Methylated Purines, and Purine Metabolism in *Tetrahymena*

by

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A dissertation submitted to the Graduate Faculty in Biology
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
The City University of New York.

1975

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

May 8, 1975
Date

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ABSTRACT

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The tRNA of *Tetrahymena pyriformis* was isolated and purified by phenol extraction and DEAE-cellulose column chromatography. Following acid hydrolysis of the tRNA, the methylated purine content was determined by Dowex-50 column chromatography, and paper chromatography. The most abundant methylated guanine derivative was found to be N^2-DMG. Also found were 1-MG, N^2-MG, and 7-MG. The most abundant methylated adenine was found to be 1-MA. No 2-MA was detected. Small amounts of the N^6-methyladenines were detected.

Whole cells of *Tetrahymena* were found to be capable of demethylating 1-MGR, with the methyl group being incorporated into lipid, probably as choline. Cell-free homogenates also demethylated 1-MGR yielding formaldehyde, which could be recovered as CO_2. The demethylation reaction apparently requires NADPH as a cofactor.

Tetrahymena pyriformis, which demonstrates an absolute growth requirement for the purine base guanine, can convert guanine to adenine. *Tetrahymena pyriformis* GL was grown on defined medium with guanine as the sole source of purine. Growth studies showed that XMP, IMP, and adenine were able to spare the guanine requirement, and furthermore, when suboptimal amounts of guanine were provided, increasing amounts of XMP resulted in increasingly better growth. When cells were grown on defined medium containing ^{3}H-8-Guanosine as the sole purine source, it was found that unlabeled XMP and IMP diluted the radioactive label which was

incorporated into nucleic acid adenine. When the Acid-Soluble Pool was analyzed after a short pulse of ^{3}H-8-Guanosine; XMP, IMP, AMP, and probably succinyl-AMP were found to be radioactive. When ^{14}C-8-GMP was incubated with a low speed cell supernatant fraction, radioactive XMP was found to accumulate with time. That the product was indeed XMP was demonstrated by column and paper chromatography, by its conversion to xanthine by acid hydrolysis, and by its conversion to uric acid with xanthine oxidase after acid hydrolysis. These results lead to the following proposed pathway for purine interconversion in *Tetrahymena pyriformis*:

GMP → XMP → IMP → succinyl-AMP → AMP

LIST OF ABBREVIATIONS

A	Adenine
1-MA	1-methyladenine
2-MA	2-methyladenine
N^6-MA	N^6-methyladenine
N^6-DMA	N^6-dimethyladenine
G	guanine
N^2-MG	N^2-methylguanine
N^2-DMG	N^2-dimethylguanine
1-MG	1-methylguanine
1-MGR	1-methylguanosine
7-MG	7-methylguanine
RNA	ribonucleic acid
tRNA	transfer ribonucleic acid
rRNA	ribosomal ribonucleic acid
mRNA	messenger ribonucleic acid
NAD	nicotinamide-adenine dinucleotide (oxidized)
NADP	nicotinamide-adenine dinucleotide phosphate (oxidized)
NADH	nicotinamide-adenine dinucleotide (reduced)
NADPH	nicotinamide-adenine dinucleotide phosphate (reduced)
Tris	tris(hydroxymethyl)amino-methane (2-amino-2-hydroxymethyl-propane-1,3-diol)
DEAE-cellulose	O-(diethylaminoethyl)-cellulose
GMP	guanosine 5′-monophosphate
GR	guanosine
IMP	inosine 5′-monophosphate
IR	inosine
XMP	xanthosine 5′-monophosphate
AMP	adenosine 5′-monophosphate
ADP	adenosine 5′-diphosphate
ATP	adenosine 5′-triphosphate
PCA	perchloric acid

MATERIALS

All chemicals used in these experiments were of the finest reagent grade available. [^{14}C-methyl] L-methionine, 10mCi/mmole, 250μCi in 0.01N HCl, were purchased from Schwarz-Mann. 1-MG, N^2-MG, 7-MG, 1-MA, and N^6-MA were purchased from Sigma Chemical Co. N^2-DMG, 2-MA, and N^6-DMA were purchased from Calbiochem Inc.

1-MGR was purchased from Sigma Chemical Co. Methyl-^{14}C Iodide, 0.25mCi, 2.4 mg, were purchased from New England Nuclear Co. The 100X's concentrated Antibiotic-Antimycotic mixture was purchased from the Grand Island Biological Co., New York. ^{3}H-8-Guanosine, 2ml, 1mCi/ml, 15Ci/mmole, 53mCi/mg, were purchased from Amersham/Searle. Guanosine-8-^{14}C-5' Monophosphate (^{14}C-8-GMP), 10μCi in 200μl, 61mCi/mmol, were purchased from Amersham/Searle. Xanthine oxidase (Xanthine: oxygen oxidoreductase) E.C. No. 1.2.3.2, Grade 1 from Buttermilk, as a suspension in 2.3M $(NH_4)_2SO_4$, was obtained from Sigma Chemical Co.

G, GR, GMP, A, AMP, succinyl-AMP, inosine, IMP, and XMP were all products of the Sigma Chemical Co.

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INTRODUCTION

Modifications of organic bases in ribonucleic acids:

Soon after tRNAs were first discovered and characterized, and their constituent nucleotides identified, it was determined that some of the organic bases were modified (1,2,3,4). These modifications took the form of methylation, thiolation, and even rather elaborate "hypermodifications" (5,6,7). Methylations were found to occur on both the organic base and on the 2'-OH group of the ribose of the nucleoside (8,9). Modified bases have been found in tRNA (5), rRNA (5), and have recently been reported in mRNA (10,11).

Since their discovery, the function of these modifications has been a field of intensive research. Much of the investigation has centered on the function of these modifications in tRNA, which contains the highest percentage of modification of any RNA. These studies have attempted to demonstrate the effect of methylation on the aminoacylation of tRNA, the binding of aminoacylated tRNA to the ribosome, and the ability of tRNA to translate mRNA into protein. Unfortunately, many of these studies have been conducted using heterologous systems, that is, the source of the synthetase enzyme or protein synthesizing system might be from liver, while the source of the undermethylated tRNA might be from *E. coli.* Such experiments are very difficult to interpret and do not really answer the question as to what is happening *in vivo.* However, some conclusions can be drawn from this information, and from experiments in which homologous systems were used. It is generally accepted that methylation of tRNA, or its lack, does not affect aminoacylation (12). Under certain conditions, an effect has been observed on the initial kinetics of aminoacylation which suggests that, initially at least, undermethylated tRNA is less efficiently aminoacylated than fully methylated tRNA (13). A decreased efficiency in binding has also been reported for methyl-deficient tRNA in a ribosome binding assay in the presence of synthetic messengers (14). The clearest evidence for the importance of tRNA base modifications

comes from studies of the ability of tRNA's to recognize specific codons. It has been clearly demonstrated that the proper modification of the adenine residue adjacent to the anticodon of the tRNA is essential for proper codon recognition (15). The importance of modification and methylation in other positions of the tRNA molecule is not yet known. It is probable that these modifications affect the overall structure of the tRNA molecule in subtle ways to increase the stability and efficiency of tRNA function.

Ribosomal RNA also contains methylated bases. That these modifications are important for the survival of the organism can be inferred from the fact that when 16s rRNA from widely differing procaryotic organisms was analyzed, it was found that the nucleotide sequence immediately adjacent to the methylated bases has been conserved during evolution. On the other hand, the nucleotide sequences which were not adjacent to methylated bases showed considerable variation (16). Mutants of *E. coli* have been isolated which have been shown to lack 1-methylguanine in their rRNA, yet these mutants are completely viable (17). This would suggest that the function of methylated bases in rRNA is to produce subtle effects in the conformation of the ribosome. That this is so is supported by the fact that a mutant of *E. coli* which is resistant to the drug kasugamycin has been isolated. The conferring of resistance has been traced to the failure to methylate two specific adenine residues near the 3′ end of 16s rRNA (18). The effects of methylation must be rather subtle, since both sensitive and resistant strains grow quite normally except when challanged with kasugamycin.

It has recently been reported that mRNA contains methylated bases, specifically N^6-methyladenine. The function of these methylated bases is as yet speculative (10,11).

Methylation and Cancer:

The methylation of nucleic acid bases is accomplished by the addition of a methyl group from S-adenosyl-methionine to a specific base residue in the preformed nucleic acid macromolecule (19). The enzymes accomplishing this transfer are known as methyl-transferases, or simply, as methylases. Numerous reports have appeared in the

literature which show that methylase activity increases in cancer cells (20,21,22). As with the studies on the function of methylation, these studies on the degree of methylase activity often utilized heterologous systems, making a determination of the *in vivo* significance of elevated methylase activity rather dubious. Subsequently, it has been shown that some of the differences observed can be abolished by adjusting the ionic strength of the reaction mixture (23). Other differences can be ascribed to the presence of competing methyl-transferase systems present in normal adult tissue, but lacking in embryonic and tumor tissue (24). When the *in vivo* tRNA methylation patterns of normal and SV 40 transformed 3T3 cells were compared, they were found to be similar both quantitatively and qualitatively (25). Recent experiments using more precise techniques show that, *in vivo,* transformation does not alter the amount of methylation, but does alter the pattern of methylation, such that, previously unmethylated positions are methylated and vice versa (26,27). These results suggest that increased tRNA methylase activity in tumor cells may represent a general derepression of host cell functions.

Methylation of ribonucleic acid in Protozoa:

Since methylated bases are formed by the addition of methyl groups to specific residues in the macromolecular RNA, it was of interest to know how an organism handles these methylated bases, when, during the normal turnover of RNA, these bases would be released in the cell. Could the organism metabolize the methylated base, by demethylating and regenerating the normal base? Or would the organism simply excrete the methylated bases? It would be best to study the metabolism of a methylated base with which the organism would normally come into contact, that is, one of the more abundant methylated bases of *Tetrahymena pyriformis* own RNA. Unfortunately, there was no data on methylated bases in *Tetrahymena* available. The methylated base content of tRNA of a wide variety of organisms has been reported (5,28). Bacteria, a number of tissues of higher organisms, and plant tissues have been examined; however, few reports have appeared on the methylated base content of Protozoans. A partial analysis of the methylated base content of the tRNA of *Euglena gracilis* revealed that N^6-MA and N^2-MG were the most abundant methylated purines (29). However, since

the tRNA was hydrolyzed under alkaline conditions, it was uncertain whether N^6-MA is present *in vivo* or whether it resulted from the rearrangement of 1-methyladenine during the isolation procedure (30). Because of the lack of information on the methylated base content of Protozoan RNA, it was decided to undertake a study of the methylated purine content of *Tetrahymena pyriformis* tRNA. Since the tRNA contains the preponderance of the methylated bases, and since the pattern of purine methylation is more complex than that of the pyrimidines, this study concentrated on the identification and quantitation of the methylated purines in the tRNA of *Tetrahymena pyriformis*.

The demethylation of aromatic compounds:

The ability of microorganisms to demethylate methylated purines has been partially explored. It has been shown that *E. coli* is capable of demethylaminating N^6-MA to yield inosine and methylamine (31,32). It has also been demonstrated that *Salmonella typhimurium* contains a similar enzyme system and that the enzymatic activity appears to be distinct from adenosine deaminase (33,34). Liver microsomal systems have also been widely studied for their ability to demethylate organic aromatic compounds (35,36,37,38,39,40,41). This work received a great impetus because of its relationship to the demethylation of a number of drugs and carcinogenic compounds. In these systems demethylation takes place with the formation of formaldehyde. The microsomal system requires NADPH as a cofactor in the reaction. Quite importantly, cancer cells do not appear to contain these demethylase systems (42,43). Since *Tetrahymena* is often used as a model mammalian system it was of some interest to determine if it contained the enzyme systems capable of carrying out demethylation. An analysis of the methylated purines of *Tetrahymena pyriformis'* tRNA revealed that 1-methylguanine was one of the more abundant methylated purines, and since it had been reported that 1-MG could substantially replace the guanine requirement of *Tetrahymena* (44), it was decided to examine the metabolism of 1-MG in *Tetrahymena pyriformis.*

Purine metabolism in *Tetrahymena*:

Early nutritional studies had shown that guanine is an absolute growth requirement for *Tetrahymena* (45). Isotope tracer studies demonstrated that guanine could be converted to adenine, but that adenine could not be converted to guanine (46,47,48). Although these facts have been known for quite some time, the pathway by which guanine is converted to adenine has eluded investigators. The direct deamination of guanine by guanase, or the direct deamination of GMP by GMP reductase have both been found not to take place in *Tetrahymena* (49,50). Since the demethylation of 1-MG would lead to the production of the free base guanine, a search was undertaken for the pathway by which guanine could be further metabolized to adenine.

METHODS

Paper Chromatography:

Throughout this work, either Whatman No. 1, or Whatman No. 3MM chromatography paper was used as noted. Chromatography was accomplished by the descending technique. At all times standards were run on the same chromatogram with the unknown experimentals, and all identifications are made on the basis of comparison with authentic standards run on the same chromatogram. The following solvents were used as noted in the text.

A	methanol – 12N HCl – water	(7:2:1)
B	*n*-butanol – acetic acid – water	(4:1:1)
C	n-butanol – water	(85:15)
D	isopropanol – water-28% aqueous NH_3	(85:15:1.3)
E	Isopropanol – 5% aqueous $(NH_4)_2SO_4$	(1:19)
F	isopropanol – 12N HCl – water	(68:17.6:14.3)
G	t-butanol – methyl ethyl ketone – water – 28% aqueous NH_3	(4:3:2:1)
H	isopropanol – water – 28% aqueous NH_3	(7:2:1)
I	isopropanol – water – 12N HCl	(65:18.4:16.6)
J	t-butanol – methyl ethyl ketone – water – diethylamine	(40:50:20:4)
K	n-butanol – glacial acetic acid – water	(2:1:1)
L	isobutyric acid – 28% aqueous NH_3 – water	(66:1:33)

Preparation of tRNA with ^{14}C-labeled methyl groups:

Tetrahymena pyriformis, strain GL, was grown in the following medium: 1.1% Proteose-Peptone, 0.1% Dextrose, 0.1% K_2HPO_4 and 2μg/l Thiamine·HCl. The pH of the medium was adjusted to pH 7.2 with 1N NaOH. Four 250ml flasks containing 75ml of medium each, were each inoculated with 5ml of log phase cells (72hrs), and

incubated with shaking at 27°C. After 3 days the cells were transferred to 1 liter of medium in each of four 2.8 liter Fernbach flasks. The cells were incubated in the dark with shaking at 27°C for 1 day, and then 62μCi of sterile [^{14}C-methyl] L-methionine, specific activity 50 mCi/mmole, were added to each flask, and incubation with shaking was continued for another 2 days.

Cells were harvested at room temperature by centrifugation in the IEC Model HN-S centrifuge, in pear-shaped oil centrifuge tubes, and washed in 0.01M Phosphate buffer (pH 7.6). The cells were then suspended in 0.01M Tris (pH 7.6) containing 0.1% 8-hydroxyquinoline, and disrupted by 75 strokes with a motor-driven Teflon Homogenizer. The homogenate was centrifuged at 14,500 X g in the SS-34 rotor in the Sorvall RC-2 Centrifuge, at 4°C for 1 hour. The supernatant was then centrifuged at 100,000 X g in the Ti-50 rotor in the Beckman L3-40 Ultracentrifuge at 4°C for 1 hour, in order to sediment the microsomes.

The supernatant was extracted with an equal volume of 90% phenol for 1 hour at room temperature with stirring (51). The emulsion was broken by centrifugation at 12,100 X g in the RC-2 for 30 min. The aqueous layer was again extracted with an equal volume of phenol, and the emulsion broken as above. The final aqueous phase was made 2% with potassium acetate, and then 66% with ethanol, and the tRNA allowed to precipitate overnight in the freezer. The ethanol precipitate was collected by centrifugation, and washed and dried with cold absolute ethanol and ether.

The tRNA preparation was dissolved in 0.1M Tris (pH 7.6) and applied to a 2.5 X 40cm DEAE-cellulose column, equilibrated with the same buffer. The column was washed with 60ml of 0.1M Tris (pH 7.6), and then with 140ml of 0.1M Tris (pH 7.6) containing 0.3M NaCl. The tRNA was then eluted with 0.1M Tris (pH 7.6) containing 0.7M NaCl. The effluent was monitored with an LKB Uvicord I at 254nm, and 20ml fractions were collected. The tubes containing tRNA were combined and lyophilized.

Isolation, identification and quantitation of methylated purines of tRNA:

The tRNA was dissolved in 20ml of 1N HCl and hydrolyzed in a boiling water bath for 1 hour. Hydrolysis was carried out in acid in order to prevent base rearrangements known to occur in alkali (30,52). A 1 X 25cm Dowex-50 column was prepared

by washing with 20 volumes of 0.1N NaOH, 1X; 2N HCl, 2X's; and distilled water, 15X's. The fines were removed by suction. The hydrolysate was applied to the Dowex-50 column, which was first washed with 20ml of 1N HCl, and then developed with 2N HCl. The effluent was monitored with an LKB Uvicord I at 254nm. and 10ml fractions were collected. A 0.1ml aliquot of each tube was neutralized with 1N NaOH, and dissolved in 10ml of Packard Permafluor (mixture B) Liquid Scintillation Fluid. Samples were counted in a Nuclear-Chicago Mark II Liquid Scintillation Counter. Tubes were combined as indicated in Figure 1, to give Fractions 1-7. The Fractions were dried under vacuum and redissolved in 0.7ml of 0.1N HCl. Each Fraction was analyzed for its content of methylated purines by descending chromatography on Whatman No. 1 paper in each of the six solvents A through F. The chromatograms were cut into 1.5 X 4cm sections, eluted in 0.5ml of 0.1N HCl for one hour with shaking, neutralized with NaOH, and counted in 10ml of Bray's liquid scintillation fluid (53). Background counts (30-35cpm) were subtracted from all values.

Growth of the organism:

For a number of subsequent experiments the growth of the organism, and the preparation of low speed supernatant fractions was as outlined below. *Tetrahymena pyriformis* GL were grown by inoculating 75ml of the defined medium of Elliott (54) in a 250ml flask with 5ml of log phase cells (72 hour). Cells were incubated for two days at 27°C in the dark with shaking. The cells were then transferred to 925ml of medium in a 2.8 liter Fernbach flask, and incubated for an additional three days. Cells were harvested by centrifuging in the IEC Model HN-S centrifuge, in pear-shaped oil centrifuge tubes, and washed twice with 0.08M Phosphate buffer, pH 7.4, resuspended in 20ml of 0.08M Phosphate buffer, pH 7.4, and disrupted by 75 strokes with a motor-driven Teflon Homogenizer. A low speed supernatant fraction was prepared by centrifuging the homogenate at 480 X g for 10 minutes at 4°C in a Sorvall RC-2 refrigerated centrifuge. The supernatant fraction was then removed and recentrifuged at 480 X g for an additional 10 minutes. This supernatant fraction was then used as the source of the low speed supernatant fraction. Variations in either the growth medium, growth conditions, or the method of preparing the homogenate, will be noted under the appropriate section.

Growth of cells on 1-methylguanosine:

Cells, which were growing on the defined medium of Elliott (54), were inoculated into a defined medium in which the sole source of purine was chromatographically pure 1-methylguanosine. Cells have been maintained on this medium with weekly transfers for over two years. After some initial problems with 1-MGR samples which were heavily contaminated with guanosine, all samples were checked for purity in Solvent H which separates guanosine from 1-MGR quite completely. Samples were also checked for trace contamination with guanosine by measurement of fluorescence at 350nm with excitation at 275nm at pH 11 using a Perkin-Elmer Fluorescence Spectrophotometer Model MPF-2A. Under these conditions guanine and guanosine are fluorescent while the 1-methyl derivative is not (55).

Isolation of nucleic acid purines:

Two liters of *Tetrahymena pyriformis* growing on defined medium containing 1-MGR as the sole purine source were harvested and washed as described above. The cells were suspended in ice cold 5% PCA and homogenized briefly. The macromolecular pellet was centrifuged and washed once with cold 5% PCA and then twice with cold 1N HCl. The pellet was then resuspended in 1N HCl and hydrolyzed at 90°C for 1 hour. This procedure liberated the purine bases from the nucleic acids, and the pyrimidine nucleotides from RNA (56). The hydrolysate was cooled, clarified by centrifugation, and lyophilized. The dried hydrolysate was then chromatographed on Whatman No. 1 paper by the descending technique in Solvent H. Ultraviolet spots were visualized with a Chromatovue. Since in this solvent, 1-methylguanine co-chromatographs with adenine, the adenine spot was cut out, eluted with 0.1N HCl, dried and rechromatographed in Solvent I. Again ultraviolet absorbing spots were visualized with a Chromatovue.

Synthesis of ^{14}C-methyl-1-methylguanosine:

^{14}C-methyl-1-methylguanosine was prepared by a modification of the procedure of Broom *et al.* (57). Methyl-^{14}C Iodide, 2.4mg, 0.25 mCi in a sealed tube were placed

in a dry ice-acetone bath for 30 minutes in order to liquify any gaseous ^{14}C-methyl iodide. The sealed tube was then opened and 10mg of guanosine and 6mg of K_2CO_3 were added. Then 100μl of dimethyl sulfoxide were added and the tube resealed. The reaction mixture was incubated at 25°C for 5 hours. After 5 hours the reaction mixture was again cooled on a dry ice-acetone bath and a water aspirator was attached. The reaction mixture was evaporated to dryness at room temperature. The residue was twice redissolved in 100% ethanol and redried, in order to be sure that all unreacted ^{14}C-methyl iodide had been removed. The residue was then redissolved in 100% ethanol and spotted on Whatman No. 3MM paper and chromatographed in Solvent H. The major radioactive spot, which co-chromatographed with 1-MGR, was cut out and eluted in water. The ultraviolet absorption spectrum of the eluted material was determined in a Beckman DB Spectrophotometer and a measured aliquot was taken for the determination of specific radioactivity. The purified ^{14}C-me-1- MGR was lyophilized and redissolved in 30ml of 0.08M Phosphate buffer, pH 7.4, 2.7 X 10^6cpm/ml, immediately before use.

Isolation and purification of macromolecular fractions (58) from cells grown on ^{14}C-me-1-MGR:

Tetrahymena pyriformis growing on 1 liter of defined medium containing 1-MGR as the sole source of purine were harvested and washed as described above. The cells were resuspended in 100ml of defined medium which contained no purine source to which 0.2ml of 100 X concentrated Antibiotic-Antimycotic mixture had been added. Approximately 15ml of the synthesized ^{14}C-me-1-MGR, 2.7 X 10^6cpm/ml, were added to the nutrient medium and the cells were grown for 18 hours at 27°C with shaking. Under these conditions cells would be expected to divide three times. The cells were harvested by centrifugation, washed, and suspended in ice cold 5% PCA. The suspension of cells in 5% PCA was homogenized briefly, and the macromolecules sedimented by centrifugation. The precipitate was washed with 1000 volumes ice cold 5% PCA and the acid soluble fraction was discarded.

Lipid Fraction: The macromolecular precipitate was extracted once with 50% and 70% ethanol, twice with 95% ethanol, 3X's with 100% ethanol, 3X's with ethanol-ether

(3:1), and 5X's with ether. All extractions were performed in the cold. All of the ethanol and ether extracts were combined and constitute the lipid fraction. This fraction was dried under reduced pressure and redissolved in 10ml of 6N HCl in methanol. This solution was then refluxed for 3 hours to liberate choline (59). The hydrolysate was dried under reduced pressure, redissolved in water, and redried several times to remove HCl, and finally redissolved in 5ml of water and filtered through a layer of glass wool-Celite at 4°C. The clear filtrate was lyophilized, dissolved in 100μl of water, suspended in 10ml of Bray's solution, and counted in a Packard Mark II Liquid Scintillation Counter.

RNA Fraction: The dried pellet, which contains RNA, DNA, and protein, was suspended in 2ml of 0.3N KOH and hydrolyzed at 37°C for 18 hours. This procedure releases the RNA nucleotides. The alkaline hydrolysate was acidified to pH 1.5 with cold, concentrated PCA. After centrifugation, the precipitate of $KClO_4$, DNA, and protein was washed with 0.1N PCA and the wash was added to the acidified hydrolysate. The pooled supernatant fraction was then neutralized with KOH, and $KClO_4$ was removed by alternately freezing, drying and centrifuging. The neutralized fraction was freeze-dried, dissolved in concentrated PCA, and hydrolyzed at 100°C for 1 hour in a sealed tube (Kontes No. K-896850). This procedure liberates the free bases. The hydrolysate was neutralized with KOH, freed of $KClO_4$ by alternately freezing, thawing and centrifuging, and finally lyophilized. The residue was dissolved in 0.1N HCl, spotted on Whatman No. 1 paper and chromatographed in Solvent J along with authentic base standards. The chromatogram was visualized for ultraviolet absorbing spots with a Chromatovue. Radioactivity was determined by cutting the experimental lanes into 2cm wide by 1cm long sections, eluting in 100μl of 0.1N HCl, neutralizing with NaOH, and counting in 10ml of Bray's solution.

DNA Fraction: The DNA and protein pellet was suspended in 10ml of 5% PCA and hydrolyzed at 100°C for 20 minutes. The hydrolysate was cooled, centrifuged, and the supernate decanted off. The pellet was again suspended in 5% PCA and heated at 100°C for 20 minutes, and the hydrolysates containing the nucleotides from DNA were combined. The insoluble protein was removed by centrifugation. The DNA bases

were obtained, chromatographed, and analyzed for radioactivity as described above for the RNA Fraction.

Protein: The remaining protein pellet, after hydrolysis of RNA and DNA, was suspended in 6N HCl, and hydrolyzed at 121°C, 20psi, in an evacuated, sealed tube (Kontes No. K-896850) for 15 hours to release the free amino acids. The hydrolysate was dried under vacuum, redissolved in water and redried several times, to remove excess HCl, and finally redissolved in water and chromatographed in Solvent K on Whatman No. 1 paper. The position of amino acid standards was determined by spraying with ninhydrin reagent (60). Radioactivity was determined by cutting the experimental lane into 2cm wide by 1cm long sections, eluting each section with 200μl of water and counting in 10ml of Bray's solution.

Metabolism of 1-MGR by *Tetrahymena pyriformis*:

Two liters of cells growing on 1-MGR as the sole source of purine were harvested and washed as described above. An homogenate was prepared as described above, and the low speed supernatant was divided equally among five 50ml side-arm flasks (Kontes No. K-882351). The remainder of the synthesized ^{14}C-me-1-MGR was dissolved in 15ml of 0.08M Phosphate buffer, pH 7.4, 2.7×10^6cpm/ml, and 10mg each of NADH and NADPH were added, and this solution divided equally among the five flasks, for a final volume of 3ml in each flask. A scintillation vial, containing a piece of filter paper soaked in 20% KOH, was attached to the side-arms, and the flasks were sealed. The flasks were incubated at 25°C for appropriate time intervals with intermittent shaking. At the end of the appropriate time period the reaction mixtures were cooled on ice and 4ml of 1N NaOH were added. At this point, the scintillation vial was removed and capped, and a fresh scintillation vial containing a piece of filter paper soaked in 20% KOH was attached to the side-arm. Formaldehyde present in the reaction mixture was converted to formic acid by the addition of 4ml of 0.1N iodine solution. The reaction mixture was adjusted to pH 4 with 3.3ml of 1N H_2SO_4, and excess iodine was reduced by the addition of 0.4ml of 0.1N thiosulfate solution. Then 0.5ml of 1N acetic acid was added and, finally 2.0ml of freshly

prepared 0.3M mercuric acetate were added, and the mixture boiled for 20 minutes. This procedure converts formic acid to CO_2 and the liberated CO_2 is trapped by KOH in the side-arm (61). The filter papers containing trapped CO_2 were suspended in 10ml of Bray's solution and the ^{14}C radioactivity determined.

From another 2 liters of cells, a cell homogenate was prepared as described above. The homogenate (8ml) was incubated with 0.08M PO_4 buffer, pH 7.4 (15ml), 1-MG (12mg), and NADPH (6mg). Another experimental containing 1-MGR (12mg), and a control to which no purine was added were also prepared. At appropriate time intervals, an aliquot of the reaction mixture was removed, diluted into 3ml of 0.08M Phosphate buffer, pH 7.4, and the absorbance at 340nm measured with a Beckman DB Spectrophotometer.

After three hours, the reaction was stopped by the addition of an equal volume of ice cold 10% PCA and the mixture was clarified by centrifugation. The supernatant fraction was neutralized with KOH, and freed of $KClO_4$ by alternately freezing, thawing and centrifuging. The neutralized supernatant was lyophilized and chromatographed in Solvent I. Ultraviolet absorbing spots were visualized with a Chromatovue, cut out and eluted in water, and the ultraviolet absorption spectrum determined in a Beckman DB Spectrophotometer.

Growth studies:

Tetrahymena pyriformis strain GL was maintained, for nine months prior to the initiation of these experiments, on the complete defined medium described by Elliott (54). Defined medium was prepared without purines and additions were made as noted in Figure 8. In another series of experiments, the additions were made as noted in Figure 9. Cells were grown in culture tubes containing 5ml of medium at 27°C with shaking. The Percent Transmission at 620nm was determined in a Spectronic 20 Colorimeter at 12 hour intervals. Actual cell counts showed that cell density was related to Percent Transmission during the course of the growth studies. The data presented are the result of third serial transfers, using the average of triplicate determinations.

Isotope dilution experiments:

Cells were grown in 500ml of defined medium containing guanosine (82.5μM) and twice the molar amounts of the compounds indicated in Table VIII, in separate 1 liter flasks, at 27°C with shaking. After two days ^{3}H-8-Guanosine, 0.3ml, 1mCi/ml, 15Ci/mmole, was added to each flask, and growth continued for an additional three days. The cells were harvested and washed as described above, and macromolecules were precipitated in the cold with a final concentration of 1N HCl. The precipitated fraction was washed twice with 10ml of 1N HCl, resuspended in 2ml of 1N HCl, and hydrolyzed at 90°C for 1 hour. This procedure releases the purine bases from nucleic acids (56). The hydrolysate was chilled and unhydrolyzed material was removed by centrifugation. The hydrolysate was then dried, redissolved in 100μl of 0.1N HCl and spotted on Whatman No. 1 paper and chromatographed in Solvent H. Ultraviolet absorbing spots were visualized with a Chromatovue, and the areas corresponding to guanine and adenine were cut out and eluted in 0.5ml of 0.1N HCl, and the ultraviolet absorption spectrum of each compound was determined. The samples were then neutralized with NaOH and counted in 15ml of Bray's solution. The specific radioactivity of the guanine and adenine from each experimental flask was calculated from this data. Additional experiments were performed in which the source of the radioisotope was again ^{3}H-8-Guanosine, but in which the defined medium contained the free base guanine (82.5μM) instead of guanosine as the purine source as indicated in Table IX. These experiments were analyzed as described above.

The Acid-Soluble Pool:

Cells were grown in one liter of defined medium which contained guanosine (82.5μM) as the sole source of purine, in a 2.8 liter Fernbach flask as described above. ^{3}H-8-Guanosine, 0.5ml, 1mCi/ml, 15Ci/mmole, was added 30 minutes prior to harvesting and washing as described above. The washed cells were suspended in ice cold 5% PCA, and homogenized briefly. The macromolecular pellets were removed by centrifugation, washed once with cold 5% PCA and the acid soluble fractions were combined. The PCA soluble fraction was neutralized with KOH and the $KClO_4$ removed by alternately freezing, thawing and centrifuging. The acid-soluble fraction

was adjusted to 0.5N HCl and applied to a Dowex-50 column (0.9 X 30cm). The Dowex-50, 200-400 mesh X8, was prepared by washing 2X's with 5N HCl, water, 5N NaOH, water, 5N HCl, water, and 0.5N HCl. The column was developed with 0.5N HCl at 4°C, and 3.3ml fractions were collected. Ultraviolet absorption at 254nm was monitored with an LKB Uvicord I with attached recorder. From each fraction 5μl was dissolved in 10ml of Bray's solution and ^{3}H radioactivity determined.

Conversion of GMP to XMP:

Two liters of *Tetrahymena pyriformis* were grown on defined medium containing guanosine (82.5μM) as the sole source of purine. Cells were harvested, washed, homogenized, and a low speed supernatant fraction prepared as described above. The low speed supernatant fraction was brought to a volume of 15ml with 0.08M Phosphate buffer, pH 7.4, and 10μCi of ^{14}C-8-GMP 61mCi/mmol, were added. At appropriate time intervals, aliquots were removed and the reaction was stopped by the addition of ice cold 4N HCl to a final concentration of 0.5N HCl. The mixture was frozen, thawed, and clarified by centrifugation. The supernatant was then applied to a Dowex-50 column, prepared as described above, and eluted at 4°C with 0.5N HCl as described above. A 5μl aliquot from each fraction was dissolved in 10ml of Bray's solution, and the ^{14}C radioactivity determined.

The radioactive material eluted from the Dowex-50 column in tubes 7-11, which co-chromatographs with XMP, was lyophilized. The dried material was redissolved in 1N HCl and an aliquot taken for chromatography in Solvents K, I, and L. The remaining material was hydrolyzed at 90°C for 1 hour, and an aliquot chromatographed in Solvent K. The remaining hydrolysate was lyophilized repeatedly to remove excess HCl and then dissolved in 0.08M Phosphate buffer, pH 7.4. One unit of xanthine oxidase (Xanthine:oxygen oxidoreductase) Grade 1 from buttermilk was added and the reaction mixture incubated at 25°C for 1 hour. The reaction was stopped by the addition of cold 4N HCl to a final concentration of 1N HCl, and the mixture clarified by centrifugation. The supernatant was then lyophilized, and the dry residue was redissolved in 0.1N HCl and chromatographed on Whatman No. 1 paper in Solvent K. All Chromatograms were dried, and ultraviolet absorbing spots visualized

with a Chromatovue in order to determine the location of the standards. Where radioactive fractions had been chromatographed, 2cm wide lanes were cut out and divided into 1cm long sections, and the position of radioactivity determined by eluting the paper sections in 0.1N HCl, neutralizing with NaOH, dissolving in 10ml of Bray's solution, and determining the ^{14}C radioactivity.

RESULTS

Isolation of tRNA with ^{14}C-labeled methyl groups:

Initial attempts to isolate tRNA from *Tetrahymena pyriformis* were complicated by two problems, (a) the co-isolation of glycogen with the RNA fraction, (b) the presence of very active ribonuclease activity. In order to solve the first problem the phenol-extracted tRNA preparation was applied to a DEAE-cellulose column. Glycogen was then eluted with buffer, residual protein with 0.3M NaCl, and tRNA with 0.7M NaCl, Figure 1 (62). Any large molecular weight RNA in the post-microsomal supernatant fraction remains bound to the column. The second problem was solved by including the ribonuclease inhibitor, 8-hydroxyquinoline, in the isolation buffers (63).

Isolation, identification and quantitation of methylated purines of tRNA:

Figure 2 shows the separation of the components of the tRNA hydrolysate on a Dowex-50 column. Under the conditions of mild acid hydrolysis the pyrimidines are released as the nucleotides, while the purines are released as the free bases. The pyrimidine nucleotides are eluted off the column first, followed by guanine and the methylated guanines (F1-F3), then followed by adenine and the methylated adenines (F4-F7). The distribution of radioactivity in the various Fractions is as indicated.

The methylated bases were identified on the basis of their co-chromatography with authentic standards on the same chromatogram. The R_f values for the standards in solvent systems A-E are given in Table I. Chromatography in Solvent F was for 4 days, with the solvent allowed to drip off the edge of the paper. The distance migrated by the standards in solvent F after 4 days is also given in Table I. Figure 3 shows drawings of some typical chromatograms by which the methylated purines were identified. The peaks of radioactivity can be seen to be clearly associated with specific methylated purines.

Figure 1. DEAE-cellulose Chromatography of tRNA from *Tetrahymena pyriformis.*

The phenol-extracted, ethanol precipitated, dried tRNA preparation was chromatographed on DEAE-cellulose as described under Methods. Separation of tRNA from glycogen and protein was achieved by eluting with buffers of different ionic strengths as noted.

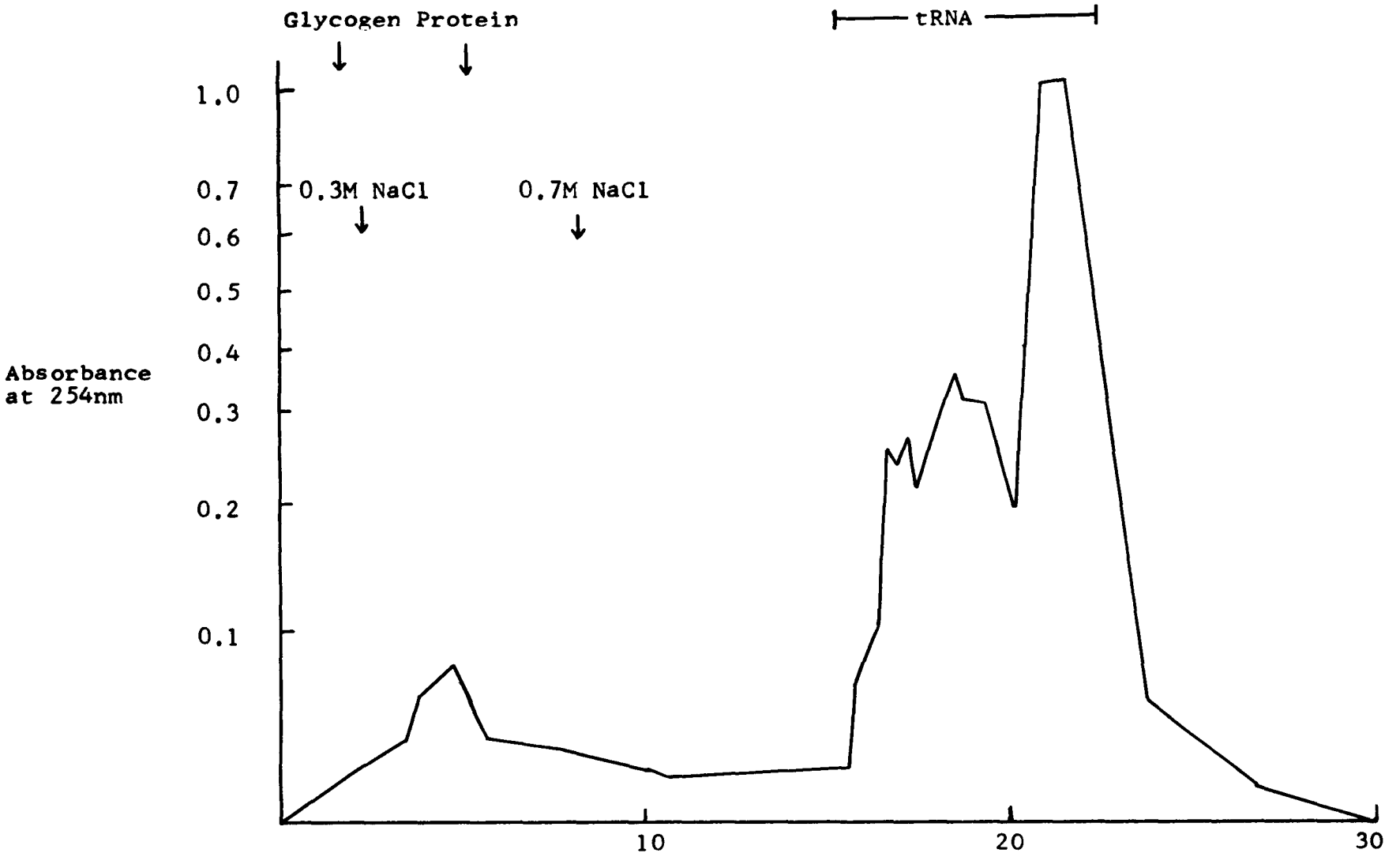
Glycogen Protein
tRNA
1.0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.3M NaCl
0.7M NaCl
Absorbance at 254nm
10
20
30
Tube Number

Figure 1

Figure 2. Dowex Column Chromatography of tRNA Hydrolysate.

The tRNA containing tubes obtained from the DEAE-cellulose column were combined and lyophilized. The dried tRNA was dissolved in 1N HCl and hydrolyzed in a boiling water bath for 1 hour. The hydrolysate was applied to a 1 X 25cm Dowex-50 column and eluted first with 20ml of 1N HCl followed by 580ml of 2N HCl. Fractions of 10ml each were collected, and aliquots from each tube were counted in Packard Permafluor Liquid Scintillation Fluid, mixture B. Tubes were combined into Fractions 1-7 as indicated, for paper chromatographic analysis. •—• A_{254}; •--• cpm ^{14}C.

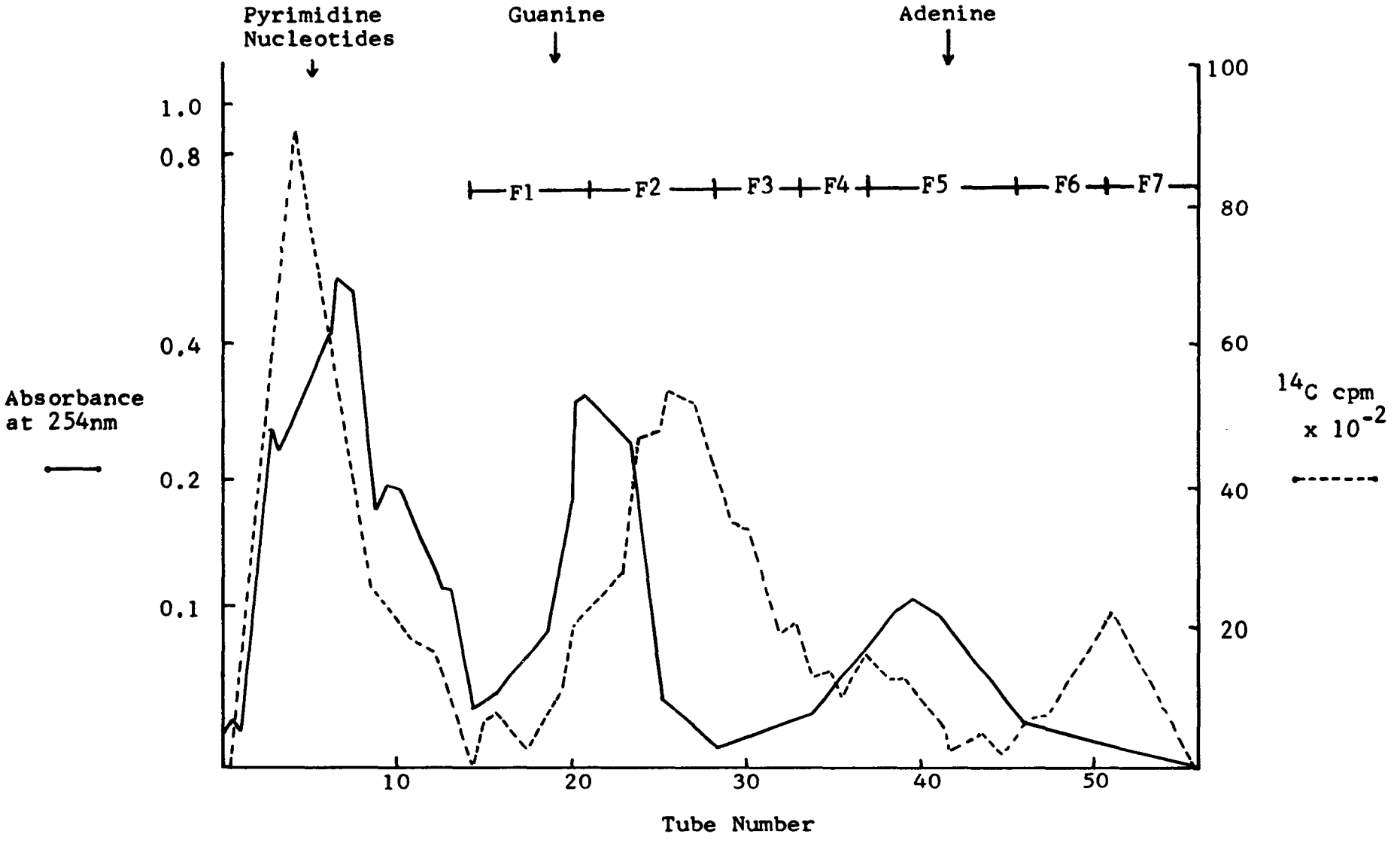
Pyrimidine Nucleotides
Guanine
Adenine
F1
F2
F3
F4
F5
F6
F7
1.0
0.8
0.4
0.2
0.1
100
80
60
40
20
Absorbance at 254nm
14C cpm x 10-2
10
20
30
40
50
Tube Number

Figure 2

Table I. Migration of Standards by Descending Chromatography.

Migration of Standards by Descending Chromatography. Each standard (5μg) was spotted on Whatman No. 1 paper, and the position of migration determined by ultraviolet illumination.

Compound	R_f Solvent A	B	C	D	E	Distance migrated in solvent F after 4 days (cm)
G	.18	.09	.14	.51	.45	15.8
1-MG	.17	.12	.18	.53	.48	14.2
N^2-MG	.35	.15	.27	.60	.57	34.9
N^2-DMG	.39	.15	.20	.69	.64	27.9
7-MG	.23	.09	.07	.65	.68	21.0
A	.28	.39	.38	.48	.45	22.8
1-MA	.37	.07	.07	.64	.57	23.5
2-MA	.43	.13	.17	.58	.73	31.1
N^6-MA	.53	.15	.19	.60	.73	36.6
N^6-DMA	.44	.16	.25	.66	.76	38.3

Figure 3. Drawings of Typical Chromatograms.

Shown are drawings of typical chromatograms indicating the method by which the radioactive methylated bases were identified. Chromatograms were cut into 1.5 X 4cm sections, eluted with 0.01N HCl, and counted in Bray's Solution. The position of migration of the standards on the same chromatogram was visualized with a Chromatovue, and is as indicated on the Figure.

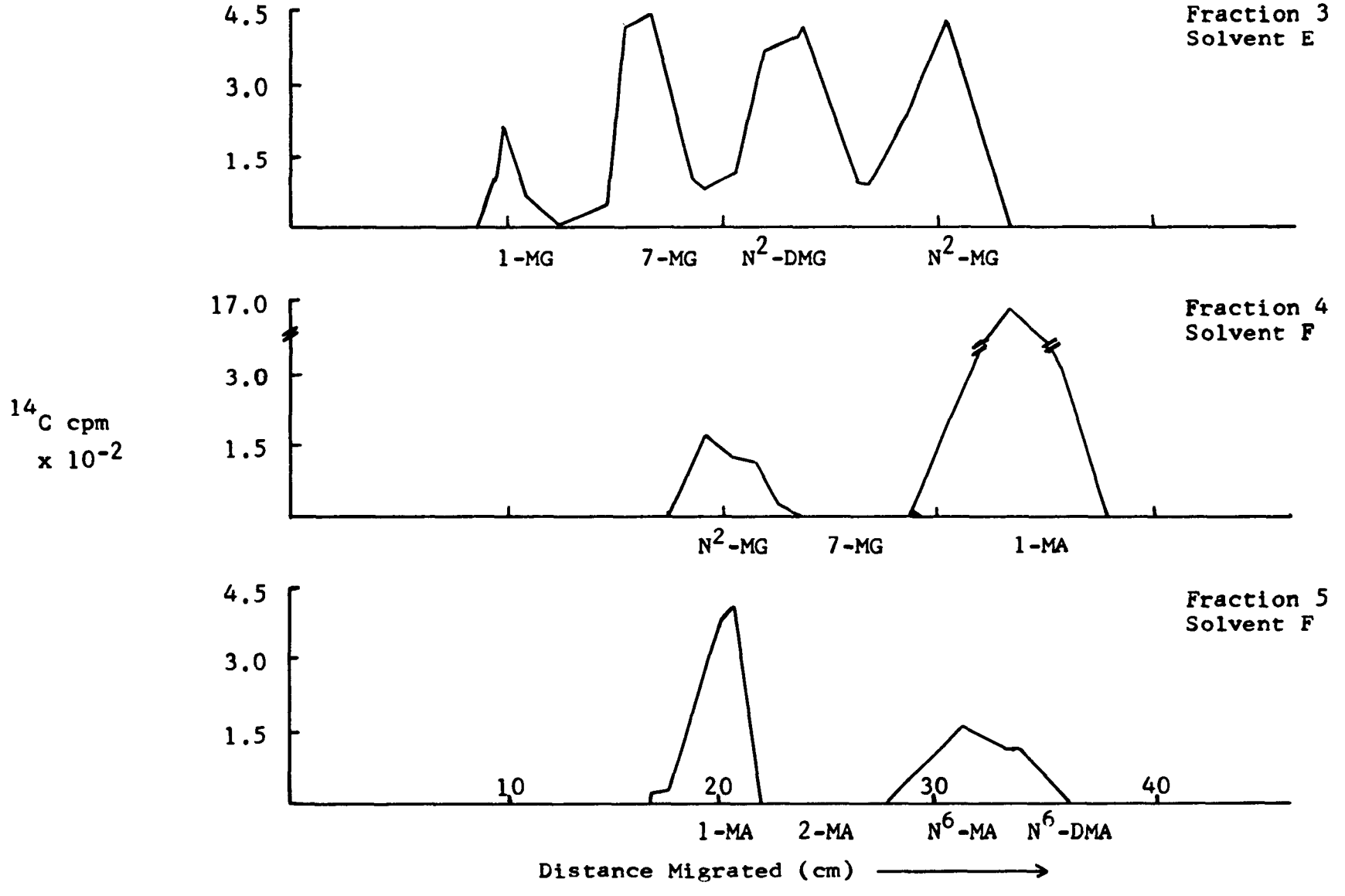
Fraction 3
Solvent E
4.5
3.0
1.5
1-MG
7-MG
N2-DMG
N2-MG
Fraction 4
Solvent F
17.0
3.0
1.5
N2-MG
7-MG
1-MA
14C cpm
x 10-2
Fraction 5
Solvent F
4.5
3.0
1.5
10
20
30
40
1-MA
2-MA
N6-MA
N6-DMA
Distance Migrated (cm)

Figure 3

Preliminary experiments had shown that N^2-DMG was present in the tRNA of *T. pyriformis*. Upon isolation it was found to be present in sufficient quantities, so that its concentration could be determined spectrophotometrically. In order to accomplish this, Fraction 2 was chromatographed in solvent F, and the spot containing N^2-DMG was cut out, eluted in 0.1N HCl, and its ultraviolet absorption spectrum determined. The spectral data obtained in acid is given in Table II along with the published data for this compound (64). The total number of cpm in N^2-DMG was determined, and on the basis of its calculated specific activity the concentration of the other methylated bases was determined. The amount of each methylated purine found in the tRNA of *T. Pyriformis* is presented in Table III.

Growth of cells on 1-MGR:

While attempting to develop a procedure for measuring the conversion of 1-MGR to guanosine, by taking advantage of the fact that 1-MGR is not fluorescent, it was noted that some commercial samples of the methylated base had a very high fluorescence. The fluorescence spectrum matched that of authentic guanosine, and subsequent paper chomatography of the samples in Solvent H turned up the fact that they were grossly contaminated with guanosine. It was finally determined that only Sigma Chemical Company preparations were authentic and guanosine free. Throughout this work only the Sigma product was used, and each new batch was analyzed for purity.

Tetrahymena pyriformis have been maintained on defined medium in which the sole purine source is 1-MGR for a period of over two years. In order to determine if the organism was demethylating the base before incorporation into nucleic acid, or if the organism incorporated the methylated base directly into nucleic acid, the nucleic acid purines were isolated from cells grown on defined medium with 1-MGR as the sole source of purine. As can be seen from Figure 4, when the nucleic acid hydrolysate is chromatographed in Solvent H, one finds three ultraviolet absorbing spots. The spot closest to the origin contains the pyrimidine nucleotides. The next spot co-chromatographs with and has the ultraviolet absorption spectrum of guanine. The fastest moving spot co-chromatographs with and has the ultraviolet absorption spectrum

Table II. Spectral Data for N^2-DMG and for Compound Identified as N^2-DMG.

Spectral Data for N^2-DMG and for Compound Identified as N^2-DMG. Fraction 2 (see Figure 1) was chromatographed in Solvent F for 4 days on Whatman No. 1 paper. The ultraviolet spot containing N^2-DMG was cut out, eluted in 0.01N HCl, and its spectra determined in a Beckman DB Spectrophotometer.

				Spectral Ratios/260					
	pH	λ_{max}^{nm}	λ_{min}^{nm}	230	240	250	270	280	290
Published Data	1	256	233	.36	.48	.92	.52	.37	.39
Experimental	1	256	233	.35	.47	.93	.52	.36	.35

Table III. The Amounts of Methylated Purines in the tRNA of *T. pyriformis.*

The Amounts of the Methylated Purines in the tRNA of *T. pyriformis.* Conditions of isolation, and methods of identification and quantitation are given in the text.

Compound	moles/100moles Guanine	moles/100moles Adenine
1-MG	0.07	0.12
N^2-MG	0.10	0.15
N^2-DMG	0.14	0.22
7-MG	0.09	0.14
1-MA	0.23	0.36
N^6-MA	0.013	0.02
N^6-DMA	0.003	0.004

Figure 4. Demonstration of the Lack of 1-MG in the Nucleic Acid of Cells Grown on 1-MGR as the Sole Purine Source.

The nucleic acids from *Tetrahymena pyriformis* growing on 1-MGR as the sole purine source, were hydrolyzed to release the free purine bases and pyrimidine nucleotides. The hydrolysate was chromatographed in Solvent H, and the ultraviolet absorbing material co-chromatographing with adenine was eluted and re-chromatographed in Solvent I. See Methods for details.

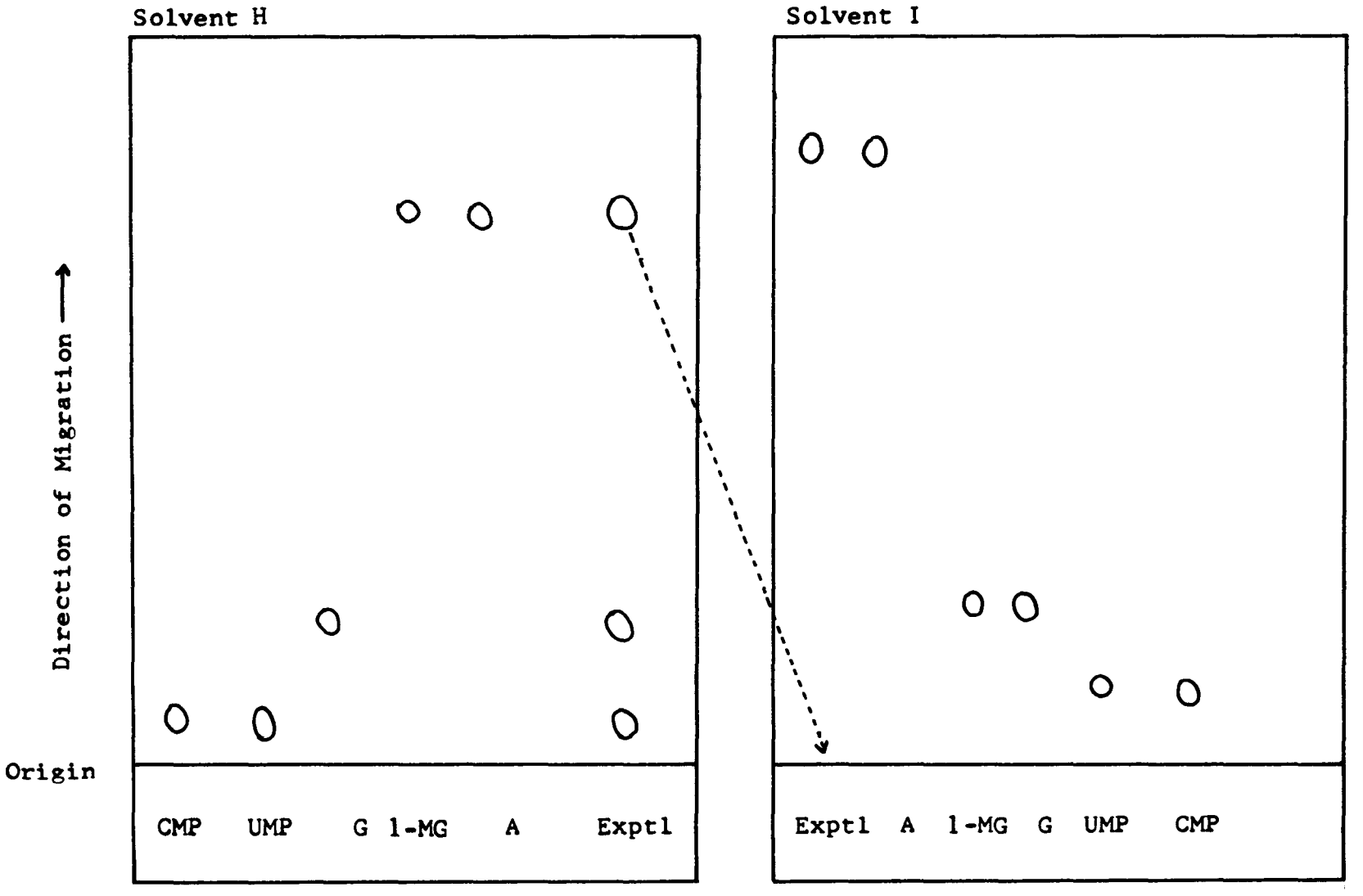
Solvent H
Solvent I
Direction of Migration
Origin
CMP
UMP
G
1-MG
A
Exptl
Exptl
A
1-MG
G
UMP
CMP

Figure 4

of adenine. Since in this solvent adenine and 1-MG have the same R_f, one could not be sure that the adenine was not masking a substantial amount of 1-MG. Therefore, the material co-chromatographing with adenine was rechromatographed in Solvent I which separates 1-MG from adenine quite completely. As can be seen in Figure 4, no 1-MG is detected. These results demonstrate that 1-MG does not form a substantial amount of the nucleic acid purines in cells growing on 1-MGR as the sole source of purine.

Synthesis of ^{14}C-me-1-MGR:

In order to determine the fate of the methyl group of 1-MGR, methyl labeled 1-MGR was prepared. When the reaction mixture was chromatographed, two radioactive spots were found, Figure 5. The fastest moving material co-chromatographed with and had the ultraviolet absorption spectrum of 1-MGR. The slower moving spot co-chromatographs with 1-MG and overlaps with unreacted guanosine, and this material also has a guanosine like spectrum. This material is probably 1-MG generated by the repeated cycle of redissolving and drying during the preparation of the sample. The specific radioactivities of these compounds were determined, and are shown in Table IV.

Metabolism of 1-MGR by *Tetrahymena pyriformis*:

In order to follow the fate of the methyl group of 1-MGR, *Tetrahymena pyriformis* were grown on a nutritive medium containing ^{14}C-me-1-MGR as the sole source of purine. When each macromolecular fraction was isolated, purified, and hydrolyzed it was found that there was no radioactivity in the nucleic acid bases of RNA or DNA, or in the amino acids of protein. Only the lipid fraction was radioactive, with the radioactivity probably in the choline as shown in Table V.

The ability of *Tetrahymena pyriformis* to demethylate 1-MGR was next examined in a cell-free homogenate. As shown in Table VI, the amount of $^{14}CO_2$ liberated from ^{14}C-1-MGR increases with time of incubation. Furthermore, when any formaldehyde or formate present in the homogenate is treated to liberate the carbon as CO_2, additional radioactive CO_2 is recovered, which also increases with time, Table

Figure 5. Purification of ^{14}C-me-1-MGR.

^{14}C-me-1-MGR was synthesized from guanosine and ^{14}C-methyl iodide and chromatographed in Solvent G as described under Methods. Radioactivity was determined by cutting a 2mm strip into 1cm sections, eluting in 200μl of water and counting in 10ml of Bray's solution.

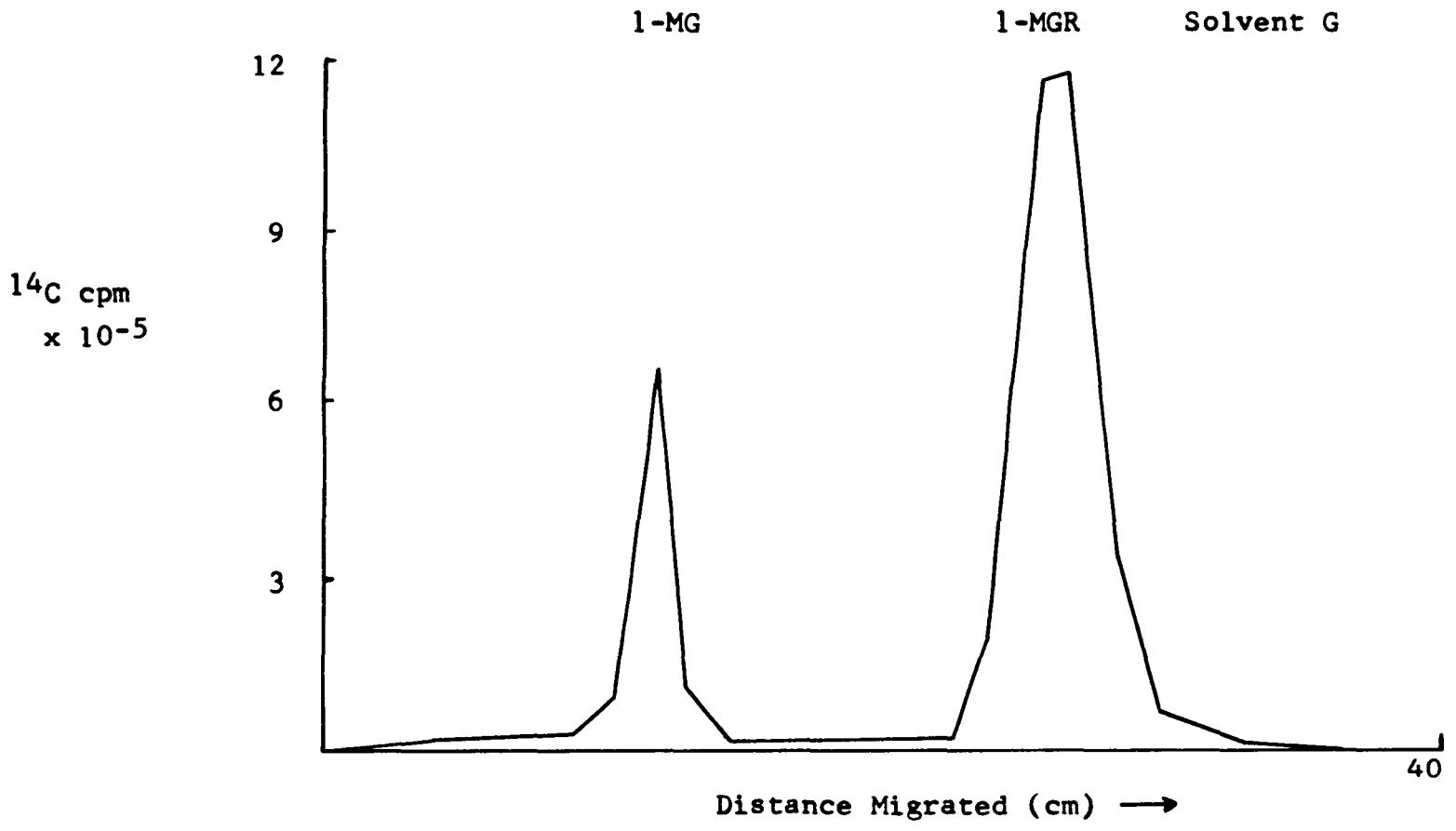
1-MG
1-MGR
Solvent G
12
9
6
3
^{14}C cpm
x 10^{-5}
40
Distance Migrated (cm) ⟶

Figure 5

Table IV. Specific Radioactivities of Methylated Guanines.

^{14}C-me-1-MGR was synthesized from guanosine and ^{14}C-methyl iodide as described under Methods. The reaction mixture was chromatographed in Solvent G, (see Figure 5) and the specific radioactivities of the radioactive peaks were determined as described under Methods.

Radioactive Compound	Specific Radioactivity
^{14}C-me-1-methylguanosine	5×10^8 cpm/mole
"^{14}C-me-1-methylguanine"	1.4×10^7 cpm/mole

Table V. Distribution of Radioactivity from ^{14}C-me-1-MGR in Macromolecular Fractions.

Tetrahymena pyriformis was grown in the presence of ^{14}C-me-1-MGR. The macromolecular fractions were prepared, and their components analyzed, as described under Methods. The radioactivity incorporated into each fraction was determined.

Fraction	Component	^{14}C cpm
RNA	Adenine	0
	Guanine	0
	Uracil	0
	Cytosine	0
DNA	Adenine	0
	Guanine	0
	Thymine	0
	Cytosine	0
Protein	Serine	0
	Methionine	0
	other amino acids	0
Lipids	"Choline"	856

Table VI. Liberation of $^{14}CO_2$ from ^{14}C-me-1-MGR by *Tetrahymena pyriformis*.

A cell homogenate was incubated with ^{14}C-me-1-MGR for various time periods, as described under Methods. Liberated $^{14}CO_2$ was trapped with KOH, and counted in 10ml of Bray's solution.

Time (minutes)	$^{14}CO_2$ cpm
0	53
30	119
60	526
120	1,125
180	1,353

VII. These results demonstrate that *Tetrahymena* is able to demethylate 1-MGR to produce a one carbon fragment which can be incorporated into choline or liberated as CO_2.

When a cell-free homogenate was incubated in the presence of 1-MGR, or 1-MG, and the co-factor NADPH, a significant decrease in absorption at 340nm was noted with time, Figure 6, indicating a disappearance of NADPH. The oxidation of NADPH was correlated with the demethylation of 1-MG, and the product, guanine, was recovered chromatographically, Figure 7.

Growth studies:

As can be seen from Figure 8, when *Tetrahymena pyriformis* are grown on a defined medium containing guanine as the sole source of purine, the cells show an initial lag in growth followed by logarithmic growth up to about the fifth day. When equimolar amounts of either XMP, IMP, or adenine are added along with the guanine, the initial lag is abolished, and the cells grow to higher final densities. Adenine shows the greatest sparing effect on the guanine requirement, followed by IMP and XMP. In order to be sure that XMP was indeed sparing the guanine requirement, cells were grown on defined medium containing a suboptimal guanine concentration (16.5μM). Tubes were set up with increasing amounts of XMP and their growth rates were monitored. The results are presented in Figure 9, and show that with increasing amounts of XMP, the growth of the organism is improved, thus indicating a true sparing of the guanine requirement by XMP.

Isotope dilution experiments:

In order to determine what compounds are intermediates in the conversion of GMP to AMP, the following experiment was devised. If *Tetrahymena pyriformis,* which has an absolute growth requirement for guanine, is grown on a defined medium containing guanine as the sole source of purine, then the cells must be converting guanine to adenine. If the guanine pool is radioactively labeled, then after a number of generations of growth, the specific radioactivity of the nucleic acid guanine and adenine should be approximately equal. If a non-radioactive compound, which is an

Table VII. Liberation of $^{14}CO_2$ from Putative Formaldehyde and Putative Formate.

The reaction mixtures from Table VI were treated in order to convert any formaldehyde or formate to $^{14}CO_2$, as described under Methods. This $^{14}CO_2$ was trapped with KOH and counted in 10ml of Bray's solution.

Time (minutes)	$^{14}CO_2$ cpm
0	906
30	3,074
60	3,505
120	4,401
180	7,417

Figure 6. Decrease in Absorbance at 340nm in the Presence of either 1-MG or 1-MGR.

A cell homogenate was incubated in the presence of NADPH and 1-MG (O—O), or 1-MGR (□—□), or with no purine (X—X). For each time period appropriate dilutions were made and the absorbance at 340nm determined.

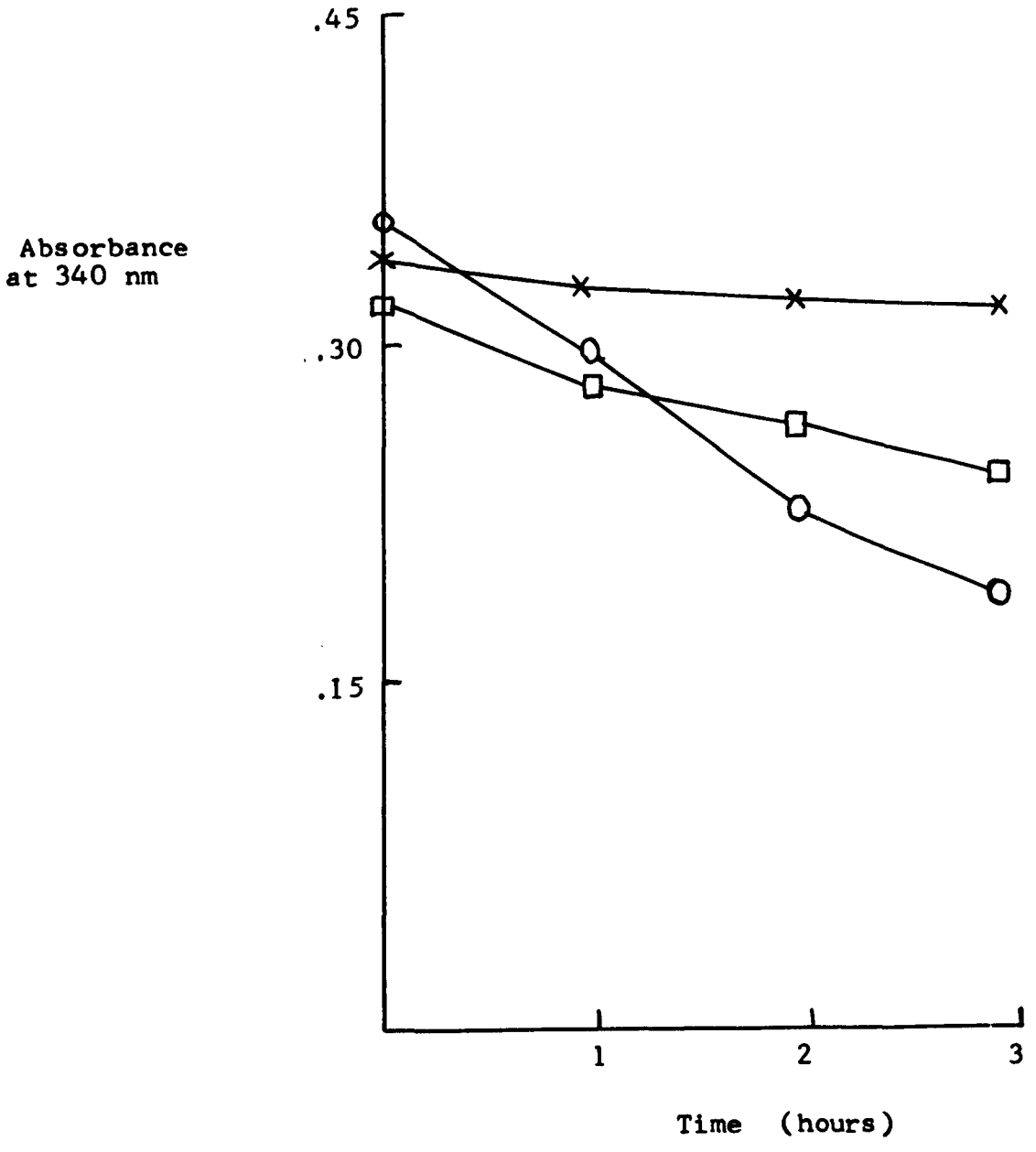

Figure 6

Figure 7. Recovery of Guanine after Incubation of 1-MG with Cell Homogenate in Presence of NADPH.

A cell-free homogenate of *Tetrahymena pyriformis* was incubated for 3 hours with 1-MG and NADPH. After 3 hours the acid-soluble supernatant fraction was chromatographed in Solvent H, and the ultraviolet absorbing spots were visualized with a Chromatovue. An ultraviolet absorbing spot co-chromatographing with guanine was cut out, eluted, and its ultraviolet absorption spectrum determined.

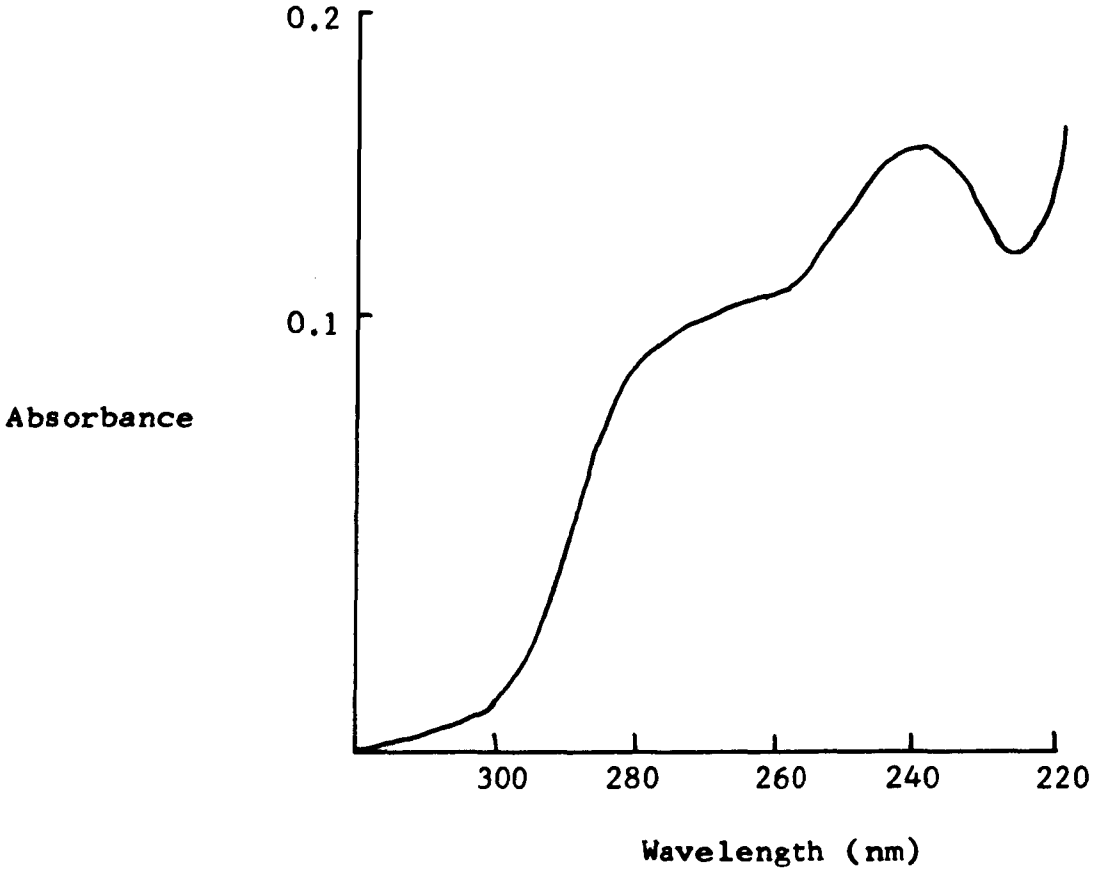

Figure 7

Figure 8. Growth Studies with *Tetrahymena pyriformis* in the Presence of Suspected Intermediates.

Tetrahymena pyriformis were maintained on a defined medium containing guanine, 82.5μM, as the sole source of purine. Adenine, IMP, and XMP were added at a final concentration of 175μM to separate series of tubes, and growth was monitored as described under Methods. X—X No additions, □—□ XMP, 0—0 IMP, Δ—Δ Adenine.

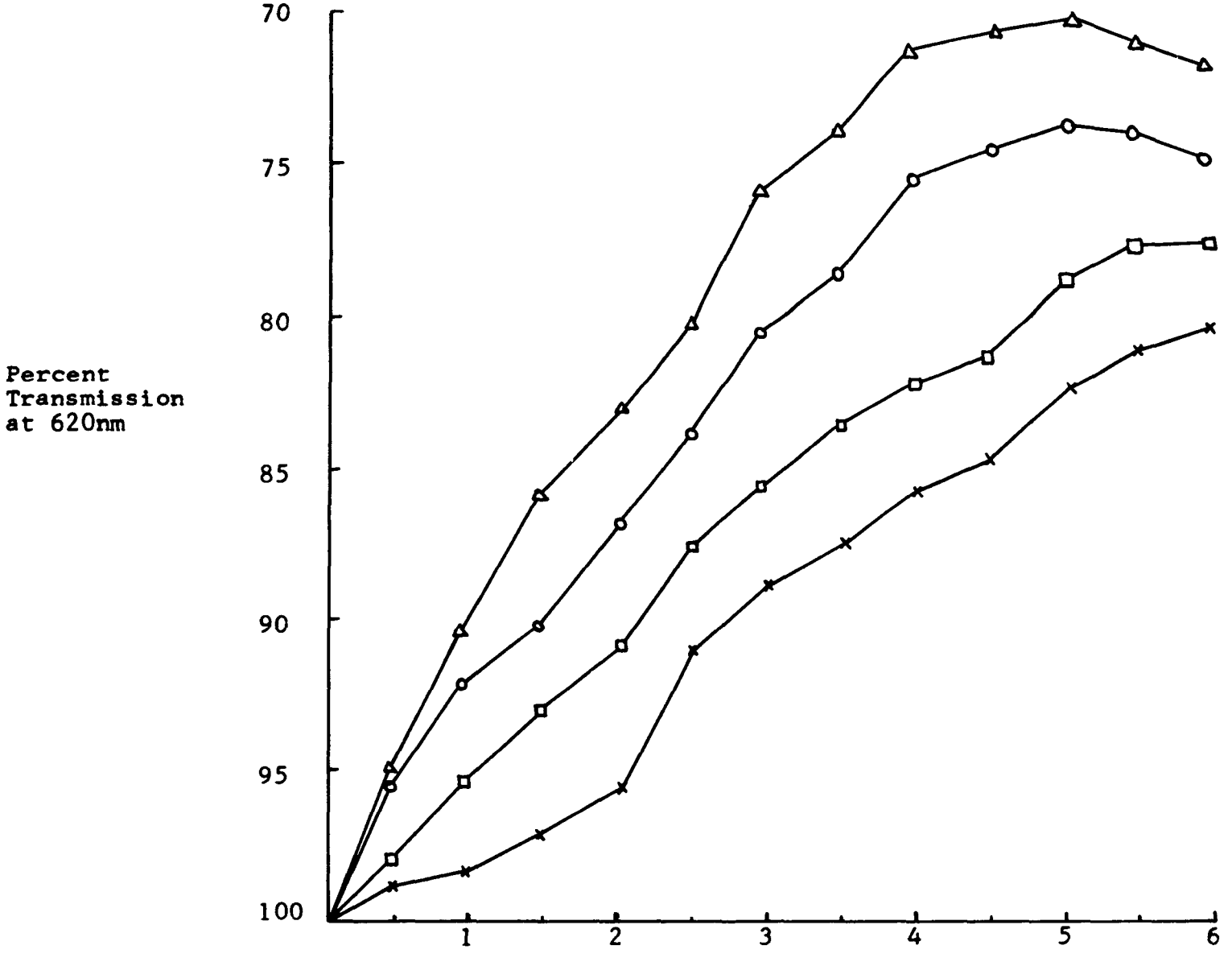
Percent
Transmission
at 620nm
70
75
80
85
90
95
100
1
2
3
4
5
6
Time (days)

Figure 8

Figure 9. Growth Studies with *Tetrahymena pyriformis* in the Presence of Increasing Amounts of XMP.

Tetrahymena pyriformis were maintained on a defined medium containing a sub-optimal amount of guanine, 16.5μM, as the sole source of purine. Increasing amounts of XMP were added to separate tubes, and growth was monitored as described under Methods. X—X No additions, □—□ 14μM XMP, 0—0 27μM XMP, Δ—Δ 67μM XMP.

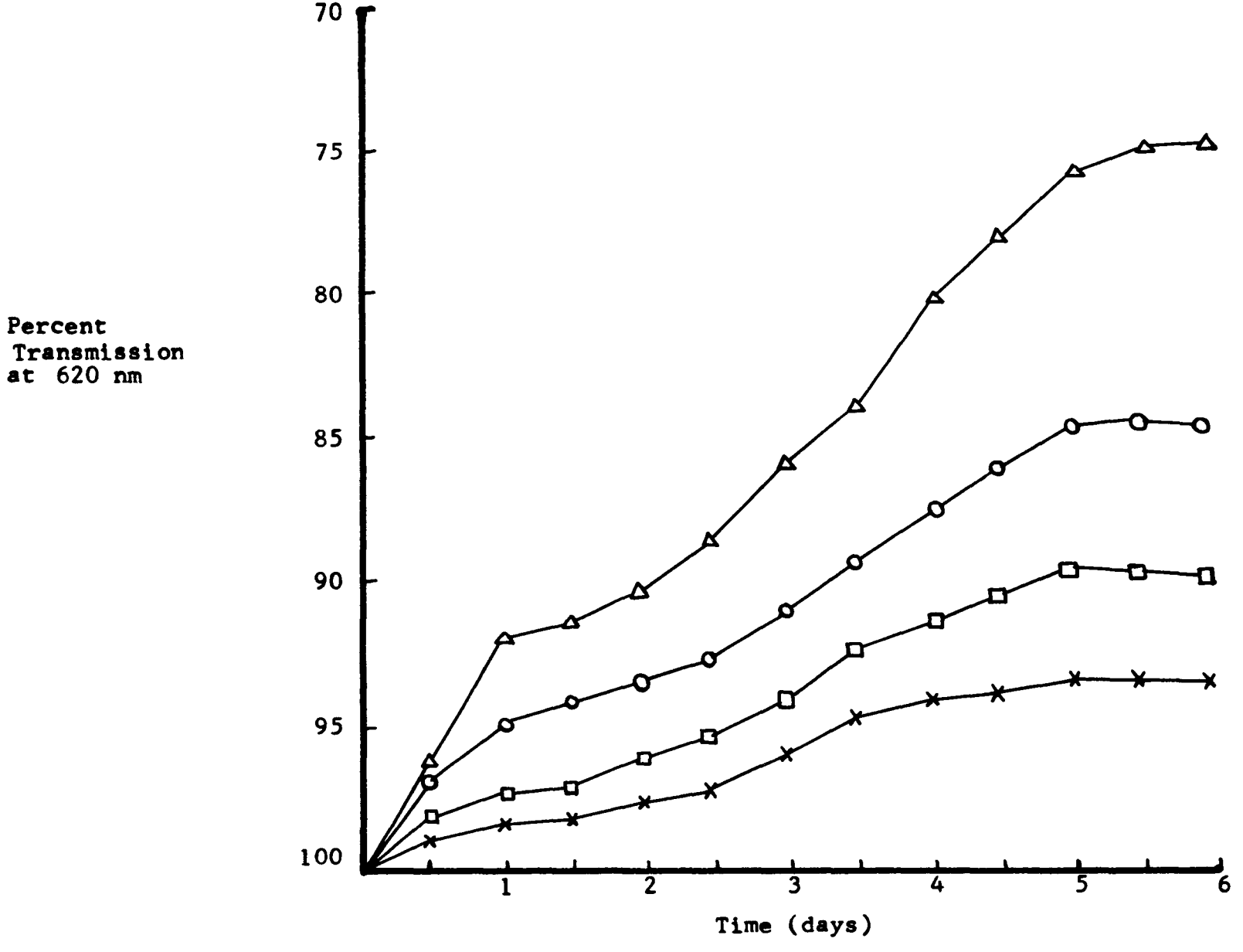
Percent
Transmission
at 620 nm
70
75
80
85
90
95
100
1
2
3
4
5
6
Time (days)

Figure 9

intermediate in the conversion, is added to the medium, then the cells would use the non-radioactive intermediate to make nucleic acid adenine, and thus dilute the label getting into the nucleic acid adenine. As a result the specific radioactivity of the nucleic acid guanine would exceed that of the nucleic acid adenine. A number of compounds were tested for their ability to dilute the adenine label. To cells growing in the presence of ^{3}H-8-guanosine, wherein guanosine (82.7μM) was the sole source of purine, twice molar amounts of each of the following unlabeled compounds were added: adenine, AMP, inosine, IMP, and XMP. The nucleic acid adenine and guanine were isolated and their respective specific radioactivities determined. The specific radioactivities, and the ratio of these specific radioactivities are shown in Table VIII. It can be seen that each of the compounds tested brings about a decrease in the specific radioactivity in the nucleic acid adenine, indicating that the unlabeled compounds are capable of being converted to nucleic acid adenine. Another series of experiments was performed in which the cells were grown on defined medium in which the free base guanine was the purine source, while the radioactive label was supplied as ^{3}H-8-guanosine. The results of this series of experiments are presented in Table IX, and the implications of these results are discussed below.

Acid-Soluble Pool:

From the results described above, it seemed likely that XMP and IMP were intermediates in the conversion of GMP to AMP. If these were indeed intermediates, it seemed likely that after a short pulse of ^{3}H-8-guanosine these compounds might be found labeled in the acid-soluble pool. A number of methods for separating the components of the acid-soluble pool were tested, and it was found that Dowex-50 column chromatography using 0.5N HCl as the eluting agent was the most acceptable system. Under these conditions, all the compounds of interest are cleanly separated from each other. Figure 10 shows the ultraviolet absorption, and the radioactivity profile obtained when the acid-soluble pool is isolated from cells grown on defined medium containing guanosine as the sole source of purine, after a 30 minute exposure to ^{3}H-8-guanosine, and chromatographed on Dowex-50. The position of elution of the standards was obtained by adding 200μg of each standard as unlabeled carrier to the

Table VIII. Specific Radioactivities of Nucleic Acid Adenine and Guanine after Growth in the Presence of Guanosine and ^{3}H-8-Guanosine, and Various Unlabeled Compounds.

Cells were grown on a defined medium containing guanosine as the sole source of purine, and ^{3}H-8-guanosine. Various compounds, proposed as intermediates in the conversion of GMP to AMP, along with appropriate controls, were added. The specific radioactivity in the nucleic acid adenine and guanine was determined as described under Methods.

Additions	Specific Radioactivities cpm X 10^{-3}/μmole Adenine	Guanine	Adenine/Guanine
None	8.19	11.37	0.72
Adenine	0.75	12.16	0.06
AMP	1.45	13.28	0.11
Inosine	1.75	16.40	0.11
IMP	1.72	12.63	0.14
XMP	6.85	15.00	0.46

Table IX. Specific Radioactivities of Nucleic Acid Adenine and Guanine after Growth in the Presence of Guanine and ^{3}H-8-Guanosine, and Various Unlabeled Compounds.

Cells were grown on a defined medium containing guanine as the sole source of purine, and ^{3}H-8-guanosine. Various compounds, proposed as intermediates in the conversion of GMP to AMP, along with appropriate controls, were added. The specific radioactivity of the nucleic acid adenine and guanine was determined as described under Methods.

Additions	Specific Radioactivities cpm X 10^{-3}/μmole Adenine	Guanine	Adenine/Guanine
None	1.01	0.96	1.05
Adenine	0.02	1.54	0.01
AMP	0.01	0.04	0.23
Inosine	0.02	0.11	0.17
IMP	0.10	0.21	0.48
XMP	0.05	0.14	0.36

Figure 10. Analysis of the Acid-Soluble Pool of *Tetrahymena pyriformis.*

The components of the Acid-Soluble Pool of *Tetrahymena pyriformis,* after a 30 minute labeling period with ^{3}H-8-guanosine, were analyzed for radioactivity on a Dowex-50 column as described under Methods. The position of elution of the standards was determined by adding 200μg of each standard to the column as unlabeled carrier, and is as indicated. •—• Absorption at 254nm, •- -• ^{3}H cpm.

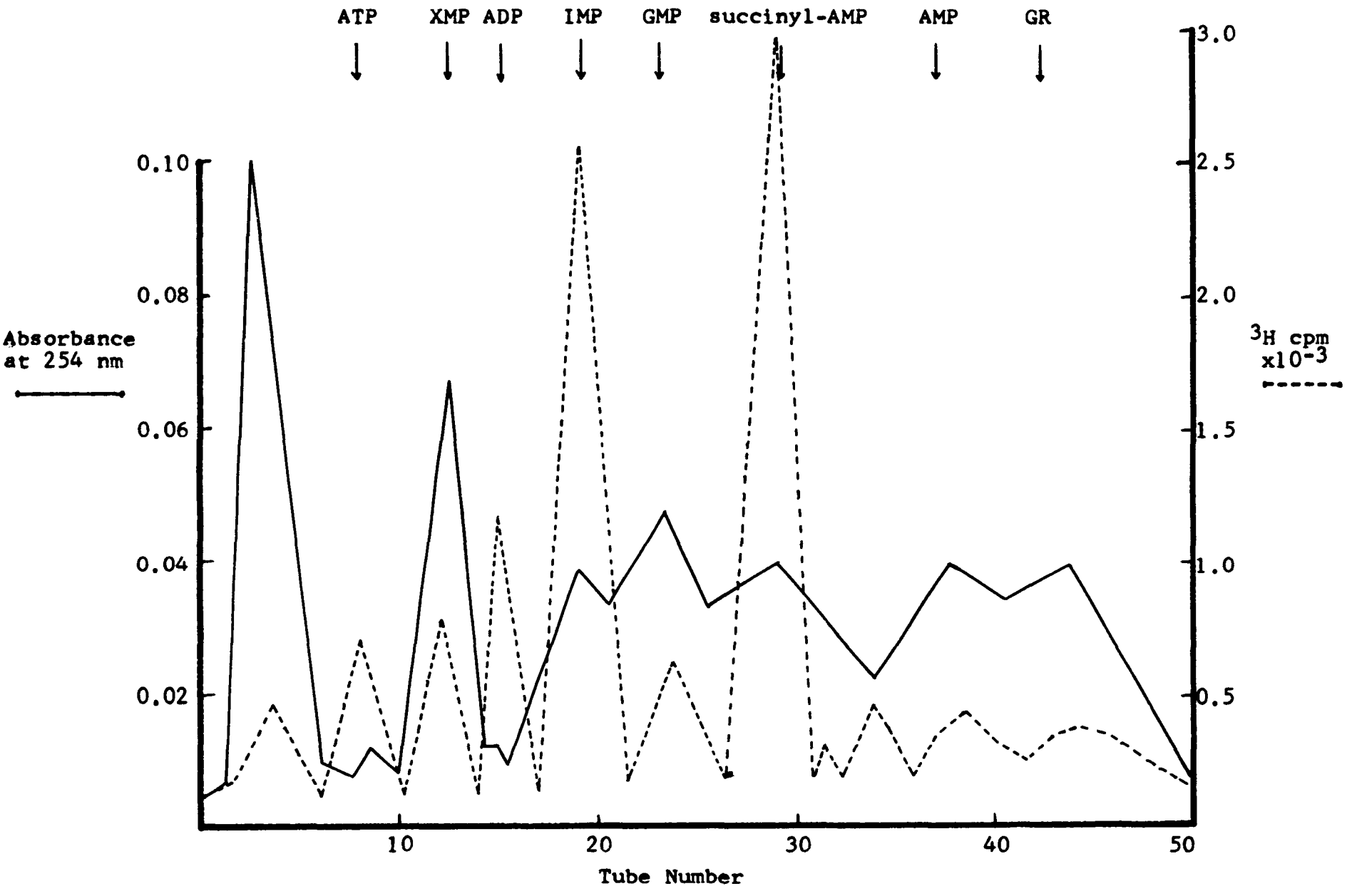
ATP
XMP
ADP
IMP
GMP
succinyl-AMP
AMP
GR
0.10
0.08
0.06
0.04
0.02
Absorbance at 254 nm
3.0
2.5
2.0
1.5
1.0
0.5
3H cpm x10-3
10
20
30
40
50
Tube Number

Figure 10

acid-soluble pool prior to chromatography. Standards were chromatographed on the Dowex-50 column periodically throughout this work, and the position of elution of the standards was found to be remarkably reproducible. From Figure 10 it can be seen that XMP, IMP, GMP, succinyl-AMP, and AMP are labeled. Other radioactive peaks are found including ATP, ADP, and guanosine. When the radioactive peaks were dried in order to do a further analysis of the material, it was found that the conditions of drying led to an exchange of the ^{3}H label and a loss of greater than 95% of the radioactivity. Consequently, it was decided to switch to the more stable ^{14}C isotope for subsequent experiments.

Conversion of GMP to XMP:

Since the evidence indicated that XMP was an intermediate in the conversion of GMP to AMP, it was decided to attempt to demonstrate the enzymatic conversion of GMP to XMP. A cell-free homogenate, prepared from cells growing on guanosine as the sole source of purine, was incubated in 0.08M Phosphate buffer, pH 7.4, with ^{14}C-8-GMP. At various time periods, aliquots were removed and prepared for chromatography on Dowex-50. A new peak of radioactivity appeared, which increased with time, and which co-chromatographed with XMP. The time course of appearance of label in this peak is shown in Figure 11. When this experiment was repeated using half the protein concentration used in the initial experiment, the result was approximately half the conversion of label to XMP as found initially. This would indicate that the reaction is enzyme dependent.

In order to be sure that the new peak of radioactivity was indeed XMP, additional analysis of the material was performed. The material was chromatographed in Solvents K, I, and L, and in each case the radioactivity was coincident with the XMP standard as shown in Figure 12. After acid hydrolysis the radioactive peak shifted on paper chromatography to a position co-chromatographing with xanthine. The material was eluted, treated with xanthine oxidase, and rechromatographed on paper. The radioactivity now co-chromatographed with uric acid, Figure 13.

Figure 11. Conversion of ^{14}C-8-GMP to ^{14}C-8-XMP by a Cell-free Homogenate of *Tetrahymena pyriformis.*

A cell-free homogenate of *Tetrahymena pyriformis* was prepared, and incubated with ^{14}C-8-GMP. At the time periods indicated, aliquots were taken, chromatographed on Dowex-50, and the % conversion of label to XMP was determined as described under Methods.

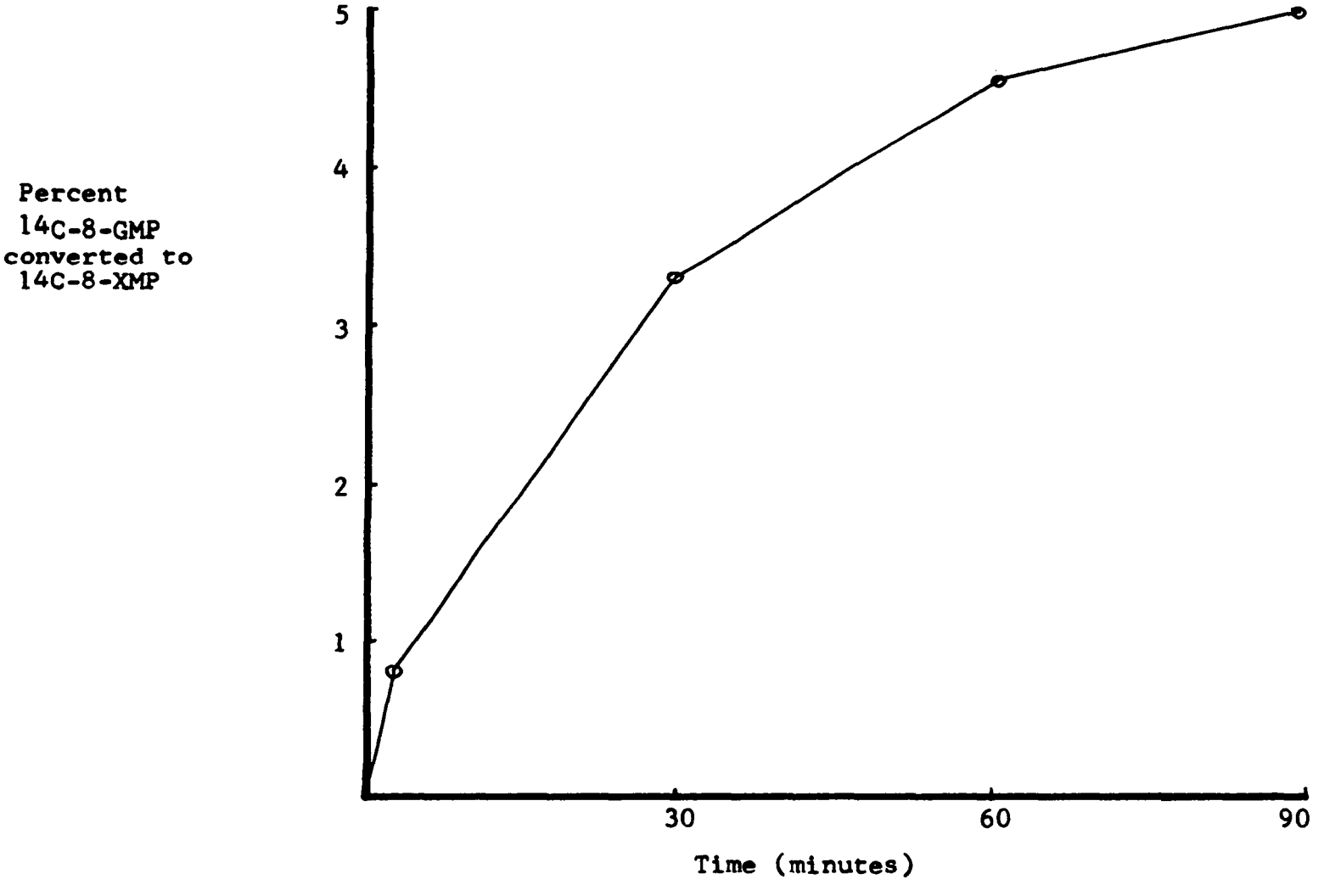
Percent
14C-8-GMP
converted to
14C-8-XMP
5
4
3
2
1
30
60
90
Time (minutes)

Figure 11

Figure 12. Identification of XMP by Paper Chromatography.

The material eluting from the Dowex-50 column in the position of XMP was chromatographed in Solvents I, K, and L. The position of radioactivity was determined as described under Methods. The position of migration of XMP standards is as indicated.

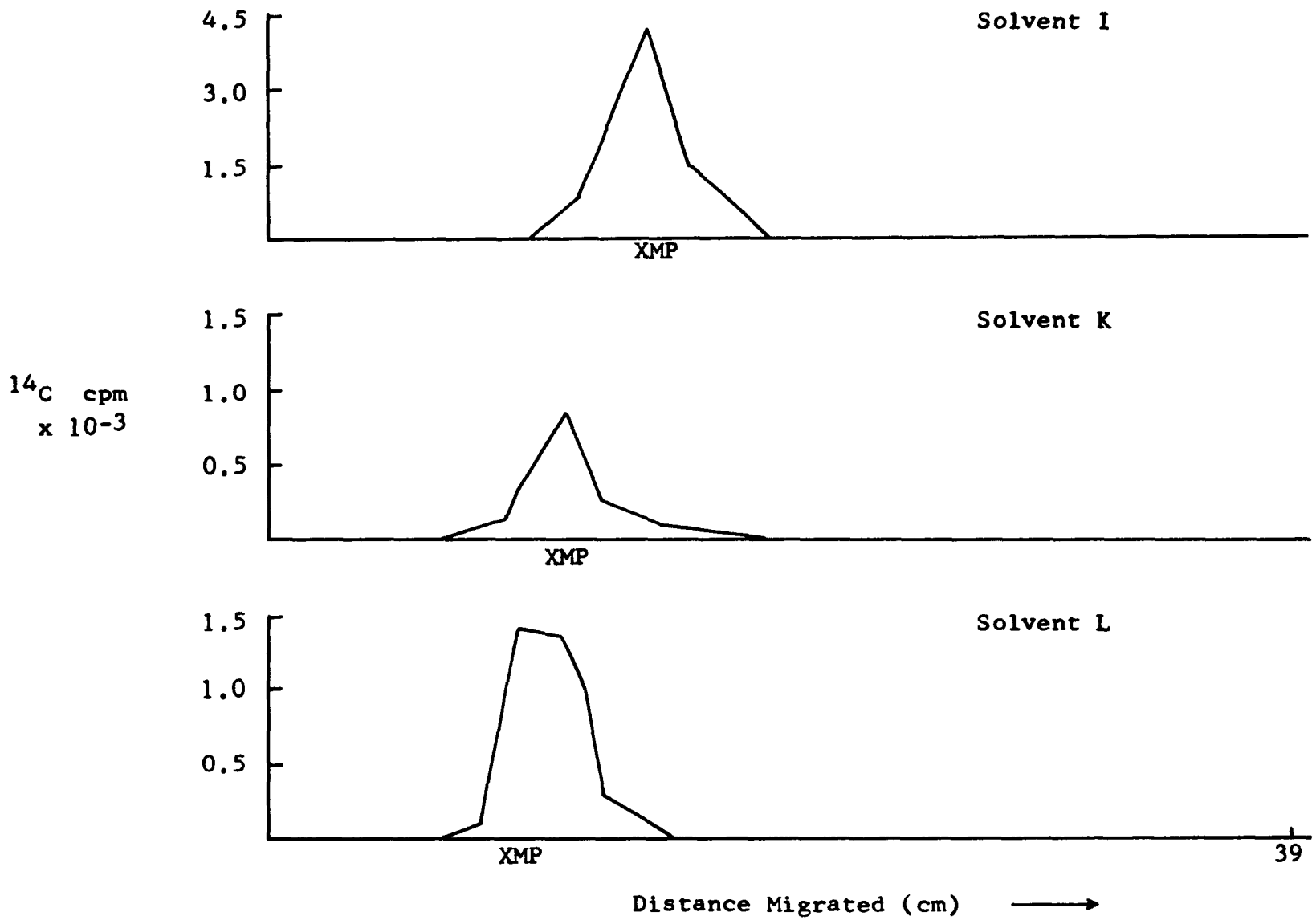
4.5
3.0
1.5
Solvent I
XMP
1.5
1.0
0.5
Solvent K
XMP
1.5
1.0
0.5
Solvent L
XMP
39
14C cpm
x 10-3
Distance Migrated (cm)

Figure 12

Figure 13. Identification of XMP by Acid Hydrolysis and Enzyme Conversion.

The material identified as XMP was further analyzed by acid hydrolysis and conversion by Xanthine oxidase as described under Methods. The products of these treatments were chromatographed in Solvents L and K as shown. The position of the standards is as noted. Material in tubes 7-11 from Dowex-50 column (A); after acid hydrolysis (B); after enzymatic conversion (C).

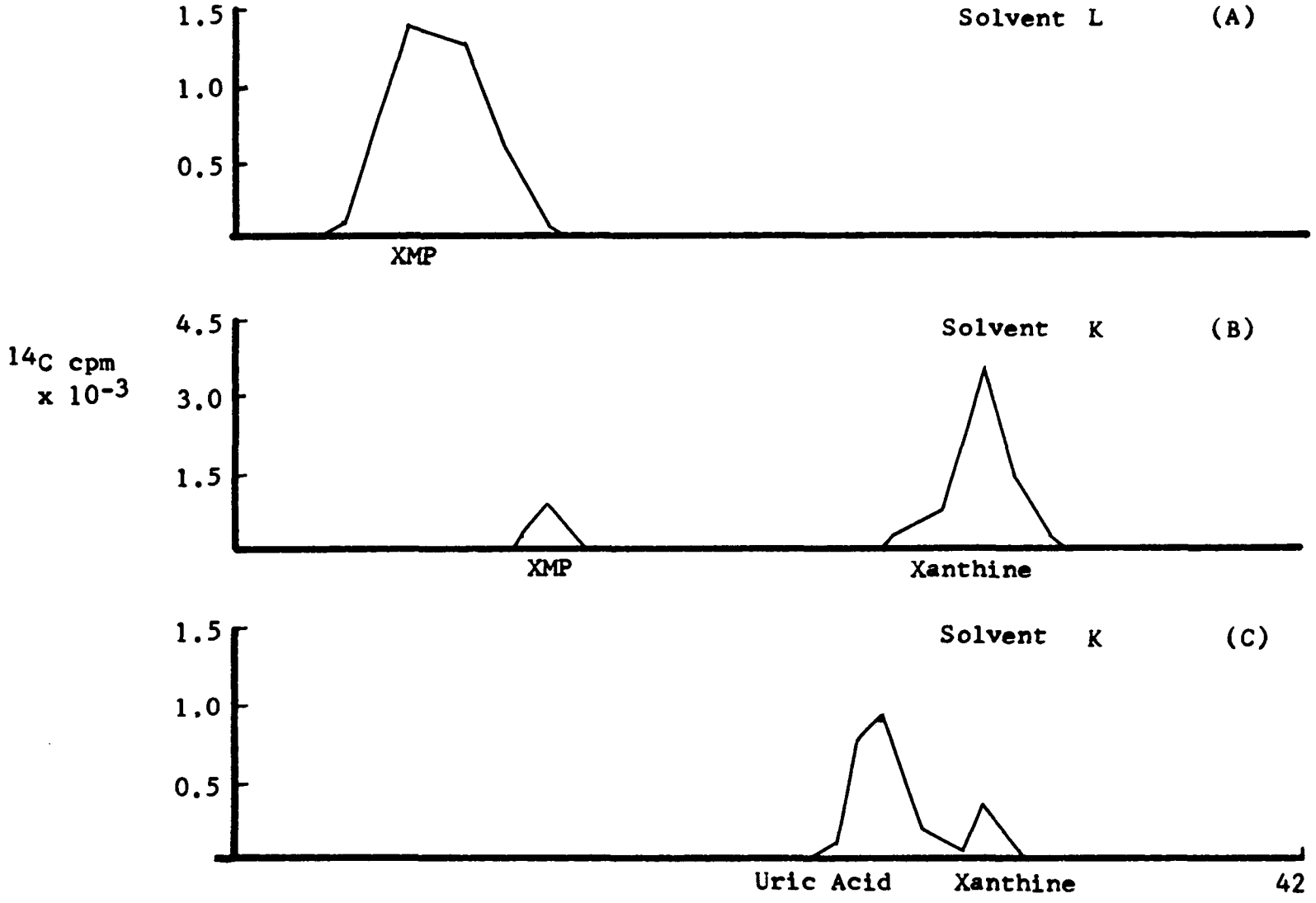
1.5
1.0
0.5
Solvent L
(A)
XMP
4.5
3.0
1.5
Solvent K
(B)
^{14}C cpm
x 10^{-3}
XMP
Xanthine
1.5
1.0
0.5
Solvent K
(C)
Uric Acid
Xanthine
42
Distance Migrated (cm)

Figure 13

DISCUSSION

The use of *Tetrahymena* as a model of a mammalian system:

Tetrahymena pyriformis has often been used as a model of a mammalian organism (65). It offers great advantages over the usual mammalian systems since as a unicellular organism it is easy and inexpensive to grow, and large amounts of material can be obtained for biochemical analysis. Furthermore, the organism can be grown on a defined medium, which is a decided advantage in the study of biochemical pathways. Recently, the organism has found use as a model system to test the effects of new drugs and other potentially harmful agents (66,67). The use of *Tetrahymena* as a test organism for agents which might be approved for human use would provide a great deal of savings in time and money. Also, numerous additional tests, for instance, using differing concentrations, differing growth conditions, and different combinations of chemical agents could be performed. Such necessary additional tests are almost prohibitively expensive, and time consuming, when mammalian systems are used as the test organism. With the development of *Tetrahymena* as a model for mammalian systems, it becomes imperative that we learn as much as possible about the normal metabolism of this organism, so that we can predict the applicability of the results obtained with *Tetrahymena* to true mammalian organisms. The first part of this work examines the pattern of methylation of the purine bases of *Tetrahymena* tRNA. The second part examines the ability of *Tetrahymena* to demethylate one of these methylated bases. The final part examines the normal metabolism of purine bases in this model of a mammalian system.

Identification of methylated purines in tRNA:

Very little was known about the pattern of base methylation in Protozoan RNA. It was of some interest to know whether the pattern of methylation was more like that found in procaryotes, or if the pattern more closely approximated that of higher

organisms. Since tRNA contains the greater number of methylated bases found in RNA, and since rRNA methylation in higher organisms is primarily of the 2'-O-ribose type (8), it was decided to examine the pattern of purine methylation in tRNA.

A number of methods were tried for the isolation of pure tRNA from *Tetrahymena pyriformis,* and phenol extraction was found to be the most satisfactory. A major problem encountered with phenol extraction, however, was the co-isolation of a substantial amount of glycogen with the tRNA fraction. To remove this troublesome glycogen, the phenol extracted tRNA was chromatographed on DEAE-cellulose. The glycogen fraction, containing few charged groups, is easily eluted with buffer, and was identified with the anthrone reagent. A small amount of residual protein was then eluted with 0.3M NaCl, and finally tRNA was eluted with 0.7M NaCl. Other types of RNA of large molecular weight (mRNA, rRNA) remain bound to the column and are not eluted under these conditions (62). The purity of the tRNA preparation was checked by chromatography on Sephadex G-75. In initial experiments, it was noted that there was a substantial ribonuclease activity present during the isolation procedure. This problem was corrected by the inclusion of the RNase inhibitor 8-hydroxyquinoline in the isolation buffers, by washing all glassware with a solution of 0.1% 8-hydroxyquinoline, and by wearing surgical gloves.

The most abundant methylated guanine derivative in the tRNA of *T. pyriformis* is N^2-DMG. Also found are 1-MG, N^2-MG, and 7-MG. An examination of the relative amounts of the various methylated guanines in a variety of organisms fails to reveal a specific pattern in so far as which methylated guanine is the predominant species (28). However, only in *E. coli* tRNA is 7-MG the predominant methylated guanine, while in the other organisms for which complete data is available, the relative amount of 7-MG is more nearly that of N^2-MG. *T. pyriformis* also differs from the *E. coli* pattern, in that, in its tRNA the relative amounts of 7-MG and N^2-MG are very similar.

When one examines the data on the relative amounts of the methylated adenines in the tRNA of various organisms, one finds that, except for *Proteus vulgaris* tRNA, 1-MA is the predominant base. The tRNA of *T. pyriformis,* likewise, has 1-MA as the predominant methylated adenine. A variety of organisms have been reported to contain the methylated base 2-MA in their bulk tRNA (28), and it has recently been reported

that 2-MA is present in the primary sequence of a number of specific *E. coli* tRNA's (68). It is surprising, therefore, to find that no 2-MA is present in the tRNA of *T. pyriformis* (see Figure 2(c)), yet this base is present in the tRNA of mammalian tissue, plant tissue and a number of bacteria. The presence of N^6-MA in RNA has been demonstrated after acid hydrolysis in *E. coli* (69) and HeLa cells (70). A small amount of N^6-MA was detected in the tRNA of *T. pyriformis.* Since conditions were kept acidic at all times following acid hydrolysis, and because of its position of elution from the Dowex-50 column, it is unlikely that this represents a rearrangement product of 1-MA. N^6-DMA was also detected.

Growth of *Tetrahymena* on 1-MGR:

Early nutritional studies by Kidder and Dewey (44) had indicated that 1-MG could substantially replace guanine for growth. Since most commercial samples of both 1-MG and 1-MGR were found to be grossly contaminated with the unmethylated base or riboside, it was suspected that the replacement observed was due to guanine contamination of the sample. Therefore, the ability of 1-MG to replace the growth requirement for guanine was reinvestigated. It was found that, indeed, chromatographically pure 1-MG and 1-MGR could replace the guanine requirement of *Tetrahymena pyriformis.* Since 1-MGR could satisfy the total purine requirement of *Tetrahymena,* two conclusions were possible. Either 1-MGR was being demethylated, or 1-MGR was being incorporated *per se* into nucleic acid. In the latter case, cells grown on 1-MGR should contain substantial amounts of 1-MG in their nucleic acid, and for that matter, substantial amounts of 1-MA should also be found. As can be seen in Figure 4, only the normal, unmethylated purines are found, and it can be concluded that the demethylation of 1-MGR is taking place. It should be noted that the amount of 1-MG naturally present in the tRNA, as reported in the first part of this work, would not be detected by the techniques used here, which are based on the absorption of ultraviolet light.

The metabolism of 1-MGR by *Tetrahymena pyriformis*:

Since aberrant methylation takes place in tumor cells (26,27), and since tumor

cells appear to lack demethylase systems (42,43), a study was undertaken to determine the mechanism of demethylation of 1-MGR by *Tetrahymena.* Since whole cells had been shown to be capable of demethylation, the fate of the methyl group was next examined in the intact organism.

Methyl-labeled 1-MGR was not commercially available, and the cost of custom synthesis was too expensive, therefore, ^{14}C-me-1-MGR was synthesized and purified as described under Methods. Figure 5 shows that two radioactive spots are obtained when the reaction mixture is chromatographed. Since other possible methylated derivatives have different R_fs in this solvent, for example, 1,7-DMGR, R_f=0.60, and 7-MGR decomposes in this solvent, it is tentatively concluded that this material is 1-MG, R_f= 0.25, generated under conditions of repeated drying at room temperature. The material might possibly be 7-MG which has an R_f of 0.24 in this solvent. In any case the lower specific radioactivity of this compound indicates that the material contains a substantial amount of unreacted guanosine, which has an R_f of 0.27.

After incubating whole cells of *Tetrahymena* with ^{14}C-me-1-MGR for 18 hours, and isolating the various macromolecular fractions, radioactivity was found to be associated only with the lipid fraction. The hydrolysis of the lipid fraction brings about the liberation of choline, and probably also some serine and ethanolamine, and it is this water soluble filtrate which contains the radioactivity, Table V. If the methyl group comes off 1-MGR as a 1-carbon moiety, it might be expected to find its way into the one-carbon pool. Consequently, one might have expected that thymine in DNA would be labeled, and that the amino acids serine and methionine in proteins would be labeled. However, Lanzetta (71) has shown that ^{14}C-me-methionine is not incorporated into thymine in this strain, and that methionine is the only amino acid labeled after growth on ^{14}C-me-methionine. These results then seem to coincide, and indicate that one-carbon metabolism in *Tetrahymena pyriformis* GL is severely restricted.

When a cell free homogenate of *Tetrahymena pyriformis* GL was incubated with ^{14}C-me-1-MGR, it was found that $^{14}CO_2$ was liberated, and that when any formaldehyde or formate produced in the reaction was oxidized, additional $^{14}CO_2$ was liberated. These results taken together show that the methyl group of 1-MGR can be

removed and liberated as CO_2 or incorporated into choline. Hogg (72) has shown that the cycle depicted in Figure 14 is operable in *Tetrahymena pyriformis.* This cycle explains the results obtained in the preceding experiments. The methyl group of 1-MGR is probably oxidized to the formyl group, which can then be picked up by tetrahydrofolate to yield N^5, N^{10}-Methylene tetrahydrofolate. This formyl group can then be transferred to glycine to yield serine, and the radioactive carbon then moves around the cycle into choline. The degradation of choline leads to the regeneration of glycine, and on the second turn of the cycle, the radioactive carbon is released as CO_2. The failure to find radioactive serine in the protein fraction, suggests that either the serine generated in this cycle is "compartmentalized", or that serine in protein did not become sufficiently labeled over the time course of this experiment. Radioactive CO_2 might also be released from ^{14}C-me-1-MGR by a cell-free homogenate of *Tetrahymena* if the formaldehyde produced is oxidized to formate and then to CO_2. At the present time, little is known about formaldehyde metabolism in *Tetrahymena.*

Similar demethylase systems (35,36,37,38,39,40,41) have been shown to produce formaldehyde, and to require NADPH as a cofactor in the reaction. In order to see if the *Tetrahymena* demethylase system was similar these parameters were examined. Attempts to measure formaldehyde formation from 1-MGR in cell free homogenates by spectrophotometric techniques proved unsuccessful, probably due to the low level of conversion. However, it was possible to take advantage of the fact that NADPH absorbs light at 340nm, while NADP does not. It was found that 1-MGR caused a significant decrease in the absorbance at 340nm over the control, Figure 6. The fact that the decrease with 1-MG is more immediate and abrupt suggests that 1-MGR is first hydrolyzed to the free base before demethylation. The fact that unmethylated guanine is recovered from the three hour experimental, Figure 7, but not from the zero time sample, indicates that the conversion of NADPH to NADP is correlated with a conversion of 1-MG to guanine. These results support the proposed pathway for the demethylation of 1-MG shown in Figure 15.

Growth studies:

If *Tetrahymena pyriformis* is to be used as a model of mammalian systems, it is

Figure 14. Restricted 1-Carbon Cycle in *Tetrahymena pyriformis* GL.

The cycling of the 1-carbon moiety into choline and its liberation as CO_2 from serine.

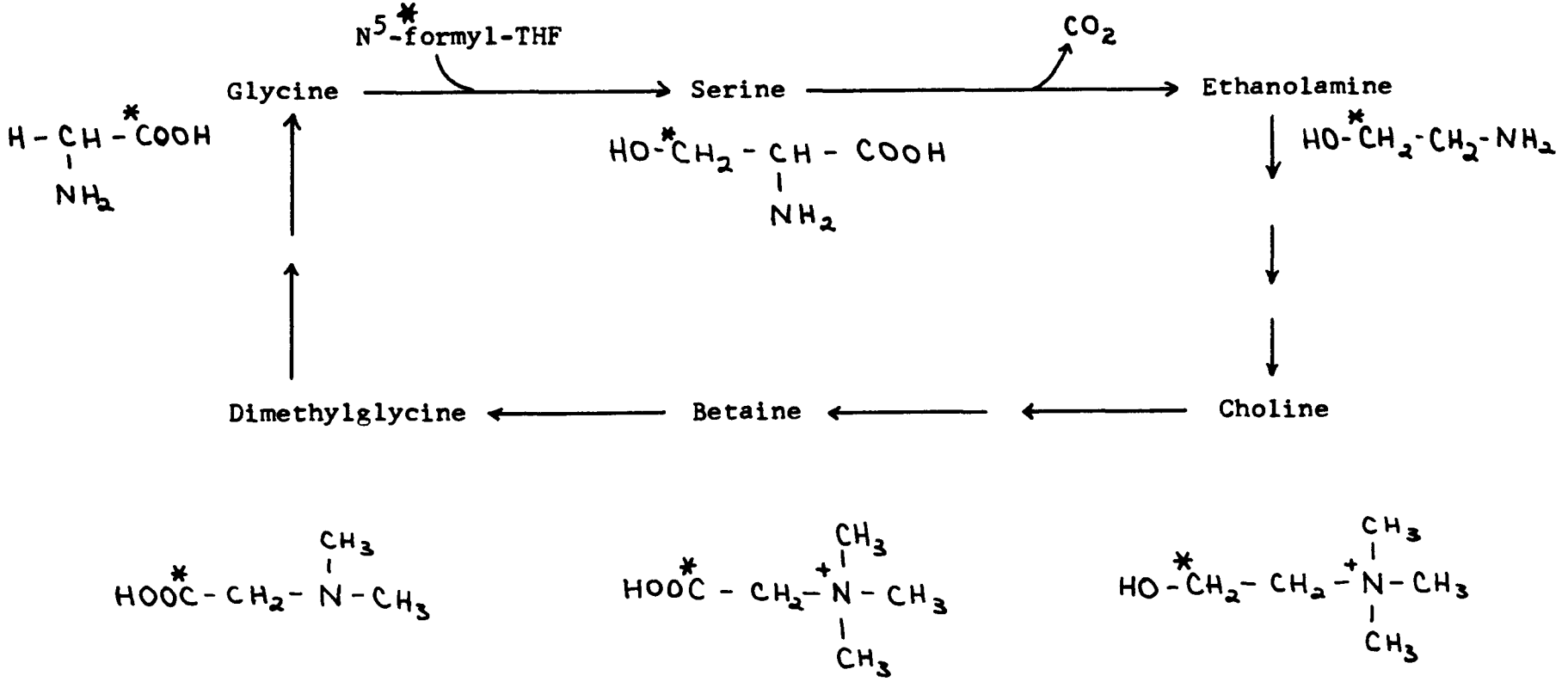
N5*-formyl-THF
CO2
Glycine
Serine
Ethanolamine
Dimethylglycine
Betaine
Choline

Figure 14

Figure 15. Proposed Pathway for the Demethylation of 1-MG by *Tetrahymena pyriformis.*

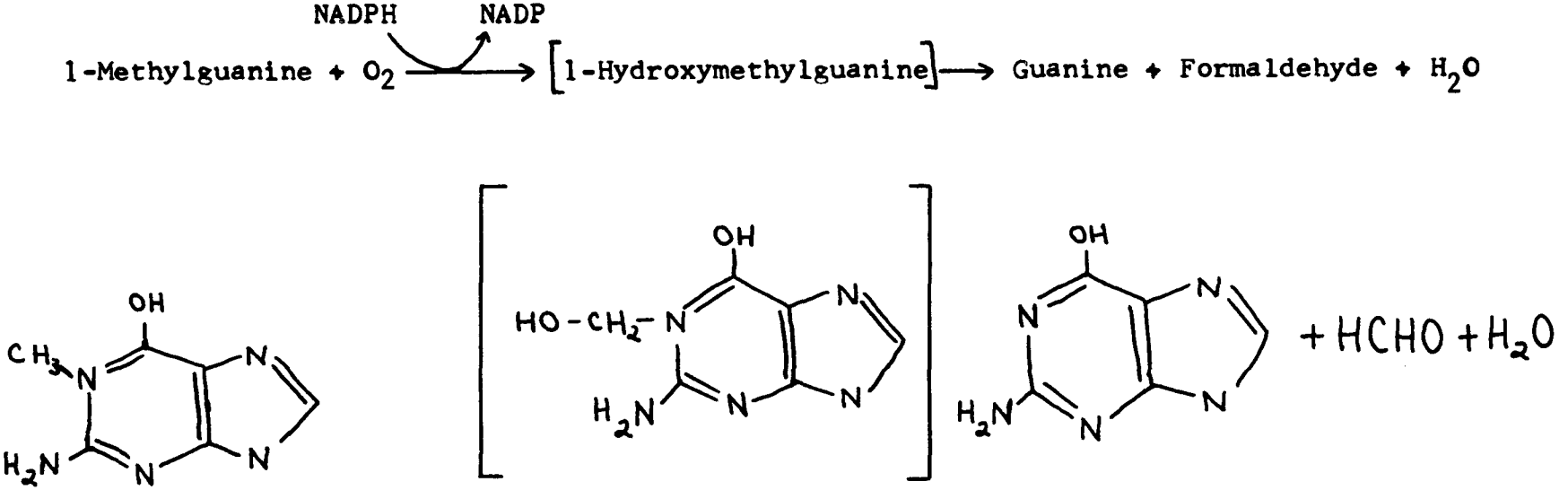
NADPH
NADP
1-Methylguanine + O_2
[1-Hydroxymethylguanine]
Guanine + Formaldehyde + H_2O
+ HCHO + H_2O

Figure 15

important to know as much as possible about normal metabolic interconversions, especially, if the organism is to be used as a screening agent for potentially harmful, and carcinogenic drugs, many of which are purine ring derivatives (66), a complete understanding of purine metabolism is essential. Furthermore, since it was possible to grow cells on 1-MGR as the sole source of purine, it was of interest to determine the metabolic fate of the free base after demethylation.

Soon after it had been shown that *Tetrahymena* can convert guanine to adenine, but not vice versa, a number of studies were undertaken to delineate the metabolic pathway by which this was accomplished (48,49,50). Attempts to demonstrate guanase or GMP reductase were unsuccessful. Since a post-mitochondrial supernatant fraction, and spectrophotometric techniques were used for most of the studies, it was thought that perhaps an enzymatic activity was being missed. Therefore, the presence of guanase, and GMP reductase in *Tetrahymena* was rigorously reinvestigated using a variety of subcellular fractions prepared by differential centrifugation. A variety of assay conditions and cofactors were tried, and incubation mixtures were analyzed by chromatography, in order to pick up even small amounts of conversion. The existence of guanosine deaminase was also examined. All these experiments failed to demonstrate the presence of guanase, guanosine deaminase, or GMP reductase in *Tetrahymena.*

After having failed to demonstrate these enzymatic activities *in vitro*, it was thought that a clue to the identity of the intermediates might be obtained from growth studies with cells growing on defined medium containing guanine as the sole source of purine. It was felt that any compound which was an intermediate in the conversion of guanine to adenine, or which could be converted to adenine, would enhance the growth of the cells. A compound which had been suggested as a possible intermediate, 2,6-diaminopurine (73), was tested, and it was found that both the free base and the riboside were inactive. Adenine, AMP, inosine and IMP were all found to enhance growth. In bacterial and mammalian systems, GMP is synthesized from IMP through the intermediate XMP (74,75,76,77,78). Although xanthine and xanthosine had been reported to be nutritionally inert in *Tetrahymena* (45,46), and this finding was confirmed in this laboratory, it was decided to try XMP in a sparing experiment. As can be seen in Figure 8, XMP shows a small, but reproducible, sparing of the guanine

requirement. This finding was confirmed when the cells were grown on very low levels of guanine, and increasing amounts of XMP were added. The increasing amounts of XMP resulted in increased sparing of the guanine requirement, Figure 9. These results suggested three possibilities, either XMP was an intermediate in the conversion of GMP to AMP, or XMP was converted to GMP which was then converted to AMP, or XMP was substantially contaminated. The last possibility was ruled out by rigorous analysis of the XMP sample by paper and column chromatography. The possibility that XMP was being converted to GMP was ruled out by the fact that cells grown in the presence of XMP as the sole purine source soon died out. Therefore, it seemed likely that XMP was a true intermediate in the conversion of GMP to AMP.

Isotope dilution experiments:

The ability of various compounds to dilute the radioactive label that is incorporated into nucleic acid adenine from radioactive guanosine, indicates that these compounds are either intermediates in the conversion of GMP to AMP, or are able to be converted to AMP. In these experiments, Table VIII, it can be seen that when no additional purine is added to the medium, the ratio of adenine to guanine specific radioactivities approaches 1, as would be expected. When adenine is added to the medium, the specific radioactivity ratio is decreased 100-fold, indicating a sharp and immediate curtailment of the incorporation of radioactive label into nucleic acid adenine from the guanine pool. When AMP is added, as a control to check for the effect of the monophosphate, a ten-fold decrease in the ratio is observed, and illustrates that the AMP is not as effective in decreasing the incorporation of label from the guanine pool. The sharp decrease noted with adenine, suggests that it nearly shuts off the pathway of conversion of GMP to AMP, and that AMP is a less effective modulator, or does not pass through the cell membrane as easily as the free base. Both inosine and IMP also showed a ten-fold decrease in the ratio, while XMP showed a two-fold decrease. In other experiments using higher concentrations of XMP, the decrease was as much as four-fold. These results then, in view of the results obtained for the control, are quite significant, and implicate XMP and IMP as intermediates in the conversion of GMP to AMP.

Some very interesting results were obtained when the experimental set-up was altered slightly. In these experiments, Table IX, the source of purine was the free base guanine, while the radioactive label was supplied as ^{3}H-8-guanosine. The same results were obtained with respect to the dilution of the label in nucleic acid adenine, but a difference was observed in the absolute specific radioactivities in nucleic acid guanine under the various experimental conditions. When the free base adenine was added to the medium, the specific radioactivity in nucleic acid guanine increased approximately two-fold. When a riboside or ribotide was added to the medium, the specific radioactivity in the nucleic acid guanine decreased about five-fold. These results were completely reproducible. These findings can be explained on the basis of two membrane carrier systems in *Tetrahymena,* one for the transport of the free bases, and one for the transport of the ribosides and ribotides, as shown in Figure 16. When guanine is present in the medium alone, guanine and ^{3}H-guanosine enter the cell by separate carrier systems and the ratio of G/*GR inside and outside the cell is unaffected. When another free base, adenine, is added to the medium, it competes with guanine for entry into the cell and, consequently, relatively more ^{3}H-guanosine gets inside the cell, resulting in the increased specific radioactivity of nucleic acid guanine. On the other hand, when a riboside or ribotide is added to the medium, it competes with the ^{3}H-guanosine, and relatively less ^{3}H-guanosine enters the cell, causing a decrease in the specific radioactivity of the nucleic acid guanine. Although these results can best be explained by the carrier model proposed in Figure 16, the actual nature of the carrier will have to be elucidated in future experiments.

Acid-Soluble Pool:

When the Acid-Soluble Pool of whole cells was analyzed after a 30 minute pulse of ^{3}H-guanosine, the pattern shown in Figure 10 was obtained. The co-chromatography of radioactive peaks with XMP and IMP reinforces the evidence that they are intermediates in the conversion of GMP to AMP. A surprising finding was the large radioactive peak associated with succinyl-AMP. This finding suggests that succinyl-AMP may be the intermediate in the conversion of IMP to AMP. However, this is based only on its co-chromatography with a succinyl-AMP standard on Dowex-50. It must

Figure 16. Proposed Membrane Carrier Model for the Transport of the Free Bases, and Nucleosides and Nucleotides in *Tetrahymena pyriformis.*

On the basis of results obtained in the isotope dilution experiments, it is proposed that free bases and nucleosides-nucleotides enter *Tetrahymena pyriformis* by separate, independent mechanisms as described in the Discussion, Isotope Dilution Experiments.

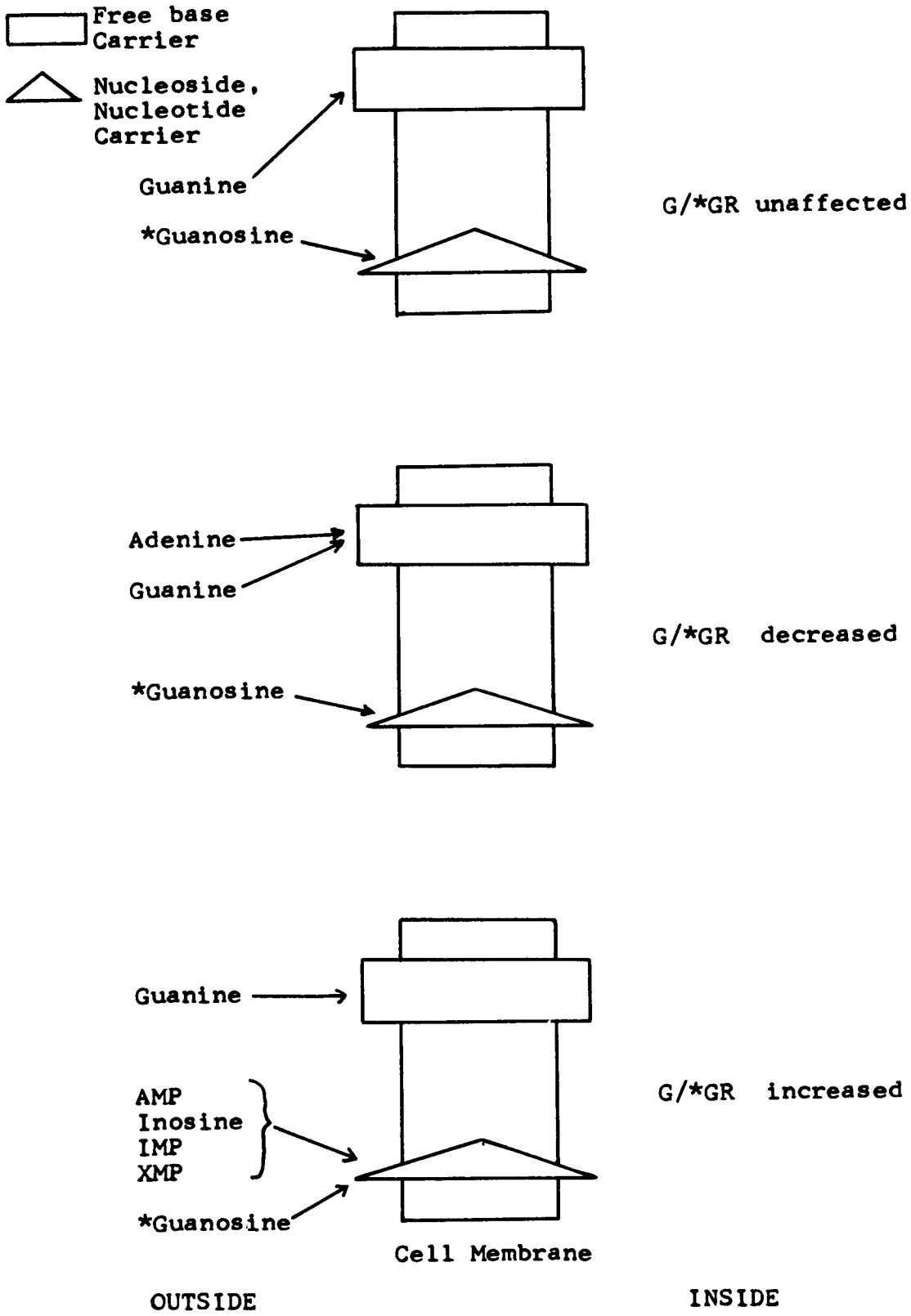
Free base Carrier
Nucleoside, Nucleotide Carrier
Guanine
*Guanosine
G/*GR unaffected
Adenine
Guanine
*Guanosine
G/*GR decreased
Guanine
AMP
Inosine
IMP
XMP
*Guanosine
G/*GR increased
Cell Membrane
OUTSIDE
INSIDE

Figure 16

be remembered that the amount of radioactivity is actually a reflection of the pool size, and the state of synthetic activity in the cell, and no conclusion can be drawn from the relative amounts of radioactivity in the various intermediates. The pulse time was sufficiently long for radioactivity to enter AMP, ADP, and ATP, from which radioactive label could enter the adenine of nucleic acids.

The enzymatic conversion of GMP to XMP:

The evidence presented above supports the proposed pathway for the conversion of guanine to adenine by *Tetrahymena pyriformis* shown in Figure 17. The first step in this pathway, the conversion of GMP to XMP, is a most unusual and unexpected enzymatic reaction. In both procaryotic and eucaryotic systems (76,77,78), the synthesis of GMP is through the amination of XMP by XMP-aminase. The enzyme, XMP-aminase, has been found to be irreversible in these systems. It was felt, therefore, that the conversion of GMP to XMP had to be definitively demonstrated *in vitro.* Figure 11, shows that an homogenate of *Tetrahymena pyriformis* can convert 5% of the GMP radioactivity to XMP in 90 minutes. The identify of XMP was rigorously shown by column and paper chromatography, and by acid and enzyme conversions. These findings show that *Tetrahymena pyriformis* contains a unique enzyme system, and this fact must be kept in mind when the organism is used as a model of mammalian systems.

Experiments have been undertaken to demonstrate the *in vitro* enzymatic conversion of XMP to IMP. ^{14}C-8-XMP was prepared from ^{14}C-8-GMP by nitrous acid deamination, and the conversion of the label was looked for under a variety of conditions. However, the proper assay conditions have not yet been found. Evidence that IMP is an intermediate comes from experiments that show that radioactive guanosine can be converted to radioactive IMP (50) as we also have shown. Hill (79) has proposed that IMP may be converted to AMP via succinyl-AMP, and the evidence from the labeling pattern of the Acid-Soluble Pool, Figure 10, tends to support this suggestion. In conclusion, the pathway shown in Figure 17 is supported by a great deal of indirect evidence, and by the demonstration of the *in vitro* conversion of GMP to XMP by *Tetrahymena pyriformis.*

Figure 17. Proposed Pathway for the Conversion of GMP to AMP by *Tetrahymena pyriformis.*

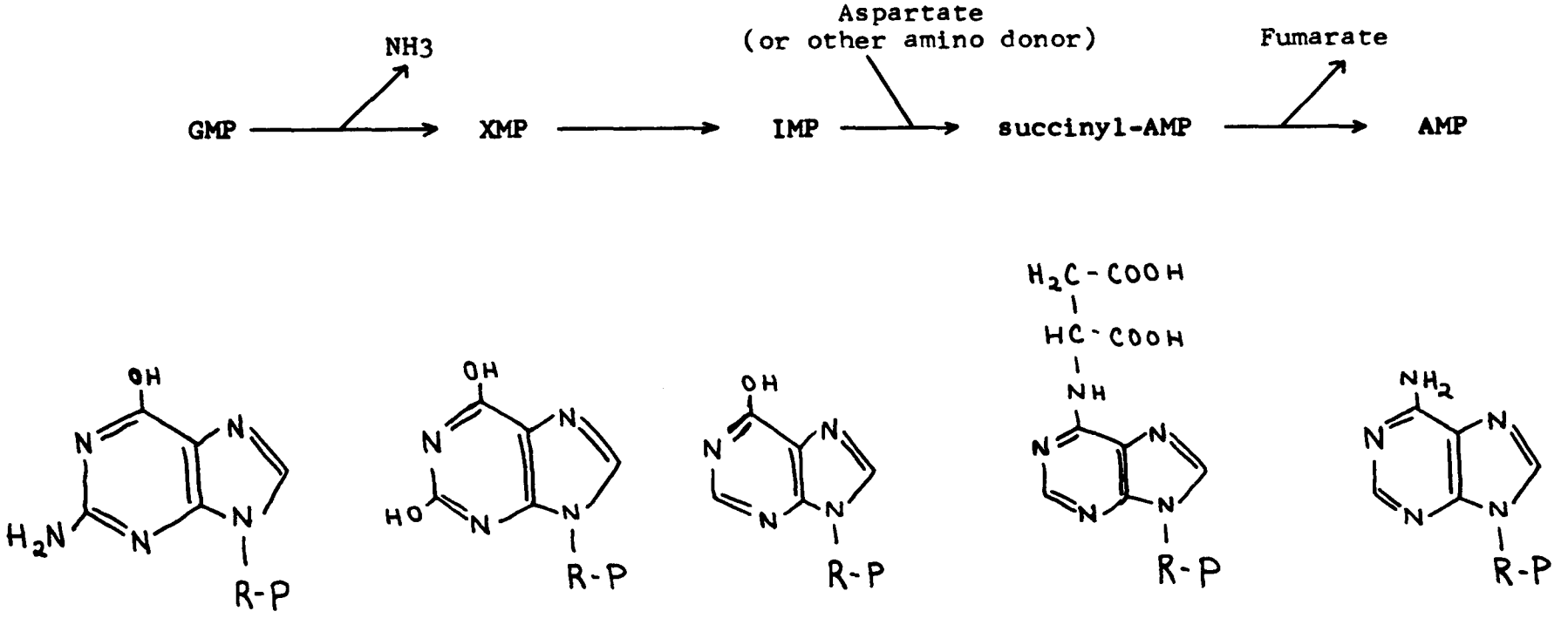
NH3
Aspartate
(or other amino donor)
Fumarate
GMP
XMP
IMP
succinyl-AMP
AMP
R-P
R-P
R-P
R-P
R-P

Figure 17

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