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Pseudouridine Formation: Evidence for RNA as an Intermediate

Abstract. *Experiments with intact or lysed spheroplasts from* Escherichia coli *indicate that conditions which impair the DNA-directed synthesis of RNA, also prevent formation of pseudouridine. Studies with uridine-labeled RNA support the concept that RNA may be a direct intermediate for pseudouridine synthesis.*

Since the description of 5-ribosyluracil (pseudouridine) as a component of cellular RNA's (*1*), several investigators have attempted to elucidate the mechanism by which this nucleoside is formed (*2*). Robbins and co-workers, using doubly labeled uridine and 5-fluorouridine, concluded that both the ribose and pyrimidine moieties of uridine are direct precursors for ψ-uridine formation in yeast (*3*). On the basis of these findings, the same authors suggested an intramolecular rearrangement of the uridine base and sugar; however, the data was insufficient to decide whether this rearrangement occurred before or after uridine incorporation into RNA.

On the other hand, several investigators have suggested that ψ-uridine might be formed by a 3,5-diribosyluracil intermediate (*4*) or by a direct condensation of uracil with ribose-5-phosphate to form ψ-UMP (*5*). The enzyme ψ-UMP synthetase which catalyzes this condensation reaction has been partially purified from extracts of *Tetrahymena pyriformis* (*5*). Although these findings indicate that ψ-uridine may be formed before nucleotide polymerization, the possibility still exists that ψ-uridine, found in the framework of cytoplasmic RNA's, is formed after polynucleotide assembly. We now report evidence that, in *Escherichia coli*, ψ-uridine formation requires the prior synthesis of RNA. Agents which inhibit ribonucleotide polymerization also prevent ψ-uridine formation.

The following experiments were conducted with either intact or lysed spheroplasts from *E. coli*. The spheroplasts were prepared as previously reported (*6*). Lysed cells were prepared by sedimenting the spheroplasts and suspending the cells in a hypotonic medium containing 0.02*M* tris, *p*H 7.5, 0.002*M* $MgCl_2$ and 0.014*M* 2-mercaptoethanol. The labeled precursors used were uracil-2-^{14}C (Schwarz BioResearch), UTP-^{14}C, or uridine-labeled cRNA-^{14}C. The UTP-^{14}C was prepared by deamination of uniformly labeled CMP (New England Nuclear) according to the procedure of Lohman (*7*), purification of the UMP product by chromatography on Dowex-1 (formate), and conversion to UTP by a yeast extract containing uridylate kinase activity (*8*). The labeled UTP was not purified from the kinase mixture but used as such after it was heated at 90°C for 3 minutes. Paper electrophoresis of this mixture indicated that more than 95 percent of the radioactivity migrated as UTP, the remainder being distributed between UMP and UDP. The above procedure was used to avoid the presence of labeled ψ-uridine nucleotides found in commercial preparations of uridine-^{14}C nucleotides. This difficulty was only partially overcome, since our preparation of UTP-^{14}C still contained some contaminant (approximately 0.05 percent) which behaved like ψ-uridine in the assay procedure and contributed to the high base lines found. The contaminant did not appear to be ψ-UTP-^{14}C since synthetic cRNA-^{14}C, prepared with the same labeled UTP, contained little radioactive ψ-uridine. Radioactive cRNA was prepared with the highly purified RNA polymerase from *Micrococcus lysodeikticus, Escherichia coli* DNA being used as template and UTP-^{14}C as the only labeled substrate (*8*). The synthetic cRNA was freed from UTP-^{14}C of the polymerase reaction mixture by repeated precipitation with ethanol and exhaustive dialysis against 0.1*M* NaCl and then against water. Pseudouridine used as carrier was isolated from human urine by the procedure of Adler and Gutman (*9*) as modified by Goldberg and Rabinowitz (*10*).

The procedure routinely used for isolating ψ-uridine was as follows: (i) The entire reaction mixture was hydrolyzed with alkali and treated with alkaline phosphatase; the total labeled nucleosides were isolated by adsorption onto Dowex-1 (acetate) and eluted with acetic acid (Table 1); (ii) carrier uridine and ψ-uridine were added to the isolated nucleoside fraction which was then concentrated and subjected to descending paper chromatography in a mixture of butanol and water (86:14); (iii) pseudouridine was eluted and passed over a column (1 by 7 cm) of Dowex-50 (H^+) in 0.05*N* HCl; the acid-eluate was neutralized and the labeled nucleoside was concentrated by treatment with Dowex-1 (acetate) as described above; (iv) the concentrate was subjected to paper chromatography in the butanol-water system a second time; (v) the ψ-uridine band was eluted and subjected to paper chromatography in a system containing isopropanol, acetic acid, and water

Table 1. Effect of actinomycin D on uracil-^{14}C incorporation into pseudouridine with *Escherichia coli* spheroplasts. The incubation mixture, final volume 10.9 ml, contained 10 ml of spheroplasts in a modified nutrient broth (*6*) (approximately 1×10^9 cells/ml) and, as labeled precursor, 0.532 μmole of uracil-2-^{14}C (64.9×10^6 count/μmole). In addition, one incubation mixture contained 250 μg of actinomycin D. After 2 hours at 37°C, the cells were sedimented by centrifugation, suspended in 2 ml of 0.4*N* KOH and held at 37°C for 18 hours. The hydrolyzate was acidified with 70 percent $HClO_4$, iced for 1 hour and centrifuged; the supernatant was exposed to the action of intestinal alkaline phosphatase (1 mg/ml) overnight at 37°C after adjustment to *p*H 9 in a final volume of 10 ml. The reaction was stopped by heat (10 minutes in a boiling-water bath, cooled, and filtered; the filtrate was made up to a volume of 100 ml and adjusted to *p*H 10.5 with NH_4OH. The ^{14}C-nucleosides were concentrated by adsorption onto a 6-cm^2 column of Dowex-1 (acetate), elution with 0.1*N* acetic acid and evaporation to dryness under vacuum. The labeled residue was dissolved in a minimum volume of water; carrier uridine and ψ-uridine were added, and the ψ-uridine was reisolated by chromatographic and Dowex-50 (H^+) treatment.

Expt. No.	Actinomycin D	Radioactivity (count/min)	
		Acid-insoluble	In ψ-uridine
1	+	0.18×10^6	775
2	−	1.54×10^6	4160

Table 2. Dependence of ψ-uridine labeling on DNA and ribonucleoside triphosphates. The basic mixture (2.0 ml) contained 200 μmole of tris, *p*H 7.5; 4 μmole each ATP, GTP and CTP; 0.075 μmole of uniformly labeled UTP-^{14}C (approximately 150 mc/mmole) as substrate; 8 μmole of phosphoenolpyruvate; 80 μg of pyruvate kinase; 6 μmole of $MnCl_2$; 4 μmole of spermidine and osmotically lysed spheroplasts (approximately 1.5 to 2.0 $\times$ 10^{11} cells). The mixtures were incubated at 30°C for 30 minutes, the reactions were stopped with 0.20 ml of 4*N* KOH, held at 37°C for 18 hours, and then ψ-uridine was isolated as described in the legend of Table 1.

Expt. No.	Additions	Radioactivity (count/min)	
		Acid-insoluble	ψ-uridine
1	None (zero time)	0.05×10^6	4,985
2	None	1.33×10^6	17,380
3	Deoxyribonuclease (50 μg)	0.11×10^6	4,820
4	Omit ATP, CTP, GTP, PEP	0.31×10^6	6,740

(6:3:1). After the second butanol-water chromatography, the radioactivity in the ψ-uridine region remained fairly constant, with little change on subsequent chromatography and little radioactivity detectable in uridine. In some experiments further discrimination between the uridine isomers was made by the action of yeast uridine nucleosidase (*11*), which specifically cleaves the glycosidic linkage of uridine but does not attack ψ-uridine (*12*).

Table 3. Formation of ψ-uridine-^{14}C from urdine-labeled cRNA-^{14}C. The complete system (2.0 ml) was similar to that described for the experiments shown in Table 2 except that UTP-^{14}C was omitted and *E. coli* cRNA-^{14}C served as the substrate with additions as indicated. The reaction mixtures were incubated at 30°C and stopped with 5-percent acid. The precipitate was washed twice with 5 percent acid, once with ethanol-ether (3:1), and analyzed for labeled ψ-uridine. In experiment 2 (but not 1), the ψ-uridine counts were subjected to the action of yeast uridine nucleosidase and then rechromatographed in the butanol-H_2O system.

Incubation (min)	Total count/min			
	cRNA-^{14}C		Uridine	Pseudouridine
	Added	Recovered		
	Expt. 1, 100 μg deoxyribonuclease			
0	6.5×10^6	5.8×10^6		336
10	11.1×10^6	6.6×10^6		1280
	Expt. 2, 30 μg actinomycin D			
0	10.6×10^6	9.5×10^6	74	110
15	17.8×10^6	10.1×10^6	39	670

Intact *E. coli* spheroplasts readily incorporate uracil-^{14}C into an acid-insoluble form, and this incorporation is quite sensitive to the presence of actinomycin D. Analysis of the total spheroplast radioactivity (acid-soluble and insoluble material) revealed that ψ-uridine labeling was far less extensive in actinomycin-treated cells than in cells which had not been exposed to the antibiotic (Table 1). Because of the inhibitory effect exerted by actinomycin D on the DNA-directed synthesis of RNA (*13*) the results of this experiment suggested the possible involvement of RNA in the conversion of uridine to ψ-uridine.

Osmotically lysed spheroplasts are highly viscous and actively catalyze ribonucleotide polymerization. Since RNA synthesis with lysed *E. coli* preparations is dependent upon fairly intact endogenous DNA, ψ-uridine formation should also be dependent upon intact DNA if RNA is involved in the conversion mechanism. Lysed preparations of *E. coli* spheroplasts actively incorporate UTP-^{14}C into an acid-precipitable form, and this incorporation is inhibited by the presence of deoxyribonuclease or by the omission of a full complement of ribonucleoside triphosphates (Table 2). Analysis of the total labeled uridine material in these reaction mixtures indicated that a significant increase in ψ-uridine labeling, as compared to the control at zero time, occurred only when UTP-^{14}C incorporation into RNA was not impaired. In a similar experiment with labeled UTP and lysed spheroplasts, actinomycin D also inhibited the formation of labeled ψ-uridine.

The experiments described provide indirect evidence that RNA may be an intermediate in the conversion of uridine to ψ-uridine and that the conversion takes place after polynucleotide synthesis. A more direct proof of this conclusion can be provided with uridine-labeled cRNA-^{14}C. If RNA is the substrate, ψ-uridine formation should be independent of agents which specifically block RNA synthesis. Upon reisolation of uridine-labeled cRNA-^{14}C after incubation with a lysed spheroplast preparation, in the presence of either deoxyribonuclease or actinomycin D, there was an increased amount of labeled ψ-uridine formed as compared to the unincubated controls (Table 3). The low ψ-uridine radioactivity cannot be attributed to contamination by labeled uridine since the latter contained negligible radioactivity (Expt. 2). Nearly twice as much cRNA-^{14}C was added to the "incubated" mixtures as to the "zero" time controls to compensate for the vigorous ribonuclease activity exhibited by the lysed bacterial preparations. Since ψ-uridine analysis was performed only on the cRNA-^{14}C recovered by acid-precipitation, and since in each experiment the amount recovered from the control and experimental mixtures was nearly similar, the excess cRNA-^{14}C which was degraded to acid-soluble material should not have altered the results, provided that the liberated uridine-^{14}C nucleotides were not reincorporated into RNA. The presence of deoxyribonuclease or actinomycin D in the incubated mixtures should have prevented this recycling process. Although the total ψ-uridine radioactivity was relatively small compared to the RNA counts analyzed, the results are consistent with those of the other experiments presented here.

Furthermore, if one makes the assumptions (i) that most of the ψ-uridine of cellular RNA is primarily located in transfer RNA; (ii) that in the RNA polymerase transcription process only 0.02 percent of the synthetic cRNA represents transfer RNA; and (iii) that 15 to 20 percent of the uridine in transfer RNA represents ψ-uridine; then the radioactivity attributed to ψ-uridine (Table 3) is within the correct order of magnitude for the amount of labeled cRNA used. The rather small formation of labeled ψ-uridine in these experiments also suggests that the enzyme(s) responsible for this reaction must be highly specific in the selection of uridine residues in the polynucleotide chain for conversions to ψ-uridine.

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References and Notes

1. The following abbreviations are used: RNA and DNA for ribo- and deoxyribonucleic acid; ψ-uridine, ψ-UMP, and ψ-UTP for 5-ribosyluracil and the mono- and triphosphate nucleotides of 5-ribosyluracil; CMP, UMP, UDP, CTP, UTP, ATP, and GTP as the mono-, di-, and triphosphate nucleotides of cytidine, uridine, adenosine, and guanosine, selectively; PEP, phosphoenolpyruvate; and cRNA (complementary RNA) for the synthetic RNA prepared with RNA polymerase.
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Iron-55 in Humans and Their Foods

Abstract. *Measurable quantities of iron-55 are present in people, animals, and food. The amount in Eskimos and caribou at Anaktuvuk, Alaska, is about eight times that in residents and cattle near Richland, Washington. Meat and grains are the foods that contribute most to the body burdens of iron-55 in human beings.*

Iron-55 is one of several activation products produced in relatively high abundance by the nuclear detonations of the U.S.S.R. and the United States in 1961–62. Periodic measurements of this radionuclide in fallout have been made at several locations in the United States (*1*). We recently found Fe^{55} in the blood of Alaskan caribou, and have since measured the content of this isotope in Eskimos, in residents of Richland, Washington, and in the food of both.

Iron-55 is a nuclide which decays by electron capture. The only easily detectable radiation it emits is a 5.9-kev x-ray in 28 percent of the disintegrations. To detect this low-energy x-ray, we used an argon-methane gas-flow proportional counter with a thin [0.00015-inch (0.0038-mm) aluminized Mylar] entrance window. The 5.9-kev photopeak counts were determined with a multichannel analyzer. To reduce the background count rate, the counter was placed between two 4- by 9⅜-inch (10- by 24.4-cm) NaI(Tl) scintillation crystals which act as anticoincidence shields, and this system was surrounded by 4 inches of lead shielding. The minimum amount of Fe^{55} detectable with this system is 0.008 nc at 99-percent confidence in a 1-hour count.

The samples for counting were ashed and dissolved in 6*M* HCl; the iron was extracted with 20 percent Alamine-336 in xylene. The iron was then stripped into 1*M* $HClO_4$, precipitated as the hydroxide, redissolved in a 6*M* H_2SO_4 solution saturated with $(NH_4)_2C_2O_4$, and electrodeposited onto 3.85-cm copper planchets by the method of Maletskos and Irvine (*2*).

Table 1 gives our measurements of the concentration of Fe^{55} in the blood of humans, caribou, and cattle; each figure represents analyses of duplicate 20-ml samples. Estimates of the total blood volumes came from the literature; we then assumed that 60 percent [values up to 73 percent have been reported (*3*)] of the iron in the body was in the blood and so estimated the total body burden.

Eskimos contain about eight times as much Fe^{55} as Richlanders. Similarly, the concentration in caribou, which the Eskimos eat, is about eight times that in cattle slaughtered near Richland. Since a steer weighs about five times as much as a caribou, the total body burdens of the animals differ by less than a factor of two. We assume that the high burdens of Fe^{55} in Eskimos and caribou result, like those of cesium-137 (*4*), from accumulation of the radionuclide on lichens.

Analysis of a sample of lichen collected at Anaktuvuk, Alaska, in September 1964 showed the Fe^{55} content to be 14 nc per kilogram of dry lichen. Eskimo body burdens range from 32 to 89 nc; those of Richland residents, from 4 to 16 nc. The average Eskimo burden of 61 nc should be compared with the maximum permissible body burden of 100,000 nc for members of the general population who are not exposed in the course of their occupations (*5*).

It seems likely that the main source of Fe^{55} for the Anaktuvuk Eskimos is caribou meat, which constitutes about 40 percent of their total diet (*6*). However, we found that Eskimos with the highest body burdens of cesium-137 did not necessarily have the highest burden of Fe^{55}; in fact two Eskimos with the highest cesium-137 burden had the lowest Fe^{55} burden. This fact corroborates the finding of other investigators, that uptake and retention of these isotopes is not the same for each individual. To determine the main sources

Table 1. Content of Fe^{55} in humans, cattle, and caribou.

Date sampled	No. of subjects/ total blood vol.*	Fe^{55} Blood (nc/ liter)†	Fe^{55} Total body content (nc)†
	Anaktuvuk Eskimos		
Jan. 1965	7/5	7.3	61
	Richland residents		
Nov. 1964	4/5	0.96	8
	Alaskan caribou		
Jan. 1965	11/5	87.4	728
	Washington cattle		
Jan. 1965	8/27	9.9	440

* Total blood volume (in liters) per subject is estimated. † Average.

Table 2. Content of Fe^{55} in various foods purchased in December 1964. Sample numbers are in parentheses; stable Fe and Fe^{55} are expressed per kilogram of sample.

Food	Content: Stable Fe (mg)	Content: Fe^{55} (nc)
Alaskan caribou		
Round steak (1)	53	3.6
Round steak (2)	43	2.9
Liver	208.8	28.7
Washington State meats		
Beef, round steak (1)	22	0.86
Beef, round steak (2)	25	.92
Beef, round steak (3)	22	.30
Beef liver (1)	95	.95
Beef liver (2)	45	1.2
Elk, lean (1)	30	1.7
Elk, lean (2)	37	1.8
Bologna	9.1	.34
Pork and ham loaf	16	.06
Pork chops	6.7	.013
Hamburger	27	.25
Weiners	12	.21
Pacific seafoods		
Smelt	8.1	7.47
Salmon	5.0	3.42
Tuna (1)	6.9	6.42
Tuna (2)	2.3	5.19
Cod	3.1	0.24
Clams	17.7	.045
Oysters	170	.087
Oysters (Atlantic)	98	.057
General		
Whole-wheat flour (1)	29	0.31
Whole-wheat flour (2)	46	.27
Bleached white flour (1)	30	.065
Bleached white flour (2)	31	.036
Bleached white flour (3)	33	.049
Bleached white flour (4)	31	.026
Wheat flakes	56	.25
40% Bran flakes	48	.37
Quaker oats	41	.15
Eggs (1)	25	.54
Eggs (2)	20	.055
Eggs (3)	23	.16
Lettuce*	4.5	.011
Carrots*	4.1	.028

*Average, 3 samples.