THE METABOLISM OF 2-C^{14}-ADENINE IN THE ADULT MALE RAT

by

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Members of the Department of Biochemistry.

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ABSTRACT

Isotopic adenine, labeled with C^{14} in position 2, has been prepared in three steps; (a) formylation of 4-amino,- 5-imidazolecarboxamidine in aqueous C^{14}-formic acid, (b) ring closure of the resulting formamido compound to form 2-C^{14}-adenine, (c) purification by sublimation in vacuo. The overall yield for the three operations was 60 percent. Proof of identity of the adenine prepared in this manner was obtained from the preparation of a derivative, combustion analysis, paper chromatography and ultraviolet spectrophotometry. The metabolism of 2-C^{14}-adenine was studied in the adult male rat. The labeled compound was administered to the experimental animals by intraperitoneal injection. The isotope of the administered adenine was found distributed in the purines of the visceral nucleic acids, the expired carbon dioxide and urine, where part of the activity was found in both urea and allantoin. Nucleic acid adenine and guanine were synthesized to the extent of 7.7 percent and 5.5 percent respectively from administered 2-C^{14}-adenine. The adenine renewal is lower than similar values derived from 1,3-N^{15}-adenine as reported in the literature. Expired carbon dioxide was found to contain over 8 percent of the administered isotope. Combustion analyses of whole urine

indicated that 28 percent of the administered isotope was contained therein. Urea and allantoin together accounted for 16-29 percent of the total radioactivity in urine. The presence of radioactive carbon dioxide in the expired air of the experimental animals, when considered in the light of other evidence, is regarded as being indicative of a biological lability in position 2 of the purines.

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TABLE OF CONTENTS

TABLES

FIGURES

INTRODUCTION

Interest in nucleic acids dates back to 1868 when Friederich Miescher began the fundamental investigations which led to their discovery. The subsequent research, which first revealed the chemical nature of the nucleic acids and which is now bringing to light something of the role these compounds play in life processes, constitutes a long and complex chapter of science. Although the original enquiries into this field were of a biochemical nature, the first important contributions to our knowledge of the nucleic acids were made by organic chemists. In the course of investigation into the properties and structure of uric acid by the early German organic chemists, the chemistry of the purines and pyrimidines became well-established. Representatives of both classes of these nitrogenous bases are present in nucleic acids. The early biochemists active in nucleic acid research were concerned principally with the nature of the nucleic acid components. The German school, notably Kossel and his group, were largely responsible for the isolation and characterization of the nitrogenous bases of the nucleic acids. The pentoses of the nucleic acids were first investigated by the German chemists, but their identity was established principally

through the efforts of the American investigators, Levene and Jacob. That the nucleic acids contained phosphorous had been known since Miescher's early investigations. Logically following this stage in the history of nucleic acid research came investigations into the manner in which the constituent parts of the nucleic acid molecule are linked to form its small structural units, the nucleosides and the nucleotides. During the last thirty years there has been considerable work done on the structure of the nucleic acid polymer employing a wide variety of techniques.

The development of analytical methods for the estimation of nucleic acids constituted the next major step in this field. Analytical techniques based on phosphorous determination and the colorimetric estimation of pentoses were the first developed and are still in wide use today. Purine estimations, at first done chemically, are now made almost exclusively by ultraviolet absorption techniques.

Two important achievements, the establishment of suitable analytical methods and the development of histochemical tests for nucleic acids, then set the stage for fundamental developments in the biology and biochemistry of the nucleic acids. The necessary chemical background having been supplied, biochemists and cytologists began examining the nucleic acids in the light of their

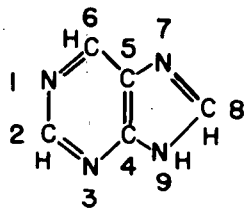
participation in processes of cell growth, cell reproduction and cell function. In recent years, the biology of the nucleic acids has advanced to the point where the fundamental importance of these compounds has been truly appreciated. For example, there is now a well established body of evidence which indicates that the chromosomes are constituted mainly of nucleoprotein (1) and that desoxyribose nucleic acid might well be the actual genic material (2). The changes in the nucleic acid content of rapidly growing tissues and the fact that the viruses and bacteriophages have invariably proven to be nucleoprotein in nature (1) indicate that the nucleic acids participate in growth processes. It is widely appreciated today that the nucleic acids play important, if not fundamental roles in the economy of the cell. Recognition of this fact by workers in the biological sciences is largely responsible for the great revival of interest in nucleic acids within the last decade.

The nucleic acids occur in the intact cell as protein conjugates termed nucleoprotein. The protein, usually a simple basic protein such as a histone or protamine, is removed during the process of isolation of the free nucleic acids. There are two principal types of nucleic acid, the chemical distinction between them being made on the basis of the sugar moiety of each type.

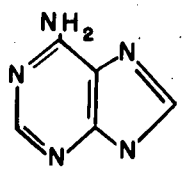
Ribonucleic acid (often abbreviated RNA) has as its characteristic sugar, β -D-ribofuranose. The other nucleic acid type, desoxyribonucleic acid (DNA), incorporates in its structure β -D-2-desoxyribofuranose. A further difference between RNA and DNA may be found in their constituent nitrogenous bases (See Figure 1). The purines, adenine and guanine, are found in both types of nucleic acid. With respect to the pyrimidines, cytosine is present in both types of nucleic acid, but uracil is found only in RNA and its methylated derivative, thymine, occurs only in DNA.

The biological distinction between DNA and RNA lies not in their sources, as early workers thought, but in their locations within the cell. Desoxyribonucleic acid is confined exclusively to the nucleus while ribonucleic acid occurs principally in the cytoplasm and to a small extent in the nucleus where it is located in the nucleolus and its vicinity.

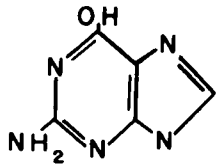
The nucleic acids are highly polymerized substances with molecular weights ranging from 300,000, as reported for tobacco virus RNA (3), to 3,000,000 for thymus gland DNA (4). The monomer units of these structures, the nucleotides, are joined by phosphoric ester linkages. Cold, weakly alkaline conditions are



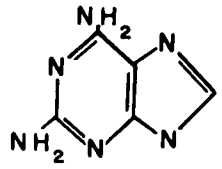
PURINE



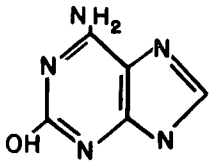
ADENINE



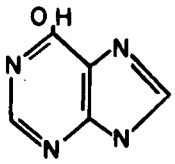
GUANINE



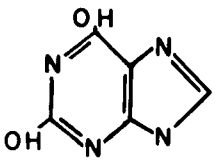
2,6-DIAMINOPURINE



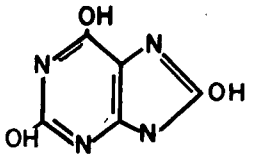
ISOGUANINE



HYPOXANTHINE

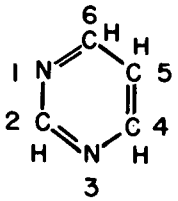


XANTHINE

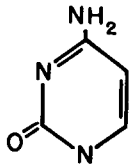


URIC ACID

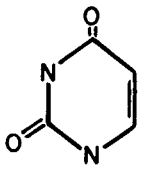
PURINES



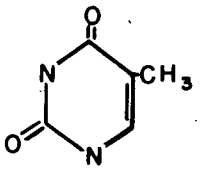
PYRIMIDINE



CYTOSINE

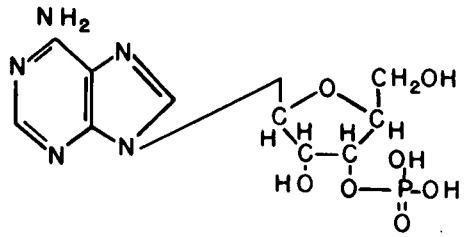


URACIL



THYMINE

PYRIMIDINES



YEAST ADENYLIC ACID
(ADENOSINE-3'-PHOSPHATE)

A RIBONUCLEOTIDE

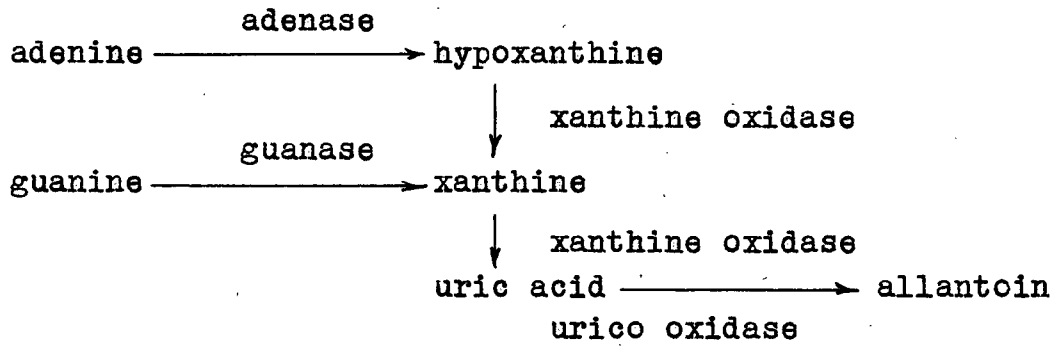
FIGURE 1. STRUCTURAL FORMULAE OF SOME PURINES AND PYRIMIDINES.

sufficient to hydrolyse RNA into its constituent nucleotides, but the more stable DNA is best depolymerized to its nucleotides by enzymatic hydrolysis. With reference to structure, the ribonucleotides (Figure 1) are purine and pyrimidine ribosides phosphorylated in position 3 of the ribose molecule. Recently, two isomeric forms for each of the ribonucleotides have been isolated; it is not known whether the new isomer, designated "a", differs from the first discovered form "b", in having the phosphate located at the 2-position in ribose or whether the difference is due to α, β isomerism about the glycosidic linkage (5,6,7). The desoxyribonucleotides are phosphorylated in the 3-position of the desoxyribose molecule. The sugar moiety in the purine nucleotides is attached to the nitrogen in position 9 (Figure 1) in both adenine and guanine; in the pyrimidine nucleosides, the sugar linkage is directed to nitrogen 3 in all cases.

Nucleic acids are common dietary constituents and investigations into the manner in which they are handled by the intact animal have provided us with much valuable information concerning the metabolism of these compounds. Dietary nucleic acids are hydrolysed to nucleotides by enzymes (nucleases) of the duodenal secretions. The nucleotides are, in turn, degraded to nucleosides and phosphoric acid by intestinal nucleosidases. The

nucleosides apparently are absorbed as such from the upper part of the intestine (8). The purine nucleosides, once absorbed, may be split into purines and sugar in various organs, notably the liver, by purine nucleosidase.

Studies of the metabolism of the free purine and pyrimidine bases have been slanted heavily in favour of the purines. Practically all that is known of the metabolic fate of the pyrimidines is that pyrimidine nitrogen is converted to urea and ammonia (9). The course of purine metabolism varies widely amongst the different animal species. The primates, birds and some reptiles convert purines to uric acid, the form in which they are excreted. In the lower mammals, purine degradation is carried a step further with uric acid being oxidized to the excretory product allantoin. In some fishes allantoin is oxidized, in an additional step, to allantoic acid; however, most of the fishes and the amphibia degrade purines beyond this point to urea and glyoxylic acid. The conversion of adenine and guanine into uric acid and allantoin may be summarized by the following diagram (10).



Purines from both exogenous and endogenous sources are metabolised in this manner. Birds not only convert purines to uric acid, but also synthesize uric acid for the purpose of nitrogen excretion. Uric acid is the principal form in which nitrogen is eliminated in birds; that portion of it arising from purine breakdown amounts to approximately 5 percent of all the nitrogen excreted (10). The purine hypoxanthine has been shown by Krebs and his co-workers (11) to be an intermediate in the biosynthesis of uric acid in birds. Thus it may be seen that purine formation is a major synthetic pathway in the bird. These facts make it apparent that the bird is a convenient animal in which to study the metabolism and biosynthesis of the purine bases. Pigeon liver homogenates and slices have been used extensively as purine-synthesizing systems, since it has been found that hypoxanthine tends to accumulate in these preparations.

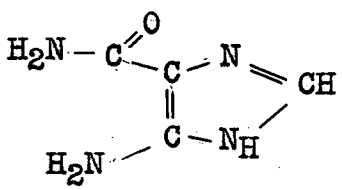
As one aspect of enquiries into the manner in which the animal is able to elaborate tissue polynucleotides, extensive studies have been made of the biosynthesis of the purines. Early approaches to this problem, made through the classical feeding techniques, have not provided much information of a specific nature concerning the precursors of the purine ring. However, it was found by such methods that the animal does not depend upon dietary

sources for purines but is able to synthesize them de novo (8). Only information of a general character has arisen from the use of these methods and it has remained for those using the recently-developed tracer techniques to provide specific evidence as to the precursors which are involved in the synthesis of the purine ring.

Since the animal has been shown to be independent of dietary sources for purines, it is evident that it must synthesize these compounds from smaller molecules. Barnes and Schoenheimer (12) demonstrated that N^{15}-labeled ammonium salts were incorporated into the tissue nucleic acids and the purine excretory products. Buchanan notes that "this paper was largely responsible for calling attention to the fact that the purines are built up from small carbon and nitrogen units rather than larger, preformed metabolic units from the diet" (13). In the course of their investigations of purine biosynthesis, Buchanan and his associates have determined the sources of the various atoms of the purine ring by feeding pigeons isotopically labeled compounds which were incorporated into uric acid. The excreted uric acid was degraded by procedures which permitted the separate examination of each of the different atoms of its molecule. Briefly, their work showed that of the nitrogen atoms, N_1, N_3 and N_9 were derived from the metabolic nitrogen pool, while N_7 was

provided by glycine. Of the carbon atoms, C_2 and C_8 were derived from formate, C_4 and C_5 from glycine, and C_6 was derived from carbon dioxide (13). Recently these findings have been extended, demonstrating that ammonium salts, glycine, formate and carbon dioxide contribute in the same manner to the biosynthesis of polynucleotide adenine and guanine in the rat and yeast (14, 15).

In order to determine what steps are intermediate in the incorporation of the early precursors into the purine molecule, various cyclic compounds have been examined to determine whether they participate in purine biosynthesis. The pyrimidines would appear to be likely precursors of the purines since the 6-membered ring is common to both. But experiments with the N^{15}-labeled pyrimidine ring have shown that these compounds do not participate in purine synthesis (9). Similarly, N^{15}-labeled histidine was shown not to be a precursor (16), in spite of early indications of involvement in purine metabolism (17). An interesting cyclic intermediate has been found in the compound 4-amino-5-imidazolecarboxamide:



The imidazolecarboxamide, when injected into rats, has been

shown to be incorporated in large measure into the tissue nucleic acid purines (18). There are strong indications that this compound, in a conjugated form, may be an intermediate in purine synthesis (13).

In a recent paper, Greenberg has elaborated on the role of the imidazolecarboxamide in purine synthesis (19). In demonstrating the de novo synthesis of hypoxanthine with C^{14}-formate in pigeon liver homogenates, he has shown that hypoxanthine is preceded by inosine-5-phosphate and inosine, in that order. Inosine-5-phosphate appeared to be the first complete purine synthesized in the system used and Greenberg postulated that the immediate precursor of this compound was a ribotide of incomplete structure, likely 4-amino-5-imidazolecarboxamide ribotide.

Although the animal is able to synthesize its own purines, it is logical to expect that administered purines would be utilized by the organism in anabolic processes. However, on the basis of feeding experiments with labeled guanine, uracil and thymine, Plentl and Schoenheimer concluded that "neither purines nor pyrimidines supplied in the diet are utilized by the body for the synthesis of nucleoproteins" (9). Because adenine is much more important in a biochemical sense than is guanine, Brown and his associates (20) considered that adenine merited a separate investigation before the general statement of

Plentl and Schoenheimer could be accepted. Accordingly, these workers synthesized adenine labeled with N^{15} in positions 1 and 3. They found in feeding experiments with this compound that the isotope was incorporated into polynucleotide adenine and guanine. Adenine, therefore, appears to be the only one of the free nucleic acid bases that participates in polynucleotide synthesis.

Because the isotope was found in positions 1 and 3 of isolated adenine and guanine, Brown and his collaborators concluded that the purine ring remained intact during the conversion of adenine to guanine. More recent evidence on the conversion of 8-C^{14}-adenine into nucleic acid guanine by yeast (21) and the rat (22) has been cited by Brown (23) as further indication that the purine ring remains intact. In experiments with adenine labeled in positions 1 and 3 with N^{15} and in position 8 with C^{14}, Marrian et al. (22) have shown that polynucleotide renewal in the rat is the same whether measured by the uptake of N^{15} or C^{14}. They concluded from this that the purine ring remains intact during the transformation of adenine into polynucleotide guanine.

Other purines, namely hypoxanthine, xanthine and isoguanine, were synthesized to contain N^{15} and were found not to participate in the biosynthesis of polynucleotide purines (24, 25). The only purine, other than adenine,

which has been shown to participate to any extent is 2,6-diaminopurine. This compound was considered by Bendich, Furst and Brown (26) to be a likely intermediate in the conversion of adenine to guanine *in vivo*. These workers synthesized the diaminopurine labeled with N^{15} in the 2-amino group and ring nitrogens 1 and 3. After oral administration of the tagged diaminopurine to rats, the polynucleotide purines were found to have taken up the isotope only in guanine. The isotope was incorporated to approximately the same extent as that from 1,3-N^{15}-labeled adenine administered in similar experiments. Hence, it was considered that the diaminopurine may participate as an intermediate in the conversion of adenine to guanine. In a parallel experiment, carried out using 2,6-diaminopurine-2-C^{13}, the isotope in the polynucleotide purines again was found to be confined to the guanine fraction but with a notably smaller incorporation (1.5 percent as opposed to 4.0 percent for the N^{15} experiment). Bendich and his co-workers did not offer an explanation for this discrepancy but Gordon (27) has suggested that it may be due to a biological lability at the 2-position in the purine ring during its incorporation. He also pointed out that the evidence offered by Brown *et al.* in support of the retention of the intact ring system does not rule out the possibility that the purine ring may open between nitrogens 1 and 3 or in the imidazole

ring.

Marsh has interpreted the findings reported in several other papers as indicating that carbon 2 of the nucleic acid purines might be relatively more labile than some of the other ring carbons (28). Barnes and Schoenheimer observed that the 2-amino nitrogen of polynucleotide guanine from pigeon viscera had a higher turnover than did the ring nitrogens (12). Confirmation of this observation comes from Reichard (29) in his experiments on the incorporation of N^{15}-labeled glycine into RNA purines. A labile 2-position in the purines could quite conceivably increase the rate of turnover of the 2-position substituent. The previously mentioned participation of 4-amino-5-imidazolecarboxamide or one of its derivatives in purine synthesis (18, 13) supports the idea of labile 2-positions in the purines. In a purely chemical sense, adenine has been shown to possess an active 2-position. In degradation studies on adenine, Cavalieri et al. (30) showed that, by hydrolysis with HCl, formate can be split out of the adenine molecule leaving 4-amino-5-imidazolecarboxamidine. Since the concept of a labile 2-position suggests an interchange between the purines and some 1-carbon compound or a labile substituent of a larger molecule in the tissues, the observation that formate is an excellent precursor of

carbon 2 and 8 of the purines (13) would appear to support this postulate.

There is, therefore, considerable indirect evidence favouring this concept, but evidence which does not depend on inference has been lacking. Since administered adenine participates in polynucleotide formation and is excreted in a manner identical to that of the endogenous purines, it seemed that a study of the metabolism of 2-labeled adenine might provide specific evidence for or against the postulated lability of carbon 2 in the purines.

A method for the synthesis of 2-C^{14}-adenine was developed using, as its basis, the formylation of 4-amino-5-imidazolecarboxamidine and the ring closure of the resulting formamido derivative to form adenine (31). Radioactive carbon was incorporated into the molecule by carrying out the formylation with C^{14}-formic acid. Cyclization of the formamido-imidazole compound resulted in the formation of 2-C^{14}-adenine as may be seen in Figure 2.

By this method, 2-C^{14}-adenine may be prepared in yields of 40-65 percent, depending on the formylating conditions used, and the excess formylating agent, C^{14}-formic acid, may be recovered easily for further use. The identity of adenine prepared in this manner was verified by paper chromatography, ultraviolet spectrophotometry and the preparation of a derivative.

Metabolic studies of 2-labeled adenine were then undertaken using the rat as the experimental animal. The postulated biological lability of the 2-carbon of the purines implies an interchange between this carbon and some 1-carbon compound (or a labile group of some larger molecule) in the tissues. If this were so, administered 2-labeled adenine and the purine intermediates in its metabolism would be expected to lose isotope from the 2-position to some tissue constituent and reincorporate non-isotopic carbon. The compound involved in the interchange reaction with carbon 2 would likely undergo oxidation in the course of its participation in other metabolic processes. For these reasons it was considered desirable to examine the expired air of the experimental animals for the presence of radioactive carbon dioxide. Therefore, 2-C^{14}-adenine was administered to rats which were placed in a metabolism cage that permitted the separate collection of expiratory carbon dioxide, urine and feces. The labeled compound was injected intraperitoneally in daily doses for four days, during which time regular collections of expiratory CO_2 and excreta were made. At the completion of the experiment the animals were sacrificed and the nucleic acids extracted from the pooled viscera. The purines, adenine and guanine, and the combined pyrimidines were prepared from the nucleic acids and assayed for radioactivity.

Analysis of the expired carbon dioxide revealed that it contained a significant amount of the radioactive carbon. Approximately 8 percent of the administered isotope appeared in the respiratory gases. This finding is considered to be direct evidence that the 2-position in the purines is biologically labile.

Allantoin, prepared from the urine of the experimental animals, had incorporated the isotope, as would be expected. It was observed that urinary urea had also incorporated some of the radioactive carbon. The specific activities of the expired carbon dioxide and urinary urea made it apparent that the isotope could not have been incorporated solely from the carbonate of the tissue fluids (32). The combined activity of the allantoin and urea accounted for 16-29 percent of the total activity in the urine.

If carbon 2 of the purine ring is labile, the renewals of polynucleotide adenine and guanine, calculated from the incorporation of isotope from administered 2-C^{14}-adenine, would be expected to be lower than the corresponding renewals measured by the uptake of isotope from 1,3-N^{15}-adenine. Accordingly, the proportions of the nucleic acid purines synthesized from 2-C^{14}-adenine in the experiments reported herein were compared with the results of similar experiments with 1,3-N^{15}-adenine reported in the

literature (20, 21, 25). This comparison is of limited value only since these latter experiments differ in the level at which the adenine was administered and in the manner of administration. It was noted that nucleic acid adenine renewal from 2-C^{14}-adenine was significantly lower than that measured by the uptake of isotope from 1,3-N^{15}-adenine (25), although guanine renewals were essentially the same for these two experiments. The reason for this apparent anomaly is not evident. The question of whether carbon 2 has a higher turnover than the other atoms of the purine ring would undoubtedly be clarified by experiments in which N^{15} and C^{14} labeled adenine were administered under identical conditions.

EXPERIMENTAL

A. METHODS

I. Synthesis of 2-C^{14}-adenine

Appraisal of the evidence cited in favour of a biologically labile 2-position in the purines showed that there had been no experiments reported in which the turnover rate of carbon 2 had been measured directly, and no reports had been made of attempts to follow carbon 2 in the course of purine metabolism. The knowledge that administered adenine participates in nucleic acid formation and is excreted apparently in the same manner as endogenous purines suggested that studies of the metabolism of adenine, labeled in the 2-position, might provide evidence relevant to the question of 2-position lability in the tissue purines. The synthesis of various purines from imidazole precursors described by Shaw (31) suggested a method by which adenine could be prepared with isotopic carbon incorporated into the 2-position.

A method for the synthesis of 2-C^{14}-adenine was developed having as its basis the formylation of 4-amino-5-imidazolecarboxamidine and cyclization of the resulting compound (31). The synthesis of the imidazolecarboxamidine from malononitrile is illustrated in Figure 2.

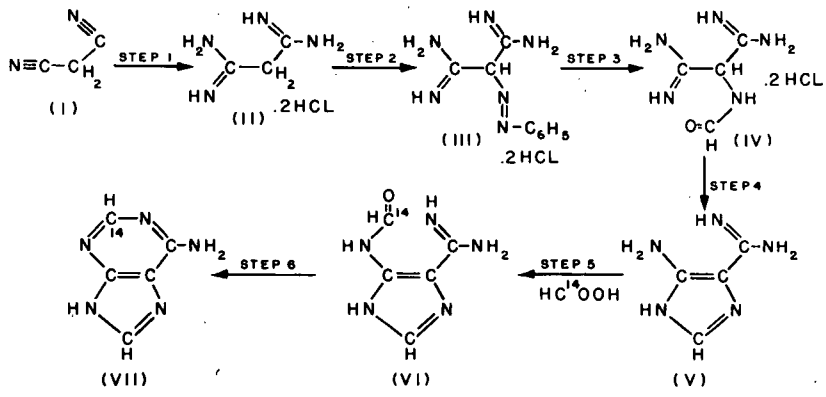


FIGURE 2. THE SYNTHESIS OF 2-C^{14}-ADENINE FROM MALONONITRILE.

Radioactive carbon is incorporated into the 2-position of adenine by formylating 4-amino-5-imidazole-carboxamidine (V) with C^{14}-formic acid (Step 5). The formylation reaction, when performed according to the method of Shaw, requires a large excess of 98 percent formic acid and acetic anhydride and, consequently, is quite impractical for the introduction of the radioactive formyl group. The cost of the isotopic formic acid required to produce adenine having a specific activity high enough to be useful, would be prohibitive if this method were followed. It was found, however, that the 4-amino group could be formylated under much milder conditions, namely, in dilute aqueous formic acid. The reduction in the amount of formic acid employed in the formylation reaction made the use of radioactive

formic acid practical, and the elimination of acetic anhydride from the reaction mixture made it possible to recover the excess C^{14}-formic acid for further use. Ring closure of the 4-formamido-C^{14}-5-imidazolecarboxamidine to form 2-C^{14}-adenine was effected by refluxing in alkaline solution as described by Shaw.

(i) Preparation of 4-amino-5-imidazole-carboxamidine dihydrochloride

The preparation of this compound, according to the method of Shaw (31), is outlined in Figure 2. Malononitrile (I) was converted to malonamidine (II) which coupled readily with benzene diazonium chloride to form phenylazomalonamidine (III). Reduction of the azo compound in formic acid produced formamidomalonamidine (IV), which underwent cyclization on heating to become 4-amino-5-imidazolecarboxamidine (V). In spite of strict adherence to the experimental procedures described by Shaw, the best yield of five attempts was 2 percent (based on malononitrile), whereas the reported yield was 25 percent. The principal difficulty appeared to be in the coupling of benzene diazonium chloride with malonamidine (Step 2). Personal communication with Dr. Shaw indicated that the coupling reaction would proceed under conditions less acidic than the value of pH 4 reported, namely, between pH 4 and 7. By carrying out the coupling reaction at a pH of 5 - 5.2

the overall yield from malononitrile was increased to 9 percent.

(ii) Preparation of C^{14}-formic acid

In preliminary experiments with non-isotopic formic acid it was demonstrated that the formylation of the amino-imidazole could be effected in dilute formic acid. The use of radioactive formic acid for the introduction of a labeled carbon atom in the 2-position of adenine therefore became feasible. In consequence, the synthesis of C^{14}-formic acid was undertaken employing the method of Melville, Rachelle and Keller (33). In this procedure radioactive potassium bicarbonate is reduced to radioactive potassium formate by hydrogen in the presence of palladium black. The reaction sequence, with barium carbonate as the starting material, is as follows:

$$BaC^{14}O_3 \xrightarrow{H^+} C^{14}O_2 \xrightarrow{KOH} KHC^{14}O_3 \xrightarrow[70^\circ C, 100\ atm.]{H_2Pd} HC^{14}OOK$$

Radioactive potassium bicarbonate was prepared from radioactive barium carbonate in a gas transfer apparatus described by the same authors. The palladium black catalyst used in preliminary hydrogenation experiments was prepared by the reduction of palladous chloride with formaldehyde in the presence of alkali (34), but use of this catalyst resulted in highly variable yields of formate. More satisfactory

yields were obtained with a palladium catalyst prepared by the method of Shriner and Adams (35). In this procedure, palladous oxide is formed from palladous chloride by oxidation with sodium nitrate. The oxide was added to the solution of radioactive bicarbonate and became reduced to the active catalyst, palladium black, when subjected to the conditions of the hydrogenation reaction. In effect, this step involved the preparation of the palladium black catalyst by reduction in the presence of the substance to be hydrogenated. The hydrogenation was carried out in an Aminco high pressure hydrogenator.#

Since the hydrogenation reactions did not go to completion, it was found necessary to purify the formate and recover the unreduced radioactive bicarbonate. This was accomplished by acidifying the hydrogenation mixture in the combustion apparatus shown in Figure 3. The procedure used was similar to that followed for combustion analysis, differing only in the respect that phosphoric acid was added in place of combustion fluid and no heat was applied. The formic acid was recovered from the acidified hydrogenation mixture by steam distillation in the apparatus shown in Figure 4. The steam distillate was then titrated with

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Permission to use this equipment was generously granted by the Department of Chemistry.

alkali and evaporated to dryness under reduced pressure. By this procedure, the isotopic formic acid was both purified and determined.

(a) Procedure for the purification and determination of formic acid

The reaction mixture from the hydrogenation was placed in Tube C of the combustion apparatus shown in Figure 3, and carbon dioxide -free 1 N sodium hydroxide (25 ml.) (36) was added to Tube D which was then quickly attached to the apparatus. The apparatus was then evacuated with a water pump and stopcock F closed. Syrupy phosphoric acid (2.5 ml.) was placed in Tube A and added dropwise to the mixture in Tube C. Thirty minutes were allowed for the absorption of the liberated carbon dioxide and then air was allowed to enter the apparatus slowly through a soda-lime tube placed on Tube A. The receiver D was lowered and, after the gas inlet tube E had been rinsed rapidly into D with freshly-boiled water, was closed tightly with a rubber stopper. The radioactive carbonate was recovered from this solution by precipitation as barium carbonate (37).

The acidified mixture in Tube C and the washings were transferred quantitatively to the apparatus illustrated in Figure 4 and C^{14}-formic acid recovered by steam distillation. During the steam distillation the volume in the flask was kept to approximately 15 ml. by

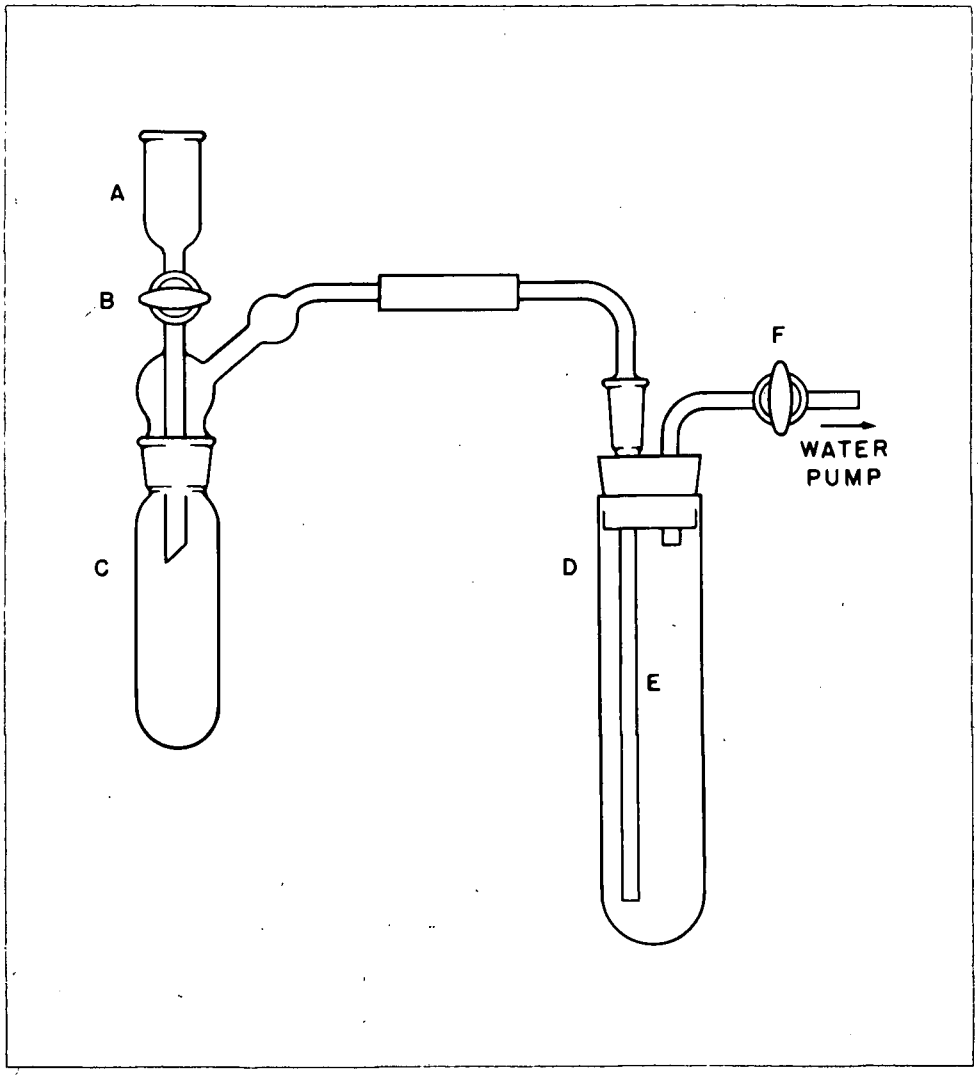

Figure 3. Apparatus for the wet combustion of organic compounds (37). This apparatus was used for the recovery of unreduced C^{14}-bicarbonate in the synthesis of C^{14}-potassium formate according to the method of Melville et al. (33).

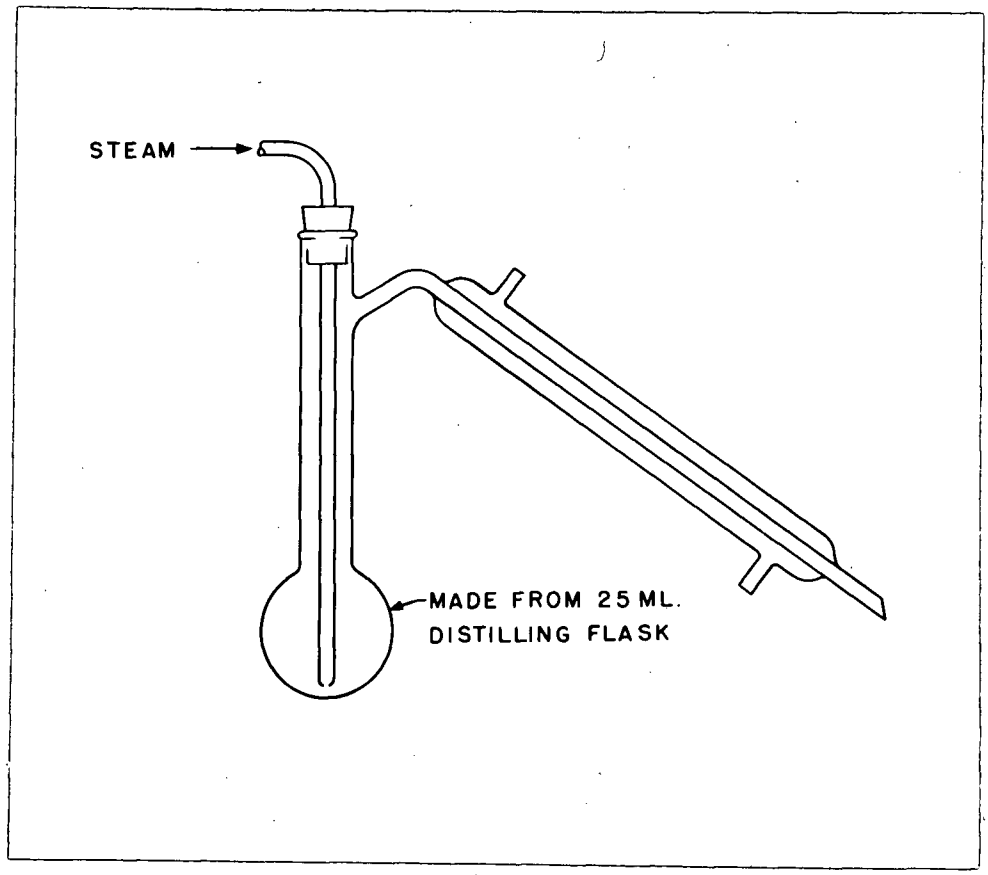

Figure 4. Apparatus for the purification and recovery of formic acid by steam distillation.

heating with a small flame. When the volume of the distillate was 50 - 60 ml. the recovery of the formic acid was essentially complete. The distillate was then titrated with standard alkali to the phenolphthalein end-point and concentrated to a small volume by evaporation in vacuo. In the development of this method, trial recoveries of added formate showed that the method was nearly quantitative. In five experiments, in which 200 mgm. of potassium formate were added, an average of 97 percent recovery was obtained.

(iii) Synthesis of 2-C^{14}-adenine

Since the incorporation of radioactive carbon into the adenine molecule is achieved primarily by the formylation of the amino group of 4-amino-5-imidazole-carboxamidine, the specific activity of the product will be the same as that of the formylating agent, C^{14}-formic acid. As adenine of a high specific activity was essential for the metabolism experiments planned, it followed that a practical method for its synthesis must employ the smallest possible amount of formylating agent. It was found, as the result of experiments with various concentrations of formic acid, that adenine could be prepared in yields of 36 - 50 percent using 14 percent formic acid in 6 molar excess; yields were increased to 61 - 65 percent by increasing the concentration of formic acid to 24 percent and the molar

excess to 11 times. As the imidazolecarboxamidine had been prepared as the dihydrochloride salt, the free base was liberated in the formylation mixture by the addition of 2 equivalents of potassium formate:

$$\text{imidazolecarboxamidine dihydrochloride} + 2\ HC^{14}OOK \longrightarrow \text{imidazolecarboxamidine} + 2\ HC^{14}OOH + 2\ KCl$$

The potassium formate used for this purpose contained all of the radioactive carbon used. The isotopic formic acid released by the above reaction became equilibrated with and increased the concentration of the formic acid solution initially added to the reaction mixture.

(a) Procedure for the synthesis of 2-C^{14}-adenine

The experiment described below was that in which the 2-C^{14}-adenine for Experiment 2 was prepared. A solution of C^{14}-potassium formate (0.17 gm., with an activity of 5.41×10^6 c.p.m.) was placed in the reaction vessel, which was a 60 mm. test-tube made from a 24/40 ℧ outer member joint, and evaporated to dryness under a stream of hot, dry air. To the residue was added 4-amino-5-imidazolecarboxamidine dihydrochloride (0.20 gm.) and 20 percent formic acid (2.0 ml.) and the solution refluxed for 4 hours. This procedure constitutes the formylation reaction.

Ring closure was effected by refluxing an alkaline solution of the formamido derivative. The formylation solution was made alkaline by the slow addition of potassium bicarbonate (1.69 gm.) and subsequently diluted to 8.0 ml., which made the solution 0.5 M with respect to bicarbonate. A boiling tube was added and the solution refluxed for 1 hour.

The solution was then almost neutralized by the addition of slightly less than the equivalent amount of hydrochloric acid. The solution was transferred to a 100 ml. R.B. flask and concentrated to 3 ml. by evaporation under reduced pressure. After cooling in an ice-bath, the precipitate of crude adenine was collected by centrifugation, washed three times with cold water (0.5 - 1.0 ml.) and dried in vacuo. The supernatant liquid and washings contained the excess C^{14}-formic acid which was recovered and determined by the previously described methods.

The crude adenine was purified by sublimation (38) in the apparatus shown in Figure 5. In preliminary experiments with this apparatus, adenine recovered in the sublimed form averaged 93 percent of the original material. The dried, crude adenine was placed in the outer tube and ground to powder with a glass rod. When the pressure was reduced to approximately 1 mm. and the temperature maintained at 220°C by heating in a wax bath, the purified adenine

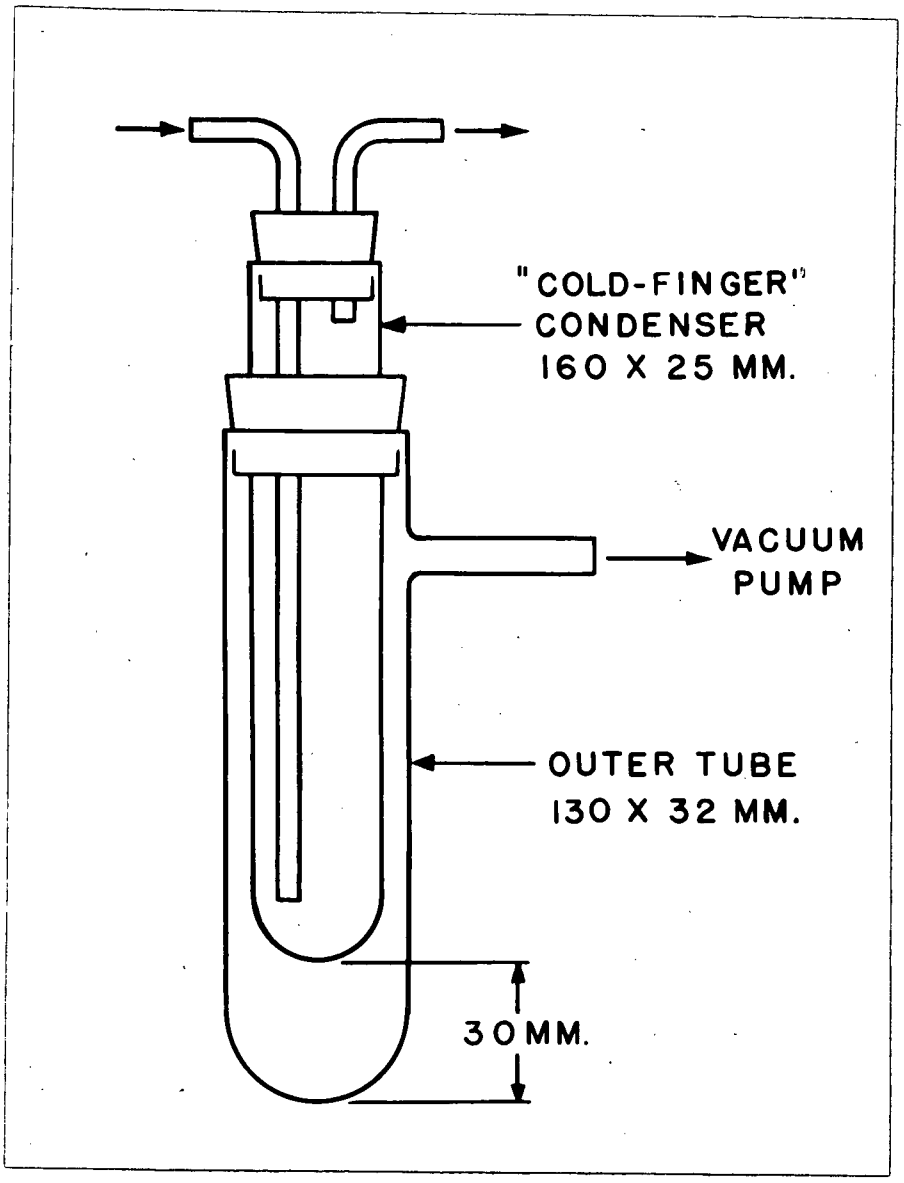

Figure 5. Apparatus for purification of adenine by sublimation.

appeared as a white deposit on the "cold finger" condenser, leaving a light, brownish residue in the outer tube. In this manner 83.0 mg. of adenine (containing 4,358 c.p.m. per mg.) were obtained, representing an overall yield of 61 percent for the three steps, formylation, cyclization and purification.

The excess C^{14}-formic acid (11.24 milliequivalents, a recovery of 93.5 percent) recovered as the potassium salt, was used in the preparation of a second lot of isotopic adenine. As in the first preparation, a solution of radioactive formate was evaporated to dryness in the reaction vessel. To this was added 4-amino-5-imidazole-carboxamidine dihydrochloride (0.200 gm.) and 5.18 N hydrochloric acid (1.77 ml.) and the solution refluxed for 4 hours. The remaining steps of this preparation were similar to those described above. The sublimed adenine from this procedure weighed 88.1 mg., an overall yield of 64.5 percent.

(iv) Proof of identity of synthetic adenine

The identity of adenine, prepared by the above method was established by combustion analysis, preparation of a derivative, paper chromatography and ultraviolet absorption spectrophotometry.

(a) Combustion analysis

Adenine: $C_5H_5N_5$. Calculated, C 44.44; found, C 44.27.

(b) Derivative

The product formed a picrate, mp. 286 - 287°C (with decomposition), and admixture of this picrate with that of an authentic sample did not depress the melting point. It must be noted that this melting point is not a particularly good criterion, since the concomitant decomposition makes the melting point difficult to observe with precision.

(c) Chromatography

Partition chromatography on filter paper, employing the techniques of Markham and Smith (39, 40) was used to verify the identity of synthetic adenine as well as adenine and guanine isolated from experimental animals and to test the purity of these compounds. The solvent system employed was the tertiary butanol-hydrochloric acid-water system described by these authors (39) and a modification of their photographic method (40) was used to locate purine components on the chromatogram. Since Whatman No. 1 filter paper is reasonably transparent to ultraviolet light, and since the purines absorb strongly in the 2600 Å region of the spectrum, it is possible to locate these compounds on a chromatogram by making a contact print of it with ultraviolet light on sensitized paper. A low pressure germicidal lamp (General Electric 15 watt Germicidal lamp) which emits 90 percent of its radiation at 2537 A was found to be a

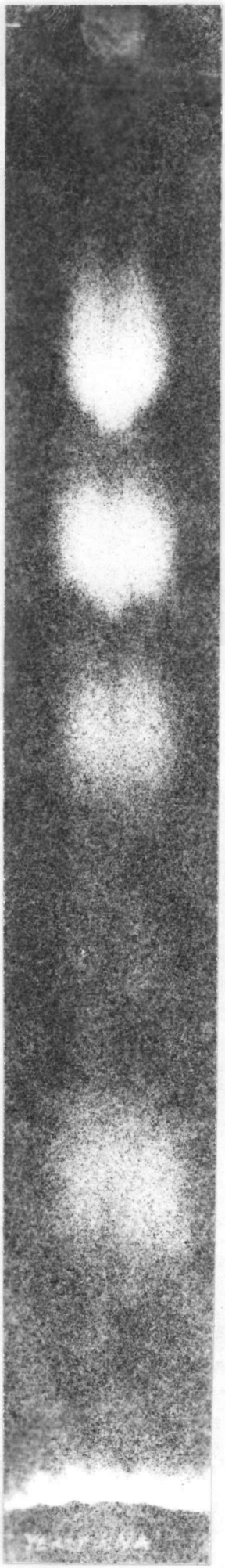

Figure 6.

Contact print of a chromatogram made with light from a General Electric 15 watt Germicidal lamp. The chromatogram is of a hydrolysate of yeast ribonucleic acid and shows, in descending order, spots of guanine, adenine, cytidylic acid and uridylic acid. The solvent system used was the tertiary butanol-HCl-water system described by Smith and Markham (39). The hydrolysate was made by placing 14.8 mg. yeast ribonucleic acid in a small sealed tube with 1 ml. N HCl and heating the mixture for 1 hour at 100°. 15.4 microlitres of the hydrolysate were used for this chromatogram.

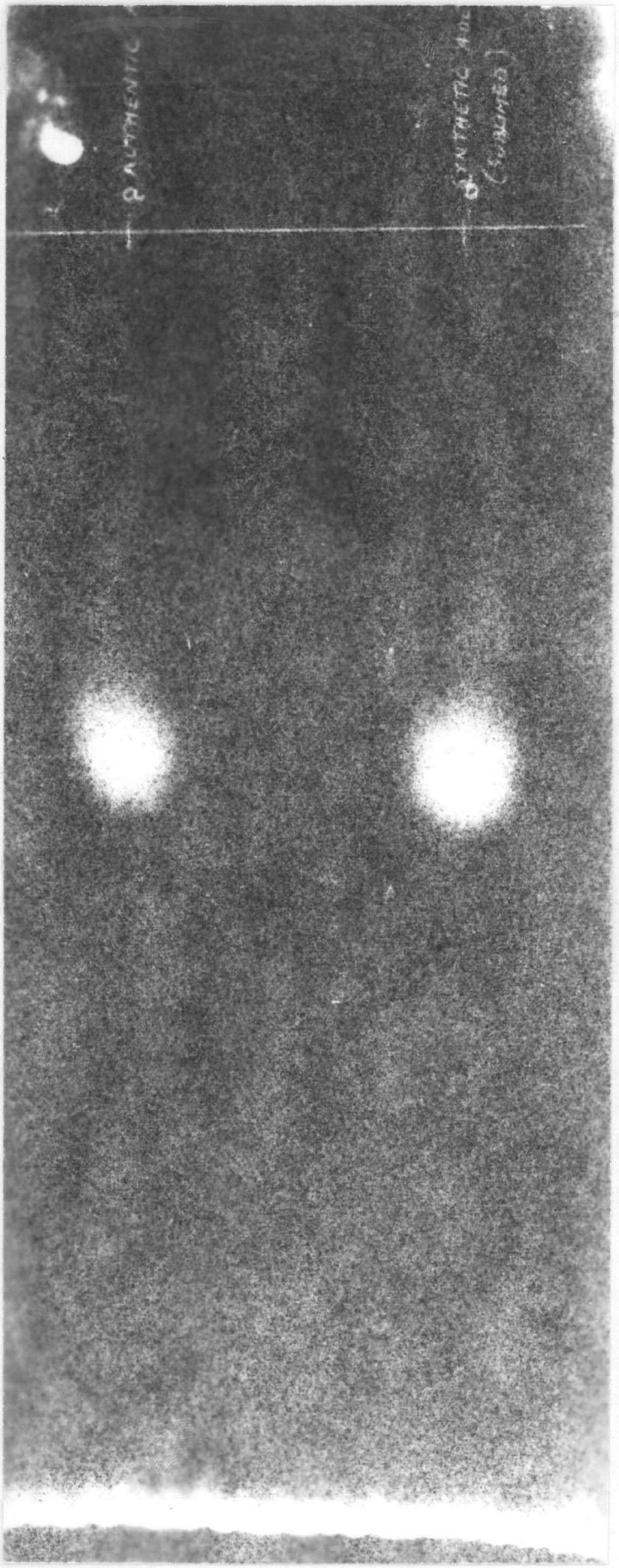

Figure 7.

Contact print of a chromatogram comparing the R_f values of adenine synthesized as described above with authentic adenine. The synthetic product (right spot) is homogeneous and has the same R_f value as the authentic adenine (left spot).

simple, cheap, and entirely satisfactory substitute for the high pressure lamp with gas and liquid filters used by Markham and Smith. Examples of chromatograms printed with a 15 watt germicidal lamp are shown in Figures 6 and 7. The photographic paper used was Ansco "Scona" Reflex paper or Kodak A-4 Kodabromide paper.

On chromatograms run in the above manner synthetic adenine, prepared and purified as described above, had the same Rf value as authentic adenine, that is, 0.40 (Figure 7). Further, these chromatograms showed only one spot for the synthetic product and radioactivity could be detected only at this location. As the Rf value of 4-amino-5-imidazolecarboxamidine in this solvent system was determined to be 0.3, the presence of this substance in the synthetic adenine would have been detected as another spot.

This technique proved valuable in confirming the identity of adenine and guanine isolated from biological sources and in determining whether such compounds were pure. A sample of adenine, for example, if it were contaminated with guanine would show two spots on its chromatogram with Rf values 0.27 (guanine) and 0.40 (adenine).

(d) Spectrophotometry

The purines, pyrimidines and their derivatives have very well-defined absorption maxima in the ultraviolet which have proven to be valuable characteristics in

analysis. Adenine and guanine have maxima in the vicinity of 2600 Å; the absorption spectra of authentic adenine and adenine prepared as described above are compared in Figure 8 and are seen to be identical. The presence of either 4-amino-5-imidazolecarboxamidine or its formamido derivative as impurities in the synthetic adenine would have distorted the shape of the absorption curve since these compounds have maxima at 2850 Å and 2720 Å respectively (31). Observations were made with the Beckman Model DU Quartz spectrophotometer.

II. Measurement of Radioactivity

All determinations of radioactive carbon were made with the carbon in the form of barium carbonate. Samples to be analysed for radioactivity were oxidized by the wet combustion method; the carbon dioxide so produced was trapped in alkali and precipitated as barium carbonate (37). In the case of respiratory carbon dioxide, the carbonate was precipitated directly from the alkali in which it was trapped. The barium carbonate samples were mounted on paper-lined, brass filtering dishes for counting. The preparation of barium carbonate samples for counting and the determination of radioactivity have been described in detail by Wright (37).

Radioactivity was determined by means of a self-

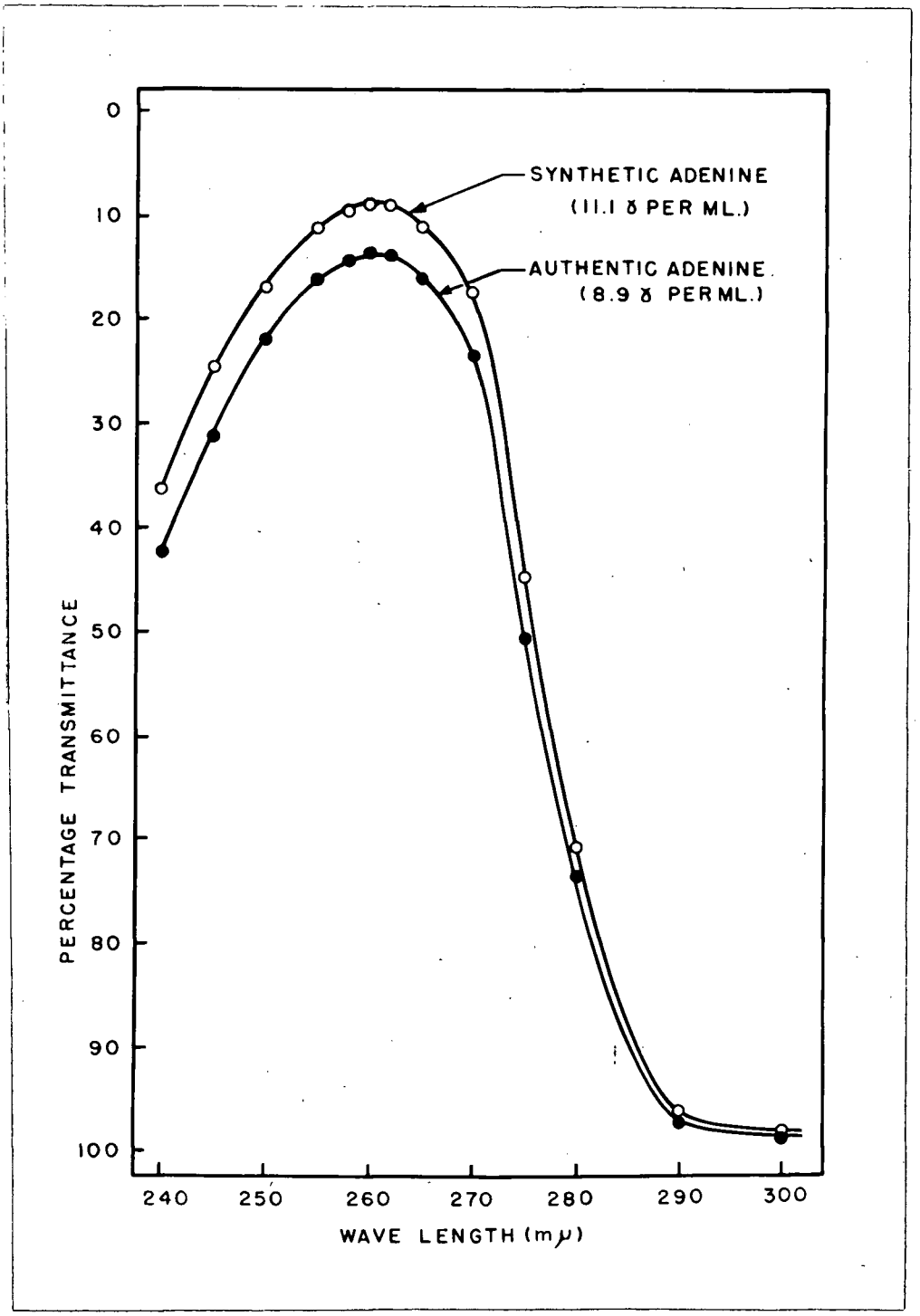

Figure 8. A comparison of the absorption curves of authentic adenine, concentration 8.9 δ per μ l. with adenine prepared as described above, concentration 11.1 δ per μ l. The solvent used in both cases was phosphate buffer, pH 6.5, and the observations were made in a Beckman quartz spectrophotometer Model DU.

quenching Geiger-Müller counter, having a mica end-window with a thickness of 1.6 mg. per sq. cm. The G.M. counter was connected to a commercial scaling unit.#

The barium carbonate samples for radioactive assay were prepared in such a manner that their weights were kept above the minimum weight required to produce an "infinitely thick" sample, which was found to be 132.7 mg. for the particular assembly used (37). Since the activity observed from an "infinitely thick" sample is proportional to the specific activity of the sample, no self-absorption corrections were necessary. All observed activities were corrected for coincidence error, background and counter performance (36). The total activity of the sample was then determined by multiplying the observed activity, corrected in this manner, by the factor

$$\frac{\text{Sample } (BaCO_3) \text{ weight in mg.}}{132.7} .$$

A sufficient number of counts was recorded to insure that the error involved in counting was not greater than 2 percent (36).

The wet combustion technique employed in the preparation of the barium carbonate samples was that described by Wright (37) and permitted the determination of both

Nuclear Instruments and Chemicals Corp. Scaling Unit Model 163.

total and radioactive carbon of organic substances. In this procedure, the sample was oxidized under reduced pressure with the Van Slyke-Folch combustion mixture and the evolved carbon dioxide was trapped in carbonate-free alkali (36) contained in a centrifuge tube. The carbonate was then precipitated as the barium salt from hot solution, washed and collected in a paper-lined, brass filtering dish. The apparatus used is shown in Figure 3.

III. Metabolism Experiments

Dietary or parenterally administered adenine (20, 25) is incorporated, in part, into the tissue nucleic acids and is degraded, in part, to the purine excretory products. In studying the metabolism of 2-C^{14}-adenine administered to rats, it was therefore necessary to isolate and examine the polynucleotide purines and the purine end-product, allantoin. The expired carbon dioxide and urinary urea from the experimental animals were also examined for radioactivity since the presence of the isotope in these compounds would indicate that the purine ring was broken *in vivo* at position 2.

The rats used in these experiments were adult males of the Wistar strain. Isotopically labeled adenine hydrochloride dissolved in rat serum was administered to each rat in daily doses by intraperitoneal injection.

During the experiments the animals were kept in a metabolism cage (Figure 9) which permitted the separate collection of expired carbon dioxide, urine and feces. The total carbon dioxide was collected for each 12-hour period of the experiment and the urine was collected for each 24-hour period. The animals were fed their usual diet (U.B.C. ration 18) ad libitum throughout the experiment.

Two experiments, each of the type described, were performed. In Experiment 1, adenine containing 75,550 c.p.m. was administered; analysis of the expired carbon dioxide indicated that a small amount of radioactive carbon was present therein. However, the observed activities were so close to background that little reliance could be placed on these findings. Methods are available for determining such activities with accuracy, but the counting times involved are so long as to make the method impractical in this case (36). Consequently, Experiment 2 was undertaken in which the amount of isotope administered was 6.6 times that given in the first experiment. The observed activity in the respiratory carbon dioxide collected in Experiment 2 was significantly higher than background.

In Experiment 1, the animals received labeled adenine at the level of 17 mg. per kilo of body weight per day, administered in single daily injections for 4 days. They showed only a slight drop in weight (1.4%) and

displayed no signs of toxicity due to the administered adenine (41, 42, 25). In the second experiment, in order to increase as much as possible the amount of radioactive carbon administered, adenine of a higher specific activity was prepared and administered at a higher level per kilo of body weight than in Experiment 1. The daily dose of adenine was increased to 42 mg. per kilo and was given at this level for 4 days. This dose is approaching the level at which toxic effects may appear. Raska (41) observed that adenine administered orally at the level of 100 mg. per kilo per day over a period of 1-2 weeks induced in dogs a syndrome which resembled avitaminosis. The oral or parenteral administration of smaller amounts of adenine (30-50 mg. per kilo per day) to both dogs and rats produced changes in the blood picture, namely, marked increases in non-protein nitrogen, urea, uric acid, and creatinine (42). Parenchymatous degeneration of the kidneys had also occurred in these animals. Extensive renal damage due to the deposition of 2,8-dioxyadenine crystals in the distal tubules has been shown to occur in rats within 5 days when adenine is injected in single doses at a level of 87 mg. per kilo per day (25). It was considered that the short term nature of this experiment and the administration of 21 mg. of adenine per kilo at 12-hour intervals rather than twice this amount at 24-

hour intervals would reduce the possibility that toxic manifestations might appear.

The animals lost 6 percent of their weight in the 4 days of the second experiment. The gross appearance of the abdominal cavity was noted at the time the animals were sacrificed. The walls of the abdominal cavity, the mesenteries and the surface of the intestines were notably reddish in appearance due to the engorgement of the blood vessels. The kidneys appeared to be slightly enlarged and the brown colour was more pronounced than usual. In the absence of a detailed examination of the blood picture and histological examinations of the kidneys, it cannot be stated with certainty whether or not these animals were showing some signs of toxicity from the administered adenine. However, it was felt that although the amount of adenine administered probably would have produced toxic effects over a longer period, the daily dose was sufficiently small that any of the above-described manifestations of adenine intoxication that might have appeared would not be sufficiently advanced to affect the validity of the experimental results obtained.

It was considered desirable to administer the adenine as a solution which was nearly neutral. Since adenine is a very weak base, solutions of adenine hydrochloride will be strongly acidic. It was found that the pH

of solutions of adenine hydrochloride could be raised to approximately 6, without the precipitation of the free base, by the addition of serum. Solutions of labeled adenine administered in these experiments were prepared by dissolving the free base in slightly more than the equivalent amount of dilute acid, followed by the addition of sufficient rat serum to raise the pH to approximately 5 or 6.

In Experiment 1, the animals received four injections of labeled adenine spaced 24 hours apart and were sacrificed 24 hours after the last injection. The adenine was administered at 12-hour intervals in Experiment 2; each animal received 8 injections and was killed 12 hours after the last injection. Each animal was anesthetized with ether and as much blood as possible obtained from the dorsal aorta by means of a syringe. The heart, lungs, thymus, liver, kidneys, spleen and small intestine were removed immediately and frozen in a dry ice-ethanol mixture. Before freezing, the small intestine was slit lengthwise, washed and cut into small pieces (20). The nucleic acids were isolated subsequently from the pooled organs.

(i) Respiratory carbon dioxide; collection and analysis

Respiratory carbon dioxide was collected for each 12-hour period of the metabolism experiments in the alkali scrubbing towers of the metabolism cage (Figure 9). One

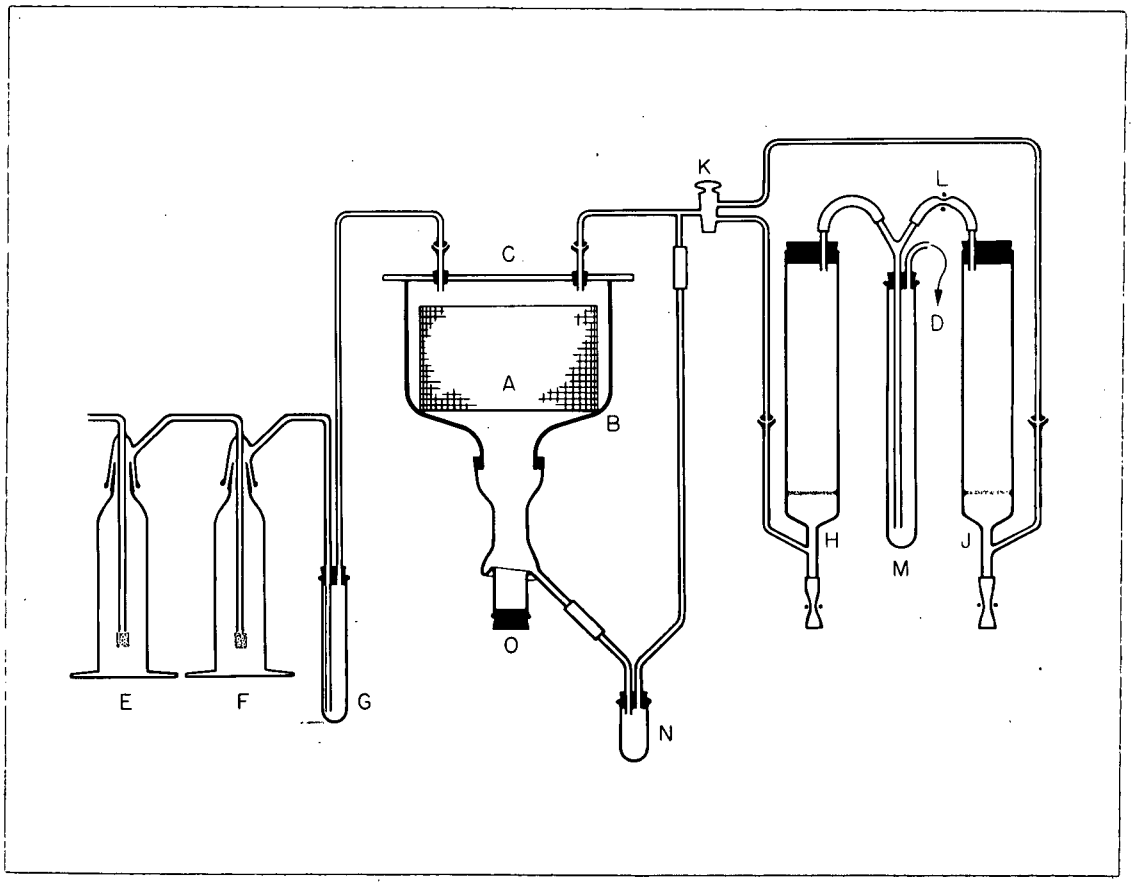

Figure 9. Metabolism cage. The experimental animals were placed in the wire basket A, resting inside vessel B which was made from a 20 litre bottle. The lucite top C was seated on a greased rubber gasket and the joint made air tight by securing the top down firmly with clamps. Tube D was attached to a water pump and air was drawn through the apparatus, passing successively through towers E and F which contained 10 percent sodium hydroxide and saturated barium hydroxide, respectively. Tube G contained saturated sodium chloride and served to control the humidity of the air. Carbon dioxide expired by the animals was swept out of the cage by the current of carbon dioxide-free air and trapped as the air passed up through the alkali scrubbing towers H or J. By manipulation of stopcock K and clamp L the air flow could be switched from one tower to the other without interrupting the experiment. Tube M contained a saturated barium hydroxide solution which indicated any incomplete absorption of CO_2 in towers H or J. Urine was collected in Tube N and feces accumulated in O with only slight contamination from urine.

tower only was used for each collection; 600 ml. of 12 percent carbonate-free sodium hydroxide was found adequate to absorb the carbon dioxide produced by the animals and yet offer a margin of safety. At the end of each collection period the air flow through the cage was switched to the alternate tower which had been filled previously with fresh alkali. The tower containing the absorbed carbon dioxide was then drained and washed; the alkali, together with the washings, was diluted to 1 litre. The carbonate from aliquots (2.0 ml.) of this solution was precipitated as barium carbonate and collected for weighing and counting as previously described.

(ii) Urine; collection and analysis

The metabolism cage (Figure 9) used in these experiments permitted the separate collection of urine and feces. As particles of food dropped by the animals frequently found their way into the urine, it was necessary to centrifuge the urine before examination. The total urine excreted for each 24-hour period of both experiments was collected, diluted to 100 ml. and aliquots taken for the preparation of allantoin, the determination of urea and the isotope content of whole urine.

(a) Whole urine. Aliquots (3.0 ml.) of each urine collection were evaporated to dryness in combustion

tubes (Figure 3, Tube C), converted to barium carbonate by the wet combustion procedure and counted, as described previously.

(b) Urea determination. Urinary urea was determined by the urease method described by Wright (37). In this procedure an aliquot of urine was incubated with glycerol-urease (43) in a phosphate-buffered solution. Urinary urea was hydrolysed to ammonium carbonate by the enzyme urease in this step. The incubation vessel was then connected to 2 alkali traps in such a way that air could be drawn through the urine-urease solution, sweeping the incubation vessel and then passing through the alkali traps. The ammonium carbonate in the urine-urease solution was decomposed by the addition of phosphoric acid and the carbon dioxide thus liberated was swept from the reaction vessel and trapped in the alkali. In this manner, the carbon of urea was converted to carbonate which then was recovered from the alkali as barium carbonate, weighed and analysed for radioactivity as previously described. The enzyme urease is highly specific in its action and determinations of total and radioactive carbon in urea by this method are in excellent agreement with analyses made using the wet combustion technique (37).

(c) Allantoin. Allantoin was isolated from urine by the method described by Brown et al. (20). The urine

was treated with phosphotungstic acid to remove interfering substances and basic lead acetate was then added to remove the excess phosphotungstic acid. Excess lead was precipitated by the addition of sulphuric acid and the solution neutralized with alkali. Allantoin was precipitated from this solution as the mercury salt. Mercury allantoinate was decomposed with hydrogen sulphide and the free allantoin recovered by crystallization. The C^{14} contents of the allantoin samples so obtained were determined by conversion to barium carbonate.

(iii) Isolation of visceral nucleic acids and the preparation of the purines

The pooled internal organs were homogenized, dried and defatted according to the method of Brown et al. (20). The remainder of the procedure followed in the preparation of the nucleic acids and purines was that described by Plentl and Schoenheimer (9). The tissue powder prepared as above was extracted with hot, 10 percent sodium chloride solution and the sodium salts of the mixed nucleic acids precipitated from this solution by the addition of ethanol. The free nucleic acids were obtained by acidification of a solution of their sodium salts. The isotopic carbon content of the free nucleic acids was determined as above.

The nucleic acids were hydrolysed by treatment

with hydrogen chloride in methanolic solution. Adenine and guanine, liberated in this process, were precipitated from solution as the hydrochloride salts. The mixture of the purine hydrochlorides was resolved by dissolving it in water and raising the pH to 5, at which point free guanine precipitated out of solution. Adenine was recovered from the solution by precipitation as the picrate. The purines prepared in this manner were purified by several recrystallizations, adenine as the picrate and guanine as the sulphate. The purity and identity of these compounds were tested by paper chromatography. For chromatography, adenine hydrochloride was prepared from the picrate by dissolving a small amount of the latter in dilute hydrochloric acid and extracting the solution with ether until colourless. Repeated applications of small volumes of this solution were placed on a strip of filter paper in a small spot until approximately 10-20 ɣ of adenine had been added. Authentic adenine was applied in an adjacent spot and the chromatogram run and printed as previously described. In the case of guanine, solutions of the sulphate were applied to the chromatogram. The pyrimidines were not isolated since only a negligible amount of radioactivity could be detected in the nucleic acid hydrolysate following the removal of the purine hydrochlorides.

B. RESULTS

I. Experiment 1

(i) Administration of 2-C^{14}-adenine. Radioactive adenine (56 mg., containing 82,550 c.p.m.) was dissolved in 0.28 N hydrochloric acid (1.5 ml.) and the solution diluted with rat serum (11.5 ml.). C^{14} determinations carried out on aliquots of this solution showed it to have an activity of 6,350 c.p.m. per ml. Two rats were each given 4 intraperitoneal injections of 1.0 ml. of this solution spaced 24 hours apart. A third rat received identical treatment except that the final injection was 0.9 ml. The combined weight of the rats was 758 gm. and they received a total of 11.9 ml. of the adenine-serum solution which contained 51.3 mg. of 2-C^{14}-adenine with an activity of 75,550 c.p.m.

(ii) Expired carbon dioxide. Analysis of the respiratory carbon dioxide indicated that some radioactive carbon was present, but, in general, the counts obtained from samples of the precipitated carbonate were so close to background that the results were unreliable. The carbon-dioxide collected from the 48-60 hour period was the only sample which displayed an activity significantly greater than background.

48-60 hour carbon dioxide: total activity 2,495 c.p.m.
spec. activity 0.42 c.p.m./mg.C

TABLE I

C^{14} analyses of urine, Experiment 1. 2-C^{14}-adenine, having an activity of 75,550 c.p.m., was administered by intraperitoneal injection to three male adult rats whose aggregate weight was 758 gm., in doses of 17 mg. per kilo per day for 4 days. The total urine excreted by these animals was collected at 24-hour intervals and the C^{14} content of urea, allantoin and whole urine in each sample was determined.

Collection Period	Urea		Allantoin		Whole Urine	
	Total activity c.p.m.	Specific activity c.p.m./mg.C	Total activity c.p.m.	Specific activity c.p.m./mg. C	Total activity c.p.m.	Specific activity c.p.m./mg.C
0-24 hours	95	0.27	553	44.6	5,410	6.32
24-48 "	77	0.21	668	73.7	5,550	7.07
48-72 "	2,390	8.13	827	80.5	5,430	8.75
72-96 "	178	0.56	1,445	84.7	5,000	6.61
Total	2,740		3,493		21,390	

This experiment showed that in any succeeding experiments which were intended to demonstrate conclusively whether the expired air contained any radioactive carbon dioxide, the amount of isotope administered must be increased by several times.

(iii) Urine. Radioactive carbon determinations on urine and urinary constituents are presented in Table I. It should be mentioned that the method employed for the isolation of allantoin was not quantitative. It will be noted that the sum of the total activities found in urea and allantoin account for 16-29 percent of the total activity in whole urine.

(iv) Nucleic acids and purines. The combined viscera of the three rats weighed 109 gm. and yielded 17.4 gm. of dried tissue powder. From this powder 1.6 gm. of crude sodium nucleic acids were extracted which when reprecipitated as the free nucleic acids weighed 0.602 gm.

The combined purine hydrochlorides obtained from the nucleic acids by hydrolysis weighed 129 mg. and resolution of this mixture yielded 44.4 mg. of guanine sulfate and 47.1 mg. of adenine picrate. By paper chromatography it was shown that the adenine was contaminated with a small amount of a substance having the same R_f value as guanine. An attempt was made to purify the adenine, but in the course of this operation it was lost. A

chromatogram of the guanine sulfate showed only one component and that had an R_f value the same as that of authentic guanine.

An examination of the nucleic acid hydrolysate after the removal of the purines showed that only negligible activity was present. Consequently, it may be concluded that the administered adenine did not contribute appreciably to pyrimidine biosyntheses.

In Table II the results of isotopic carbon determinations on the free nucleic acids and guanine are shown.

II. Experiment 2

(i) Administration of 2-C^{14}-adenine. Radioactive adenine (122.5 mg., with an activity of 4358 c.p.m. per mg.) was dissolved in 0.343 N hydrochloric acid (2.80 ml.) and diluted with rat serum (14.2 ml.). As a small amount of particulate matter appeared on standing, the solution was heated to 45°C and clarified by centrifugation. Aliquots of this solution analysed for C^{14} were shown to have an activity of 32,630 c.p.m. per ml. The two rats used in this experiment had a combined weight of 692 gm.; each rat received 7 intraperitoneal injections of 1.0 ml. of this solution at 12-hour intervals. An eighth injection of 0.65 ml. was given to each rat 12 hours later and the animals sacrificed at the end of the next 12-hour period.

TABLE II

C^{14} content of visceral nucleic acids, guanine and adenine# isolated in Experiment 1. 2-C^{14}-adenine having an activity of 75,550 c.p.m. was administered by intraperitoneal injection to three adult male rats, whose aggregate weight was 758 gm., each receiving four daily doses of 17 mg. per kilo. The animals were sacrificed 24 hours after the last injection, their viscera immediately removed and frozen. The mixed nucleic acids and nucleic acid purines were prepared from the pooled organs.

Substance isolated	C^{14} Content	
	Total c.p.m.	Specific activity c.p.m./mg. C
mixed nucleic acids	2,814	23.5
guanine sulfate	347	26.3
adenine picrate#	805	49.5

#The adenine picrate sample was lost during purification. The values reported are calculated using the analytical data of Plentl and Schoenheimer (9) and the C^{14} contents observed for the nucleic acids and guanine.

The rats received, therefore, a total of 15.3 ml. of the injection solution which had an activity of 499,300 c.p.m. The adenine was administered at a level of 41.3 mg. per kilo per day.

(ii) Expired carbon dioxide. The amount of isotope administered in this experiment was 6.6 times greater than that given in Experiment 1. The presence of C^{14} in the expired carbon dioxide, indicated in the first experiment, was confirmed and measured; the results obtained are presented in Table III.

(iii) Urine. Determinations of the radioactivity present in urine and urinary constituents are shown in Table IV.

(iv) Nucleic acids and purines. The pooled viscera, when homogenized, dried and defatted, yielded 11.1 gm. of tissue powder. The crude sodium nucleic acids extracted from this powder weighed 1.27 gm. and when re-precipitated as the free nucleic acids weighed 0.569 gm. The combined purine hydrochlorides, obtained from the nucleic acids by hydrolysis, weighed 161 mg. and yielded 48.6 mg. of adenine picrate and 55.7 mg. of guanine sulfate. Paper chromatography was used to verify the identity of these compounds and showed them to be pure.

An examination of the nucleic acid hydrolysate

TABLE III

C^{14} content of expired carbon dioxide, Experiment 2. 2-C^{14}-adenine, having an activity of 499,300 c.p.m. was administered by intraperitoneal injection to two male adult rats with an aggregate weight of 692 gm.; each rat received 8 injections of adenine at the level of 21 mg. per kilo per 12-hour period. The total expired carbon dioxide for each 12-hour period following injection was trapped in the alkali towers of a metabolism cage (Figure 9), diluted to 1 litre and aliquots taken for analysis.

Collection period	Expired CO_2 Total activity c.p.m.	Specific activity c.p.m./mg. C
0-12 hours	2,250	0.68
12-24 "	1,650	0.38
24-36 "	3,000	0.73
36-48 "	4,500	0.91
48-60 "	12,000	3.03
60-72 "	5,750	0.99
72-84 "	6,250	1.02
84-96 "	7,000	1.46
Total	42,400	

TABLE IV

C^{14} analyses of urine, Experiment 2. 2-C^{14}-adenine, having an activity of 499,300 c.p.m. was administered by intraperitoneal injection to two male adult rats whose aggregate weight was 692 gm. Each rat received 8 doses adenine given at the level of 21 mg. per kilo per 12-hour period. The total urine excreted was collected at 24-hour intervals and the C^{14} content of allantoin, urea and whole urine determined.

Collection Period	Urea		Allantoin		Whole Urine	
	Total activity c.p.m.	Specific activity c.p.m./mg.C	Total activity c.p.m.	Specific activity c.p.m./mg.C	Total activity c.p.m.	Specific activity c.p.m./mg.C
0-24 hours	363	1.29	3,860	694	41,000	59.7
24-48 "	225	0.76	3,505	431	29,260	56.4
48-72 "	3,300	10.90	5,040	909	28,450	44.5
72-96 "	2,687	9.79	4,370	872	43,300	71.2
Total	6,575		16,775		142,010	

after the removal of the purines showed that only a small amount of radioactivity was present. Since this was probably due to residual amounts of purines, it was considered that the administered adenine had not contributed to pyrimidine synthesis.

The "total activities" for the mixed nucleic acids and the combined purine hydrochlorides reported in Table V provide a measurement of the recovery of the purine hydrochlorides from the nucleic acid hydrolysate. The C^{14} content of the purine fraction is seen to be 80 percent of that of the nucleic acids. If the assumption is made that there has been no incorporation of the isotope into the pyrimidines of the nucleic acids, this value may be said to represent the fraction of the purines recovered from the nucleic acid hydrolysate.

TABLE V

C^{14} content of visceral nucleic acids and purines isolated in Experiment 2. 2-C^{14}-adenine, having an activity of 499,300 c.p.m., was administered by intraperitoneal injection to two adult male rats whose aggregate weight was 692 gm. Each rat received 8 injections of adenine at the level of 21 mg. per kilo per 12-hour period. The animals were sacrificed 12 hours after the last injection, their viscera immediately removed and frozen. Nucleic acids and nucleic acid purines were prepared from the pooled organs.

Substance isolated	C^{14} Content	
	Total activity c.p.m.	Specific activity c.p.m./mg. C
free nucleic acids	21,070	88.5
combined purines	16,940	322
guanine sulfate	4,150	270
adenine picrate	4,759	344
adenine (calculated from picrate)		757

DISCUSSION

Brown and his co-workers observed that adenine, labeled in nitrogens 1 and 3 with N^{15}, when fed to rats is incorporated into adenine and guanine of the tissue nucleic acids. The nitrogen isotope of the nucleic acid guanine was found also in positions 1 and 3; from this fact Brown et al. concluded that the purine ring remained intact during the conversion of adenine to guanine (20). Further evidence that the ring remains intact has recently been furnished by Marrian et al. who fed rats adenine labeled with N^{15} in positions 1 and 3, and with C^{14} in position 8 (1,3-N^{15}, 8-C^{14}-adenine) and showed that there was an essentially equal incorporation of the N^{15} and C^{14} isotopes into polynucleotide guanine (22). However, as has been discussed previously, this concept was questioned by Gordon (27) and Marsh (28) who suggested that the purine ring may not remain intact during metabolic processes, but rather may be biologically labile at position 2. If the concept of a biologically labile 2-position in the purines is valid, a loss of isotope from this carbon would be expected when administered 2-C^{14}-adenine underwent any metabolic transformations, such as incorporation into the nucleic acid purines. A comparison is indicated, therefore, between experiments with 1,3-labeled adenine and 2-labeled adenine which measure the extent to

which the isotope of the administered adenine is incorporated into the polynucleotide purines. Such a comparison is made in Table VI between the experiments of Brown and collaborators who used 1,3-N^{15}-adenine and the experiments on the metabolism of 2-C^{14}-adenine described herein. This comparison is of limited value since the method of administration of the labeled adenine and the amounts administered per kilo of body weight vary in the experiments described. It will be noted that the percentage of nucleic acid adenine synthesized from injected 2-C^{14}-adenine is lower than the corresponding value reported for injected 1,3-N^{15}-adenine (25). Guanine renewals are essentially the same in these two experiments. The reason for the difference between the adenine and guanine renewals is not apparent. Before significance is attached to this observation, confirmation by further experiments of a similar nature is necessary. The question of the 2-position lability could undoubtedly be clarified by an experiment in which a solution of 1,3-N^{15}-adenine and 2-C^{14}-adenine is administered to the experimental animals. Adenine administered in this manner would be, in effect, doubly labeled. A comparison made of the nucleic acid purine renewals calculated from N^{15} and C^{14} uptake very likely would reveal any differences due to the proposed lability of carbon 2.

TABLE VI

A comparison of the renewal of polynucleotide adenine and guanine as measured by the incorporation of the isotope from 1,3-N^{15}-adenine and 2-C^{14}-adenine. The values shown are the percentages of the nucleic acid purines derived from the administered labeled adenine, calculated in the case of C^{14} labeling, as the ratio of the specific activity per mole of the polynucleotide purine to that of administered adenine, expressed as a percent. The renewals shown for N^{15} labeling are calculated as the ratio of "atom percent excess N^{15}" of the isolated purine to that of the administered adenine, expressed as a percent. All of the experiments were 4 days in length and the rats used were adult males.

	2-C^{14}-adenine, intraperitoneal		1,3-N^{15}-adenine, intraperitoneal	1,3-N^{15}-adenine, oral		
	17 mg. per kilo per day Expt. 1.	42 mg. per kilo per day Expt. 2.	87 mg. per kilo per day (Ref.25)	27 mg. per kilo per day (Ref.20)	38 mg. per kilo per day (Ref.21)	200 mg. per kilo per day (Ref.20)
Adenine		7.69	11.7	5.4	2.1	13.7
Guanine	1.1	5.51	5.2	3.2	1.3	8.2

Bendich et al. (25) observed a higher renewal of polynucleotide guanine from dietary 2,6-diaminopurine-1,3-N^{15} than from dietary 2,6-diaminopurine-2-C^{13} in a parallel experiment. Approximately 4.0 percent of the nucleic acid guanine was synthesized from N^{15}-labeled diaminopurine, whereas only 1.5 percent was derived from the C^{13}-labeled compound, as measured by the uptake of isotope in each case. This difference is cited by Gordon (27) as evidence for a labile 2-position.

In the preceding discussion it was suggested that there may well be a loss of isotope in the tissues from administered 2-labeled adenine or the compounds intermediary in its metabolism. This would imply that the purine ring was broken at position 2 and that the isotopically labeled carbon 2, as a labile substituent, was partly transferred to some compound in the tissues and replaced by non-isotopic carbon. This is in accord with the concept of a dynamic equilibrium of the tissue constituents (44). Some of the C^{14}-label of adenine presumably would be found in the tissues as a 1-carbon compound, or as a labile substituent of some larger molecule, and thereby would become distributed throughout the organism according to whatever metabolic processes it underwent. For these reasons, the expired air of the experimental animals was examined for the presence of radioactive carbon dioxide. As may be seen in Table VII,

TABLE VII

Distribution of C^{14} following the administration of 2-C^{14}-adenine by intraperitoneal injection.

	Experiment 1		Experiment 2	
	Total c.p.m.	Percent of administered adenine	Total c.p.m.	Percent of administered adenine
Adenine (injected)	75,550	100	499,300	100
Expired carbon dioxide	trace		42,400	8.4
Urea	2,740	3.62	6,450	1.29
Allantoin	3,493	4.62	16,775	3.36
Whole urine	21,390	28.3	142,010	28.40
Combined nucleic acids	2,814	3.82	21,070	4.22

the expired carbon dioxide collected in Experiment 2 contained 8.4 percent of the isotope administered.

In considering what compound was the immediate precursor of the C^{14}-carbon dioxide in the expired air, note must be made of the presence of the isotope in urinary urea. Recently it has been shown that urea is active in intermediary metabolism and may contribute part of its carbon to the carbon dioxide of the tissues. Wright (37) has shown that C^{14}-urea, when injected into rats, may contribute as much as 30 percent of its isotope to expired carbon dioxide. Since, in the experiments described herein, the expired carbon dioxide contained almost 7 times the amount of isotope found in urinary urea (Table VII), it is considered that the expired carbon dioxide was not derived principally from urea.

The metabolic fate of adenine, as far as is known, appears to consist of transformations into the nucleic acid purines, allantoin, adenosine triphosphate, and 2,8-dioxyadenine (23). The possibility also exists that the adenine molecule could be extensively degraded and the carbon atoms oxidized to carbon dioxide. Such a process could conceivably account for the presence of C^{14} in the expired carbon dioxide observed in Experiment 2. However, it appears unlikely that a sizeable breakdown of adenine occurred since, in the experiments of Brown et al. (45), only fractional percentages of ammonia and urea were

derived from N^{15}-labeled adenine, isoguanine, hypoxanthine, xanthine and uric acid.

The presence of the radioisotope in expired carbon dioxide can be explained by the postulated lability of carbon 2 in the purines. As suggested previously, it is likely that the C^{14}-carbon dioxide arose from the oxidation of some tissue constituent which picked up C^{14} by interchange with the labile 2-carbon of 2-C^{14}-adenine or with some compound intermediate in its metabolism. The demonstration that formate is an excellent precursor to position 2 and the implication of 4-amino-5-imidazolecarboxamide in purine biosynthesis (13) suggest that formate or some metabolic derivate of formate, may be the compound in equilibrium with the labile 2-carbon of the purines. Recent work has shown that formate is an important intermediate in metabolism (13) and that it may be produced in the body from the α carbon of glycine (46) and the β carbon of serine (47). The presence of free formaldehyde and formate have been demonstrated in the tissues by Mackenzie (48). Formate has been shown to be oxidized by the rat but the mechanism of this process has not yet been elucidated (46). An experiment which might shed some light on the postulated interchange reaction between the purine 2-carbon and some tissue constituent is suggested by the work of du Vigneaud et al. (49). These authors showed that C^{14}-labeled methanol,

formaldehyde and formate were utilized by the rat in the biosynthesis of the labile methyl group in choline; C^{14}-bicarbonate was not used for this process. If the appearance of C^{14} in the methyl groups of choline were found as a result of the metabolism of 2-C^{14}-adenine, this would suggest that formate, or a derivative of formate, was liberated from position 2 by the opening of the purine ring.

The finding of C^{14}-urea is somewhat more difficult to interpret. Since the total amount of C^{14} in urea was only 1-4 percent of that present in the administered adenine (Table VII), the labeled urea could have arisen from several minor reactions. Urinary urea consistently showed a small uptake of isotope in metabolism experiments with various N^{15}-labeled purines (45) and with 1,3-N^{15}-adenine, administered both orally and parenterally (20, 25). In these experiments the isotope could have been derived from a small degradation of the purine or of any of the intermediates in the course of its oxidation to allantoin. A portion of the C^{14} found in urea in Experiments 1 and 2 described herein could be due to such factors. It is well established that carbon dioxide of the tissues is incorporated into urea (50, 32); a large part of the C^{14} found in the urea could be accounted for by this process. However, it must be noted that the specific activity of this urea was higher than that of the expired carbon dioxide of the corresponding

period. This does not agree with the results obtained by Mackenzie and du Vigneaud (32) who found that rats, which had received methionine labeled with C^{14} in the methyl group, excreted urea and expired carbon dioxide having the same specific activities during the same period. This anomaly may be due, in part, to the fact that the C^{14} in the urea isolated in these experiments is undoubtedly derived from several precursors. It will be noted in Tables I and III that there is a decided increase in the specific activity of urea in the 48-72 hour period of both Experiments 1 and 2; it is probably significant that a similar increase occurs in the 48-60 hour carbon dioxide of Experiment 2. The only significantly radioactive carbon dioxide found in the first experiment was that collected in this same period. This parallel increase in the radioactivity of carbon dioxide and urea suggests that at least part of the urea was derived from carbon dioxide.

As may be seen in Tables I and II, the sum of the radioactivity present in the urea and allantoin fractions of urine falls considerably short of the total radioactivity present in whole urine. An investigation of the nature of the other radioactive compounds present in urine is therefore indicated.

Although the finding of C^{14} in the expired carbon dioxide of animals to which 2-C^{14}-adenine had been

administered would seem to be direct evidence of a biological lability in the 2-position of the purines, a more definite answer to this question would undoubtedly be provided by studies of the renewals of the polynucleotide purines measured by C^{14} and N^{15} uptake from doubly-labeled adenine (1,3-N^{15}, 2-C^{14}-adenine).

SUMMARY

1. A method has been developed for the synthesis of 2-C^{14}-adenine. This compound was prepared by the formylation of 4-amino-5-imidazolecarboxamidine in aqueous C^{14}-formic acid, followed by cyclization of the resulting formamido compound. This procedure is a modification of the synthesis of adenine described by Shaw (31), which in its original form was unsuitable for the incorporation of isotopic carbon into position 2 of the adenine molecule. The formylation procedure described herein permits an almost complete recovery of the excess formylating agent, C^{14}-formic acid.

   Isotopic adenine, having an activity of 4,358 c.p.m. per mg., was prepared in 60 percent yield by this method, with a 93 percent recovery of the unused C^{14}-formic acid.

2. A method for the purification and determination of small amounts of formic acid has been developed for use in the preparation of C^{14}-formic acid required in the synthesis of 2-C^{14}-adenine.

3. A modification of the photographic technique of Markham and Smith (40) has been used for the location of nucleic acid derivatives on filter paper chromatograms. The apparatus used by these authors has been greatly

simplified by using as a source of ultraviolet light a low pressure germicidal lamp which emits 90 percent of its radiation at 2537 Å. The use of this lamp obviates the need for a system of gas and liquid filters.

4. The metabolism of 2-C^{14}-adenine was studied in the adult male rat. Isotopic adenine, administered by intraperitoneal injection, was incorporated into adenine and guanine of the nucleic acids, confirming the findings of Bendich et al. (25). Radioactive carbon dioxide was found in significant amounts in the expired air of the experimental animals and accounted for 8 percent of the administered radioactivity. Whole urine was found to contain 28 percent of the administered isotope, while urinary urea and allantoin together accounted for 16-29 percent of the total activity found in urine.

5. The renewals of nucleic acid purines, measured by the uptake of C^{14} from 2-C^{14}-adenine, were compared with renewals measured by the uptake of N^{15} from 1,3-N^{15}-adenine as reported in the literature (20, 21, 25). These findings and the presence of C^{14} carbon dioxide in expired air are discussed in connection with the postulated biological lability of the 2-position in the purines.

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