## The origin of thymine and cytosine in *Tetrahymena**

The ciliated protozoan *Tetrahymena pyriformis* W requires a pyrimidine for growth. The pyrimidine requirement is met by uracil, uridine, uridylic acid, cytidine or cytidylic acid. On the other hand, cytosine, orotic acid, thymine or thymidine do not support growth of the organism in a pyrimidine-deficient medium[1,2]. The failure of thymine and thymidine even to spare the pyrimidine requirement was interpreted[2] as indicating that thymine was synthesized from non-pyrimidine precursors, rather than by utilization of the preformed ring. Subsequent nucleic acid analyses of the organisms showed the concentration of deoxyribonucleic acid (DNA) to be very low, approximately 10% of the concentration of ribonucleic acid (RNA) (DNA 12 $\mu$g, RNA 82 $\mu$g per mg lipid-free dry weight in organisms grown on synthetic medium). The amount of thymine which would be required in the synthesis of this DNA is so small that the sparing effect on the total pyrimidine requirement might not be detectable.

The problem has now been studied by the use of labeled uracil and thymine. In the experiments described below it was found that thymine is synthesized from uracil without dilution of the isotope, when uracil is the only pyrimidine supplied. Labeled thymine itself is utilized to only a slight extent as a precursor of DNA thymine.

*Labeled uracil.* 1 l of medium A[3] was prepared, but without Tween or pyrimidines. 2 g casein was added for its apparent growth-stimulating effect. (Casein was omitted in later experiments because it accumulated in the organisms and complicated the isolations.) To the medium were added 5 mg unlabeled uracil and 5 mg uracil-2-$^{14}$C (Bio-Rad Laboratories, 0.42 mc/mmole). The medium was autoclaved in a 12-l Pyrex bottle, inoculated with *Tetrahymena pyriformis* strain W, and incubated in the dark at room temperature with the bottle rotated on its side at 10 r.p.m. Sterile air was supplied by an aquarium pump, and outgoing air was passed through barium hydroxide-barium chloride solution to collect carbon dioxide. As has been found with several compounds, a small amount of labeled uracil decomposed during autoclaving, leading to slight radioactivity in the early carbon dioxide samples. This activity decreased in samples collected up to about 60 h, then increased, perhaps indicating a small amount of catabolism. The activities of "infinitely thick" barium carbonate plates, expressed as counts/min/mg C, were: 39 h - 160, 50 h - 56, 61 h - 42, 72 h - 49, 85 h - 111.

After 3½ days growth, the organisms were centrifuged off and washed twice with water. Acid-soluble nucleotides and lipids were removed essentially as previously described[4]. Combined nucleic acids were extracted with three portions of hot 10% NaCl at pH 7. Extracts were chilled, precipitated with 3 volumes of ethanol, and the precipitate washed with ethanol and ether, and dried. The nucleates were hydrolyzed to free purines and pyrimidines with 72% perchloric acid[5] followed by neutralization of the acid with KOH and removal of $KClO_4$. The supernatant was chromatographed in bands on Whatman No. 1 paper in 65% *iso*propyl alcohol-2 *N* HCl[6]. The five bands, containing thymine, uracil, cytosine, adenine, and guanine, were cut out, eluted, and the eluate evaporated to dryness. Each compound was then rechromatographed three times in 86% aqueous *n*-butanol. Radioactivity was determined by cutting five 14-mm discs from the band of the chromatogram, and counting both sides of each disc in a windowless Geiger counter to obtain an average count. The five discs were then eluted in 4.0 ml 0.01 *N* HCl and the solution read in the Beckman DU spectrophotometer. Adenine and guanine contained no activity; the specific activity of the pyrimidines is shown in Table I. It is evident that all the pyrimidines of the organism are derived exclusively from the administered uracil. The specific activity of cytosine is approximately 3% below that of the other two pyrimidines. This is probably due to a trace of impurity, although the possibility exists that some cytosine fraction (*e.g.* DNA) is synthesized from precursors other than uracil.

*Labeled thymine.* Although thymine may be synthesized from uracil in *Tetrahymena*, as shown in the above experiment, exogenous thymine is also utilized. This is shown by the sparing of the

TABLE I

| | *Specific activity c.p.m./µmole* |
|---|---|
| Administered uracil-2-$^{14}$C before dilution | 106,700 |
| Nucleic acid uracil | 59,800 |
| Nucleic acid cytosine | 57,400 |
| Nucleic acid thymine | 59,200 |

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folic acid requirement by thymine or thymidine[2]. The incorporation was confirmed by use of a portion of the thymine-2-$^{14}C$ obtained from the previous experiment. Organisms were grown in 100 ml of medium A[3] without Tween, with 0.12 mg of labeled thymine added. After 4 days growth, pyrimidines and purines were isolated from the combined nucleic acids as described above. Of these, thymine was the only compound with detectable activity, 100 counts/min/$\mu$mole, as compared with a starting activity of 59,200. The small degree of incorporation of thymine is in agreement with the results of FRIEDKIN AND WOOD[7] in this organism and others. They have found that thymidine incorporation is 27 times that of thymine in *Tetrahymena*.

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## Quantitative aspects of $CO_2$ fixation during protein synthesis from ammonium acetate

Micro-organisms, growing on synthetic media containing ammonium acetate as sole carbon source, rapidly incorporate isotope from added $^{14}CH_3COONH_4$ into protein[1,2]. In the presence of unlabelled $NaHCO_3$, this synthesised protein is less radioactive than in its absence. Short-term experiments[2] indicated that the protein initially synthesised under such conditions may derive as much as 70% of its carbon from the unlabelled bicarbonate.

These results could be explained either by the occurrence of a $CO_2$-fixation reaction of hitherto unsuspected magnitude, or by an increase in the steady-state concentration, due to the addition of bicarbonate, of a compound in ready equilibrium with $CO_2$ lying on one of the earliest stages of the pathway from acetate to protein. The following experiments support the latter explanation.

Growing cultures of acetate-grown *Pseudomonas* KB1[3] were suspended at pH 7.0 in 15 ml of medium containing 25 m*M* ammonium acetate (Flask A) or 25 m*M* ammonium acetate + 10 m*M* $NaHCO_3$ (Flask B) as carbon source. The 500 ml flask A was gassed with 100% $O_2$ and stoppered tightly, whilst 5% $CO_2$/95% $O_2$ was continuously bubbled through the medium in the similar flask B. At zero time, 0.75 ml of a solution of $^{14}CH_3COONa$, containing 30 $\mu$moles of acetate and giving $1 \cdot 10^7$ counts/min under the conditions of radioassay used, was added to each flask. Samples (2 ml) were removed every 30 min and the protein, precipitated with trichloroacetic acid, was hydrolysed in sealed tubes at 110° for 18 hours. The amino acid nitrogen concentration was estimated colorimetrically[4] and the radioactivity of the amino acids was determined by direct assay on paper after autoradiography.

At both 22° and 30° (Table I), the specific activities of the protein synthesised in the presence of $^{12}CO_2$ were 83–85% of that synthesised in its absence. This shows that $CO_2$-fixation accounts for only 15–17% of the carbon incorporation in the synthesis of protein from ammonium acetate, and is of an order consistent with the known $C_3 + C_1$ condensations[5,6].

Recent experiments with acetate-grown *Pseudomonas*[7,8] have shown that acetate enters the tricarboxylic acid cycle at two sites, to form citrate at one and malate at the other. The present results and those obtained previously[2] are consistent with these findings. Cell-free extracts of *Pseudomonas* KB1 contain malic enzyme[10] and can form malate from pyruvate and $CO_2$. It is therefore likely that the addition of $NaH^{12}CO_3$ temporarily raises the steady-state concentration of malate or oxaloacetate (Fig. 1), causing the protein synthesised in the earliest stages of the short-term experiments to be derived from a "pool" of $C_4$-compounds of low radioactivity.