# PURINE SYNTHESIS IN SUSPENSIONS OF MUCOSA FROM THE SMALL INTESTINE OF THE RAT

by

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We accept this thesis as conforming to the standard required from candidates for the degree of DOCTOR OF PHILOSOPHY.

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Faculty of Graduate Studies

PROGRAMME OF THE

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## FOR THE DEGREE OF
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*of*

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# PURINE SYNTHESIS IN SUSPENSIONS OF MUCOSA FROM THE SMALL INTESTINE OF THE RAT


## ABSTRACT

Purine biosynthesis has been studied *in vitro* using surviving, whole cell suspensions of the mucosa from the small intestine of the rat. The *de novo* synthesis of purines by this tissue system was demonstrated by measuring the incorporation of $C^{14}$-labelled purine precursors into adenine and guanine of the acid-soluble (AS) nucleotides and the nucleic acids. Essential parts of this demonstration consisted of establishing the radiochemical purity of the isolated purines, and of showing that the isotope incorporation was not attributable to bacteria present in the tissue suspension. Upon incubation of mucosal suspensions with formate-$C^{14}$, adenine and guanine of the AS nucleotides displayed a rapid renewal, being approximately 40 and 14 times as radioactive, respectively, as the adenine and guanine of the mixed nucleic acids.

Incorporation of formate-$C^{14}$ by exchange reactions (as opposed to *de novo* synthetics) appeared to be excluded as a major process by the demonstration that two other purine precursors, glycine-1-$C^{14}$ and bicarbonate-$C^{14}$, also became incorporated into the purines. The theoretical incorporation of 2 molecules of formate for each glycine molecule used in the process of *de novo* purine synthesis was approached in these experiments by the observed incorporation of 2-4 molecules of formate per molecule of glycine. This is sufficiently close to the theoretical value to exclude a major incorporation of formate-$C^{14}$ by known exchange reactions.

Time studies indicated that the renewal of AS adenine and guanine proceeded at an approximately constant rate with a decline in rate beginning at 3-4 hours of incubation. In several experiments an initial lag in the rate of synthesis of nucleic acid purines was apparent.

The rate of purine synthesis as measured by the incorporation of formate-$C^{14}$ was not significantly affected by the addition of non-isotopic glycine or glutamine to the incubation medium. Similarly, glycinamide and 4-amino-5-imidazolecarboxamidine, both of which resemble known intermediates in purine biosynthesis, did not alter the incorporation of formate-$C^{14}$ into the purines.

Renewal of either AS or nucleic acid purines from formate-$C^{14}$ did not take place in homogenates of intestinal mucosa.

The incorporation of formate-$C^{14}$ by the purines of the intestinal mucosa of the intact rat was measured 24 hours after administration of the isotope. In contrast to the *in vitro* experiments, the large differences between the specific activities of the AS and nucleic acid purines were absent in the intact animal. A comparison of purine synthesis in the intact animal with that of the *in vitro* system suggested that guanine synthesis was suppressed in the latter.

## PUBLICATIONS

"The Preparation of 2-$C^{14}$-Adenine." A. R. P. Paterson and S. H. Zbarsky. Journal of the American Chemical Society, *75,* 5753 (1953).

"*In vitro* Synthesis of Purines by Rat Intestinal Mucosa." A. R. P. Paterson and S. H. Zbarsky. Biochimica et Biophysica Acta, *18,* 441 (1955).

"Ribonucleic Acid Synthesis in Tumor Humogenates." A. R. P. Paterson and G. A. LePage. Federation Proceedings, *15,* 324 (1956).

"The Metabolism of 2-$C^{14}$-Adenine in the Rat." S. H. Zbarsky and A. R. P. Paterson. Accepted for publication in the Canadian Journal of Biochemistry and Physiology.

## GRADUATE STUDIES

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## ABSTRACT

Purine biosynthesis has been studied *in vitro* using surviving, whole cell suspensions of the mucosa from the small intestine of the rat. The preparation and use of such suspensions have been described as have been certain features of their metabolic behaviour. The techniques used to isolate and measure the radioactivity of the several purine fractions of mucosal suspensions containing 150-200 mg. of fresh tissue have been outlined.

The *de novo* synthesis of purines by this tissue system was demonstrated by measuring the incorporation of several $C^{14}$-labelled purine precursors into adenine and guanine of the acid-soluble (AS) nucleotides and the nucleic acids. Essential parts of this demonstration consisted of establishing the radiochemical purity of the isolated purines, and of showing that the isotope incorporation was not attributable to bacteria present in the tissue suspension. Upon incubation of mucosal suspensions with formate-$C^{14}$, adenine and guanine of the AS nucleotides displayed a rapid renewal, being approximately 40 and 14 times as radioactive, respectively, as the adenine and guanine of the mixed nucleic acids. The specific activity of AS adenine was approximately 6 times greater than that of AS guanine, and nucleic acid (NA) adenine was 2 - 3 times as active as NA guanine. Following incubation of the mucosal preparation, the suspending medium contained adenine, guanine and uric acid, but since these purines were only

slightly radioactive they were regarded as breakdown products.

Incorporation of formate-$C^{14}$ by exchange reactions (as opposed to de novo synthesis) appeared to be excluded as a major process by the demonstration that two other purine precursors, glycine-1-$C^{14}$ and bicarbonate-$C^{14}$, also became incorporated into the purines. The theoretical incorporation of 2 molecules of formate for each glycine molecule used in the process of de novo purine synthesis was approached in these experiments by the observed incorporation of 2 - 4 molecules of formate per molecule of glycine. This is sufficiently close to the theoretical value to exclude a major incorporation by known exchange reactions.

Time studies indicated that the renewal of AS adenine and guanine proceeded at an approximately constant rate with a decline in rate starting at 3 - 4 hours of incubation. In several experiments an initial lag in the rate of synthesis of NA purines was apparent.

The rate of purine synthesis as measured by the incorporation of formate-$C^{14}$ was not significantly affected by the addition of non-isotopic glycine or glutamine to the incubation medium. Similarly, glycinamide and 4-amino-5-imidazolecarboxamidine, both of which resemble known intermediates in purine biosynthesis, did not alter the incorporation of formate-$C^{14}$ into the purines.

Renewal of either AS or NA purines from formate-$C^{14}$ did not take place in homogenates of intestinal mucosa.

The incorporation of formate-$C^{14}$ by the purines of the intestinal mucosa of the intact rat was measured 24 hours after administration of the isotope. In contrast to the *in vitro* experiments, the large differences between the specific activities of the acid-soluble and NA purines were absent in the intact animal, the levelling effect very probably being due to the 24 hour interval. A comparison of purine synthesis in the intact animal with that of the *in vitro* system suggested that guanine synthesis was suppressed in the latter.

The use of intestinal mucosa for *in vitro* studies of purine metabolism has not hitherto been reported. In view of the demonstrated rapid synthesis of soluble purine nucleotides in this system, particularly of adenine nucleotides, it is felt that suspensions of mucosa may have useful applications in *in vitro* studies of purine biosynthesis.

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THE FOLLOWING LIST DEFINES THE ABBREVIATIONS USED IN THIS THESIS

RNA ribonucleic acid

DNA desoxyribonucleic acid

AS acid-soluble

NA nucleic acid

AMP adenosine monophosphate

ATP adenosine triphosphate

DPN diphosphopyridine nucleotide

TPN triphosphopyridine nucleotide

carboxamide 4-amino-5-imidazolecarboxamide

PRPP 5-phosphoribosylpyrophosphate

GAR glycinamide ribotide

FGAR ∝ N-formylglycinamidine ribotide

FGAM ∝ N-formylglycinamidine ribotide

AIR 5-aminoimidazole ribotide

AICAR 5-amino-4-imidazolecarboxamide ribotide ("carboxamide" ribotide)

# INTRODUCTION

In early investigations of the biosynthesis of nucleic acids, classical feeding techniques and nutrition studies were employed. These studies provided clearcut evidence that the higher forms of life are able to synthesize nucleic acids from simple precursors, but gave little specific information about the identity of the ultimate precursors. Within the last fifteen years, the advent of the isotopic tracer technique and the availability of isotopes in quantities large enough for chemical and biological experimentation have provided the basic methodology for what has proven to be a very fruitful attack on the problem of nucleic acid biosynthesis.

Phosphate and ammonium ions were the first primary substances shown by isotopic techniques to be incorporated into nucleic acids. Subsequently, more specific experiments, employing metabolites labelled with isotopic carbon, revealed the identity of the compounds from which the carbon atoms of the purine and pyrimidine bases originate. There is much to suggest

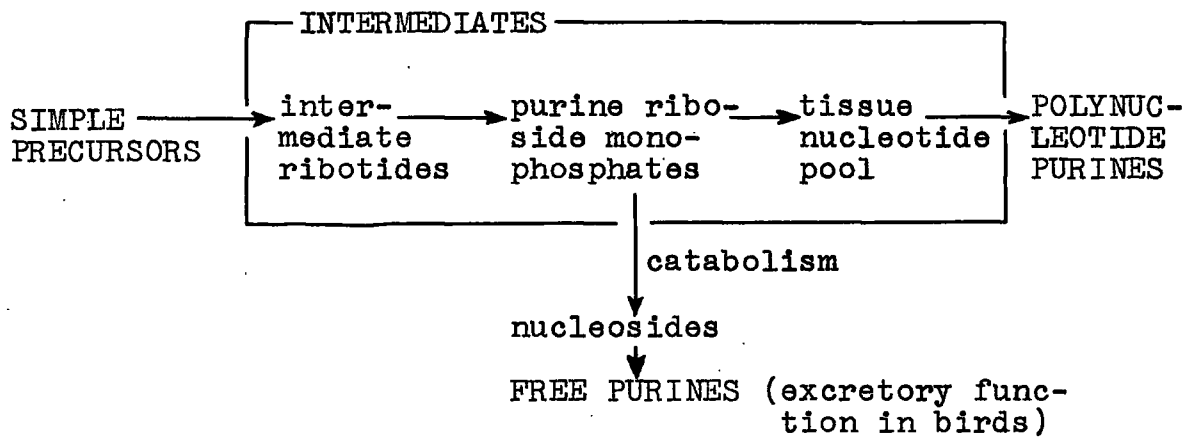
that the nucleic acids are not built up by the stepwise integration of these simple compounds, but rather that they are polymers of more complex sub-units, the purine and pyrimidine ribotides. These sub-units are presently considered to be derived from the free ribotides found in the "acid-soluble fraction" of tissues, which is an extract usually made with cold, dilute perchloric or trichloracetic acid. The composition of the acid-soluble fraction, which includes a complex mixture of the purine and pyrimidine riboside-5'-mono-, di-, and triphosphates, has been studied by Hurlbert et al. (1,2). This fraction is currently receiving much attention because of its key position in the biosynthesis of nucleic acids. Isotopic precursors of the purines (3 - 6) and pyrimidines (7,8) of the nucleic acids appear rapidly in this fraction which seems to be intermediate between the simple or early precursors and the nucleic acids (9). For this discussion, nucleic acid biosynthesis is considered under two principal topics, nucleotide (ribotide) synthesis and polynucleotide synthesis. As this investigation is concerned primarily with purine metabolism, the biosynthesis of the pyrimidines will not be discussed. The latter topic has been reviewed recently by Reichard (10).

## 1. PURINE NUCLEOTIDE SYNTHESIS

Prior to 1950, it was implicit in most discussions of nucleic acid biosynthesis that the bases were elaborated as separate entities, and experimental work was undertaken from this point of view. However, it has become apparent in recent years that neither the free bases nor their ribose derivatives, the ribosides, are intermediates in either ribonucleic acid or

in ribotide synthesis. The purine ring is assembled via reaction sequences in which the intermediate compounds are ribotides, and the first complete purine structure on the synthetic pathway is a riboside monophosphate. Thus, the process of purine biosynthesis is actually one of purine nucleotide synthesis.

The following scheme illustrates, in general terms, the relationships between the early precursors, the nucleotides and the polynucleotides and demonstrates that purine biosynthesis is actually purine nucleotide biosynthesis.

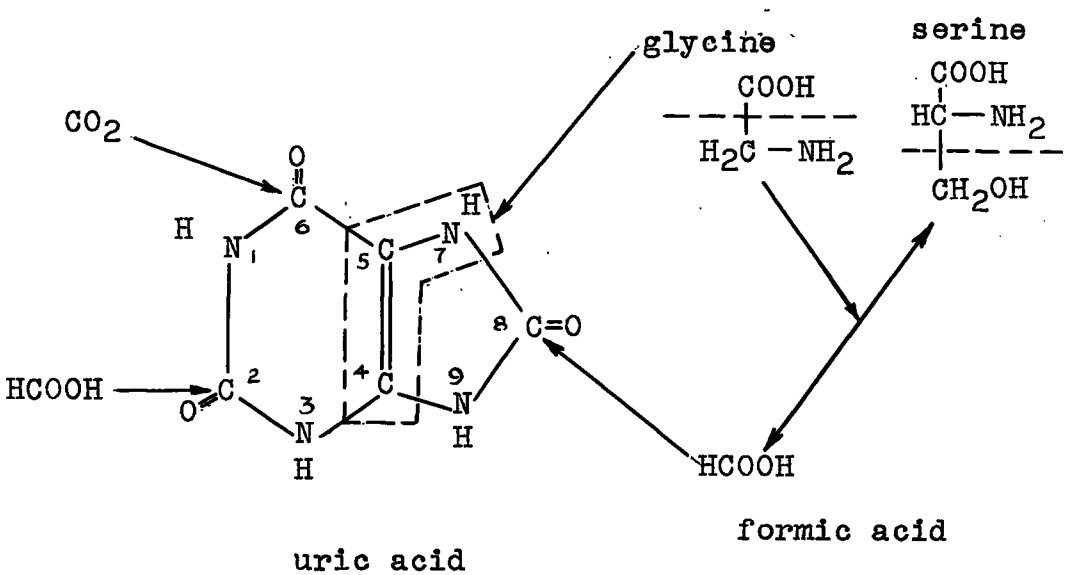



A. Early precursors of the purine carbon atoms.

The basic experiments on the precursors of uric acid carbon atoms have been adequately reviewed by several authors (10 - 14) and are summarized in Figure 1, which shows the relationships between the principal precursors and their ultimate locations in the uric acid molecule. The basic experiments on this topic were performed by Buchanan and his colleagues who administered carbon-labelled compounds to pigeons and determined the distribution of the isotope among the carbon atoms of the excreted uric acid.

Inherent in the application of these studies to purine metabolism in general, was the assumption that uric acid, in addition to being the chief excretory product of nitrogen metabolism in the bird, was also the end product of purine catabolism. The further assumption was made that uric acid, synthesized for the purpose of nitrogen elimination, was elaborated in the same manner as the purines, adenine and guanine, employed in functional roles as coenzyme and nucleic acid components. These apparently reasonable assumptions have been substantiated to the extent that isotopic precursors were found to label the polynucleotide purines of the rat, the pigeon and yeast cells in the same pattern as found in uric acid (10,14).

FIGURE 1. THE PRINCIPAL PRECURSORS OF THE CARBON ATOMS OF URIC ACID.



The purine precursors shown in Figure 1 are merely early precursors and do not represent the actual compounds which participate in purine ring synthesis. Intact animals were employed in the basic studies, but more recently, *in vitro* systems have been used to great advantage, particularly in studies of the intermediary processes in purine synthesis. Pigeon liver preparations have been favoured because of the prominence of hypoxanthine synthesis in this tissue. In the bird, the liver is the principal site of uric acid synthesis. This process, in its final stages, passes through hypoxanthine, xanthine and has as the final product, uric acid. As pigeon liver lacks the enzyme xanthine oxidase, purine synthesis stops at the hypoxanthine stage. Hypoxanthine synthesis was shown to occur *in vitro* in slices and homogenates of pigeon liver, but a most important step in the development of these studies was the discovery that the enzymes responsible for this synthesis are soluble (15,16). Schulman *et al.*(17) showed that in the synthesis of hypoxanthine by pigeon liver extracts, glycine, formate and carbon dioxide are used in the ratio 1:2:1, providing excellent confirmation of the results of the *in vivo* experiments.

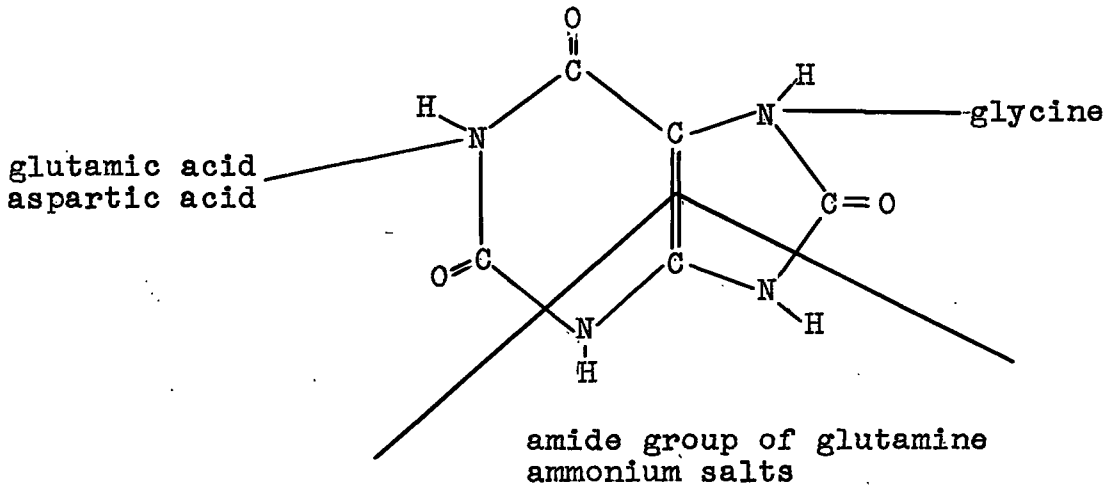
B. Early precursors of the purine nitrogen atoms.

This topic has been reviewed by Reichard (10). The early observations that ammonium salts contributed to the purine nitrogens were made more meaningful by the recent experiments of Lagerkvist (18) who degraded uric acid by a method which permitted the measurement of the isotope content of each nitrogen atom. Lagerkvist showed that in pigeons, ammonium salts contributed

preferentially to positions 3 and 9 of excreted uric acid. This distribution was very likely effected by conversion of the ammonia to the amide nitrogen of glutamine.

Pigeon liver extracts were employed in the important experiments of Sonne et al. (19,20) who demonstrated that in the synthesis of hypoxanthine, glycine and glutamic (or aspartic) acid each contributed one nitrogen atom per molecule of hypoxanthine formed, while the amide group of glutamine contributed 2 molecules of nitrogen. In these experiments degradation methods for uric acid were not available by which nitrogen atoms 1 and 3 could be separated for isotopic analysis, however it appeared that the nitrogen atoms at positions 3 and 9 were derived from the amide group of glutamine, while aspartic and glutamic amino nitrogens contributed to position 1. The glycine amino group formed purine nitrogen 7. The contribution of the glutamine amide group to positions 3 and 9 was further substantiated by the report that glutamine is required for the synthesis of glycinamide ribotide in pigeon liver extracts (21). Recently Levenberg and his collaborators have employed newly developed degradation techniques which permitted the separate isolation of purine nitrogens 3 and 9 in experiments which directly confirmed the above inferences regarding the metabolic origins of these nitrogens (22). This topic is summarized in Figure 2. Glycine and ammonium salts appear to be the only purine precursors that have been studied as sources of the nitrogen atoms in polynucleotide purines (10).

FIGURE 2. THE PRINCIPAL PRECURSORS OF THE NITROGEN ATOMS OF THE PURINE RING.




C. Intermediary compounds.

The isolation and characterization of the incompleted purine, 4-amino-5-imidazolecarboxamide ( "carboxamide" ), from sulfonamide-inhibited cultures of E. coli was an important step in the isolation of intermediates in purine nucleotide synthesis (23). The structure of carboxamide suggested a role in purine biosynthesis, as did the utilization of this compound in the place of purines by certain purine-requiring bacteria (10, see ref. 51 - 53). Labelled carboxamide was incorporated into nucleic acid purines of the rat (24) and yeast (25). Broken-cell preparations of pigeon liver (26) and yeast (27) incorporated $C^{14}$-carboxamide into hypoxanthine and inosinic acid, respectively. Thus, the participation of carboxamide in purine synthesis was established, but trapping and dilution experiments designed to detect the formation of labelled carboxamide from isotopic precursors of hypoxanthine demonstrated that carboxamide per se was not an intermediate (27,28).

A balance study of formate incorporation into hypoxanthine in pigeon liver extracts, reported at that time by Greenberg (28), showed that inosinic acid was the precursor of inosine which, in turn, formed hypoxanthine. This work indicated that the first complete purine structure formed de novo in this system was a ribotide. The exclusion of carboxamide as an intermediate was entirely compatible with the idea that ribotide formation was an integral part of purine synthesis and prompted the suggestion that carboxamide ribotide was the immediate precursor of inosinic acid in the pigeon liver system. The carboxamide riboside has been isolated from sulfadiazine-inhibited E. coli cultures (29). Phosphorylation of the riboside to form the proposed intermediate ribotide has been demonstrated in pigeon liver extracts and yeast autolysates (30). The observations of Buchanan and his colleagues (31) on the enzymatic exchange reaction of formate with position 2 of inosinic acid agreed well with the participation of the carboxamide ribotide in purine synthesis. Further, these authors have presented evidence for the formation of carboxamide ribotide from hypoxanthine in fractionated pigeon liver extracts (31). Thus far, the known intermediates in uric acid synthesis in the pigeon liver system were as follows:

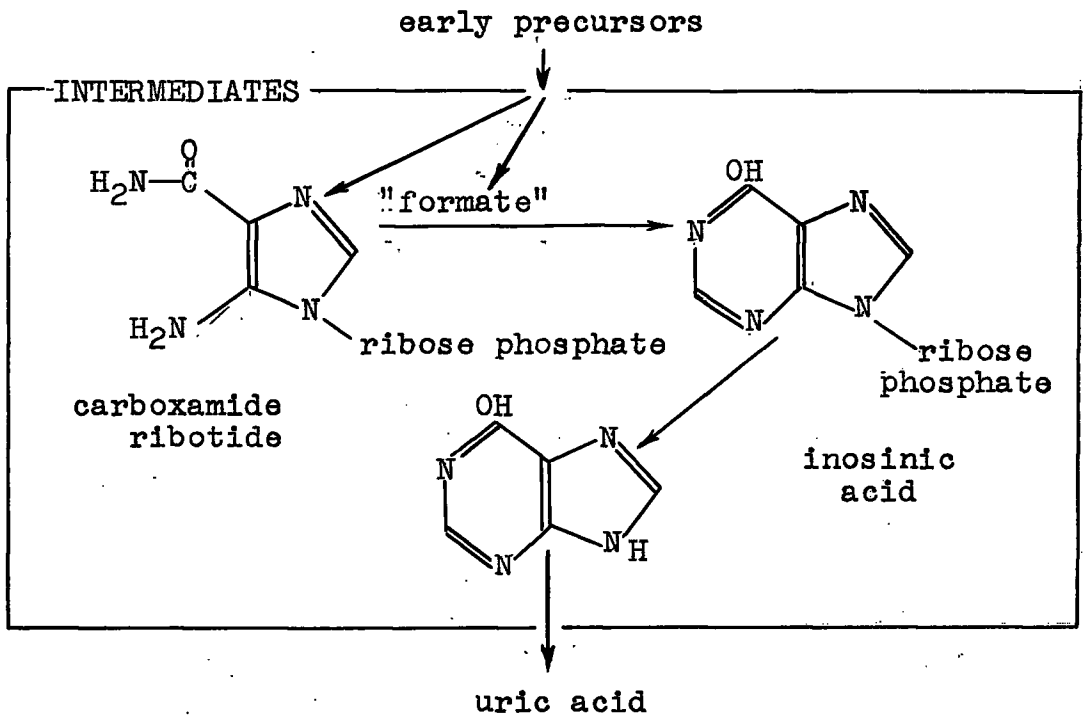



FIGURE 3. INTERMEDIATES IN URIC ACID BIOSYNTHESIS

In 1953 Greenberg proposed that acyclic ribotide derivatives of glycine were likely precursors of carboxamide ribotide (32) and recently, such intermediates have been isolated and characterized. The isolation of glycinamide ribotide (GAR) and its N-formyl derivative (FGAR) from pigeon liver extracts was first reported by Greenberg and his coworkers (33). This was confirmed by Hartman et al. who showed subsequently that these compounds accumulate in the presence of L-azaserine (21). These authors also demonstrated that 5-phosphoribosylpyrophosphate (PRPP) was the donor of the ribosylphosphate portion of the GAR and FGAR, replacing ribose-5-phosphate and adenosine triphosphate (ATP) in their system.

Kornberg et al. have shown with purified yeast enzymes that PRPP is a key intermediate in the synthesis of nucleotides from the free purine and pyrimidine bases (34). Recently Buchanan and his coworkers have partially purified enzymes from pigeon liver which will condense PRPP and the free purines, adenine and hypoxanthine, to form adenylic and inosinic acids. A separate fraction contains the enzyme responsible for the formation of PRPP from ribose-5-phosphate and ATP (35,36).

Hartman et al. showed that the amide nitrogen of GAR and FGAR was derived from the amide nitrogen of glutamine (21). From this work L-azaserine would appear to block the incorporation of the glutamine amide nitrogen designated for position 9 of the purine ring and thus, to prevent formation of the imidazole ring. GAR and FGAR are efficient precursors of inosinic acid in the pigeon liver system.

The demonstration that glycine was incorporated by way of a phosphoribosyl derivative of glycinamide raised the question of the participation of the amide per se as an intermediate in purine synthesis. In the present investigation, glycinamide was shown to have no significant effect on purine synthesis in suspensions of intestinal mucosa, although permeability effects were not excluded. A recent report by Goldthwait et al. (37) makes it unlikely that glycinamide participates in purine synthesis directly since these authors demonstrate that the amide nitrogen of GAR formed in pigeon liver extracts was introduced from 5-phosphoribosylamine. This compound was formed from glutamine and PRPP.

A number of ribotides which represent intermediate stages in inosinic acid synthesis have recently been isolated by Buchanan and his coworkers. They have employed fractionated pigeon liver extracts which, in effect, were portions of the enzyme system which synthesized inosinic acid. The enzyme preparations carried out only certain steps in this process and in this way permitted the isolation of intermediates. Levenberg and Buchanan (38) isolated a fraction from pigeon liver extracts which converted FGAR to 5-aminoimidazolecarboxamide ribotide (AIR) in the presence of glutamine and ATP. Further fractionation of the AIR-forming enzyme system yielded two preparations, one of which carried the reaction of FGAR with glutamine and ATP to another intermediate stage, forming ∝ N-formylglycinamidine ribotide (FGAM). The other enzyme fraction converted FGAM to AIR without further additions. The new intermediates were characterized chemically and both were readily converted to inosinic acid by pigeon liver enzymes. In this work the site of action of L-azaserine was localized to the inhibition of the conversion of FGAR to FGAM. The addition of the carboxamide group to AIR, forming 5-amino-4-imidazolecarboxamide ribotide (AICAR)[1], was shown by Lukens and Buchanan (39) to take place in a fractionated pigeon liver extract and required ATP, aspartic acid, and bicarbonate. This enzyme system was also fractionated into two preparations, one of which accumulated

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1 Following the abbreviation used in the literature cited, this compound has been referred to above as "carboxamide ribotide".

a new intermediate. The second enzyme preparation converted this intermediate to AICAR without further additions. Proof of structure is lacking, but the analytical data suggest that the intermediate is 5-amino-4-imidazole-(N-succinoylcarboxamide) ribotide.

Warren and Flaks (40) have studied formylation reactions in this series of intermediates and have shown that pigeon liver enzymes in the presence of serine, triphosphopyridine nucleotide (TPN), and leucovorin will convert AICAR to inosinic acid. The formyl derivative of AICAR (5-formylamino-4-imidazolecarboxamide ribotide) has been prepared chemically, and pigeon liver enzyme preparations catalyse its conversion to inosinic acid without further additions.

Our present understanding of the intermediate compounds of purine synthesis may be summarized in the scheme shown in Figure 4.

The processes of de novo synthesis of the purine nucleotides lead to the tissue pool of soluble nucleotides (the acid-soluble fraction). This fraction is a very complex mixture, containing the 5'-mono, di-, and triphosphates of the ribosides of adenine, guanine, cytosine and uracil. The purine riboside phosphates of this fraction are known to have functional roles as coenzymes in intermediary metabolic processes, largely those of energy transfer through phosphorylation reactions. The polyphosphates turn over rapidly and are maintained by glycolysis and oxidative phosphorylation (41). Isotopic nucleic acid precursors have been shown repeatedly to cause a rapid labelling

of the acid-soluble pool. Early in the incorporation process, isotope concentrations in the nucleic acids are invariably subordinate to those of the free nucleotides, and for this reason the polynucleotides are believed to be derived from the free nucleotide pool.

The above scheme (Figure 4) is based largely on in vitro experiments with pigeon liver preparations, but no experimental evidence has been reported to establish the relationships between the biosynthetic pathway and the purine nucleoside phosphate pool (acid-soluble fraction) in this tissue system. It is not known, for example, if carboxamide ribotide (AICAR) or inosinic acid are obligatory intermediates in adenylic or guanylic acid biosynthesis. Abrams and Bentley have described the transformation of inosinic acid to adenylic and guanylic acids in a soluble enzyme extract from rabbit bone marrow (45). This work establishes the connection between inosinic acid and the adenine and guanine nucleoside phosphates, but it does not indicate any sequential relationships between them in biosynthesis. There has been a recent suggestion that adenylic acid may be formed by way of a carboxamidine derivative of aminoimidazole ribotide (46). If such were the case, inosinic acid would be excluded as an obligatory intermediate in purine synthesis.

At present there is little to suggest that the facts of purine biosynthesis that apply specifically to pigeon liver systems, are not of general applicability to mammalian systems. Indeed, the pattern of labelling in the purines from precursors such as formate, glycine, and $CO_2$, being the same in yeast,

FIGURE 4. SCHEME FOR PURINE BIOSYNTHESIS

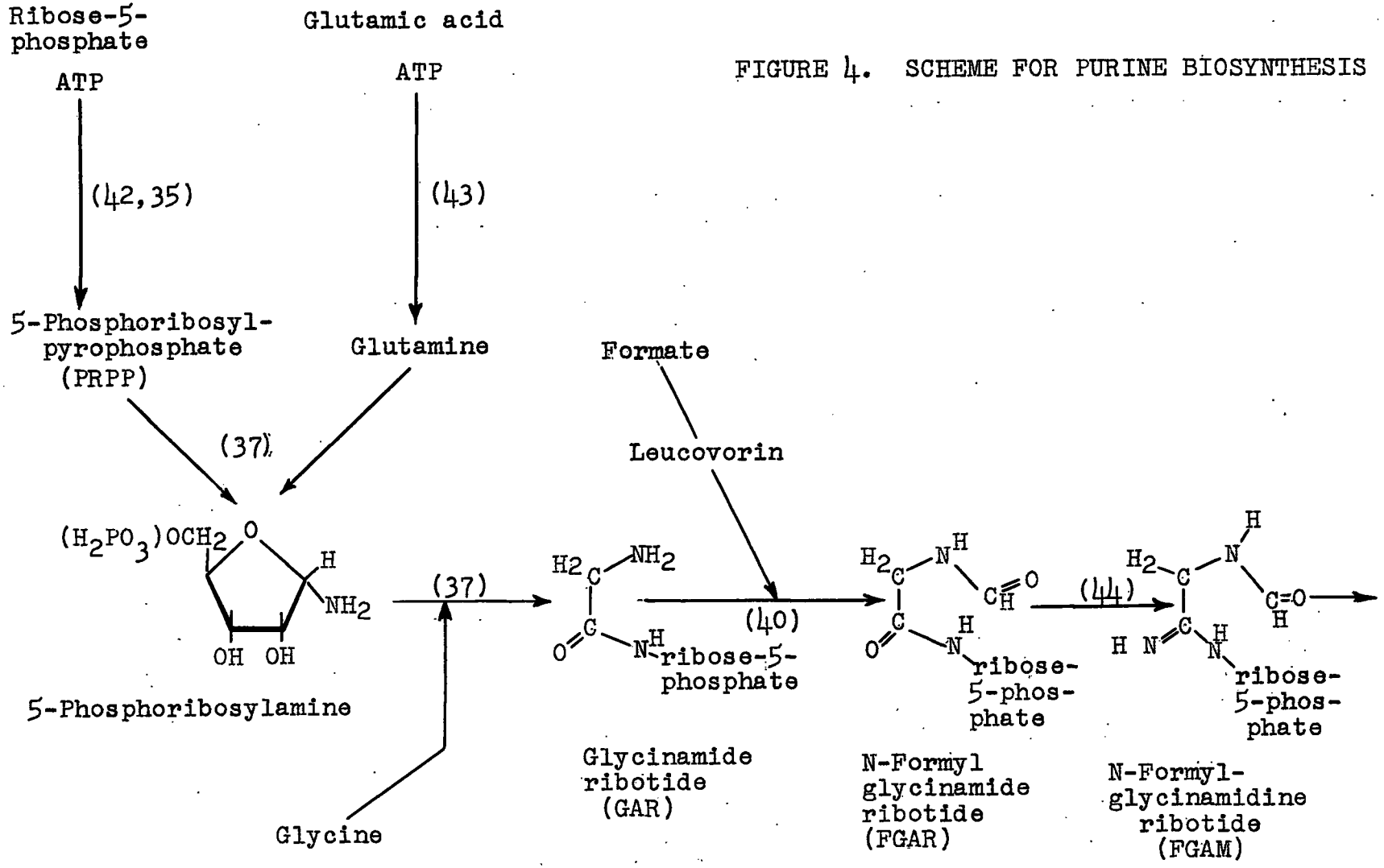
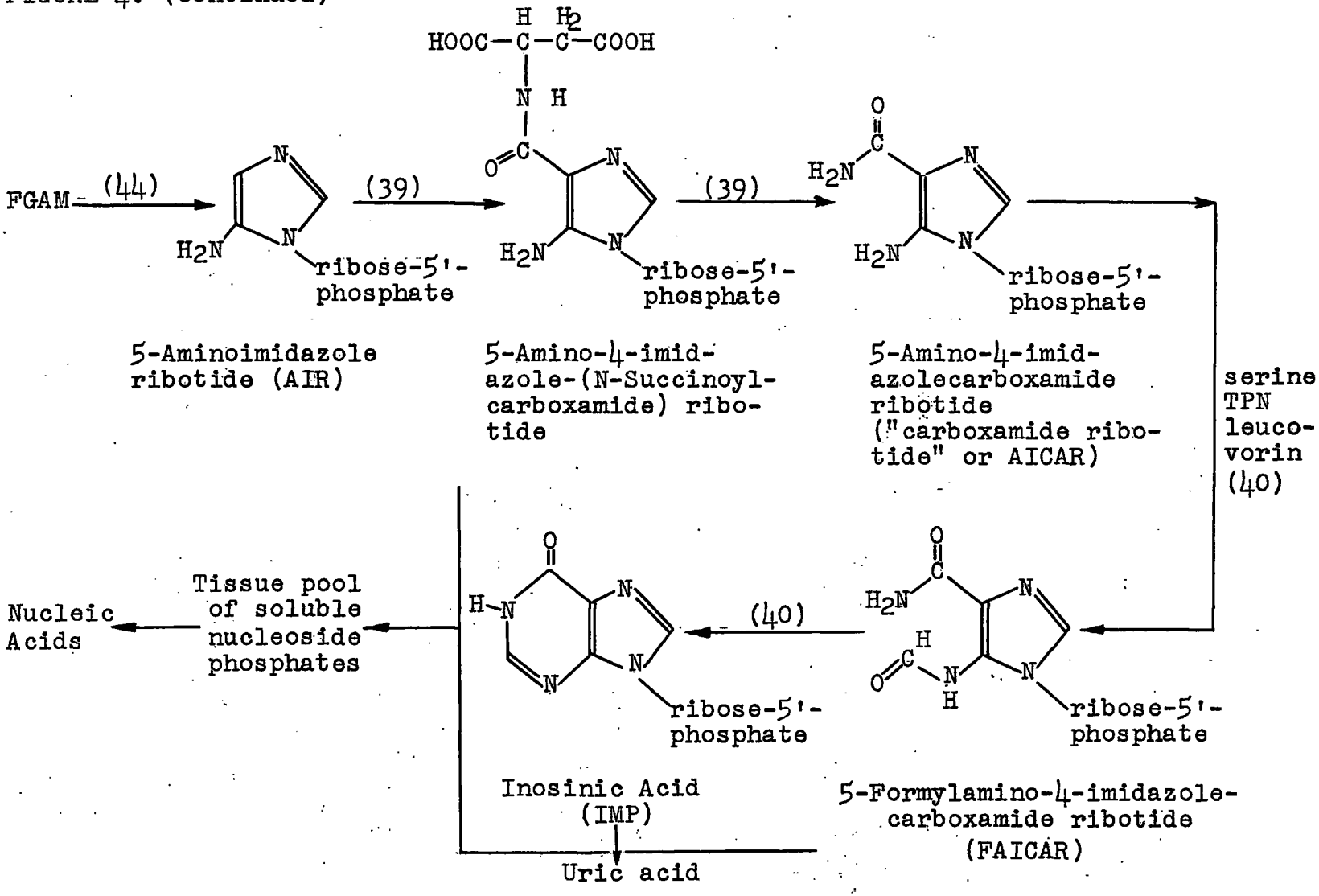


FIGURE 4. (continued)



bacteria, birds, and mammals, would suggest that the same basic mechanisms apply for purine biosynthesis. However, in avian liver the synthesis of inosinate is a major pathway directed at the production of urate, the excretory form of nitrogen in the bird. The synthesis of purine molecules, having as their fate nucleic acid components, nucleotides and coenzymes, constitutes the major purine synthetic function in other avian tissues and in other animals which do not excrete purines as their principal nitrogenous waste products. Therefore, the question arises: are the findings from pigeon liver experiments, where the urate production mechanism is being exploited, applicable to the biosynthesis of nucleic acid and coenzyme purines? The basic steps of ring synthesis are probably similar, but the points at which purine components are drawn off for nucleic acid, nucleotide, and coenzyme synthesis remain undiscovered.

Edmonds and LePage have observed in short term experiments with whole animals that inosinate, adenylate, and guanylate appear to have separate origins (5). They conclude that the nucleotides arise from a common precursor at some early stage of synthesis and are probably not metabolized along a common pathway. This point is introduced to emphasize that avian liver preparations are adequate for studies of inosinate synthesis, but the basic purine precursor may not be inosinic acid which, with subsequent oxidation and amination, would form adenylic and guanylic acids (45). These functional groups may be introduced prior to the complete cyclization of the purine ring (46). Thus, there appears to be a distinct need for parallel, comparative studies of purine biosynthesis in mammalian systems.

## 2. POLYNUCLEOTIDE SYNTHESIS.

Nucleic acid synthesis is considered to take place by the union of sub-units, the nucleotides. This concept is derived in part from structural and enzymatic studies which have shown that nucleic acids can be hydrolysed chemically and enzymatically into nucleotides. Identification of nucleoside-5'-phosphates as the proximal or immediate precursors of the nucleic acid polymer (rather than still smaller sub-units such as free bases, ribose, and phosphate) has come about through studies of the composition of the soluble nucleotide fraction of tissues and the metabolic relationships between this pool and the nucleic acids. Strictly, this mechanism can be applied only to RNA synthesis; however, in view of the similarities in DNA and RNA structure, its extension to DNA synthesis seems reasonable. Studies in DNA synthesis are not yet advanced as far as those in the RNA field, particularly in regard to the isolation of precursors.

Studies of nucleic acid synthesis using tracer techniques have employed two principal approaches, (a) through the use of labelled precursors which gain access to the acid-soluble pool by the de novo pathways, (b) through the use of labelled purines, pyrimidines and their derivatives, the nucleosides and nucleotides. The extensive literature on this latter topic has been reviewed by several authors (11 - 14), most completely by Brown and Roll (47).

### A. Desoxyribonucleic Acid Synthesis

Small molecule precursors of the purines of DNA are, in general, incorporated to a several fold smaller extent than

into the purines of RNA. DNA synthesis, as measured by the incorporation of labelled precursor molecules, occurs at higher rates in proliferating tissues than in non-growing tissues. DNA purines are derived from adenine to an extent which is directly related to the rate of cell division in the tissue under consideration, the incorporation being slight in non-proliferating tissues such as liver and kidney, but approaching that of RNA in intestine, bone marrow, and regenerating liver which are proliferating tissues. In comparisons of the renewal of DNA purines in non-growing and proliferating tissues, adenine incorporation is enhanced in the latter case to a much greater extent than is seen with "de novo" precursors, formate and glycine. Adenosine and the isomers of adenylic acid are poor precursors of RNA adenine relative to the free base and are very likely degraded to this form before incorporation (48). However, adenosine has been shown to be incorporated into DNA with the N-glycosyl bond intact (48). Guanine is utilized only slightly for nucleic acid synthesis by the rat, but, in contrast to adenine and its derivatives, the nucleotide, guanylic acid, is a superior RNA precursor. Guanylic acid is used to a small extent in DNA renewal, and in this process, the N-glycosyl bond is broken (48). In general, there is little known of the immediate precursors of the DNA purines.

Studies of DNA synthesis, in which pyrimidine derivatives are utilized, offer more definite evidence of nucleotide participation. The known precursors of DNA pyrimidines display patterns of utilization different from those of

adenine and its derivatives in the respect that the free bases of the polynucleotides are ineffective precursors, while the nucleosides and nucleotides are well incorporated. Orotic acid is readily converted into the pyrimidine bases of RNA and DNA and must pass through the acid-soluble nucleotide pool as orotodylic and uridylic acids in this process. Desoxycytidine and thymidine are unique in that they are specific DNA precursors, showing no conversion to RNA pyrimidines in the rat (49,50).

The phosphorylation of desoxyribonucleosides in an in vitro system has been demonstrated (51), and small amounts of free desoxyribonucleosides and desoxyribonucleotides have been shown to be present in liver (52). Rose and Schweigert have shown that the N-glycosyl bond of cytidine remained intact in its incorporation into desoxycytidine and thymidine of DNA (53). The knowledge that thymidine is a specific precursor for DNA, and the recent development of in vitro systems in which DNA synthesis take place (50) promise advances in the field of DNA synthesis. The immediate precursors of the desoxyribopolynucleotides are not yet known, but the evidence cited above, and the analogy with RNA synthesis suggest that desoxyribonucleotides are likely precursors.

B. Ribonucleic Acid Synthesis

The renewal of RNA bases from isotopically labelled bases has an extensive literature and will be treated only summarily in this discussion, with the objective of pointing out evidence that bears on the identity of the immediate precursors of the ribonucleic acid macromolecule. In general,

whole animal experiments have shown that adenine is well incorporated into RNA purines, whereas guanine and hypoxanthine are poorly taken up. The purine ribosides and ribotides have proven to be inferior to the free bases as RNA precursors in whole animal experiments. Such is not the case with the pyrimidine bases, where the nucleosides and nucleotides of cytosine and uracil are better incorporated into RNA than are the free bases. Early studies in this area produced little information on the nature of the intermediate nucleic acid precursors because the importance of the acid-soluble fraction in this respect was then unrecognized. In recent *in vivo* studies, the isotope contents of the acid-soluble nucleotides derived from labelled purines (5,9) and pyrimidines (1,2) have been measured and compared, showing, in general, that turnover was rapid in the nucleotides and slower in the nucleic acids. Thus, the pathways of *de novo* synthesis and the anabolism of the preformed bases appear to have a confluence in the acid-soluble fraction. The free purines are probably converted to the nucleotide form by reaction with PRPP (34 - 36).

The relative importance of the *de novo* and preformed pathways has been compared in liver and intestine, with a surprising difference being noted. In the rat, the synthesis of RNA in the liver from adenine is favoured over the *de novo* process, whereas the reverse is true in intestine.

Developments in the technique of ion-exchange chromatography have been applied with great success to the resolution of the complex acid-soluble fraction of tissues.

The nucleotide composition of this fraction has been studied by the use of gradient elution chromatography (1,2). The adenosine phosphates are particularly prominent in this complex mixture of nucleoside phosphates. As Brown and Roll point out, "the abundance of the adenosine phosphates lead to the tacit assumption that tissue adenosine monophosphate (AMP) or its polyphosphates probably serve as a source of purines, at least of adenine, for polynucleotide synthesis " (47).

Adenine-8-$C^{14}$ has been found to be incorporated by the soluble adenosine phosphates at much higher rates than by polynucleotide adenine in the mouse (9) and the rat (4). Hurlbert and Potter in their study of the incorporation of $C^{14}$-orotic acid into RNA, showed that this compound is rapidly incorporated into uridine phosphates of the acid-soluble nucleotide pool and thence into RNA (7,8). Nuclear RNA was more rapidly renewed than cytoplasmic RNA. The suthors concluded that soluble uridine phosphates were the immediate precursors of the uracil of nuclear RNA. A time study of adenine incorporation into acid-soluble adenine and nuclear RNA adenine by Fresco and Marshak (54) indicated that a general precursor-product relationship may exist between the two.

Recently, studies of RNA synthesis with cell-free systems have shown that it may be possible to study polynucleotide synthesis at the enzyme level. Goldwasser has demonstrated that RNA adenine can be derived from AMP labelled with $P^{32}$ or $C^{14}$ in homogenates of pigeon liver (55,56). Heidelberger et al. have shown a similar incorporation of $P^{32}$-labelled AMP

in homogenates of regenerating liver (57). Incorporation of $C^{14}$-AMP into the RNA of glycolysing tumour homogenates has been reported by Paterson and LePage (58). In general, the homogenate systems show a greater ability to incorporate the ribotides than free bases or early precursors. Tissue preparations, in which high-energy phosphate compounds are maintained by energy-producing processes such as glycolysis, will phosphorylate labelled nucleotide substrates, making it difficult to ascertain at what level of phosphorylation the substrate is incorporated into the nucleic acid polymer. The very important contributions of Ochoa and his coworkers suggest that RNA is synthesized from nucleoside diphosphates (59). These workers have shown that a partly purified bacterial enzyme preparation will form RNA-like polymers from the 5'-diphosphates of adenosine, guanosine, inosine and cytidine. This important discovery has provided a system in which polynucleotide synthesis can be studied at the enzyme level. It remains to be established whether these RNA-like polymers truly resemble the native polynucleotides, or whether the similarity is superficial, specific arrangements of the component nucleotides perhaps being absent.

3. PURINE SYNTHESIS IN INTESTINAL MUCOSA.

Actively proliferating tissues such as regenerating liver (60), bone marrow (61,62), and tumour (3) have been employed for in vitro studies of nucleic acid purine synthesis. In the whole animal, such tissues display a rapid turnover of nucleic acid components relative to tissues with low rates of

cell division. Avian liver has been favoured for the *in vitro* investigation of purine synthesis, since this tissue has as a particularly prominent metabolic function, the synthesis of uric acid. The rate of nucleic acid synthesis in intestinal mucosa is high in the intact animal, very probably, because of the high rate of cell division in this tissue. This suggests that intestinal mucosa would be a suitable tissue for *in vitro* studies of purine metabolism. Such a use has not been reported prior to the present work.

Hevesy and his coworkers (63) first reported that a correlation exists between the rate of cell division in a tissue and the uptake of $P^{32}$-phosphate by DNA. Their data show that intestine had the highest phosphate renewal of the principal tissues of the rat. This is not merely a feature of phosphate metabolism, as other DNA precursors such as formate and glycine have since been shown to be rapidly incorporated into the DNA of intestine and other proliferating tissues (47, 64 - 66). Stevens *et al.* have measured the cell renewal rates in several tissues of the rat, as shown in the following table:

TABLE I

RATE OF NEW CELL FORMATION IN RAT TISSUES (64)

| Tissue | Per cent daily formation of cells |
|---|---|
| liver | 0.71 |
| lung | 4.1 |
| intestinal mucosa | 54.3 |

Their work demonstrated that parallel to this very rapid proliferation of the mucosal epithelium of the intestine, there was a correspondingly high rate of incorporation of inorganic phosphate into DNA.

The RNA of intestinal mucosa also displays a vigourous renewal. The incorporation of isotopic precursors (ammonia, phosphate, formate, glycine, and adenine) by the RNA of intestine, when compared with that of liver and other tissues, shows that intestine has one of the most rapid renewal rates of RNA in the body (see Tables II and III of the review by Brown and Roll (47) ). This feature may be related to the high rate of cell division, although tissue function must also be considered in this respect.

Comparisons of formate and adenine incorporation into intestine and liver RNA (47,67) have revealed that in the latter tissue, adenine is utilized in preference to the incorporation of simple precursors via the de novo pathways of purine synthesis. The converse is true for intestine where the de novo pathway is favoured.

In the metabolism of carbohydrate intestinal mucosa has an unusual feature in which respect it bears a resemblance to tumour tissue. In most normal tissues glycolysis is supressed under aerobic conditions. A significant production of lactate from pyruvate occurs in most tissues only in the absence of oxygen. The supression of glycolysis, as measured by lactate formation, when respiration takes place is well known as the Pasteur effect. A high glycolytic rate under both

aerobic and anaerobic conditions is considered to be characteristic of tumours, with certain normal tissues displaying this feature to a similar extent (68). Dickens and Weil-Malherbe have reported a complete lack of the Pasteur effect in intestinal mucosa (69). Rosenthal has subsequently reported that the high rate of aerobic glycolysis may be, in part, attributable to damage incurred during preparation of the tissue (70). Dickens and Weil-Malherbe studied the respiration and glycolysis of strips of intestinal mucosa and their data place jejunal mucosa as one of the most actively respiring tissues of the body (69). In their experiments, respiratory rates for mucosa from various parts of the gut showed a marked decline with progress down the intestine. With respect to time, Dickens and Weil-Malherbe found that the respiration of this tissue was more stable than glycolysis. The instability of glycolysis may be related to the abundance of phosphatases in this tissue.

Lowenstein and Cohen have assayed various tissues of the rat for the enzyme carbamylphosphate-aspartate transcarbamylase, and report that intestinal mucosa has the highest activity (71). This enzyme catalyses the formation of carbamylaspartate (ureidosuccinate), an important intermediate in pyrimidine biosynthesis. The data of Allfrey et al. (72) show that intestinal mucosa is particularly rich in certain enzymes which are involved in the catabolism of nucleic acids, namely:

| | |
|---|---|
| adenosine deaminase | ATPase |
| alkaline phosphatase | adenosine-5'-phosphatase |
| DNAase II | adenosine-3'-phosphatase |

These enzymes are mainly cytoplasmic in distribution. Xanthine oxidase is also abundant in mucosa (73). The prominence of these catabolic enzymes may be related to the failure to detect purine synthesis in homogenates of intestinal mucosa in the experiments described subsequently.

While this manuscript was in preparation several reports appeared in which the *in vitro* use of intestinal mucosa has been described. Heidelberger and Harbers have reported the incorporation of orotic acid into the uridine nucleotides of the high speed supernatant fraction of homogenates of several rat tissues, including intestinal mucosa (74). The effect of aminopterin on intestinal mucosa in the intact rat and on the respiration of this tissue *in vitro* has been described by Vitale *et al.* (75,76).

In initiating the present studies, recognition was made of the need for a study of the mechanisms of *de novo* adenine and guanine synthesis in mammalian tissues. More particularly, it is important to know whether or not the known pathways for inosinic acid biosynthesis in avian liver are also primarily involved in adenylic and guanylic acid biosynthesis in mammalian tissues. The use of *in vitro* systems and tissue extracts in such studies is indicated since this approach has been fruitful in the isolation of intermediates in inosinic acid biosynthesis. Certain of the features discussed previously recommended intestinal mucosa as a likely mammalian tissue for *in vitro* studies of purine metabolism: (a) the very high rate of cell division, (b) the rapid renewal of DNA and RNA, (c) the

prominence of de novo pathways of purine renewal, (d) the high rate of respiration and glycolysis in vitro.

In the present studies a method was developed for preparing the mucosa of the small intestine of the rat as a suspension in Krebs-Ringer buffers. In this form the tissue survived for several hours of incubation, respiring actively. The de novo synthesis of acid-soluble purines was shown to occur in such suspensions by measuring the incorporation of isotopic formate, glycine, and carbon dioxide into this fraction. The renewal of soluble adenine nucleotides was prominent, being approximately 6 times that of the guanine nucleotides. The synthesis of polynucleotide purines also occured during incubation. The specific activity relationships between the isotopic precursors and the products suggested that the acid-soluble nucleotides were intermediates in nucleic acid synthesis. The demonstration that rat intestinal mucosa is capable of a rapid synthesis of adenine and guanine in vitro suggests that this system may be applied profitably to a study of the intermediates involved in purine biosynthesis as it occurs in mammalian tissue.

# EXPERIMENTAL

## 1. ANALYTICAL METHODS

In this section analytical methods are discussed from the technical point of view. Their application to biological material and the resulting problems are discussed in subsequent sections.

### A. Ion exchange chromatography

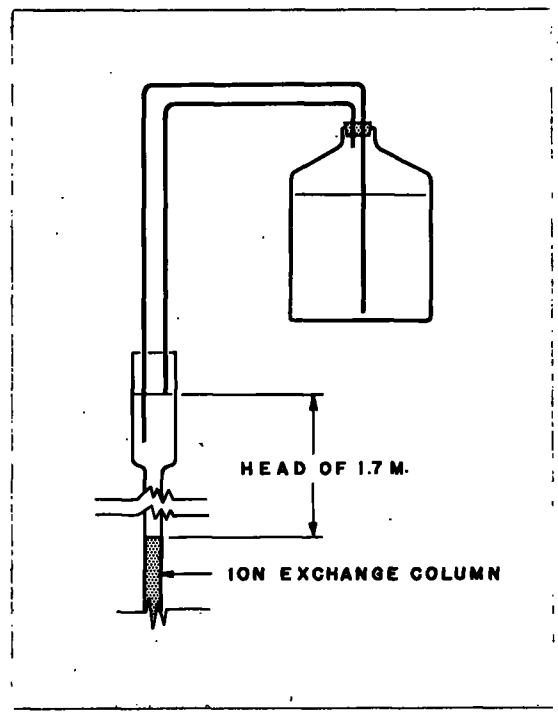
(i) The separation of adenine, guanine, hypoxanthine, xanthine, xanthine and uric acid Solutions of these purines were resolved into their components on columns of Dowex-50 ion exchange resin by first eluting with 0.1N HCl to remove the oxy-purines, which emerged in the following sequence: uric acid (not retained), xanthine, and hypoxanthine. By changing the eluent to 1.5N HCl, guanine was removed, followed by adenine. A similar scheme was recently described by Abrams and Bentley (77).

Procedure Dowex-50 cation exchange resin (200-400 mesh) was prepared for use by first removing the "fines" by decantation

from a water suspension of the resin. Removal of the "fines" increased the rate of flow through the resin. Several volumes of 6N HCl were used to wash the resin and the excess acid was removed by several rinses with water. The resin was then washed with 6N NaOH followed by several rinses with water. Finally, the resin was washed again with 6N HCl, followed by washes with water which were continued until the washings were neutral. This washing procedure freed the resin of a brownish, soluble impurity.

A solution of the above purines in 0.1N HCl was poured onto a 1 x 7 cm. Dowex-50 column (200-400 mesh) and the effluent was collected in 5 ml. fractions using a Technicon automatic fraction collector. The purines were located in the effluent by measuring the optical density of each fraction at 260 mμ in the Beckman Model DU Spectrophotometer and were identified by their absorption spectra. The column was first eluted with 0.1N HCl, flowing at 0.5 ml. per min., until hypoxanthine was removed. The eluent was then changed to 1.5N HCl. The flow rate was achieved by attaching to the column an eluent reservoir made from glass tubing high enough to give a hydrostatic head of 1.7 m. The eluent level in the reservoir was kept approximately constant by the filling device shown in Figure 5.

FIGURE 5. ION EXCHANGE COLUMN WITH SELF-FILLING PRESSURIZING RESERVOIR




(ii) Isolation of adenine and guanine from intestinal mucosa Adenine and guanine were routinely isolated from perchloric acid extracts of intestinal mucosa using the ion exchange technique described by LePage (3).

Procedure The purine-containing extracts were passed through small columns (6 x 15 mm.) of Dowex-50 resin (200-400 mesh) which retain the purines. After washing the resin with 2.0 ml. of 0.1N HCl, followed by 2.0 ml. of water, adenine and guanine were recovered together by eluting with 4.0 ml. of 6N HCl. The 6N HCl fractions were evaporated to dryness in vacuo over sulfuric acid and sodium hydroxide.

B. Paper chromatography

Descending paper chromatography on Whatman No. 1 filter paper was used throughout these experiments.

(i) Solvent systems The choice of solvent systems for chromatography was influenced by two factors: the behaviour of guanine, and the contamination of the isolated purines by amino acids, especially glycine. Strongly acidic systems such as the tertiary-butanol-HCl solvent of Smith and Markham (78) and the iso-propanol-HCl system of Wyatt (79) have proven to be among the most satisfactory solvents for the chromatography of guanine, having the capacity for moving larger amounts of guanine without streaking than neutral or basic systems (80). The Wyatt system was used routinely as the first solvent in the chromatography of the acid-soluble fraction. The overlapping of glycine and adenine spots introduced the problem of radiochemical purity since glycine-1-$C^{14}$ was used as a substrate in certain experiments. The iso-amyl alcohol-disodium phosphate solvent of Carter (81) separated glycine ($R_f$ 0.70) and adenine ($R_f$ 0.44) but gave poor results with guanine. In this solvent guanine formed double spots and streaked readily if overloaded on the paper. Guanine has a low solubility in this solvent and accordingly, does not move from the point of application. It is usually not distributed uniformly throughout the area of application, more accumulating on the side of the paper to which it was applied than on the side opposite[2].

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2 LePage, G.A., personal communication.

This behaviour of guanine has obvious disadvantages if purine areas of chromatograms are to be assayed for radioactivity directly.

The coincidence of ninhydrin-positive areas with purine areas was observed on chromatograms of the acid-soluble purines which were run in Wyatt's solvent. This raised the possibility of radioactive contamination. The n-butanol-ethanol solvent of Berry et al.(82) was found to leave near the origin the amino acids which accompany adenine and guanine in the iso-propanol solvent.

For these reasons, in the majority of the experiments described herein, the acid-soluble purines were first chromatographed in Wyatt's iso-propanol-HCl solvent. The purines from these chromatograms were recovered by extraction with 0.1N KCl and rechromatographed in n-butanol-ethanol. This procedure separated the purines from accompanying amino acids and increased the specific activity of adenine. Unless otherwise noted, adenine and guanine were purified in this manner.

Other systems were tried but with less satisfactory results. Chromatograms run first in butanol-ammonia (83) showed considerable loss of activity in the region of guanine when rechromatographed in iso-propanol-HCl.

(ii) Visualizing purine areas The nucleic acid bases were located on paper chromatograms by the conventional ultraviolet techniques which employ the high absorbancy displayed by these compounds in the region of 260 mu. In order to outline the purine areas in pencil, chromatograms were examined in transmitted light from an ultraviolet printing box

which had a germicidal lamp[3] as a light source. The ultraviolet light was filtered through a large glass filter[4]. This apparatus was far superior to the widely recommended Mineralight lamp for surveying chromatograms. The definition of the purine spots, which appear as dark areas against the faint fluorescence of the paper, was especially improved by the use of transmitted light. The outlining of the spots was facilitated by using the filter as a surface against which to draw. Contact prints of purine chromatograms, made with unfiltered light from the germicidal lamp, were prepared routinely for record purposes and to aid in cutting out the purine areas for radiocarbon assay and spectrophotometry. For the detection of ultraviolet absorbing areas, the photographic print was much more sensitive than the visual examination. Since pencil marks were recorded clearly on such prints, purine areas that had been outlined in pencil were located and cut out with precision.

With this technique adenine and guanine in amounts of 1 $\gamma$, as chromatogram spots of ordinary size (2-3 cm.$^2$), were manipulated with ease. Uric acid spots were only faintly recorded with this technique because this purine does not absorb strongly in the spectral region in which the germicidal lamp has its principal emmision. To confirm the identity of

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3 A General Electric 15 watt germicidal lamp, which emits 90 per cent of its radiation at 253.7 m$\mu$, was found to be a satisfactory ultraviolet source for scanning and printing paper chromatograms.

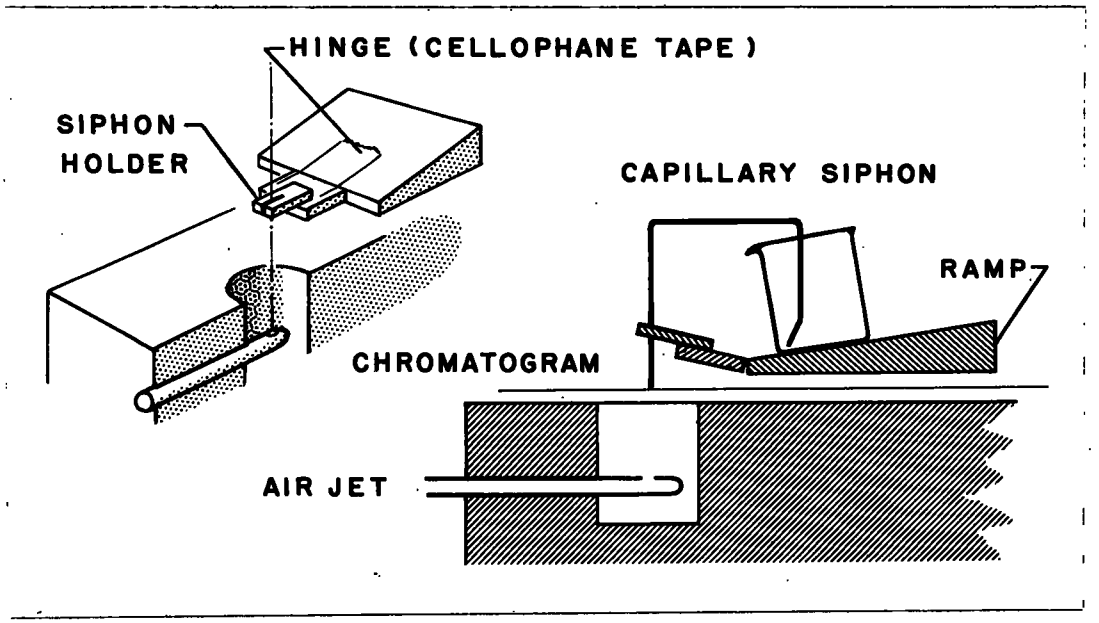
4 Corning No. 9863 glass filter

the faintly absorbing uric acid spots, samples of authentic uric acid were chromatographed on the same paper. The spots were located by cutting out the lane and spraying it with ammoniacal silver nitrate. The striking blue fluorescence of guanine hydrochloride in the light from the germicidal lamp enabled this purine to be distinguished from others on the chromatogram. The fluorescent spot is more difficult to delineate than the dark spot obtained when the same amount of guanine is present as the free base.

(iii) Capillary siphon applicator Prior to purification by chromatography, adenine and guanine were obtained as a residue left by evaporation of the 6N HCl eluate in the ion exchange step of their isolation procedure. As the quantitative transfer of this material to chromatograms proved to be a tedious operation, an apparatus utilizing capillary siphons was constructed which would automatically apply the purine solutions to the chromatograms. The device, which is illustrated in Figure 6 employed a jet of warm air directed against the chromatograms, immediately below the point at which each siphon was applied. By controlling the flow of air and its temperature, the purine solution was evaporated as fast as it was applied and the area of application kept to approximately 1 cm. in diameter.

An apparatus consisting of four of the applicator units shown in Figure 6 was used in this investigation. In use, the capillary siphons were first "primed" by filling them with water and were held in position against the paper by

FIGURE 6. CAPILLARY SIPHON APPLICATOR FOR PREPARING CHROMATOGRAMS.




The capillary siphons were made from heavy-walled pyrex tubing (7 mm. O.D. x 2 mm. I.D) drawn out to 0.5 mm. O.D. The siphon holder was cut from heavy rubber sheet, 4 mm. thick, and was stapled to a piece of heavy solid cardboard. This unit was attached to the ramp, as illustrated, by a hinge of cellophane tape. The surface of the ramp was painted with rubber-base paper adhesive to prevent the sample beakers from sliding out of position. The air jets were made from 7 mm. O.D. glass tubing with a 2 mm. hole and were positioned in the apparatus with the hole aligned directly below the point at which the capillary siphon was applied to the papergrams. Air supplied to the jets was warmed by passage through a pyrex tube heated by a bunsen flame. A wire gauze protected the portion of the tube against which the bunsen flame was directed.

the rubber fingers of the siphon holder assembly. The sample to be chromatographed was contained in a 5 or 10 ml. beaker. With the air temperature between 40 and 50°C. the apparatus applied aqueous solutions at approximately 2 ml. per hour. Filling and cleaning the siphons was greatly facilitated by use of a piece of small diameter polyethylene tubing, which, with careful heating, was drawn out to make a "neck" near one end, with a diameter of less than 0.5 mm. The siphons were inserted into the tube and gently forced into the "neck", making a seal. By applying suction to the plastic tube, water was drawn into the tube for filling or cleaning.

C. Spectrophotometry

(i) Ultraviolet absorption spectra The high absorbancy in the ultraviolet region which is characteristic of the nucleic acid bases and the development of the photoelectric spectrophotometer have provided the basis for very sensitive methods of analysis for these compounds. The absorption spectra, being characteristic for each base, are widely used to identify purines, pyrimidines and their compounds. Their optical properties have been recently compiled in a comprehensive review by Beavan, Holiday and Johnson (84).

The Beckman Model DU quartz spectrophotometer was used for the measurement of optical densities throughout this work. The absorption spectra of adenine, guanine and uric acid were determined in 0.1N HCl and are shown in Figures 7, 8, and 9. The purines used for this purpose were commercial products and were purified in the following manner:

Adenine Adenine (Nutritional Biochemicals Corp.), shown to be chromatographically pure, was twice crystallized as the sulfate from water. The free base was prepared from a solution of the sulfate by the addition of excess sodium bicarbonate. The precipitated adenine was washed with water until the washings were free of carbonate and sulfate.

Guanine Guanine (Nutritional Biochemicals Corp.), known to contain adenine, was dissolved in dilute HCl. Guanine was selectively precipitated by adjusting the acidity of this solution with sodium hydroxide until it was acid to litmus yet alkaline to congo red (85) and was then recrystallized three times as the sulfate. The free base was prepared from a solution of the sulfate by the addition of excess ammonium hydroxide and the guanine precipitate was washed until free of sulfate.

Uric acid Uric acid (Kodak) was dissolved in warm concentrated sulfuric acid and recovered from this solution by diluting with water. After repeating this operation, the uric acid was washed free of sulfate and recrystallized from water.

Prior to use, the purified purines were dried for 2 hours, *in vacuo*, at 100° C. over $P_2O_5$. Known amounts of the purines were dissolved in 0.1N HCl and the optical densities of the solutions measured at various wavelengths. From these data, molar extinction coefficients were calculated and are plotted in Figures 7, 8 and 9. There is close agreement between these curves and published data (84,86).

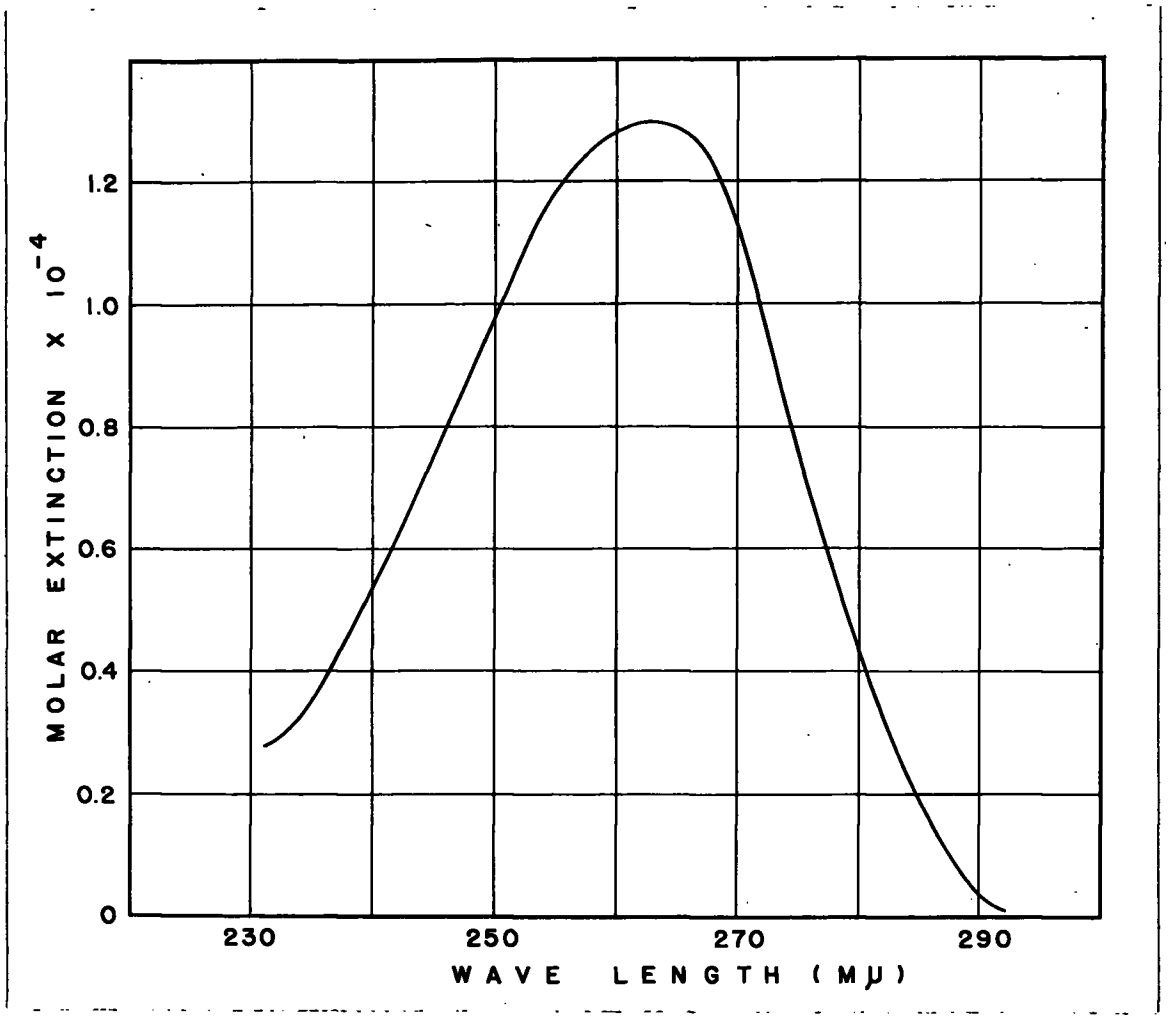



FIGURE 7. ABSORPTION SPECTRUM OF ADENINE IN 0.1N HCl.

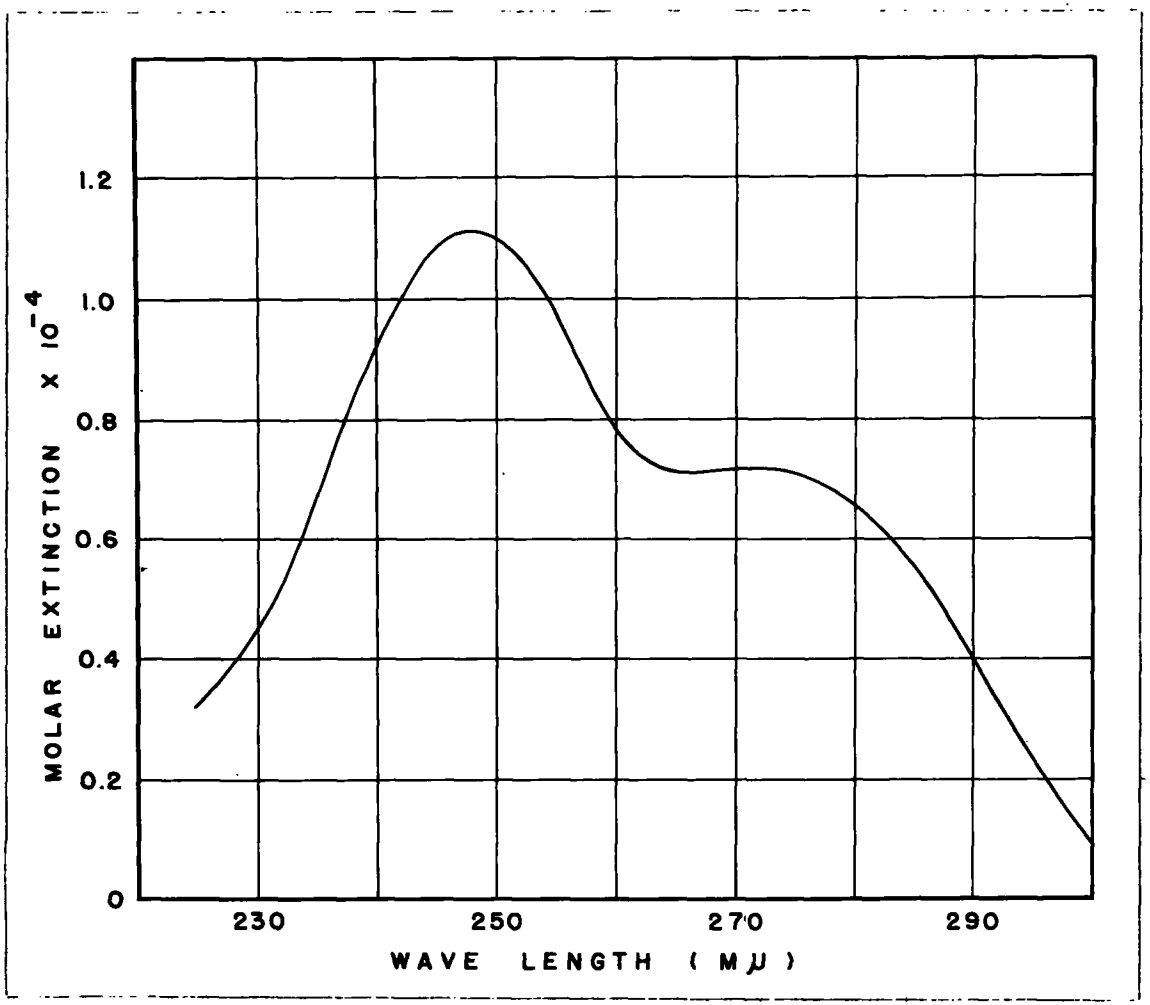



FIGURE 8. ABSORPTION SPECTRUM OF GUANINE IN 0.1N HCl

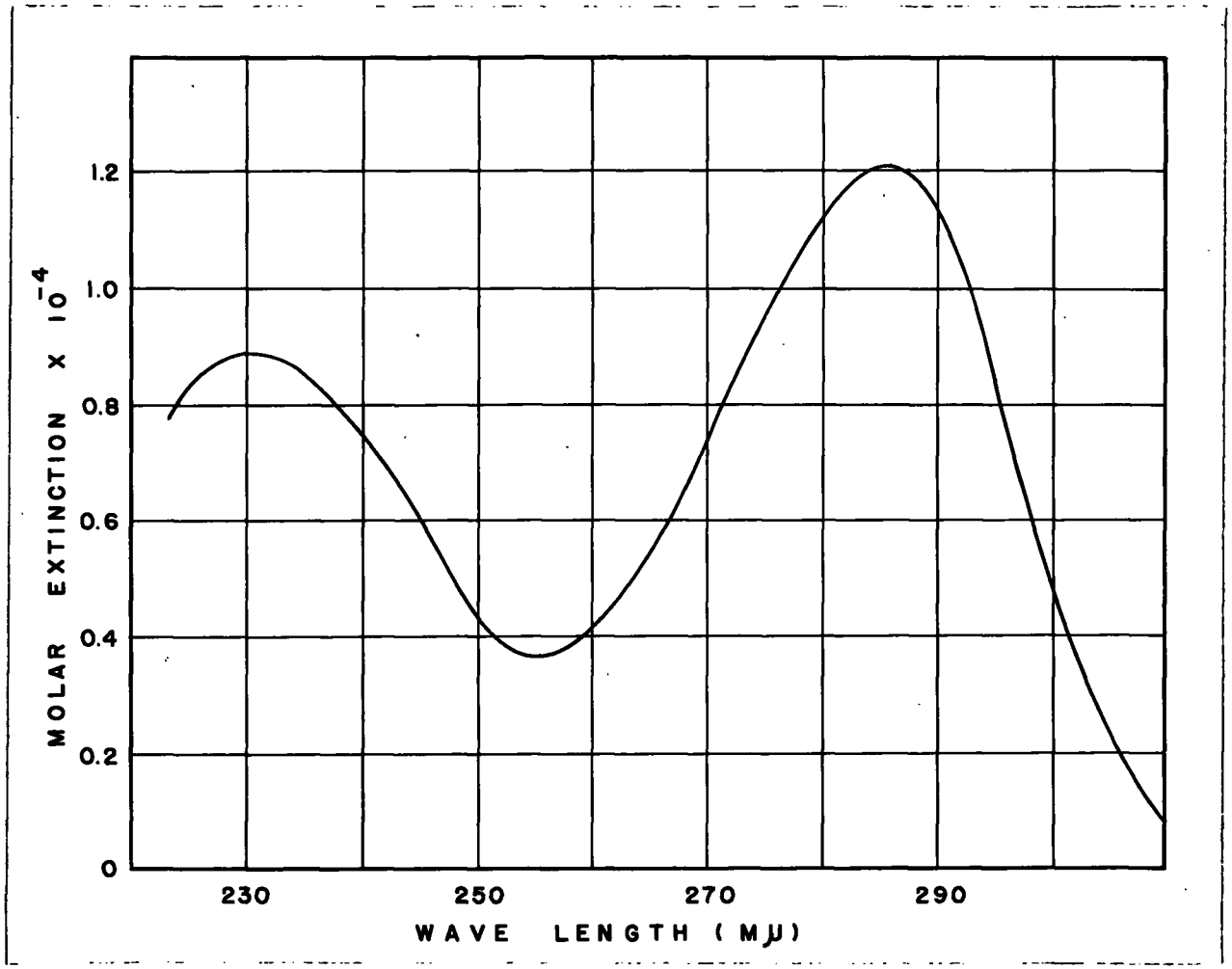



FIGURE 9. ABSORPTION SPECTRUM OF URIC ACID IN 0.1N HCl.

(ii) Determination of purines The purine content os discs punched from chromatograms was estimated spectrophotometrically after the measurements of radioactivity. This widely used method of analysis (80) consists of extracting the purine-containing disc of paper with 0.1N HCl and measuring the ultraviolet absorption of the extract at certain appropriate wavelengths.

The paper disc was cut into small pieces, placed in a test tube with 4.0 ml. of 0.1N HCl, allowed to stand overnight at room temperature, and then shaken for at least one half hour in a mechanical shaker. The extraction of purine was virtually quantitative by this method, as trial recovery experiments showed recoveries of 94 - 103 per cent for adenine and 94 - 105 per cent for guanine.

For estimation of the purine content of the extract the difference was measured between the optical densities at certain wavelengths which are indicated in Table II. By applying the appropriate molar extinction value, $\epsilon_{\Delta}$, the purine content of the extract was calculated. All optical density measurements were made against blanks which consisted of extracts of discs cut from blank lanes of the same chromatogram.

The use of extinctions measured between the spectral maximum and a reference wavelength where the absorption is low (as opposed to measurements made only at the maximum) was intended to compensate for the error introduced by the presence of non-purine ultraviolet-absorbing impurities which occur to

TABLE II

ULTRAVIOLET ABSORPTION DATA USED IN THE DETERMINATION OF PURINES

| Purine | $\lambda_M$ | $\lambda_R$ | $\epsilon_\Delta$ |
|---|---|---|---|
| Adenine | 262.5 mμ | 290 mμ | $1{\cdot}25 \times 10^4$ |
| Guanine | 248 " | 300 mu | 1·02 " |
| Uric acid | 284 " | 310 mu | 1·13 " |

1. $\lambda_M$ is the wavelength of maximum optical density.
2. $\lambda_R$ is the reference wavelength, arbitrarily chosen at a point where the extinction is low, yet definite.
3. $\epsilon_\Delta$ is the difference between the molar extinction coefficients measured at $\lambda_M$ and $\lambda_R$.

varying extents in the filter paper extracts. With this method the difference in extinction between the two wavelengths is a specific property of the purines and is unaffected by other absorption in this region providing the latter is equal at both wavelengths. In preliminary experiments with extracts of replicate adenine spots on chromatograms, the variation between analyses based on $\epsilon_\Delta$ was reduced to one half of that when $\epsilon_{262.5}$ was used. In addition, purine contents calculated with $\epsilon_{262.5}$ were 4 - 5 per cent higher than those calculated with $\epsilon_\Delta$.

(iii) Accuracy of purine analysis The error inherent in this analytical method was estimated by comparing analyses of 10 replicate purine spots from the same chromatogram. At the level of 2.2 γ of adenine per spot, the extreme

values differed from the mean by 3 and 5 per cent. With guanine, at the level of 4.1 γ per spot, the analagous experiment indicated a larger variation, the extremes of the analyses being 11 per cent from the means. In a similar experiment, a lower variation was noted when 14.4 γ of adenine per spot was used, the extremes differing from the mean of 17 analyses by less than 2 per cent.

Thus, it would appear that when this method is applied to filter paper discs containing 2 - 5 γ of purine, duplicate analyses could be expected to vary approximately 5 per cent from their mean in the case of adenine and to the larger extent of approximately 10 per cent with guanine.

D. Radioactivity measurements

(i) Instrumentation Carbon$^{14}$ radioactivity was assayed in either of two gas flow counters[5] connected with commercial scaling units[6]. The counters were operated in the proportional region and used a gas mixture of argon and methane (90:10). Differences between the two counting units and individual daily variations in counter and scaler performance were compensated for by the use of a standard radiocarbon source. Each count was corrected by a factor consisting of the counting rate of the standard source at some arbitrarily chosen time (this term is constant) divided by the counting rate of the standard source at the time of the observed count. (87).

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5 Counter Model CFIS-2, manufactured by Atomic Energy of Canada Limited.

6 Nuclear Instrument and Chemical Corporation scaler, Model 163; Tracerlab Incorporated, Model SC-32 Ampliscaler.

(ii) Methods of mounting samples Three types of sample mounting were employed in $C^{14}$ assay, "infinitely-thin" samples mounted on aluminum planchets, samples contained in filter paper discs (usually cut from chromatograms), and "infinitely-thick" samples of barium carbonate. Purine samples were routinely counted on filter paper discs and carbonates were counted in the form of barium carbonate. The only exceptions to this procedure are noted in the text.

(a) "Infinitely-thin" samples This type of mounting was used in several preliminary experiments but was subsequently replaced by the more convenient filter paper disc method. Solutions of the radioactive sample, containing submilligram amounts of material, were placed in flat aluminum planchets which were provided with a fine ring of wax to confine the liquid to a definite area. A spreading agent 100 λ of a 1:1000 dilution of Triton X-100[7] (8) was used to distribute the solution uniformly over the wax-rimmed area and promoted the deposition of the sample as a uniform layer upon drying. An attempt to relate activities of samples mounted in this fashion with activities of identical samples mounted on filter paper discs indicated that the thin-film samples were subjected to a space charge effect. This effect was manifested as an inability to get acceptable agreement between replicate counts on the same sample, the discrepancy usually being in the form of a diminishing activity with successive counts. The scatter

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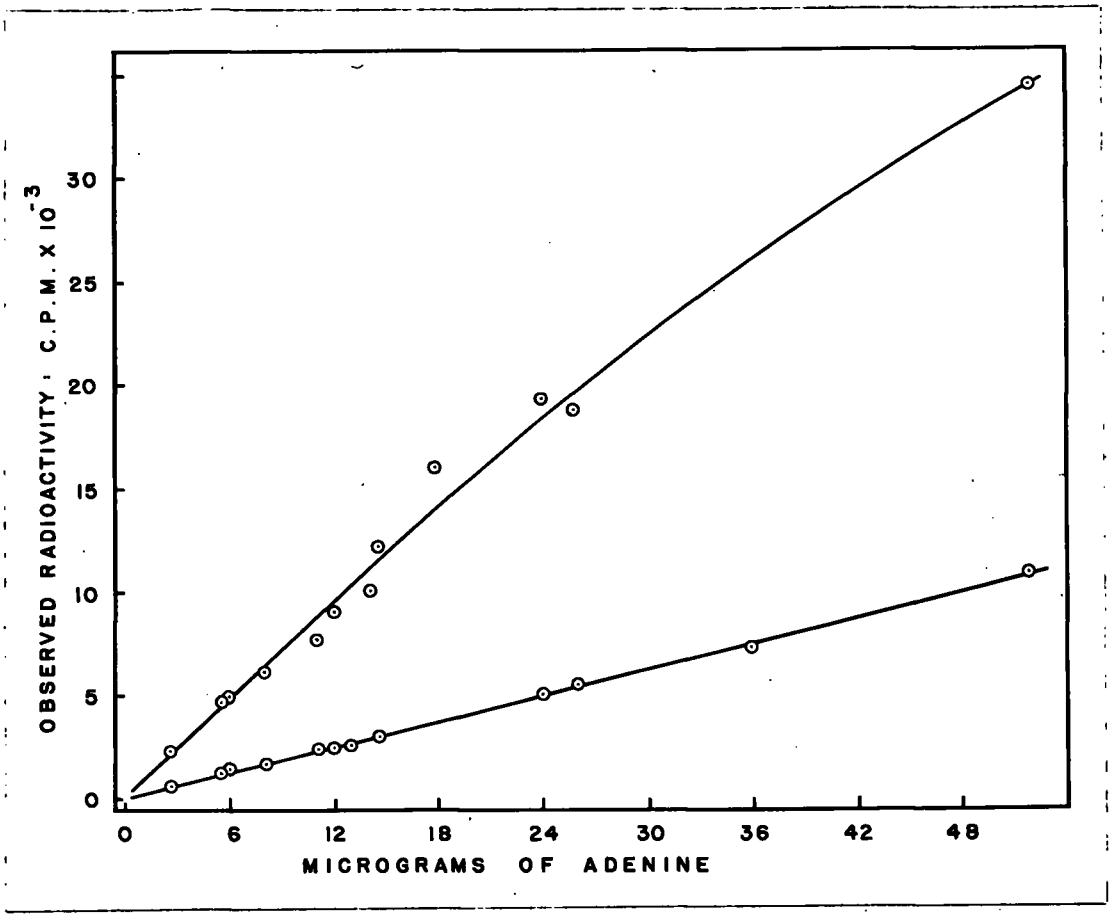
7 A product of the Rohm and Haas Company.

of the points in the upper curve in Figure 10 illustrates the poor reproducability with this type of sample mount when used in the above mentioned counter. The departure from a linear relationship between counting rate and amount of sample was interpreted as being due to a space charge. In the case of the filter paper-mounted samples, prepared from the same solution of adenine-$C^{14}$, the excellent linearity shown in the lower curve of Figure 10 indicates that this effect was absent. The application of colloidal graphite to thin-sample planchets, as suggested by Reid and Robbins (88), may possibly overcome this difficulty.

(b) Samples mounted on filter paper discs This method of sample mounting has an obvious application to the assay of radioactive substances on chromatograms and was examined with this use in mind. In the experiment illustrated in Figure 10, excellent linearity was obtained between the observed radioactivity and the amount of radioactive material present on the paper disc, indicating the suitability of this type of sample mounting for the assay of radiocarbon. The difference in the slopes of the two curves in Figure 10 shows that absorption by the filter paper reduces the radioactivity by a factor of approximately 4. This factor could not be evaluated accurately from these data, but the trend towards linearity in the thin sample curve suggests that it would be constant in the absence of the space charge effect. Experiments of the same type with biologically labelled guanine gave similar results.

FIGURE 10. A COMPARISON BETWEEN "INFINITELY-THIN" AND FILTER PAPER-MOUNTED SAMPLES OF ADENINE-$C^{14}$.




Samples of labelled adenine (obtained from intestinal mucosa which had been incubated in vitro with $C^{14}$-formate) were prepared as "infinitely-thin" samples mounted on aluminum planchets. Duplicate samples were placed on discs of Whatman no. 1 filter paper, 26 mm. in diameter. During counting, the paper discs were mounted on aluminum planchets which were provided with tabs designed to hold the disc by the edges. The same preparation of adenine was used for all data shown.

Reid and Robbins (88) state that a conducting surface on the radioactive sample improves counting reproducability in the gas flow type of counter. For this reason the apparent absence of the charge effect in filter paper-mounted samples may be due to conductivity of the paper, attributable perhaps to its water content[8]. Purine areas from chromatograms were routinely assayed for radioactivity in the form of paper discs and unless otherwise mentioned, it may be assumed that this procedure was followed.

This type of sample mounting has been employed by LePage (3) and Hurlbert (8). A similar type of sample mounting technique has been described recently (89,90) in which lens paper was used to facilitate uniform distribution of the sample on the planchet.

To prevent a possible loss of activity through volatility caused by acid vapours in the laboratory air, sodium formate-$C^{14}$ samples were assayed on paper discs punched from filter paper that had been previously sprayed with 0.5 M $K_2HPO_4$ (69).

(c) Infinitely-thick barium carbonate samples This method of sample mounting was used for radiocarbon assay of the bicarbonate substrate, carbonate trapped in the centre wells of Warburg vessels and carbonate produced from formate by oxidation with mercuric sulfate (91). The two techniques described previously cannot be used for carbonates since they

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8 Polglase, W.J., personal communication.

undergo a rapid exchange with the carbon dioxide of the air (87). The carbonate sample for assay was precipitated as barium carbonate and prepared for counting by a modification of the procedure previously followed in this laboratory (92,93) which consists essentially in collecting the sample by filtration in paper-lined, brass filtering dishes. In the modification employed here, the barium carbonate samples were collected on single discs of filter paper placed on the perforated bottom of the brass dishes. This established direct contact of the carbonate sample with the brass dish (the lining of filter paper used previously prevented this). This modification and the use of filter paper discs which had been previously impregnated with colloidal graphite[9] improved the reproductibility of counting rates when compared with samples prepared by the earlier technique[10].

(iii) Specific activity Specific activity was defined as counts per minute per milligram of sample (c.p.m. per mg.).

(iv) Estimation of errors The statistical errors inherent in the counting procedure were estimated with the following formula for the percentage error of a net activity (87):

$$E = \frac{100\ K\sqrt{(N_b + N_s)}}{N_s + N_b}$$

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9 Filter paper discs were dipped in a 1:25 dilution of concentrated Aquadag (Acheson Colloids Company, Port Huron, Michigan, U.S.A.) and dried, with the process being repeated until they had a uniform grey-black appearance. Two dips were usually required.

10 S. H. Zbarsky, unpublished information.

where, E = error of net activity

$N_b$ = background counts

$N_s$ = total of background and sample counts

K = probability constant (K = 1, standard error, was used in these experiments).

For use in this formula, background and the unknown activity must be counted for equal periods.

Unless otherwise noted counting errors are less than 5 per cent.

E. Respirometry

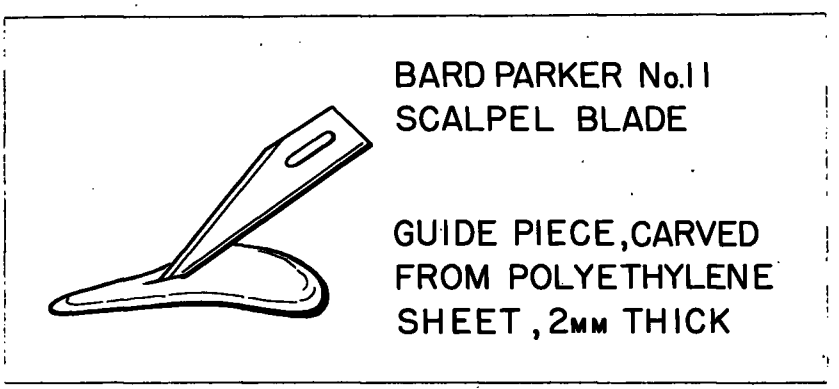
Oxygen consumption was measured by the conventional Warburg technique (94) and results are expressed in terms of $Q_{O_2}$ (microlitres of oxygen consumed per mg. of tissue, dry weight per hour).

2. THE PREPARATION OF SUSPENSIONS OF INTESTINAL MUCOSA

The *in vitro* experiments of Dickens and Weil-Malherbe (69) with strips of intestinal mucosa showed that the metabolic activity of this tissue decreases progressively along the length of the small intestine. Consequently, in studies of the metabolism of mucosa *in vitro* that require comparisons between replicate samples of this tissue, it becomes essential that some way be found to make these samples identical. It was found that relatively uniform, whole-cell suspensions of mucosa could be obtained by gently syringing a suspension of mucosal strips. Of course, homogenates will provide completely identical aliquots, but such preparations were found to be inactive in respect to purine synthesis.

In obtaining the strips of intestinal mucosa, the technique suggested by Dickens and Weil-Malherbe (69) was followed. Male Wister rats were killed by a blow on the head and decapitated. The small intestine was removed immediately, chilled and cut into 10 cm. segments. These were flushed free of contents with the Krebs-Ringer medium described below. The segments were next split open and applied to a chilled glass slab with the mucosa surface upwards. The splitting device illustrated in Figure 11 greatly facilitated this step, splitting the segment and applying it to the plate in one operation.

FIGURE 11. INTESTINE SPLITTER




While pinning the muscularis down with a microscope slide, the mucosa was stripped off with another slide, using a scraping motion. If the stripping slide had a straight, sharp edge, such as that obtained on a freshly broken slide, the mucosa was easily removed as a continuous strip, leaving the muscularis applied to the plate.

The strips of mucosa were placed in 10-15 ml. of ice-cold medium and freed by gently mixing with a stirring rod. The tissue was washed several times by centrifuging the preparation at low speed, followed by resuspension in fresh medium. The strips were then fragmented by gently and repeatedly drawing the suspension into, and expelling it from, a 10 ml. syringe. The resulting suspension was then strained through one thickness of gauze, the tissue collected by centrifugation and resuspended in the cold medium, making the final volume 25 ml. With this procedure the small intestine of a 200 g. rat provided a suspension containing approximately 10 mg. dry weight of tissue per ml.

3. THE PURINES OF THE INTESTINAL MUCOSA SUSPENSION

In preliminary experiments the purine composition of the mucosa suspension was examined by the ion exchange procedure described above. The total purine content of the suspension was isolated by means of the hot perchloric acid extraction method of Scheider (95), followed by precipitation as silver salts.

Procedure A suspension of rat intestinal mucosa was incubated at 37° C. in Krebs-Ringer bicarbonate medium (94) for 2.5 hours, at which time it was made 4 per cent in perchloric acid and heated at 90° C. for 30 minutes (95). The tissue residue was separated from the extract by centrifugation and washed with 2 per cent perchloric acid. The combined extract and washings were neutralized with ammonium hydroxide, treated with an excess of magnesia mixture (96) and allowed to stand in the refrigerator overnight. The precipitate was removed by

filtration, washed with dilute ammonium hydroxide, and the combined filtrate and washings were treated with an excess of the ammoniacal silver nitrate reagent of Graves and Kober (97). After standing overnight in the refrigerator, the gelatinous precipitate of the silver purines was recovered by centrifugation and washed twice with cold, dilute ammonium hydroxide. The silver purine precipitate was then decomposed by heating with 0.5 M HCl and, after cooling, the precipitate of silver chloride was removed by centrifugation. The purine solution was diluted with 4 parts of water and chromatographed by the ion exchange procedure described above. The only purines found in this extract were uric acid, guanine and adenine. They were identified by their position in the elution sequence, their ultraviolet absorption spectra and by paper chromatography in tertiary-butanol-HCl (78). In all subsequent experiments, these three were the only purines identified.

4. USE OF THE MUCOSAL SUSPENSION

A. Media

Krebs-Ringer carbonate or phosphate solutions (94), containing glucose (0.2%) and aureomycin (10 parts per million), were used as the suspending media. The inclusion of aureomycin in the medium was intended to minimize bacterial growth and is discussed in subsequent sections. In the phosphate-buffered saline, prepared in the designated manner (94), calcium precipitated to varying degrees and for this reason its concentration was reduced to one half of the recommended value. Elliot mentions similar difficulties with phosphate-buffered

media and omits calcium entirely (98). The bicarbonate buffer appeared to be the most satisfactory with this particular tissue, giving better pH control during incubation than the phosphate buffer. The fall in pH during incubation (post incubation values of pH 7 and slightly below were observed) could be minimized in future work with this system by using media with initial pH values of 7.6 - 7.8 (98) and by using more dilute suspensions of mucosa. This latter course would have to be followed by the combination of several replicate flasks (or by use of larger flasks) to provide sufficiently large amounts of acid-soluble purines for isolation and analysis.

B. Procedure for a typical experiment

(i) Incubation In a typical experiment, 3.0 ml. portions of the mucosal suspension were added to Warburg flasks which contained the substrates under test. The preparation was then incubated in the Warburg respirometer at 37° C. for 3 hours, except in the time study experiments. When possible, the respiration of the tissue was followed during the incubation.

(ii) Isolation and analysis of purines For the isolation of the purines the suspending medium was recovered by centrifuging the flask contents and stored in the frozen state for subsequent examination. To isolate the acid-soluble nucleotide fraction by the method of LePage (3), the tissue was first dispersed in 3.0 ml. of cold 2 per cent perchloric acid and kept in ice for 15 - 20 minutes. The tissue residue

was recovered by centrifugation and the cold extraction was repeated twice with 2.0 ml. portions of 2 per cent perchloric acid. The combined cold perchloric extracts were called the acid-soluble (AS) fraction.

The tissue residue was extracted with 3.0 ml. of 4 per cent perchloric acid for 30 minutes at 90° C. After cooling, the residue was spun down and washed twice with 2 ml. portions of 2 per cent perchloric acid. The combined extract and washings contained the nucleic acid (NA) purines, liberated by hydrolysis from the polynucleotides. The AS fraction extracts were also heated at 90° for 30 minutes to liberate the purines from the nucleotides.

The purines were isolated from the perchloric acid extracts on the small Dowex-50 columns described previously. Adenine and guanine were contained in the 6N HCl eluates which were evaporated to dryness at reduced pressure over sulfuric acid and sodium hydroxide.

The purine-containing residues were taken up in approximately 0.5 ml. of water and placed on chromatograms with the applicator described earlier. Chromatograms were run first in Wyatt's iso-propanol-HCl solvent (79). It was essential to dry thoroughly such chromatograms prior to radiocarbon assay as traces of this solvent remaining on the filter paper discs caused the appearance of spurious counts. Adequate drying was achieved by hanging the papers in a current of air (such as produced in a powerful fume closet) for at least 15 hours.

Purine areas on the chromatograms were located by the combined visual scanning and contact printing methods with ultraviolet light, and then punched out as discs, 26 mm. in diameter. The discs were assayed for carbon$^{14}$, and their purine contents determined spectrophotometrically. From these data specific activities were calculated.

In preliminary experiments, the 0.1N HCl extracts from the spectrophotometric analysis were evaporated to dryness, rechromatographed in the same or other solvents and the counting, and purine analysis repeated. In subsequent experiments, specific activities were measured only on the rechromatographed purines.

The purines contained in the medium of the mucosa suspension were isolated by making the medium 2 per cent in perchloric acid and proceeding as in the case of the AS fraction. Uric acid was isolated from the combined initial effluents from the Dowex-50 columns of the AS and the medium fractions by using the ammoniacal silver nitrate method described above. When free of silver chloride, the uric acid solutions were diluted to 10 ml. and the ultraviolet absorption spectrum determined. Very close agreement with the spectrum of pure uric acid was shown. Aliquots of the uric acid solution were evaporated for transfer to chromatograms and for mounting as infinitely-thin samples for $C^{14}$ assay. Chromatography with butanol-acetic acid solvent (82) did not alter the specific activity of the uric acid.

(iii) Analysis of centre well contents[11] Oxidation of the isotopic substrates was measured by the determination of carbonate-$C^{14}$ trapped in the centre well of the Warburg cups. This was done only when phosphate buffers were used as the suspending medium.

The alkali from the centre wells was transferred to volumetric flasks with 10 - 12 washings. The flasks were made up to volume and suitable portions removed for radiocarbon analysis by the thick-sample method. Carrier carbonate was added to each sample before precipitation as barium carbonate.

5. ISOTOPICALLY LABELLED SUBSTRATES

A. Sodium Formate-$C^{14}$

Radioactive sodium formate was obtained from Atomic Energy of Canada Limited. Chromatography by the method of Kennedy and Barker (99) and radioautography showed the preparation had all of its radioactivity present as formate. The radioactivity of formate was measured with the sample mounted on filter paper to permit comparison with purine activities which were measured in a similar fashion. The specific activity of formate was $1.59 \times 10^6$ c.p.m. per $\mu$M and was used routinely in 2.25 $\mu$M portions per Warburg flask.

To permit comparison with the radioactivity of carbon dioxide derived from the biological oxidation of formate-$C^{14}$, The specific activity of the stock formate was determined after oxidation to carbon dioxide by the mercuric sulfate method (91).

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11 These assays were very kindly performed by Dr. S. H. Zbarsky.

The specific activity, measured on "infinitely-thick" samples was found to be $0.32 \times 10^6$ c.p.m. per $\mu$M.

B. Glycine-1-$C^{14}$

Carboxyl-labelled glycine was prepared by the reaction of isotopic cyanide with N-chlormethylphthalimide (100). The isotopic cyanide for this reaction was synthesized from barium carbonate-$C^{14}$ by the method of McCarter (101) as modified by Jeanes (102). The labelled glycine produced in this way shown to be chromatographically pure. Radioautographs prepared from the chromatograms showed only one radioactive spot which coincided exactly with the ninhydrin spot.

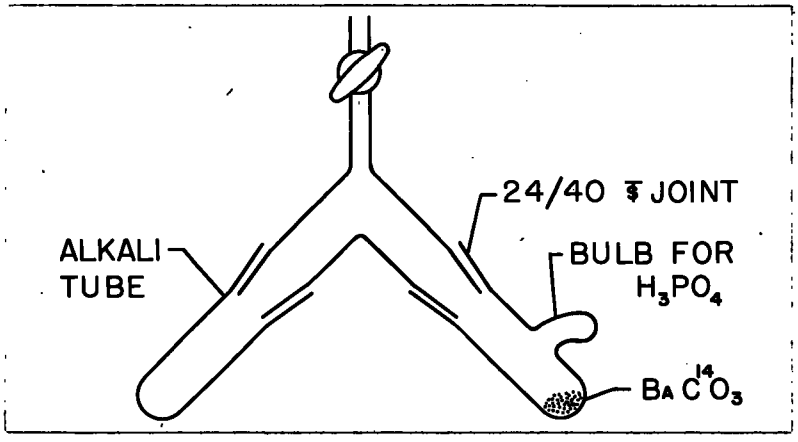
Preparation A. Counted in the form of "infinitely-thin" samples, this preparation had a specific activity of $2.84 \times 10^5$ c.p.m. per $\mu$M.

Preparation B. Samples were mounted on filter paper and indicated a specific activity of $2.64 \times 10^5$ c.p.m. per $\mu$M.

C. Sodium Bicarbonate-$C^{14}$

Sodium bicarbonate-$C^{14}$ was prepared by trapping $C^{14}$-carbon dioxide, generated from barium carbonate-$C^{14}$, in the stoichiometric amount of sodium hydroxide. The reaction was conducted in the apparatus illustrated in Figure 12.

FIGURE 12. APPARATUS FOR THE PREPARATION OF SODIUM BICARBONATE-$C^{14}$



Procedure. Isotopic barium carbonate was placed in the tube having the side bulb, which contained an excess of syrupy phosphoric acid. A volume of sodium hydroxide, exactly equivalent to the number of moles of barium carbonate present, was placed in the other tube and frozen with a Dry Ice-alcohol mixture. The apparatus was then evacuated to approximately 1 mm. of mercury and the phosphoric acid tipped onto the carbonate very cautiously. After the reaction was complete, the apparatus was allowed to stand at room temperature for at least 12 hours to allow complete absorption of carbon dioxide. In preliminary experiments almost quantitative yields of bicarbonate were obtained. Bicarbonate having a specific activity of $1.12 \times 10^6$ c.p.m. per $\mu$M was prepared in this manner.

6. NON-ISOTOPIC SUBSTRATES

A. Glycinamide hydrochloride

The ammonolysis of glycine ethylester hydrochloride was used to prepare glycinamide hydrochloride (103). The product was recrystallized from a methanol solution to which ether was added until crystallization was incipient. On paper chromatograms the product showed a single yellow spot with ninhydrin reagent.

B. 4-amino-5-imidazolecarboxamidine dihydrochloride

This compound was synthesized by the method of Shaw (82) and was recrystallized twice from methanol-ether, as above. The identity of the product was established by melting point (242-244° C., (104) ) and by the fact that it was converted to adenine by formylation and cyclization (105).

## RESULTS

### 1. CHARACTERISTICS OF THE INTESTINAL MUCOSA SUSPENSION

#### A. Physical characteristics

The mucosal suspensions consist of small fragments of epithelial tissue ranging in size from clumps of a few cells to sheets of cells 4 - 6 $mm.^2$ in area. Low-power photomicrographs[12] of a typical preparation are shown in Figures 13 and 14. Diffusion of gases to the deepest lying cells presents no problem in these preparations since the largest fragments are thinner than the limiting thickness of 0.5 mm. (69,98).

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12 The photomicrographs were very kindly prepared by Dr. H. E. Taylor of the Department of Pathology.

PHOTOMICROGRAPHS OF A TYPICAL PREPARATION
OF RAT INTESTINAL MUCOSA

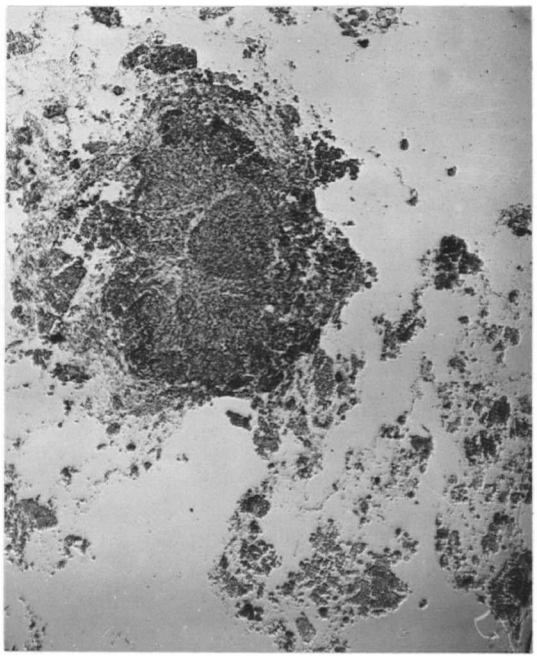

FIGURE 13

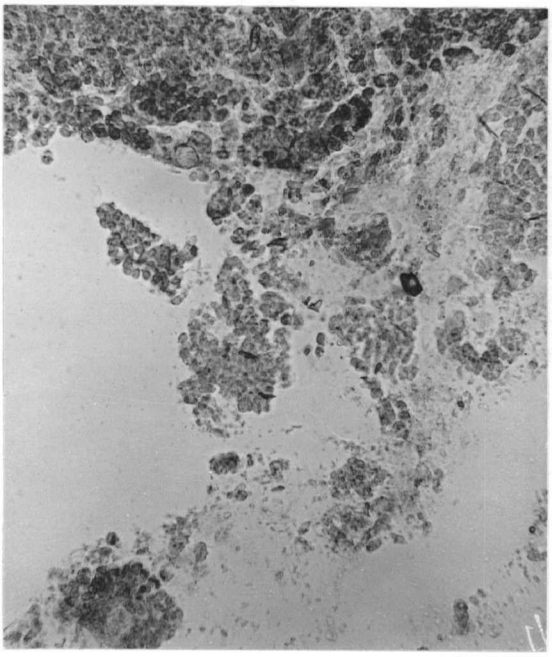

FIGURE 14

B. Respiratory activity

The mucosa suspensions respired actively for 3 - 4 hours at gradually declining rates. Respiratory quotients ($Q_{O_2}$), which averaged approximately 7 (values of 11 and 12 were also recorded), were comparable with the range of values Dickens and Weil-Malherbe (69) reported for mucosa from various regions of the smal intestine. Aureomycin (10 p.p.m.) had no measurable effect on oxygen uptake in these preparations.

C. Bacterial contamination

Bacterial contamination was small in these preparations. In preliminary experiments, in which the tissue

suspension was not washed, bacterial counts[13] did not exceed $13 \times 10^6$ per ml. at the end of the incubation period. The suspension was routinely washed several times in all subsequent experiments. The bacterial counts did not exceed $2 \times 10^6$ bacteria per ml. in the washed preparations. The bacterial counts varied greatly below these limits and were generally lowered by the addition of 10 p.p.m. of aureomycin to the medium. Although probably unnecessary, aureomycin was routinely used in the medium as a safeguard against bacterial growth during incubation.

The bacterial contamination was considered to exert no significant influence on the metabolism of the preparations in view of the fact that homogenates of this tissue were uniformly inactive with respect to the uptake of formate into the purines. The fact that oxygen consumption decreased with time supports this conclusion.

D. The purines of the mucosa suspension

(i) Whole suspension Analysis of the total purine content of the incubated mucosal preparation by the ion exchange procedure outlined above showed that only adenine, guanine, and uric acid were present in demonstrable amounts.

(ii) Medium The suspending medium, when recovered after incubation, was found to be rich in uric acid. Adenine and guanine were present also and were presumed to originate from the lysis of some of the mucosal cells during incubation.

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13 Miss Aiko Hori very kindly performed the bacterial counts.

The pyrimidine cytosine was found in some experiments.

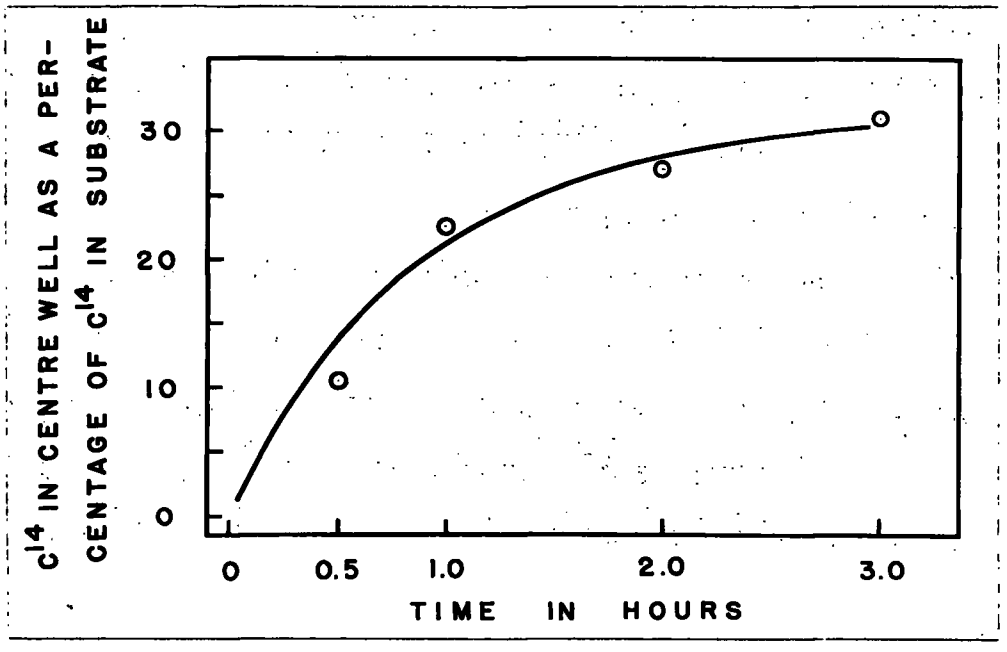
(iii) Acid-soluble fraction The acid-soluble fraction of tissues contains a complex mixture of nucleotides of the purine and pyrimidine bases (1,2). Adenine and guanine were released from their nucleotides by the hydrolytic treatment mentioned previously.

(iv) Polynucleotide fraction Adenine and guanine were liberated from DNA and RNA by the hot perchloric acid extraction of the tissue residue.

E. The oxidation of glycine-1-$C^{14}$ and formate-$C^{14}$

Analysis of the contents of the centre wells of the respirometer flasks showed that the mucosal suspension oxidized formate readily. In Figure 15 the radioactivity recovered as carbonate from the centre wells is expressed as a percentage of the total radioactivity originally present as formate and is plotted against time of incubation.

FIGURE 15. THE OXIDATION OF $C^{14}$-FORMATE TO $C^{14}O_2$ BY A SUSPENSION OF INTESTINAL MUCOSA



Nakada and Weinhouse, who have studied the oxidation of formate in individual tissues of the rat (106), report that homogenates of liver and kidney were the most active of the tissues tested. As whole cell preparations were not employed in their study and intestine was not examined, comparisons with the present results can be only approximate. As may be seen in Figure 15, after 1 hour incubation approximately 20 per cent of the formate substrate was oxidized, giving a conversion capacity ("C.C."; microatoms of substrate converted to product per hour per gram of dry tissue (106) of approximately 15. Nakada and Weinhouse report C.C. values for liver and kidney homogenates of 48 and 23 respectively.

TABLE III

THE OXIDATION OF FORMATE-$C^{14}$ TO CARBON DIOXIDE-$C^{14}$

| Expt. | Number of centre wells analysed | $Q_{O_2}$ | Average percentage of formate-$C^{14}$ activity in centre well |
|---|---|---|---|
| 1 | 6 | ca. 1 | 30.8 |
| 2 | 2 | 3 | 31.4 |
| 3 | 5 | 6 | 18.7 |

Substrate: 2.25 μm sodium formate-$C^{14}$ ($0.72 \times 10^6$ c.p.m.) per flask.

It would appear from the data of Table III that the ability of the mucosal suspension to oxidize formate does not depend on its respiratory activity. The respiration of the tissue preparation was suboptimal in the first two experiments cited in Table III, yet the oxidation of formate was twice that observed when respiration occurred at a higher rate of Experiment 3.

Nakada and Weinhouse (106) propose that formate oxidation occurs through a peroxidatic activity of catalase. They further suggest that the hydrogen peroxide necessary for this reaction may originate in the oxidation of purines by xanthine dehydrogenase. The present results may be explained on this basis. As an adequate energy supply has been shown to be necessary for the maintenance of nucleic acid structure (107), the low respiration rates probably indicate some breakdown of the tissue system. A greater availability of purines might therefore be expected in the poorly respiring preparations, which then could give rise to increased amounts of hydrogen peroxide through the action of xanthine dehydrogenase. Intestine has a high xanthine dehydrogenase activity (73). Glycine-1-$C^{14}$ was oxidized to only a very slight extent (approximately 0.2%) under these conditions.

2. PURINE SYNTHESIS IN MUCOSA SUSPENSIONS

Purine synthesis was studied by incubating the suspensions with the purine precursors, formate, glycine, and bicarbonate, each labelled with radiocarbon. An observed incorporation of isotope from these compounds by the purines

of the mucosa cells was considered to indicate that purine synthesis was taking place by the so-called "de novo pathway".

The purines of the preparation were found to be radioactive after incubation with formate-$C^{14}$, but before significance could be attached to this observation, it was essential to establish their radiochemical purity. This was done by comparing the specific activities of the isolated purines before and after rechromatography in different solvents. The specific activities of the NA purines were unaltered by rechromatography, while those of AS adenine were increased. In this way the $C^{14}$ content of the isolated purines was shown to be real and not due to accompanying contaminants. This evidence indicated that de novo purine synthesis was occurring, but did not exclude the possibility of the incorporation of $C^{14}$-formate by known exchange reactions. The latter process was shown to be of minor importance by a comparison of purine synthesis from glycine-1-$C^{14}$ and formate-$C^{14}$.

A. Radiochemical purity

A comparison was made of the specific activities of adenine and guanine of both AS and NA fractions, before and after a second chromatographic purification as shown in Table IV.

AS Purines It was noted that iso-propanol-HCl chromatograms of the AS fraction had large ninhydrin positive areas in the vicinity of the purine spots. In order to avoid the possibility of interference from this material (presumably amino acids), the purine areas were routinely rechromatographed in

the n-butanol-ethanol system. This solvent was found to separate the ninhydrin-positive material from the purines, leaving it almost entirely at the origin. These amino acid areas were absent from chromatograms of the NA fraction. The data of Table IV indicated that rechromatography of the AS purines was desirable.

Rechromatography in the butanol solvent made the specific activity data of the AS fraction more consistent, causing duplicate determinations to agree more closely (not shown) and in some cases increasing specific activities. This would suggest that ultraviolet absorbing impurities were present on the iso-propanol chromatograms.

NA Purines As can be seen from Table IV, rechromatography of the NA purines obtained from iso-propanol-HCl chromatograms was found to be unnecessary since the redetermined specific activities were not significantly different from the initial values.

On the basis of these results, it was concluded that the radioactivity associated with the purine areas on the chromatograms was part of the purine structure and not due to accompanying contaminants. This suggested that purine synthesis took place in the suspension.

The AS purines isolated from iso-propanol-HCl chromatograms required an additional purification, but the specific activity of the nucleic acid purines was unaltered by rechromatography. Unless otherwise noted, the AS purines were rechromatographed routinely in n-butanol-ethanol.

## TABLE IV

RADIOCHEMICAL PURITY OF AS AND NA PURINES DERIVED FROM FORMATE-$C^{14}$. A COMPARISON OF SPECIFIC ACTIVITIES BEFORE AND AFTER RECHROMATOGRAPHY

| | Specific Activity (c.p.m. per mg.) x $10^{-4}$ [1] | | | |
|---|---|---|---|---|
| | A. Initial Determination[2] | | B. Determined after Rechromatography[3] | |
| | adenine | guanine | adenine | guanine |
| AS Purines[4] | | | | |
| Experiment 1 | 12.6<br>15.1<br>23.5 | 2.7<br>4.2<br>6.8 | 13.2<br>13.0<br>24.0 | 1.9<br>1.7<br>7.1 |
| Experiment[5] 2 | 3.3<br>5.2<br>5.5<br>8.9 | 0.6<br>0.7<br>0.7<br>0.8 | 5.5<br>6.1<br>7.7<br>9.6 | 0.7<br>0.8<br>0.7<br>0.9 |
| NA Purines | | | | |
| Experiment 3 | 0.90<br>0.57<br>0.20<br>0.15 | | 0.91<br>0.54<br>0.18<br>0.16 | |
| Experiment 4 | | 1.03<br>1.11<br>1.38<br>1.13 | | 1.17<br>1.05<br>1.44<br>1.19 |

1 Spectrophotometric errors were probably not in excess of 5% for adenine determination and 10% for guanine determination.

2 Specific activity from initial iso-propanol-HCl chromatograms (see, "Isolation and analysis of purines", p.53.

3 Specific activities of purines from (A) were redetermined after being rechromatographed in n-butanol-ethanol except in Experiment 1 where iso-propanol-HCl was used.

4 Counted by thin-sample method.

5 Error of net activity was less than ±10% for guanine.

B. Incorporation of formate, glycine and carbonate

The probability that the de novo synthesis of purines occurred in the mucosal preparations received support from experiments in which purine precursors other than formate were incorporated. Glycine-1-$C^{14}$ and bicarbonate-$C^{14}$ labelled the purines of mucosal suspensions which were also incorporating formate, as illustrated in the experiments of Tables V and VI.

When radioglycine was used as the labelled substrate, rechromatography of the AS purines was essential since a small amount of glycine accompanied the purines through their isolation. On chromatograms run in the iso-propanol-HCl solvent, glycine had an $R_f$ value sufficiently close to that of adenine that their spots overlapped. A solvent system which isolated this highly active glycine contaminant was obviously essential. Carter's iso-amyl-disodium phosphate (81) or n-butanol-ethanol (82), in which glycine and $R_f$ values of 0.7 and 0.1 respectively, were found to be effective solvent systems in this respect.

In the experiment described in Table V, the $C^{14}$-formate substrate had a specific activity approximately 7 times that of the $C^{14}$-glycine substrate. Assuming no dilution from endogenous sources, the formate should give rise to purines approximately 14 times as active as those derived from glycine, since 2 molecules of formate are incorporated in the purine ring (positions 2 and 8) for every molecule of glycine (positions 4, 5 and 7). From a comparison of the specific activities of the adenine derived from these substrates, it

TABLE V

A COMPARISON OF FORMATE-$C^{14}$ AND GLYCINE-1-$C^{14}$ INCORPORATION BY THE AS PURINES

| flask | Substrates | Specific Activity (c.p.m. per mg.) x $10^4$ | |
|---|---|---|---|
| | | AS adenine | AS guanine |
| 1 | $C^{14}$-formate | 20.8 | 4.07 |
| 2 | $C^{14}$-formate glycine | 25.6 | 3.93 |
| 3 | $C^{14}$-glycine | 0.75 | 0.18 * |
| 4 | $C^{14}$-glycine formate | 0.85 | 0.18 |

medium: Krebs-Ringer carbonate
substrates, amount per flask:
glycine-1-$C^{14}$, 8.5$\mu$m (2.5 x $10^6$ c.p.m.)
sodium formate-$C^{14}$, 9.5$\mu$m (19.7 x $10^6$ c.p.m.)
glycine, 10$\mu$m
sodium formate, 10$\mu$m
$C^{14}$ assay: * error of net activity approximately $\pm$ 20%

TABLE VI

A COMPARISON OF FORMATE-$C^{14}$, GLYCINE, AND BICARBONATE-$C^{14}$ INCORPORATION BY ADENINE

| Substrates | Specific Activity (c.p.m. per mg.)x $10^{-4}$ | |
|---|---|---|
| | AS adenine | NA adenine |
| 1. $C^{14}$-formate, glycine and bicarbonate | 1.10 | 0.19 |
| 2. $C^{14}$-glycine, formate and bicarbonate | 0.10 | 0.08 * |
| 3. $C^{14}$-bicarbonate, formate and glycine | 0.14 | 0.15 ** |

Medium: Krebs-Ringer phosphate (calcium omitted)
substrates (amount per flask):
sodium formate-$C^{14}$, 2.25 µm ($3.58 \times 10^6$ c.p.m.)
glycine-1-$C^{14}$, 2.24 µm ($5.92 \times 10^6$ c.p.m.)
sodium bicarbonate-$C^{14}$, 0.99 µm ($1.12 \times 10^6$ c.p.m.)
non-isotopic substrates were present in the same amounts.
$C^{14}$ assay: formate-derived purines, error of net activity less than ±10%; glycine-and carbonate-derived purines, error of net activity approximately ±30%.

* error of net activity less than ±19%.
** error of net activity less than ±13%

appeared that glycine was used approximately one half of the amount expected from the observed incorporation of formate. The results with guanine were similar and indicated that approximately 3 molecules of formate were used for each glycine molecule instead of the theoretical 2:1 ratio.

The experiment outlined in Table VI indicates that approximately 2 molecules of formate were used for each molecule of glycine in the formation of AS adenine. It shows also a significant uptake of isotope by adenine during incubation with bicarbonate-$C^{14}$. The incorporation of formate into AS adenine in this experiment was less than one tenth of the usual value (compare with succeeding experiments) and was likely related to the low oxygen uptake ($Q_{O_2}$ less than 1). It appeared that the preparation was functioning atypically and for this reason, comparisons between the three substrates may be unreliable. The contribution of bicarbonate cannot be compared with those of the other substrates because of the differences in counting techniques and further, because the bicarbonate content diminished progressively during incubation because of acid production by the tissue. With phosphate media the pH usually fell during incubation, in some cases to as low as pH 6.9. Elliot (98) has commented on the fall of pH with similar media during the incubation of slices. In spite of the poor conditions of this experiment, significant uptake of bicarbonate-$C^{14}$ was observed.

The departure from the theoretical ratio of 2:1 for the incorporation of formate and glycine may be attributable

to dilution of the radioactivity of the substrates by endogenous pools of these metabolites, or possibly to some inosinic acid transformylase activity (31). This enzyme system has been shown to catalyse an exchange reaction between the 2-position of inosinic acid and formate. By the interrelationships between the purine nucleotides outlined previously, such an exchange could place more formate isotope in purine position 2 of adenosine and guanosine phosphates than was derived by synthesis alone.

## 3. SOME CHARACTERISTICS OF PURINE SYNTHESIS IN INTESTINAL MUCOSA SUSPENSIONS

### A. Uniformity of the suspension

The errors inherent in $C^{14}$ assay and purine analysis have been discussed in previous sections, but an additional factor also influences the specific activities of the purines of replicate samples; namely, the homogeniety of the suspension. The existence of differences in metabolic activity between the mucosa from various regions of the small gut, measured in terms of $Q_{O_2}$ by Dickens and Weil-Malherbe (69) make it essential that the suspension be made completely homogeneous when identical samples are to be taken.

A measure of the uniformity of the preparation and of the manner in which it was sampled is found in the data of Table VII. The variation in the replicates, which do not vary more than 15 per cent from the mean, suggests that the homogeniety of the preparation could be improved. The agreement between duplicate samples in subsequent experiments was

variable but frequently much closer than that in Table VII. It would seem desirable to fragment the tissue more finely, the object, of course, being to obtain a free-cell suspension. The use of chelating agents (108) may facilitate the fragmentation of the mucosal strips and avoid the mechanical damage which would increase with additional manipulation of the preparation.

TABLE VII

A COMPARISON OF REPLICATE SAMPLES

| Warburg vessel | Specific Activity (c.p.m. per mg.) x $10^{-4}$ | | | | % of total formate-$C^{14}$ in centre well |
|---|---|---|---|---|---|
| | AS adenine | AS guanine | NA adenine | NA guanine* | |
| 1 | 13.5 | 1.15 | 0.14 | 0.051 | 18.6 |
| 2 | 14.3 | 1.00 | 0.14 | 0.045 | 18.3 |
| 3 | 13.3 | 1.07 | 0.12 | 0.042 | 18.7 |
| 4 | 11.2 | 0.95 | 0.11 | 0.039 | 18.7 |
| 5 | 12.4 | 0.84 | 0.13 | 0.045 | 19.0 |
| Average | 12.5 | 1.00 | 0.13 | 0.044 | 18.7 |

medium: Krebs-Ringer phosphate
substrate: sodium formate-$C^{14}$, 2.25 µm ($3.58 \times 10^6$ c.p.m. per flask
solvent system for chromatography: iso-propanol-HCl
$C^{14}$ assay: * error of net activity less than ± 9%.
Values for NA purines are averages of duplicate analyses.

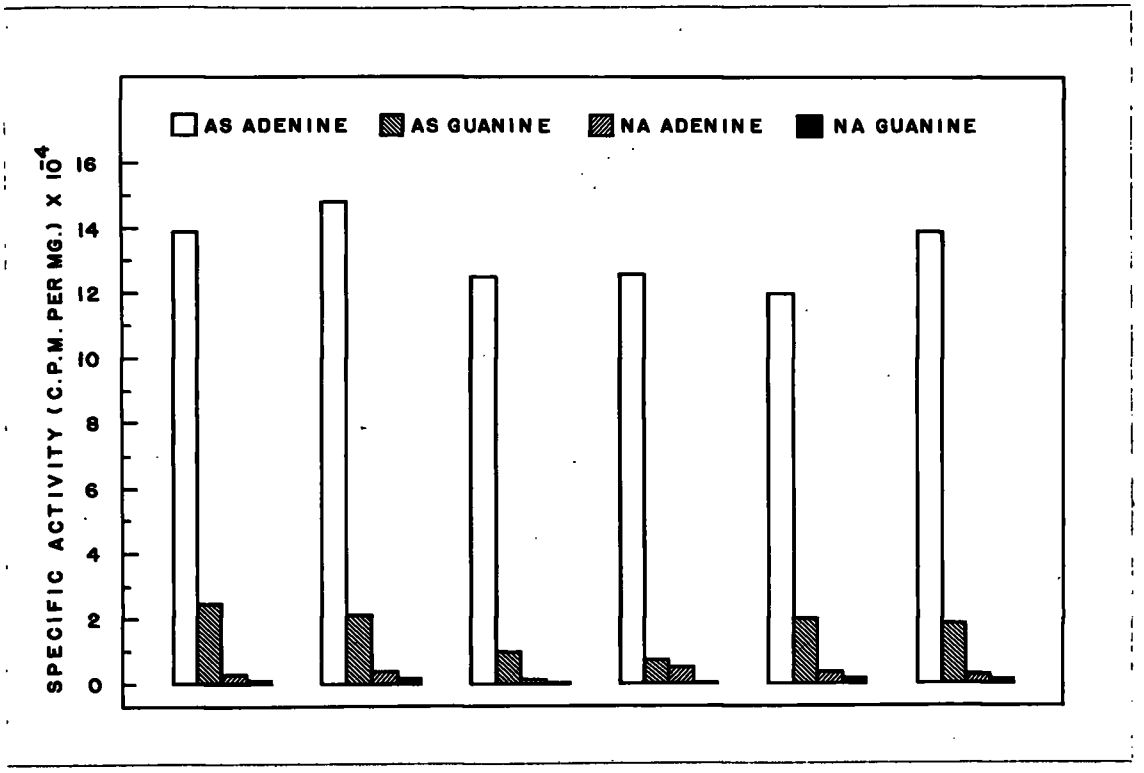
B. A comparison of the specific activities of the several purine fractions

(i) Acid-soluble and nucleic acid purines Typical values for the AS and NA purines are arranged for comparison in Figure 12. Isotopic formate was used as the substrate in all cases and $Q_{O_2}$ values were similar. Several deviations occurred from these general values as is noted subsequently. The acid-soluble purines were highly labelled, the adenine and guanine of this fraction being approximately 40 and 14 times more active, respectively, than the corresponding nucleic acid purines. AS adenine, which consistently displayed the highest renewal rates, was 6 or more times as active as AS quanine. NA adenine was usually 2 - 3 times as active as NA guanine.

(ii) Purines in the medium Uric acid occurred in relatively large amounts (60 - 70 γ) in the suspending medium following incubation, but it was only slightly radioactive. Adenine and guanine were also present in the medium, in amounts 5 to 10 times larger than in the AS fraction. This adenine fraction was only slightly radioactive and the guanine was non-radioactive suggesting that these compounds arose from the breakdown of tissue during incubation and were, therefore, not participants in the synthetic processes. Cytosine (non-radioactive) was also found on chromatograms of the supernatant fraction.

Table VIII and IX permit a comparison of the specific activities of the purines in the medium with other purine

FIGURE 16 TYPICAL SPECIFIC ACTIVITIES OF THE PURINE FRACTIONS




fractions. It is to be noted in Table IX, which summarizes the results of a time study experiment, that the values of the NA purines are atypical, being approximately 10 times higher than usual. This activity remained unchanged on rechromatography.

The purines of the medium were apparently catabolites, as suggested by their specific activities. The specific activities of uric acid and adenine of the medium were approximately 3 and 1 per cent, respectively, of the AS adenine. In Table VIII the observation that uric acid has a higher activity than NA adenine suggests that it was derived partly from the acid-soluble fraction of the surviving cells. The data do not enable any conclusions to be made as to whether

the $C^{14}$-uric acid was produced endogenously and passed to the medium or whether it was produced exogenously from purine metabolites of formate which had "leaked-out" of the mucosa cells, as in the case of Ehrlich carcinoma cells (109).

TABLE VIII

A COMPARISON OF THE SPECIFIC ACTIVITIES OF URIC ACID OF THE MEDIUM, AS AND NA PURINES

| Specific activity (c.p.m. per mg.) x $10^{-4}$ | | | | |
|---|---|---|---|---|
| uric acid | AS adenine | AS guanine | NA adenine | NA guanine |
| 0.33 | 13.3 | 1.9 | 0.29 | 0.16 |

medium: Krebs-Ringer carbonate
substrate: sodium formate-$C^{14}$, 2.25 µm ($3.58 \times 10^6$ c.p.m.)
uric acid: was counted as the free acid on planchets and discs cut from chromatograms run in butanol-acetic acid (83), with agreement between both results.

C. Formate incorporation with respect to time

The individual results of four time study experiments are shown in Figures 17 to 20. The averaged values of the AS purines for the four experiments are shown in Figure 21. There was considerable variation between the shape of individual curves, but the averaged values indicated a trend towards a linear uptake with respect to time. Figure 18 indicated a declining rate of incorporation after four hours of incubation. The preparation was deteriorating at this time (falling pH and rate of respiration).

## TABLE IX

A COMPARISON OF THE SPECIFIC ACTIVITIES OF ACID-SOLUBLE AND NUCLEIC ACID PURINES AND THE PURINES OF THE MEDIUM

| period of incubation | Specific activity (c.p.m. per mg.) x $10^{-4}$ | | | | | | | % of formate-$C^{14}$ in centre well |
|---|---|---|---|---|---|---|---|---|
| | AS adenine | AS guanine | NA adenine | NA guanine | Med.*** adenine | Med. guanine | Med.** uric | |
| 0.5 hr. | 5.5 | 0.73* | 2.50 | 1.03 | 0.04 | inactive | -- | 10.2 |
| 1.0 hr. | 6.10 | 0.82 | 3.64 | 1.11 | 0.05 | " | 0.27 | 23.3 |
| 2.0 hr. | 7.71 | 0.65 | 5.51 | 1.38 | 0.07 | " | 0.26 | 27.0 |
| 3.0 hr. | 9.62 | 0.88 | 5.61 | 1.26 | 0.09 | " | 0.29 | 31.4 |

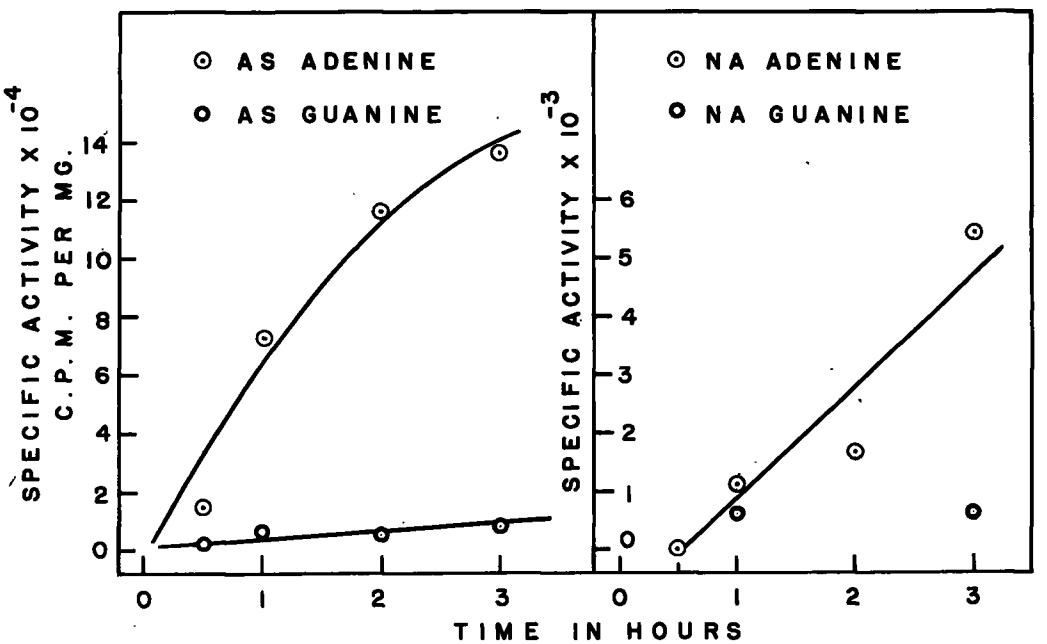
medium: Krebs-Ringer phosphate

substrate: sodium formate-$C^{14}$, 2.25 µm ($3.58 \times 10^6$ c.p.m.) per flask

solvent system for uric acid chromatography was n-butanol-ammonia (83).

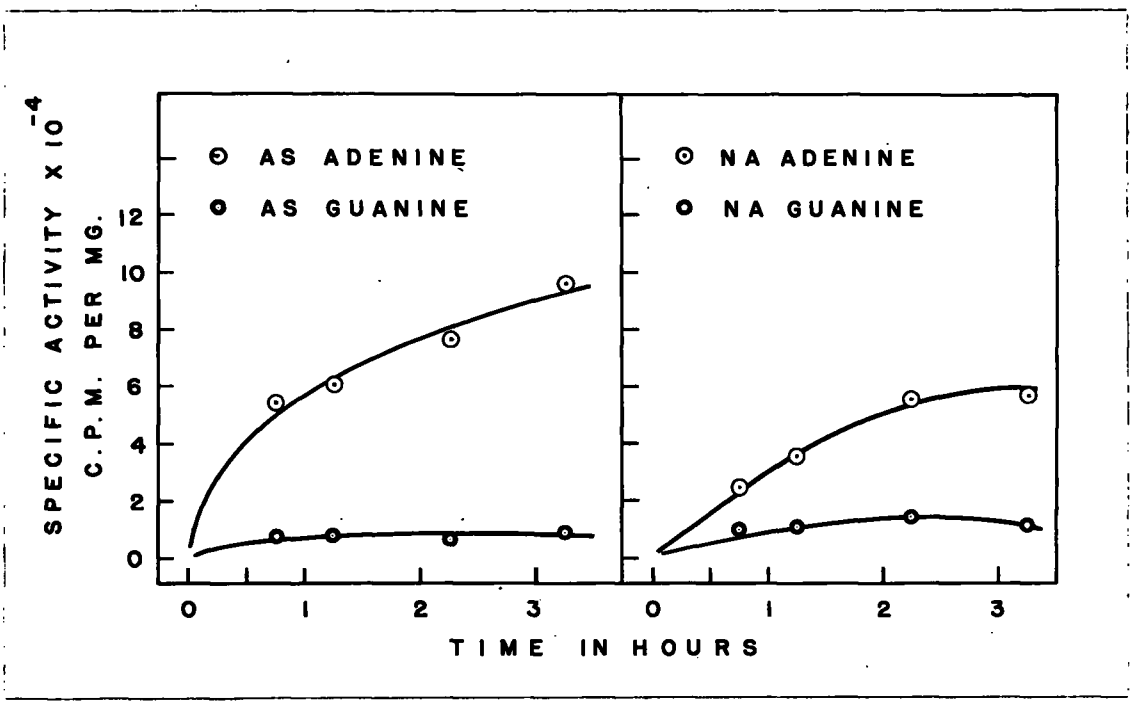
$C^{14}$ assay: *error of net activity less than ± 10%
** " " " " " " ± 15%
*** " " " " " " ± 20%

FIGURE 17. A TIME STUDY OF THE INCORPORATION OF FORMATE-$C^{14}$ INTO ADENINE AND GUANINE



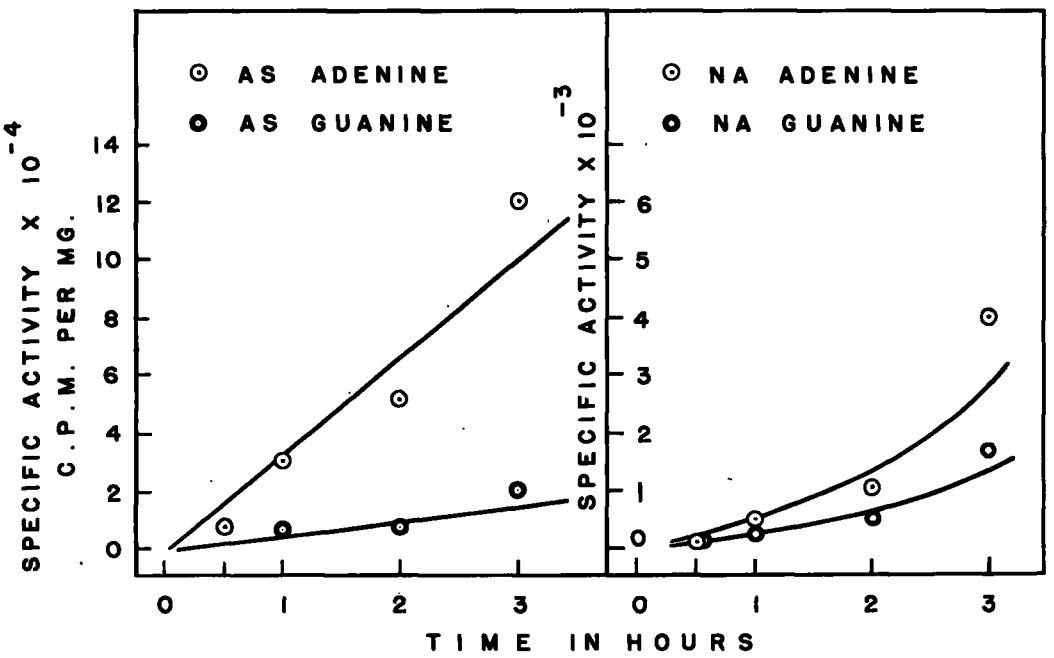

medium: Krebs-Ringer phosphate
substrate: 2.25 µm sodium formate-$C^{14}$ ($3.58 \times 10^6$ c.p.m.) per flask
$C^{14}$ assay: -the 30 and 180 min. NA purine specific activities were determined from single incubations while the other values were averaged from duplicate incubations
-errors of the net activity were less than ±10% for the AS guanine and less than ±15% for the NA purines

FIGURE 18. A TIME STUDY OF THE INCORPORATION OF FORMATE-$C^{14}$ INTO ADENINE AND GUANINE



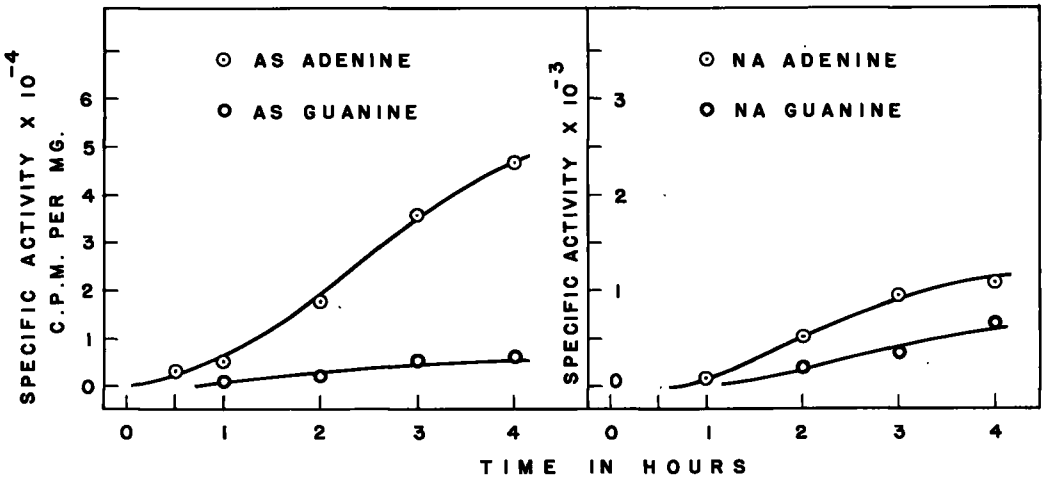

medium: Krebs-Ringer phosphate
substrate: 2.25 μm sodium formate-$C^{14}$ ($3.58 \times 10^6$ c.p.m.) per flask
$C^{14}$ assay: error of the net activity was less than ±10% for 65 min. AS guanine
The values plotted are averages obtained from duplicate incubations.

FIGURE 19. A TIME STUDY OF THE INCORPORATION OF FORMATE-$C^{14}$ INTO ADENINE AND GUANINE




medium: Krebs-Ringer carbonate
substrate: 2.25 μm sodium formate-$C^{14}$ (3.58 x $10^6$ c.p.m.) per flask
$C^{14}$ assay: error of the net activity was less than ±10% for the NA fraction

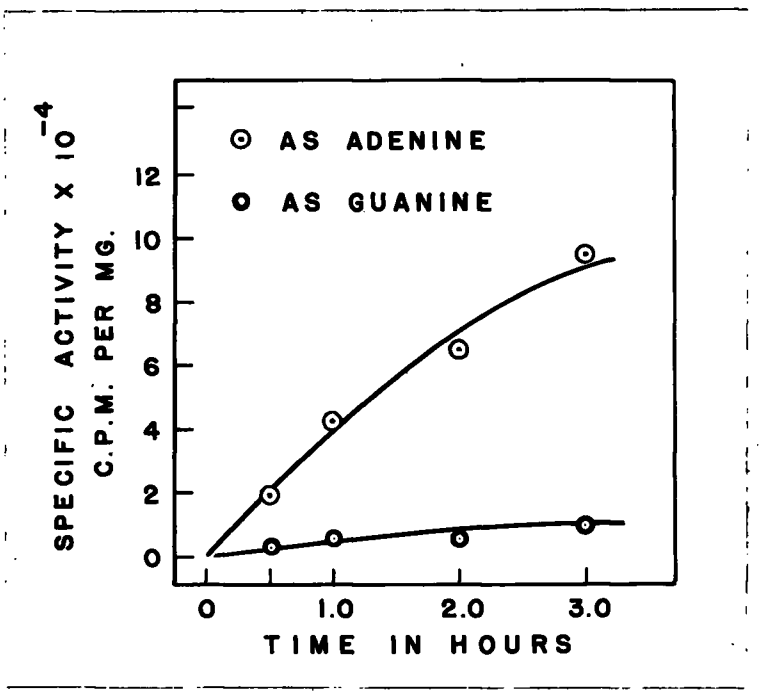
FIGURE 20. A TIME STUDY OF THE INCORPORATION OF FORMATE-$C^{14}$ INTO ADENINE AND GUANINE




medium: Krebs-Ringer carbonate
substrate: 2.25 $\mu$m of sodium formate-$C^{14}$ (3.58 x $10^6$ c.p.m.) per flask
$C^{14}$ assay: error of net activity is less than $\pm$10% for adenine fractions and less than $\pm$15% for guanine fractions

All values are averages of two incubations, one with aureomycin and one without. Since there were no significant differences between these pairs, they were treated as duplicates and averaged.

FIGURE 21. A TIME STUDY OF THE UPTAKE OF FORMATE-$C^{14}$ INTO ADENINE AND GUANINE

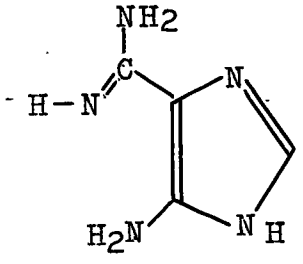



The data shown in this figure were obtained by averaging the specific activities for each particular time interval from the four preceding time studies.

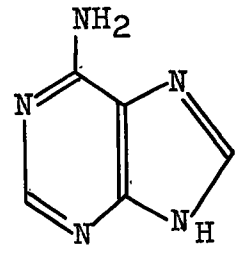
LePage has demonstrated with glycolysing homogenates that the production of high energy phosphate is necessary for the maintenance of nucleic acid structures (107). For these reasons and also because the possibility of bacterial interference increases with time, the incubation periods were not extended beyond three hours. Excluding the results of Figure 18, which are atypical, there seems to be a time lag in the appearance of radioactivity in the nucleic acid purines. This may be another manifestation of the observation made in experiments with intact animals that nucleic acid precursors pass through the acid-soluble nucleotide pool before entering the nucleic acid fraction.

4. THE EFFECT OF ADDITIVES ON PURINE SYNTHESIS

An attempt was madeto influence the purine synthesis of the mucosal suspension by the addition of certain compounds which were involved in purine biosynthesis. Glycine and glutamine, known to contribute to the purine ring, were tested in this way, as were glycinamide and 4-amino-5-imidazolecarboxamidine. The latter compound was considered likely to influence purine synthesis because of its structural resemblance to adenine which may be seen in the following formulas.



4-amino-5-imidazole-carboxamidine



adenine

The ribotide of glycinamide and its formyl derivative have recently been implicated as intermediates in inosinic acid biosynthesis (21,33). If they were to have any effect, glycine, glutamine and glycinamide were expected to exert a stimulating influence on formate-$C^{14}$ uptake by the purines. A lowering in formate-$C^{14}$ uptake was expected with 4-amino-5-imidazole-carboxamidine if this compound was incorporated into the purines, since it requires the addition of only one formate molecule to complete the purine ring.

The data of Tables X - XII indicate that the addition of any of the above compounds to the medium did not significantly influence purine synthesis in the mucosal system. The variations observed in these data could be explained by the relative inhomogeniety of the suspensions of mucosa.

TABLE X

THE EFFECTS OF GLYCINE, GLUTAMINE, AND GLYCINAMIDE ON PURINE SYNTHESIS

| flask No. | Additives (10 M per flask) | | | Specific activity (c.p.m. per mg.) x $10^{-4}$ | | | |
|---|---|---|---|---|---|---|---|
| | | | | acid-soluble fraction | | nucleic acid fraction | |
| | glycine | glycin-amide | glutamine | adenine | guanine | adenine | guanine |
| 1 | -- | -- | -- | 15.1 | 2.37 | 0.46 | 0.20 |
| 2 | + | -- | + | 12.8 | lost | 0.24 | 0.14 |
| 3 | + | -- | -- | 12.5 | 2.58 | 0.28 | 0.14 |
| 4 | + | -- | + | 10.7 | 1.94 | 0.22 | 0.12 |
| 5 | -- | -- | + | 14.4 | 2.56 | 0.38 | 0.11 |
| 6 | -- | + | + | 17.9 | 2.96 | 0.33 | 0.14 |
| 7* | -- | -- | -- | 17.0 | 2.09 | 0.49 | 0.22 |
| 8* | -- | -- | + | 18.5 | 2.03 | 0.40 | 0.24 |

* flasks 7 and 8 contained a different preparation of mucosa.

medium: Krebs-Ringer bicarbonate
substrate: sodium formate-$C^{14}$, 2.25 $\mu$M ($3.58 \times 10^6$ c.p.m.) per flask
solvent system for chromatography: Iso-propanol-HCl

## TABLE XI

THE EFFECT OF GLYCINAMIDE ON PURINE SYNTHESIS

| Substrate | Specific Activity (c.p.m. per mg.) x $10^{-4}$ | |
|---|---|---|
| | AS adenine | AS guanine |
| formate-$C^{14}$ | 13.2 | 1.9 |
| formate-$C^{14}$ glycinamide | 13.0 | 3.8 |

medium: Krebs-Ringer carbonate
substrate (amount per flask): sodium formate-$C^{14}$, 9.75 µm ($1.97 \times 10^6$ c.p.m.)
glycinamide, 10 µm
$C^{14}$ assay: infinitely-thin samples

TABLE XII

THE EFFECT OF 4-AMINO-5-IMIDAZOLECARBOXAMIDINE ON PURINE SYNTHESIS

| Substrate | Specific activity (c.p.m. per mg.) x $10^{-4}$ | | | |
|---|---|---|---|---|
| | AS fraction | | NA fraction | |
| | adenine | guanine | adenine | guanine |
| formate-$C^{14}$ | 0.7 | 0.1 | 0.2 | 0.2 |
| formate-$C^{14}$ plus imidazolecarboxamidine | 1.0 | 0.2 | 0.2 | 0.1 |

medium: Krebs-Ringer phosphate ($Ca^{++}$ omitted)
substrate (amount per flask): sodium formate-$C^{14}$, 2.25 µm ($3.58 \times 10^6$ c.p.m.)
imidazolecarboxamidine, 2 µm
$C^{14}$ assay: values are averages obtained from duplicate incubations.

5. PURINE SYNTHESIS IN HOMOGENATES

To avoid the possibility of cellular impermeability to the test substances, homogenates of mucosa were employed. The homogenates were prepared in all-glass homogenizers of the Potter-Elvehjem type (110) in an isotonic potassium chloride buffer of the following composition (111):

| | |
|---|---|
| Potassium chloride, | 0.13 M |
| Potassium phosphate buffer (pH 7.4), | 0.03 M |
| Magnesium chloride, | 0.003 M |
| Potassium bicarbonate, | 0.04 M |
| Glucose, | 0.01 M |

The homogenates respired at a very low rate ($Q_{O_2}$ less than 1) and uniformly failed to incorporate formate into adenine and guanine of either the AS or NA fractions. Uric acid, also not radioactive, was found in large amounts (approximately 400 γ per flask, a sixfold increase over the amount ordinarily found in the suspensions). Wenner et al. have noted that kidney, heart and liver homogenates have an absolute requirement for diphosphopyridine nucleotide (112). Following their suggestions, the mucosal homogenates were fortified with:

| | |
|---|---|
| Diphosphopyridine nucleotide, | 0.0002 M |
| Cytochrome C, | 0.00004 M |
| Adenosine triphosphate, | 0.002 M |

In addition, the following compounds were included in the incubation mixture, to insure an adequate supply of components for de novo purine synthesis:

| | |
|---|---|
| Ribose-5-phosphate, | 0.005 M |
| Glutamine, | 0.01 M |
| Glycine, | 0.01 M |

These additives had no stimulating effect on purine synthesis and caused only a slight increase in oxygen consumption. The inactivity of the homogenates with respect to purine synthesis probably was connected with the inability of the system to maintain respiration. Increasing the final dilution of the homogenate (by using less tissue in larger vessels) from approximately 1:10, as used above, to 1:100 would probably improve the respiratory activity of the preparation (113). Paterson and LePage have observed that the de novo synthesis of AS and NA purines from glycine-2-$C^{14}$ in tumour homogenates occurred only to a small extent under conditions in which $C^{14}$-adenine was well incorporated into both fractions (58).

6. INCORPORATION OF FORMATE-$C^{14}$ BY THE PURINES OF INTESTINAL MUCOSA IN THE INTACT RAT

The distribution of formate-derived radioactivity in the AS and NA purines of intestinal mucosa from the intact animal is included for comparison with the results of the in vitro experiments. This comparison must be of a superficial nature only for several reasons, such as the differences in the experimental conditions for the tissue, and the differences in the time interval during which formate was available to the mucosa.

The intestinal tissue used in this experiment was obtained from an experiment conducted by $D_r$. S.H. Zbarsky in which male Wistar rats bearing the Novikoff hepatoma were injected with $C^{14}$-formate. These animals received a single dose of sodium formate-$C^{14}$ administered subcutaneously on the back.

The data of Table XIII do not show the large differences between the specific activities of the AS and NA fractions observed in the in vitro experiments described in this thesis and in other investigations (3,5). Time studies with intact animals have shown that very rapidly after the administration of a labelled nucleic acid precursor, the isotope concentration in the acid-soluble nucleotides passes through a sharp maximum followed by a more gradual decline (7, 8, 114). The appearance of the isotope in the nucleic acid fraction is more gradual, the maximum specific activity appearing well after that of the nucleotide fraction. Bennett has reported that in the nucleotide fraction of mouse stomach and intestine, AMP reached a maximum specific activity within 2 hours after the administration of adenine-$C^{14}$ (114). The specific activity of nucleic acid adenine showed a rapid initial rise, but thereafter rose slowly to the maximum value within 24 hours and then declined slowly at a first order rate. In the present experiment, because of the interval of 24 hours between the administration of the isotope and the sacrifice of the animal, the specific activities of the acid-soluble purines had undoubtedly fallen below their maximum values and were approaching those of the nucleic acid purines.

The ratio of the specific activities of AS adenine to AS guanine was approximately 1.4, whereas in the in vitro experiments, this value was approximately 6. This discrepancy may indicate some impairment of guanine synthesis in the in vitro system. The ratio of the specific activities of NA

adenine to NA guanine were similar to those reported by Drochmans et al. (67).

TABLE XIII

THE INCORPORATION OF FORMATE-$C^{14}$ BY THE PURINES OF INTESTINAL MUCOSA IN THE INTACT RAT

| Rat | Specific activities in c.p.m. per mg. x $10^{-4}$ | | | |
|---|---|---|---|---|
| | Acid-soluble fraction | | Nucleic acid fraction | |
| | adenine | guanine | adenine | guanine |
| 1.(tumour) | 4.17 | 2.97 | 2.23 | 2.03 |
| 2.(control) | 3.62 | 2.52 | 2.11 | 2.14 |
| 3.(tumour) | 4.46 | 2.84 | 2.86 | 2.76 |
| 4.(control) | 4.18 | 3.77 | 3.37 | 3.30 |

Each horizontal row of values was obtained from 1 rat which received subcutaneously 225. $\mu$m sodium formate-$C^{14}$ ($3.58 \times 10^8$ c.p.m.). Animals were sacrificed 24 hours later by bleeding under ether anaesthesia. Intestinal mucosa was placed in 2% perchloric acid and the purines prepared in the usual fashion. AS fractions, except No. 4 were rechromatographed in n-butanol-ethanol.

## DISCUSSION

The general field of nucleotide and nucleic acid metabolism is very active at present. The principal areas of interest and expansion at this time involve the pathways of purine and pyrimidine biosynthesis, the interrelationships between the components of the acid-soluble fraction, and the functional roles of these compounds as coenzymes and building blocks in polynucleotide synthesis. Ribo- and desoxyribonucleic acid biosynthesis and the biological functions of the nucleic acids are areas in which expansion is imminent. Many of these studies have progressed to the extent that use of the intact animal must be bypassed in favour of systems which give the experimentalist greater control over the reaction system under study. For such reasons, the use of tissue preparations *in vitro* has become essential to many aspects of studies of nucleic acid metabolism.

Intestinal mucosa has not hitherto been utilized for *in vitro* studies of purine metabolism. The demonstration that

both acid-soluble and polynucleotide purines are rapidly synthesized in the mucosal suspension suggests that this preparation may be of value in studies of purine nucleotide biosynthesis and metabolism. In this field, comparisons between avian and mammalian systems are distinctly needed and intestinal mucosa suspensions may have a useful function in this respect. In vitro systems have been used in metabolic studies of anti-metabolites (62, 115) with the tumour chemotherapy as a possible objective; it is suggested that the mucosal suspension could be employed similarly.

The apparent absence of hypoxanthine in these preparations and the large amounts of uric acid found present, especially in homogenates, are indicative of the high level of xanthine oxidase in the mucosa of the small intestine (73). The occurrence of inosinic acid in the acid-soluble fraction of liver and tumour has been shown by Hurlbert et al. (1), and by Edmonds and LePage (5), but was not detected in these experiments where it would appear as hypoxanthine in the hydrolysed AS fraction.

The oxidation of formate by the mucosal preparation is to be expected in view of the apparent ease of this reaction in most tissues (116). As much as 30 per cent of the formate substrate was oxidized to carbon dioxide in the incubation period of 3 hours. This conversion is not as great as that observed by Nakada and Weinhouse (106) for formate oxidation in liver and kidney homogenates, but compares favourably with values reported for other tissues by these authors (intestine

was not investigated). Nakada and Weinhouse consider that formate is oxidized through a peroxidatic activity of catalase, the peroxide arising by the action of xanthine oxidase, presumably on purines. The low level of glycine oxidation observed in this work is in accord with Greenberg's report that tissue preparations decompose glycine to carbon dioxide to a small extent (116). Nakada and Weinhouse have reported high rates of glycine oxidation for liver and kidney preparations, but low rates for other tissues (intestine was not examined).

The study of nucleic acid metabolism in intestine has been confined to observations of the incorporation of isotope from labelled precursors in the intact animal (4, 9, 64, 65, 67, 117 - 123). Of the viscera, intestine displays the highest rate of synthesis of DNA and RNA purines from small molecule precursors such as formate (67, 117, 120, 121), glycine (118, 119, 123), and ammonia (118). Relative to liver, intestine has a smaller ability to incorporate preformed purines into the nucleic acids (67, 123). In comparison with other viscera, intestine is superior in its ability to incorporate isotopic adenine into the acid-soluble purine nucleotide fraction (4,9). The prominence of nucleic acid synthesis in this tissue is probably related to the very high mitotic activity (64, 65)

The vigorous purine synthesis demonstrated in the intestine of the intact animal manifested itself in the mucosal suspension <u>in vitro</u> by the synthesis of both acid-

soluble and nucleic acid purines. That the radioactivity associated with the purines of the preparation was truly incorporated in the purine molecule and was not due to radioactive contaminants was established by demonstrating the radiochemical purity of the purines. The conversion of the substrate radioactivity to adenine and guanine could not be attributed to bacterial activity, as shown by the low bacterial counts, declining respiratory activity and, most conclusively, by the inability of homogenates to perform this conversion.

It is unlikely that the incorporation of formate-$C^{14}$ into adenine and guanine was due to an exchange type of reaction such as that observed between formate and position 2 of inosinic acid by Buchanan and his coworkers (13, 31 (see ref. 106, 107, 109)). The exchange took place in pigeon liver preparations in which inosinic acid synthesis was limited by suboptimal amounts of bicarbonate, and was detected by an extreme elevation of the ratio of formate to glycine incorporation. In the mucosal preparations, glycine was incorporated less efficiently than formate, but the relative amounts incorporated of these precursors were sufficiently close to the theoretical proportions to exclude a major incorporation of formate by an exchange reaction. A comparison of the relative incorporation of labelled formate and glycine is complicated by the endogenous occurrence of these compounds which, of course, has the effect of lowering the specific activity of the isotopic substrates. That the incorporation of glycine relative to formate was observed to be less than

theoretical in the present experiment may be due to dilution of the labelled substrate by endogenous glycine. A comparison between bicarbonate and the other precursors cannot be made because of the different $C^{14}$-counting techniques employed, and because the endogenous production of carbon dioxide greatly affected the specific activity of the bicarbonate-$C^{14}$ substrate. The data presented for the uptake of $C^{14}$-bicarbonate by the purines had a large statistical error, but indicated a significant incorporation of this purine precursor by nucleic acid adenine. The incorporation of these three precursors by adenine and guanine is regarded as evidence of a de novo synthesis of purines by the mucosal suspension.

In the growing number of reports on nucleotide metabolism, glycine-2-$C^{14}$ has been used to demonstrate the rapid turnover of the AS purines in vivo (5) and in isolated tissue preparations (3). Adenine-$C^{14}$ has also been used similarly in whole animal experiments (9, 4) and in in vitro studies (55). The mucosal suspension displayed this characteristic also, acid-soluble adenine and guanine being 43 and 14 times more active, respectively, than the corresponding nucleic acid purines. In the in vitro experiments reported by LePage (3, see Table V), the AS : NA ratios for adenine and guanine of mouse liver cells are 170 and 220, respectively. For adenine and guanine of the Ehrlich mouse ascites cell carcinoma, these ratios are 30 and 60, respectively.

In the mucosal suspension, the synthesis of adenine from formate-$C^{14}$ is more rapid than that of guanine, this

being particularly noticeable in the acid-soluble fraction. LePage found the reverse to be true in suspensions of mouse tumour and liver cells with glycine-2-$C^{14}$ as the labelled substrate (3). Totter and Best (62) have investigated formate incorporation into the nucleic acids of rabbit bone marrow cells in vitro and report specific activities for RNA adenine 3 - 5 times those of of RNA guanine. The reason for the divergence between the relative rates of adenine and guanine synthesis in these reports is not apparent, but may be due, in part, to differences in the sizes of the pools of endogenous nucleotides.

In the in vivo experiments described in Table XIII, the purines of intestinal mucosa all had very similar specific activities, in both the AS and NA fractions. As explained previously, this is probably due to the time interval between the administration of the formate-$C^{14}$ and the isolation of the purines. The specific activity of the AS adenine was 1.1 to 1.5 times higher than that of AS guanine (compare with the 6 - 16 fold difference between these compounds in the in vitro experiments summarized in Figure 16. Assuming that the specific activity of the NA purines is related in more or less direct fashion to that of the AS purines, this may be interpreted as meaning that guanine synthesis is suppressed in the in vitro preparations.

The low level of activity found in the uric acid fraction suggests that it is a degradation product. Similarly, the weak activity of the adenine and the absence of isotope

in the guanine of the medium indicate that these purines probably arise by catabolic processes.

The combined results of the time studies shown in Figure 21 demonstrate that the AS purines are synthesized at an approximately linear rate and underline the difference between the rates of synthesis of adenine and guanine. The data of Figure 20 indicate a decline in the rate of synthesis at 3 - 4 hours of incubation, by which time the system was undoubtedly breaking down. Incubation periods cannot be extended without risking bacterial growth. The atypically high specific activities of the NA purines in Figure 18 is unexplained.

The addition of the several compounds to the incubation mixture was undertaken in the hope that formate incorporation would be influenced in such a way as to indicate an involvement of the additive in purine synthesis. However, formate incorporation was not affected significantly by additions of glycine, glutamine, glycinamide and 4-amino-5-imidazolecarboxamidine. Subsequent to the initiation of these studies, several publications have shown that it is unlikely that <u>free</u> glycinamide is involved in purine synthesis (21, 31, 33, 37). Present indications are that glycine, by interaction with 5-phosphoribosylamine, enters the biosynthetic pathway as glycinamide ribotide. The negative findings with glycinamide in the mucosal system are compatible with these concepts. Abrams has shown that 4-amino-5-imidazolecarboxamide has no effect on formate uptake by bone marrow slices (61).

The amidine of this compound was tested in the mucosal suspension because of its structural relationship to possible adenine precursors and the indication that it may have a metabolic function in certain protozoa (38).

The lack of significant effects on purine synthesis by the above additions may be due to factors such as the following:

(1) non-involvement of the compound in purine synthesis

(2) permeability effects

(3) an already adequate endogenous pool of the additive or its metabolites

(4) the reaction sequences of purine synthesis may have rate-limiting steps in this system subsequent to the point at which the additive enters.

In view of the above demonstration that a rapid de novo synthesis of purines takes place in the suspensions of intestinal mucosa, several applications of this system suggest themselves. As noted previously, there is a need for comparison of the pathways of purine biosynthesis in mammalian tissue with the known pathways in avian liver; the mucosal preparation may have a useful function in this respect. This system may have an application in the testing of anti-metabolites directed at nucleic acid metabolism, with the objective of selecting compounds which are potentially useful in cancer chemotherapy. In certain respects, intestinal mucosa bears a resemblance to cancer tissue, notably, in carbohydrate metabolism, in the high rate of cell division,

and in the prominence of nucleic acid renewal; the latter two features are related to the apparently continuous growth of intestinal mucosa in the rat (124). For these reasons, this system has a potential application to studies of the metabolic features which are characteristic of rapidly growing tissues and neoplasms.

## SUMMARY

The preparation of suspensions of mucosa from the small intestine of the rat is described and methods are reported for the use of this system in *in vitro* studies of nucleic acid metabolism. Some features of purine metabolism in these preparations are described and a demonstration of *de novo* purine synthesis is presented.

The mucosal suspension is a whole cell preparation consisting of small fragments of mucosa ranging in size from clumps of a few cells to sheets 4 - 6 $mm.^2$. The suspensions of mucosa respired actively for 3 - 4 hours with initial $Q_{O_2}$ values of approximately 8. Bacterial contamination was not great in these preparations and was shown to have no significant influence on the measurements of purine synthesis. The mucosal suspensions oxidized glycine-1-$C^{14}$ to a small extent only, but vigorously oxidized formate-$C^{14}$ to carbon dioxide-$C^{14}$.

A procedure is presented for the separation, by ion exchange chromatography of the individual purines from solutions

containing uric acid, xanthine, hypoxanthine, guanine and adenine. When this method was applied to incubated suspensions of intestinal mucosa, the only purines demonstrable were adenine, guanine and uric acid. Techniques were developed for routine use which permitted the isolation, determination, and radiocarbon assay of the purines of the acid-soluble, nucleic acid and medium fractions of mucosal suspensions containing 150 - 200 mg. of fresh tissue.

The de novo synthesis of purines by this system was demonstrated by measuring the incorporation of several $C^{14}$-labelled purine precursors into the tissue purines. It was necessary to establish the following three points which are fundamental to the conclusion that the observed incorporation was due to de novo purine synthesis by the mucosal tissue:

(a) radiochemical purity of the isolated purines

(b) non-participation of exchange reactions

(c) exclusion of bacterial influence

The criterion of radiochemical purity employed was the unchanged specific activity of the purines after being successively chromatographed on filter paper in different solvent systems. The conclusion that the incorporation of formate-$C^{14}$ took place by de novo purine synthesis rather than by a process of exchange was based on the observation that glycine-$C^{14}$ and bicarbonate-$C^{14}$ were also incorporated into the purines. In one experiment, 3 - 4 molecules of formate were incorporated for each molecule of glycine used in purine synthesis and values approximating the theoretical 2:1 ratio

were observed in another experiment. These values are sufficiently close to the theoretical ratio of formate to glycine incorporation to exclude a major incorporation of formate by exchange reactions of the inosinic acid transformylase type. The inability of homogenates of mucosa to incorporate isotopic formate rules out the participation of bacteria in this process.

Adenine, guanine and uric acid were found in the suspending medium, but were only weakly radioactive, suggesting that they originated largely in the breakdown of tissue during incubation and were not participants in anabolic processes. The acid-soluble purines were highly labelled, the adenine and guanine of this fraction having specific activities approximately 40 and 14 times those of nucleic acid adenine and guanine, respectively. In the acid-soluble fraction, adenine consistently had the highest renewal rate, being 6 or more times as active as guanine. The rapid turnover of the acid-soluble purines relative to the nucleic acid purines suggested that the former bears a precursor relationship towards the latter.

The incorporation of formate-$C^{14}$ by purines with respect to time showed an approximately linear trend, with a decline in rate appearing after 3 - 4 hours of incubation. In several experiments an initial lag in the rate of synthesis of nucleic acid purines was apparent.

Purine synthesis, as measured by the uptake of formate-$C^{14}$ into purines was unaffected by the addition of the

following substances to the incubation mixture: glycine, glycinamide, glutamine, and 4-amino-5-imidazolecarboxamidine.

Homogenates of intestinal mucosa, prepared in buffered isotonic potassium chloride and fortified with DPN, cytochrome C, and ATP, failed to incorporate formate-$C^{14}$ into purines.

The purines were prepared from the intestinal mucosa of intact animals which had received subcutaneously administered formate-$C^{14}$ 24 hours prior to sacrifice. Adenine and guanine of both the acid-soluble and nucleic acid fractions were shown to have very similar specific activities. The relative lowering of the activities of the acid-soluble purines in this experiment was attributed to the 24 hour interval. Comparison of purine synthesis from formate-$C^{14}$ in the intact animal with that of the in vitro system suggested that guanine synthesis was suppressed in the latter.

The possible application of this tissue system to studies of purine biosynthesis in the mammal is discussed, and its use in studies of nucleic acid antimetabolites and in the screening of anti-tumour compounds is suggested.

## BIBLIOGRAPHY


1. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., J. Biol. Chem., 209, 23 (1954).

2. Schmitz, H., Hurlbert, R. B., and Potter, V. R., J. Biol. Chem., 209, 41 (1954).

3. LePage, G. A., Cancer Res., 13, 178 (1953).

4. Marrian, D. H., Biochim. et Biophys. Acta, 13,282 (1954).

5. Edmonds, M. P., and LePage, G. A., Cancer Res., 15, 93 (1955).

6. Bennett, E. L., and Krueckel, B. J., Biochim. et Biophys. Acta, 17, 515 (1955).

7. Hurlbert, R. B., and Potter, V. R., J. Biol. Chem., 209, 1 (1954).

8. Hurlbert, R. B., and Potter, V. R., J. Biol. Chem., 195, 257 (1952).

9. Bennett, E. L., and Krueckel, B. J., Biochem. et Biophys. Acta, 17, 503 (1955).

10. Reichard, P., The nucleic acids (Chargaff, E., and Davidson, J. N., ed.), Vol. II, Academic Press Inc., New York (1955).

11. Christman, A. A., Physiol. Rev., 22, 303 (1952).

12. Davidson, J. N., The biochemistry of the nucleic acids, 2nd. ed'n., Methuen and Co., London (1953).

13. Schulman, M.P., Chemical pathways of metabolism, Greenberg, D.m., ed.). Vol. II, Academic Press Inc., New York (1954).

14. Buchanan, J. M., J. Cell. and Comp. Physiol., 38, Supp. 1, 143 (1951).

15. Greenberg, G. R., Federation Proc., 10, 192 (1951).

16. Schulman, M.P., and Buchanan, J.M., Federation Proc., 10, 244 (1951).

17. Schulman, M. P., Sonne, J. C., and Buchanan, J. M., J. Biol. Chem., 196, 449 (1952).

18. Lagerkvist, U., Arkiv Kemi, 5, 569 (1953).

19. Sonne, J. C., Lin, I., and Buchanan, J. M., J. Am. Chem. Soc., 75, 1516 (1953).

20. Sonne, J. C., Lin, I., and Buchanan, J. M., J. Biol. Chem., 220, 369 (1956).

21. Hartman, S. C., Levenberg, B., and Buchanan, J. M., J. Am. Chem. Soc., 77, 501 (1955).

22. Levenberg, B., Hartman, S. C., and Buchanan, J. M., J. Biol. Chem., 220, 379 (1956).

23. Shive, W., Ackermann, W. W., Gordon, M., Getzendaner, M. E., and Eakin, R. E., J. Am. Chem. Soc., 69, 725 (1947).

24. Miller, C. S., Gurin, S., and Wilson, D. W., Science, 112, 654 (1950).

25. Williams, W. J., and Buchanan, J. M., J. Biol. Chem., 202, 253 (1953).

26. Schulman, M. P., Buchanan, J. M., and Miller, C. S., Federation Proc., 9, 225 (1950).

27. Schulman, M. P., and Buchanan, J. M., J. Biol. Chem., 196, 513 (1952).

28. Greenberg, G. R., J. Biol. Chem., 190, 611 (1951).

29. Greenberg, G. R., J. Am. Chem. Soc., 74, 6307 (1952).

30. Greenberg, G. R., Federation Proc., 12, 211 (1953).

31. Buchanan, J. M., Levenberg, B., Flaks, J. G., and Gladner, J. A., A symposium on amino acid metabolism (McElroy, W. D. and Glass, H. B., ed.) The Johns Hopkins Press, Baltimore (1955).

32. Greenberg, G. R., Federation Proc., 12, 651 (1953).

33. Goldthwait, D. A., Peabody, R. A., and Greenberg, G. R., J. Am. Chem. Soc., 76, 5258 (1954).

34. Kornberg, A., Lieberman, I., and Simms, E. S., J. Biol. Chem., 215, 417 (1955).

35. Remy, C. N., Remy, W. T., and Buchanan, J. M., J. Biol. Chem., 217, 885 (1955).

36. Korn, E. D., Remy, C. M., Wasilejko, H. C., and Buchanan, J. M., J. Biol. Chem., 217, 875 (1955).

37. Goldthwait, D. A., Greenberg, G. R., and Peabody, R.A., Biochim. et Biophys. Acta, 18, 148 (1955).

38. Aaronson, S., and Nathan, H. A., Biochim. et Biophys. Acta, 15, 306 (1954).

39. Lukens, L. N., and Buchanan, J. M., Federation Proc., 15, 305 (1956).

40. Warren, L., and Flaks, J. G., Federation Proc., 15, 379 (1956).

41. Brumm, A. F., Potter, V. R., and Siekevitz, P., J. Biol. Chem., 220, 713 (1956).

42. Kornberg, A., Lieberman, I., and E. S., J. Biol. Chem., 215, 389 (1955).

43. Speck, J. F., J. Biol. Chem., 179, 1405 (1949).

44. Levenberg, B., and Buchanan, J. M., J. Am. Chem. Soc., 78, 504 (1956).

45. Arabms, R., and Bentley, M., J. Am. Chem. Soc., 77, 4179 (1955).

46. Buchanan, J. M., Levenberg, B., and Lukens, N. L., Abst. Am. Chem. Soc., 128th mtg., 126 (1955).

47. Brown, G. B., and Roll, P. M., The nucleic acids (Chargaff, E., and Davidson, J. N., ed.), Vol. II, Academic Press Inc., New York (1955).

48. Roll, P.M., Weinfeld, H., Carroll, E., and Brown, G. B., J. Biol. Chem., 220, 439 (1956).

49. Reichard, P., and Estborn, B., J. Biol. Chem., 188, 839 (1951).

50. Friedkin, M., and Wood, H., IV, J. Biol. Chem., 220, 639 (1956).

51. Hecht, L. I., Potter, V. R., and Herbert, E., Biochim. et Biophys. Acta, 15, 134 (1954).

52. Schneider, W. C., and Brownell, L. W., Federation Proc., 15, 349 (1956).

53. Rose, I. A., and Schweigert, B. S., J. Biol. Chem., 202, 635 (1953).

54. Fresco, J. R., and Marshak, A., J. Biol. Chem., 205, 585 (1953).

55. Goldwasser, E., Nature, 171, 126 (1953).

56. Goldwasser, E., Federation Proc., 15, 263 (1956).

57. Heidelberger, C., Harbers, E., Liebman, K. C., Takagi, Y., and Potter, V. R., Biochem. et Biophys. Acta, 20, 445 (1956).

58. Paterson, A. R. P., and LePage, G. A., Federation Proc., 15, 324 (1956).

59. Grunberg-Manago, M., Ortiz, P. J., and Ochoa, S., Science, 122, 907 (1955).

60. Reichard, P., and Bergstrom, S., Acta Chem. Scand., 190, 5 (1951).

61. Abrams, R., and Goldinger, J. M., Arch. Biochem. and Biophys., 35, 243 (1952).

62. Totter, J. R., and Best, A. N., Arch. Biochem. and Biophys., 54, 318 (1955).

63. Ahlstrom, L., von Euler, H., and Hevesy, G., Arkiv Kemi, Minerol. Geol., A19, No. 9 (1944).

64. Stevens, C. E., Daoust, R., and LeBlond, C. P., J. Biol. Chem., 202, 177 (1953).

65. Stevens, C. E., Daoust, R., and LeBlond, C. P., Can. J. Med. Sci., 31, 263 (1953).

66. Smellie, R. M. S., The nucleic acids (Chargaff, E., and Davidson, J. N., ed.), Vol. II, Academic Press Inc., New York (1955).

67. Dochmans, P., Marrian, D. H., and Brown, G. B., Arch. Biochem. and Biophys., 39, 310 (1952).

68. Greenstein, J. P., Biochemistry of cancer, 2nd ed'n., Academic Press Inc., New York (1954).

69. Dickens, F., and Weil-Malherbe, H., Biochem. J.,

70. Rosenthal, O., Federation Proc., 6, 286 (1947).

71. Lowenstein, J. M., and Cohen, P. P., J. Biol. Chem., 220, 57 (1956).

72. Allfrey, V. G., Mirsky, A. E., and Stern, H., Advances in Enzymol., 16, 441 (1955).

73. Dietrich, L. S., and Borries, E., J. Biol. Chem., 208, 287 (1954).

74. Heidelberger, C., and Harbers, E., Federation Proc., 15, 271 (1956).

75. Vitale, J. J., Gershoff, S. N., Sinesterra, L., Hegsted, D. M., and Zamchek, N., J. Biol. Chem., 220, 363 (1956).

76. Gill, T. J., III, Zamchek, N., Vitale, J. J., and Hegsted, D. M., Federation Proc., 15, 516 (1956).

77. Abrams, R., and Bentley, M., Arch. Biochem. and Biophys., 56, 184 (1955).

78. Smith, J. D., and Markham, R., Biochem. J., 46, 509 (1950).

79. Wyatt, G. R., Biochem. J., 48, 584 (1951).

80. Wyatt, G. R., The nucleic acids (Chargaff, E., and Davidson, J. N., ed.), Vol. II, Academic Press Inc., New York (1955).

81. Carter, C. E., J.Am. Chem. Soc., 72, 1466 (1950).

82. Berry, H. K., Sutton, H. E., Cain, L., and Berry, J.S., The Univ. of Texas Publication, No. 5109, 23 (1951).

83. Chargaff, E., Lipshitz, R., Green, C., and Hodes, M.E., J. Biol. Chem., 192, 225 (1951).

84. Beavan, G. H., Holiday, E. R., and Johnson, E. A., The nucleic acids (Chargaff, E., and Davidson, J. N., ed.), Vol. I, Academic Press Inc., New York (1955).

85. Plentl, A. A., and Schoenheimer, R., J. Biol. Chem., 153, 203 (1944).

86. Johnson, E. A., Biochem. J., 51, 133 (1952).

87. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. F., Isotopic carbon, John Wiley and Sons Inc., New York (1949).

88. Reid, A. F., and Robbins, M. C., Science, 116, 148 (1952).

89. Main, R. K., Cole, L. J., and Bond, V. P., Arch. Biochem. and Biophys., 56, 143 (1955).

90. Garrow, J., and Piper, E. A., Biochem. J., 60, 526 (1955).

91. Weinhouse, S., and Friedmann, B., J. Biol. Chem., 197, 733 (1952).

92. Wright, W. D., Master of Arts Thesis, The University of British Columbia, (1952.).

93. Zbarsky, S. H., and Wright, W. D., Can. J. Med. Sci., 31, 151 (1953).

94. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and tissue metabolism, Burgess Publishing Co., Minneapolis, U.S.A. (1949).

95. Schneider, W. C., J. Biol. Chem., 161, 293 (1945).

96. Hawk, R. B., Oser, B. L., and Summerson, W. H., Practical physiological chemistry, 12th edn., The Blakiston Co., New York, (1951).

97. Graves, S. S., and Kober, P.A., J.Am. Chem. Soc., 37, 2430 (1915).

98. Elliot, K. A. C., Methods in enzymology, Vol. I, Academic Press Inc., New York (1955).

99. Kennedy, E. P., and Barker, H. A., Anal. Chem., 23, 1033 (1951).

100. Sakami, W., Evans, W. E., and Gurin, S., J. Am. Chem. Soc., 69, 1110 (1947).

101. McCarter, J.A., J.Am. Chem. Soc., 73, 483 (1951).

102. Jeannes, J. K., Science, 118, 3076 (1953).

103. Yang, P. S., and Rising, M., J. Am. Chem. Soc., 53, 3183 (1931).

104. Shaw, E., J. Biol. Chem., 185, 439 (1950).

105. Paterson, A. R. P., and Zbarsky, S. H., J.Am. Chem. Soc., 75, 5753 (1953).

106. Nakada, H. I., and Weinhouse, S., Arch. Biochem. and Biophys., 42, 257 (1953).

107. LePage, G. A., Cancer Res., 9, 297 (1949).

108. Anderson, N. G., Science, 117, 627 (1953).

109. Edmonds, M.P., and LePage, G. A., Cancer Res., 16, 222 (1956).

110. Potter, V. R., Methods in enzymology, Vol. I, (Colwick, S. P., and Kaplan, N. O., ed.), Academic Press Inc., New York (1955).

111. Miller, Z., and Warren, L., J. Biol. Chem., 205, 331 (1953).

112. Wenner, C. E., Dunn, D. F., and Weinhouse, S., J. Biol. Chem., 205, 409 (1953).

113. Potter, V. R., Methods in medical research, Vol. I, The Year Book Publishers Inc., Chicago (1948).

114. Bennett, E. L., Biochim. et Biophys. Acta, 11, 487 (1953).

115. LePage, G. A., and Greenlees, J. L., Cancer Res. Supp., 3, 102 (1955).

116. Greenberg, D. M., Chemical pathways of metabolism, Vol. II, (Greenberg, D. M., ed.), Academic Press Inc., New York (1954).

117. Barclay, R. K., Garfinkel, E., and Stock, C. C., J. Biol. Chem., 208, 875 (1954).

118. Abrams, R., Arch. Biochem. and Biophys., 33, 436 (1951).

119. Reichard, P., J. Biol. Chem., 179,773 (1949).

120. Goldthwait, D. A., and Bendich, A., J. Biol. Chem., 196, 841 (1952).

121. Martin, J. L., and Totter, J. R., Proc. Soc. Exp. Biol. Med., 86, 41 (1954).

122. Abrams, R., Arch. Biochem. and Biophys., 30, 90 (1931).

123. Furst, S. S., Roll, P. M., and Brown, G. B., J. Biol. Chem., 183, 251 (1950).

124. Leblond, C. P., and Stevens, C. E., Anat. Rec., 100, 357 (1948).

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# *In vitro* synthesis of purines by rat intestinal mucosa*

*In vitro* studies of purine and nucleic acid synthesis from precursors such as glycine, formate and carbon dioxide have been carried out principally with pigeon liver preparations. In the small number of such investigations involving mammalian tissues, regenerating liver[1], bone marrow[2,3] and tumor cells[4] have been utilized. Certain characteristics of rat intestinal mucosa suggested that this tissue would be suitable for studies of purine biosynthesis, e.g., the very high rate of cell division[5], the high uptake of purine precursors in the intact animal[6,7,8] and the high metabolic activity *in vitro*[9]. Further, the mucosa can be prepared with little damage as a suspension in which the tissue particles are sufficiently small so that diffusion is not limiting. This report describes the preparation of such suspensions and some observations of purine biosynthesis in the acid-soluble nucleotides and the mixed nucleic acid fractions of these preparations.

A male Wistar rat was killed by a blow on the head. The small intestine was removed immediately, chilled and cut into 10 cm segments. These were flushed free of contents with the ice-cold medium described below and, after being split open, were applied to a cold glass slab, mucosa upwards. The mucosa was then removed with a microscope slide, as described by DICKENS[9], suspended in 4–5 volumes of ice-cold medium and the strips of mucosa freed by gentle mixing. The tissue was washed several times by centrifuging at low speed followed by resuspension in the cold medium. The volume was made up to approximately 25 ml and the mucosa strips converted to a fairly homogeneous suspension by gently and repeatedly drawing the preparation into and expelling it from a syringe without a needle. The material was then strained through gauze. Throughout all operations the preparation was kept cold. The small intestine from a 200 g rat provided a suspension containing 25–45 mg dry weight of tissue per 3.0 ml portion.

Krebs-Ringer phosphate or bicarbonate solutions containing aureomycin (10 ppm) and glucose (2 mg/ml) were used as the suspending medium. The mucosa suspensions respired actively at a gradually declining rate for 3–4 hours with initial $Q_{O_2}$'s of 6–8. Bacterial counts**, which did not exceed $4–5 \cdot 10^6$ bacteria per ml at the end of 3 hours incubation, indicated that bacterial contamination in these preparations was not great and would not affect the results significantly.

The suspension of mucosa was added in 3.0 ml portions to Warburg cups which contained 2.25 $\mu M$ of sodium formate-$^{14}$C ($8.1 \cdot 10^6$ cpm) and during incubation at 37° C, oxygen consumption was routinely followed. After incubation, the tissue was recovered by centrifugation of the cup contents and the purines of the acid-soluble and nucleic acid fractions obtained by the perchloric acid extraction method described by LE PAGE[4].

The purines of each fraction were separated by paper chromatography using *iso*propanol-HCl[10]. The specific activities of the purines of the nucleic acid fraction were unaltered by rechromatography in butanol–ethanol–water[11], but it was found necessary to rechromatograph the acid-soluble purines in this solvent.

The purine areas on the chromatograms were located with U.V. light, and for analysis a disc, 26 mm in diameter, was punched out of each area. After determining the radioactivity of the discs, they were extracted with 0.1 *M* HCl and the purine content of the extracts measured in the usual way by U.V. absorption. The radioactivity determinations were corrected for absorption by the paper discs with empirically determined factors.

The uptake of formate in the purines was considered to be a measure of *de novo* synthesis in this system. That radioactive formate was readily incorporated into the purines of both the acid-soluble nucleotides and nucleic acids is shown in the data of Table I which is typical of a

TABLE I

PURINE SYNTHESIS BY REPLICATE SAMPLES OF AN INTESTINAL MUCOSA SUSPENSION*

| *Vessel Number* | *Specific activity in cpm/mg · 10$^{-4}$* | | | |
|---|---|---|---|---|
| | *Acid-soluble fraction*** | | *Nucleic acid fraction**** | |
| | *adenine* | *guanine* | *adenine* | *guanine* |
| 1 | 50.7 | 3.7 | 0.41 | 0.16 |
| 2 | 53.7 | 3.3 | 0.40 | 0.15 |
| 3 | 49.9 | 3.5 | 0.35 | 0.14 |
| 4 | 42.0 | 3.1 | 0.34 | 0.13 |
| 5 | 46.5 | 2.4 | 0.40 | 0.15 |

* Incubated at 37° C for 3 h with 2.25 $\mu M$ sodium formate-$^{14}$C ($8.1 \cdot 10^6$ cpm).
** Chromatographed in *iso*propanol-HCl only.
*** Average of duplicate determinations.

number of such experiments. The variation in the replicates indicates that the present method of preparation does not produce a completely homogeneous suspension. Aureomycin was shown to have no effect on the rate of incorporation of formate by the purines.

Glycine-1-$^{14}$C is also incorporated into the purines of this system but at about half the rate of formate. The addition of glycine, glycinamide and glutamine, singly or in combination, appeared to have no significant effect on the incorporation of formate.

The data of Table I and the rate study shown in Table II (in which a different mucosa preparation was used) indicate that the purines of the acid-soluble fraction are synthesized rapidly. This observation is in accord with reports of the early labelling of the nucleotide pool *in vivo*[12,13,14] and *in vitro* by purine precursors[4] and free purines[15]. Adenine was more active than guanine in both fractions, a finding in agreement with the observations of TOTTER[3] and ABRAMS[8] for nucleic acid purines derived from formate-$^{14}$C in their *in vitro* experiments with bone marrow. In contrast, LE PAGE[4] has reported that in mouse liver and mouse tumors glycine-2-$^{14}$C is incorporated to a greater extent in guanine.

TABLE II

RATE OF PURINE SYNTHESIS IN AN INTESTINAL MUCOSA SUSPENSION*

| *Time of incubation h* | *Specific activity in cpm/mg · 10$^{-4}$* | | | |
|---|---|---|---|---|
| | *Acid-soluble fraction* | | *Nucleic acid fraction* | |
| | *adenine* | *guanine* | *adenine* | *guanine* |
| 0.5 | 1.2 | 0 | trace | 0 |
| 1.0 | 1.8 | trace | trace | trace |
| 2.0 | 6.5 | 0.7 | 0.16 | 0.07 |
| 3.0 | 13.6 | 1.7 | 0.29 | 0.12 |
| 4.0 | 17.6 | 1.9 | 0.33 | 0.22 |

* The values presented are averages of duplicate incubations made for each period. Each vessel contained 2.25 $\mu M$ of sodium formate-$^{14}$C ($8.1 \cdot 10^6$ cpm).

It has been shown that rat intestinal mucosa, prepared in the form of a suspension, will synthesize acid-soluble and nucleic acid purines and appears to be a useful mammalian system for *in vitro* studies of the synthesis of nucleic acids and their components.

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1 P. REICHARD AND S. BERGSTRÖM, *Acta Chem. Scand.*, 190 (1951) 5.
2 R. ABRAMS AND J. M. GOLDINGER, *Arch. Biochem.*, 30 (1951) 261.
3 J. R. TOTTER AND A. N. BEST, *Arch. Biochem. Biophys.*, 54 (1955) 318.
4 G. A. LE PAGE, *Cancer Research*, 13 (1953) 178.
5 C. E. STEVENS, R. DAOUST AND C. P. LEBLOND, *J. Biol. Chem.*, 202 (1953) 177.
6 P. DROCHMANS, D. H. MARRIAN AND G. B. BROWN, *Arch. Biochem. Biophys.*, 39 (1952) 310.
7 P. REICHARD, *J. Biol. Chem.*, 179 (1949) 733.
8 R. ABRAMS, *Arch. Biochem. Biophys.*, 33 (1951) 436.
9 F. DICKENS AND H. WEIL-MALHERBE, *Biochem. J.*, 35 (1941) 7.
10 G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
11 H. K. BERRY, H. E. SUTTON, L. CAIN AND J. S. BERRY, *University of Texas Publication*, No. 5109 (1951) 22.
12 R. B. HURLBERT AND V. R. POTTER, *J. Biol. Chem.*, 195 (1952) 257.
13 D. H. MARRIAN, *Biochim. Biophys. Acta*, 13 (1954) 282.
14 E. L. BENNETT, *Biochim. Biophys. Acta*, 11 (1953) 487.
15 E. GOLDWASSER, *Nature*, 171 (1953) 126.

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# The Preparation of 2-$C^{14}$-Adenine

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As a preliminary to a study of the metabolism of the purines, with especial reference to the 2-position of the ring, the synthesis of adenine labeled in the 2-position with $C^{14}$ was undertaken. The method described by Shaw,[1] in which 4-amino-5-imidazolecarboxamidine is formylated and the product cyclized to give adenine, appeared to be suitable since by using $C^{14}$-formic acid for the formylation 2-labeled adenine would be obtained. An advantage of this method is that the isotope would be introduced at a late step in the synthesis, thereby minimizing losses of radioactive material. The undesirable feature of the method, however, as far as economy of radioactive material is concerned, is that the formylation is carried out with a large excess of 98% formic acid in the presence of acetic anhydride. This would necessitate the use of an inordinately large amount of $C^{14}$-formate in order to obtain adenine with appreciable radioactivity.

In order to avoid the use of such a large excess of formic acid, experiments were carried out to study the feasibility of formylating the carboxamidine with an aqueous solution of formic acid, since such conditions have been used to formylate other amines.[2,3] The formylation reaction was found to proceed in 6 *M* formic acid, and by using this modification it was possible to obtain 2-$C^{14}$-adenine in yields of 60–65%, based on the carboxamidine used. The unreacted $C^{14}$-formate can be recovered almost quantitatively and used for further preparations of labeled adenine.

**Method.**—A solution of 0.200 g. of 4-amino-5-imidazolecarboxamidine dihydrochloride[1] in 2.0 ml. of 20% formic acid was placed in a reaction tube made from the outer member of a 24/40 standard taper joint. To this solution was added 0.170 g. of potassium formate, making the solution 6.3 *M* with respect to formate. The solution was then boiled gently under reflux for 4 hours. The formamido derivative was not isolated but was cyclized to adenine by diluting the solution to 8 ml. with water, adding sufficient potassium bicarbonate to neutralize the formic acid and to make the solution 0.5 *M* in bicarbonate, and then boiling under reflux for 1 hour. An amount of hydrochloric acid slightly less than that required to neutralize the solution was added, and the solution was concentrated under reduced pressure to a volume of 2–3 ml. On placing the solution in the refrigerator for several hours crude adenine precipitated. This material was collected by centrifugation, washed 3 times with ice-cold water and dried *in vacuo*. The supernatant and wash liquids were saved for the recovery of unreacted formate. The crude material was sublimed at 220° and a pressure of 1 mm. to give 0.083 g. of pure adenine, a yield of 61% based on the carboxamidine. Yields of 40–42% were obtained when the formylation was carried out with 4.0 *M* formic acid.

*Anal.* Calcd. for $C_5H_5N_5$: C, 44.44. Found: C, 44.27.

The compound formed a picrate which melted with decomposition at 286–287°.[1] Admixture with picrate prepared from authentic adenine did not depress the m.p. The ultraviolet absorption spectrum and $R_f$ values obtained by paper chromatography[4] were identical with those of authentic adenine.

2-$C^{14}$-Adenine was prepared by using $C^{14}$-potassium formate in the above procedure. In a typical experiment, adenine having a specific activity of $1.055 \times 10^6$ c.p.m. per m*M* was synthesized and the formate recovered from the reaction mixture had a specific activity of $1.025 \times 10^6$ c.p.m. per m*M*.

The unreacted $C^{14}$-formate in the supernatant fluid and washings after separation of the crude adenine was recovered almost quantitatively by steam distillation.[5] For further use in preparing radioactive adenine, the steam distillate was titrated with standard potassium hydroxide solution and concentrated to small volume under reduced pressure. The concentrate was then transferred to the reaction tube and evaporated to dryness. The appropriate amount of 4-amino-5-imidazolecarboxamidine dihydrochloride was added, followed by hydrochloric acid equivalent to the formate present less the amount of hydrochloric acid present as the dihydrochloride salt. The procedure outlined above was then followed for the remainder of the synthesis.

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(1) E. Shaw, *J. Biol. Chem.*, **186**, 439 (1950).
(2) V. M. Clark and H. M. Kalckar, *J. Chem. Soc.*, 1029 (1950).
(3) R. Abrams and L. Clark, This Journal, **73**, 4609 (1951).
(4) J. D. Smith and R. Markham, *Biochem. J.*, **46**, 509 (1950).
(5) S. Weinhouse and B. Friedmann, *J. Biol. Chem.*, **197**, 733 (1952).

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