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THYMIDINE CATABOLISM AND REUTILIZATION OF THE DEGRADATIVE PRODUCTS IN *TETRAHYMENA*

by

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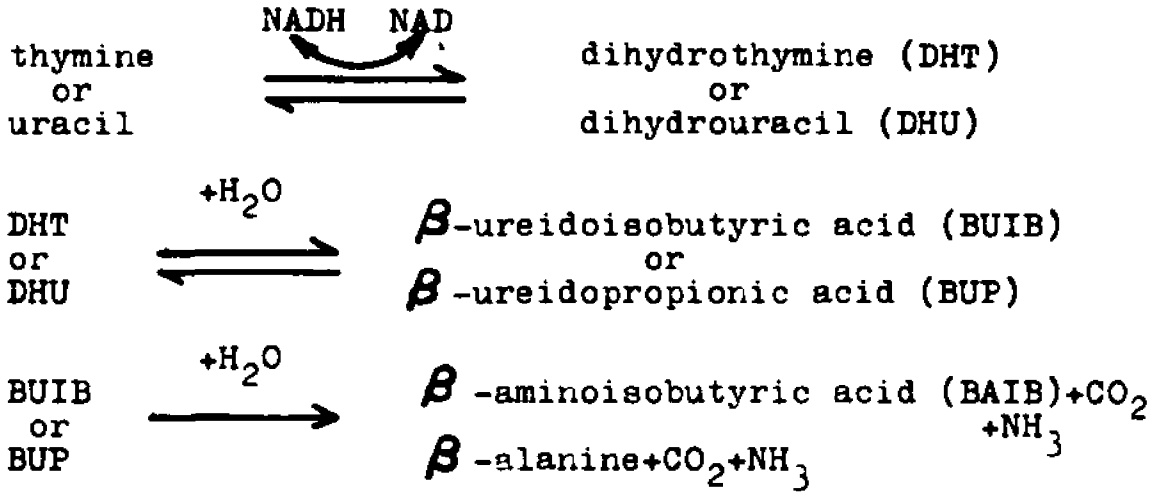
INTRODUCTION

There are many claims and counterclaims in the literature about utilization of thymidine for other than DNA synthesis. Early autoradiographic studies with protozoa grown in media containing tritiated thymidine revealed considerable isotope incorporation into extranuclear material (1,2,3,4,5). Even though DNA has been reported to be present in kinetosomes, kinetoplasts, chloroplasts, mitochondria and pellicular material the interpretation of the autoradiographic results has been difficult (6,7,8,9,10,11,12,13). Use of enzyme digests on material prepared for autoradiograph has led to highly variable results. In some cases the cytoplasmic label was sensitive to deoxyribonuclease, in others to ribonuclease and in still others to neither enzyme (5,13,14).

Berech in a preliminary experiment found evidence that radioactive uridylic acid could be isolated from RNA prepared from Paramecia grown in media with tritiated thymine, while Albach found evidence that in Tetrahymena there is essentially no non-DNA incorporation of tritiated thymidine (1,15). Brachet found C^{14} labeled thymidine was converted to uridylic acid and cytidylic acid in Acetabularea mediterrania (16). Fink and others have

reported that in Neurospora, and possibly mouse liver, thymine can be demethylated to uracil in a two step process involving the production of hydroxymethyl uracil. Some of the uracil is converted to cytosine and thymine is therefore effectively transformed into both of the RNA pyrimidines (17,18,19,20). Berry et al have reported a similar enzyme system in the silkworm Hyalophora cecropia which demethylates thymine through hydroxymethyldeoxyuridine and remethylates the deoxyuridylic acid to deoxythymidylic acid (21).

Work on reductive pyrimidine catabolism has not adequately connected pyrimidine ring breakdown with possible reincorporation of the products into molecules other than DNA. Enzymatic pathways of uracil and thymine degradation have been described in whole or in part in mammals (22, 23,24,25,26,27,28,29,30,31), plants (32,33,34, 35,36), bacteria (37,38,39,40), rat tapeworm (41), and some parasitic flatworms (42). Little work has been reported on the protozoa; Soldo's isolation of dihydrouracil from paramecia represents the only report of a pyrimidine catabolic product in this group (43). The pyrimidine catabolic scheme can be summarized for most organisms as follows:



Most studies on animal production of thymine degradation products have been concerned with the urinary excretion of β-aminoisobutyric acid, particularly from the point of view of human cancer research (44,45,46,47). Few studies consider the possibility for the incorporation of any of the above products (or their possible derivatives) into any of the common macromolecules. Dihydrouracil and dihydrothymine are known constituents of t-RNA and chromosomal RNA (48,49,50). In fact, Grisolia and Carr have described the incorporation of dihydrouridine monophosphate directly into the liver and brain RNA of the pigeon (51). Sakai and Kihara report that, one hour after the administration of uniformly tritium labeled uridine, mouse liver proteins will accumulate an amount of radioactivity almost equal to that found in the nucleic acids (52). Bryant reported the incorporation of tritium from methyl labeled thymidine into protein of the mouse (53). Piko has isolated a radioactive polysaccharide from mouse embryos incubated in uniformly labeled C^{14}-thymidine (54).

Greenhouse has described the presence of silver grains over periodic acid Schiff-positive regions of mouse embryos treated with tritiated thymidine (55). Lanzetta and Berech have recovered radioactive glucose from glycogen isolated from Tetrahymena grown in the presence of thymidine-methyl-H^3 (56). Fink found radioactive glucose in the soluble fraction of mouse liver slices incubated with thymidine-methyl-C^{14} (23). Therefore a study of the metabolic pathways of radiolabeled thymidine was undertaken in an attempt to clarify some of the confusion caused by the many seemingly inexplicable reports.

The most likely pathway for the introduction of radiolabel of thymine into carbohydrates, RNA and proteins is by the reductive catabolic scheme of thymine. This scheme leads to β-aminoisobutyric acid which we feel is then converted to methylmalonic acid. Methylmalonic acid as the CoA derivative can be converted succesively into succinyl CoA and propionyl CoA (57). Succinyl CoA, along with being a precursor for porphyrin biosynthesis, can lead to phosphoenol pyruvate and ultimately glucose and glycogen through the normally operative gluconeogenic pathway (58). Propionyl CoA can lead to acetate which along with the propionyl CoA can be incorporated into fatty acids (59). In addition several tricarboxylic intermediates resulting from succinyl CoA can be transaminated into common amino acids. If the above

scheme is operative thymine can be a carbon source for proteins (amino acids), fatty acids, and carbohydrates as well as a nucleic acid precursor. Another possible mechanism for the introduction of carbons from thymine into molecules other than DNA is a destructive (to the ring) demethylation and introduction of the methyl carbon into the one carbon pool. The other previously described demethylation involving hydroxymethyluracil appears extremely unlikely because it is the methyl carbon which finds its way into the glucose molecules. It is felt that the possibility for a destructive demethylation is very remote because it has not been described in the literature and would represent a biologically wasteful process. A third possibility is the oxidative pyrimidine catabolic pathway which leads to methylbarbituric acid (from thymine) and ultimately to urea and methylmalonic acid. This pathway was originally described by Hayaishi and Kornberg in Corynebacterium and Mycobacterium; no evidence for its existence in Tetrahymena could be obtained (60). The evidence presented herein is an attempt to establish the existence of a pyrimidine reductive pathway in a protozoan, and also to establish that in protozoa, and possibly other organisms, the pathway need not end with the excretion of β-aminoisobutyric acid but that this latter compound may lead to all varieties of biologically useful derivatives.

MATERIALS AND METHODS

Cultures

Stock cultures of Tetrahymena pyriformis strain GL (kindly provided by Dr. James F. Hogg) were maintained on an undefined medium containing 1.1% proteose peptone, 0.01% yeast extract, 0.1% glucose, 0.1% K_2HPO_4 and 0.002 micrograms per ml of thiamine·HCl. The medium used is the author's slight modification of Elliott's medium (61). Cells were harvested by centrifuging at 4°C at 500 - 1000 x g for ten minutes. Cells were washed in Ryley Ringer Phosphate buffer consisting of 0.047M NaCl, 0.002M KCl, 0.001M $MgSO_4$ and 0.012M phosphate at pH 7.3 (62). Culture growth was monitored in nepholometer flasks at 660 nm with a Spectronic 20 colorimeter. Cell numbers were determined using appropriate dilutions and counting with a hemocytometer. Flasks were inoculated with five ml of media from tube cultures grown to mid-log stage (200,000 cells/ml). For washed cell suspensions, cell concentrations by volume were determined by centrifuging one ml of the cell suspension in a constable protein tube at 1000 x g for ten minutes.

Growth in Radioactive Media

Cells were grown in above media containing either methionine-methyl-H^3 (5.0 uCi/ml), thymidine-2-C^{14} (1.25 uCi/ml), thymidine-methyl-C^{14} (2.5 uCi/ml), thymidine-

6-H^3 (5.0 uCi/ml), or thymidine methyl-H^3 (5.0 uCi/ml) for 48 - 72 hours at 27°C (until the cultures reached late log phase). Cells were also grown for one, two and five generations to check any differences between long and short term growth in the radioactive materials. For experiments measuring the release of $C^{14}O_2$ cells were collected from mid-log cultures, pooled, washed and suspended in either sterile media or sterile Ryley Ringer Phosphate to make 2 - 5% cell suspensions (3.0 - 6.5 x 10^6 cells/ml). Three milliliter portions of the cell suspension were pipetted into 25 ml erlenmyer flasks and usually 0.5 uCi of either thymidine-2-C^{14} or thymidine-methyl-C^{14} was added. The flasks were stoppered with serum stoppers fitted with plastic dippers containing 0.1 ml of either 5% KOH or NCS solubilizer (Nuclear Chicago) and a piece of fluted filter paper (1 x 1.5 cm). The flasks were placed on a Dubnoff metabolic shaker and maintained at 27°C and slow shaker speed (approximately 36 cycles/min.) for appropriate time intervals.

Cell Fractionation

Cells were harvested by centrifuging at slow speeds (500 x g) for 10 minutes in the cold. After washing with Ryley Ringer Phosphate the organisms were fractionated essentially by the procedure of Schmidt and Tannhauser (63). Cells were suspended in an equal volume of cold 10% trichloroacetic acid (TCA). The homogenate was then centrifuged and the precipitate washed twice with a volume

at least equivalent to that of the precipitate of cold 5% TCA. After centrifugation the wash was added to the original 10% TCA extract and the fraction labeled as the acid soluble fraction (ASF). To the acid soluble fraction 1.1 volumes of cold absolute ethanol was added and the precipitate collected by centrifugation. The precipitate, presumably glycogen, was dissolved in water and again precipitated by the addition of 1.1 volumes of ethanol. The precipitate was dissolved in water, brought to volume and labeled glycogen. The supernatant liquids from the centrifugations were pooled, measured, and relabeled acid soluble fraction. The remaining pellet from the original TCA extract which contains RNA, DNA, protein and lipids was extracted with 5 volumes of cold absolute ethanol and centrifuged. The precipitate was subsequently extracted twice with equal volumes of a 3:1 (v/v) ether/absolute alcohol solution and finally with absolute ether. The alcohol, ether/alcohol, and ether extracts were pooled and the fraction labeled the lipid fraction. The remaining lipid-extracted pellet was weighed after air drying; it was then incubated for 18 hours at 37°C with 0.3N KOH (1 ml/50 mg pellet weight). The pellet was crushed with a glass rod to facilitate hydrolysis. After 18 hours the alkaline hydrolysate was acidified (to pH 1.5) with cold concentrated perchloric acid (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 material was then neutralized with KOH and

frozen, thawed and centrifuged to remove as much salt as possible. The neutralized hydrolysate labeled RNA was brought to volume and the total number of 260 nm absorbance (OD) units calculated after measurement of a portion of the sample in a Beckman DB spectrophotometer. The acid precipitated pellet was suspended in 5 volumes of 5% TCA and heated for 20 minutes at 100°C. The hydrolysate was centrifuged and the supernate decanted off and the precipitate again treated with 5% TCA for 20 minutes at 100°C. The hydrolysate was centrifuged and the supernate added to the previous one. The precipitate was washed with cold 5% TCA and the wash added to the above supernates. The remaining pellet which is protein was dissolved in 0.3N KOH and reprecipitated with 50% TCA. The supernate was pooled with the previous ones brought to volume and labeled DNA. The remaining pellet was dissolved and brought to volume in 1N KOH and labeled protein. Some small amount of cross contamination of fractions was evident. The amount was variable from experiment to experiment. The fraction most consistently affected was the RNA fraction. It contained glycogen which escaped the cold TCA extraction and a small amount of DNA resulting probably from some small fragments of the DNA broken off by the procedure. The protein fraction was also, on occasion, contaminated by a small amount of DNA. The RNA fraction therefore needed some further purification.

Recovery of Intermediates of Thymidine Catabolism

Cells incubated in modified Cavett flasks (Erlenmyer flasks fitted with serum stoppers) to trap $C^{14}O_2$ were collected by centrifugation, washed in buffer and the buffer wash added to the original supernate. The cells were extracted with 5 volumes of absolute ethanol:acetic acid (3:1 v/v) for 2 hours at room temperature and then with 2 volumes of 70% aqueous ethanol (32). The extracts were pooled and concentrated to one ml in vacuo. The pooled supernates were lyophilized and brought to one ml.

Column Chromatography

The RNA fraction was subjected to DEAE cellulose column chromatography after the methods of Morisawa and Chargaff, Tomlinson and Tener (64,65). DEAE cellulose which was washed in 0.5N NaOH until the wash was negative for chloride was then suspended and settled in glass distilled water until most of the fine particles were removed and the pH lowered to 9 or 10. It was then washed on a Buchner funnel until the pH was neutral, suspended in 0.1N acetic acid (volume dependent upon milliequivalents of DEAE to be equilibrated), filtered, and finally suspended in 0.01M lithium acetate buffer pH 5.0. A column 1.5 x 30 cm was loaded with DEAE cellulose to a height of at least 19 cm. The RNA fraction was loaded on top of the bed and allowed to percolate down. Several ml of buffer were allowed to percolate after the RNA application. The column was then

filled with buffer and attached to a reservoir of buffer and 20 ml fractions collected. The effluent was continuously monitored at 253 nm with an LKB Uvicord I. The contaminating carbohydrate came off the column first followed shortly thereafter by the mononucleosides. The buffer in the column reservoir was then changed to 0.01M lithium acetate with 0.04M LiCl. The mononucleotides were then eluted off the column and at this point the buffer was changed again to 1.0M LiCl in 0.01M lithium acetate. The last salt elution eluted all of the oligonucleotides and most of the contaminating DNA off the column. The absorbance of all fractions was then redetermined with a Beckman DB spectrophotometer.

Isotope Measurements

All cell fractions and column fractions were assayed for radioactivity by placing appropriate aliquots of material in Bray's counting solution and counting in a Beckman LS200 liquid scintillation counter (66). Paper strips (1 - 2 cm x 1 - 2 cm) from chromatograms were placed in counting vials with 1 ml water and shaken for one hour before Bray's scintillation fluid was added. Paper wicks from incubation flasks were placed directly in vials with the counting fluid. Radiochemicals were obtained from Schwarz BioResearch, Amersham/Searle, New England Nuclear and Calatomic. Radiochemical purity was checked by paper chromatography followed by liquid scintillation counting of 1 cm segments of the chromatogram.

Colorimetric Assays

Carbohydrate content of various fractions was determined by the addition of 1 ml anthrone reagent (0.2% anthrone in concentrated H_2SO_4) to 0.1 ml of sample in 0.4 ml water. The mixture was heated for 10 minutes at 100°C and read at 620 nm. Standard glucose solutions (5 - 20 ug/ml) were always run simultaneously (67). Both the glycogen isolated from the cells and the fractions from the DEAE column were assayed with the anthrone colorimetric test. It was found on the assay of the column effluent that the mononucleotide ribose reacted very little with the anthrone reagent; this may be due to the fact that there was far less ribose than glycogen. However, ribose color is reported to be destroyed when concentration of anthrone is above 0.05% (68).

Protein concentration was quantitiated by the Lowry technique (69). Human serum albumin (10 - 50 ug/ml) samples were used as standards.

DNA concentration was determined by the diphenylamine technique of Dische (70). DNA standards of 50 - 150 ug/ml were run simultaneously with the samples. In addition the diphenylamine test was used to determine DNA and RNA content together by using the Pederson modification of the Dische technique (71).

Hydrolysis

The mononucleotide and oligonucleotide fractions from the DEAE cellulose column were lyophilized and then desalted by dissolving the lyophilized sample in absolute methanol and precipitating with excess acetone (5 volumes) (72). The mononucleotides and oligonucleotides were then redissolved in methanol and reprecipitated with acetone to remove all lithium chloride.

The mononucleoside/glycogen fractions were lyophilized, dissolved in water and the glycogen precipitated with 1.1 volumes of cold absolute ethanol. The precipitated glycogen was added to that material obtained from the cold TCA extract and the combined fractions brought to volume in water, analyzed by anthrone test, and a portion reprecipitated with ethanol. The precipitate 1 ml of 1N H_2SO_4 was added and the solution heated for 3 hours at 100°C (67). The hydrolysate was neutralized with $Ba(OH)_2$ (saturated solution) and the solution frozen, thawed and centrifuged to remove salt. The neutralized hydrolysate was then chromatographed on paper in several solvent systems.

The protein fraction, after quantitation by the Lowry method, was divided and a small portion precipitated along with carrier bovine serum albumin in a hydrolysis tube. The precipitation was affected by adding 40% TCA dropwise to the protein solution. The tube was evacuated and sealed

after one ml 6N HCl was added to the precipitate. The tube was then placed in an autoclave at 20 pounds pressure and 121°C for 15 hours. The HCl was removed by repeated lyophilization after the addition of water and the final residue brought to volume with water.

The RNA (obtained as the alkaline hydrolysate by the procedure given previously) was further hydrolyzed by two methods. To obtain ribose the RNA mononucleotides were hydrolyzed with 1N H_2SO_4 for 1 hour at 100°C. This procedure released ribose from the purine mononucleotides. The hydrolysate was neutralized and simultaneously desalted with $Ba(OH)_2$ and then lyophilized. The lyophilized sample was then extracted with hot pyridine. The pyridine contained the ribose. The mononucleotides of RNA were also pyrolyzed with concentrated PCA in a sealed tube for 1 hour at 100°C to obtain the free purine and pyrimidine bases. The pyrolysate was neutralized and desalted with KOH and the supernate containing the free bases lyophilized and brought to volume.

The purine and pyrimidine bases of DNA were obtained by using the same procedure applied to RNA (73).

Paper Chromatography

RNA mononucleotides were chromatographed two dimensionally: first direction solvent A; second direction solvent B. The mononucleotides were visualized

by U.V. quenching in a "Chromatoview" light box. Purine and pyrimidine bases of both DNA and RNA were chromatographed unidimensionally in solvents C and B, and visualized in the "Chromatoview".

Protein hydrolysates were chromatographed in solvents D, E, F, and G. All protein hydrolysates, amino acid and β-amino acid chromatograms were visualized with a variation of the ninhydrin technique. To 100 ml of a 0.2% ninhydrin solution in aboslute ethanol 20 ml of glacial acetic acid, 4 ml of s-collidine and 7.5 ml of a 1% $Cu(NO_3)_2$ solution in ethanol were added (74). Chromatograms were sprayed and heated at 100°C for five minutes to develop color.

Glycogen hydrolysates were chromatographed in solvents A, B and I and the glucose visualized by spraying the chromatograms with a solution of 0.2% p-anisidine·HCl in ethanol with 4% orthophosphoric acid and additional concentrated HCl until the solution was clear. The chromatograms were heated in a drying oven at 100°C for 2 minutes.

Ethanol:acetic acid extracts of the cells incubated for radioactive CO_2 measurement were chromatographed in solvents F, G and H. Standards run with the extracts included: methylmalonic acid visualized by spraying chromatogram with a solution of bromcresol purple (40 mg

in 100 ml of 50% pH 10 ethanol); urea, β-ureidoisobutyric acid, dihydrothymine and dihydrouracil - visualized by spraying chromatogram with 0.5N NaOH, drying and the spraying with a solution of p-dimethylaminobenzaldehyde (1 gm in 100 ml 100% ethanol and 10 ml concentrated HCl); thymine and thymidine - seen as ultraviolet quenching spots; β-aminoisobutyric acid, alanine and β-alanine - identified as ninhydrin positive spots (27,30).

Solvent Systems

A. isobutyric acid (66):conc. NH_4OH (1):H_2O (33) - (75).

B. isopropanol (68):11.6N HCl (17.6):H_2O (14.4) - (76).

C. butanol saturated with water, 5% NH_3 in vapor phase - (77).

D. absolute ethanol (60):88% formic acid (5):t-butanol (20): H_2O (15) - (78).

E. butanol (55) then water saturated:glacial acetic acid (15) - (78).

F. t-butanol (40):methyl ethyl ketone (30):H_2O (20): NH_4OH (10) - (30).

G. upper phase of ethyl acetate (60):H_2O (35):formic acid (5) - (30).

H. t-butanol (44):methyl ethyl ketone (44):H_2O (11): formic acid (0.264) - (30).

I. butanol (4):acetic acid (1):water (5) - upper phase - (79).

Methoxyl Determination

The methoxyl groups of the RNA oligonucleotides (resulting from the o-methylation of the 2 position of RNA ribose) were determined by a modification of the method of Dekker (80). RNA oligonucleotides obtained from a DEAE

cellulose column fractionation of the RNA alkaline hydrolysate were pyrolyzed with concentrated PCA in a tightly sealed pyrolysis tube. After one hour at 100°C the tube was cooled and placed in a liquid nitrogen bath. The tube was opened and 2 mls of ice cold 50% methanol were added and mixed with the contents of the tube. One ml of material was transferred to a round bottom flask of a microdistillation apparatus. The flask was then heated to 65°C and carefully maintained at that temperature. The condenser arm of the apparatus was connected to the pump of a Forma constant temperature bath which was circulating a water/ethylene glycol mixture at -15°C. The collecting flask was placed in a dry ice-2-methoxypropanol bath. After several drops of methanol were collected, aliquots were removed and counted in Bray's solution on a liquid scintillation counter.

All experiments involving growth in the presence of radioactive nutrients were repeated at least once. Values from the replicate experiments were essentially the same as those of the original and revealed identical incorporation patterns into the various cell fractions. Incubations in isotopes for short lengths of time were generally carried out in triplicate with the values given representing the average of the two closest values. In none of the experiments did the values in any of the replicates vary appreciably (more than 10% of the higher number).

RESULTS

Preliminary Results

Tetrahymena was grown on the non-defined media described in the materials and methods section. The optical density at 660 nm and cell counts were taken at regular intervals over a sixty hour period. The generation time calculated for the culture in logarithmic phase was three and one half hours.

Cultures were grown to late log phase in the presence of thymidine-methyl-H^3 (Schwarz BioResearch) collected and fractionated as described except the extracted RNA was not further fractionated. The RNA fraction was found to contain about five percent of the total activity incorporated. When cultures in the stationary phase were inoculated with the same radioisotope, 24 percent of the total activity incorporated was found in the RNA fraction. In addition the lipid fraction from both of the above experiments contained considerable radioactivity -- greater than 18% of the total activity incorporated. Cells were also grown to late log phase in the presence of thymidine-6-H^3 and found to have almost no activity in the RNA fraction while the lipid fraction had less than half the activity of the lipid fraction from the methyl label experiment. "Pooling"

of the thymine methyl group was indicated because: 1) the methyl label tritium found its way into RNA and lipids while ring position tritium did not do so. 2) Stationary phase cells incorporated five times more label into RNA than log phase cells (little DNA synthesis was presumably taking place and thymine could serve more readily as a methyl donor).

Growth With Radiolabeled Methionine

In an attempt to become more familiar with the one carbon metabolism of Tetrahymena that involves the methylation of RNA (for which methionine is a classic methyl group contributor) cultures were grown to late log phase in the presence of methionine-methyl-H^3 (81). Cells were collected, washed and fractionated as described in the methods section. The distribution of radioactivity incorporated into various cell fractions is given in Table I.

The RNA fraction was placed on a DEAE cellulose column and separated into mononucleosides, mononucleotides and oligonucleotides with batch elutions of buffer containing various amounts of LiCl salt. The results of the column fractionation are given in fig. 1.

The mononucleosides were heavily contaminated with glycogen. The mononucleotide and oligonucleotide fractions, despite their ribose content, gave negligible color with

TABLE I

Incorporation of Tritium From Methionine-Methyl-H^3 Into Various Cell Fractions of *Tetrahymena*

Fraction	% Incorporation of Isotope
Acid Soluble	21.7
Lipid	41.5
RNA	5.6
DNA	8.7
Protein	16.3
Carbohydrate	6.6

Figure 1. DEAE cellulose column fractionation of alkaline hydrolyzed RNA from cells grown in methionine-methyl-H^3. Material eluted with 0.01 M lithium acetate - pH 5.0 buffer, 0.04 M LiCl, and 1.0 M LiCl at points indicated. Fractions were assayed for 260 nm absorbance with an LKB Uvicord I and a Beckman DB spectrophotometer. From each fraction 100 ul samples were assayed with anthrone reagent and measured at 620 nm. From each fraction additional 100 ul samples were removed and counted in 10 ml of Bray's solution in a Beckman LS200 scintillation counter. A_{260} • • • A_{620} -•-•-•- CPM/100 ul ——•——•——.

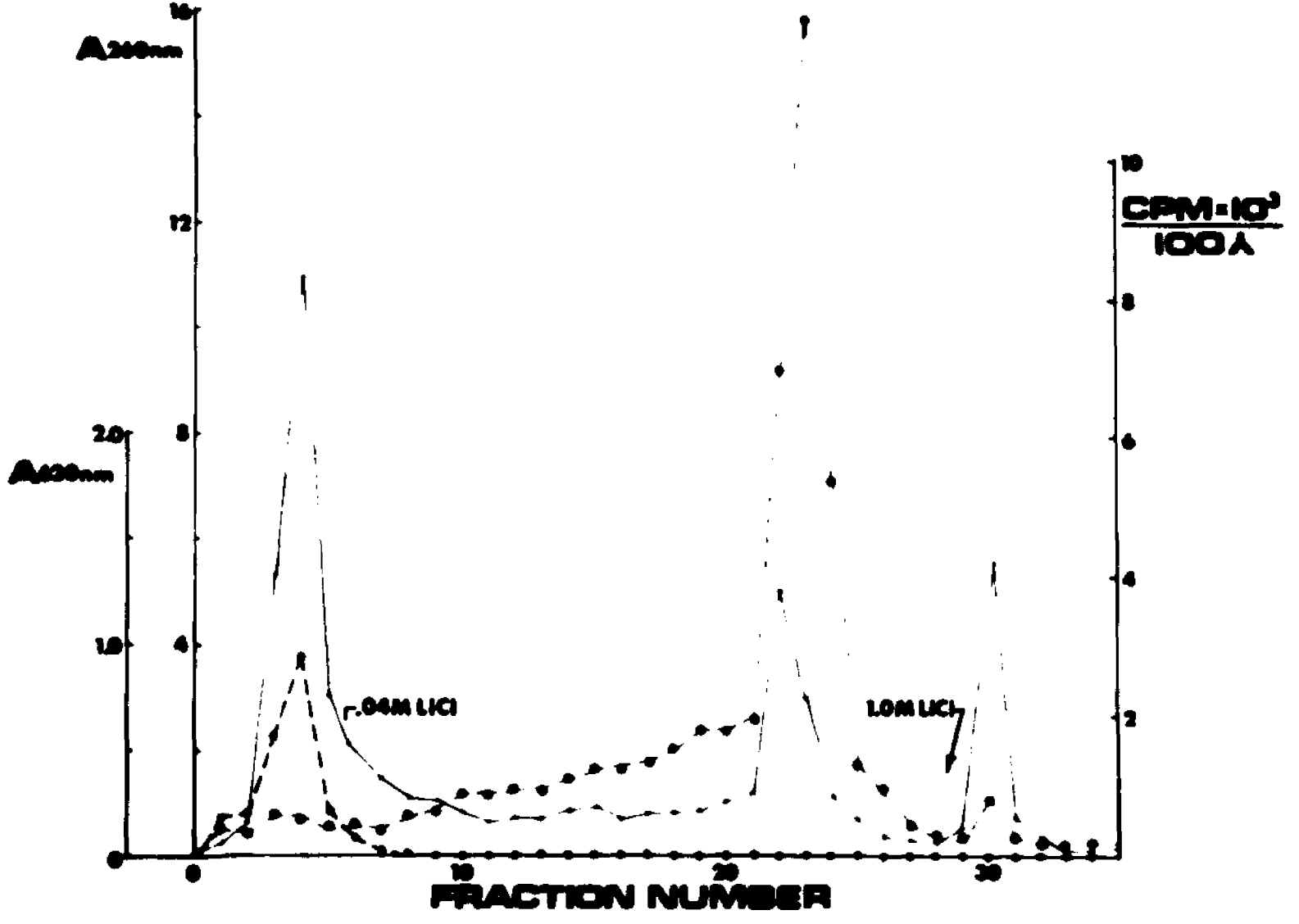

A280nm
A400nm
CPM×10³ / 100 λ
.04M LiCl
1.0M LiCl
FRACTION NUMBER

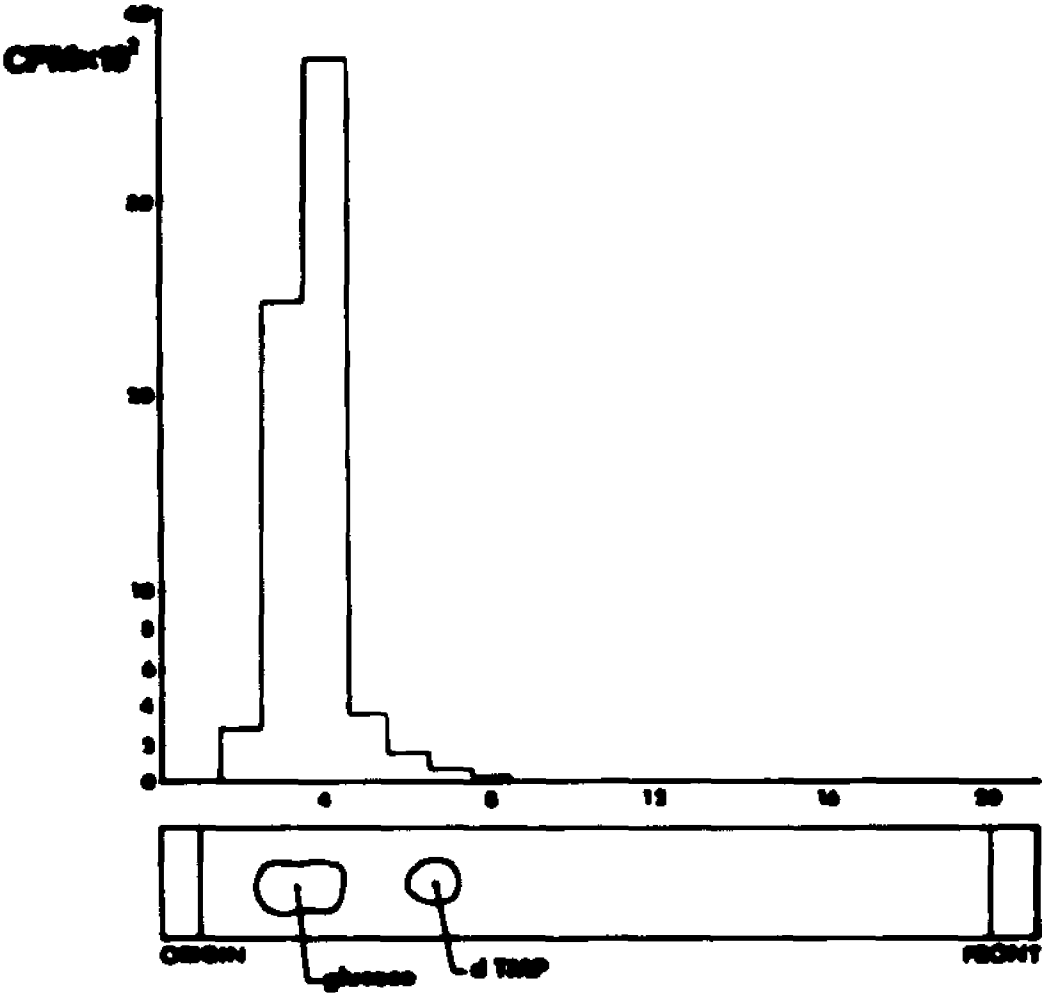
FIGURE 1

anthrone reagent. The fractions from the column which contained mononucleosides and glycogen were pooled and lyophilized. The lyophilisate was dissolved in a small amount of water and 1.1 volumes of ethanol added to precipitate the polysaccharide. The precipitate was added to that carbohydrate material obtained from the acid soluble fraction. A portion of the carbohydrate was precipitated and hydrolyzed, neutralized/desalted, and chromatographed. The results are given in fig. 2.

Deoxythymidylic acid (dTMP) was run simultaneously in all carbohydrate chromatograms because Counts and Flamm have reported that glycogen specifically binds dTMP (82). In addition solvent A was found to separate glucose from all the major ribonucleotides (and dTMP and thymidine), ribonucleosides, and free bases and was therefore used as an additional check for the correspondence of radioactivity with glucose. Ninety-nine percent of the radioactivity in the mononucleoside fraction was in glucose. The glycogen had a specific activity of 140,700 cpm/mg of glucose.

The mononucleotide-containing fractions from the column were pooled, lyophilized and dissolved in a small amount of water. Thirty microliters containing 13,000 cpm were spotted in one corner of a large sheet of Whatman #1 filter paper and chromatographed two-dimensionally in solvents A and B. The chromatogram was cut up in

Figure 2. Distribution of radioactivity on a chromatographic strip. Polysaccharide obtained from cells grown in methionine-methyl-H^3 was hydrolyzed and chromatographed in solvent A. Chromatogram was cut into 2 cm strips and each strip placed in a vial with one ml of water and shaken for one hour. After shaking 10 ml of Bray's scintillation fluid was added to the vial and the solution counted in Beckman LS200 scintillation counter. Deoxythymidylic acid (dTMP) added to polysaccharide hydrolysate as a marker to test if the glycogen had bound any thymidylate (see text). Material containing approximately 14,000 cpm was spotted at origin. Areas containing carbohydrate material were visualized by running a second chromatogram in an adjacent lane and after development in solvent spraying with p-anisidine reagent. Thymidylic acid was seen as U.V. quenching area.



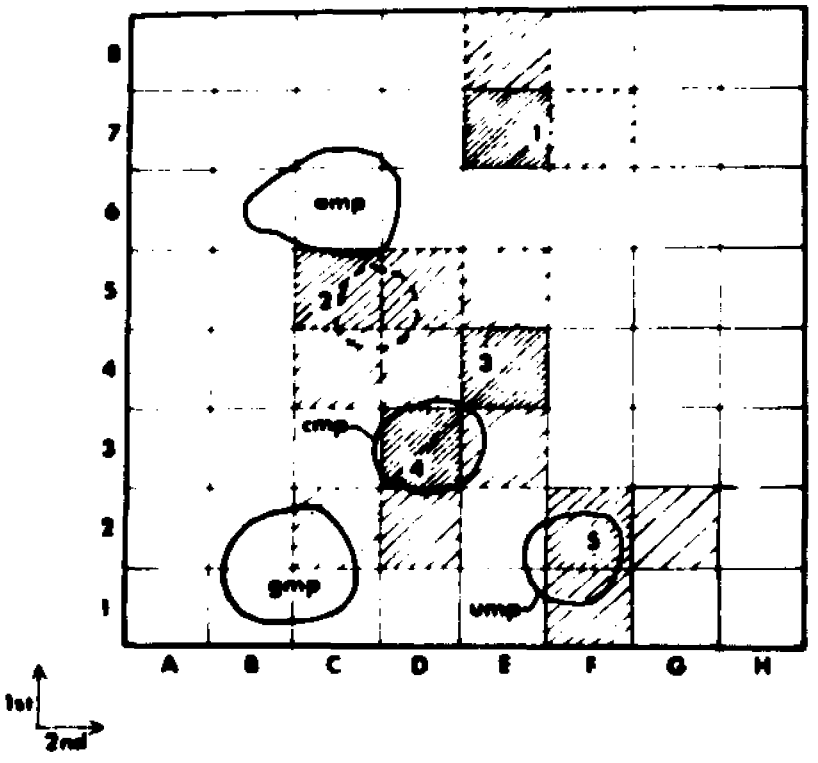
"checkerboard" fashion after locating the four major ribonucleotides by ultraviolet quenching (253 nm). The sections which were 2 cm^2 were assayed for radioactivity and the results given in fig. 3.

The radioactive sections correspond to methylated derivatives of the normal ribonucleotides. From replicate chromatograms, areas corresponding to methyladenylic acid were eluted, lyophilized, hydrolyzed and rechromatographed in one dimension. The activity was located solely with the methyladenines and not with the ribose or adenine. Pyrolysis of a portion of the mononucleotides with PCA and subsequent chromatography in solvents B and C also revealed the methylated derivatives of the four major bases.

The oligonucleotide fractions contained little 260 nm absorbing material. The fractions were pooled and lyophilized. The lyophilisate was pyrolyzed with concentrated PCA in a sealed tube, frozen, opened, carrier methanol added and placed in a microdistillation apparatus. The procedure was repeated on a sample of the mononucleotides. It was found that the oligonucleotides contained 22.3% of their radioactivity in distillable methanol after pyrolysis and the mononucleotides 7.7%. The high value of the mononucleotides can possibly be explained by the fact that tritium label was used and some distillable products other than methanol may have been formed during pyrolysis.

Figure 3. Distribution of radioactivity on a two dimensional chromatogram. Mononucleotides resulting from the alkaline hydrolysis and DEAE fractionation of RNA from cells grown in methionine-methyl-H^3 were chromatographed first in solvent A and the second direction in solvent B. Approximately 13,000 cpm was spotted in the lower left hand corner of the Whatman paper. The chromatogram was cut into 2 cm^2 strips and counted as before. Spots indicated are areas of either quenching or flourescence under short wave U.V. light and correspond to the four major mononucleotides (2'-3' isomers). Hatched squares correspond to radioactive areas of the chromatogram with each line representing 20 cpm above background. Only squares with 50 cpm or more above background have hatched lines. Spots: 1--corresponds to the methyladenylic acids; 2--seen as faint blue flourescence corresponds to 7-methylgunylic acids; 3--methylcytidylic acid; 4--methylguanylic acids; 5--methyluridylic acid (ribothymidylic acid).

FIGURE 3



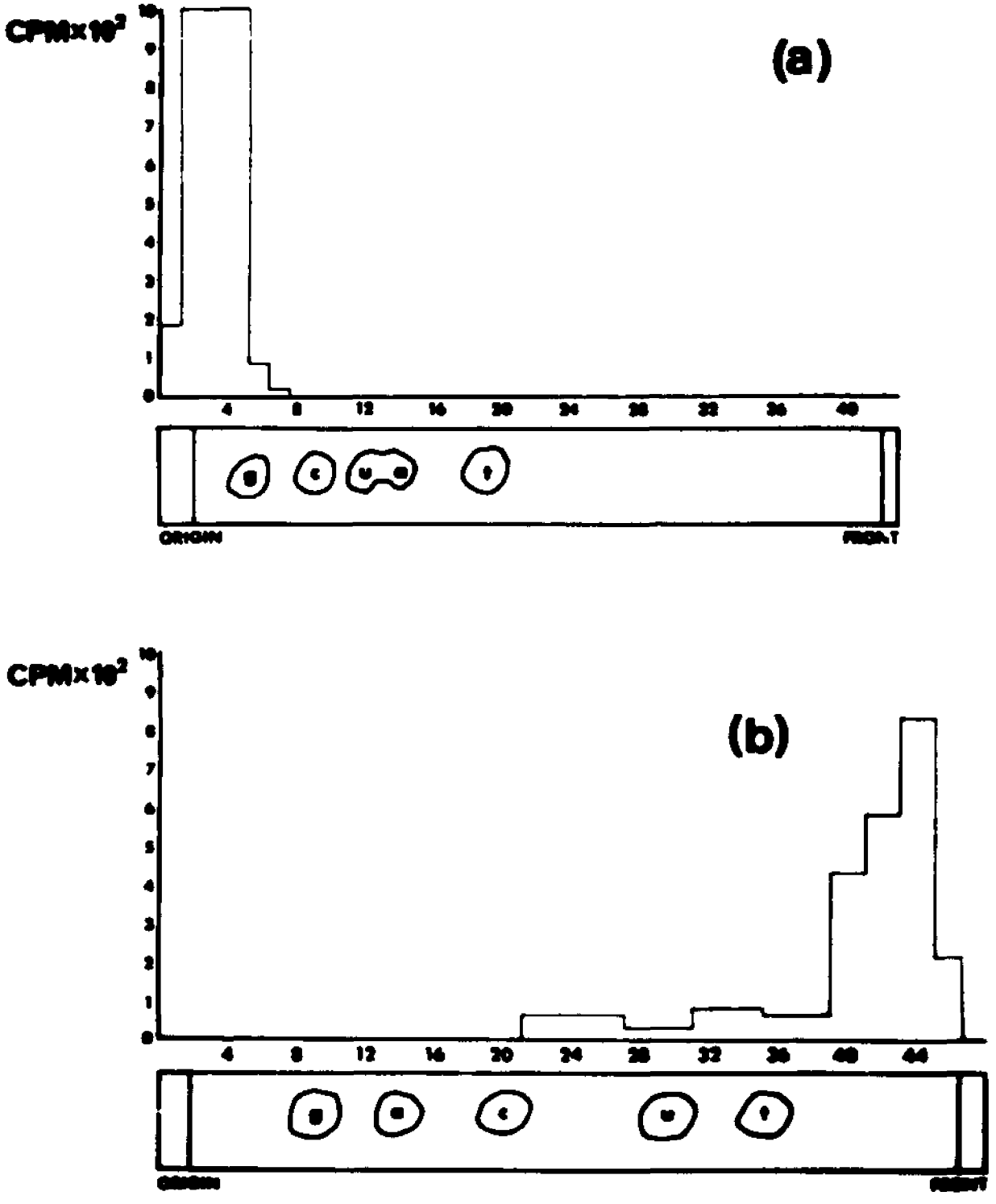
It is therefore concluded that at least 15% of the radioactivity of the oligonucleotides is due to the 2-O-methyl groups on the ribose moiety of RNA.

The DNA fraction was pyrolyzed and chromatographed in several solvent systems; no radioactivity was found in thymine. The bulk of the radioactivity was found at the origin in solvent system C and streaked with some accumulation of products towards the fron in system B (fig. 4). Past experience has revealed that some sugar hydrolysis products not carbonized during pyrolysis chromatograph in a similar fashion. Although the sugar was not isolated per se, the chromatographs indicate the activity in the deoxyribosenucleic acid may very well be in the deoxyribose moiety. In addition the oligonucleotide fraction after hydrolysis and chromatography showed considerable radioactivity in those same areas. This is consistent with our finding that small amounts of DNA contaminate the RNA fraction and are eluted from the column with the oligonucleotides.

The protein fraction revealed mostly radioactive methionine after hydrolysis and chromatography. There was some background activity throughout the chromatograms but with only one ninhydrin positive spot containing as much as four percent of the activity of methionine spot. The spot was not identified.

Figure 4. Distribution of radioactivity on chromatographic strips. DNA from cells grown in methionine-methyl-H^3 was pyrolyzed and 9600 cpm spotted on each of two chromatographic strips along with carrier purines and pyrimidines. Chromatograms were developed in solvents C (strip a) and A (strip b). Chromatogram (a) was cut into 1 cm strips while (b) was cut into 2 cm strips. Counting procedure as in figure 2.

FIGURE 4


CPM×10²
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Incubation With Thymidine-Methyl-H^3

With some knowledge gained of Tetrahymena's one carbon metabolism, cells were again incubated in thymidine-methyl-H^3 (Schwarz BioResearch) to late log phase, collected and fractionated as described. The distribution of radioactivity is given in table II.

The RNA fraction was placed on a DEAE cellulose column to remove contaminating carbohydrate and DNA. The results of the column fractionation are given in fig. 5.

The polysaccharide was separated, hydrolyzed, and chromatographed as in the previous experiment. The results are given in fig. 6.

The mononucleotide-containing fractions were pooled, concentrated and chromatographed two dimensionally. The chromatogram is reproduced in fig. 7.

The UMP spot was cut out from replicate chromatograms and eluted; the nucleotide was pyrolyzed, neutralized and chromatographed in several systems. Since ribothymidylic acid chromatographs closely to UMP in our two dimensional system, solvent systems were employed that separate uracil from thymine. The results are given in fig. 8.

TABLE II

Incorporation of Tritium From Thymidine-Methyl-H^3 (Schwarz) Into the Various Cell Fractions of Tetrahymena

Fraction	%Incorporation
Acid Soluble	5.4
Lipid	16.3
RNA	3.3
DNA	54.5
Protein	16.7
Carbohydrate	2.0

Figure 5. DEAE cellulose column fractionation of alkaline hydrolyzed RNA from cells grown in thymidine-methyl-H^3 (Schwarz). Details as in fig. 1.

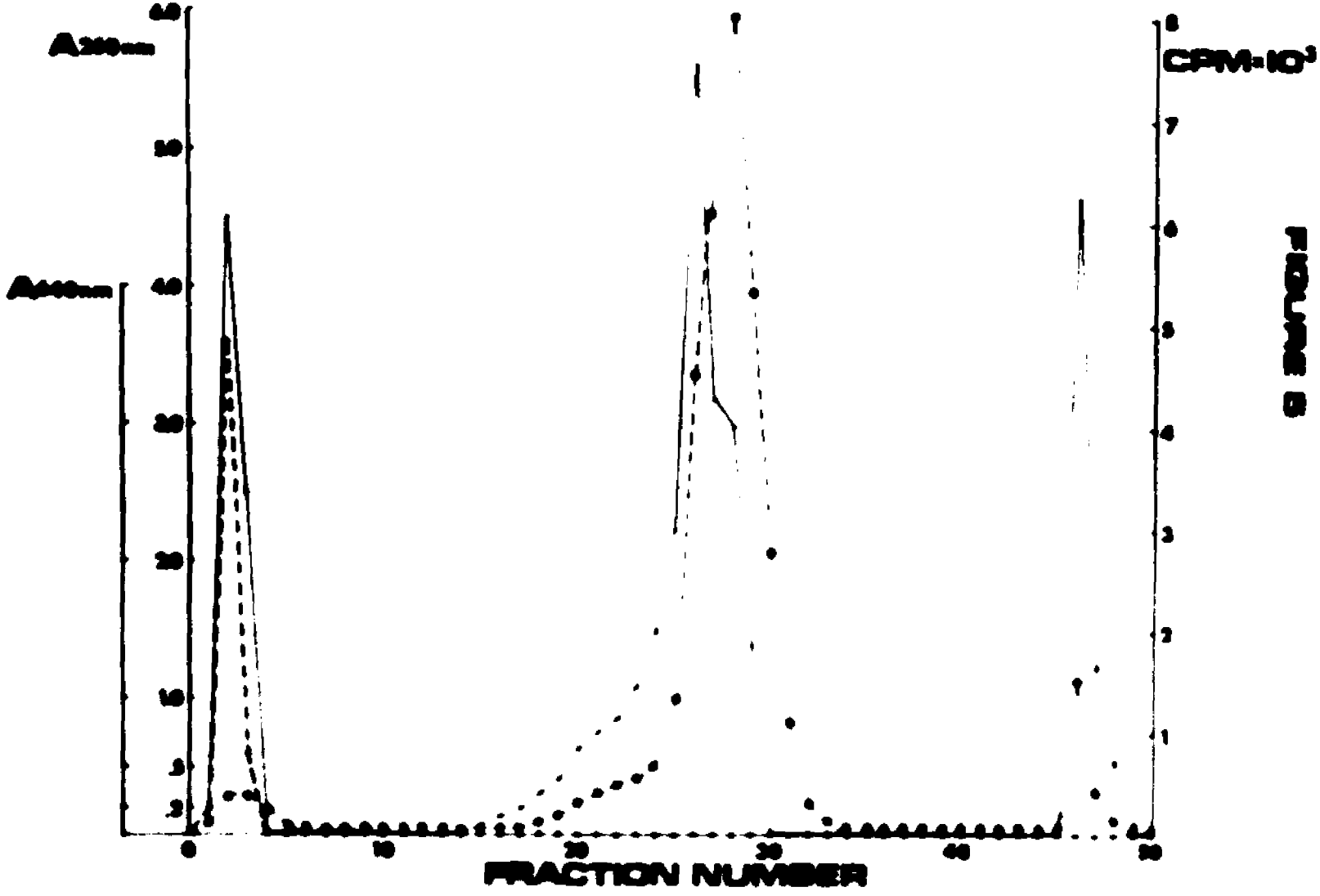


FIGURE 8

Figure 6. Distribution of radioactivity along a chromatographic strip. Polysaccharide obtained from cells grown in thymidine-methyl-H^3 (Schwarz) was hydrolyzed and a sample with 3500 cpm was chromatographed in Solvent A. Details as in figure 2.

FIGURE 6

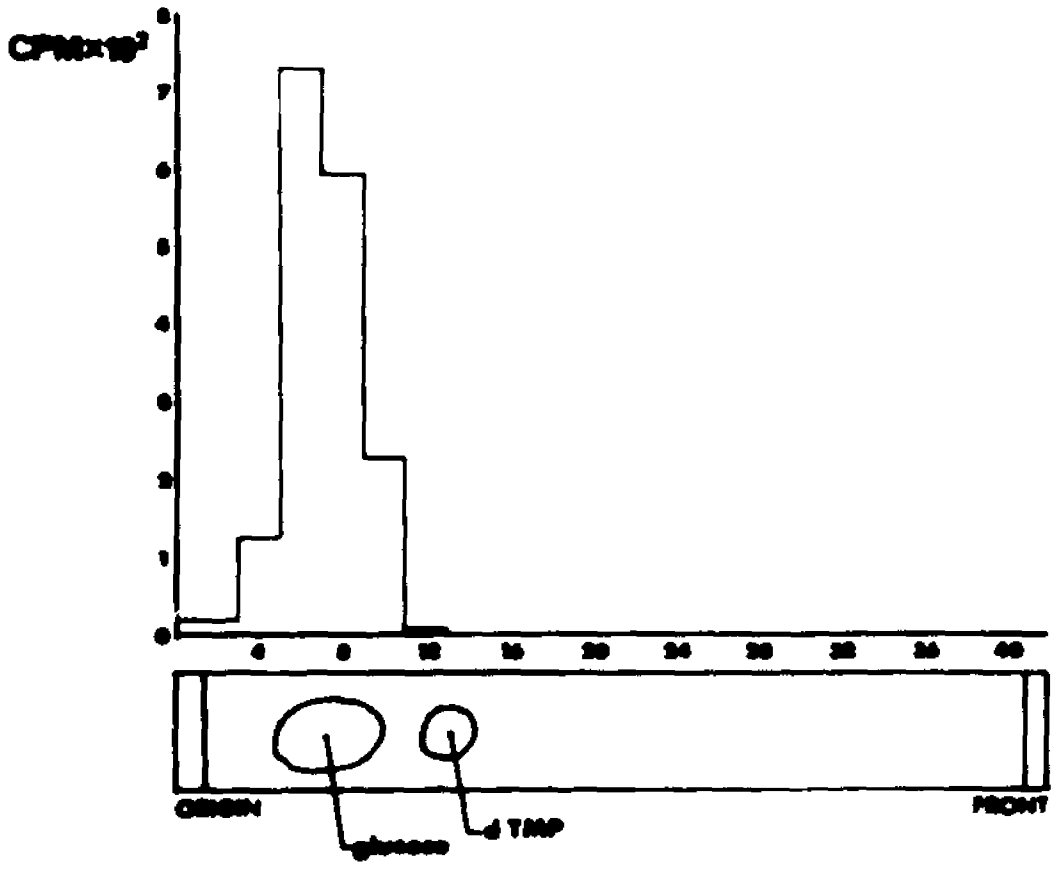


Figure 7. Distribution of radioactivity on a two dimensional chromatogram. Mononucleotides resulting from the alkaline hydrolysis and DEAE fractionation of RNA from cells grown in thymidine-methyl-H^3 (Schwarz) were chromatographed first in solvent A and secondly in solvent B. A sample of the mononucleotides containing approximately 5,000 cpm was spotted in the lower left hand corner of the Whatman #1 sheet. Counting procedure as in figure 3.

FIGURE 7

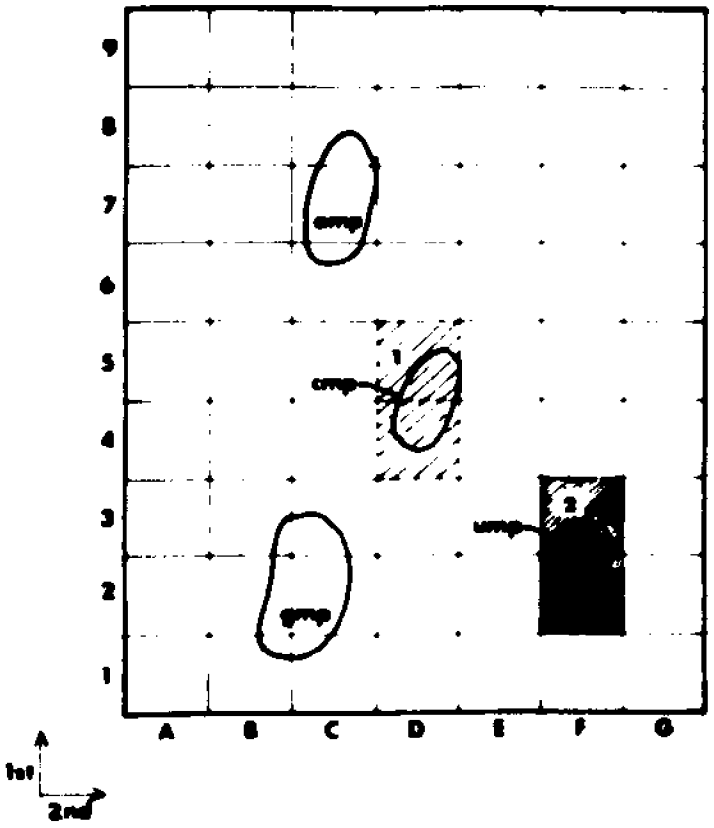
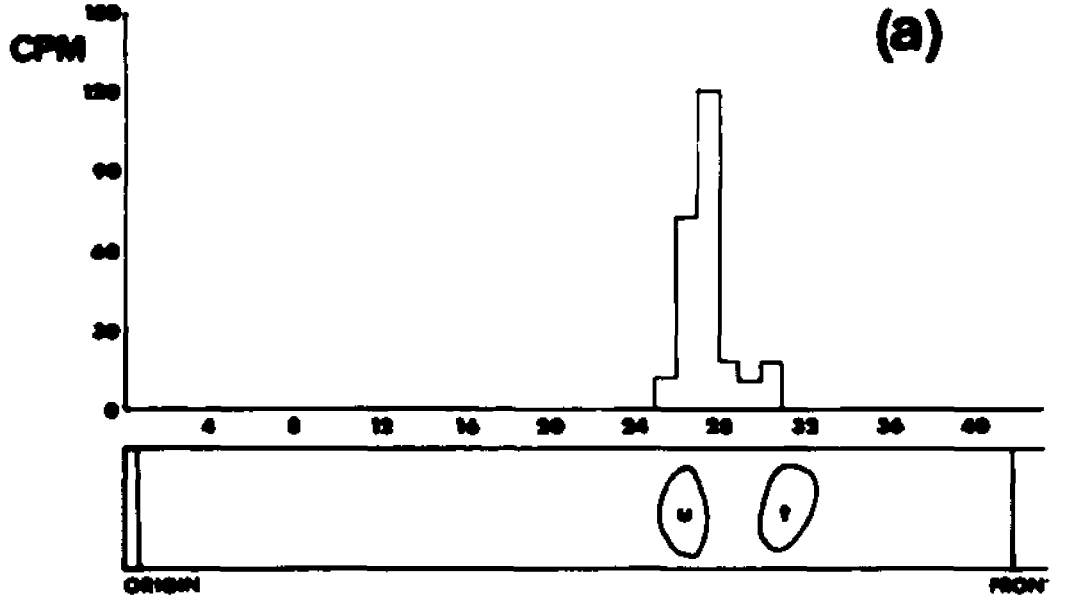
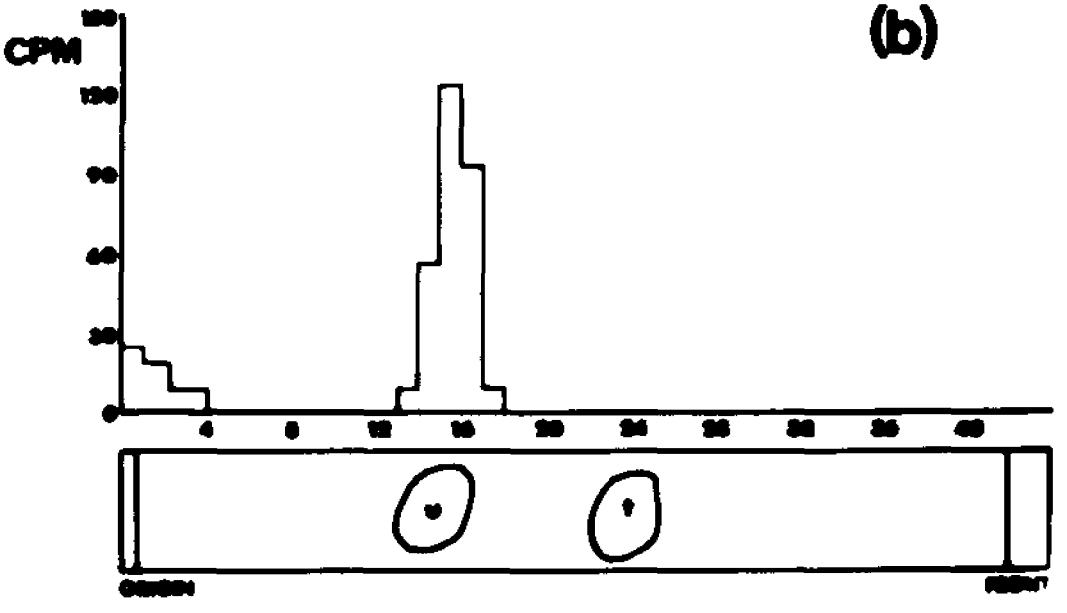


Figure 8. Distribution of radioactivity along several chromatographic stripe. The spot corresonding to UMP on a two dimensional chromatogram was cut out, eluted, pyrolyzed, neutralized and chromatographed in two solvent systems. Strip (a) was developed in solvent B and strip (b) in solvent C. Counting procedure as in figure 2.

FIGURE 8





Direct pyrolysis of mononucleotides gave similar results -- uracil and cytosine were the only radioactive spots. In addition no radioactivity was found in the ribose extracted from the RNA.

The oligonucleotide fraction was pyrolyzed and tested for any distillable radioactivity and none was detected. The pyrolysate was chromatographed and revealed mostly radioactive thymine. These results indicate that DNA contamination of the RNA fraction is the major source of radioactivity in the oligoribonucleotides. There was no detectable thymine in either the mononucleoside or mononucleotide fractions, the oligonucleotides alone contained that contamination. The amount of DNA present in that fraction was small and below the limits of detection by the diphenylamine test. A portion of the DNA fraction was pyrolyzed and it contained only thymine as a radioactive base.

A chromatographic check, made before the experiment was performed, revealed that better than 99% of the radioactivity was in thymine, but the chromatogram was assayed using a Baird Atomic 4 Pi scanner and considerable tritium activity in other areas could have been missed. The manufacturer's assay showed thymine as the only detectable contaminant, and it contained less than one percent of the total activity. The thymidine was still suspect. The experiment was

repeated with Schwarz thymidine and the results were essentially identical. The experiment was then repeated using thymidine-methyl-H^3 from New England Nuclear Corporation. The results are listed in table III.

Great care was taken to limit cross contamination. The mononucleotides and oligonucleotides contained almost no radioactivity. The carbohydrate fraction upon hydrolysis and subsequent chromatography revealed only radioactive glucose. DNA was hydrolyzed and it was found to contain thymine as the only radioactive component. When the experiment was repeated with thymidine-methyl-H^3 supplied by Amersham/Searle essentially identical results to those of the New England Nuclear experiment were obtained. The introduction of radioactivity into the glycogen of Tetrahymena was consistent no matter what the commercial source of thymidine-methyl-H^3. Cells were next grown for one, two and five generations in the presence of methyl labeled thymidine to test for any differences in long and short term label exposure. After three hours ($<$ one generation) the glycogen fraction had a specific activity of 3,384 cpm/gm which doubled after eight hours (two generations) and leveled off thereafter. It was concluded the pathway to glycogen from thymidine was real and not a fortuitous event of long exposure to the radioactive material. It was further concluded that the labeling of RNA uracil and cytosine was due to an artifact of Schwarz thymidine

TABLE III

Incorporation of Tritium From Thymidine-Methyl-H^3 (New England Nuclear) Into the Various Cell Fractions of Tetrahymena

Fraction	% Incorporation
Acid Soluble	12.2
Lipid	6.6
RNA	< 0.1
DNA	75.7
Protein	1.9
Carbohydrate	3.6

(probably a uracil contaminant) and that there is no relation between the methyl group of thymine and the one carbon pool of Tetrahymena.

Pyrimidine Catabolism

The problem of connecting pyrimidine metabolism to carbohydrate and lipid metabolism still remained. The logical pathway from thymine to glucose would be through the pyrimidine catabolic scheme. Pyrimidine breakdown is not described in protozoa and it was necessary to establish the existence of such a pathway and whether it is a reductive or oxidative one. Mid-log cells were therefore collected, pooled and suspended in fresh media to make a 1.5% cell suspension (2×10^6 cells/ml). Three mls of the cell suspension were placed in each of six modified Cavett flasks. To three of the flasks 1 uC of thymidine-2-C^{14} was added and to the remaining three flasks 1 uC of thymidine-methyl-C^{14} was added. Into two flasks, one with ring label and the other with methyl-labeled thymidine, was pipetted 1 ml of 40% TCA. The remaining flasks were shaken at 27°C in a Dubnoff shaker for three hours before the reaction was stopped by injecting 1 ml of 40% TCA. After the addition of the acid the flasks were shaken for an additional 15 minutes then the KOH soaked paper wicks were removed and counted. The results are in table IV.

TABLE IV

$C^{14}O_2$ Released From Cells Incubated in Radiolabeled Thymidine

	$C^{14}O_2$ Released (CPM)
Thymidine-methyl-C^{14}	6,000
Thymidine-2-C^{14}	50,000

1 uCu incubated for three hours with a 1.5% cell suspension (2 x 10^6 cells/ml) at 27°C. Values are corrected for both background and zero time counts.

The above experiment was repeated using thymidine-2-C^{14} and varying amounts of dihydrothymine, dihydrouracil and β-aminoisobutyric acid in an attempt to dilute out the production of $C^{14}O_2$ with nonradioactive intermediates of the thymine and uracil degradative scheme -- the results are given in table V. Dihydrouracil was used since Grisolia has shown that in several organisms the reduction or uracil to DHU and thymine to DHT involves the same enzyme at different efficiencies (28). As a further check of the ability of the enzyme system to handle uracil a cell suspension in Cavett flasks was incubated with uridine-2-C^{14}. Dihydrothymine and dihydrouracil were added to several flasks in an attempt to dilute the $C^{14}O_2$ production if the 2-position carbon of uracil could indeed be released as CO_2. The results are given in table VI.

Since thymine is being degraded by the reductive pathway it was reasoned that one or more of the intermediates could be isolated. Cells were grown to mid-log stage, collected, washed, and suspended in buffer (Ryley Ringer phosphate) to make a 4.0% suspension (5.5×10^6 cells/ml). Cells were placed in two groups; half inoculated with thymidine-2-C^{14} and half with thymidine-methyl-C^{14}. After 45 minutes incubation at 27°C the cells were spun down and washed and the wash plus the original supernate pooled and lyophilized. The cells were then extracted for 2 hours at room temperature with 5 volumes

TABLE V

Effect of Increasing Concentrations of Nonradioactive DHT, DHU and BAIB on Release of $C^{14}O_2$ From Thymidine-2-C^{14}

Nonradioactive DHT (u moles)	$C^{14}O_2$ Release (CPM)
0	11,000
5	400
10	211
20	130
Nonradioactive DHU (u moles)	
0	11,000
5	6,300
10	3,030
20	2,450
Nonradioactive BAIB (u moles)	
0	13,100
5	13,100
10	15,400
20	13, 270

2% cell suspension incubated 30 minutes at 27°C with 0.5 uCi of radiolabeled material in Ryley Ringer Phosphate

TABLE VI

$C^{14}O_2$ Released From Cells Incubated in Uridine-2-C^{14} and the Effect of Dihydrothymine and Dihydrouracil on that Release

	$C^{14}O_2$ Released (CPM)
Uridine-2-C^{14}	9,330
Uridine-2-C^{14}+10 uM DHT	260
Uridine-2-C^{14}+10 uM DHU	1,100

4% cell suspension incubated one hour at 27°C with 0.5 uCi of radiolabeled material in Ryley Ringer phosphate.

of a mixture of 3 parts absolute ethanol to 1 part glacial acetic acid (4). The mixture was centrifuged and the precipitated cells washed in 2 parts of 70% ethanol. The wash was added to the extract and the mixture concentrated to one ml. The lyophilized supernatants were each brought to one ml. Samples of the supernates of cells grown in ring and methyl labeled thymidine were then chromatographed in three systems: Fink's solvents 1, 5 and 8 (our solvents F, G and H). Similarly alcohol/acetic acid extracts of the cells grown in ring and methyl labeled thymidine were chromatographed in the same systems. It can be seen from the chromatograms in figs. 9, 10 and 11 that thymidine-methyl-C^{14} does lead to β-aminoisobutyric acid and that some of this material is excreted into the media. No detectable DHT was obtained from thymidine-2-C^{14} but it can be seen from figs. 9, 10 and 11 that all the thymidine is degraded to thymine. In another experiment it was found that after 15 minutes incubation virtually 100% of the thymidine was converted to thymine by Tetrahymena.

Reincorporation of Degradative Products

Since thymidine-methyl-C^{14} was converted into β-aminoisobutyric acid and previous experiments have shown that the carbon skeleton could be converted to glucose it was felt that the possible connecting link was β-aminoisobutyric acid being converted to methylmalonic acid and subsequently to succinyl CoA. Since essentially no radioactive methylmalonic acid was discovered in the chromatograms of figs. 9

Figure 9. Distribution of radioactivity along two chromatographic strips. Strip (a) is a chromatogram of the incubation medium of cells grown for 45 minutes in thymidine-methyl-C^{14} (6,600 cpm spotted). Chromatogram (b) is of an alcohol:acetic acid extract of the cells themselves (4,600 cpm spotted). Both chromatograms were developed in solvent H. Standards including methyl-malonate, glucose, urea, thymidine, β-ureidoisobutyrate, α-alanine, β-alanine and dihydrothymine were run in adjacent lanes simultaneously, and the spots were visualized by various reagent sprays indicated in text. All spots other than BAIB and thymine contained little or no activity and are therefore omitted for the sake of clarity.

FIGURE 8

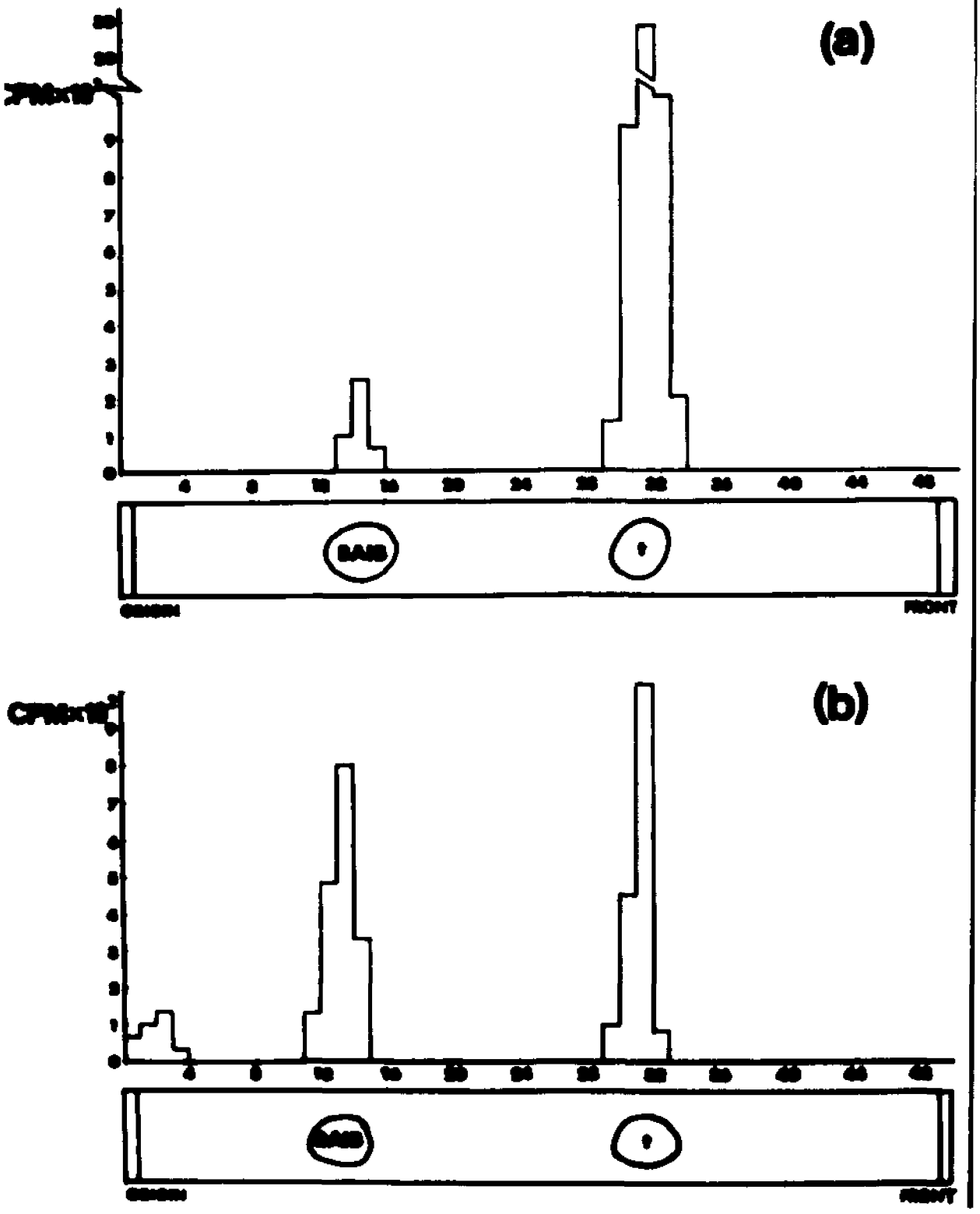


Figure 10. Distribution of radioactivity along two chromatographic strips. Same as figure 9 only the chromatograms were developed in solvent F.

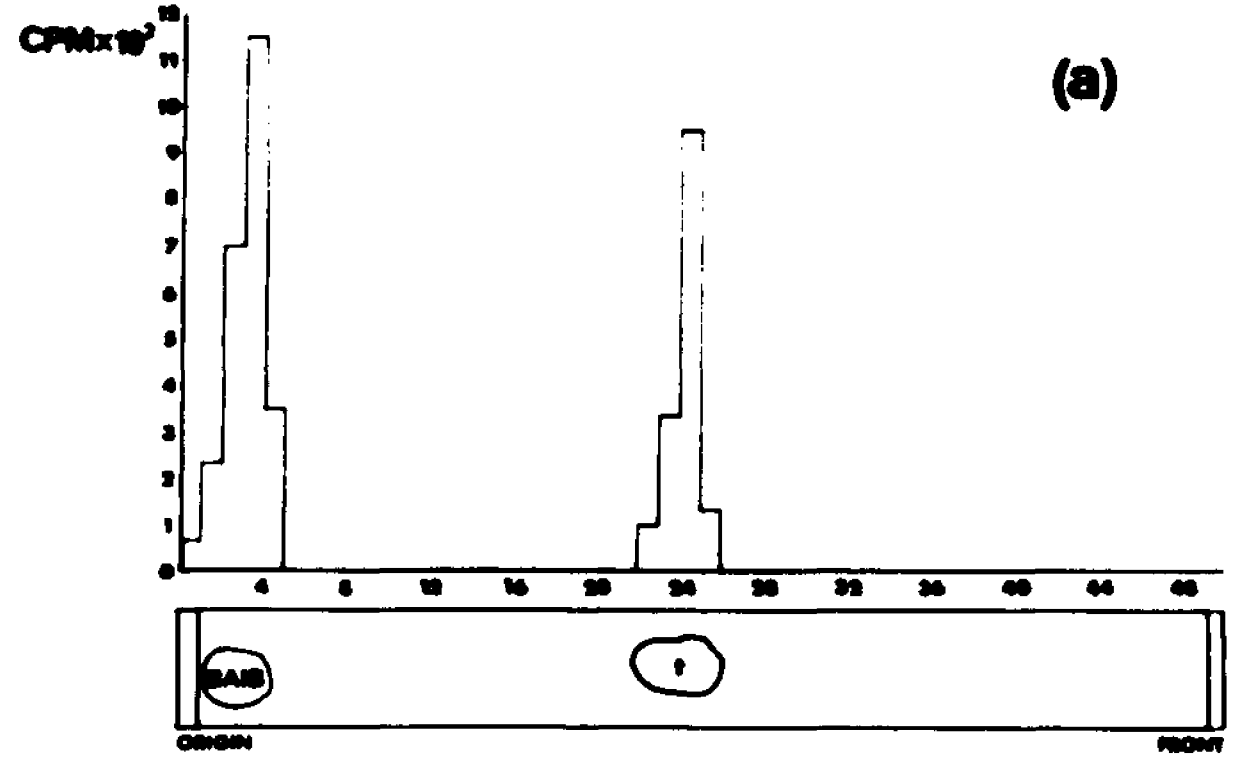
(a)

(b)

FIGURE 10

Figure 11. Distribution of radioactivity along two chromatographic strips. Strip (a) is a chromatogram of the ethanol:acetic acid extract of the cells incubated in thymidine-methyl-C^{14} (4,600 cpm spotted). Strip (b) is a chromatogram of material eluted at the level of BAIB from a chromatogram of cell extract as in figure 10(b). Both chromatograms were developed in solvent G. Details as in figure 9.

FIGURE 11


CPMx10²
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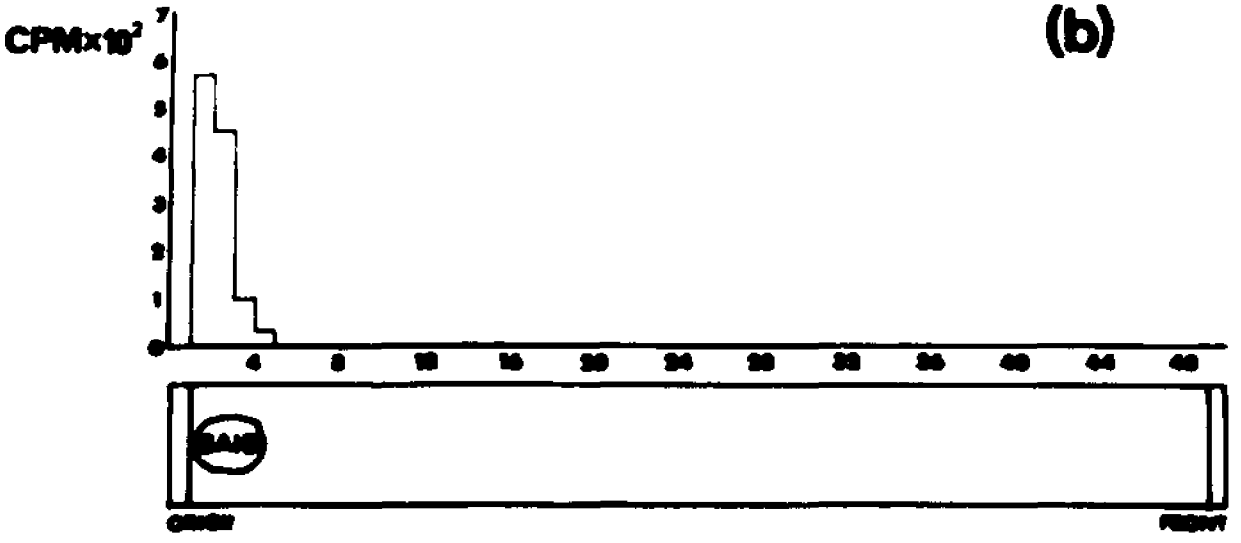
CPMx10²
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and 10 other evidence was required. It was reasoned that if thymidine-methyl-C^{14} could be converted to succinyl CoA, under conditions of incubation in buffer (i.e. starvation) the succinyl CoA could then be directed into the Krebs' cycle rather than into gluconeogenesis. A 4.0% suspension of Tetrahymena was pipetted in 12 Cavett flasks. Half were inoculated with 0.5 uC thymidine-methyl-C^{14} and half with 0.5 uC thymidine-2-C^{14}. The flasks were incubated for 0, 15, 30, 60, 90 and 120 minutes and the amount of $C^{14}O_2$ released determined as before. The results are given in fig. 12. Cells were also incubated in modified Cavett flasks with thymidine-methyl-C^{14} and nonradioactive β-aminoisobutyric acid and methylmalonic acid in an attempt to dilute the radioactive CO_2 being released. The results are given in table VII.

It can be seen that in time an increasing amount of the methyl carbon of thymidine is released as $C^{14}O_2$. Tetrahymena seems to be able to use the carbon skeleton of thymine both for gluconeogenesis and as an immediate carbon source for energy. It may be argued that several of the Krebs' cycle intermediates which normally transaminate to form alanine, glutamic acid and aspartic acid would do so and yield the respective radioactive molecules if the cells were incubated with thymidine-methyl-C^{14}. Two groups of cells were grown to mid log phase in the presence of ring (2 position) and methyl-labeled C^{14} thymidine.

Figure 12. Release of $C^{14}O_2$ by cells incubated in C^{14}-labeled thymidine. The graph compares the rate of $C^{14}O_2$ released by 4% suspensions of Tetrahymena incubated in buffer with thymidine-2-C^{14} —■—■—■—■— and thymidine-methyl-C^{14} —●—●—●—●—

FIGURE 12

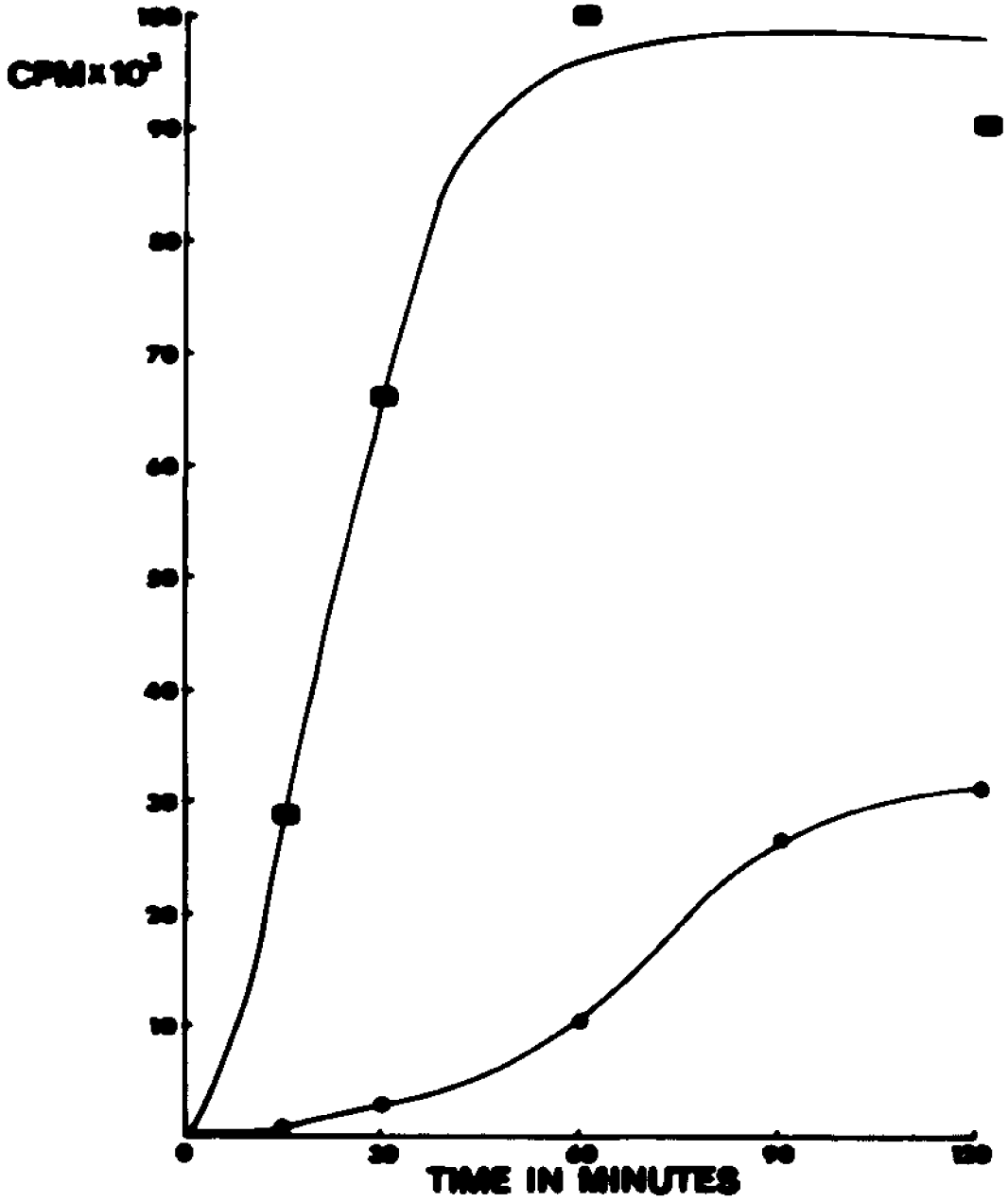


TABLE VII

$C^{14}O_2$ Released From Cells Incubated in Thymidine-Methyl-C^{14} and the Effect of BAIB and Methylmalonic Acid (MMA) on that Release

	$C^{14}O_2$ Released (CPM)
Thymidine-methyl-C^{14}	9,223
Thymidine-methyl-C^{14}+10 uM BAIB	3,402
Thymidine-methyl-C^{14}+25 uM MMA	4,745

2% cell suspension incubated two hours at 27°C with 0.5 uCi of radiolabeled material in Ryley Ringer phosphate.

Cells were collected and worked up as before, and a comparison of incorporation into various fractions follows in table VIII.

The RNA fractions were chromatographed on DEAE cellulose and separated into mononucleoside, mononucleotide and oligonucleotide. All three fractions above from the methyl-label experiment were radioactive. The material from the ring label experiment contained little radioactivity in any of the fractions. The mononucleosides from the methyl-labeled experiment contained glycogen which was pooled with the material from the acid soluble fraction, hydrolyzed and chromatographed. Figure 13 illustrates that glucose was the only radioactive component.

The mononucleotide fraction of the methyl-label experiment was pyrolyzed and yielded no radioactive bases but a great deal of radioactivity in the areas of the chromatograms which contained undefined material. The mononucleotides were chromatographed two dimensionally in systems A and B and each of the four mononucleotides was found to be radioactive. Figure 14 illustrates the two dimensional chromatogram. AMP was eluted from a replicate two dimensional chromatogram, hydrolyzed for one hour at 100°C in 1N H_2SO_4, neutralized and chromatographed in solvent C with carrier adenine and ribose; all the activity was found associated with the ribose spot. Samples of the mononucleotide and oligonucleo-

TABLE VIII

Percent of Total C^{14} Activity Incorporated Into Various Cell Fractions of Tetrahymena Incubated in Thymidine-Methyl-C^{14} and Thymidine-2-C^{14}

Fraction	Methyl	Ring (2 Position)
Acid Soluble	1.1	11.2
Lipid	21.8	1.3
RNA	5.5	0.8
DNA	64.3	85.6
Protein	4.9	0.2
Carbohydrate	2.5	0.9

Figure 13. Distribution of radioactivity along a chromatographic strip. Polysaccharide obtained from cells grown in thymidine-methyl-C^{14} was hydrolyzed and chromatographed in solvent B. Details as in figure 2.

FIGURE 1B

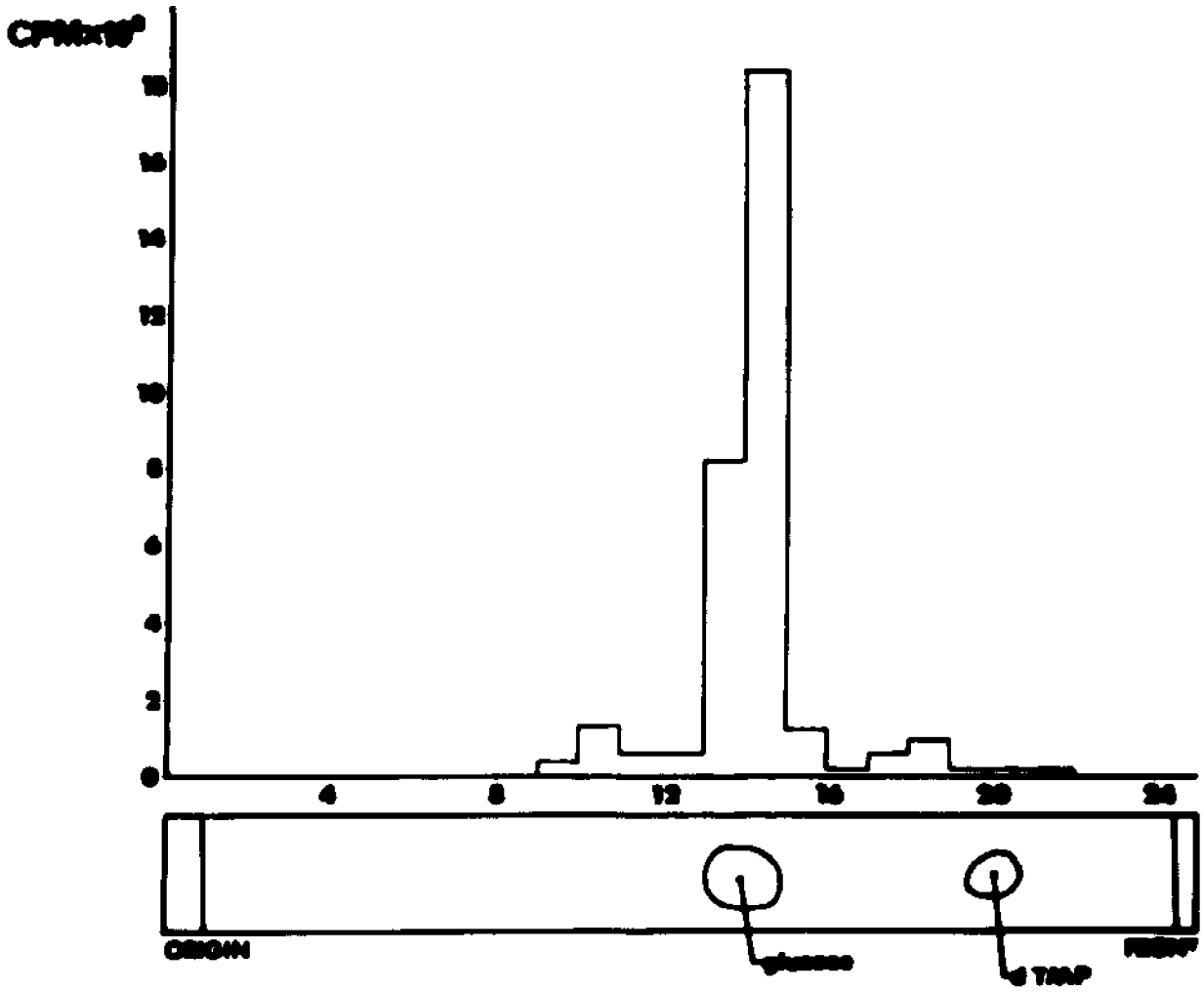
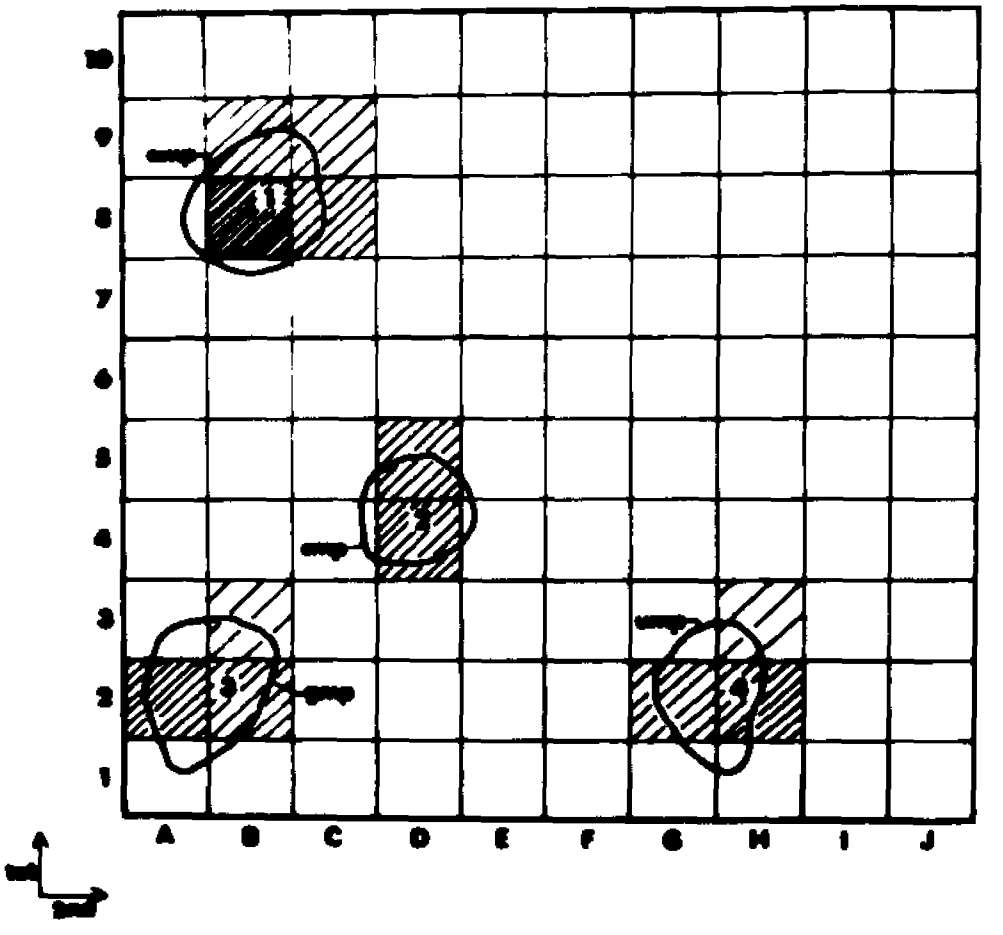


Figure 14. Distribution of radioactivity on a two dimensional chromatogram. Mononucleotides resulting from the alkaline hydrolysis of and DEAE fractionation of RNA from cells grown in thymidine-methyl-C^{14} were chromatographed first in solvent A and the second direction in solvent B. Approximately 5,750 cpm was spotted in the lower left hand corner of the sheet of Whatman #1 paper. Counting details as in figure 3.

FIGURE 14



tide fractions were then lyophilized, hydrolyzed in 1N H_2SO_4, neutralized, relyophilized and extracted with warm pyridine. The pyridine extracts were chromatographed in solvents C and I. The mononucleotides were found to contain radioactive ribose but the oligonucleotides had very little radioactive material extractable with pyridine. Results are given in fig. 15.

The protein fractions from the two experiments were hydrolyzed along with 10 mg of Bovine Serum Albumin with 6N HCl at 121^{o}C for 15 hours. The hydrolysate was lyophilized several times to remove the HCl and then brought to 500 microliters with water. The material was chromatographed in solvents D, F, and G and scanned for radioactivity. The results are given in figures 16 and 17. It can be seen that the alanine, glutamic acid and aspartic acid are the only amino acids that are radioactive. These three amino acids can be derived from pyruvate, α-ketoglutaric acid and oxaloacetic acid.

Figure 15. Distribution of radioactivity along a chromatographic strip. Mononucleotides from RNA of cells incubated in thymidine-methyl-C^{14} were treated with 1N H_2SO_4 for one hour at 100°C, neutralized with $Ba(OH)_2$, lyophilized and then extracted with hot pyridine. Chromatogram was developed in solvent C. Counting details as in figure 2.

FIGURE 15

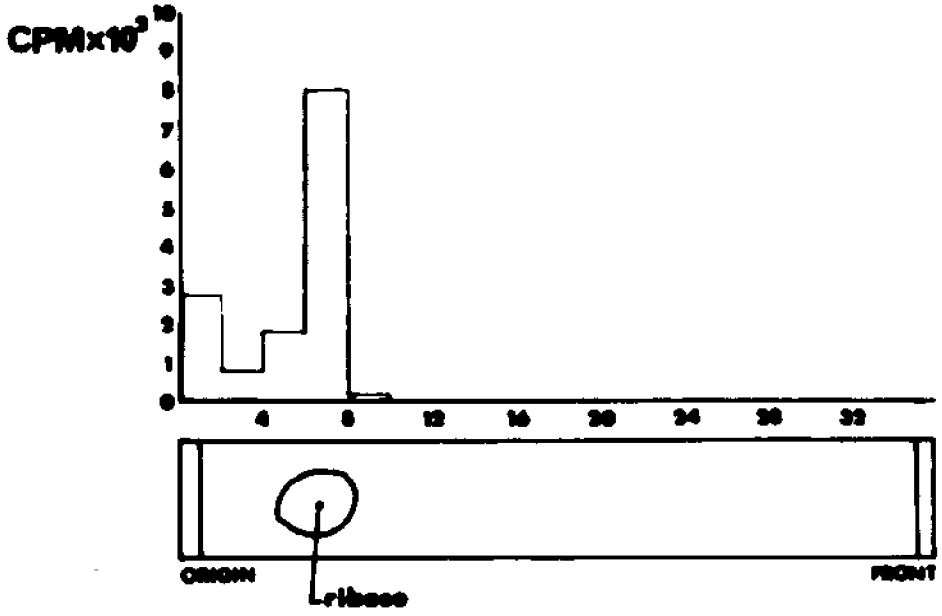
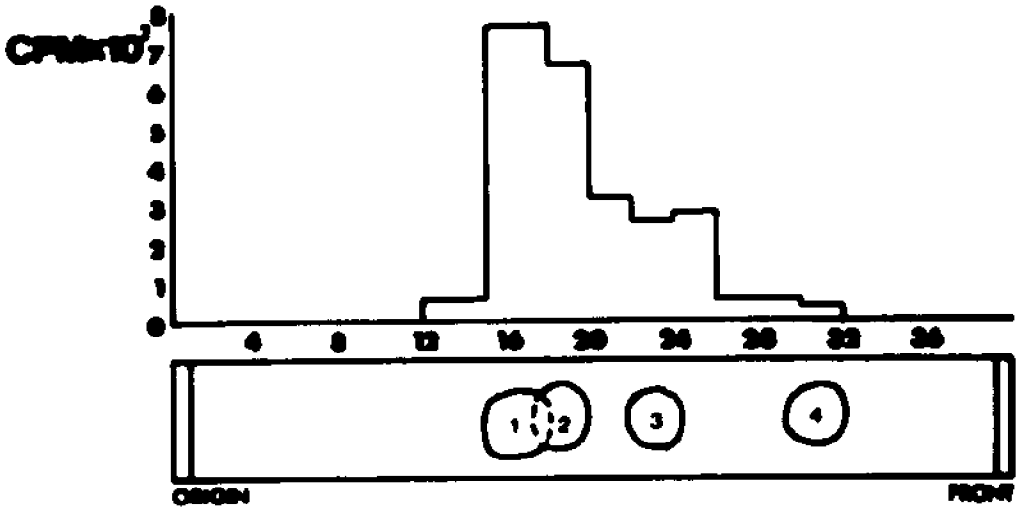


Figure 16. Distribution of radioactivity along two chromatographic strips. Protein from cells incubated in thymidine-methyl-C^{14} was hydrolyzed and neutralized. Strip (a) was spotted with approximately 2,500 cpm, and was developed in solvent F, while strip (b) was spotted with approximately 1,800 cpm and was developed in solvent D. Amino acids were visualized by running both protein hydrolysates and standard amino acids in adjacent lanes and spraying after development with a ninhydrin mixture given in text. Thymine was seen as a U.V. quenching area. Spots: 1--aspartic acid; 2--glutamic acid; 3--alanine; 4--thymine.

FIGURE 1B


CPM x 10⁷
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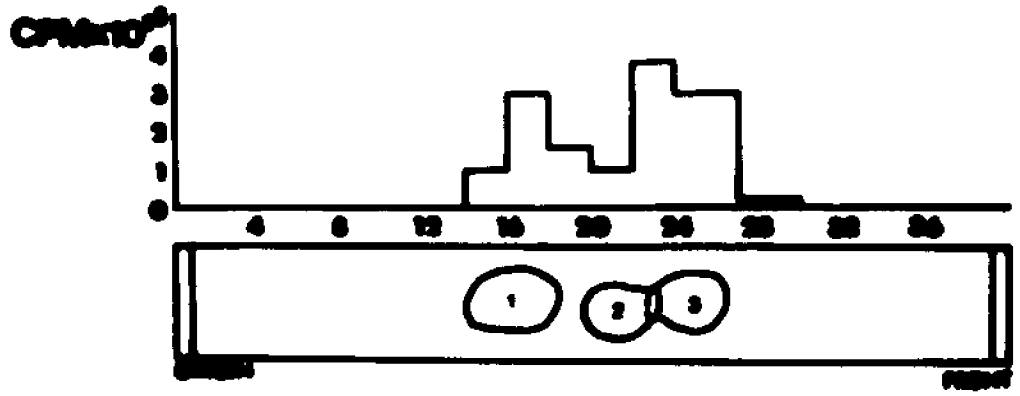
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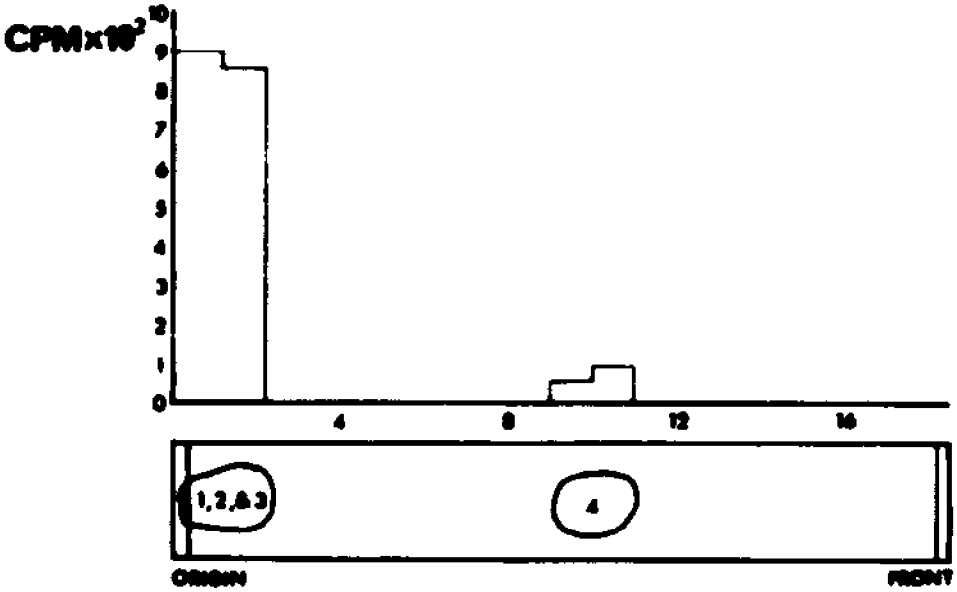
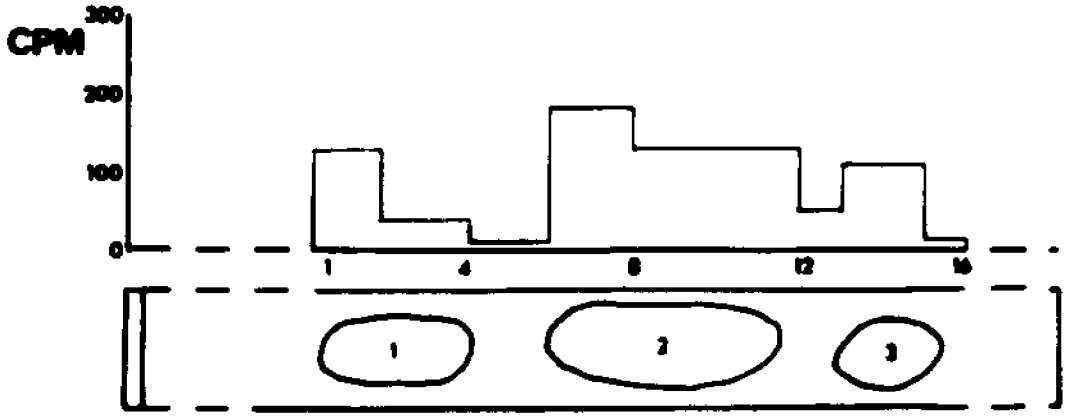
Figure 17. Distribution of radioactivity along two chromatographic strips. Protein lydrolysate from cells incubated in thymidine-methyl-C^{14} (about 2,500 cpm) was spotted on two adjacent lanes of Whatman #1 paper and developed in solvent G. One lane was assayed for radioactivity and the results given in strip (a). A strip of paper representing the first five centimeters of the second lane was cut out and eluted with water. Strip (b) represents a chromatogram of that eluant developed in solvent D until solvent front ran off the paper. Other details as in figure 16.

FIGURE 17


CPM x 10²
10
9
8
7
6
5
4
3
2
1
0
4
8
12
16
1, 2, & 3
4
ORIGIN
FRONT


CPM
200
100
0
1
4
8
12
16
1
2
3

DISCUSSION

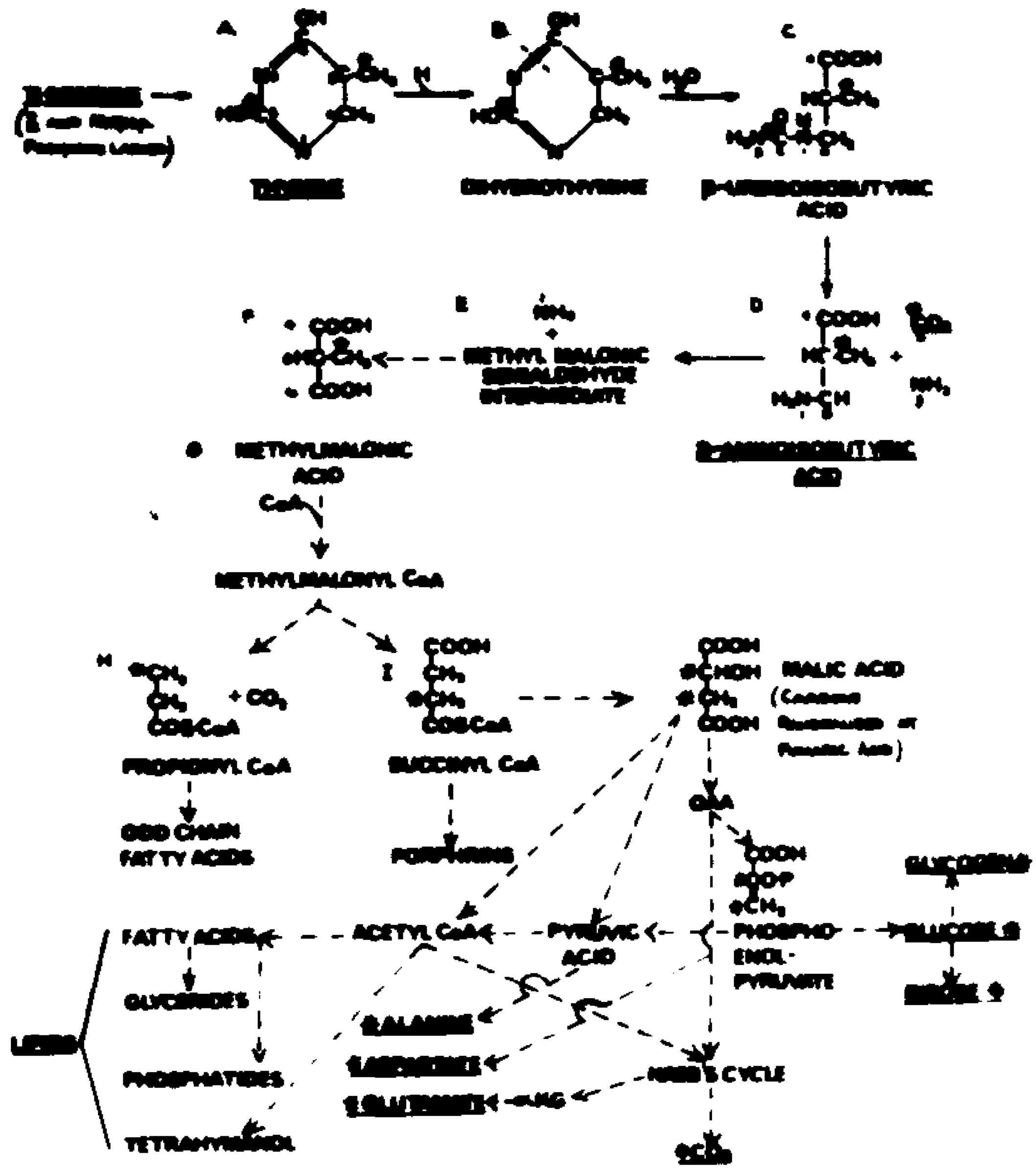
Reductive Pyrimidine Catabolism

There are several unusual features of pyrimidine metabolism in Tetrahymena which are summarized in papers by Hill and Chambers and Wykes and Prescott (83,84). Uracil can be reversibly converted into uridine, but the uridine in turn cannot be phosphorylated into uridylic acid. However, uracil can be directly converted into UMP by the action of UMP pyrophosphoylase (85,86). In addition cytidine can be oxidatively deaminated to uridine but can form neither cytosine nor cytidylic acid (87). Cline and Conner have indicated that uracil, through the deamination of cytidine, is the pyrimidine excretory produce of ribonucleic acid breakdown (88). Our evidence shows that uracil can be reductively degraded to some extent. Little is said or known of the role of thymidine in the pyrimidine metabolism of Tetrahymena. It is well established that thymine is not required for growth by Tetrahymena and that it cannot spare uracil and therefore cannot serve as a source of nucleic acid pyrimidines (89). Thymidine phosphorylase and kinase have been described along with some non specific phosphatases capable of removing the phosphate group of TMP (90,91,92). Catabolism of thymine, as well as uracil, has not been reported in protozoa at

all. We think that the reductive catabolic scheme outlined in the introduction and which was described in mammals, bacteria, plants and several other categories of organisms operates fully in Tetrahymena. Figure 18 combines the known pyrimidine reductive pathway with the reactions we think can connect that catabolism with the anabolic pathways for glycogen, fatty acids and protein. Thymidine was found by us and others to be rapidly converted to thymine (84). We believe the conversion of thymidine to thymine may occur in the culture medium. At present, attempts to demonstrate the enzyme's presence in the incubation medium have not been successful. If the enzyme proves to operate wholly within the cell, thymine may also represent a catabolic waste product and the deoxyribose moiety may be used in the organism's carbohydrate metabolism. Leboy et. al. showed that the excretion of pentose (ribose) by Tetrahymena strain W could be increased by the addition of iodoacetate to the cells in a starvation medium. They have also shown that the pentose release could be diminished by the addition of glucose to that same medium (93). The thymine excreted by the cells might be taken up again for further degradation or incorporation into DNA according to the prevailing incubation conditions. Cells incubated in thymidine-2-C^{14} degraded the nucleoside at the approximate rate of 10% of the added material per hour. The degradation, measured as the amount of $C^{14}O_2$ released, reached its maximum rate after one hour of incubation of mid-log cells in Ryley Ringer

Figure 18. Proposed pathway for thymine's reductive catabolism and reutilization. All steps are known to exist in various organisms. Steps A through D are well known degradative steps which it is felt may now be ascribed to protozoa. All steps from D on are known to exist but have not been proven herein; they are given as possible pathways to describe results thus far obtained. All compounds placed in rectangular boxes have been isolated during the course of this work.

FIGURE 18


DIHYDROTHYMINE
ACID
METHYLMALONIC
ACID
METHYLMALONYL CoA
MALIC ACID
PROPIONYL CoA
SUCCINYL CoA
ODD CHAIN
FATTY ACIDS
FATTY ACIDS
ACETYL CoA
PYRUVIC
ACID
PHOSPHO-
ENOL-
PYRUVATE
GLYCERIDES
PHOSPHATIDES
LIPIDS
KREB'S CYCLE

phosphate buffer. Cells incubated in regular growth medium and the radioactive nucleoside released $C^{14}O_2$ at approximately one half the rate of the cells in buffer.

Radioactive β-aminoisobutyric acid was the only intermediate of the thymine catabolic pathway that was isolated. This material accumulated within mid-log cells that were incubated in Ryley Ringer phosphate buffer containing thymidine-methyl-C^{14}.

It was calculated that less than 25% of the β-amino-isobutyrate produced by the above cells was released to the medium. The bulk of the thymine produced was in the medium, and no thymidine could be found either inside or outside the cells. We conclude that the thymidine is quickly converted to thymine by Tetrahymena, and then, according to incubation conditions, the thymine is reductively degraded to β-amino-isobutyrate at a much slower rate. The catabolism of uridine (to presumably β-alanine) proceeded at about 20% of the rate of thymine catabolism. Since dihydrothymine reduced $C^{14}O_2$ release much more effectively than dihydro-uracil from both thymidine and uridine-2-C^{14}, thymine may be the usual substrate for the catabolic enzyme system. Thus it appears that uracil is degraded at least in part by the same enzymes which degrade thymine. It therefore appears that uracil (which can serve as the sole source of nucleic acid pyrimidines) is excreted as a product of RNA

catabolism with little further degradation. Thymine on the other hand can be excreted *per se* and/or reductively degraded. Grisolia and others have shown that in mammalian systems both substrates are reduced but these systems seem to degrade uracil at a greater rate than thymine (28,31). In bacteria Campbell has shown the pyrimidine reductive system to be able to handle uracil only (37,38). The pyrimidine catabolism of *Tetrahymena* appears to be unique among those reported in the literature.

Reutilization of Degradation Product

Our evidence indicates that β-aminoisobutyric acid is not the end product of the thymine degradative pathway as is generally described in other organisms, but, that it leads to a variety of other biologically useful compounds. In figure 18 the reductive pathway is outlined, and we follow the methyl carbon of thymine through what we think may be the possible connecting links between β-aminoisobutyric acid and macromolecules other than DNA. Those presumed steps are indicated by dotted arrows. White, Handler and Smith indicate (without reference) that BAIB could be converted to methylmalonic acid and then to succinyl CoA (57).

Cells grown to late log phase in thymidine-methyl-C^{14} yielded radioactive glucose, ribose, alanine, aspartic acid and glutamic acid from their respective macromolecules -- glycogen, RNA, and protein. After the DNA fraction the

lipids from that experiment contained the greatest amount of radioactivity. Cells incubated in thymidine-2-C^{14} had almost no activity in the non-DNA fractions as would be expected from the reactions of fig. 18. We would like to discuss each of the fractions individually.

Lipid Fraction

Albach in his paper showing essentially no non-DNA incorporation of thymidine-methyl-H^3 found some activity in his lipid extract. He localized the activity mostly in the triglycerides and phospholipids and to a somewhat smaller extent in the free fatty acids, cholesterol, cholesterol-esters, mono and diglycerides (15). Some of Albach's data was found to be in error by a factor of 10 and his lipid fraction therefore contained ten times more radioactivity than he indicated. In addition he localizes some of the activity in the lipid fraction in cholesterol and cholesterol-esters but cholesterol is not synthesized by Tetrahymena. Albach's data is still interesting because the lipid fractions in our experiments contained 7% of the total radioactivity incorporated from tritium labeled material (New England Nuclear) and 22% from C^{14} labeled material. Following the scheme in figure 18 thymine's methyl group can effectively (more C^{14} than H^3) become part of acetyl CoA and thereby enter fatty acid metabolism. Also Feller and Fiest have shown that mouse adipose tissue incorporates propionic acid-1-C^{14} into long chain fatty acids as efficiently as they incorporate acetate. In

addition C^{14}-labeled methylmalonate was incorporated just as effectively as propionate. Since they could not isolate radioactive methylmalonate, from the propionic acid-1-C^{14} experiment, they concluded that propionate is an intermediate in the conversion of methylmalonate to fatty acids (94). In addition to serving as a source for the carbon units of even numbered fatty acids, propionate can serve as the starting unit for odd chain fatty acids. James et. al. have shown that in perfused cow's udder odd-chain fatty acids are formed by condensation of propionate and acetate (95). Horning and coworkers have shown the same in the soluble enzyme system of rat adipose tissue (96). In addition succinyl CoA serves as a critical starting compound in porphyrin biosynthesis. It can be seen that several of the intermediates proposed in figure 18 could serve as points of entry into lipid and porphyrin metabolism.

Carbohydrates

We have found that during late logarithmic phase and early stationary phase Tetrahymena makes large quantities of glycogen. This phenomenon has also been reported by others (58). Albach reported that stationary phase Tetrahymena strain W incorporates some tritium into all fractions from thymidine-methyl-H^3 (15). He found that the acid soluble RNA and DNA fractions had approximately 38, 18, and 29 percent of the incorporated activity respectively. He assumes the activity of the RNA fraction is due to the

partial degradation and spillover of DNA into that fraction. The activity in the acid soluble fraction is attributed to some thymidine degradation products which are excreted by the cells. Albach's interpretation of his results (no non-DNA incorporation of thymine) leads him to offer that Bryant's finding tritium from thymidine-methyl-H^3 in mouse protein and Sagan's report that tritium from the same source winds up in the RNA of Euglena are due to some small amount of transmethylation of the methyl groups of RNA and protein with thymine. Our preliminary experiments with stationary phase cells and thymidine-methyl-H^3 gave us 39% of the total activity incorporated into the acid soluble fraction, 24% into RNA and 25% into DNA. We came to a conclusion somewhat similar to that of Albach; we thought the methyl group of the thymine was entering a "one carbon pool". Further investigation revealed that the activity in our acid soluble fraction was mostly due to glycogen while the activity in the RNA fraction was due to spillover of some glycogen and only a minor DNA contaminant. In several subsequent experiments we found that even logarithmic phase cells incorporate tritium from methyl labeled thymidine into glycogen. If, however, cells were grown on plain 2% proteose peptone, incorporation of label into glycogen was minimal because glycogen biosynthesis is lower than in the enriched medium and no spillover into RNA was noticed. In the modified Elliott's medium (gluconeogenic), very large amounts of glycogen were made by log

phase Tetrahymena, so much so that quantitative removal by cold TCA was impossible without "chasing" some degraded DNA into the RNA fraction. Experiments utilizing thymidine-methyl-C^{14} revealed the same pattern of incorporation into the glycogen as with tritium label. All of the radioactivity in the glycogen fraction was found in glucose (none was due to any non-specific binding of the molecules). Cells grown in thymidine-2-C^{14} did not incorporate any C^{14} into the glucose of the glycogen fraction. This result fits our hypothetical pathway well. When cells were grown in thymidine-6-H^3 (according to our pathway none of the 6 position tritium should find its way into glucose) some radioactive glycogen was detected. The glycogen fraction contained 0.6% of the incorporated activity, and again the radioactivity was localized in the glucose molecule. Even this small amount was bothersome until it was learned that up to 20% of the tritium in ring labeled thymidine is in the methyl group (97).

As was noted in the introduction the incorporation of activity into carbohydrate from radiolabeled thymine is not totally unknown. Piko' has extracted radioactive polysaccharide from mouse embryos incubated in uniformally C^{14}-labeled thymidine (54). Greenhouse's autoradiographic experiments and Fink's in vitro work were both done on mouse tissue and both revealed incorporation into carbohydrate material (30,55). Since the mouse can reductively degrade thymine it is conceivable that it is

also reincorporating some of thymine's carbons by a pathway similar to the one we suggest here.

Our data indicates that glucose derived from thymine and the intermediates in that pathway leading to glucose can serve as carbon sources for energy. It was shown that the methyl group of thymine was released as CO_2 by Tetrahymena incubated in buffer alone. The release was at first slow and then increased 100 fold over a 100 minute incubation period. The CO_2 released from thymidine-methyl-C^{14} was diluted in another experiment with the addition of nonradioactive BAIB and methylmalonic acid to the incubation buffer. The latter result further substantiates our proposed pathway and at the same time reduces the possibility that a completely novel biochemical pathway from thymine to glucose exists. It is interesting to note that Fritzon and Pihl found that rats could convert 83% of administered uridine-6-C^{14} into $C^{14}O_2$ within 10 hours (29). Tsai and Axelrod found that Rape seedlings could reductively degrade uracil and thymine. They also found that β-alanine gave rise to pyruvic, malic, acetic and citric acids, and was incorporated into lipids and sterols (34). The interconversions of pyrimidines and carbohydrates may therefore be more common than has previously been assumed.

Proteins

β-alanine is an important component of carnosine, anserine and panthothenic acids (98). It can also

transaminate in some systems with pyruvate and α-keto-glutarate to yield malonic semialdehyde which can then proceed through further conversions to acetyl CoA and perhaps eventually to other more common amino acids (34, 99). The fate of β-aminoisobutyrate on the other hand is very rarely discussed (57). Hogg and Wu found that Tetrahymena has a large pool of alanine and glutamate and a somewhat large pool af aspartate, glycine and glutamate in conjugated form (100). Alanine, aspartate and glutamate can easily arise from transaminations of tricarboxylic intermediates (which we think can arise eventually from thymine). Since Tetrahymena may be geared to make high levels of these compounds, it was natural to look for these amino acids in the protein hydrolysates of cells grown in thymidine-methyl-C^{14}. The clearly labeled alanine, aspartate and glutamate were only found when C^{14} was used as the label. Bryant found radioactive protein after growing mouse cells in thymidine-methyl-H^3. In our cells grown on the tritiated nucleoside most of the activity in the protein fraction was due to a very small amount of DNA contaminant.

RNA

The labeling of the ribonucleic acid fraction of Tetrahymena can really be viewed as an extension of carbohydrate metabolism. Tetrahymena seems to be able to make glucose out of almost any available carbon. It can then either store the glucose as glycogen or use it to

make other necessary sugars like ribose. Leboy, et. al. have shown that Tetrahymena can even utilize ribose as a carbon source during a period of starvation when RNA is being degraded (93). Radioactive ribose was made only when cells were grown in methyl-C^{14}-labeled thymidine, and none was detected when cells were grown with methyl-tritium labeled thymidine. The probability of methyl tritium surviving the gluconeogenic pathway and the pentose phosphate shunt is very low while the methyl carbon (especially after randomization) has a much greater opportunity of being converted into ribose.

The earlier results with Schwarz thymidine-methyl-H^3 seemed to be due to the presence of tritiated uracil as a contaminant of their product. The contaminant has recently been noted (not publicly) by others (101). We have been assured by the manufacturer that they have changed their process and thymidine is now absolutely "clean". A recent quick check of a gift sample donated by Schwarz BioResearch (courtesy of Mr. T. Castro) revealed no radioactive uracil recoverable from RNA of Tetrahymena grown in the thymidine-methyl-H^3.

All our results with radiolabeled thymidine fit our proposed pathway very well. The results also seem to explain many unusual results reported in the literature about thymidine's possible introduction into cell fractions other than DNA. It has recently been noted by Standish

Hartman (without evidence or reference) that "pyrimidine degradation often serves to provide a substantial fraction of the elementary nutrients needed by cells (microorganisms) for growth" (102). Hutner has voiced similar opinions about nucleotides serving as general carbon sources in microorganisms (103). It may well be that the metabolic pathways of thymine we propose in Tetrahymena are present to some extent in higher eucaryotes as well as microorganisms and important metabolic interconversions have thus far been overlooked.

SUMMARY

1. The methyl group of methionine was found in Tetrahymena pyriformis strain GL to:

a) methylate both the bases and sugar of RNA

b) become incorporated into glucose

c) readily enter lipid metabolism

An unusual result obtained was that methionine's methyl group did not become involved in that part of the "one carbon pool" which methylates dUMP to dTMP. Thymine's methyl group was found not to become part of the organism's one carbon metabolism.

2. Both tritium and C^{14} labels on thymine's methyl group were recovered in the glycogen and lipid fractions after the cells were grown in their presence. Carbon fourteen was also recovered in ribose and the amino acids alanine, aspartic acid and glutamic acid. Ring position labels (6-tritium and 2-C^{14}) were limited to the DNA fraction.

3. Tetrahymena was found to be able to reductively degrade thymine and to a lesser extent uracil. In addition the main thymine reductive product, β-aminoisobutyric acid was found to be converted to a variety of molecules used in anabolic pathways.

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