Purine metabolism of heterotrophic bacteria

William B. Sutton

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PURINE METABOLISM OF HETEROTROPHIC BACTERIA

by

William B. Sutton

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
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INTRODUCTION

Comparatively little is known concerning the mechanism of synthesis or degradation of bacterial purines. Nevertheless, it has been well established that certain of the heterotrophic bacteria are not dependent upon exogenous purines for the formation of nucleic acids.

The attention of the bacterial physiologist has been limited to determining the presence of the various purine bases in bacterial nucleic acids, and to the study of the purine base requirements of those organisms unable to synthesize these compounds.

Important results have been recently achieved in the study of animal purine metabolism by the application of isotopic techniques. These studies have revealed in part the nature of certain of the intermediary compounds participating in the formation of purine bases in the animal body. In addition, these studies will tend to establish a basis for future investigation of the enzymes bringing about the synthesis of purine bases.

The purpose of this investigation was to obtain information concerning the synthesis and degradation of purine bases by heterotrophic bacteria, and to correlate the purine metabolism of these organisms with that found in animal tissue.
REVIEW OF LITERATURE

Purine Bases of Bacterial Nucleic Acids

The extensive literature in the field of nucleic acid chemistry contains many references to the presence of nucleic acids in bacterial cells. In a few cases the nature of the more complex constituents of these acids has been determined and appears to be similar to that found in plant and animal cells.

Chemical studies have indicated the presence of the various known purine and pyrimidine bases in bacterial nucleic acid material. Cultural studies have helped to establish the purine and pyrimidine requirements of those bacteria unable to synthesize these compounds. The problem of the synthesis of purine and pyrimidine bases by heterotrophic bacteria has been almost completely neglected. In addition, the degradation of these bases by bacterial cells has received almost no attention in recent years.

Purine chemistry began with the important discovery of uric acid by Scheele in 1776. This was followed considerably later by the isolation of guanine by Unger in 1846. Meischer (1871) is credited with the discovery of nucleic acids and Kossel (1885) with the isolation of adenine. One of the first
investigators to report the presence of adenine, guanine, xanthine, and hypoxanthine in bacteria was Nishimura in 1893.

The bacterial cell is characterized by having a very high percentage of both protein and nucleic acid material. It is of interest to note that the quantity of nucleic acid varies with the age of the bacterial cell, with younger organisms containing the highest percentage of nucleic acid material. In this connection, Belozersky (1947, p.1) wrote,

... one may suppose that the accumulation of great quantities of these important substances is one of the factors responsible for the exceptional vitality of the bacterial cell, which finds expression in very intensive metabolism, rapidity of reproduction, and remarkable adaptability to the environment...

In 1900 Aronson demonstrated the presence of xanthine in nucleoprotein material extracted from diphtheria bacilli. Levene (1904) first demonstrated the presence of thymine and uracil in bacterial nucleic acids obtained from Mycobacterium tuberculosis. The most extensive early work in this field was that by Long (1931), using the organism M. tuberculosis. This worker was able to isolate adenine and guanine from the nucleic acid material, but did not find xanthine or hypoxanthine. The organisms were cultured in a simple glycerol ammonium sulfate medium and the nucleic acid content ranged from 2.0 to 3.5 per cent on a dry weight basis. Further work by Johnson and Brown (1922) established the presence of cytosine and thymine but not uracil in this same organism.
Thus in the very early studies of bacterial nucleic acids a conflict over the presence or absence of the pyrimidine, uracil, in the nucleic acid material of *M. tuberculosis* was in the making. Such differences as this appear frequently in the literature and can, in most cases, be attributed to the different methods used in extraction and hydrolysis of the nucleic acid material.

The presence of nucleic acid in *Escherichia coli* was first demonstrated by Schaffer, Folkoff, and Bayne-Jones (1922). These workers were able to isolate guanine from extracted nucleic acid. Their failure to detect the presence of pentose using the orcinol test is questionable in light of more recent studies involving this organism.

Lancefield (1938) found a nucleoprotein in *Streptococcus pyogenes* and demonstrated that it was of the ribose type. Sevag, Smolens, and Lackman (1940) more recently found that in this organism 10 to 30 per cent of the nucleic acid material was of the desoxyribose type, the remainder being of the ribose type.

Boivin and Mesrobeanu (1934) made extensive studies of the purine bases present in *Proteus vulgaris, E. coli*, and other organisms. They found that adenine constituted 70 per cent of the purine nucleoside present in *E. coli* and 63 per cent in *P. vulgaris*. Guthrie (1949) isolated a mutant strain of *E. coli* which required preformed purines for growth.
(adenine, guanine, xanthine, and hypoxanthine). The growth of this mutant was proportional to the concentration of the purine base added to the basal medium.

More recent studies by Akasi (1938-1939), Boivin (1942), Boivin and Vendrely (1943-1946), Vendrely and Lehault (1946) have demonstrated that both the ribose and deoxyribose types of nucleic acids are present in bacteria, but no one type is common to all bacteria.

Bacterial Purine Degradation

In man the final product of purine degradation is known to be uric acid. In the rat and certain other mammals, allantoin, which is an oxidation product of uric acid, has been found to be the final product. One of the first reported studies on bacterial degradation of a purine compound was that by Liebert (1908). This study of the degradation of uric acid, using an aerobic bacterium, showed the final products to be carbon dioxide and ammonia. The intermediary products were allantoin, urea, and oxalic acid. The anaerobe, Bacillus acidi (Clostridium acidi-urici), Liebert found, formed carbon dioxide, ammonia, and acetic acid as the final products.

Morris and Ecker (1924) studied the disappearance of ingested uric acid and found that this base was utilized by intestinal organisms. In addition, in vitro studies were carried out, using a mixed bacterial flora, and it was found
that utilization of uric acid occurred. Koser (1918) and Mitchell and Levene (1938), using this fact, differentiated *Aerobacter aerogenes* from *E. coli* on the basis of their metabolic action on uric acid. Most strains of *A. aerogenes* can utilize uric acid, whereas, those of *E. coli* cannot. Hanzal and Ecker (1931) found that the anaerobic organism *Clostridium acidi-urici* metabolized 30 per cent of added uric acid in twelve hours. *A. aerogenes* was able to completely metabolize added uric acid within 48 hours at 37°C. but was unable to attack various substituted forms of uric acid.

The degradation of various purines by *Cl. acidi-urici* and *Clostridium cylindrosporum* has been studied by Barker and Beck (1941-1942). These investigators have found that these uric acid-fermenting anaerobic bacteria metabolize no other organic compounds but the purine derivatives with the exception of glycine. Uric acid, xanthine, and guanine were broken down rapidly and completely by cell suspensions of these organisms. Hypoxanthine and adenine were incompletely metabolized. The final products, as the result of the degradation of the purines by *Cl. acidi-urici*, were the same as those reported by Liebert, whereas *Cl. cylindrosporum* formed, in addition, small amounts of glycine. Both organisms metabolized glycine when a fermentable purine was available. Barker and Beck suggested that the glycine is apparently an intermediate in either purine degradation or functions as a hydrogen donor.
in carbon dioxide reduction. It appears, according to Barker and Beck, that the enzyme systems and intermediary compounds known to be involved in purine metabolism of animal and plant tissue, and aerobic microorganisms are evidently not involved in the degradation process of these anaerobic bacteria. Although the detailed mechanism of these processes is obscure, Barker and Beck believed that they represent oxidations in which carbon dioxide acts as the ultimate hydrogen acceptor and is reduced to acetic acid. Glucose would not serve as an energy source for these organisms.

Barker and Elden (1947) also studied the degradation of purines by *Gl. cylindrosporus* using $^{14}O_2$. The isotope was found in both the glycine and the acetic acid formed by this organism. By studying the distribution of the isotope, they concluded that these compounds are formed by different reactions and are not interconvertible. The methyl carbon of the acetic acid and the carboxyl carbon of the glycine may be derived from carbon dioxide.

Lutwak-Mann (1936) carried out a study to determine the extent of bacterial action on adenine and adenine derivatives. Using a twenty-hour tryptic broth agar growth, Lutwak-Mann was able to demonstrate the deamination (55 per cent) of adenine by *E. coli*. The cell suspensions (30-160 mg wet weight) were incubated in the presence of 0.085-0.120 mg of adenine at pH 7. The deamination of adenine occurred under either aerobic or
anaerobic conditions. Aliquots were withdrawn from the tubes at various times and treated with 20 per cent trichloroacetic acid solution. The extent of the deamination was determined by measuring the amount of ammonia present in the aliquot.

The final product of the deamination of adenine by *E. coli* was identified as hypoxanthine by the preparation of the silver-picrotate derivative and biologically by the use of xanthine oxidase.

Stephenson and Trim (1938), preliminary to their study of the mode of action of adenine compounds as coenzymes in bacterial metabolism, studied the changes sustained by these adenine compounds in the presence of the various enzymes of *E. coli*. The experimental procedures used were similar to those of Lutwak-Mann. As expected, their results were much the same as Lutwak-Mann's, except that they found the rate of deamination of adenine in M/3 phosphate buffer was very slow. This rate was increased two to six fold in the presence of adenosine or inosine. Stephenson and Trim proposed that the amino group of adenine is transferred to inosine which is then rapidly deaminated. The final product of the deamination of adenine was again found to be hypoxanthine.

Chargaff and Kream (1948) have also reported that *E. coli* can deaminate adenine to form hypoxanthine. Balis and Brown (1951) have demonstrated that *Lactobacillus casei* was able to transform guanine to adenine. The interconversion of these
two purines proceeded in the presence of adequate supplies of each base. Balis and Brown found that after growth of the organism in a medium containing guanine-8-C$_{14}$, both the adenine and guanine isolated from the pentose nucleic acid contained the same activity as the guanine placed in the medium. Kerr, Seraidarian, and Brown (1951) reported that Torulopsis utilis cannot accomplish this transformation. The mechanism of the interconversion of adenine into guanine was demonstrated earlier in the rat by Brown, et al., (1948).

Biosynthesis of Purine Bases

4(5)-Amino-5(4)-imidazolecarboxamide

Studies on the role of p-aminobenzoic acid and pteroyl-glutamic acid in the synthesis and degradation of purine bases have not revealed the exact nature of their activity in these mechanisms. The isolation of possible intermediates in purine synthesis has been limited to the work of Fox (1942) and Stetten and Fox (1945). These workers observed an accumulation in a sulfonamide-containing medium of a non-acetylatable diazotizable amine. Stetten and Fox cultured E. coli on a synthetic medium containing amino acids, glucose, and inorganic salts in the presence of bacteriostatic concentrations of sulfonamides. They found that the production of the amine was not limited to E. coli nor to the presence of any specific
sulfonamide. The amount of the amine produced was not proportional to the concentration of the sulfonamide used, but the quantity formed was of the same order of magnitude when bacteriostatic concentrations of different sulfonamides were used. The amount of the amine formed was greatest in the presence of sulfadiazine. Para-aminobenzoic acid, in concentrations sufficient to block the action of the sulfonamides, prevented the formation of the amine.

Stetten and Fox indicated that the amine was a heterocyclic orthodiamine \((C_4H_8N_4O)\) and its properties were probably those of 2-hydroxy-5,6-diaminopyrazine. Shive, et al., (1947) compared the biologically synthesized amine with various compounds and found the amine to be 4(5)-amino-5(4)-imidazole-carboxamide. This compound has the following structural formula:

\[
\begin{align*}
&\text{H}_2\text{N}-\text{C}=\text{O} \\
&\text{C}-\text{N}=\text{H} \\
&\text{H}_2\text{N}-\text{C}-\text{N} \\
\end{align*}
\]

This compound was first synthesized by Windaus and Langenbeck in 1923.

The extensive inhibition analysis studies of Shive and Roberts (1946) and Rogers and Shive (1948) indicated that purines tend to reverse the inhibition of sulfonamides and methyllumic acid of certain bacteria. Shive and Roberts
believed that the sulfonamides prevent p-aminobenzoic acid from functioning as a coenzyme, or from being converted to a coenzyme, involved in the synthesis of purines. Ravel, Eakin, and Shive (1948) indicated that under conditions of sulfonamide bacteriostasis of *E. coli* the accumulation of 4(5)-amino-5(4)-imidazolecarboxamide represents a purine precursor.

Ravel, *et al.*, investigated the effects of amino acids on the quantity of the amine accumulating in the medium as the result of an 18-hour growth of *E. coli*. Only a small amount of the amine was formed in a medium restricted to glucose and inorganic salts. The medium was assayed for non-acetylatable diazotizable amine by the method of Bratton and Marshall (1939), after treatment with 3 per cent acetic anhydride as described by Rosenthal and Bauer (1939).

In the presence of 50 µg of sulfadiazine, the greatest increase in the production of the amine was found when glycine was added to the medium. The increase in the amount of the amine formed was proportional to the amount of added glycine. This amino acid was somewhat more stimulatory in the production of the amine than threonine, glutamic acid, or leucine. Ravel, *et al.*, found no stimulatory action with serine. They suggested that this lack of stimulation on the part of added serine might arise from the inhibition of its conversion to glycine by the sulfadiazine present.
The increased yield of the amine resulting from the addition of a mixture of glycine and glutamic acid, and to a lesser extent with leucine and other amino acids with glutamic acid, indicated that glutamic acid was involved in the amination reactions leading to the production of the amine from glycine. In this connection, the nitrogen atoms of glutamic acid and purines isolated from rat tissue have been reported by Barnes and Schoenheimer (1943) to contain similar amounts of isotopic nitrogen after the feeding of ammonium citrate containing an excess of N\textsuperscript{15} isotope.

Ravel, et al., also found that the activity of threonine in the production of the amine indicated that this amino acid can be converted to glycine by \textit{E. coli}. Rossi and Gennamo (1945) investigated the synthesis of threonine from glycine by yeast. Ravel, et al., stated that this finding leaves in doubt which precedes the other in the normal course of biosynthesis of these amino acids.

Gots (1950) reported the isolation of a purine-requiring \textit{E. coli} mutant which produced 4(5)-amino-5(4)-imidazole-carboxamide in a salts-glucose medium containing purine. This strain, unlike other purine-requiring mutants, also utilized xanthine for growth. The yield of the amine was greatest in the presence of this base. Neither synthetic nor isolated 4(5)-amino-5(4)-imidazolecarboxamide supported the growth of any of the purine-requiring mutants. Gots has
suggested that there is little doubt that the amine is associated with the reactions leading to the biosynthesis of purines.

In the course of a study on bacterial stimulation, Straughn and Sevag (1949) tested the effect of histamine and other imidazole compounds on the growth of *Klebsiella pneumoniae*. This organism is thought to be closely related to *A. aerogenes*, and can be cultured in a simple salts-glucose medium. The results obtained indicated a stimulatory effect by 4(5)-amino-5(4)-imidazolecarboxamide as well as with histamine. In both cases, no measurable utilization of either of these compounds was detected.

Pigeon liver homogenates have been reported to convert 4(5)-amino-5(4)-imidazolecarboxamide to hypoxanthine by Schulman, Buchanan, and Miller (1950); whereas, the intact pigeon transformed this amine into uric acid. Miller, Gurin, and Wilson (1950) administered labeled $^{14}O$-4(5)-amino-5(4)-imidazolecarboxamide subcutaneously to rats. The nucleic acids were isolated and the purines obtained by hydrolysis and chromatographic separation. The greatest percentage of the amine was found in the collected urine samples. The remainder of the isotope appeared in the isolated adenine and guanine obtained from the nucleic acid material. Some of the unchanged amine appeared in the urine with its radioactivity undiluted. Miller, et al., suggested that this
indicated there is no pool of the amine compound in the body of the rat. They believed that the amine is not formed as a normal intermediate in purine metabolism.

Buchanan (1951) reported that 4(5)-amino-5(4)-imidazolecarboxamide was not an intermediate per se in purine biosynthesis. Buchanan suggested that ribose is added to the amine structure before the six-membered ring is closed. Therefore, a ribose compound of 4(5)-amino-5(4)-imidazolecarboxamide would be the common intermediate in the conversion of the amine and glycine to hypoxanthine.

**Glycine, formate, and carbon dioxide**

The question of the biosynthesis of the purine bases is essentially a matter of determining the precursors of these bases. Recently a great deal of information as to the various compounds which are precursors of purines has come from the study of uric acid formation in pigeons. Sonne, Buchanan, and Delluva (1946) have extended the early observations of Barnes and Schoenheimer. These investigators found that isotopically labeled glycine, or a metabolic derivative of glycine, when fed to pigeons, was involved in the synthesis of uric acid. The carboxyl carbon of glycine supplied the number four-carbon of uric acid. The following is the structure of uric acid with the various atoms numbered according to accepted practice:
Buchanan and Sonne (1946) found that formate contributed its carbon atom to positions two and eight of uric acid. Shemin and Rittenberg (1947) fed $\text{CH}_3\text{N}^{15}\text{H}_2\text{COOH}$ to human adults and found that the nitrogen in the seven position arises from the amino group of glycine. Sonne, et al., (1948) carried out a very extensive series of experiments using the following isotopically labeled compounds: $^{13}\text{C}_2\text{O}_2$, $^{13}\text{H}_2\text{COOH}$, $^{13}\text{CH}_2\text{OOC}$, $^{13}\text{CH}_2\text{NH}_2\text{OOC}$, $^{13}\text{CH}_3\text{CHOOC}$, and $^{13}\text{H}_2\text{C}^{13}\text{HDHOOC}$. The uric acid excreted by pigeons used in these experiments was collected and the location of the isotope determined by stepwise degradation. It was found that the carboxyl carbon of acetate and formate and the $\alpha$-(or $\beta$-) carbon of lactate participated in ureide synthesis (carbon atoms 2 and 8 of uric acid). The labeled atoms of the other compounds did not participate in the synthesis of the ureide carbons. These workers believed that acetate is the biological source of the ureide carbons and that formate is either an intermediate or may be readily converted to an intermediate of the reaction. Sonne, et al., suggested that the isotopic carbon of the $\alpha,\beta$-labeled lactate is converted to ureide carbon by
virtue of the fact that the carboxyl carbon of acetate may be derived metabolically from the α-carbon of lactate. The facts that formate was found to be an important source of the ureide carbon of uric acid, and carbon dioxide does not enter into this reaction to any measurable extent, indicated that carbon dioxide may not be reduced to formate in the pigeon as it is in certain bacterial preparations as reported by Woods (1936). In additional experiments Sonne, et al., found that the carboxyl group of acetate was not a direct precursor of urea. They assumed that these experiments demonstrated that the carbon of urea and the carbon (ureide) of uric acid have different metabolic origins; an assumption later not substantiated.

Elwyn and Sprinson (1950a) investigated the role of acetate in uric acid formation in connection with their serine studies. These workers were unable to confirm the incorporation of the carboxyl carbon of acetate into the ureide carbons of uric acid. More recently, Schulman, et al., have reported that the acetate used in the experiments of Sonne, Buchman, and Deluva was contaminated with labeled formate. They indicated that the findings of Elwyn and Sprinson are correct. It has been shown that α-labeled glycine was converted in the rat to acetate labeled in both carbon atoms by Sprinson (1949). The reverse of this reaction, acetate to glycine, has been demonstrated in the rat by Arnstein and Neuberger (1949).
Buchanan, Sonne, and Dellauba (1949) also studied the role of lactate, glycine, and carbon dioxide as precursors of the carbon chain of uric acid excreted by pigeons. The metabolic derivatives of proteins, fats, and carbohydrates have been investigated by various workers as possible precursors of the carbon chain of uric acid (carbon atoms 4, 5, and 6). Örström, Örström, and Krebs (1939) have demonstrated that asparagine, glutamine, pyruvate, and oxalacetate may stimulate the formation of hypoxanthine using pigeon liver slices. This investigation of the stimulatory effect of these compounds does not necessarily indicate that their carbon chains participate directly in purine biosynthesis. It is known that energy producing oxidations must accompany many biological syntheses.

The study of Buchanan, et al., was particularly important in light of the failure of previous attempts to establish the precursors of the carbon chain of uric acid. These workers have shown by isotopic studies that carbon dioxide was the source of carbon-six of uric acid and that the carboxyl carbon of glycine was the source of carbon-four in the uric acid excreted by the pigeon. By the use of indirect experiments with N\(^{15}\)H\(_4\)Cl, they demonstrated that the amino nitrogen of glycine was probably the source of nitrogen atom seven of uric acid. Therefore, carbon-five would probably arise from the \(\alpha\)-carbon of glycine. Buchanan, et al.,
believed the incorporation of carbon dioxide into position six of uric acid occurred through a carbon dioxide fixation reaction.

In addition, it was found that the carboxyl and \(\alpha\)-carbons of lactate were also incorporated into positions four and five respectively of uric acid. This incorporation was to a lesser extent than that from the carboxyl carbon of glycine into position four. Buchanan, et al., suggested that the lactate is converted to glycine by reactions similar to those involving the conversion of serine to glycine. Furthermore, glycine and acetate are not directly interconvertible in the metabolism of the pigeon. These investigators suggested that the formation of a three-carbon intermediate of carbohydrate metabolism by a reaction involving carbon dioxide and the \(\alpha\)-carbon of glycine might be a step in the oxidative and glycogenic metabolism of glycine.

The incorporation of dietary purines into nucleoproteins was investigated by Plentl and Schoenheimer (1944) by feeding labeled guanine-N\(^15\) to rats and pigeons. They found no incorporation of the isotope into the purines isolated from the tissues. The guanine was extensively converted to allantoin in the case of the rat and to uric acid in the case of the pigeon. From these results, it was concluded that purines supplied in the diet are not utilized for synthesis of nucleoproteins. Brown, et al., investigated the problem further
and concluded that ingested guanine was not incorporated into nucleic acid material. Nevertheless, ingested isotopically labeled adenine was converted to guanine. Balis and Brown have demonstrated a transformation of guanine to adenine by \textit{Lactobacillus casei}. The problem of the conversion of adenine to guanine and the reverse of this reaction remains unsettled.

Getler, \textit{et al.}, (1949) have found that dietary xanthine and hypoxanthine are ineffective precursors of nucleic acids in the rat, but are extensively converted to allantoin. Greenberg (1948) has reported the incorporation of labeled carbon dioxide and formate into hypoxanthine in the presence of pigeon liver homogenates. Buchanan found that for every two moles of formate utilized for hypoxanthine synthesis one mole of glycine was also used by pigeon liver homogenates.

Abrams, Hammarsten, and Shemin (1948) have cultivated yeast in the presence of \(N^{15}\)-labeled glycine and found the label in both the adenine and guanine isolated from the cell nucleic acid material. By degrading the isolated guanine to glycine, which represents the four, five, and seven atoms of the guanine, they determined that \(N^{15}\)-labeled glycine was a precursor of the seven-nitrogen atom of the guanine. The weakness in this procedure lies in the failure to demonstrate that deamination, with subsequent incorporation of the released ammonia, did not occur. Unless the carbon chain, as well as the amino nitrogen, of the glycine is labeled, the con-
clusions that can be drawn are not completely valid. The uptake of the isotopic nitrogen from the labeled glycine was greater in the guanine than in the isolated adenine. The following compounds are a few of the larger type of molecules which might be direct precursors of purines: aminomalonic acid, aminomalonic acid diamide, and aminomalonamidine. These compounds have been prepared with incorporated isotopes and tested with pigeon liver homogenates and in the intact rat by Buchanan. They were not utilized in purine synthesis. Animal studies have demonstrated that the four, five, and seven positions of uric acid are derived from glycine, carbons two and eight from formate, carbon six from carbon dioxide, and nitrogen atoms one, three, and nine from still unknown nitrogen donor or donors.

Serine and threonine

The relationship of the hydroxyamino acids serine and threonine to the biosynthesis of the purine bases has received considerable attention recently. The conversion in vivo of serine to hippuric acid, thus to glycine, was first demonstrated by Shemin (1946). The reverse of this reaction, the synthesis of serine from glycine, has been observed by Goldsworthy, Winnick, and Greenberg (1949) in vivo following the administration of C\(^{14}\)-labeled glycine to rats. In an earlier study, Greenberg and Winnick (1948) failed to observe the
formation of serine from labeled glycine. This failure, Goldsworthy, et al., believed was due to the relatively low specific radioactivity of the glycine used. Winnick, et al., (1949) demonstrated the transformation of C\textsuperscript{14}-glycine to serine in rat liver homogenates. A similar transformation has been demonstrated in yeast grown in a medium containing C\textsuperscript{15}-labeled glycine by Ehrensvard, et al., (1947). These investigators, studying the metabolic connection between proline and glycine in amino acid utilization of \textit{Torulopsis utilis}, found isotopically labeled C\textsuperscript{15}-alanine transferred its label to the various amino acids with an excess appearing only in the isolated alanine. Glycine preponderantly transferred its label to serine and proline. The explanation offered for the apparent transcarboxylation is that carboxylation from a common pool of fixed carbon dioxide was involved in the synthesis of peptide linkages; thus the excess C\textsuperscript{13} in the respiratory carbon dioxide will, in part, appear as carboxyl groups in the amino acid residues.

Rats fed C\textsuperscript{13}-carboxyl labeled glycine and C\textsuperscript{14}-labeled formate by Sakami (1948) showed the presence of both isotopes in the serine and glycogen isolated from the liver tissue. In the serine, the C\textsuperscript{13} isotope was found in the carboxyl group and the C\textsuperscript{14} isotope in the \(\beta\)-carbon. In glycogen, the C\textsuperscript{13} isotope was found in positions three and four, while the C\textsuperscript{14} isotope was found in all of the positions of the glycogen
carbon chain. The greatest C\textsuperscript{14} isotopic concentrations appeared in the one and six positions. Sakami postulated that glycogen is formed via pyruvate and serine from formate and glycine. Anker (1948) fed pyruvate, labeled in the carboxyl and carbonyl carbons, to rats. The glycine, obtained from the hippuric acid, contained the isotope in the carboxyl and methylene carbon atoms.

Sakami (1949) has demonstrated in vivo that formate condenses with the α-carbon of glycine to form serine. Rats were fed glycine labeled with C\textsuperscript{14} in the α-carbon atom. The serine isolated from the rat liver tissue contained C\textsuperscript{14} in both the α-carbon and β-carbon atoms but essentially none in the carboxyl carbon. This result supported the hypothesis that glycine is converted to serine by condensation with formate, the latter being formed by deamination and subsequent decarboxylation of glycine. Siekevitz and Greenberg (1949) found that with carboxyl labeled glycine, only the carboxyl carbon of serine was labeled; with α-labeled glycine, both the α- and β-carbons of serine were labeled. These investigators proposed the following overall reaction:

\[
\text{C}^{14}\text{H}_2\text{NH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow (\text{C}^{14}\text{H}_2\text{COOH}) \rightarrow \text{C}^{14}\text{H}_2\text{OОН} + \text{CO}_2 + \text{NH}_3 + 4\text{H}
\]

\[
\text{HC}^{14}\text{OOH} + \text{C}^{14}\text{H}_2\text{NH}_2\text{COOH} + 2\text{H} \rightarrow \text{C}^{14}\text{H}_2\text{OHC}^{14}\text{HNH}_2\text{COOH} + \text{H}_2\text{O}
\]

Siekevitz and Greenberg found that the carbon atoms of glycine behave differently in that the carboxyl carbon was metabolized
to carbon dioxide and the α-carbon to formate. The production of formate from the α-carbon of glycine was not reversible, and carbon dioxide was not reduced to formate by rat liver slices. In addition, the synthesis of serine occurred both aerobically and anaerobically. The percentage of radioactivity in the β-carbon of serine, relative to the radioactivity in the α-carbon of the glycine, indicated that another source of formate existed in the rat liver slices.

Elwyn and Sprinsson (1950a) have suggested that the demonstration of the conversion of serine to glycine and formate provides ground for expecting the ureide carbons of uric acid to arise from the β-carbon atom of serine. Formate would enter these positions through condensation with glycine to form serine, which would act as a formate carrier in the organism. In order to test this scheme, Elwyn and Sprinsson synthesized serine labeled with N\(^{15}\) and C\(^{14}\) in the β-carbon atom, and deuterium in the α and β positions. The labeled serine was injected intraperitoneally into pigeons and the excreted uric acid collected. The β-carbon atom of serine was utilized for the ureide carbon atoms of uric acid to about the same extent as the N\(^{15}\) was for position seven. The utilization of the nitrogen of serine for the nitrogen atom seven of uric acid was more than twice as great as for the other nitrogen atoms. This result was in accord with the known conversion of serine to glycine, and the utilization of glycine for positions four, five, and seven of uric acid.
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control rate. These workers suggested that these results
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This result supported the view that glyoxylic acid is converted to glycine and the latter utilized in the biosynthesis of uric acid, but failed to support the view that the decarboxylation of glyoxylic acid is an intermediate step as suggested by Siekevitz and Greenberg.

Meltzer and Sprinson (1950) have found that threonine can be cleaved to acetate and glycine. Threonine, containing C\textsuperscript{14} in the α-carbon atom and N\textsuperscript{15} in the amino group, was fed to rats. These workers also demonstrated that threonine was decarboxylated in the rat to a three-carbon compound. This three-carbon compound was split to yield acetate and formate.

Rat liver slices, incubated in the presence of C\textsuperscript{14}-formate and C\textsuperscript{14}-methyl labeled choline, incorporated the isotopes into the β-carbon of serine. Kruhöffer (1951) showed further that rat liver homogenates failed to carry out this incorporation, though incorporation of labeled glycine into the serine occurred. The formate was decarboxylated to carbon dioxide by the rat liver homogenates. Kruhöffer suggested that formate is not the only, or is not the immediate, precursor of the β-carbon of serine formed in the rat liver.

 Chargaff and Sprinson (1943a-1943b) studied the rapid deamination of serine by a number of different microorganisms. In the case of \textit{E. coli}, they found serine to be deaminated anaerobically to pyruvic acid and suggested the reaction proceeded by way of a dehydration. These investigators also
carried out the deamination aerobically and found the final product to be pyruvic acid. Sprinson and Chargaff (1946) reported that mammalian kidney, liver, and muscle tissue oxidatively deaminated dl-serine to hydroxypyruvic acid. They were unable to find hydroxypyruvic acid when l-serine was used. Threonine was not oxidatively deaminated.

Para-aminobenzoic acid


. . . a para-aminobenzoic acid coenzyme is required for the reductive condensation by which serine is formed from glycine and formic derivatives, or for the reverse oxidative cleavage by which glycine is produced from serine, because: serine increases the sulfonamide para-aminobenzoic acid ratio needed to inhibit bacteria; serine is a precursor of glycine, but in the presence of sulfonamides this conversion cannot take place; a synthesis of serine from formic acid and glycine has been demonstrated; and folic acid has been shown to be involved in the interconversion of glycine to serine . . . .

The synthesis of serine from formic acid and glycine and the role of folic acid in the interconversion of glycine to serine have both been demonstrated in animals but not in bacteria. Eakin indicates that the conversion of serine to glycine cannot take place in the presence of sulfonamides. Ravel, Eakin, and Shive suggested that the inactivity of serine in the production of 4(5)-amino-5(4)-imidazolecarboxamide by E. coli arose from the inhibition of the conversion of serine to glycine. Glycine was found to stimulate
the production of the amine. These authors offered no further proof to support this suggestion. Eakin, however, has indicated without reservation that a p-aminobenzoic acid coenzyme is required. This statement was based on the suggestion of Ravel, Eakin, and Shive.

Lascelles and Woods (1950) have studied the formation of serine by microorganisms. These investigators have found that serine was partially replaced by glycine in the presence of pyridoxal and carbon dioxide, and the replacement was almost complete on the further addition of p-aminobenzoic acid for the growth of Leuconostoc mesenteroides. In addition, they found in washed cell experiments, using Streptococcus faecalis, that pteroylglutamic acid was needed to optimal synthesis of serine in the absence of p-aminobenzoic acid. They suggested that pteroylglutamic acid has an essential function in the condensation of glycine and a one-carbon compound to yield serine. Furthermore, the results obtained with Leuconostoc mesenteroides suggested that carbon dioxide may serve as a precursor of the one-carbon compound in serine synthesis.

Pteroylglutamic acid solutions are not stable, but decompose yielding diazotizable amines and other growth factors. Kof, et al., (1950) have found that aged pteroylglutamic acid solutions were 100-fold more active in growth stimulation than fresh solutions for Lactobacillus arabinosis.
Para-aminobenzoic acid was also 100-fold more active than fresh solutions of pteroylglutamic acid. These workers believed that the disintegration of pteroylglutamic acid to other factors was essential for the stimulation of growth of the L. arabinosus. This finding may in part explain the pteroylglutamic acid requirement of S. faecalis in the absence of p-aminobenzoic acid.

The role of p-aminobenzoic acid and its derivatives in connection with serine and purine biosynthesis remains in a state of flux. The relationship of the findings in the case of bacteria with those in animal tissue have not been established.
METHODS

Organisms and Medium

The organisms employed in the following investigations were *Aerobacter aerogenes* (strain 199) and *Escherichia coli* (strain E-36). These organisms were grown in a basal medium of the following composition: 2 per cent dextrose (Bacto or Pfanstiehl), 0.8 per cent K$_2$HPO$_4$, 0.8 per cent (NH$_4$)$_2$SO$_4$, 10 per cent tap water, made to volume with distilled water. The phosphate was dissolved in a small volume of distilled water and the pH of the solution adjusted to 6.7-7.0. The solutions were sterilized by autoclaving at 15 pounds pressure for 45 minutes, or at 15 pounds pressure for 20 minutes in the case of small volumes of the medium. The sterilized phosphate solution was added aseptically to the other portion of the medium.

*Aerogenes* was inoculated into 10 ml of a 1 per cent glucose-yeast extract medium; after incubation for 16 hours at 37°C., the 10 ml culture was added to 100 ml of the basal medium described above. This was incubated for 8 hours at 37°C. and then added to 1 liter of the basal medium. This culture was incubated in a similar manner and then inoculated into 9 liters of the basal medium contained in a 13 liter
Florence flask. The medium was vigorously aerated and incubated by means of sterile air passing through a fritted glass tube for 8 hours. The cells were harvested in a Sharples centrifuge, washed, and lyophilized. The most effective means of resuspending the cell paste was to treat the mixture in a Waring blender for several minutes. This treatment proved to be very useful in resuspending the gummy-mass of A. aerogenes cells. When resting cell experiments were to be carried out, the washed cells were used immediately without resorting to lyophilization. The dried cells were stored over CaCl₂ at 4 to 10°C.

Cell-free extracts were prepared by mixing fresh cells with glass powder in a ratio of 10 grams of the cells to 1 gram of the glass. This material was suspended in either M/15 phosphate buffer or distilled water and then subjected to sonic vibration from 30 to 40 minutes using a Raytheon 9-ka Magnetostriction Oscillator. The cell debris and glass were removed from the bacterial juice by centrifuging the material in a refrigerated centrifuge at 0°C. and 10,000 r.p.m. for 30 minutes. The juice was removed from the centrifuge tubes and frozen. The E. coli was treated in the same manner as the A. aerogenes.

The methods for culturing Proteus vulgaris, Streptococcus faecalis, Micrococcus pyogenes var. aureus, Micrococcus pyogenes var. albus, Lactobacillus arabinosis, and Lacto-
bacillus casei will be described in connection with the experiments in which they were used.

Analytical Methods

Glycine

Glycine was determined by a modification of the method of MacFadyen (1945) and Alexander, et al., (1945). The determination is based on the reaction of ninhydrin (triketo-hydrindene hydrate) with glycine, in which the amino acid is decarboxylated and deaminated to form formaldehyde. The reaction is quantitative and the formaldehyde is determined colorimetrically after its reaction with chromotropic acid (1,8-dihyronaphthalene-3,6-disulfonic acid).

Convenient aliquots of the bacterial suspensions were deproteinized, either by treatment with one volume of 10 percent sodium tungstate, followed by the addition of one volume of 2/3 N sulfuric acid, or by the addition of sufficient 10 N sulfuric acid to make the aliquot 0.3 N with respect to the sulfuric acid and then heating the mixture for 20 minutes in a boiling water bath. The precipitated proteins were removed by centrifugation and the supernatant liquor filtered through Whatman no. 2 filter paper.

A suitable aliquot was removed and placed in a distillation apparatus constructed from a 25 ml Kjeldahl flask con-
nected to a condenser. Sufficient distilled water was added to bring the volume to 7.3 ml. A phosphate buffer of pH 5.5 was prepared by dissolving 3.5 gm of K$_2$PO$_4$ in 100 ml of a 20 per cent solution of KH$_2$PO$_4$. Two ml of this buffer and 1 ml of a freshly prepared solution of 1 per cent ninhydrin were added to the aliquot. The solution was rapidly distilled into a 10 ml volumetric flask immersed in an ice bath. When approximately 7.0 ml of the distillate had been collected, the neck of the distillation flask was also heated until a total of 10 ml of distillate had been collected. The receiving flask was removed and the distillate mixed by inversion. A 5.0 ml aliquot was removed and placed in a Pyrex test tube. The test tube was placed in an ice bath and 4 ml of concentrated sulfuric acid (sp. g. 1.84) were added slowly in 0.5 ml portions. The contents of the test tube were mixed after each addition of acid. Following the addition of the acid, the test tube was removed from the ice bath and allowed to come to room temperature. Three drops of a freshly prepared solution of 5 per cent chromotropic acid were added. The test tube was stoppered with a glass marble and placed in a boiling water bath for 30 minutes.

The test tube was removed from the water bath and placed in an ice bath. After the solution had cooled, it was quantitatively transferred to a Klett colorimeter tube. The
volume was made to 10 ml and the color intensity of the solution measured in a Klett-Summerson photoelectric colorimeter using a 540 μm filter. A reagent blank was run in addition to a solution containing a known amount of glycine. Due to the low volatility of formaldehyde in dilute aqueous solution, it was found that the distillation must be carried to near dryness. A standard curve was prepared using glycine. The effective range of this method is from 10 to 50 μg. Formaldehyde may be determined, using the same standard curve, without the ninhydrin treatment. The effective range for the formaldehyde determination is from 4 to 20 μg.

Serine

Serine was determined chemically by the method of Boyd and Logan (1942). The reaction is based on the oxidation of serine by periodic acid. On treatment with periodic acid, serine quantitatively yields one molecule of formaldehyde per molecule of the amino acid. Threonine yields instead acetaldehyde. By distillation under proper conditions, the two aldehydes are collected and the formaldehyde determined colorimetrically by reaction with chromotropic acid.

A suitable deproteinized aliquot, prepared in the same manner as described in connection with the glycine procedure, was neutralized with dilute NaOH and then transferred to a roundbottom flask. Three drops of a saturated solution of
methyl red in 0.05 N HCl were added. Four ml of a 25 per cent solution of sodium arsenite were added to the flask, and 2.5 to 2.8 ml of a 0.5 M periodic acid solution added gradually while shaking the flask. When the solution turned acid to methyl red, the addition of the periodic acid was stopped. A small amount of talc and sufficient water to make a total volume of 70 ml were added. The flask was attached to a condenser by means of a ground glass joint. The outlet was dipped into a small volume of water in the receiver. Distillation was carried out until approximately 5.0 ml of solution remained in the distilling flask. The distillate was finally diluted to a volume of 100 ml.

The determination of the distilled formaldehyde was carried out as previously described under the glycine procedure. The effective range for serine using this method is from 14 to 70 µg. It was found necessary to purify the chromotropic acid because of the deep brown color of the reagent, which interfered with the purple color-complex formed with formaldehyde. Twenty-five gm of the acid were dissolved in 100 ml of warm water. Two gm of PbCO₃ were added to the solution and allowed to dissolve. The lead ions were removed by treatment with H₂S. The supernatant liquid was pale yellow after the removal of the dissolved H₂S. The solution was placed at 4°C. and the chromotropic acid allowed to crystallize. The crystalline material was washed first with ethanol and
then with ether. The nearly white product was stored in a vacuum desiccator.

**Ribonucleic acid**

Ribonucleic acid was determined by a modification of the methods of Mejbaum (1938), and Kerr and Seraldarian (1945) which are based on an adaptation of the orcinol reaction of Bial. The determination of ribonucleic acid and ribose-containing split products depends on the formation of furfural upon treatment with hot acid. The furfural is coupled with a color-yielding phenol, orcinol, in the presence of ferric ion catalyst. As little as 0.1 micromole of ribose or ribose-containing material will produce a stable green color in the presence of orcinol.

A suitable aliquot of the pentose-containing material was placed in a test tube and made to a volume of 1.5 ml by the addition of water. The orcinol reagent was prepared by adding 0.1 per cent FeCl₃ and 1.0 per cent orcinol to concentrated HCl. One and one-half ml of this freshly prepared reagent were added to the diluted aliquot. The contents of the tube were mixed and stoppered with a glass marble. The tube was then placed in a boiling water bath for 40 minutes. After cooling, the contents of the tube were quantitatively transferred to a Klett colorimeter tube and the volume adjusted to 5.0 ml. The color intensity of the solution was measured in a Klett-Summerson colorimeter at 660 μμ.
The commercial samples of orcinol contain impurities which cause difficulty in obtaining the desired stable green color. To overcome this difficulty, the orcinol used was recrystallized from chloroform. A standard curve was prepared using l-arabinose (Pfanstiehl). Any pentose may be used as a reference standard under the conditions described in the aforementioned procedure. The effective range of this procedure is from 0 to 20 μg.

Desoxyribonucleic acid

The determination of desoxyribonucleic acid was carried out by the method of Dische (1930). The reaction depends upon the formation of a purple to violet color-complex of desoxyribose and diphenylamine in the presence of glacial acetic acid and sulfuric acid. The test is not as sensitive as the orcinol reaction for ribose and the presence of protein material tends to enhance the color intensity. The lack of available desoxyribose for study has helped to prevent the complete understanding of the nature of the color-complex formed with diphenylamine.

A suitable aliquot of the material to be examined was placed in a test tube and brought to a volume of 3.0 ml. A solution containing 1.0 gm of diphenylamine, 2.0 ml of concentrated sulfuric acid, and 98 ml of glacial acetic acid was prepared. Three ml of this freshly prepared reagent were
added to the diluted aliquot. The contents of the test tube were mixed and placed in a boiling water bath for 30 minutes. After the solution had been removed and cooled, it was quantitatively transferred to a Klett colorimeter tube. The volume was made to 5.0 ml and the intensity of the color measured in a Klett-Summerson instrument using a 540 mp filter.

It was found necessary to recrystallize the commercial diphenylamine in order to reduce the reagent-blank color. The recrystallization was carried out by dissolving the diphenylamine in absolute alcohol by heating on the steam bath. This hot solution was decolorized by the addition of Norite "A" and the Norite was then removed by filtration. The solution was allowed to cool and the diphenylamine crystallized out by the addition of water. The crystallized material was removed by filtration, washed with water, and dried in a vacuum desiccator.

Desoxyribonucleic acid, prepared from calf thymus gland (Kirshell Lot no. 481009), was used as a reference standard for the diphenylamine reaction. The acid was suspended in 1 N KOH and incubated at 37°C. for 48 hours. For each mg of the desoxyribonucleic acid, 1.0 ml of the alkali was used. The small amount of undissolved material remaining after incubation was removed by filtration. The filtrate was neutralized with 1 N HCl and then made to volume. Aliquots of this solution were examined for inorganic phosphate by the method of Fiske and SubbaRow (1925). It was found
necessary to purify the 1,2,4-aminonaphtholsulfonic acid as suggested by Fiske and Subbarow. The inorganic phosphate concentration was taken as a measure of the amount of deoxyribonucleic acid present in the solution. A standard curve was then prepared from this solution using the diphenylamine reaction.

Pyruvate

Pyruvate was determined by the chemical method of Straub (1936). This colorimetric determination of pyruvate involves the formation of a burnt-orange color upon condensation of this compound with salicylaldehyde. To 1.0 ml of the sulfuric acid deproteinized material, there were added 1.0 ml of a KOH solution (100 gm of KOH in 60 ml of water) and 0.5 ml of a salicylaldehyde solution (2.0 per cent by volume in 95 per cent alcohol). The contents of the tube were mixed by inversion and allowed to stand in a water bath at 37°C. for 10 minutes, after which the tube was cooled to room temperature. The solution was quantitatively transferred to a Klett colorimeter tube and diluted to 10 ml. The intensity of the color was measured in a Klett-Summersen colorimeter using a 470 μm filter. A reagent blank was included and a standard curve was prepared using sodium pyruvate. The most effective range for this method is from 0 to 150 μg. Acetymethylecarbinol, acetone, and acetaldehyde may interfere with this color
reaction. Lactic acid does not interfere and oxalacetic acid will not, provided the color intensity is determined immediately after dilution.

4(5)-Amino-5(4)-imidazolcarboxamide

A modification of the Bratton and Marshall (1939) method for diazotizable amines by Rosenthal and Bauer (1939) provides a convenient method for the determination of a 4(5)-amino-5(4)-imidazolcarboxamide, a non-acetylatable diazotizable amine. The determination of this amine in the presence of sulfonamides depends on the acetylation of the sulfonamides by acetic anhydride.

The principle of the Bratton and Marshall method is based on the treatment of the sulfonamide with nitrous acid to diazotize the compound. The excess nitrous acid is destroyed by the addition of ammonium sulfamate. The diazotized sulfonamide is then coupled with N-(1-naphthyl)-ethylenediamine to form a stable red color. The effective range of the procedure is from 0 to 10 µg.

A suitable deproteinized aliquot was treated with a 2.0 per cent concentration of acetic anhydride for 30 minutes at room temperature. Following this treatment, 4.0 ml of 0.2 N H₂SO₄ and 0.5 ml of a 0.1 per cent sodium nitrite solution were added to 1.0 ml of the acetic anhydride material. After 5 minutes, the excess nitrous acid was destroyed by the addi-
tion of 0.5 ml of a 0.5 per cent ammonium sulfamate solution. Three minutes later, 0.5 ml of a 0.1 per cent solution of N-(1-naphthyl)-ethylenediamine dihydrochloride was added. The color was allowed to develop at room temperature and, after 10 minutes, the solution was diluted to a volume of 10 ml. The color intensity was read in a Klett-Summerson photoelectric colorimeter using a 540 μm filter.

Synthetic 4(5)-amino-5(4)-imidazolecarboxamide was not used to prepare a standard curve because the available supply was limited. Instead, para-aminobenzoic acid was used to prepare a standard curve. The values obtained were converted to those for the sulfonamides and 4(5)-amino-5(4)-imidazolecarboxamide through the use of the following factors: Sulfadiazine 1.81, sulfathiazole 1.88, and 4(5)-amino-5(4)-imidazolecarboxamide 0.92.

Methylamine

A modification of the method of Ormsby and Johnson (1950) was used to determine methylamine. A reaction between methylamine and lactose in alkaline solution produces a red color. The addition of ammonia increases the sensitivity of this colorimetric method. The effective range of this method is from 0.01 to 0.15 mg of methylamine-N.

To a suitable aliquot in a test tube, 0.5 ml of a 6.6 per cent solution of (NH₄)₂SO₄ and 0.5 ml of a 3.0 per cent
lactose solution were added. Following these additions, 0.3 ml of a 20 per cent NaOH solution was added and the contents of the test tube mixed by inversion. Excessive aeration had to be avoided to prevent a decrease in the intensity of the final color. The tube was stoppered with a glass marble and then heated in a 56°C. water bath for 30 minutes. The contents of the tube were cooled and transferred quantitatively to a Klett colorimeter tube. The volume was made to 10 ml and the color intensity read at 540 m\u. A standard curve was prepared from a solution of methylamine hydrochloride.

**Nitrogen**

Cell nitrogen was determined by the micro-Kjeldahl method. Digestion of an appropriate sample was carried out in the presence of a copper-potassium sulfate mixture and sulfuric acid, (Baker's Special, ammonia free). The digested sample was treated with saturated NaOH solution, and the liberated ammonia steam distilled. The ammonia was collected in 5.0 ml of a 2.0 per cent boric acid solution and then titrated with 0.01 N HCl using a mixed indicator. The advantage of this mixed indicator lies in the fact that a dead grey color is obtained at the titration end point. The indicator was prepared by dissolving 17 mg of methyl red and 83 mg of brom cresol green in 100 ml of 95 per cent alcohol.
Hypoxanthine

Hypoxanthine was determined by the differential enzymatic spectrophotometric method of Kalckar (1947a-1947b). The method is based on the measurement of the changes in extinction, at two or more different wave lengths, resulting from the enzymatic action of xanthine oxidase. A model DU Beckman spectrophotometer was used in these determinations. The xanthine oxidase enzyme oxidizes hypoxanthine or xanthine to uric acid and the changes in extinction are measured. The xanthine oxidase for this procedure was prepared by using a modification of the method of Ball (1939).

To each 100 gm of cream, 100 ml of a 0.2 M solution of Na₃HPO₄ were added and the mixture was stirred while incubated for two hours at 37°C. The mixture was then centrifuged at 0°C. and the aqueous portion removed from beneath the fat layer, which solidified at the top of the centrifuge container. To each 100 ml of aqueous extract, 100 mg of trypsin powder 1-110 (Pfanstiehl) were added and the mixture incubated at 37°C. for three hours. The mixture was then cooled to 20°C. and sufficient 0.5 M solution of CaCl₂ added to precipitate suspended material. After standing at 20°C. for 15 minutes, the mixture was centrifuged at 0°C. for 30 minutes.

The supernatant liquid was saturated (80 per cent) with \((\text{NH}_4\)₃)²SO₄ and placed in the refrigerator at 10°C. overnight. The precipitate formed was removed by centrifuging at 0°C.
and then dissolved in cold distilled water (1/10 of the original volume). This solution was brought to 42 per cent (NH₄)₂SO₄ saturation and stored at 10°C until used.

An aliquot of this suspension was tested for activity. The precipitate was removed by centrifuging at 0°C and dissolved in a small volume of cold distilled water. One-half ml of the enzyme preparation and 1.0 ml of 0.1 M phosphate buffer at pH 7.2 were placed in a conventional Warburg flask equipped with 0.3 ml of a 5.0 per cent KOH solution in the center well. One-half ml of a 0.05 M solution of hypoxanthine in 0.05 M NaOH was added from the sidearm and the oxygen uptake was measured. The activity determined was about 75 per cent of that found by Ball with similar xanthine oxidase preparations.

Synthesis of Compounds

Isotopically labeled glycine

The H₃N⁺CH₂C¹³OOH and H₃NCH₂C¹³OOH used for the metabolic experiments were synthesized by a modification of the procedure described by Sakami, Evans, and Gurin (1947). The N-hydroxymethylphthalimide used was prepared by the procedure of Pucher and Johnson (1922). The preparation of this compound by the procedure of Sachs (1893), as recommended by Sakami, et al., proved very unsatisfactory, particularly in product yield.
In the preparation of the $H_2N\ CH_2\ COOH$, the starting compound was phthalic anhydride. One hundred gm of this compound were placed in a 1 liter round bottom flask, which was connected with an ammonia generator through a wash bottle containing a half-saturated solution of NaOH and a drying tube charged with calcium chloride. The flask containing the phthalic anhydride was heated in an oil-bath to 140°C. and when the anhydride was melted a stream of $NH_3$ gas was passed over the surface of the liquid. The gas was rapidly absorbed, and the temperature of the bath was finally raised to 160°C., where it was held for 2 hours. The phthalimide formed solidified in the flask on cooling, and it was recrystallized from hot water and dried under vacuum.

N-Hydroxymethylphthalimide was prepared by suspending 100 gm of phthalimide in a solution of 80 ml of 40 per cent formaldehyde and 300 ml of distilled water. This suspension was heated in a round bottom flask under a reflux condenser at 103-108°C. for 4 hours. As the reaction proceeded, the phthalimide gradually dissolved and when this material had completely disappeared, the reaction was judged to be complete. The hot solution was filtered through Whatman no. 1 filter paper and allowed to crystallize at room temperature. The N-hydroxymethylphthalimide was recrystallized from benzene. The compound melted at 143°C. The melting point reported in the literature is 141-143°C. The yield of N-hydroxymethylphthalimide was 90 per cent of the theoretical.
N-Chloromethylyphthalimide was prepared by allowing 22.5 gm of N-hydroxymethylyphthalimide to stand in a round bottom flask with an excess (45 ml) of thionyl chloride (sulfurous oxychloride). The thionyl chloride had previously been purified by distillation from a mixture of quinoline and boiled linseed oil in a system protected from moisture. This distillate was fractionated and the fraction, boiling at 78-80°C, was collected.

After the mixture of N-hydroxymethylyphthalimide and thionyl chloride had been allowed to stand at room temperature for 2 hours, it was heated in a boiling water bath for 30 minutes. The excess thionyl chloride was removed under vacuum. The remaining crystalline material was dissolved in hot toluene, filtered, and allowed to crystallize. The N-chloromethylyphthalimide was recrystallized from hot dioxane. The compound melted at 135°C. The melting point reported in the literature is 134-135°C. The yield of N-chloromethylyphthalimide was 76 per cent of the theoretical.

Fifteen and one-tenth gm of N-chloromethylyphthalimide, dissolved in 60 ml of warm dioxane, were added to a solution of 4.3 gm of isotopic potassium cyanide (10.5 atom per cent excess C\(^{13}\)-Eastman, CR233D) in 260 ml of acetone-free methanol. This mixture was allowed to stand at room temperature for 2 hours and then evaporated to dryness on a steam bath. The residue was successively extracted with 113, 76, and 58 ml
portions of warm dioxane. The extracts were combined and evaporated to dryness on a steam bath. The hydrolysis of this material was carried out by refluxing for 15 hours in the presence of a mixture of 85 ml glacial acetic acid, 93 ml concentrated HCl, and 100 ml of distilled water.

The mixture was cooled to 0°C. and the phthalic acid removed by filtration. The filtrate was evaporated to dryness under vacuum at 60-70°C. The residue was dissolved in 100 ml of distilled water with heat. An excess of freshly prepared silver carbonate was added to remove the chloride ions from the solution. The silver chloride was removed by filtration and the excess silver ions by treatment with H₂S. The Ag₂S formed by this treatment was removed by filtration and washed with hot water. The filtrate and washings were combined and the volume reduced to dryness under vacuum at 60-70°C. The residue was taken up in a few ml of water and the glycine precipitated by the addition of 3 volumes of ethanol. The glycine was removed by filtration and recrystallized by dissolving it in water and precipitating it by the addition of ethanol. The product was dried under vacuum and the purity appraised by the chemical method of Alexander et al., (1945) and a melting point determination. The yield of labeled glycine was 50.2 per cent (based on the isotopic potassium cyanide used).

In the preparation of H₂N₁⁸O⁻·CH₂·C¹⁸O₂H, the starting compound was isotopic potassium phthalimide (33 atom per
cent excess N_{15} - Eastman, E940-25B-974094). Fifteen gm of the isotopic potassium phthalimide were dissolved in 50 ml of distilled water. Tank carbon dioxide was bubbled through this solution for 5 minutes. The solution was then placed in the refrigerator overnight. The phthalimide that crystallized from the solution was removed by filtration and the filtrate again saturated with carbon dioxide. The final yield of phthalimide was 84 per cent of the theoretical value. The remainder of the synthesis of the H_{3}N_{15} \cdot CH_{2} \cdot C_{13}^{13} COOH was carried out in a manner similar to that described for the H_{3}N \cdot CH_{2} \cdot C_{13}^{13} COOH.

Isotopically labeled sodium acetate

The synthesis of carboxyl-labeled sodium acetate from isotopic carbon dioxide and cyanide has been reported by Olsen, Hemmingway, and Nier (1943) and Weinhouse, Medes, and Floyd (1945) respectively. The method of synthesis of the sodium acetate, used for the metabolic experiments to be described, differs to some extent from those reported.

Twelve gm of clean magnesium turnings, and 80 ml of anhydrous ether were placed in a three-necked Wolff reaction flask to which was attached a dropping funnel, a water cooled condenser, and a motor driven stirrer. This system was flushed out with tank nitrogen for 10 minutes at 5 pounds pressure. Thirty-four ml of methyl iodide were mixed with
180 ml of dry ether and placed in the dropping funnel. The stirrer was started and 5.0 ml of the \( \text{CH}_3\text{I} \)-ether solution added rapidly. The remainder of this solution was added drop-wise at the same rate at which the ether dropped from the condenser. The magnesium was completely reacted after a period of 3 hours.

The condenser was removed from the three-necked flask and a gas manometer connected through a T-tube to the flask. The dropping funnel was replaced by a drying tube charged with calcium chloride and connected to a carbon dioxide generator. The generator consisted of a dropping funnel, containing 145 ml of 6N HCl, attached to a round bottom flask containing 70 gm \( \text{C}^{13} \)-barium carbonate in 50 ml of distilled water. The complete apparatus was evacuated by means of an oil pump through the T-tube. Following the evacuation, the system was filled with tank nitrogen and the process of evacuation repeated to insure complete removal of carbon dioxide and water vapor from the reaction flask. The reaction flask containing the Grignard reagent was immersed in a salt-ice bath and allowed to cool for 15 minutes. The stirrer was then started and the generation of the carbon dioxide begun. The system was kept under a slight positive carbon dioxide pressure by controlling the rate of addition of the acid to the barium carbonate. As the reaction progressed, a white precipitate of \( \text{CH}_3\text{C}^{13}\text{OONa} \) formed. When the Grignard reagent no longer would take up carbon dioxide, the reaction was considered complete. This
step in the synthesis required 4.5 hours. The residual $^{13}\text{C}_2\text{O}_3$ was recovered by passing the gas through CO$_2$-free alkali.

The hydrolysis of the CH$_2$C$^{13}$OOMgI was accomplished by the addition of small pieces of ice until the reaction ceased. One hundred ml of 5N H$_2$SO$_4$ were added to dissolve the magnesium salt. The ether phase was separated from the aqueous phase after making the mixture acid to congo red with additional 5N H$_2$SO$_4$. The aqueous phase was extracted with fresh ether three additional times. The ether extracts were combined and shaken with 5N NaOH until the washings remained alkaline to phenolphthalein. The alkaline extracts were combined and placed in a steam distilling flask, and acidified to congo red with H$_2$SO$_4$. The steam distillate (10 volumes) was neutralized with NaOH and the volume reduced to 10 ml on a steam bath. This concentrated solution was made acid to congo red and steam distilled. The distillate was assayed quantitatively for acetic acid by the partition method of Osburn, et al., (1936). The yield of acetic acid was 86 per cent of the theoretical, based on the barium carbonate used. The acetic acid was neutralized and sodium acetate recovered in the usual manner.

Isotopically labeled sodium bicarbonate

The isotopic C$^{13}$ available was in the form of barium carbonate. It was necessary to convert the barium carbonate
to sodium bicarbonate before use. Ten grams of barium carbonate (15.6 atom per cent excess Cl\textsuperscript{38}-Eastman, 940-57-900263) were suspended in 200 ml of CO\textsubscript{2}-free distilled water in a two-necked flask. A dropping funnel was so arranged that the tip was below the liquid level in the flask. Fifty ml of 35 per cent perchloric acid solution were added dropwise over a period of 4 hours. The carbon dioxide released by the addition of the acid to the barium carbonate was absorbed in 50 ml of 0.97 N NaOH in a bead tower, which was attached to the two-necked flask. After absorption of the carbon dioxide, the solution of sodium bicarbonate was tightly stoppered and stored at 10°C.

\textbf{4(5)-Amino-5(4)-imidazolecarboxamide}

The synthesis of 4(5)-amino-5(4)-imidazolecarboxamide was based on a modification of the synthesis of 4(5)-methylimidazole reported by Windaus and Knoop (1905), and the synthesis of 4(5)-amino-5(4)-imidazolecarboxamide from 4(5)-methylimidazole as reported by Windaus and Langenbeck (1923). Since this synthesis was carried out, Shaw and Woolley (1949) have reported a method starting with ethyl cyanoacetate which is converted in five steps to the desired compound with an over-all yield of 30 per cent. On the basis of this reported yield, the method of Shaw and Woolley would appear somewhat more advantageous than that to be described.
The synthesis of 4(5)-methylimidazole was carried out using dextrose, and ammonium and zinc hydroxides as the starting materials. Eight hundred gm of zinc sulfate were dissolved in 1300 ml of distilled water, and to this solution was added sufficient saturated sodium hydroxide solution to precipitate the zinc as zinc hydroxide. The precipitate was washed until the washings no longer turned phenolphthalein red. The zinc hydroxide was air dried and then dissolved in 1.3 Kg of concentrated ammonium hydroxide, to which 800 gm of Clinton dextrose had been added. This mixture was incubated at room temperature for 6 weeks. A dark brown precipitate began to form after several days incubation and continued to increase in quantity during the remainder of the incubation period.

The precipitate was removed by filtration and washed to remove excess ammonium hydroxide and dextrose. The moist precipitate was suspended in a liter of hot distilled water, which had been acidified with glacial acetic acid. Hydrogen sulfide gas was passed into the suspension and the zinc sulfide formed removed by filtration. Several treatments with H₂S were required to remove all traces of the zinc. The filtrates and hot water washings from the zinc sulfide were combined and the volume reduced under vacuum at 50-55°C.

Potassium carbonate was added to the solution (300 ml) until it was saturated. This saturated solution was placed
in a continuous ether extraction apparatus. After 73 hours extraction, the ether was evaporated under vacuum leaving a dark yellow oil. The yield was 95 ml and no attempt was made to crystallize the 4(5)-methylimidazole oil.

The synthesis of 4(5)-methyl-5(4)-nitroimidazole was conducted by placing 85 ml of the 4(5)-methylimidazole in a round bottom flask connected to a reflux condenser and adding 170 ml of concentrated nitric acid (d.1.42) from a dropping funnel. The flask was cooled in a dry ice-acetone mixture during the addition of the nitric acid. One hundred and seventy ml of concentrated H₂SO₄ were added slowly. The temperature of the reaction flask was allowed to return to room temperature. The flask was then heated for 2 hours in a boiling saline-water bath. Following this preliminary heating, the reaction mixture was refluxed at 150-160°C. for one-half hour, using a glycerol bath.

The reaction mixture was cooled to room temperature and then poured over cracked ice. Crystallization failed to occur until the pH was adjusted to 4.5 by the addition of sodium hydroxide. The crystalline material was removed by filtration. The yield was 8.3 gm and the colorless prisms melted at 250°C. The melting point of 4(5)-methyl-5(4)-nitroimidazole reported in the literature is 248°C. Additional 4(5)-methyl-5(4)-nitroimidazole was synthesized from 4(5)-methylimidazole.
The synthesis of 4(5)-nitro-5(4)-styrylimidazole was conducted by adding 31.2 gm of freshly distilled benzaldehyde, and 1.6 ml of piperidine to 15.6 gm of 4(5)-methyl-5(4)-nitroimidazole. This mixture was refluxed for 2 hours at 150-160°C. The mixture was then placed at 10°C. overnight and a golden-yellow mass of crystalline material formed. To remove tarry by-products, the crystalline material was extracted with hot ethanol and then several times with boiling water. The crystalline material was dissolved in hot glacial acetic acid and filtered. The styryl compound readily crystallized upon cooling and was removed by filtration. The final yield was 12.2 gm (46 per cent of theory), and the compound melted at 300-305°C. with decomposition. The literature reports the melting point to be 303°C.

The oxidation of the benzene side-chain of 4(5)-nitro-5(4)-styrylimidazole was carried out in the presence of alkaline potassium permanganate solution. Ten gm of the styryl compound were dissolved in 50 ml of 1N sodium hydroxide solution. The solution was placed in a salt-ice bath and 300 gm of crushed ice were added to the solution. Four hundred ml of a 5 per cent potassium permanganate solution were added slowly from a dropping funnel while the mixture was stirred. The addition of the permanganate solution required 3 hours and the slight excess remaining at the end of the reaction was destroyed by the addition of 0.5 ml of
formic acid. The mixture was heated on a steam bath and then
the manganese dioxide removed by filtration. The precipitate
was extracted with hot water and the washings added to the
filtrate. The volume of this solution was reduced under
vacuum at pH 4.5.

The reduced volume of the solution was cooled and
extracted with three volumes of ether to remove the benzoic
acid formed during the oxidation. The aqueous phase was
placed at 10°C. overnight, and the crystalline material that
formed was removed by filtration. The product sublimed at
225°C., and melted with decomposition at 260-264°C. The
yield was 6.3 gm (89 per cent of theory). The melting point
reported in the literature is 302-303°C. The subsequent
preparation of the methyl ester indicated that the reported
melting point may be incorrect.

The esterification of 4(5)-nitroimidazole-5(4)-carboxylic
acid was carried out by adding 4.3 gm of the compound to 83
ml of dry methanol containing 5 per cent dry hydrogen chloride.
The mixture was refluxed for one hour and on cooling, the
4(5)-nitroimidazole-5(4)-methylcarboxylate crystallized out.
The compound was removed by filtration and washed with
methanol. The pale yellow crystals sublimed at 160° and
melted at 210-212°C. The melting point reported in the
literature is 212-213°C.
To synthesize 4(5)-nitro-5(4)-imidazolecarboxamide, 1.0 gm of the ester was dissolved in 10 ml of 13.5 N ammonium hydroxide and allowed to stand at room temperature overnight. Orange-yellow needle crystals separated from this solution, which melted over a range of 285-295°C. The melting point reported in the literature is 291°C. Several recrystallizations failed to change the melting range of the compound. The yield was 0.5 gm (53 per cent of theory).

The reduction of the nitro group of 4(5)-nitroimidazole-5(4)-carboxamide was carried out using Adam's platinum catalyst and hydrogen. The yield was 8.6 per cent of the theoretical. A second hydrogenation was carried out under 2 atmospheres of hydrogen for 1.5 hours. The yield was 6.7 per cent of the theoretical. The solvent used for both hydrogenations was ethanol. The 4(5)-amino-5(4)-imidazolecarboxamide was crystallized as the hydrochloride, which melted at 255-256°C. The literature reports that the melting point is 256°C. The reactions in the synthesis of 4(5)-amino-5(4) imidazolecarboxamide are summarized by Figure 1.

Degradation Methods

Adenine and guanine

The results obtained by using C^{13} and N^{15} isotopes are dependent upon the methods of degrading isolated compounds.
Figure 1. Synthesis of 4(5)-amino-5(4)-imidazolecarboxamide
In this study, the purine bases, adenine and guanine, were the principle compounds degraded. The isotopic analyses were made on a Nier mass spectrometer. This instrument is somewhat less sensitive than the various counting instruments used for detecting radioactivity; therefore, samples of sufficient size to prevent dilution of isotopes beyond the precision of the mass spectrometer were degraded.

The isolated purine bases were pyrolyzed in a CuO combustion train with oxygen. The carbon dioxide formed was absorbed in 3N CO₂-free alkali and precipitated as barium carbonate by adding a saturated solution of barium nitrate. The collected samples were centrifuged, washed, and dried at 110°C. Additional samples of the bases were digested in the presence of concentrated H₂SO₄. The ammonium sulfate formed was treated with concentrated NaOH in a micro-Kjeldahl apparatus. The ammonia formed was distilled off and collected in a measured amount of H₂SO₄. These solutions were reduced to dryness at 110°C. The resulting ammonium sulfate was examined for N¹⁵ content.

**Stepwise degradation of guanine**

The stepwise degradation of guanine was carried out by two different procedures: (1) hydrolysis with hydrochloric acid to glycine, in which the carboxyl and methylene carbons are derived from carbons 4 and 5 of guanine, and (2) perman-
ganate oxidation to carbon dioxide, urea, and guanidine. The hydrolysis was carried out by a modification of the procedure of Wulff (1893) and the oxidation by the method described by Heinrich and Wilson (1950).

The hydrolysis of about 100 mg of isolated guanine hydrochloride was carried out by heating the compound with 7.0 ml of concentrated HCl in a sealed tube at 180-190°C. for 20 hours. After hydrolysis the chloride ions were removed by treating the solution with an excess of silver sulfate. The precipitate formed was removed by centrifugation, and the excess silver ions removed by H₂S treatment. An excess of freshly prepared cupric hydroxide was added to the filtrate and the mixture heated at 80-90°C. for 15 minutes. The excess cupric hydroxide was removed by filtration and the filtrate and washings were combined and reduced to a small volume on a steam bath. Three volumes of ethanol were added to the solution and the material placed at 10°C. overnight. The precipitated copper-glycine salt was removed by filtration and dissolved in a small amount of water. This solution was acidified and treated with H₂S to remove the copper ions. After filtration, the glycine was precipitated by the addition of cold ethanol. The glycine was removed and dried under vacuum.

A sample of this glycine was pyrolyzed as previously described. An additional sample was digested and the nitrogen
recovered as ammonium sulfate. The pyrolyzed sample represents the average concentration of isotopes from carbons 4 and 5 of the original guanine. Another portion of the glycine was decarboxylated with ninhydrin and the resulting carbon dioxide collected in 3N CO$_2$-free alkali. Barium carbonate was prepared from this solution and analyzed for C$^{13}$ content. This sample represents the isotope concentration from carbon 4 of the original guanine.

The permanganate oxidation of guanine gives rise to guanidine containing carbon 2, urea (mainly carbon 8), and carbon dioxide principally from carbons 4, 5, and 6. A sample of the isolated guanine hydrochloride was dissolved in CO$_2$-free water and placed in a small two-necked flask fitted with a dropping funnel with the inlet tube extending to the bottom. The flask was placed in an ice bath and 0.4 N potassium permanganate solution added slowly until it was no longer decolorized. The liberated carbon dioxide was aerated through 3 N CO$_2$-free sodium hydroxide. Finally, the contents of the flask were acidified and then heated briefly to drive off any additional carbon dioxide. The absorbed carbon dioxide was precipitated by the addition of a saturated solution of barium nitrate. The samples were centrifuged, washed, and dried at 110°C. This sample represents carbons 4, 5, and 6 of the original guanine. The isotopic content of carbon 6 was obtained by difference.
EXPERIMENTAL

Purine Degradation by Heterotrophic Bacteria

The limited studies of Lutwak-Mann (1936) and Stephenson and Trim (1938) on the extent of bacterial action on adenine and adenine derivatives indicated a need for further investigation of the degradation of purine bases by heterotrophic organisms. The elaboration of methods for the separation and identification of purine bases by partition chromatography by Vischer and Chargaff (1947), and Hotchkiss (1948), provided a means for determining possible intermediates in purine degradation not available to Lutwak-Mann and Stephenson and Trim at the time of their studies.

Cells of *Aerobacter aerogenes* and *Escherichia coli* were grown on dextrose-tryptose agar in Roux flasks for 20 hours at 37°C. The cells were harvested with 0.85 per cent saline solution and washed two times. A 10 per cent suspension (wet weight) was inoculated into test tubes containing adenine hydrochloride and phosphate buffer. These tubes were incubated at 37°C. for 4 hours. The amino-nitrogen produced by the hydrolytic deamination of the adenine was determined by the aeration method of Van Slyke and Cullen (1914) (Table 1).

Lutwak-Mann found that the hydrolytic deamination of adenine by *E. coli* never exceeded 30 per cent during a 1 hour
incubation period in the absence of added phosphate. In the presence of 0.1 M phosphate buffer, the percentage of hydrolytic deamination increased to 55. The results (Table 1) indicate a much lower rate of hydrolytic deamination from that found by Lutwak-Mann using similar cell concentrations.

Table 1
Deamination of Adenine by Non-proliferating
Aerobacter aerogenes and Escherichia coli

<table>
<thead>
<tr>
<th></th>
<th>Aerobacter aerogenes</th>
<th></th>
<th>Escherichia coli</th>
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<tbody>
<tr>
<td></td>
<td>mM NH₃</td>
<td>Per cent</td>
<td>mM NH₃</td>
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<tr>
<td>I</td>
<td>1.2 x 10⁻³</td>
<td>7.0</td>
<td>4.6 x 10⁻³</td>
</tr>
<tr>
<td>II</td>
<td>1.6 x 10⁻³</td>
<td>9.4</td>
<td>5.7 x 10⁻³</td>
</tr>
</tbody>
</table>

Each tube contained 2.0 ml of a 10 per cent cell suspension, 1.0 ml of a 0.1 M phosphate buffer, pH 7.1 (Experiment I), or 1.0 ml of a 1.0 M phosphate buffer, pH 7.1 (Experiment II), and 1.0 ml of a 0.017 mM solution of adenine (C₅H₇N₃ · HCl · 1/2 H₂O). Final volume = 4.0 ml. Temperature = 37°C. Incubation time = 240 minutes.

To further investigate the nature of the hydrolytic deamination of adenine, Warburg respirometer experiments, using lyophilized cells of E. coli, were carried out. The activity of lyophilized cells was determined using dextrose as the substrate. The cells used were grown in dextrose ammonium sulfate medium at 30°C. for 20 hours. The harvested cells were washed and lyophilized. The results are presented in Table 2. No oxygen was utilized following the hydrolytic
desamination of the adenine, nor did oxidation of hypoxanthine
occur. The oxidation of hypoxanthine is to be expected, if
the degradation of this compound occurs by way of xanthine
and uric acid as in animal tissue.

Table 2

Oxidation of Purine Bases by Non-proliferating
Escherichia coli

<table>
<thead>
<tr>
<th>µl O₂</th>
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<th>None</th>
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<tr>
<td>2.2 µM Adenine</td>
<td>2.6 µM Guanine</td>
<td>2.3 µM Hypoxanthine</td>
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</table>

Each Warburg flask contained 0.5 ml (50 mg dry weight)
of E. coli cell suspension, 0.5 ml of a 0.1 M phosphate
buffer solution, pH 7.0, 0.3 ml of a 5.0 per cent KOH
solution in the center well, and 0.5 ml of substrate.
Final volume = 2.3 ml. Temperature = 30.4°C. Incuba-
tion time = 240 minutes.

The cup contents were diluted to a volume of 10 ml and
0.03 ml aliquots were placed on filter paper strips. The
strips were partitioned using a butanol-water system (1 to 1)
at 30°C. for 24 hours. Since the mercuric nitrate-ammonium
sulfide treatment of Vischer and Chargaff proved to be
unsatisfactory using known amounts of the purine bases, the
method of Hotchkiss was used.

The dried chromatogram strips were sectioned and each 10
mm strip was extracted with 3.5 ml of a 0.01 M phosphate
buffer solution, pH 7.0. The extract was examined in a
Beckman spectrophotometer at 260 μM. The results obtained were inconclusive.

The experimental procedure was repeated using higher concentrations of adenine (7.4 μM) and hypoxanthine (6.6 μM) per Warburg flask. Aliquots of 0.05 ml of the diluted cup contents were partitioned and extracted as previously described. The results are shown in Table 3. A plot of the extinction values against distance on the chromatogram shows a peak at 90 mm with an absorption maximum at 245 μm in the case of adenine. This peak and absorption maximum suggested the presence of either hypoxanthine or guanine at this point on the chromatogram. The adenine maximum obtained on the Warburg flask contents was shifted slightly from that of the non-incubated control material. In addition, no peaks were found on the hypoxanthine chromatogram.

An experiment extending over a 24-hour period was carried out using *E. coli*, *A. aerogenes*, and *Proteus vulgaris*. These organisms were cultured on nutrient agar slants at 37°C for 16 hours. The growth from each slant was suspended in sterile saline solution using aseptic technique. The density of these suspensions was adjusted to the same turbidity using a Klett-Summerson instrument. One ml of the respective suspensions was then added to test tubes containing 1.0 ml of a 0.1 M phosphate buffer solution at pH 7.0, 2.0 ml of adenine solution (7.4 μM), and 1.0 ml of a 5.0 percent dextrose solution.
<table>
<thead>
<tr>
<th>Strip</th>
<th>Indicated Maximum Extinction</th>
<th>Extinction Value at 360 Mm</th>
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<td>0.0038</td>
</tr>
<tr>
<td>7.0</td>
<td>0.00</td>
<td>0.0038</td>
</tr>
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</tr>
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</tr>
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<td>0.0038</td>
</tr>
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</tr>
<tr>
<td>21.0</td>
<td>0.00</td>
<td>0.0038</td>
</tr>
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</table>

**Table 2**
This mixture had previously been sterilized at 15 pounds pressure for 30 minutes. The inoculated tubes were incubated at 37°C. Selected tubes were removed from the incubator after 3 hours, 8 hours, and 24 hours incubation. The contents of the tubes were centrifuged and 0.05 ml aliquot then removed and added to 3.5 ml of a 0.01 M phosphate buffer solution at pH 7.0. The absorption spectrum was determined on each aliquot using the Beckman spectrophotometer. The results obtained are indicated on Figures 2 and 3.

The graph of the absorption spectrum in the case of the *E. coli* cultures showed a shift in the absorption maximum from 260 μ to 255 μ after 8 hours' incubation, and from 255 μ to 250 μ after 24 hours' incubation. With the *A. aerogenes* cultures, the height of the absorption curve was lower after 8 hours' incubation and no shift in the peak was observed.

The reduction in height with both the *E. coli* and *A. aerogenes* curves may be attributed to the utilization of the adenine for the synthesis of cell material. In addition, the absorption peak returned to the control level after 24 hours' incubation in the *A. aerogenes* cultures. This result may possibly be explained by the formation of another compound having the same absorption maximum as adenine. The *Proteus vulgaris* aliquots gave the same picture as obtained with *A. aerogenes*.

An attempt was made to increase the rate of the shift of the absorption peak, using *E. coli*, at different pH levels
Figure 3. Absorption Spectrum on Aliquots from *Escherichia coli* Cultures
Figure 3. Absorption Spectrum on Aliquots from *Aerobacter aerogenes* Cultures
during an 8-hour period of incubation under the conditions previously indicated. It was found that the shift occurs somewhat more rapidly at pH 6.0 than at 7.0 or 8.0. Figure 4 indicates the results obtained with a similar type of an experiment carried out at pH 4.5. The shift in the absorption maximum from 260 μ to 255 μ was indicated after 7 hours' incubation. The aliquot examined for the preparation of this curve was treated with 0.06 ml of 5.0 N sodium hydroxide and then with 0.05 ml of 12 N HCl. The absorption spectrum was taken after each addition and the maximum shifted to 265 μ in the presence of the alkali and returned to 255 μ in the presence of the acid.

Hotchkiss has found that under such conditions the adenine peak at 260 μ will shift to 270 μ in the presence of alkali and return to 260 μ upon the addition of the acid. Guanine at 245 μ will shift to 275 μ in alkali and to 250 μ in acid. Hypoxanthine at 250 μ will shift to 280-265 μ in alkali and return to 250 μ in the presence of acid.

Xanthine has an absorption maximum at 270 μ. The values obtained on the 7-hour aliquot failed to indicate that the compound was any of the known purine bases.

The aliquot examined after 12 hours incubation had an absorption maximum at 250 μ. On the addition of alkali the peak shifted to 265 μ and returned to 250 μ on the addition of acid. These shifts were indicative of hypoxanthine. Similar results were obtained after 24 hours' incubation. A
Figure 4: Absorption Spectrum of the Products of Metabolism by Enzyme Data Set.

- Curve 1: Control
- Curve 2: 7 Hours
- Curve 3: 12 Hours
- Curve 4: 24 Hours

Absorption Wavelength, nm:
- 220
- 230
- 240
- 250
- 260
- 270
- 280
parallel experiment, in which no dextrose was added to the tubes, showed no reduction in the amount of adenine present nor any shift in absorption maximum throughout the entire 24-hour incubation period.

An aliquot of the 12 hour material was treated with xanthine oxidase prepared from cream by the method of Ball (1939). One-half ml of the previously tested xanthine oxidase preparation was added to the Beckman cell and the extinction values determined at 248 μ and 290 μ as described by Kalokar (1947a). The results obtained are indicated in Table 4.

The reaction as carried out depends on the oxidation of hypoxanthine to uric acid in the presence of the xanthine oxidase. As the reaction proceeds, there is a corresponding increase in the absorption maximum at 290 μ and a decrease at 248 μ. Uric acid has an absorption maximum at 290 μ.

The final product of the action of E. coli on adenine appears to be hypoxanthine. Occurrence of the compound formed after 7 to 8 hours incubation with an absorption maximum at 355 μ requires identification.

The possibility that a mixture of adenine and hypoxanthine might produce an absorption peak at 255 μ was considered. An equimolar solution of the two purine bases was prepared and the absorption spectrum determined in the Beckman spectrophotometer. The results are presented in Table 5. On addition of alkali the absorption maximum shifted to 265 μ and on addi-
Table 4
Action of Xanthine Oxidase on Aliquot from 12 Hour Culture

<table>
<thead>
<tr>
<th>Time Minutes</th>
<th>348 μm</th>
<th>290 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.712</td>
<td>0.037</td>
</tr>
<tr>
<td>3</td>
<td>0.660</td>
<td>0.060</td>
</tr>
<tr>
<td>6</td>
<td>0.635</td>
<td>0.090</td>
</tr>
<tr>
<td>9</td>
<td>0.610</td>
<td>0.125</td>
</tr>
<tr>
<td>12</td>
<td>0.597</td>
<td>0.165</td>
</tr>
<tr>
<td>15</td>
<td>0.575</td>
<td>0.213</td>
</tr>
<tr>
<td>20</td>
<td>0.560</td>
<td>0.252</td>
</tr>
<tr>
<td>25</td>
<td>0.540</td>
<td>0.323</td>
</tr>
<tr>
<td>35</td>
<td>0.513</td>
<td>0.415</td>
</tr>
<tr>
<td>45</td>
<td>0.500</td>
<td>0.510</td>
</tr>
<tr>
<td>55</td>
<td>0.488</td>
<td>0.560</td>
</tr>
<tr>
<td>60</td>
<td>0.480</td>
<td>0.605</td>
</tr>
</tbody>
</table>

The Beckman cell contained 0.05 ml aliquot of 12 hour culture, 3.5 ml of a 0.01 M phosphate buffer solution, pH 7.0, and 0.5 ml of xanthine oxidase preparation. Figures given are extinction values.
<table>
<thead>
<tr>
<th>μm</th>
<th>Adenine</th>
<th>Hypoxanthine</th>
<th>Adenine + Hypoxanthine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neutral</td>
</tr>
<tr>
<td>220</td>
<td>0.133</td>
<td>0.115</td>
<td>0.312</td>
</tr>
<tr>
<td>225</td>
<td>0.087</td>
<td>0.116</td>
<td>0.172</td>
</tr>
<tr>
<td>230</td>
<td>0.097</td>
<td>0.153</td>
<td>0.218</td>
</tr>
<tr>
<td>235</td>
<td>0.127</td>
<td>0.210</td>
<td>0.308</td>
</tr>
<tr>
<td>240</td>
<td>0.175</td>
<td>0.285</td>
<td>0.428</td>
</tr>
<tr>
<td>245</td>
<td>0.235</td>
<td>0.337</td>
<td>0.543</td>
</tr>
<tr>
<td>250</td>
<td>0.297</td>
<td>*0.353</td>
<td>0.630</td>
</tr>
<tr>
<td>255</td>
<td>0.347</td>
<td>0.322</td>
<td>*0.645</td>
</tr>
<tr>
<td>260</td>
<td>*0.372</td>
<td>0.270</td>
<td>0.625</td>
</tr>
<tr>
<td>265</td>
<td>0.327</td>
<td>0.200</td>
<td>0.510</td>
</tr>
<tr>
<td>270</td>
<td>0.247</td>
<td>0.133</td>
<td>0.370</td>
</tr>
</tbody>
</table>

*Indicates maximum absorption.
tion of acid returned to 255 μ. These results indicated that a mixture of adenine and hypoxanthine was present after 7 to 8 hours' incubation and this mixture produced an absorption maximum at 255 μ.

The results obtained are in agreement with those of Lutwak-Mann and Stephenson and Trim in that the final product of the action of E. coli on adenine is hypoxanthine. The failure to find additional breakdown products of hypoxanthine indicated that this purine base is the terminal product of the metabolism of adenine by E. coli but not with A. aerogenes.

In connection with experiments on the function of pteroyl-glutamic acid, not reported here, it was found that high concentrations of adenine have an inhibitory action on the growth of certain organisms. The basal medium of Roberts and Snell (1946), composed of hydrolyzed casein with added growth factors, was used in these experiments. The results obtained are reported in Tables 6 and 7.

It can be seen (Table 6) with Lactobacillus arabinosus (assay organism for PGA) that high concentrations of adenine had an inhibitory action on the growth obtained in the basal medium in the presence of PGA and PABA. In the presence of PABA alone, increased growth was obtained, and in the absence of this growth factor, the amount of growth obtained was less. The addition of PABA reduced the inhibitory action of the adenine.
Table 6

| Concentration | Adherence
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>191</td>
<td>178</td>
</tr>
<tr>
<td>385</td>
<td>196</td>
</tr>
<tr>
<td>313</td>
<td>388</td>
</tr>
<tr>
<td>580</td>
<td>375</td>
</tr>
<tr>
<td>131</td>
<td>505</td>
</tr>
<tr>
<td>320</td>
<td>475</td>
</tr>
<tr>
<td>323</td>
<td>444</td>
</tr>
<tr>
<td>380</td>
<td>388</td>
</tr>
<tr>
<td>380</td>
<td>470</td>
</tr>
</tbody>
</table>

**Table 6**

Effect of High Concentrations of Adherence

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Adherence</th>
<th>Strainpno Coupe</th>
<th>Leuadoploalline</th>
<th>Leuadopealine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Adherence, H2O1

on Bacterial Growth

Effect of High Concentrations of Adherence
<table>
<thead>
<tr>
<th>Organism</th>
<th>Micrococcus pyogenes var. aureus</th>
<th>Escherichia coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine·HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µg</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93</td>
</tr>
<tr>
<td>50 µg</td>
<td>40</td>
<td>85</td>
</tr>
<tr>
<td>100 µg</td>
<td>37</td>
<td>86</td>
</tr>
<tr>
<td>No Added Adenine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Added Adenine, PABA, and PGA</td>
<td>38</td>
<td>86</td>
</tr>
</tbody>
</table>

Tube contents: As indicated on Table 6.

<sup>a</sup>Klett-Summerson readings at 660 µm.
The growth of *Streptococcus faecalis* appeared to be dependent on the presence of PGA and was not influenced by the presence of either adenine or PABA. The situation appeared to be similar in the case of *Lactobacillus casei*. Feeney and Strong (1943) have reported an inhibitory effect by 100 µg of adenine or guanine upon the growth of *L. casei*. The data obtained does not indicate such a result. The growth of *Micrococcus pyogenes* var. *sueus* and *E. coli* (Table 7) appeared to be independent of the adenine, PABA, and PGA. In addition, the concentration of the adenine did not have any pronounced inhibitory action on the growth of either organism.

In connection with these determinations an additional experiment was conducted in which the effects of PABA and PGA on the growth of *M. pyogenes* var. *sueus* and *S. faecalis* were determined. The organisms were cultured in the basal medium of Roberts and Shell in the absence of PGA and PABA. The results of this experiment (Table 8) indicate that *S. faecalis* cannot synthesize PGA from PABA. Furthermore, *M. pyogenes* var. *sueus* does not require preformed PGA for its metabolism. These results are in keeping with those of Lampen and Jones (1946) who reported that PGA cannot overcome the sulfonamide inhibition in organisms; i.e., *M. pyogenes* var. *sueus* and *E. coli*, which do not require preformed PGA.
Table 8

Effects of PABA and PGA on the Growth
Micrococcus pyogenes var. aureus and
Streptococcus faecalis

<table>
<thead>
<tr>
<th>Organism</th>
<th>M. pyogenes var. aureus</th>
<th>Streptococcus faecalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Medium (Adenine 100 μg)</td>
<td>117&lt;sup&gt;a&lt;/sup&gt;</td>
<td>149</td>
</tr>
<tr>
<td>Without PGA</td>
<td>127</td>
<td>26</td>
</tr>
<tr>
<td>Without PABA</td>
<td>88</td>
<td>155</td>
</tr>
<tr>
<td>Without PGA and PABA</td>
<td>70</td>
<td>15</td>
</tr>
</tbody>
</table>

Tube contents: Inoculated with 0.06 ml of a 10 per cent washed saline suspension from a 24 hour culture grown in the complete basal medium. PABA concentration = 1.0 μg. PGA concentration = 0.02 μg. Final volume = 10 ml. Incubation time = 48 hours. Incubation temperature = 37°C. for M. p. var. aureus and 30°C. for S. faecalis.<sup>a</sup>

<sup>a</sup>Klett-Summerson readings at 660 μμ.
Purine Synthesis by *Aerobacter aerogenes* and *Escherichia coli*

**Role of 4(5)-amino-5(4)-imidazolecarboxamide**

The isolation of a non-acetylatable diazotizable amine from sulfonamide-containing cultures of *E. coli* has been reported by Stetten and Fox (1945). Shive, *et al.*, (1947) identified the amine as 4(5)-amino-5(4)-imidazolecarboxamide and Ravel, Eakin, and Shive (1948) suggested that under conditions of sulfonamide bacteriostasis of *E. coli*, the accumulation of 4(5)-amino-5(4)-imidazolecarboxamide represents a purine precursor. This view is based on inhibition studies in which these workers demonstrated that purines tend to reverse the inhibition effects of sulfonamides.

Since, up to this time, no attempts to substitute 4(5)-amino-5(4)-imidazolecarboxamide for adenine or the other purine requirements of various organisms had been reported, experiments were conducted to elaborate the possibility of such a substitution. The biological synthesis of 4(5)-amino-5(4)-imidazolecarboxamide with *E. coli* was attempted using the medium described by Stetten and Fox. The available strain of *E. coli* failed to produce the amine in the presence of 0.4 per cent of sulfadiazone. Stetten and Fox reported that the yield of the amine was increased by expanding the surface area of the culture exposed to the air. Ten liters of the medium of Stetten and Fox were prepared, consisting of 5.0 gm of
ammonium citrate, 3.0 gm of KH₂PO₄, 3.0 gm of Na₂CO₃, 2.0 gm of NaCl, 0.2 gm of MgSO₄, 0.005 gm of nicotinic acid, 2.0 gm of dextrose, and 10 ml of casein hydrolysate made to one liter. To this was added sufficient sulfadiazine to make a concentration of 0.4 per cent.

Nine liters of the medium were inoculated with a one-liter culture of E. coli in the same medium. The 10-liter culture was incubated at 30°C. while being vigorously aerated with sterile nitrogen for 18 hours. Aliquots removed before and after incubation were assayed for the presence of 4(5)-amino-5(4)-imidazolecarboxamide by the Rosenthal and Bauer (1939) modification of the Bratton and Marshall method for the determination of sulfonamides. The culture failed to produce the desired amine.

The publication of the experimental results of Ravel, et al., indicated that the addition of glycine to the culture medium produced a proportional increase in the amount of the amine formed. A buffered basal medium consisting of dextrose and ammonium sulfate was prepared, with 25 mg of sulfadiazine and 1.0 gm of glycine added. The 10 liters of medium were inoculated with E. coli and incubated at 30°C. for 18 hours without nitrogen aeration. This culture, also, failed to produce the desired amine.

A culture of A. aerogenes, under the same conditions, produced 4.9 μg of the amine per ml of medium. This yield
was comparable to that found by Stetten and Fox using *E. coli*, but somewhat less than that obtained by Ravel, *et al.* The culture procedure was repeated using nitrogen aeration and the amount of the amine formed under these conditions was 23.1 μg per ml of the culture medium.

An attempt was made to isolate the 4(5)-amino-5(4)-imidazolecarboxamide from the aerated culture medium using the method described by Stetten and Fox; however, the resulting yield was not sufficient to warrant further attempts at biological synthesis and isolation.

The chemical synthesis of 4(5)-amino-5(4)-imidazolecarboxamide had been described by Windaus and Langenbeck in 1923. The synthesis of 4(5)-amino-5(4)-imidazolecarboxamide was carried out as indicated in an earlier part of this thesis.

Experiments were conducted to determine whether certain organisms could utilize the synthesized 4(5)-amino-5(4)-imidazolecarboxamide. The organisms used were cultured in 1 per cent dextrose yeast extract medium and then inoculated into a basal medium containing dextrose and ammonium sulfate with added 4(5)-amino-5(4)-imidazolecarboxamide. The cultures were incubated at 37°C for 24 hours and then assayed for the amine. The results are shown in Table 9. The data indicated no utilization of the amine under the conditions of the experiment. Shive (1949) has since reported that 4(5)-amino-5(4)-imidazolecarboxamide can substitute for approximately 10
per cent of the purine requirements of certain *Lactobacilli*. All additional attempts to demonstrate utilization of the amine by various organisms under a variety of conditions failed.

### Table 9

**Utilization of 4(5)-amino-5(4)-imidazolecarboxamide**

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Non-incubated</th>
<th>Incubated</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>193&lt;sup&gt;a&lt;/sup&gt;</td>
<td>195</td>
</tr>
<tr>
<td><em>Aerobacter aerogenes</em></td>
<td>300</td>
<td>208</td>
</tr>
<tr>
<td><em>Lactobacillus arabinosus</em></td>
<td>191</td>
<td>202</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>194</td>
<td>197</td>
</tr>
<tr>
<td><em>Micrococcus pyogenes</em></td>
<td>193</td>
<td>185</td>
</tr>
<tr>
<td><em>var. aureus</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Klett-Summerson readings at 540 mμ.

### Glycine utilization

The reported findings by Ravel, *et al.*, that glycine stimulates the production of 4(5)-amino-5(4)-imidazolecarboxamide by *E. coli* in the presence of bacteriostatic concentrations of sulfonamide suggested that this amino acid might function as a precursor in the formation of bacterial purines. The investigations of Buchanan, *et al.*, with animal tissue, as well as the findings reported by Abrams, Hammersten, and Shemin with yeast, supported the possibility of glycine playing such a role in bacterial purine synthesis.
Earlier experiments had indicated that the available strain of *E. coli* did not produce 4(5)-amino-5(4)-imidazole-carboxamide. For this reason, preliminary experiments to determine glycine utilization were carried out with *A. aerogenes*. This organism cultured in small volumes of dextrose ammonium sulfate medium utilized 90 to 95 per cent of added glycine in a period of 8 hours at 30°C.

Ten liters of the above medium were prepared with 0.04 per cent added glycine. The complete procedure used has previously been described under Methods. The culture was vigorously aerated by means of sterile air passing through a fritted glass tube. After 8 hours' incubation at 30°C., the cells were harvested in a Sharples centrifuge and lyophilized. The wet weight of the cells was 83.8 gm and the lyophilized weight (dry weight) was 18.7 gm. Comparable yields of cells were obtained in the absence of added glycine. This indicated the added amino acid was not in itself stimulatory. The amount of glycine present in the culture medium after incubation was determined on aliquots of the cell-free medium by the method of Alexander, *et al.*, (1945) as previously described.

The yield of cell material (18.7 gm dry weight) indicated that, provided a suitable method could be found, the isolation of the purine bases from the nucleic acids might be feasible. Belozersky (1947) has reported that 5 hour cultures of *E. coli* contain 20 to 32 per cent nucleic acid, 20 hour cultures 14
per cent, and 48 hour cultures 10 per cent. These results indicate that the greatest concentration of nucleic acid material in the *E. coli* cells occurs during the early stages of the logarithmic growth phase, i.e., 4 to 8 hours. For this reason, *A. aerogenes* cultures were incubated at 30°C for 8 hours. The logarithmic growth phase for this organism is from 4 to 6 hours at 32 to 37°C, according to Jennison (1935).

The possibility of demonstrating glycine utilization by non-proliferating *A. aerogenes* cells was examined. A 10-liter culture of the organism was grown and the fresh washed cells were suspended in a small volume of the basal medium. Each ml of medium contained 0.5 gm (wet weight) of cells. To 100 ml of the cell suspension 0.5 gm of glycine was added. Control flasks were also included without added glycine. The flasks were incubated at 37°C. for 2 hours.

The contents of each flask were deproteinized with 10 per cent TCA (trichloroacetic acid) solution and then made to volume. Using the orcinol test for ribose, it was determined that no increase of RNA had occurred during the incubation period. Glycine utilization was determined using the method of Alexander, *et al.*, and it was found that 45.2 per cent of the added glycine had been utilized. These results indicated that a resting cell procedure would not produce added nucleic acid material and thus no incorporation of glycine into the cell nucleic acid.
Experimental details of purine isolation procedure

Preliminary to the study of glycine utilization as a purine precursor, it was found necessary to establish the completeness of various nucleic acid extraction procedures. The literature reports considerable variation in the amounts of nucleic acid material extracted from the same species of organism. The amount obtained depended upon the method used in each case, and exact procedures for \textit{A. aerogenes} and \textit{E. coli} were not given.

\textit{A. aerogenes} and \textit{E. coli} were grown as previously described in the presence of 0.04 per cent added glycine. The cells were harvested after 8 hours' incubation, and lyophilized. The wet weight of \textit{A. aerogenes} obtained from 20 liters of medium, incubated at 37°C., was 96 gm and the dry weight 21.5 gm. The wet weight of \textit{E. coli} cells was 56.9 gm and the dry weight 11.4 gm. The \textit{E. coli} cells utilized 53.5 per cent of the added glycine, whereas, the \textit{A. aerogenes} utilized the added glycine completely.

The estimation of the effectiveness of the various extraction procedures was based on the orcinol reaction for ribose and the diphenylamine reaction for deoxyribose. Five hundred mg of the dried cell material were used with each extraction procedure. To establish a base value, the cell material was suspended in 10 per cent TCA and immediately one-half of the suspended material was centrifuged. Suitable
dilutions of the supernatant liquid and the uncentrifuged portion were made and the amounts of RNA and DNA present determined. The results of these determinations are presented in Table 10.

Table 10

<table>
<thead>
<tr>
<th></th>
<th>Aerobacter aerogenes (μg RNA)</th>
<th>Aerobacter aerogenes (μg DNA)</th>
<th>Escherichia coli (μg RNA)</th>
<th>Escherichia coli (μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspended cell</td>
<td>379.4</td>
<td>595.9</td>
<td>380.3</td>
<td>787.8</td>
</tr>
<tr>
<td>Material</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>90.2</td>
<td>227.3</td>
<td>43.5</td>
<td>227.3</td>
</tr>
<tr>
<td>Total</td>
<td>469.6</td>
<td>823.2</td>
<td>423.8</td>
<td>1015.1</td>
</tr>
</tbody>
</table>

All values are based on one ml amounts and on an assumed average molecular weight for DNA-monomonucleotide and RNA-monomonucleotide.

The dry cell material was subjected to various TCA treatments. In particular, those of Morse and Carter (1949), Schneider (1945), and Abrams, et al., were used. The values obtained are presented with explanatory note on the methods used in Tables 11 and 12. The extraction method of Sevag, et al., (1940) was also carried out; however, the presence of acid during the drying steps in this procedure resulted in carmelization of part of the carbohydrate material present and correspondingly low values for RNA and DNA were the result.
Table 11
Application of Various Nucleic Acid Extraction Procedures to Bacteria

<table>
<thead>
<tr>
<th>Method and Material Examined</th>
<th>µg RNA</th>
<th>µg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morse and Carter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% TCA (r.t.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% TCA (hot)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Debris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. aerogenes</td>
<td>106.5</td>
<td>343.3</td>
</tr>
<tr>
<td>E. coli</td>
<td>86.9</td>
<td>412.9</td>
</tr>
</tbody>
</table>

| Schneider                   |        |        |
| 7% TCA (ice cold)           |        |        |
| 5% TCA (wash)               |        |        |
| 5% TCA (hot)                |        |        |
| 5% TCA (hot)                |        |        |
| Cell Debris                 |        |        |
| A. aerogenes                | 86.9   | 286.8  |
| E. coli                     | 69.5   | 326.0  |

- a5 per cent TCA heated 30 minutes in boiling water bath.
- b7.0 per cent TCA cooled in salt ice-bath for 2 hours with stirring.
- c5.0 per cent TCA ice cold wash.
- d5.0 per cent TCA heated 15 minutes at 90°C and repeated.
Table 12

Application of Abrams, et al., Extraction Procedure

<table>
<thead>
<tr>
<th>Material Examined</th>
<th>EtOH-(^a)</th>
<th>H(_2)O(^b)</th>
<th>0.05N H(_2)O(^c)</th>
<th>7% TCA(^d) (hot)</th>
<th>Cell Debris</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ether Wash</td>
<td></td>
<td>Extraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. aerogenes</td>
<td>33.5</td>
<td>83.0</td>
<td>0</td>
<td>234.7</td>
<td>0</td>
</tr>
<tr>
<td>E. coli</td>
<td>31.9</td>
<td>84.5</td>
<td>0</td>
<td>247.7</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th>µg RNA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A. aerogenes</td>
<td>55.6</td>
<td>197.0</td>
<td>0</td>
<td>197.0</td>
<td>121.3</td>
</tr>
<tr>
<td>E. coli</td>
<td>20.9</td>
<td>224.7</td>
<td>20.0</td>
<td>338.4</td>
<td>146.5</td>
</tr>
</tbody>
</table>

|                   |            |               |                      | µg DNA             |            |

\(^a\)Sonic treated cells (one hour at 9 kc.) extracted with hot 3 to 1 alcohol-ether mixture.

\(^b\)Cell precipitate washed with water and centrifuged.

\(^c\)Acid extraction of cell precipitate.

\(^d\)Dissolved in 0.1N NaOH and heated with 7.0 per cent TCA for 15 minutes at 90°C.
Additional methods examined were extraction with 0.1 N HCl, 0.09 N NaOH, and enzymatic digestion (trypsin). In the case of the trypsin, the cell material was digested in 0.8 per cent NaHCO₃ solution containing 100 mg of trypsin powder (Pfanstiehl 1-110) at 37°C. for 48 hours under toluene. The *A. aerogenes* sample showed 286.8 µg of RNA and the *E. coli* 291.2 µg. The DNA determinations were unsatisfactory due to interfering substances. The results of the acid and alkaline extractions are shown in Table 13. A summary of the various values obtained with the different extraction treatments is presented in Table 14.

It has been noted that nucleic acid material can be but poorly extracted from certain bacterial species. The ease or difficulty encountered in such extractions depends to a large measure upon the composition and/or structure of the bacterial cell wall. To overcome in part some of these difficulties, the cells used in examining the various extraction procedures were lyophilized. Such cells have increased permeability and may easily be made into a homogeneous suspension.

It can be seen from Table 14 that the use of 0.09 N NaOH with heating in a boiling water bath for 10 minutes yielded the greatest amount of RNA from *A. aerogenes*. On the other hand, the yield of DNA was greatest in the 5.0 per cent TCA extraction procedure of Schneider. With *E. coli*, 5.0 per cent TCA extraction and 0.09 N NaOH proved to be equally
Table 13

Extraction of Nucleic Acids from Bacteria by Acid or Alkali Treatment

<table>
<thead>
<tr>
<th></th>
<th>A. aerogenes</th>
<th></th>
<th>E. coli</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg RNA</td>
<td>μg DNA</td>
<td>μg RNA</td>
<td>μg DNA</td>
</tr>
<tr>
<td>Cells + 0.1 N HCl</td>
<td>65.2</td>
<td>40.4</td>
<td>39.1</td>
<td>60.6</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells + 0.1 N HCl</td>
<td>356.4</td>
<td>292.9</td>
<td>347.7</td>
<td>348.5</td>
</tr>
<tr>
<td>(heated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells + 0.09 N NaOH</td>
<td>369.4</td>
<td>303.0</td>
<td>382.5</td>
<td>440.4</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells + 0.09 N NaOH</td>
<td>473.7</td>
<td>424.3</td>
<td>408.5</td>
<td>272.7</td>
</tr>
<tr>
<td>(heated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experimental conditions:

a Cells suspended in 0.1 N HCl and then heated for 10 minutes in a boiling water bath and then centrifuged. Control samples were not heated. Samples heated 20 minutes gave similar results.

b Cells suspended in 0.09 N NaOH and then heated for 10 minutes in a boiling water bath. After centrifuging 5.0 ml aliquot was neutralized with HCl. Control samples were not heated. Samples heated 20 minutes gave similar results.
<table>
<thead>
<tr>
<th>Method</th>
<th>A. aerogenes</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg RNA</td>
<td>µg DNA</td>
</tr>
<tr>
<td>Base</td>
<td>469.6</td>
<td>823.2</td>
</tr>
<tr>
<td>Morse and Carter</td>
<td>343.3</td>
<td>393.9</td>
</tr>
<tr>
<td>Schneider</td>
<td>286.8</td>
<td>494.9</td>
</tr>
<tr>
<td>Abrams, et al.</td>
<td>234.7</td>
<td>197.0</td>
</tr>
<tr>
<td>0.1 N HCl</td>
<td>356.4</td>
<td>392.9</td>
</tr>
<tr>
<td>Heated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 N HCl</td>
<td>65.3</td>
<td>40.4</td>
</tr>
<tr>
<td>Heated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.09 N NaOH</td>
<td>473.7</td>
<td>424.2</td>
</tr>
<tr>
<td>Heated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.09 N NaOH</td>
<td>369.4</td>
<td>303.0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>286.8</td>
<td></td>
</tr>
</tbody>
</table>

*The amount of RNA or DNA in the principle extracted portion.*
effective for RNA. The DNA yield was greatest in the 5.0 per cent TCA extraction of the Schneider procedure. On the basis of the results, and without resorting to preliminary cold TCA treatment, the extraction of the cell material with hot TCA (5.0 per cent) yielded the greatest combined quantity of DNA and RNA from the cells in question.

It should be noted that neither sonic nor enzymatic treatment materially increased the amount of extractable nucleic acid material obtained from the _A. aerogenes_ and _E. coli_ cells.

To determine the effectiveness of TCA extraction on large volumes of cells, 10 gm of dried _A. aerogenes_ were suspended in 100 ml of a 5.0 per cent solution of TCA and heated 30 minutes in a boiling water bath. The material was cooled and centrifuged and the extraction repeated on the cell debris three additional times. Aliquots of the supernatant were assayed for RNA by the orcinol test. The results showed that 66.4 per cent of the total extracted RNA was contained in the supernatant of the first extraction and a total of 94 per cent had been removed after the second extraction.

It was of interest to note that the supernatant solutions were a bright yellow color. The greatest amount appeared in the first extract and lesser amounts in subsequent extracts. Aliquots of the supernatants were examined in the Klett-Summerson colorimeter and it was found that the color intensi-
ties paralleled the amounts of extracted RNA. This yellow pigment could be adsorbed with Norite "A". Attempts to determine an absorption maximum in the visible range were not successful.

Levene and Jorpes (1939) studied the rates of hydrolysis of nucleotides under a variety of conditions and found that heating with 1.0 N H₂SO₄ in a sealed tube for 60 minutes at 100°C was generally the most effective hydrolytic procedure. Vischer and Chargaff (1948) reported that 1.0 N H₂SO₄ hydrolysis of yeast nucleic acid produced more consistent results than the cold methanol-gaseous HCl treatment reported by Levene and Bass (1931). Furst, Roll, and Brown (1950) reported the use of 0.4 N H₂SO₄ with refluxing for 2 hours as a method for the hydrolysis of RNA and DNA extracted from rat tissue.

The effect of 1.0 N H₂SO₄ and 1.0 N HCl, under refluxing conditions, on adenine and guanine was determined by measuring the amount of the bases present before and after treatment. The measurements were carried out on a Beckman spectrophotometer and the results indicated no loss of material under the conditions of the experiment. An aliquot of the TCA extract of A. aerogenes cells was hydrolyzed with 1.0 N H₂SO₄ by refluxing for one hour. It is well known that the liberation of pyrimidines from nucleic acids requires extreme drastic treatment; i.e., autoclaving at 175°C for several hours in the presence of 20 per cent HCl or 35 per cent H₂SO₄.
Refluxing of the cell extract with 1.0 N \( \text{H}_2\text{SO}_4 \) for one hour permitted the liberation of only the purines from the nucleic acid material.

Aliquots of the hydrolyzed cell extract were treated by two different methods to precipitate the purines as copper or silver salts. The precipitation of the copper purines is based on the method described by Hitchings and Fiske (1941) in which \( \text{NaHSO}_3 \) and \( \text{CuSO}_4 \) are used. The silver precipitation is based on a modification of the procedure described by Levene (1922). Following the precipitation, the salts were removed by centrifugation and washed. The free purines were obtained by dissolving the salts in a small amount of 3 N \( \text{H}_2\text{SO}_4 \), the copper or silver ions removed by precipitation with \( \text{H}_2\text{S} \), and the sulfides washed with additional hot dilute acid. The filtrates were aerated to remove all traces of \( \text{H}_2\text{S} \) and then made to volume. Aliquots were examined in the Beckman spectrophotometer and on the basis of the final concentration of the purine base present, the silver precipitation method was more effective.

The problem at this point was whether sufficient quantities of adenine and guanine could be isolated from 1.0 gm (dry weight) of \textit{A. aerogenes} to make isotopic experiments feasible. The future limitation on the amount of cell material obtainable will be the supply of isotopically labeled glycine available. The synthesis of this compound had been previously described under Methods.
The 18 gm of cell material was divided into two portions and each one extracted with 100 ml of a 5.0 per cent TCA solution three times by heating in a boiling water bath. The combined cell debris was extracted two additional times in a similar manner. After each heating, the cell debris was removed by centrifugation and finally the combined extracts were filtered through Whatman no. 2 filter paper.

The volume of the combined extracts was reduced under vacuum at 55 to 60°C. to 300 ml. This convenient volume of solution was hydrolyzed by refluxing for one hour after the addition of sufficient 10 N H₂SO₄ to make the extract 1.0 N with respect to the H₂SO₄. Following hydrolysis, the extract was adjusted to pH 1.5 by the addition of solid barium hydroxide, and the barium sulfate formed, removed by centrifugation and washed with hot water. The washings and supernatant liquid were combined. An aliquot of this solution was examined on the Beckman spectrophotometer and the extinction maximum was found to be at 255 mp.

The silver purines were precipitated by the addition of an excess of silver nitrate. The silver salts of the purines are insoluble in acid solution, while the silver salts of pyrimidines are soluble under these conditions. The characteristic solubility differences of the silver salts of the purines and pyrimidines have been determined by Kossel (1898). The silver purines were collected by centrifugation, and washed
several times with hot water. The supernatant and washings showed an absorption maximum at 270 μm.

The silver purines were suspended in 100 ml of distilled water and then dissolved by the addition of 6.0 N HCl. The solution was treated with H₂S and the precipitate removed by filtration. The Ag₂S was washed repeatedly with boiling water and the washings added to the filtrate. An aliquot of this solution showed an absorption maximum at 255 μm. The pH of the solution was adjusted to 4.0 to 4.5 by the addition of dilute NaOH. The precipitate that formed (guanine) was removed by centrifugation and washed with hot water. The supernatant liquid was combined with the washings and showed an absorption maximum at 360 μm. The adenine present in this solution was precipitated as the picrate.

The precipitated guanine was suspended in a small volume of water and then dissolved by the addition of 6.0 N HCl. The pH was adjusted to 4.0 to 4.5 by the addition of dilute NaOH and the precipitated guanine removed and washed with water. The washed precipitate was dissolved in 4.0 ml of 6.0 N HCl by heating. After the solution had cooled an equal volume of ethanol was added and the solution placed in the refrigerator overnight. The guanine formed was removed and recrystallized in the same manner as described above. The final yield was 509 mg of guanine hydrochloride.
The adenine picrate was suspended in 0.1 N HCl and, after continuous ether extraction for 72 hours, a solution of adenine was obtained. This solution was evaporated to dryness on a steam bath and the residue formed dissolved in 4.0 ml of 6.0 N HCl. The procedure from this point was the same as described for the guanine. The final yield after recrystallization was 384 mg of adenine hydrochloride.

The purity of the isolated adenine and guanine was determined on the Beckman spectrophotometer, using the method of Hotchkiss. The results of these determinations are shown in Table 15.

The pyrimidines were isolated from the supernatant liquid remaining after the precipitation of the silver purines. The excess silver ions were removed by H₂S treatment. The volume was reduced under vacuum to 60 ml. To 30 ml of this solution there were added 15 ml of 20 N HCOOH and this mixture sealed in a Carius combustion tube and heated at 175-180°C for 3 hours. Eight ml of concentrated H₂SO₄ were added to the remainder (30 ml) of the solution and this mixture was also treated in a Carius combustion tube as indicated above. After cooling, the tubes were opened and the contents filtered. The pyrimidines were precipitated at pH 10 to 11 as the silver salts. These silver salts were purified in the usual manner.
Table 15
Absorption Data on Isolated Adenine and Guanine

<table>
<thead>
<tr>
<th>Åµ</th>
<th>Guanine</th>
<th></th>
<th></th>
<th>Adenine</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutral</td>
<td>Alkaline</td>
<td>Acid</td>
<td>Neutral</td>
<td>Alkaline</td>
<td>Acid</td>
</tr>
<tr>
<td>240</td>
<td>0.212</td>
<td>0.185</td>
<td>0.204</td>
<td>0.107</td>
<td>0.073</td>
<td>0.112</td>
</tr>
<tr>
<td>245</td>
<td>*0.243</td>
<td>0.188</td>
<td>0.237</td>
<td>0.152</td>
<td>0.093</td>
<td>0.158</td>
</tr>
<tr>
<td>250</td>
<td>0.242</td>
<td>0.132</td>
<td>*0.246</td>
<td>0.199</td>
<td>0.123</td>
<td>0.305</td>
</tr>
<tr>
<td>255</td>
<td>0.209</td>
<td>0.141</td>
<td>0.220</td>
<td>0.238</td>
<td>0.160</td>
<td>0.245</td>
</tr>
<tr>
<td>260</td>
<td>0.172</td>
<td>0.155</td>
<td>0.180</td>
<td>*0.259</td>
<td>0.203</td>
<td>0.264</td>
</tr>
<tr>
<td>265</td>
<td>0.159</td>
<td>0.178</td>
<td>0.162</td>
<td>0.233</td>
<td>0.233</td>
<td>*0.265</td>
</tr>
<tr>
<td>270</td>
<td>0.168</td>
<td>0.199</td>
<td>0.162</td>
<td>0.173</td>
<td>*0.235</td>
<td>0.227</td>
</tr>
<tr>
<td>275</td>
<td>0.167</td>
<td>*0.204</td>
<td>0.158</td>
<td>0.072</td>
<td>0.177</td>
<td>0.163</td>
</tr>
<tr>
<td>280</td>
<td>0.153</td>
<td>0.185</td>
<td>0.145</td>
<td>0.023</td>
<td>0.110</td>
<td>0.103</td>
</tr>
<tr>
<td>285</td>
<td>0.123</td>
<td>0.143</td>
<td>0.121</td>
<td>0.026</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>290</td>
<td>0.084</td>
<td>0.087</td>
<td>0.086</td>
<td>0.007</td>
<td>0.021</td>
<td></td>
</tr>
</tbody>
</table>

*Indicates absorption maximum.
Metabolic origin of atoms 4, 5, and 7 of adenine and guanine

The extent of incorporation of isotopically labeled glycine in purine bases of bacterial nucleic acids was determined by the isolation of adenine and guanine from lyophilized cell material. The isotopically labeled glycine used contained 7.92 per cent excess $^{13}$C in the carboxyl group. The glycine was synthesized from $^{13}$C-KCN as described in the section on Methods. The *A. aerogenes* were grown in a dextrose ammonium sulfate medium with 0.04 per cent added glycine. The yield of cells obtained from 10 liters of medium was 86.5 gm (wet weight) and 19.3 gm (dry weight). The cell nitrogen was 10.6 per cent and 92 per cent of the added glycine was utilized.

The isolated cellular adenine and guanine were pyrolyzed in a CuO combustion train with oxygen. The carbon dioxide formed was absorbed in 3 N CO$_2$-free alkali and precipitated as barium carbonate by adding a saturated solution of barium nitrate. The carbonate samples were centrifuged, washed, and then dried at 110°C. The isotopic analyses were made on a Nier mass spectrometer. The results of these analyses are presented in Table 16. The results obtained clearly indicate an incorporation of the $^{13}$C isotope into both the adenine and guanine isolated from the cell material. No $^{13}$C was found in the pyrimidines isolated from the nucleic acids from the *A. aerogenes*. 
The immediate question was whether the added glycine was decarboxylated and the resulting carbon dioxide incorporated in the purine bases. To answer this question in part, doubly labeled glycine was synthesized from C\(^{13}\)-KCN and N\(^{15}\)-potassium phthalimide as previously described. The glycine was added to 20 liters of dextrose ammonium sulfate medium.

**Table 16**

Incorporation of Carboxyl Labeled Glycine in Bacterial Purines

<table>
<thead>
<tr>
<th>Glycine(^{13})</th>
<th>Adenine</th>
<th>Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{13})C</td>
<td>(^{13})C</td>
<td>Per Cent</td>
</tr>
<tr>
<td>Per Cent Incorporation</td>
<td>Incorporation</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>7.92(^{b})</td>
<td>1.55</td>
<td>19.5</td>
</tr>
</tbody>
</table>

\(^{a}\)Carboxyl \(^{13}\)C-labeled glycine.

\(^{b}\)All values are given as atom per cent excess \(^{13}\)C.

*A. aerogenes* was cultured as previously noted in the presence of 0.04 per cent of the doubly labeled glycine (carboxyl-\(^{13}\)C and amino-nitrogen \(^{15}\)N). The yield of cells from 20 liters of medium was 131 gm (wet weight) and 30.6 gm (dry weight). The utilization of the glycine was 83 per cent in one flask, and for the other, 86 per cent. The adenine and guanine were isolated and pyrolyzed as previously described. Additional samples of the bases were digested in the presence of concentrated H\(_2\)SO\(_4\). The ammonia formed was distilled off in a micro-Kjeldahl apparatus and collected in
a measured amount of $\text{H}_2\text{SO}_4$. These solutions were reduced to dryness at 110°C. The resulting ammonium sulfate was examined for $^15N$ content. The results of the mass spectrometer analyses are presented in Table 17. A second experiment was also conducted with doubly labeled glycine using a concentration of 0.016 per cent. The results are also reported in Table 17. The experimental evidence demonstrated that the isotopes are both incorporated into the adenine and guanine of the bacterial nucleic acids.

In order to more completely determine whether glycine is incorporated as such into adenine and guanine, a series of degradation procedures were carried out. The isolated guanine was degraded by the method of Wulff (1893) to yield atoms 4, 5, and 7 of this base. The method depends on the hydrolysis of guanine with concentrated HCl in a sealed tube. The reaction is conducted at 190°C. for 30 hours. The products of the hydrolysis are formic acid, carbon dioxide, ammonia, and glycine. The glycine carboxyl carbon and methylene carbon are derived from carbons 4 and 5 of the guanine. The amino group arises from nitrogen atom 7. The glycine formed was isolated as described in the section on Methods.

Samples of the glycine were pyrolyzed and the carbon dioxide collected and analyzed for isotope content. Additional samples were digested and the ammonia formed examined for $^15N$ content. The guanine used in this particular degradation was that isolated in Experiment I (Table 17). The results are
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Glycine</th>
<th>N\textsubscript{150}</th>
<th>C\textsubscript{14} per Cent</th>
<th>N\textsubscript{15} per Cent</th>
<th>Guanine</th>
<th>N\textsubscript{15} per Cent</th>
<th>Incorpor.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10.9</td>
<td>3.65</td>
<td>39.3</td>
<td>3.35</td>
<td>30.7</td>
<td>3.95</td>
<td>42.5</td>
</tr>
<tr>
<td>III</td>
<td>10.9</td>
<td>2.24</td>
<td>34.1</td>
<td>1.50</td>
<td>13.7</td>
<td>1.13</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Values given as atom per cent excess C\textsubscript{14} and N\textsubscript{15} in carboxyl-C\textsubscript{14}, amino group N\textsubscript{15} glycine.

0.04 per cent added glycine.

40.076 per cent added glycine.
presented in Table 18. In addition, the guanine from Experiment II was hydrolyzed and the isolated glycine decarboxylated with ninhydrin. The liberated carbon dioxide was collected in 3 N CO₂-free alkali and precipitated as barium carbonate.

Table 18

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Carbon Atoms 4 plus 5</th>
<th>Carbon Atom 4</th>
<th>Nitrogen Atom 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C¹³ Per Cent Found</td>
<td>C¹³ Per Cent Found</td>
<td>N¹⁵ Per Cent Found</td>
</tr>
<tr>
<td>I</td>
<td>3.76ᵃ (3.95ᵇ) 95.1</td>
<td>2.96 (4.06)</td>
<td>73.0</td>
</tr>
<tr>
<td>II</td>
<td>1.05 (1.12) 93.7</td>
<td>1.27 (1.78)</td>
<td>71.3</td>
</tr>
</tbody>
</table>

ᵃAll values are given as atom per cent excess.
ᵇThe values given in parenthesis are for total incorporation as shown in Table 17.

Another portion of this glycine was digested for ammonia and the resulting ammonium sulfate examined for N¹⁵ content. The results of the isotopic analyses are also reported in Table 18. The incorporation figures obtained for N¹⁵ and C¹³ from the same sample of glycine do not agree on the basis of percentages of recovery; however, the agreement between the glycine samples from Experiments I and II is of the same order. It can therefore be assumed that the differences in percentages are ones of method and not a true
difference in the incorporation of the two isotopes into the guanine.

To determine whether acetic acid might be aminated to form glycine and then incorporated into the bacterial purines, an experiment was carried out in which A. aerogenes was cultured in the presence of carboxyl labeled sodium acetate. The isotopic compound used in this experiment was synthesized from isotopic carbon dioxide. The details of the method used are presented under Methods. The organism was grown in a dextrose ammonium sulfate medium with 3.5 gm of sodium acetate added to each 10 liters of the medium. The sodium acetate contained 3.03 per cent excess C\textsuperscript{13} in the carboxyl group. The yield of cells from 20 liters of medium was 88 gm (wet weight) and 30.5 gm (dry weight). The adenine and guanine were isolated in the previously described manner.

The carbonate samples from the purine bases showed that no incorporation of the C\textsuperscript{13} had occurred. These results can be explained in one of two ways. Either the acetate was not aminated to form glycine, or the acetate added was diluted sufficiently by acetate formed by the cells to place the samples analyzed beyond the analytical limits of the mass spectrometer. Steam distillation of aliquots of the cell-free culture medium after incubation showed a four-fold increase in the amount of acid present over that at the start of the incubation period.
Metabolic origin of carbon 6 of adenine and guanine

The studies of Buchanan, et al., have shown that glycine, or a metabolic derivative of glycine, is involved in the synthesis of uric acid isolated from pigeons. Heinrick and Wilson (1950) have demonstrated the incorporation of labeled glycine into both adenine and guanine isolated from rat tissue. These workers have also demonstrated the utilization of carbon dioxide for purine synthesis in animals. In consideration of these findings and that of the incorporation of glycine into bacterial purines, it appeared likely that carbon dioxide might also be incorporated into bacterial purines. With such a possibility in mind, an experiment using C\textsuperscript{13}-sodium bicarbonate was carried out.

The available isotopic barium carbonate was converted to sodium bicarbonate by treating the carbonate with perchloric acid and collecting the liberated carbon dioxide in a measured amount of 0.09 N NaOH solution. The concentration of the sodium bicarbonate solution used was 0.3 M and the isotopic content was 15.6 atom per cent excess C\textsuperscript{13}. The bicarbonate solution was sterilized by Seitz filtration. Twenty ml of the 0.3 M solution of sodium bicarbonate were used for each 10 liters of dextrose ammonium sulfate medium. The A. aerogenes was cultured as previously described and the yield of cells was 118.5 gm (wet weight) and 27.3 gm (dry weight). The dried cell material was extracted and the
adenine and guanine isolated as previously described. In
addition to pyrolyzed samples, the isolated guanine was
degraded by the method of Wulff.

A portion of the isolated glycine was pyrolyzed and the
carbonate formed was derived from carbons 4 and 5 of the
original guanine. In addition, a portion of the guanine was
oxidized with permanganate solution to carbon dioxide, urea,
and guanidine. The carbon dioxide formed was collected in
3.0 N CO₂-free alkali and the carbonate prepared. This
material represents carbons 4, 5, and 6 of the original
guanine. The results of the mass spectrometer determinations
are shown in Table 19.

Table 19
Incorporation of Labeled Carbon Dioxide
in Bacterial Purines

<table>
<thead>
<tr>
<th>Bicarbonate</th>
<th>Adenine</th>
<th>Guanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>C¹³</td>
<td>C¹³</td>
</tr>
<tr>
<td></td>
<td>Per Cent</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>Incorp.</td>
<td>C¹³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C¹³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C¹³</td>
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<tr>
<td></td>
<td></td>
<td>C¹³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C¹³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C¹³</td>
</tr>
</tbody>
</table>

15.6ᵇ 0.80  5.1  0.28  1.8  0.04  0.23  0.19

ᵃCalculated value.
ᵇAll values are given as atom per cent excess.

The results indicate that 67.8 per cent of the total
isotopic concentration of the isolated guanine was present
in carbon 6, carbons 4 plus 5 contained 14.2 per cent of the
isotope, and carbons 3 plus 8 contained the remainder, or 18 per cent. The significance of these results will be discussed.

**Role of threonine and serine**

The relationship of the hydroxyamino acids to the biosynthesis of the purine bases has been studied by Elwyn, Sprinson, Shemin, and others. Chargaff and Sprinson (1943a) reported the conversion of serine to pyruvate by *E. coli* under anaerobic conditions. Shemin (1946) found that serine may be converted to glycine and formate, and Elwyn and Sprinson (1950b) have directly related this reaction to uric acid synthesis in the pigeon. In light of these reports, a series of experiments were conducted to determine whether serine and/or threonine might serve as the source of the glycine utilized for purine synthesis by *A. aerogenes*.

The organism was grown in dextrose ammonium sulfate medium as previously described. The cells were harvested, washed, and then suspended in 1.5 liters of an 0.8 per cent solution of K₂HPO₄ and (NH₄)₂SO₄. The final cell concentration was 60 mg per ml of solution. One hundred ml of this suspension were added to a flask containing 100 mg of dl-serine. Suitable controls without added serine were prepared. The flasks were incubated with shaking for 3 hours at 30°C. Following incubation, the flask contents were deproteinized with H₂SO₄ and the filtrate made to volume. The extent of
serine utilization was determined by the method of Boyd and Logan (1942). The filtrates were also examined for the presence of glycine, using the method of Alexander, et al.

The added serine was completely utilized by the organisms during the 2-hour incubation period. No detectable amount of glycine was found. Comparable cell suspensions utilized 10 per cent of added glycine during the same incubation period. These results tend to indicate at least two possible reactions might have occurred during the same incubation period. One reaction was the split of serine to form glycine and formate, and the second one did not result in the formation of glycine. If all of the added serine was split to glycine and formate, some of the resulting glycine should not have been metabolized. This is indicated by the limited utilization of the glycine in a parallel experiment. On the other hand, no glycine would appear if the serine was metabolized by the second mechanism. An additional possibility exists in that a large part of the serine was metabolized by a mechanism not resulting in the formation of glycine, and the remainder of the serine was split to glycine and formate. The small amount of glycine thus formed would be immediately metabolized resulting in none being found in the cell-free filtrates.

Siekevitz and Greenberg (1949) reported the condensation of glycine and formate to form serine, using rat liver slices.
Ravel, et al., have suggested the reason that *E. coli* was not stimulated in the production of 4(5)-amino-5(4)-imidazolecarboxamide by serine, and was stimulated, in the presence of bacteriostatic concentrations of sulfonamides, by glycine arose from the inhibition of the serine splitting reaction by the sulfonamides. Ravel, et al., did not offer any experimental evidence for this suggestion. An experiment was carried out to determine whether the presence of sulfadiazine would inhibit or reduce the rate of utilization of serine.

*A. aerogenes* cells were cultured as previously described and then suspended in a solution of 0.8 per cent *K*₂*H*₂*PO₄* and 

\((\text{NH}_4)_2\text{SO}_4\). The final cell concentration was 112 mg per ml. One hundred ml of this suspension were incubated with 100 mg dl-serine. Five hundred µg of sulfadiazine were added to additional flasks containing serine. All the flasks were incubated, with shaking, at 30°C. for 2 hours. Aliquots of the flask contents were removed at different times during the incubation period. The aliquots were deproteinized and then assayed for serine. The results indicated that the serine was utilized at the same rate in the presence or absence of sulfadiazine. If the serine splitting reaction was functioning, the rate of utilization of serine in the presence of the sulfadiazine should have been lower. The filtrates from the flasks containing sulfadiazine were examined for 4(5)-amino-5(4)-imidazolecarboxamide and none
was found. No glycine or pyruvate was found in the filtrates.

It was considered that the failure of the sulfadiazine to reduce the reaction rate might be due in part to permeability factors. Fresh cells of A. aerogenes were mixed with powdered glass and distilled water and treated in a sonic vibrator at 9 kc for 30 minutes. The cell-free extract was removed by centrifugation. The details of the preparation have been previously described.

The cell extract was added to serine and sulfadiazine contained in large Warburg flasks. The results of the serine determinations following incubation at 30.4°C. are indicated in Table 20. The values obtained show a more rapid utilization of serine in the presence of sulfadiazine.

To obtain a better picture of the reaction or reactions occurring during the metabolism of the serine, several Warburg experiments were conducted. The first experiment was carried out with a cell-free extract. The results are presented in Table 21. The oxygen uptake, and the carbon dioxide produced, indicate that for each mole of serine metabolized one-half of a mole of oxygen was utilized and one mole of carbon dioxide was produced. The possible reactions may be represented by the equations:

\[
\begin{align*}
(1) & \quad \text{CH}_2\text{OHCHNH}_2\text{COOH} + 0.5 \text{O}_2 \rightarrow \text{HCOOH} + \text{CH}_3\text{NH}_2\text{COOH} \\
(1A) & \quad \text{CH}_3\text{NH}_2\text{COOH} \rightarrow \text{CO}_2 + \text{CH}_3\text{NH}_2 \\
(2) & \quad \text{CH}_2\text{OHCHNH}_2\text{COOH} \rightarrow \text{CO}_2 + \text{CH}_3\text{OHCH}_2\text{NH}_2 \\
(2A) & \quad \text{CH}_3\text{OHCH}_2\text{NH}_2 + 0.5 \text{O}_2 \rightarrow \text{HCOOH} + \text{CH}_3\text{NH}_2
\end{align*}
\]
Table 20

Serine Utilization by *Aerobacter aerogenes*
Cell-free Extract

<table>
<thead>
<tr>
<th>mM Serine</th>
<th>mM Serine After 30 Minutes</th>
<th>mM Serine After 60 Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.238</td>
<td>0.150</td>
</tr>
<tr>
<td>1A</td>
<td>0.238</td>
<td>0.135</td>
</tr>
<tr>
<td>2</td>
<td>0.238</td>
<td>0.048</td>
</tr>
<tr>
<td>2A</td>
<td>0.238</td>
<td>0.057</td>
</tr>
</tbody>
</table>

Each flask contained 5.0 ml of the cell-free extract, 5.0 ml of 0.8 per cent ammonium sulfate-phosphate mixture, pH 7.0, 5.0 ml of serine solution. Flasks 1A and 2A contained 500 µg of sulfadiazine. Temperature 30.4°C.

Table 21

Dissimilation of Serine by Cell-free Extract of *Aerobacter aerogenes*

<table>
<thead>
<tr>
<th>Serine Concentration</th>
<th>Utilized µl O₂</th>
<th>Produced µl CO₂</th>
<th>R.Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>98</td>
<td>197</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Each cup contained 0.5 ml of cell-free extract, 0.5 ml of M/15 phosphate buffer, pH 7.0, 1.0 ml of substrate. All values are corrected for endogenous respiration. Final volume = 2.0 ml. Time = 120 minutes. Temperature = 30.4°C.
The products of reaction (1) would be formate and glycine, with the glycine being decarboxylated to methylamine (1A). Reaction (2) would produce ethanolamine (cholamine) and carbon dioxide. The ethanolamine would be further metabolized to formate and methylamine as shown in equation (2A), or to glycolaldehyde and ammonia as shown in equation (2B). Reaction (3) would result in the formation of hydroxypyruvic acid and ammonia. The hydroxypyruvic acid would be further metabolized to glycolaldehyde and carbon dioxide as shown in equation (3A). To examine these possibilities further, Warburg experiments were carried out using the various possible final products as substrates. The results are presented in Table 22.

From the values obtained, glycine could be expected to accumulate; however, this previously had been demonstrated not to be the case. In addition, any formate produced would be further metabolized requiring an additional one-half mole of oxygen (R.Q. 3.0). Reaction (1) would be improbable, as well as reaction (2A). In this latter case, the formate would also be further metabolized and no methylamine was detected using the method of Ormsby and Johnson (1950).
Table 23

Dissimilation of Various Substrates by Aerobacter aerogenes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Utilized</th>
<th>Produced</th>
<th>R.Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>μlO₂</td>
<td>μlCO₂</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>10.0</td>
<td>99</td>
<td>201</td>
<td>2.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.0</td>
<td>30</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Formate</td>
<td>10.0</td>
<td>118</td>
<td>229</td>
<td>1.9</td>
</tr>
<tr>
<td>Methylamine</td>
<td>10.0</td>
<td>26</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>10.0</td>
<td>34</td>
<td>66</td>
<td>1.9</td>
</tr>
<tr>
<td>Glycolaldehyde</td>
<td>10.0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>10.0</td>
<td>17</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Each flask contained 0.5 ml of cell suspension (350 mg), 0.5 ml M/15 phosphate buffer, pH 7.0, 1.0 ml of substrate. Values corrected for endogenous respiration. Final volume = 2.0 ml. Time = 130 minutes. Temperature = 30.4°C.
Equation (28) does hold, since the ethanolamine is metabolized giving an R.Q. of 1.9. This, plus the mole of carbon dioxide of the original decarboxylation, would give an overall R.Q. of 3.0, except the R.Q. of the serine results from one-half mole of oxygen, not one mole. Equation (3), however, appears to be the most likely possibility. Attempts, using chemical methods (Dische and Borenfreund) to determine the presence of glycolaldehyde on the cup contents were not successful due to the presence of interfering substances.

Of interest in connection with the dissimilation of serine was the effect of sulfadiazine. Table 23 presents the results of a Warburg experiment in which serine was metabolized in the presence of sulfadiazine. The flasks containing sulfadiazine required 391 \( \mu \)l more oxygen than those without. This amount is 261 \( \mu \)l greater than the theoretical based on an R. Q. of 2.0. Partition chromatograms (phenol-water) of the flask contents indicated that no glycine was formed.
Table 23
Effect of Sulfadiazine on Dissimilation of Serine by Aerobacter aerogenes

<table>
<thead>
<tr>
<th>Serine Concentration</th>
<th>Sulfadiazine Concentration</th>
<th>Utilized</th>
<th>Produced</th>
<th>R.Q.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>µg</td>
<td>µl O₂</td>
<td>µl CO₂</td>
<td></td>
</tr>
<tr>
<td>0.238</td>
<td>none</td>
<td>2558</td>
<td>4971</td>
<td>1.94</td>
</tr>
<tr>
<td>0.238</td>
<td>500</td>
<td>2949</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each flask contained 5.0 ml of a 20 per cent cell suspension in phosphate buffer, pH 7.0, 5.0 ml of substrate, 5.0 ml of a sulfadiazine solution or 5.0 ml of distilled water. Values are corrected for endogenous respiration. Final volume = 15.0 ml. Time = 150 minutes. Temperature = 30.4°C. Sulfadiazine concentration 500 µg per flask.
DISCUSSION

Purine Synthesis by Heterotrophic Bacteria

It has been well known for many years, mainly through the efforts of bacterial cytologists, that one of the principal constituents of the bacterial cell is nucleic acid. In addition, the nature of the type of nucleic acid present has been in part established, as well as the presence of certain of the purine and pyrimidine bases. The advent of biological assay procedures necessitated the determination of the purine and pyrimidine requirements of those organisms, i.e., Lactobacilli, unable to synthesize these compounds. Comparatively little is known concerning the synthesis of nucleic acids or nucleic acid constituents by organisms not requiring preformed components.

Prior to this investigation the fact had been established that certain of the heterotrophic bacteria are not dependent on exogenous purines for nucleic acid synthesis. In addition, it was known that Aerobacter aerogenes and Escherichia coli could be grown on a medium containing dextrose as the sole source of carbon and ammonium sulfate as the sole source of nitrogen. These facts suggested that information could be obtained concerning the bacterial synthesis of purine bases
by these organisms through the application of isotopic procedures involving the addition of labeled compounds to a dextrose ammonium sulfate medium, and the isolation of the purine bases from the cellular nucleic acid material formed as a result of growth.

Preliminary to the study of the utilization of various isotopically labeled compounds as possible purine precursors, it was found necessary to establish a procedure for the isolation of the purine bases from bacterial nucleic acids. Adenine and guanine had previously been shown to be present in the nucleic acid material of *E. coli* and *A. aerogenes*. An isolation procedure for these bases from the nucleic acid material of *A. aerogenes* was devised with sufficient amount of the purine bases being obtained to permit stepwise degradation.

Ravel, et al., (1948) reported that glycine stimulated the production of 4(5)-amino-5(4)-imidazolcarboxamide by *E. coli* in the presence of bacteriostatic concentrations of sulfonamides. On the basis of this finding and in consideration of the isotopic incorporation studies of Buchanan, Sonne, Shemin, and others, Ravel, et al., suggested that glycine functions as a precursor in the formation of bacterial purines. In the course of this investigation, it was found that the laboratory strain of *A. aerogenes* produced 4(5)-amino-5(4)-imidazolcarboxamide, whereas, the available strain
of E. coli did not. The addition of glycine to the culture medium produced the stimulation reported by Ravel, et al. In addition it was found that the A. aerogenes utilized 90 to 95 per cent of the added glycine in 8 hours.

Studies were then carried out with carboxyl labeled glycine and the isotope was found in both the isolated adenine and guanine. The procedure used did not establish the position of the isotope in the bases; however, on the basis of the studies on uric acid excreted by pigeons fed labeled glycine, it is reasonable to assume that the isotope was present in the number 4 position of the purine bases. The amino group of labeled glycine had been found by Shemin and Rittenberg (1947) to contribute nitrogen 7 in the formation of uric acid by man, as well as in the nucleic acid purines of yeast by Abrams, Hammarsten, and Shemin (1948). Heinrich and Wilson (1950) had found the incorporation of doubly labeled glycine (carbon atoms) into the 4 and 5 positions of adenine and guanine isolated from rat liver tissue. None of these studies had completely ruled out the possibility of the decarboxylation of the carboxyl labeled glycine and the subsequent incorporation of the label through other pathways, or the possibility of deamination of the glycine with incorporation of the released nitrogen atom into position seven, nor the deamination and the subsequent incorporation of the doubly labeled carbon fragment into positions 4 and 5.
In light of these possibilities, glycine was synthesized with the carboxyl carbon and the nitrogen atom of the amino group labeled. This compound was added to the culture medium and the adenine and guanine isolated from the cellular nucleic acids. The isotopes ($^{13}C$ and $^{15}N$) were found to have been incorporated in the bases. Stepwise degradation demonstrated that the carboxyl carbon was in position 4 and the $^{15}N$ from the amino group in position 7 of the purine bases. These results tend to strengthen the view that glycine is directly incorporated as such in the purine bases. More important, it correlated the findings in *A. aerogenes* with those of various workers using animal tissue, thus permitting the possible assignment of the findings in tissue to the problem of synthesis of purines by heterotrophic bacteria.

The greater incorporation of $^{13}C$ than $^{15}N$ in both the adenine and guanine requires examination. This is particularly true if the incorporation of glycine, as such, is to be accepted. A possible explanation of this finding may lie in the reductive deamination of glycine. Stephenson and Gale (1937) have reported that the presence of 2 per cent dextrose in the growth medium inhibited 95 per cent of the deaminase formation in *E. coli* and related organisms. Under the conditions of growth used, reductive deamination of the isotopic glycine probably did not occur to an appreciable extent. The incorporated glycine would have to be diluted with glycine labeled only in the carboxyl group, produced by the amination
of labeled acetic acid with non-labeled ammonia, to give the isotopic values obtained (Table 17). The experimental data obtained with incorporation studies of labeled acetate indicate that this compound is not involved in the synthesis of the purine bases. Elwyn and Sprinson (1950a) have found that labeled acetate is not incorporated into uric acid excreted by pigeons. The possibility of the amination of acetate to form glycine does not appear to be very likely.

The probable explanation of the findings would be the oxidative deamination of glycine to form glyoxylic acid as reported by Janke and Tayenthal (1937). It has been shown by Sprinson (1951) that glyoxylic acid, labeled with C¹³ in the aldehyde group, gave a similar isotope distribution as did α-labeled glycine in the formation of uric acid. In addition, Wright (1951) has reported the substitution of glyoxylic acid for the glycine and serine requirements of a Neurospora mutant. These findings indicate that glyoxylic acid may substitute for glycine in purine formation. If a portion of the isotopically labeled glycine added to the culture medium was oxidatively deaminated and the resulting glyoxylic acid incorporated, as well as isotopic glycine, one would expect a greater concentration of C¹³ in position 4 than N¹⁵ in position 7 in both the adenine and guanine. This, however, does not explain why the concentration of N¹⁵ in position 7 is greater in the guanine than in the adenine.
Brown (1949) and Rittenberg (1949) have reported that the uptake of isotopic nitrogen from labeled glycine was twice as great in guanine as it was in adenine. The data obtained with doubly labeled glycine in Experiments I and II (Table 17) substantiate this finding. The values obtained, however, do not represent a two-fold difference as reported by Brown and by Rittenberg. The explanation for such a difference is at present not available. The possibility should be considered, in light of the method used to determine the isotopic content, that in the shift of the amino group from position 6 in adenine to position 2 in guanine, additional labeled nitrogen may be incorporated from the ammonia pool present in the medium. The extent of the dilution with nitrogen from ammonium sulfate would tend to invalidate such a possibility. If rearrangement of adenine to form guanine proceeds through a number of unknown intermediates, the opportunity for differences in $N^{15}$ content might easily arise.

If the adenine and guanine are formed at the same rate from a common intermediate, the percentage of isotope in both bases should be the same. The data obtained supports this premise in the case of the $C^{13}$ isotope (Tables 16 and 17). It fails in the case of the $N^{15}$ isotope and even with the $C^{13}$ when a lower concentration of glycine was added to the culture medium (Experiment II, Table 17). This indicates that guanine was possibly formed after the synthesis of adenine or that both were not formed at the same rate from a common inter-
mediate. An interconversion of adenine and guanine has been reported by Brown, et al., (1948) and Baltis and Brown (1951) in *Lactobacillus casei*. These studies were limited to the determination of the total isotope concentration of a single label (C\(^{14}\)). It would be of interest, starting with adenine labeled with N\(^{15}\) in position 7 and C\(^{13}\) in position 4, to determine the distribution of the isotopes in both the adenine and guanine after a period of incubation.

The metabolic origin of position 6 of both the adenine and guanine isolated from the bacterial nucleic acid material was found to be carbon dioxide. The studies of Buchanan, et al., (1948) and those of Heinrick and Wilson have also demonstrated such an incorporation in the uric acid excreted by pigeons and in the adenine and guanine isolated from the liver tissue of the rat. The origin of position 6 appears to be the same in the purines isolated from both animal tissue and *A. aerogenes*. Of more interest is the finding of the label in positions 4 plus 5 and 2 plus 8. Heinrick and Wilson found no C\(^{14}\) from labeled carbon dioxide in positions 2 and 8 and relatively little in positions 4 plus 5 of guanine isolated from rat liver tissue. Buchanan, et al., reported some incorporation in positions 4 plus 5 and in 2 plus 8 of uric acid from pigeons. The data obtained here and reported in Table 19, indicates that 14.2 per cent of the C\(^{13}\) was present in positions 4 plus 5 and 18 per cent in positions 2 plus 8.
These values indicate that carbon dioxide may be involved in the synthesis of glycine. Marr and Wilson (1951) have reported the incorporation of $\text{C}^{14}$-carbon dioxide into glycine and serine isolated from the cell protein of *Brucella abortus* grown under 0.02 atmospheres of carbon dioxide. This finding suggests the possible reason for the presence of $\text{C}^{13}$ in positions 4 plus 5.

The presence of 18 per cent of the incorporated isotope in positions 2 plus 8 may be explained on the basis that formate has been shown to be the metabolic precursor for these positions in both uric acid excreted by pigeons, and in adenine and guanine isolated from rat liver tissue. The reduction of carbon dioxide to formic acid has been demonstrated by Woods (1936) in *A. aerogenes*. Such a reduction has not been demonstrated in animal tissue. The isotopic findings, therefore, suggest that the carbon dioxide may have been reduced to formic acid and then incorporated into positions 2 plus 8.

It has been demonstrated that *A. aerogenes* is able to incorporate glycine into the 4, 5, and 7 atoms of adenine and guanine. This was reported by Sutton, Schlenk, and Werkman (1951). Carbon dioxide is incorporated into position 6, and into positions 2 and 8 indirectly through formic acid. Nitrogens 1, 3, and 9 arise from ammonium sulfate nitrogen (sole source of nitrogen in culture medium) either directly or through some donor system.
The isolation of 4(5)-amino-5(4)-imidazolecarboxamide suggests that the five membered ring of the purine molecule is formed first. In addition, the incorporation of glycine into adenine and guanine and not into the pyrimidines of bacterial nucleic acids, as indicated by the experimental data, supports the belief that the formation of the five membered ring precedes that of the six membered ring. Of interest is whether carbon dioxide may be fixed to the methylene carbon of glycine as the initial step in the formation of the five membered ring. In this connection, experiments not reported here indicated that such a fixation does not occur. If such a fixation did occur, the initial product would be aminomalonic acid. Buchanan (1951) has reported that aminomalonic acid, aminomalonic acid diamide, and aminomalonamidine do not serve as precursors of purines. These findings tend to indicate that perhaps the five membered imidazole ring is formed and then carbon dioxide is fixed to carbon 4(5).

Serine and threonine were investigated as possible sources of glycine for the bacterial synthesis of adenine and guanine. Shemin (1946) found that serine may be converted to glycine and formate. Elwyn and Sprinson (1950a) have directly related this reaction to uric acid synthesis in the pigeon. The failure of serine to stimulate the production of 4(5)-amino-5(4)-imidazolecarboxamide by E. coli suggested to Ravel, et al., that this failure was due to the inhibition of the
serine splitting reaction by sulfonamides. The 4(5)-amino-5(4)-imidazolecarboxamide producing strain of A. aerogenes metabolized serine in the presence of bacteriostatic concentrations of sulfadiazine. The data obtained indicates a failure of the sulfadiazine to inhibit the utilization of the added serine. As a matter of fact, the rate of utilization appears to be even greater in the presence of the sulfonamide. Respirometer studies indicated that the serine was decarboxylated and deaminated.

Stephenson (1949) indicated that the bacterial deaminases have maximal activity at pH 8.0 and the decarboxylases at pH 4.0. The minimal activity of both enzymes is in the neighborhood of pH 6.0 to 6.5. The serine utilization experiments were carried out at pH 6.8 to 7.0; therefore, the splitting reaction would be favored under these conditions, and not the deamination and the decarboxylation reactions. This precaution did not result in the detection of the splitting reaction with either serine or threonine. The relationship of the splitting reaction in animal tissue to purine synthesis was not established in A. aerogenes.

Purine Degradation by Heterotrophic Bacteria

The studies of Lutwak-Mann (1936), and Stephenson and Trim (1938), have shown that adenine was hydrolytically deaminated to hypoxanthine by both A. aerogenes and E. coli.
Koser (1918), and Mitchell and Levene (1938), had earlier established the fact that *A. aerogenes* can utilize uric acid as a source of nitrogen, whereas, *E. coli* cannot. These findings suggested that the enzyme systems and intermediate compounds known to be involved in the breakdown of purines by animal tissue might be found in *A. aerogenes* and *E. coli*. Partition chromatography and the Beckman spectrophotometer provided a means of locating possible intermediates in the breakdown of the purines by these organisms, which were not available to these earlier workers. The strains of *A. aerogenes* and *E. coli* used were found to deaminate adenine, but not as readily as reported by Lutwak-Mann. Respirometer experiments indicated that *E. coli* would not oxidize hypoxanthine, but the chromatograms of the Warburg flask contents indicated the formation of hypoxanthine from adenine. Subsequent experiments indicated a rapid hydrolytic deamination of adenine by *E. coli*, but the Beckman values indicated that no further purine intermediates were formed other than hypoxanthine. Hypoxanthine could not be found when adenine was metabolized by *A. aerogenes*. The adenine concentration was reduced during the first 8 hours of the experiment; however, no shift in the absorption maximum occurred as was found in the case of *E. coli* (Figures 2 and 3). Since the adenine was deaminated by *A. aerogenes* (Table 1), it may be assumed that the hypoxanthine formed was immediately utilized by the
cells. No explanation can be suggested for the absorption maximum at 260 μμ after 24 hours incubation in the case of the *A. aerogenes*.

The failure of *E. coli* to oxidize hypoxanthine indicates the absence of an oxidative enzyme(s) comparable to xanthine oxidase found in animal tissue. This enzyme(s) oxidizes hypoxanthine or xanthine to uric acid. All further attempts to demonstrate possible intermediates in the breakdown of adenine by *E. coli* or *A. aerogenes* failed to indicate such compounds. The findings indicate that the final product of the action of *E. coli* on adenine is hypoxanthine. The final product of the action of *A. aerogenes* on adenine remains in doubt. Both organisms apparently do not possess the complete degradative enzyme systems present in animal tissue.
SUMMARY AND CONCLUSIONS

1. The biosynthesis of purines by *Aerobacter aerogenes* was studied by culturing the organism in a dextrose ammonium sulfate medium with added isotopically labeled compounds. Glycine is incorporated into the 4, 5, and 7 positions of adenine and guanine of bacterial nucleic acids. Carbon dioxide is incorporated into position 6 of these purine bases, and into positions 3 and 8 through reduction to formate. Acetate does not play a role in bacterial purine synthesis. Nitrogen atoms in positions 1, 3, and 9 arise from the nitrogen of ammonium sulfate either directly from ammonia or through some donor system. The synthesis of adenine and guanine by the same mechanisms present in animal tissue is strongly suggested.

2. The existence of a serine or threonine splitting mechanism in *Aerobacter aerogenes* was not demonstrated.

3. Purines are not completely degraded by *Aerobacter aerogenes* or *Escherichia coli* through the same mechanisms present in animal tissue.

4. 4(5)-amino-5(4)-imidazolecarboxamide is not a utilisable intermediate in the formation of bacterial purines.
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