Pseudouridine, Isolation and Biosynthesis of the Nucleoside Isolated from the Culture Filtrates of *Streptoverticillium ladakanus*†

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ABSTRACT: 5-Azacytidine, pseudouridine, uridine, and uracil have been isolated from the culture filtrates of *Streptoverticillium ladakanus*. Two methods describe the isolation of pure, crystalline β-D-pseudouridine from the culture filtrates of *S. ladakanus* in yields of 70 mg/l. The physical, chemical, and biological properties of the β-D isomer of pseudouridine are described. The purity of pseudouridine as the β-D isomer was determined by microbioassay, gas chromatography, and nuclear magnetic resonance spectroscopy. The pseudouridine was shown to be 97–100% β-D isomer. The yields and specific activities of pseudouridine isolated from the culture filtrates containing whole dried yeast from [U-^{14}C]uridine experiments were the same as yields and specific activities of pseudouridine isolated from the filtrates to which alkaline hydrolyzed dried yeast was added. Therefore, *S. ladakanus* must be excreting large amounts of pseudouridine into the culture filtrate. The data on the biosynthesis of ψ show that the uracil moiety, but not the ribose moiety, of the exogenously added [U-^{14}C]-uridine is incorporated into pseudouridine isolated from the culture filtrates. Uridine phosphorylase has been assayed in extracts of *S. ladakanus*. Cell-free extracts of a 20–70% ammonium sulfate fraction of *S. ladakanus* did not show any pseudouridine 5′-monophosphate synthetase activity. There was no inhibitor of pseudouridylate synthetase in the cell-free extracts of *S. ladakanus*. The turnover of RNA in the the cell results in the formation of purine and pyrimdine nucleotides which are hydrolyzed to the free base. The free base can then be reutilized by the cell. However, the pseudouridine 5′-phosphate can not be converted to pseudouridine and ribose 5-phosphate by pseudouridylate synthetase since this degradative enzyme is absent in *S. ladakanus*. It appears that pseudouridine 5′-phosphate is hydrolyzed to pseudouridine and then excreted into the medium.

5-Azacytidine is a *s*-triazene nucleoside antibiotic elaborated by *Streptoverticillium ladakanus* (Hanka *et al.*, 1966; Bergy and Herr, 1966). While studying the biosynthesis of 5-azacytidine by *S. ladakanus*, we observed three additional ultraviolet-absorbing compounds on paper chromatograms. A more detailed study has revealed that one of these compounds is pseudouridine.[1] The yield of crystalline ψ isolated from the culture filtrates of *S. ladakanus* is 70 mg/l. The other two ultraviolet-absorbing compounds have been identified as uracil and uridine.

The presence of such large amounts of ψ in the culture filtrates of *S. ladakanus* provides an excellent system to determine if the biosynthesis of ψ proceeds *via* pseudouridylate synthetase or if this ψ is the result of the turnover of cellular RNA. The biosynthesis of ψ and ψ5′P have been proposed to occur by either (1) rearrangement of uridine residues in tRNA or (2) condensation of uracil and ribose-5-P or (3) the formation of 1,5-diribosyluracil. Experimental evidence for the first two proposals has been reported by Johnson and Söll (1970), Heinrikson and Goldwasser (1964), Suzuki and Hochster (1966), and Breitman (1970, 1971). The third proposal has been tested by Dugajczyk and Eiler (1966).

The present paper describes two methods for the isolation and crystallization of ψ from the culture filtrates of *S. ladakanus*. The purity, physical and chemical properties, and biosynthesis of ψ are also described. Preliminary reports of this work have been published (Suhadolnik, 1970; Suhadolnik and Uematsu, 1971). Three excellent reviews on the chemistry and biochemistry of ψ have been reported (Chambers, 1967; Goldwasser and Heinrikson, 1967; Söll, 1971).

Materials and Methods

[2-^{14}C]Uracil was obtained from New England Nuclear; [U-^{14}C]uridine from Schwarz BioResearch Inc.; D-[1-^{14}C]-ribose from Calatomic; [U-^{14}C]adenosine from International Chemical and Nuclear Corp.; all other compounds were the purest available from commercial sources. Coconut charcoal was purchased from Pittsburgh Activated Carbon Co., silicic acid (100 mesh, analytical reagent) from Mallinckrodt Chemical Works, AG 1-X8 (formate, 200–400 mesh) and AG 50-X8 (H^+, 100–200 mesh) from Bio-Rad Laboratories, Dowex 50-X8 (H^+, 20–50 mesh) from J. T. Baker Chemical Co.; alkaline phosphatase (chicken intenstine) was obtained from Worthington Biochemical Corp.

Paper and Thin-Layer Chromatography. Whatman No. 3MM chromatograms were employed for descending development in the following systems: (A) isobutyric acid–concentrated ammonium hydroxide–water (66:1:33, v/v), (B) 1-butanol–concentrated ammonium hydroxide–water (86:7:7, v/v), (C) 1-butanol–water (86:14, v/v), and (D) 1-butanol–acetic acid–water (4:1:5, v/v). Avicel-F thin-layer plates

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[1] Abbreviations used are: ψ, the β-D isomer of pseudouridine; ψ5′P, pseudouridine 5′-monophosphate.

TABLE I: R_F Values of the Pyrimidines and Pyrimidine Derivatives.

Compound	Solvent Systems[a]			
	A[b]	B[c]	C[d]	D[e]
Uracil	0.87	0.31	0.55	0.46
5-Formyluracil	0.67	0.12	0.12	0.41
5-Carboxyuracil	0.49	0.00	0.08	0.36
Uridine	0.62	0.15	0.12	0.31
Pseudouridine	0.51	0.08	0.03	0.20
5-β-D-Ribofuranosyluridine[f]		0.05		
Uridine 5′-monophosphate	0.25	0.00	0.00	0.03

[a] See Materials and Methods for description of solvents. [b] Dugajczyk and Eiler (1966). [c] Cohen (1960). [d] Chambers *et al.* (1963). [e] Hall (1965). [f] Brown *et al.* (1968).

(250 μ thick) (Anal. Tech. Inc., Wilmington, Del.) were developed in solvent A. The ultraviolet areas on the chromatograms were visualized under an ultraviolet lamp. The R_F values of the nucleosides on the thin-layer chromatograms were the same as those observed by paper chromatography (Table I). Elemental analyses were performed by the Greenwood Laboratories. Three criteria were used to determine the per cent purity of β-D-ψ. They were microbioassay, gas chromatography, and nuclear magnetic resonance (nmr) spectroscopy. The microbioassay method used the pyrimidine auxotroph of *E. coli* (mutant B 5RU) (Breitman, 1970). This mutant utilizes only the β-D-isomer of ψ. These bioassays were performed by Dr. T. Breitman, National Cancer Institute, National Institutes of Health.

The R_F values and ultraviolet spectral properties (in 0.1 N HCl, water, and 0.1 N NaOH) of the uracil and uridine, isolated from the culture filtrates of *S. ladakanus*, were identical with those reported for the authentic compounds. Radioactive measurements were carried out in a Packard liquid scintillation spectrometer Model 314 EX with Bray's scintillation solution (Bray, 1960).

Analytical Techniques. Optical measurements, nmr spectra, and infrared spectra were made with a Gilford recording spectrophotometer, Model 2400, a Beckman DB monochrometer system, an A_{60} nmr spectrometer using tetramethylsilane as the external standard and a Beckman infrared spectrophotometer. Gas chromatographic measurements of ψ were made on a Hewitt-Packard Model 5750. The column dimensions were 6 ft × 0.5 in.; the support was Chromasorb W; stationary phase 3% OV-17; solvent, trisil; column temperature, 210°; injection port temperature, 310°; flame detector temperature, 340°. Melting points were obtained with a Thomas-Hoover melting point apparatus. Melting points were not corrected.

Growth of Agrobacterium tumefaciens. Cultures of *A. tumefaciens* (strain II BNY6) were obtained from Dr. A. C. Braun, Rockefeller University, New York. The organism was grown by the method described by Suzuki and Hochster (1966).

Preparation of Cell-Free Extracts. The cells (*S. ladakanus* (40 g wet cells) or *A. tumefaciens* (44 g wet cells)) were collected and broken with a French press at 16,000 psi. All other procedures for the isolation of ψ5′P-synthetase were the same as those described by Suzuki and Hochster (1966). The crude extract of *S. ladakanus* contained 8.8 mg of protein/ml; the 20–70% ammonium fraction contained 25 mg/ml. The crude extract of *A. tumefaciens* used for the ψ5′P-synthetase contained 3.5 mg of protein/ml.

Assay Procedure for Synthesis of ψ5′P. Two procedures were used to assay for ψ5′P-synthetase. In method A, ψ5′P-synthetase activity was determined by measuring the tritium oxide formed when [5-^{3}H]uracil condenses with ribose 5-phosphate (Breitman, 1970). These assays were performed by Dr. T. Breitman (National Cancer Institute, National Institutes of Health). In method B, ψ5′P-synthetase was measured according to the procedure as described by Heinrikson and Goldwasser (1964) and Suzuki and Hochester (1966).

Procedure for Hydrolysis of 5′-Nucleotides Formed from Pseudouridylate Synthetase. The ψ5′P formed following incubation with pseudouridylate synthetase was hydrolyzed to ψ by the addition of alkaline phosphatase. The pseudouridylate synthetase incubation mixtures were inactivated by heating at 100° for 5 min.

Growth of S. ladakanus. The cultures of *S. ladakanus* were obtained from the Upjohn Co. Stock cultures were kept as spore preparations in sterile soil (method A) and agar straws (method B).

METHOD A. The composition of the seed medium was as follows: 5 g of D-glucose and 5 g of pharmamedia in 200 ml of tap water (adjusted to pH 7.2 with 0.1 N NaOH) in a 2-l. nonstippled flask. This medium was inoculated with spores from sterile soil and incubated on a New Brunswick rotary shaker at 28°. The production medium for ψ contained 12 g of D-glucose, 8 g of pharmamedia, and 8 g of Philadelphia whole dried yeast in 400 ml of tap water (adjusted to pH 7.2 with 0.1 N NaOH) in a 2-l. nonstippled flask. The seed culture (20 ml, grown for 48 hr) was transferred to the production medium and incubated on a New Brunswick rotary shaker at 28°.

METHOD B. A 0.5-ml aliquot of the production medium, grown for 72 hr, was transferred to a nutrient agar plate and maintained at room temperature for 4 days. The spores on the agar plate were forced into sterilized drinking straws and maintained at −20° until use. One straw was added to each production flask and incubated at 28° on a rotary shaker.

Isolation of ψ. Two methods for the isolation of ψ are described.

METHOD 1. Five flasks were incubated for 101 hr. The culture medium was centrifuged and the supernatant was passed through a Dowex 50 (H^+) column (250 ml, 20–50 mesh) and washed with water. The effluent was adjusted to pH 5.0 and concentrated to 50 ml under vacuum at 45°. The concentrate was mixed with 300 ml of 95% ethanol, refluxed, cooled, and the supernatant was decanted. The process was repeated. The combined alcohol extracts were evaporated to a syrup under vacuum at 45°. Water (20 ml) and silicic acid (5 g) were added and lyophilized. The dried silicic acid sample was added to a silicic acid column (80 ml, 25 × 1.7 cm) which was packed with chloroform. The column was washed with 300 ml of chloroform, 600 ml of chloroform–ethanol (85:15, v/v), 300 ml of chloroform–ethanol (70:30, v/v), and 500 ml of chloroform–ethanol (40:60, v/v). The ψ was detected by thin-layer chromatography using solvent A. The solvent was evaporated and the residue was treated with 30 ml of water and filtered. The filtrate was added to a charcoal–Celite

column (4:4, w/w) and washed with 1.5 l. of water. ψ was eluted with water–ethanol (85:15, v/v) at room temperature (**Caution:** if this elution were carried out at 0–4°, this concentration of alcohol would not bring off the ψ.) The fractions containing ψ were combined and evaporated under vacuum at 45° to give 67 mg of a white powder. ψ was crystallized from 75% ethanol: yield 16 mg, mp 221°, lit. (Cohn, 1960; Dugajczyk and Eiler, 1969; Shapiro and Chambers, 1961) mp 221–231°. The per cent of β-D-ψ isomer as determined by gas chromatography or microbioassay was 96.5 and 100%, respectively. The nmr spectrum of the ψ (D_2O, pD 6.3, 0.12 M, 30°) showed the resonance of the anomeric $H_{1'}$ with a quartet centered at 4.67 ppm (lit.(Hruska *et al.*, 1970; Grey *et al.*, 1970) 4.67). There was no detectable proton chemical shift for the ψ sample at 4.99 ppm. This is the spectrum for the anomeric $H_{1'}$ proton for the α-D isomer (Grey *et al.*, 1970). The nmr indicates that the ψ isolated is the pure β-D isomer. The ultraviolet and infrared spectra of the isolated crystalline β-D isomer of ψ were identical with reported values (Sober, 1968; Suzuki and Hochster, 1964; Cohn, 1960; Hruska *et al.*, 1970; Grey *et al.*, 1970). Elemental analyses were in agreement with the structure $C_9H_{12}N_2O_6$.

METHOD 2. Three flasks were incubated for 108 hr. The medium was centrifuged and the supernatant was passed through 160 g of coconut charcoal, washed with 15 l. of water, and treated with 2.5 l. of acetone–water (60:40, v/v). The acetone extract was concentrated to a syrup and taken up in 25 ml of water, adjusted to pH 6.8 with saturated barium hydroxide and added to ten volumes of 95% ethanol and filtered. The filtrates were evaporated to a syrup under vacuum at 45°, dissolved in 20 ml of water, mixed with 5 g of silicic acid, and lyophilized. The dried mixture was added to the top of a silicic acid column (50 ml) which was packed to a constant height with chloroform. After washing with 200 ml of chloroform, the column was treated with ethanol–chloroform (30:70, v/v). Fractions of 20 ml were collected. ψ was in fractions 14–34. The ψ fractions were combined and evaporated to dryness under vacuum. The residue was dissolved in a minimum volume of water and evaporated to 1 ml by an air stream on a water bath. The crystals that separated out were washed with 2 ml of cold 95% ethanol three times. The solid was dissolved in 0.5 ml hot water followed by the addition of 4.5 ml of hot ethanol and stored for 5 hr at −20°: yield 45 mg, mp 224°. The mother liquor was concentrated to dryness under vacuum at 45°. The residue was taken up in 10 ml of water and mixed with AG 50-W-X8 (H^+) resin (2 ml); pH of supernatant was 2.5. The resin was removed by centrifugation and washed with 5-ml volumes of water. The combined supernatant and washings were adjusted to pH 4.5 with solid ammonium bicarbonate or DEAE-52 cellulose (hydrogen carbonate form). The supernatant was evaporated to dryness. The residue was dissolved in water and concentrated to 0.5 ml. The solution was allowed to stand for 10 min in an ice bath. The ψ was collected by centrifugation, washed with cold 95% ethanol, and recrystallized from 90% ethanol: yield 25 mg, mp 224°. The total yield of crystalline ψ from three flasks was 70 mg. The physical and chemical properties of ψ isolated by Method 2 were identical with those of ψ isolated by Method 1.

Periodate Oxidation of ψ. ψ was oxidized with periodate to 5-carboxyuracil and 5-formyluracil by the procedure of Dugajczyk and Eiler (1969). The uv properties of the 5-formyluracil are in agreement with those reported by Cline *et al.* (1959) and Dugajczyk and Eiler (1969). The yield of 5-carboxyuracil was 18.6 μmoles, 41% yield was based on OD_{272}. These spectral properties are in excellent agreement with those reported by Dugajczyk and Eiler (1969).

Decarboxylation of 5-Carboxyuracil to Uracil. Isono and Suzuki (1970) reported on the bisulfite-catalyzed facile decarboxylation reaction of 5-carboxyuracil.

Sodium bisulfite (104 mg; 1 mmole) was added to 12 μmoles of 5-carboxyuracil in 2 ml of water and maintained at 50–55° for 1.5 hr on a water bath. In another experiment, it was observed that the decarboxylation was complete after 30 min. The reaction was stopped by the addition of 1 ml of concentrated ammonium hydroxide. The reaction mixture was spotted on Whatman No. 3MM paper and developed by solvent B. The chromatogram showed one ultraviolet area that had the same R_F as uracil. For isotope labeling experiments, a second paper chromatogram was employed using solvent D. The ultraviolet spectra showed λ_{max} at 259 mμ (in water) and λ_{min} at 228 mμ; ratios of ϵ_{250}, ϵ_{280}, and ϵ_{290}–ϵ_{260} were 0.88, 0.17, and 0.01, respectively (reported λ_{max} 259.5 mμ and λ_{min} 227 mμ, ratios of ϵ_{250}, ϵ_{280}, and ϵ_{290}–ϵ_{260} were 0.84, 0.17, and 0.01, respectively (Sober, 1968).

Protein concentration was determined by the method of Murphy and Kies (1960).

Results

Isolation of ψ, *Uracil, and Uridine from the Culture Filtrates of S. ladakanus.* Two methods are described for the isolation and crystallization of the β-D isomer of ψ from the culture filtrates of *S. ladakanus*. The yield of ψ by Method 2 is 70 mg/l. The yield of ψ from the control experiment (Table II, expt 2) was 45 μmoles/l.; the yield of ψ from the alkaline hydrolyzed whole dried yeast experiment (Table II, expt 2) was 40 μmoles/l. Therefore, the production of ψ in medium containing whole dried yeast is the same as the production of ψ in medium in which the ψ in the RNA of the whole dried yeast was removed by alkaline hydrolysis. Monier *et al.* (1966) and Chao and Schachman (1956) reported the yield of tRNA in fresh pressed baker's yeast to be 0.08–0.3%. The experiments described here utilized 20 g of dried yeast/l. of medium. This 20 g of dried yeast would contain about 2 mg of ψ/l. This is considerably lower than the 70 mg of crystalline pseudouridine isolated per l. of medium. The physical and chemical properties of ψ described here are in agreement with those reported in the literature. The purity of ψ as the β-D isomer was determined by three methods. First, the microbioassay method using the pyrimidine auxotroph of *Escherichia coli*, which utilizes only the β-D isomer of ψ, showed the ψ to be 100% β-D isomer. Second, the nmr and the infrared spectra were identical with that reported for the β-D isomer; third, gas chromatographic analysis showed that the ψ isolated was 97% β-D isomer and 3.0% α-D isomer. Although ψ has been isolated from the hydrolysates of yeast RNA (Cohn, 1957; Davis and Allen, 1957), human urine (Adler and Gutman, 1959), and the culture filtrates of *A. tumefaciens* (Suzuki and Hochster, 1964), this is the first report on the isolation of ψ as a naturally occurring nucleoside in yields of 70 mg/l. The yields of uracil and uridine isolated 84 hr after inoculation were 1.5 and 0.6 μmoles per l., respectively.

Incorporation of Radioactivity from ^{14}*C-labeled Uracil, Uridine, Ribose, and Adenosine into* ψ, *Uracil, and Uridine by S. ladakanus.* The data in Table II show that ^{14}C from [2-^{14}C]-uracil, [U-^{14}C]uridine, and [1-^{14}C]ribose is incorporated into the ψ, uracil, and uridine isolated from the culture filtrates of *S. ladakanus*. The specific activities and incorporation efficien-

TABLE II: Incorporation of ^{14}C from [2-^{14}C]Uracil, [U-^{14}C]Uridine, [1-^{14}C]Ribose, and [U-^{14}C]Adenosine into Pseudouridine, Uracil, and Uridine by *S. ladakanus*.

Compound Isolated from the Culture Filtrates	Compound Added					
	Expt 1 [U-^{14}C]-Uridine[a]	Expt 2 [U-^{14}C]Uridine		[2-^{14}C]-Uracil[a]	[1-^{14}C]Ribose[a]	[U-^{14}C]-Adenosine[a]
		1[b]	2[c]			
Pseudouridine						
μmoles/l.	280	45	40	37	180	135
mCi/mole	0.19	3.7	3.7	7.7	0.03	0.06
Incorporation efficiency[d]	0.009	0.75	0.75	0.02	0.0003	0.00002
Uracil						
μmoles/l.				1.5		
mCi/mole				1.8		
Incorporation efficiency				0.005		
Uridine[e]						
μmoles/l.				0.6		
mCi/mole				1.3		
Incorporation efficiency				0.004		

[a] The amount of ^{14}C-labeled compound added was as follows: [U-^{14}C]uridine (6.0 μCi, 2.8 μmoles, 2170 mCi/mole), to five flasks; [2-^{14}C]uracil (20 μCi, 0.584 μmole, 34,240 mCi/mole), to two flasks at 84 hr; [1-^{14}C]ribose (13.6 μCi, 1.53 μmoles, 8900 mCi/mole), to three flasks; [U-^{14}C]adenosine (12 μCi; 0.048 μmole, 250,000 mCi/mole), to two flasks; additions were made at 84 hr ψ, uracil, and uridine from the uracil and uridine experiments were isolated 6 hr after the addition of the labeled compounds; ψ from the ribose and adenosine experiments was isolated 24 hr after the addition of the labeled compounds. [b] The amount of [U-^{14}C]uridine added to one flask 81 hr after inoculation was as follows: 10 μCi, 20 μmoles; 500 mCi/mole. ψ was isolated 6 hr later. [c] This flask contained 10 g of dried residue following alkaline hydrolysis of dried yeast. The dried yeast (100 g) was treated with 1200 ml of 0.3 N KOH, 18 hr, 37°, to hydrolyze the RNA and remove the ψ in the yeast tRNA. The hydrolysate was made acidic with trichloroacetic acid. The insoluble material was removed by centrifugation, washed with ethanol, centrifuged, and dried; yield 40 g. Ten grams of this dried, hydrolyzed yeast extract was added per flask. Pharmamedia and glucose were added as described in the Experimental Section. [U-^{14}C]Uridine was added to one flask 81 hr after inoculation: 10 μCi, 20 μmoles, 500 mCi/mole. ψ was isolated 6 hr later. [d] This term is defined as 100 times the specific activity of ψ divided by the specific activity of the compound tested. [e] Ribose (1×10^{-3} M) was added to the culture flasks simultaneously with the [U-^{14}C]uridine.

cies of ψ varied with the amount and specific activity of the [U-^{14}C]uridine added.

The incorporation efficiency of the ψ was 0.009 when the amount of [U-^{14}C]uridine added to five flasks were 2.8 μmoles (2170 mCi/mole). When 20 μmoles of [U-^{14}C]uridine (500 mCi/mole) was added to 300 ml of medium, the incorporation efficiency was 0.75 (Table II). To determine if the ψ in the whole dried yeast added to the medium was contributing to the ψ isolated from the culture filtrate, experiments were performed in which the RNA in the yeast was removed by alkaline hydrolysis. In this case, the specific activities of the ψ in the control flasks were the same as in the flask in which the ψ in the RNA was removed by alkaline hydrolysis, *i.e.*, 3.7 mCi/mole. Therefore, the ψ isolated from the culture medium does not result from hydrolysis of the ψ in the RNA of the yeast extract. The high dilution of the ^{14}C-labeled uracil and uridine into ψ when the concentration was 0.17 and 0.56 μmole per 300 ml of medium, respectively, can be explained as follows. First, 2.8 μmoles of [U-^{14}C]uridine was added to five flasks and 280 μmoles of crystalline ψ was isolated. The conversion of the labeled uridine to ψ represents a 100-fold dilution. Second, the incorporation efficiencies of the ^{14}C-labeled uracil and uridine isolated from the culture medium, from the [2-^{14}C]uracil experiment, were 0.005 and 0.004, respectively. These high dilution data indicate that the *S. ladakanus* is continuously producing uracil and uridine. Third, under identical experimental conditions, the dilution of [U-^{14}C]uridine could be changed appreciably. For example, when the amount of [U-^{14}C]uridine added to 300 ml of medium was increased from 0.56 to 20 μmoles, the incorporation efficiency of ψ increased from 0.009 to 0.75 (Table II).

[U-^{14}C]Adenosine was added to the ψ producing *S. ladakanus* to determine if the ribosyl moiety of a purine nucleoside or nucleotide undergoes a transribosylation to form ψ. The transribosylation reaction does not appear to be important for ψ biosynthesis as evidenced by the lack of incorporation of ^{14}C-labeled ribose in the ψ isolated from the [U-^{14}C]adenosine experiments. The distribution of ^{14}C in the adenine: ribose of the [U-^{14}C]adenosine used in these experiments was 46:54.

Distribution of ^{14}C in ψ from the [2-^{14}C]Uracil, [U-^{14}C]-Uridine, and [1-^{14}C]Ribose Experiments. The data in Table III show that the distribution of ^{14}C in the uracil: ribose of the ψ isolated from the [U-^{14}C]uridine experiments was 100:0; the distribution of the ^{14}C in the uracil: ribose of the [U-^{14}C]-uridine added to the culture flasks was 40: 60. All of the ^{14}C from the [2-^{14}C]uracil experiment resided in the uracil moiety of ψ. The specific activity of the uracil did not change following the decarboxylation of 5-carboxyuracil. This indicates that C-1′ of the ribose moiety of ψ was not radioactive.

TABLE III: Distribution of ^{14}C in Pseudouridine Isolated from the [2-^{14}C]Uracil, [U-^{14}C]Uridine, and D-[1-^{14}C]Ribose Experiments.[a]

Compound Tested	Pseudouridine Isolated (mCi/mole)	Degradation Products: 5-Carboxyuracil mCi/mole	5-Carboxyuracil %	Uracil mCi/mole	Uracil %
[2-^{14}C]Uracil	3.1	3.08	99	3.1	100
[U-^{14}C]Uridine	0.38	0.33	87	0.34	89
[U-^{14}C]Uridine[b]	0.2	0.2	100	0.2	100
[1-^{14}C]Ribose	0.3	0.2	67	0.02	8

[a] Experimental conditions are identical with those in Table II. Degradations were done as described under Materials and Methods. [b] D-Ribose (1×10^{-3} M) was added to the culture flasks simultaneously with the [U-^{14}C]uridine.

TABLE IV: Activity of Uridine Phosphorylase in Crude Extracts of *S. ladakanus*.[a]

Incubation Mixture	Uridine Formation (nmoles)	Uracil Formation (nmoles)
Experiment A		
[2-^{14}C]Uracil + uridine		
Minus phosphate	12	
Plus phosphate	22	
Boiled control	0	
Experiment B		
[U-^{14}C]Uridine		
Minus phosphate		11
Plus phosphate		24
Boiled control		0

[a] The reaction 0.5 ml, contained 4.8×10^{-4} M [U-^{14}C]-uridine (430 mCi/mole) or 8.9×10^{-4} M [2-^{14}C]uracil (270 mCi/mole) in 8×10^{-2} M 2-(*N*-morpholino)ethanesulfonic acid–sodium hydroxide buffer (pH 6.8) and 1.4 mg of protein (dialyzed-lyophilized cell-free extract of *S. ladakanus*). Unlabeled uridine (4.8×10^{-4} M) or phosphate (4×10^{-4} M) was added as shown. Incubations were done at 37° for 30 min. Reactions were stopped by heating on a steam bath for 3 min. Uracil or uridine was determined by adding 0.1-ml aliquot of incubation mixture to a Whatman No. 3MM paper chromatogram and developing in 1-butanol–water (86:14, v/v). The uracil and uridine areas on the paper chromatograms were cut out, eluted with hot water (two times), and counted. The ratio of ^{14}C in the uracil:ribose moieties of [U-^{14}C]uridine was 37:63, respectively.

Degradation of the ψ from the [1-^{14}C]ribose experiment showed that 92% of the ^{14}C resides in the ribose moiety and 8% resides in the uracil. It was expected that all of the ^{14}C in the ribose of ψ from the [1-^{14}C]ribose experiment would reside in C-1′. However, only 59% of the ^{14}C was found in C-1′ of ψ (Table III). The remaining 33% of the ^{14}C in the ribose of ψ from the [1-^{14}C]ribose is in C-2′, C-3′, C-4′, and/or C-5′.

In Vitro Synthesis of ψ. When either the whole homogenates of the 10,000*g* supernatant or the 20–70% ammonium sulfate fraction of *S. ladakanus* was incubated with [5-^{3}H]uracil and ribose 5-phosphate, no tritium oxide was formed. Similarly, incubations with [2-^{14}C]uracil and ribose, ribose 5-phosphate, ribose 1-phosphate, or phosphoribosyl pyrophosphate followed by the addition of alkaline phosphatase, did not yield ^{14}C-labeled ψ. ψ was synthesized and isolated when the supernatant of cell-free extracts of *A. tumefaciens* or *E. coli* were incubated with uracil and ribose 5-phosphate as described by Suzuki and Hochster (1966) and Breitman (1970). To determine if an inhibitor of ψ were present in the supernatant of *S. ladakanus* aliquots of the 10,000*g* supernatant from *S. ladakanus* were added to the ψ5′P-synthesizing incubations of either *A. tumefaciens* or *E. coli*. There was no inhibition of ψ formation.

Uridine Phosphorylase Activity with Crude Extracts from S. ladakanus. Because all the ^{14}C from the [U-^{14}C]uridine experiments was located in the uracil moiety of the ψ isolated from the culture filtrates of *S. ladakanus* (Table III), it was necessary to determine if this change in ^{14}C ratio in the uracil:ribose moieties were attributed to an active uridine phosphorylase. Krenitsky (1968) reported that uridine phosphorylase involved the formation of free pentose 1-phosphate which in turn reacts with a free base to form a new nucleoside. This reaction requires inorganic phosphate. The data in Table IV show that there is a transfer of the ribosyl moiety from uridine to [2-^{14}C]uracil to form [2-^{14}C]uridine (expt A).

When the incubations were done with [U-^{14}C]uridine, [U-^{14}C]uracil was isolated (expt B). The direct transfer reaction and the hydrolysis of [U-^{14}C]uridine were both increased by the addition of inorganic phosphate. Similar assays were performed in incubations with dialyzed, lyophilized samples of the culture filtrates. There was no uridine phosphorylase nor uridine hydrolase activity in the culture filtrate.

Discussion

The data presented firmly establish that, in addition to 5-azacytidine, *S. ladakanus* also excreted ψ, uridine, and uracil into the medium. Seventy milligrams of crystalline ψ was isolated per liter of culture filtrate of *S. ladakanus*. The yield and specific activities of ψ in flasks containing whole dried yeast was essentially the same as the yield of ψ in flasks to which alkaline hydrolyzed dried yeast was added. Therefore, *S. ladakanus* must be excreting large amounts of ψ into the culture filtrate. Pseudouridylate synthetase activity was not detected in cell-free extracts of *S. ladakanus*. The biosynthesis of ψ has been suggested to proceed *via* pseudouridylate synthetase or by rearrangement of uridine residues in RNA (Heinrikson and Goldwasser, 1964; Suzuki and Hochster, 1966; Chambers, 1967; Ginsberg and Davis, 1968; Chirikdjian and Davis, 1970; Breitman, 1970; Matsushita and Davis, 1971; Johnson and Söll, 1970). The possibility that the role of pseudouridylate synthetase in cellular reactions is to act as a degradative enzyme to hydrolyze ψ to uracil and ribose 5-phosphate following turnover of cellular RNA has also been considered. Breitman (1970, 1971) has shown that pseudouridylate synthetase in pyrimidine auxotrophs of *E. coli* can grow on the β-D isomer of ψ. This *E. coli* mutant hydrolyzes ψ5′P to uracil and ribose 5-phosphate. The uracil

and ribose 5-phosphate is used for essential cellular reactions. These findings by Breitman strongly suggest that the ability of those *E. coli* strains to grow equally well on uracil or the β-D isomer of ψ appears to be the biochemical basis for pseudouridylate synthetase activity.

The absence of pseudouridylate synthetase in extracts of *S. ladakanus* along with the isolation of large amounts of ψ in the culture filtrates is taken as additional evidence that the role of pseudouridylate synthetase is to act as a degradative enzyme to remove the ψ5′P formed following turnover of cellular RNA.

The data presented show that the uracil moiety but not the ribose moiety of [U-^{14}C]uridine serves as the aglycone for the biosynthesis of ψ. The isolation of crystalline ^{14}C-labeled ψ from the [U-^{14}C]uridine, [2-^{14}C]uracil, and [1-^{14}C]ribose experiments is added proof that the ψ in the culture filtrates is synthesized by *S. ladakanus*. The ψ is not formed following hydrolysis of the RNA from the yeast that is added to the growth medium. The isotope dilution when [U-^{14}C]uridine was incorporated into ψ was markedly changed by increasing the amount of [U-^{14}C]uridine from 0.56 to 20 μmoles per 300 ml. ψ was synthesized by *S. ladakanus* and not from the ψ in the RNA from the whole dried yeast added to the medium. This is based on the data showing that the specific activities of the ψ isolated from the flasks containing 20 g of yeast in 1 l. of medium was the same as the specific activity of the ψ isolated from the flasks in which the ψ in the RNA was removed by alkaline hydrolysis.

The labeling pattern observed in the ψ from the [U-^{14}C]-uridine experiments (*i.e.*, all ^{14}C in ψ is located in the uracil) can be explained as follows. There is a very active uridine phosphorylase in *S. ladakanus*. Since the reaction is reversible, the effect of phosphate for the synthesis of uridine (Table III, expt A) and the hydrolysis of uridine (expt B) was studied. Phosphate stimulated the synthesis and hydrolysis reactions.

References

Adler, M., and Gutman, A. B. (1959), *Science 130*, 862.

Bergy, M. E., and Herr, R. R. (1966), *Antimicrob. Ag. Chemother.*, 625.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Breitman, T. R. (1970), *J. Bacteriol. 103*, 264.

Breitman, T. R. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 257.

Brown, D. M., Burdon, M. G., and Slatcher, R. P. (1968), *J. Chem. Soc. C*, 1051.

Chambers, R. W. (1967), *Progr. Nucl. Acid Res. Mol. Biol. 5*, 349.

Chambers, R. W., Kurkov, N., and Shapiro, R. (1963), *Biochemistry 2*, 1192.

Chao, F. C., and Schachman, H. K. (1956), *Arch. Biochem. Biophys. 61*, 220.

Chirikdjian, J. G., and Davis, F. F. (1970), *J. Biol. Chem. 245*, 1296.

Cline, R. E., Fink, R. M., and Fink, K. (1959), *J. Amer. Chem. Soc. 81*, 2521.

Cohn, W. E. (1957), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 16*, 166.

Cohn, W. E. (1960), *J. Biol. Chem. 235*, 1488.

Davis, F. F., and Allen, F. W. (1957), *J. Biol. Chem. 227*, 907.

Dugajczyk, A., and Eiler, J. (1966), *Biochim. Biophys. Acta 119*, 11.

Dugajczyk, A., and Eiler, J. (1969), *J. Biol. Chem. 244*, 2750.

Elstner, E. F., and Suhadolnik, R. J. (1972), *Biochemistry 11*, 2578.

Ginsberg, T., and Davis, F. F. (1968), *J. Biol. Chem. 243*, 6300.

Goldwasser, E., and Heinrikson, R. L. (1967), *Progr. Nucl. Acid Res. Mol. Biol. 5*, 399.

Grey, A. A., Smith, I. C. P., and Hruska, F. E. (1971), *J. Amer. Chem. Soc. 93*, 1765.

Hall, R. (1965), *Biochemistry 4*, 661.

Hanka, L. J., Evans, J. S., Mason, D. J., and Dietz, A. (1966), *Antimicrob. Ag. Chemother.*, 619.

Heinrikson, R. L., and Goldwasser, E. (1964), *J. Biol. Chem. 239*, 1177.

Hruska, F. E., Grey, A. A., and Smith, I. C. P. (1970), *J. Amer. Chem. Soc. 92*, 214.

Isono, K., and Suzuki, S. (1970), *Tetrahedron Lett.*, 425.

Johnson, L., and Söll, D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 943.

Krenitsky, T. A. (1968), *J. Biol. Chem. 243*, 2871.

Monier, R., Stephenson, M. L., and Zamecnik, P. C. (1960), *Biochim. Biophys. Acta 43*, 1.

Murphy, J. B., and Kies, M. W. (1960), *Biochim. Biophys. Acta 45*, 382.

Shapiro, R., and Chambers, R. W. (1961), *J. Amer. Chem. Soc. 83*, 3920.

Sober, H. A., Ed. (1968), *in* Handbook of Biochemistry, Cleveland, Ohio, The Chemical Rubber Co., p G-42.

Söll, D. (1971), *Science 173*, 293.

Suhadolnik, R. J. (1970), Nucleoside Antibiotics, New York, N. Y., John Wiley and Sons, p 273.

Suhadolnik, R. J., and Uematsu, T. (1971), 162nd National Meeting of the American Chemical Society, Washington, D. C., Sept, *Abstract* BIOL.

Suzuki, T., and Hochster, R. M. (1964), *Can. J. Microbiol. 10*, 867.

Suzuki, T., and Hochster, R. M. (1966), *Can. J. Biochem. 44*, 259.