>Inosine</td>
<td>1.49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Adenosine</td>
<td>0</td>
<td>Adenosine</td>
<td>1.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Adenine</td>
<td>0</td>
<td>Adenine</td>
<td>20,960</td>
<td>1.46</td>
<td></td>
<td>14,300</td>
</tr>
<tr>
<td></td>
<td>Inosine</td>
<td>0</td>
<td>Inosine</td>
<td>1.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Adenine</td>
<td>0</td>
<td>Adenine</td>
<td>19,700</td>
<td>1.41</td>
<td></td>
<td>14,000</td>
</tr>
<tr>
<td></td>
<td>Adenosine</td>
<td>0</td>
<td>Adenosine</td>
<td>1.78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>30</td>
<td>None</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Adenine</td>
<td>30</td>
<td>Adenine</td>
<td>19,700</td>
<td>1.39</td>
<td>- 0.02</td>
<td>14,200</td>
</tr>
<tr>
<td>9</td>
<td>Inosine</td>
<td>30</td>
<td>Inosine</td>
<td>1.32</td>
<td></td>
<td>- 0.17</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Adenosine</td>
<td>30</td>
<td>Adenosine</td>
<td>1.62</td>
<td></td>
<td>- 0.18</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Adenine</td>
<td>30</td>
<td>Adenine</td>
<td>11,780</td>
<td>0.82</td>
<td>- 0.64</td>
<td>14,350</td>
</tr>
<tr>
<td></td>
<td>Adenosine</td>
<td>30</td>
<td>Adenosine</td>
<td>9,120</td>
<td>0.66</td>
<td>+ 0.66</td>
<td>13,800</td>
</tr>
<tr>
<td></td>
<td>Inosine</td>
<td>30</td>
<td>Hypoxanthine</td>
<td>0</td>
<td>0.58</td>
<td>+ 0.58</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Adenine</td>
<td>30</td>
<td>Adenine</td>
<td>11,200</td>
<td>1.45</td>
<td>+ 0.04</td>
<td>7,750</td>
</tr>
<tr>
<td></td>
<td>Adenosine</td>
<td>30</td>
<td>Adenosine</td>
<td>8,980</td>
<td>1.72</td>
<td>- 0.06</td>
<td>5,210</td>
</tr>
</tbody>
</table>

*Experimental conditions: pyrophosphate buffer, pH 6.5, 90 micromoles; substrates, as indicated; cell-free extract, 0.8 mg of protein; total volume, 0.9 ml.; temperature, 37°C; time, 30 minutes. Compounds determined by chromatographic method.

*bAll counts corrected for background.
The flask that contained adenine-3-C\textsubscript{14} and inosine as substrate (flask 11) showed the formation of adenosine and hypoxanthine. Adenosine and adenine were the only radioactive compounds, with adenosine having essentially the same specific activity (13,800 counts/min./micromole) as the adenine (14,300 counts/min./micromole). The sum of the radioactivity found in the two compounds, 20,900 counts/min., corresponds quite well with the activity in the corresponding flask at zero time, i.e., flask 5 with 20,960 counts/min. This complete recovery of radioactivity indicates that there were no other radioactive compounds formed from adenine. Comparison of the results of flask 11 with flask 5 shows that there is essentially a 1:1 ratio of reactants disappearing to products formed, i.e., the stoichiometry of the reaction is not affected by the use of adenine-3-C\textsubscript{14}.

An exchange of radioactivity occurred between adenine and adenosine in the presence of enzyme (flask 12). Essentially no change in concentration occurred during the incubation. Radioactive equilibrium was not reached in 30 minutes. At equilibrium the specific activity of each should be one-half of the specific activity of the original adenine or approximately 7,000 counts/min./micromole.

The formation of radioactive adenosine from adenine-3-C\textsubscript{14} and inosine is shown by the radioautogram reproduced in Figure 12. With adenine alone, no other radioactive spot was formed. In the presence of inosine and enzyme adenine-
Figure 12. Radioautogram of the formation of adenosine and hypoxanthine from adenine-$\text{S-C}^{14}$ and inosine.

Experimental conditions: same as given in Table 30. Spots shown in outline were those visible under the Mineralight. Film was exposed to the chromatogram for 3 days.
SOLVENT FRONT $R_F 1.0$

INOSINE $R_F 0.69$

HYPOXANTHINE $R_F 0.60$

ADENOSINE $R_F 0.46$

ADENINE $R_F 0.33$

ORIGIN $R_F 0.0$

FLASK
$\text{C}^{14}$ formed radioactive adenosine. The spots corresponding to hypoxanthine and inosine were devoid of activity. The exchange of radioactivity is also shown on the radioautogram.
DISCUSSION

The deamination of adenosine by resting cell suspensions of *Escherichia coli* was demonstrated by Lutwak-Mann (1936), Stephenson and Trim (1938), Marmur (1951), and Freedman and Gots (1952). Gale (1938) demonstrated that cell-free preparations of *E. coli* also deaminated adenosine.

The presence of an adenosine deaminase in cell-free extracts of *E. coli* has been confirmed by Ott and Werkman (1951). The optimum activity occurred at pH 8. Stephenson and Trim (1938) found the optimum activity at pH 7.75 and Lutwak-Mann (1936) reported pH 8.8 as the optimum for adenosine deaminase. The differences in the pH optima are not of great magnitude. They may reflect strain differences only. In all cases, the optimum was on the alkaline side of neutrality.

Lutwak-Mann (1936) found that adenosine deaminase was activated by phosphate ions. This could not be confirmed with the cell-free preparation. Phosphate had no appreciable effect on the deamination of adenosine. The reason for this difference is not known.

Deamination occurred under both aerobic and anaerobic conditions. No gaseous products were formed during the deamination of adenosine. Lutwak-Mann (1936) and Stephenson and Trim (1933) found that ribose was fermented by the cell suspensions with the formation of gaseous products. Combined ribose in the form of nucleosides was fermented at a greater
rate than free ribose. The cell-free extract (Ott and Werkman, 1954) apparently lacks the enzymes required for ribose fermentation, either as free ribose or in the form of nucleosides. The inability to ferment ribose may be due to the absence of the enzyme system in the strain of E. coli or to destruction of the enzyme system during the preparation of the cell-free extract. These possibilities were not investigated.

The formation of ammonia from adenine by cell suspensions of E. coli was demonstrated by Lutwak-Mann (1936), Stephenson and Trim (1938), Chargaff and Kream (1943, 1952), Sutton (1951), Freedman and Gots (1952) and Rashba and Krochko (1954). The deamination of adenine by a cell-free preparation has not been reported. Lutwak-Mann (1936) and Stephenson and Trim (1938) studied the rate of deamination of adenine as compared with the rate of deamination of adenosine. Both reported that adenosine deaminase was considerably more active than adenine deaminase. Stephenson and Trim (1933) found that the deamination of adenine was stimulated by the addition of catalytic amounts of adenosine or inosine.

The formation of ammonia from adenine was catalyzed by cell-free extracts of E. coli (Ott and Werkman, 1954). The addition of inosine increased the quantity of ammonia formed from adenine. When the reaction was conducted at pH 7.0, the stimulatory effect of inosine was quite marked. This finding tended to confirm the proposal of Stephenson and Trim
(1938) that the formation of ammonia from adenine in the presence of inosine is due to the formation of adenosine and the action of adenosine deaminase. The proposed equations were:

(1) Adenine + Inosine $\rightleftharpoons$ Adenosine + Hypoxanthine and
(2) Adenosine + Water $\rightarrow$ Inosine + Ammonia

The validity of equation (1) was supported by the demonstration of the formation of adenosine from adenine and inosine (Ott and Werkman, 1954). The formation of adenosine was determined by use of an adenosine deaminase prepared from calf intestinal mucosa (Brady, 1942). The mammalian adenosine deaminase was specific for adenosine and adenine deoxyriboside (Brady, 1942) and was inactive on adenine. The optimum activity for reaction (1) was found to be at pH 6.5. The adenosine deaminase, also present in the cell-free extract, had no appreciable activity at this pH. The activity of the adenosine synthesizing enzyme system was considerably greater than the activity of the adenosine deaminase. These two factors made it possible to study reaction (1) in the absence of reaction (2) by conducting the experiments at pH 6.5 and by using small quantities of cell-free extract.

The question of whether an adenine deaminase exists in the cell-free preparation cannot be definitely answered. At pH 7 or pH 8 ammonia was formed from adenine in the absence of added inosine. The presence of catalytic amounts of inosine in the cell-free preparation could account for reaction
(1) and reaction (2). In this case, adenine would react with inosine. The actual deamination step would involve adenosine rather than adenine. Although reaction (1) and reaction (2) could account for the ammonia formed from adenine, the possibility exists that a direct hydrolytic deamination of adenine also takes place. The isolation of an enzyme system that does not catalyze reaction (1) but does form ammonia from adenine would be required to show that adenine deaminase is present in \textit{E. coli}. No attempt has been made to isolate such an enzyme system.

Dialysis of the cell-free extract and preliminary fractionation with ammonium sulfate indicated that there was no readily dissociable organic co-factor required for reaction (1). Orthophosphate was required for maximum activity (Ott and Werkman, 1955). The stimulatory effect of orthophosphate indicated the possible action of a nucleoside phosphorylase in reaction (1).

Purine nucleosidase activity was first described by Levene and Medigreceanu (1911 a, b), Klein (1935) and Dische (1938) demonstrated an activation of nucleosidase by phosphate ions. The role of phosphate in nucleosidase activity was demonstrated by Kalckar (1945 b, 1947 a, b, c, d). The action was shown to be phosphorolytic in nature. The following reactions were demonstrated.

\[
\text{Inosine} + \text{Orthophosphate} \rightleftharpoons \text{Hypoxanthine} + \text{Ribose-1-phosphate}
\]

\[
\text{Guanosine} + \text{Orthophosphate} \rightleftharpoons \text{Guanine} + \text{Ribose-1-phosphate}
\]
Ribose-1-phosphate was isolated and characterized. Kalckar (1947 d) also demonstrated the synthesis of nucleosides from hypoxanthine and guanine and ribose-1-phosphate. The experiments reported by Kalckar were carried out with a nucleoside phosphorylase prepared from rat liver. Nucleoside phosphorylase preparations catalyzed similar reactions with the deoxyribosides (Friedkin and Kalckar, 1950; Friedkin, 1950). Nucleoside phosphorylase activity was demonstrated in E. coli by Paege and Schlenk (1950), Manson and Lampen (1950, 1951 b), Lampen (1952) and Hoffmann (1952) and in Bacterium cadaveris by Williams and McIntyre (1955).

The cell-free extract from E. coli (Ott and Werkman, 1955) catalyzed the cleavage of adenosine and inosine. The rate was increased by the addition of orthophosphate. The isolation of ribose-1-phosphate from the action of the extract on inosine in the presence of large quantities of orthophosphate is further evidence of the nucleoside phosphorylase activity. Reaction (1) could be the result of nucleoside phosphorylase acting in a coupled reaction. These reactions are indicated by the following equations.

\[(3) \text{Inosine} + \text{Orthophosphate} \rightarrow \text{Hypoxanthine} + \text{Ribose-1-phosphate}\]

\[(4) \text{Ribose-1-phosphate} + \text{Adenine} \rightarrow \text{Adenosine} + \text{Orthophosphate}\]

Coupled nucleoside phosphorylase reactions with ribose-1-phosphate as the intermediate is proposed as the mechanism for the formation of adenosine and hypoxanthine from adenine and
inosine. The similar rates of reaction (4) and the reverse of reaction (3) with the indication that reaction (4) may proceed at a slightly greater rate than the reverse of reaction (3) supports the proposed mechanism.

The mechanism indicates that orthophosphate is required in only catalytic amounts. It would cycle during the complete reaction. The stimulatory effect of increased orthophosphate would result from forcing reaction (3) to the right. The apparent equilibrium for reactions (3) and (4) is toward synthesis of the nucleosides. Increasing orthophosphate concentration would tend to decrease adenosine formation in reaction (4), but at the same time increase the ribose-1-phosphate concentration from reaction (3). Reaction (1) would not occur unless ribose-1-phosphate was formed from inosine. From the data obtained it would appear that orthophosphate has more relative effect in shifting reaction (3) to the right than in shifting reaction (4) to the left.

The cell-free extract contained an enzyme system that hydrolyzed ribose-1-phosphate. The rate of hydrolysis was not great. The stoichiometry of reaction (1) in which essentially one mole of adenosine and hypoxanthine was formed for each mole of adenine and inosine that disappeared, adds further evidence that the hydrolysis of ribose-1-phosphate precedes at a fairly slow rate. Reaction (4) would appear to proceed at a rate sufficient to remove ribose-1-phosphate as it is formed and before hydrolysis can take place. The ina-
bility of free ribose to replace ribose-1-phosphate in reaction (1) indicates that hydrolysis of ribose-1-phosphate decreases the formation of adenosine. Appreciable hydrolysis of ribose-1-phosphate should result in the disappearance of more inosine than could be accounted for by adenine transformed into adenosine. The stoichiometry of the reaction leads to the conclusion that hydrolysis of ribose-1-phosphate does not occur to any great extent when both adenine and inosine are present.

The inhibitory action of certain divalent cations (Ott and Werkman, 1955) is explained by the proposed mechanism. The removal of orthophosphate by the formation of precipitates with the cations, decreases the formation of ribose-1-phosphate and as a consequence decreases the amount of adenosine formed. The divalent cations have no effect on the stoichiometry of the reaction. They do not, therefore, act as co-factors for side reactions that might effect reaction (1) by removing either reactants or products.

A direct transfer of the deoxyribosyl group from one purine or pyrimidine base to another without the involvement of deoxyribose-1-phosphate was demonstrated in _L. helveticus_ by MacNutt (1952) and in _E. coli_ by Hoffmann (1952). The analogous reactions with the ribosyl group of ribose nucleosides could not be demonstrated due to nucleoside phosphorylase activity and adenosine deaminase action (Kalckar, _et al._, 1952). The possibility of a direct transfer of the ribosyl
group from inosine to adenine or from adenosine to hypoxanthine exists as a mechanism for reaction (1). Support for this mechanism is found in the ability of a fractionated and dialyzed cell-free extract to catalyze reaction (1) in the absence of added orthophosphate. This extract, however, contained small quantities of orthophosphate. As indicated previously, only catalytic amounts of orthophosphate are required for the coupled nucleoside phosphorylase reactions. The fractionated and dialyzed extract split adenosine and inosine and hydrolyzed ribose-1-phosphate at a slow rate. Although the kinetics of these reactions could not be determined because of side reactions, that such reactions are catalyzed by the extract indicates reactions (3) and (4) can occur and account for reaction (1). These data do not eliminate the presence of a trans-N-ribosidase in the cell-free extract. The demonstration of such activity would require the preparation of extracts that catalyze reaction (1) and are not affected by orthophosphate or the presence of ribose-1-phosphate. No attempts have been made to prepare such extracts.

Stephenson and Trim (1938) proposed reaction (1) to account for the effect of inosine on the deamination of adenine. They cautiously postulated transamination as a mechanism for the reaction. No attempt was made to substantiate the hypothesis. The trans-N-glycosidase action of \textit{L. helveticus} on hypoxanthine deoxyriboside and adenine as
described by MacNutt (1952) could result from a transamination rather than from trans-$N$-glycosidation by a mechanism analogous to the one proposed by Stephenson and Trim (1938). Kalckar, et al. (1952) using adenine-$8-C^{14}$ demonstrated the reaction with *L. helveticus* involved trans-$N$-glycosidase action and was not a transamination reaction.

The analogous reaction with the ribosyl compounds was catalyzed by the cell-free extracts of *E. coli* (Ott and Werkman, 1954). Although the coupled nucleoside phosphorylase reactions demonstrated in the extracts accounts for reaction (1), the possibility exists that transamination also takes place. The results of carrying out reaction (1) with adenine-$8-C^{14}$ as one substrate showed the formation of radioactive adenosine. If transamination had occurred, hypoxanthine would have been radioactive. The hypoxanthine isolated from the reaction was devoid of radioactivity. The specific activity of the adenosine was essentially the same as that of adenine. The undiluted radioactivity of the adenosine indicates that it was formed only from adenine-$8-C^{14}$ and that there were few, if any, side reactions. The exchange of radioactivity between adenine and adenosine in the absence of inosine gives further evidence on the catalysis of reaction (3) by the cell-free extract. These results supplement the finding of Kalckar, et al. (1952) that transamination does not play a role in the transfer of the deoxyribosyl group in *L. helveticus*. The results with the *E. coli* extract
do not distinguish between a direct transfer of the ribosyl group from inosine to adenine and a transfer involving ribose-1-phosphate as the intermediate. The results eliminate transamination as a mechanism for the reaction.

The role of the coupled nucleoside phosphorylase reaction in nucleic acid metabolism in *E. coli* is not known. Studies with isolated enzymes have indicated that the ribosyl (or deoxyribosyl) group and possible phosphate are joined to other precursors before ring closure to form the purine nucleosides or nucleotides (Greenberg, 1953). In most cases inosinic acid appeared to be the key intermediate. The amino purine compounds, adenosine and guanosine, that are present in nucleic acids have not been synthesized in these reactions.

Gunsalus (1952) and Gunsalus and Tonzetich (1952) demonstrated a transamination between glutamate and hypoxanthine and between glutamate and xanthine in the presence of pyridoxal phosphate and extracts of *E. coli*. The transamination did not occur with nucleosides or nucleotides (Gunsalus, 1952).

A possible role of the coupled nucleoside phosphorylase reactions in nucleic acid synthesis can be postulated as shown in Figure 13. The reactions indicated in the figure from the purine precursors, glycine, CO₂ and ammonia, to the formation of hypoxanthine and inosine are those given by Reichard (1955). Many of the reactions shown in one step must involve several steps in the actual synthesis. The re-
Figure 13. Proposed scheme of nucleic acid synthesis
actions are a composite picture from studies with enzymes from several sources, mainly liver and yeast. Few of these steps have been demonstrated with enzymes from E. coli. Many comparable reactions in nucleic acid metabolism that have been studied in both tissues and microorganisms have been shown to be similar. Thus an analogy can probably be drawn between the reactions shown in the upper part of Figure 13 and the reactions that occur in E. coli. The proposed formation of adenine from hypoxanthine is based on the results of the transamination experiments carried out by Gunsalus (1952) with extracts of E. coli. Adenosine could then be formed from adenine and inosine by the coupled nucleoside phosphorylase reactions described in this investigation. The steps involved in the incorporation of adenosine into nucleic acids are not known. The proposed scheme accounts for the formation of adenine and adenosine from precursors by means of known reactions. The transamination from glutamate to hypoxanthine is the key step in the formation of the amino purines found in nucleic acid. The transfer of the ribosyl group from inosine to adenine through the coupled nucleoside phosphorylase reaction could be another key step in the synthesis of the nucleosides as precursors for nucleic acid.
SUMMARY AND CONCLUSIONS

1. Cell-free extracts from *Escherichia coli* deaminate adenosine. Optimum activity occurs at pH 8.0. Orthophosphate has no effect on deamination.

2. The same extracts catalyze the formation of adenosine and hypoxanthine from adenine and inosine. Optimum activity occurs at pH 6.5. At this pH adenosine deaminase shows only slight activity. The enzyme system catalyzing the formation of adenosine is considerably more active than the adenosine deaminase. By conducting the reactions at pH 6.5 in the presence of small amounts of extract, the reaction involving the formation of adenosine and hypoxanthine from adenine and inosine can be studied in the absence of deamination of adenosine.

3. The formation of ammonia from adenine can be accounted for by the formation of adenosine from adenine and inosine and subsequent deamination by adenosine deaminase.

4. The formation of adenosine and hypoxanthine from adenine and inosine is stimulated by orthophosphate. Certain divalent cations inhibit the reaction. This inhibition can be overcome by orthophosphate.

5. The cell-free extracts catalyze the conversion of adenine and inosine to equimolar quantities of adenosine and hypoxanthine. The reaction is reversible. The equilibrium constant and kinetics of the reaction have been determined.
6. In the presence of orthophosphate the cell-free extracts catalyze the formation of ribose-1-phosphate from inosine. The ribose-1-phosphate was isolated and characterized. Adenosine and inosine are formed in the presence of cell-free extract, ribose-1-phosphate, and the corresponding purine bases. The stoichiometry, equilibrium constants, and kinetics for the nucleoside phosphorylase reactions could not be determined because of complicating side reactions.

7. Coupled nucleoside phosphorylase reactions with ribose-1-phosphate as the intermediate is proposed as the mechanism for the conversion of adenine and inosine to adenosine and hypoxanthine. The possibility of trans-N-ribosidation as a reaction mechanism is discussed.

8. When the reaction is carried out with adenine-8-C\textsuperscript{14} as one substrate, only radioactive adenosine is formed. Hypoxanthine is devoid of activity. This further confirms the proposed mechanism of reaction and eliminates transamination as a mechanism for the reaction.

9. A role for the coupled nucleoside phosphorylase reactions in nucleic acid synthesis is proposed.
Abrams, A. and Klenow, H.


Bendich, A., Brown, G. B., Philips, F. S. and Thiersch, J. B.

Block, R. J., Durrum, E. L. and Zweig, G.

Borsook, H. and Dubnoff, J. W.

and 1939 b. A specific enzyme deaminizing free and combined adenylic acid. Enzymologia 7, 256.

Brady, T.

Carter, C. E.
1951. Partial purification of a non-phosphorylytic uridine nucleosidase from yeast. J. Am. Chem. Soc. 73, 1508-1510.

Chargaff, E. and Davidson, J. N., editors.

and Kream, J.
1943. Procedure for the study of certain enzymes in minute amounts and its application to the investigation of cytosine deaminase. J. Biol. Chem. 175, 993-994.
Conway, E. J. and Cooke, R.

Deutsch, W. and Laser, R.

Dische, Z.

Dixon, M. and Lemberg, R.

and Thurlow, S.

Fink, R. M. and Fink, K.

Fiske, C. H. and Subbarow, Y.

Florkin, M. and Frappez, C.

Friedkin, M.

Friedkin, M.
1952 b. Enzymatic synthesis of several uncommon ribosides and deoxyribosides. Federation Proc. 11, 216.


and Kalckar, H. M.

and Hoff-Jørgensen, E.

and Roberts, D.

and

and

Friedman, S. and Gots, J. S.

Cale, E. F.
Greenberg, C. R.

Gunsalus, C. F.

and Tonzetich, J.

Gyorgy, P. and Rother, H.

Heppel, L. A.

Hoff-Jorgensen, E., Friedkin, M. and Kalckar, H. M.

Hoffmann, C. E.

and Lampen, J. O.
Johnson, E. A.

Jones, W.
1911. On the physiological agents which are concerned in the nuclein fermentation, with special reference to four independent desamidases. J. Biol. Chem. 2, 169-180.


Kalckar, H. M.


Kalckar, H. M. and Klenow, H.  
Biochemistry and metabolism of phosphorus  

MacNutt, W. S. and Hoff-Jørgensen, E.  
1952.  Trans-N-glycosidase studied with radioactive  

and Rittenberg, D.  
1947.  Rejuvenation of muscle adenylic acid nitrogen  
in vivo studied with isotopic nitrogen.  J. Biol.  
Chem. 170, 455-459.

Kalnitsky, G., Utter, M. F. and Werkman, C. H.  
1945.  Active enzyme preparations from bacteria.  

Kaplan, N. O., Colowick, S. P. and Ciotti, M. M.  
J. Biol. Chem. 194, 579-591.

Kerr, S. E. and Seraidarian, K.  
1945.  The separation of purine nucleotides from free  
purines and the determination of the purines  
and ribose in these fractions.  J. Biol. Chem.  
152, 211-225.

and Wargon, M.  
1949.  Studies on ribonucleic acid. II. Methods of  

Klein, W.  
XXXVII. Über Nucleosidase.  Hoppe-Seyler's Z.  
physiol. Chem. 231, 125-149.

Klenow, H.  
1952.  The enzymic oxidation and assay of adenine.  

Korn, E. D., Charalampous, F. C. and Buchanan, J. M.  
1953.  Enzymatic synthesis of 4-amino-5-imidazolecar-  
boxamide riboside from 4-amino-5-imidazolecar-  
boxamide and riboside-1-phosphate.  J. Am. Chem.  
Soc. 75, 3610-3611.

Kream, J. and Chargaff, E.  
1952.  Procedures for the study of purine and pyrimidine  
74, 4274-4277.
Kritskiy, G. A.


and Malik-Sarkisyan, S. S.

Lampen, J. O.

and Wang, T. P.

Levenbook, L.

Levene, P. A. and Bass, L. W.

and Medigreceanu, F.

and

and
Lineweaver, H. and Burk, D.

Loring, H. S., Fairley, J. L., Portner, H. W. and Seagran, H. L.

and Ploeser, J. M.

Lowry, O. H. and Lopez, J. A.

Lutwak-Mann, C.

McElroy, W. D. and Mitchell, H. K.

MacNutt, W. S.

Manson, L. A. and Lampen, J. O.


Mardashev, S. R. and Pavlova, N. A.

Marmur, J.

Nejbaum, W.

Mitchell, H. K. and McElroy, W. D.

Nicolaier, A.

Ott, J. L. and Werkman, C. H.

and


Paege, L. M.

and Schlenk, F.
Rashba, O. Ya. and Krochko, L. S.  

Reichard, P.  

Richert, D. A. and Westerfeld, W. W.  

Rowen, J. W. and Kornberg, A.  

Schaedel, M. L., Waldvogel, M. J. and Schlenk, F.  

Schmidt, G.  


Stephenson, M. and Trim, A. R.  

Sutton, W. B.  

Tsukada, Y. and Takafulu, G.  
Tsukada, Y. and Takaguki, G.  

Visher, E. and Chargaff, E.  

Wang, T. P.  

and Lampen, J. O.  

Weichselbaum, T. E.  

Weil-Malherbe, H.  

and Green, R. H.  

Williams, V. R. and McIntyre, R. T.  

Wyatt, G. R.  

Zittle, C. A.  
ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. C. H. Werkman for his guidance, constructive criticism, and inspiration and for the facilities made available to him for advanced study.

Grateful acknowledgment is made to Mitchell Korzenovsky, Dan Hug, Charles Claridge, George Nelson, and Robert Brennan, fellow graduate students, for aid in technical matters and for countless hours of discussion.

The author owes special gratitude to his wife for her patience and understanding during the course of this investigation.

The author wishes to thank the Atomic Energy Commission and the Agricultural Experiment Station for financial support of this work.