CONVERSION OF URACIL TO THYMINE BY STRAINS OF BACILLUS SUBTILIS

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From replacement studies (1) and inhibition analysis (2) with microorganisms requiring folic acid, it has been inferred that this vitamin is concerned in the biosynthesis of purines and of thymine. More directly, folic acid has been shown to be involved in nucleic acid synthesis in *Lactobacillus casei* (3, 4). According to Buchanan and Schulman (5), the formation of purine derivatives would seem to require a folic acid-activated enzyme. The observed effectiveness of uracil in large doses in pernicious anemia in relapse has been interpreted to mean that it may be a precursor of thymine (6). Interchangeability of certain pyrimidines in the nutrition of microorganisms is known (7–9). The present study relates to the ability of several strains of *Bacillus subtilis* to convert uracil to thymine and to the probable rôle of folic acid and vitamin B_{12} in this transformation.

EXPERIMENTAL

Organisms—Three strains of *B. subtilis*, two isolated locally and one from the Central Drug Research Laboratory, Lucknow (CDRL No. 8314), were used in this study. These were carried on peptone-agar slants with monthly transfers. All the strains were observed to effect conversion of uracil to thymine, although a major part of the studies reported relates only to one of the cultures isolated.

Culturing and Harvesting—The organism was grown in the inorganic salts-dextrose synthetic medium of Green and Sevag (10). The pellicle was broken up by shaking after 24 hours growth at 30°, separated by centrifugation, and washed twice with ice-cold distilled water. The cells were then resuspended in a suitable volume of distilled water and dispersed homogeneously by vigorous shaking. Dry cell weights were determined on aliquots of this suspension.

Procedure—A cell suspension equivalent to about 10 mg., dry weight, unless otherwise stated, was incubated with 1 mg. of uracil (1 mg. per ml. in 0.004 N NaOH) in the presence of 3 ml. of 0.1 M phosphate buffer, pH 7.2, at 37° for 4 hours, total volume 6 ml. The reaction was stopped with 1 ml. of 10 per cent trichloroacetic acid solution. The sediment obtained by centrifugation was stirred with 2 ml. of water and centrifuged again.

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The combined supernatant solution was neutralized with dilute NaOH solution and made to volume. Aliquots of this were used for the determination of thymine (Table I). An aliquot of cell suspension heated on a boiling water bath for 5 minutes failed to effect any transformation of uracil to thymine.

Determination of Thymine—The method is based on the reaction of thymine with diazotized sulfanilic acid, yielding red coloration on reduction (11), and was adapted from the procedure of Woodhouse (12). Specificity of the reaction has been reported by others (13, 14) and was checked in preliminary studies. Interference due to certain aldehydic breakdown products of deoxypentose could be removed by extraction with distilled ether (12, 14). Uracil and cytosine give a yellowish tinge.

In carrying out the determinations, appropriate blanks were kept to allow for unidentified interfering substances which, in contrast to thymine,

TABLE I

Synthesis of Thymine by Resting Cells of Bacillus subtilis

Experiment No.	Thymine formed in 4 hrs.		
	10 mg. cells	10 mg. cells + 1 mg. uracil	Net synthesis
	γ	γ	γ
1	7.6	98.0	90.4
2	11.4	88.8	77.4
3	9.4	106.2	96.8
4	9.7	90.7	81.0

produced only slight coloration before reduction with hydroxylamine. The increase in color intensity after reduction was, therefore, taken as a measure of thymine. The controls included sets for endogenous reaction as well as for reaction stopped at zero time.

Identification of Thymine on Paper Chromatogram—Ascending paper strip chromatography was carried out with *n*-butanol saturated with water as the mobile phase, on Eaton-Dikeman No. 613 paper strips. Separation was allowed for 18 to 20 hours. Positions of the separated pyrimidines on the chromatogram were spotted by the method of Vischer and Chargaff (15), modified by use of a 5 per cent silver nitrate solution instead of mercuric acetate solution. R_F values observed for uracil and thymine were 0.30 and 0.43, respectively. Added thymine could not be differentiated on the chromatogram from thymine in the test solution.

Results

Optimal pH Conditions—The reaction was studied in the following buffers: acetate (0.1 M), pH 4.6; phosphate (0.1 M), pH 6, 6.6, 7.2, and 8.0;

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pyrophosphate (0.02 M), pH 8.6; and bicarbonate, pH 7.2. Phosphate buffer, pH 7.2, was the most satisfactory and was employed in all the work. There was no activity at pH 4.6 and 8.6.

Effects of Methyl Precursors—Since transformation of uracil to thymine involves methyl linkage to carbon at position 5, conversion was expected to be enhanced if methyl precursors were provided. A number of known and probable precursors were tried. These were added to the system in equimolar concentrations, 1 ml. of 0.013 M solutions being used. For comparison two different experiments were run with controls and sets to which glycine had been added. The results are given in Table II.

TABLE II

Effects of Methyl Precursors

Addition	Thymine in 4 hrs.			Per cent net change
	10 mg. cells	10 mg. cells + 2 mg. uracil	Net	
	γ	γ	γ	
None	7.2	76.8	69.6	
Glycine	9.5	124.5	115.0	+65
Serine	7.7	101.3	93.6	+35
Methionine	8.3	90.0	81.7	+17
Choline chloride (1 mg.)	6.5	70.1	63.6	−9
Formate	8.2	85.1	76.9	+10
Formate + cysteine	4.7	62.3	57.6	−17
Threonine	9.2	100.7	91.5	+31
None	8.8	68.8	60.0	
Glycine	10.4	111.4	101.0	+68
Sarcosine	10.1	84.5	74.4	+24
Formaldehyde	8.8	72.2	63.4	+6
Methanol	6.8	54.0	47.2	−21
Tryptophan	8.0	66.5	58.5	−3
Histidine	7.4	72.0	64.6	+8

Glycine produced the greatest thymine formation. Serine and threonine and to a lesser extent sarcosine and methionine also had an appreciable effect. The depressing effect of methanol may be due to slight toxicity for the organism. In subsequent experiments, glycine (1 ml., 0.013 M) was included in the medium.

Various members of the tricarboxylic acid cycle (succinate, fumarate, α-ketoglutarate, and acetate) were tried as "sparking" compounds, but did not influence thymine synthesis.

Effects of Aminopterin and Vitamin B_{12} Oxidation Product—Addition of folic acid (pteroylglutamic acid, 10 γ) or vitamin B_{12} (100 mμgm.) to the

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reaction system had no effect on thymine formation. This was to be expected, since the organism is known to elaborate these vitamins in good quantity. The effects of antagonists of the two vitamins were therefore studied.

The vitamin B_{12} antagonist was prepared by oxidation of the crystalline vitamin with hydrogen peroxide according to Beiler (16). Either this or aminopterin was added to the reaction mixture containing cells, uracil, and glycine in phosphate buffer, pH 7.2. The results are given in Table III.

The vitamin B_{12} oxidation product could block the transformation of uracil to thymine almost completely at the 10 γ level. Aminopterin was less effective.

TABLE III

Effects of Aminopterin and Vitamin B_{12} Oxidation Product

Addition	Thymine in 4 hrs.			Per cent net change
	8 mg. cells	8 mg. cells + 1 mg. uracil	Net	
	γ	γ	γ	
None	5.7	54.6	48.9	
Aminopterin, γ				
10	5.0	51.3	46.3	−5
50	4.9	42.2	37.3	−24
100	3.9	25.6	21.7	−55
Vitamin B_{12} oxidation product ($\equiv \gamma$ vitamin B_{12})				
1	3.9	36.5	32.6	−33
10	0.5	7.7	7.2	−85
50	0	0	0	0

Reversal of inhibition was not observed with pteroylglutamic acid (100 γ) or leucovorin (50 γ) and with vitamin B_{12} (up to 10 γ) added simultaneously with aminopterin (50 γ) and the vitamin B_{12} oxidation product (1 γ). Culture filtrates and a cold water extract of crushed cells of *B. subtilis* were also ineffective in this respect.

Other Observations—When *B. subtilis* was grown on peptone-agar, the harvested cells were, surprisingly, without any activity for thymine synthesis. When the synthetic agar medium was employed, it was ascertained that this lack of activity was not due to the solid medium used; besides, cells grown in peptone-water also lacked this activity.

Ability to effect this transformation could not be observed in *L. casei*, *Streptococcus faecalis* R, and wild as well as vitamin B_{12}-requiring mutant strains of *Escherichia coli*.

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DISCUSSION

The incorporation of labeled formate into thymine-methyl has been shown to be influenced by dietary folic acid in mice (17) and in rats (18). In microorganisms there has been no such demonstration. In the microbial conversion of uracil to thymine reported here, no direct effect of folic acid or vitamin B_{12} could be shown; however, the depression of the conversion by the specific antagonists might point to a rôle for both vitamins.

Attempts have been made (19, 20) to define the loci of action of folic acid and vitamin B_{12} in thymidine synthesis. It would seem from the present observations that the two vitamins are also involved in thymine synthesis. Presumably they exert their effects at different points in the formation of the active 1-carbon fragment from glycine and in the subsequent methyl synthesis.

The observation reported here may not typify the general microbial mechanism of biosynthesis of thymine. Mitchell and Houlahan (21), working with *Neurospora* mutants, suggested that the free pyrimidines are not the natural intermediates and that conjugation with the sugar (pentose or deoxypentose) occurs at an early stage of biosynthesis. A similar conjugation of 4-amino-5-imidazolecarboxamide into the corresponding ribotide before conversion into purine ribotide has been demonstrated (22, 23). However, the interchangeability in utilization of pyrimidines exhibited by several bacteria shows that intact pyrimidines are interconvertible. The present observations demonstrate such an interconversion.

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SUMMARY

The resting cells of *Bacillus subtilis* are shown to bring about transformation of uracil to thymine.

Glycine and, to a lesser extent, serine, threonine, and sarcosine augment this transformation, and presumably serve to supply the single carbon unit precursor of the methyl group.

A vitamin B_{12} oxidation product and aminopterin inhibit the reaction, the effect of the former being more marked.

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