Sarin, P. C., and Zamecnik, P. C. (1964), *Biochim. Biophys. Acta 91*, 653.
Sharma, O. K., and Borek, E. (1970), *J. Bacteriol. 101*, 705.
Shugart, L., Chastain, B. H., Novelli, G. D., and Stulberg, M. P. (1968), *Biochem. Biophys. Res. Commun. 31*, 404.
Tonoue, T., Eaton, J., and Frieden, E. (1969), *Biochem. Biophys. Res. Commun. 37*, 81.
Turkington, R. W. (1969), *J. Biol. Chem. 244*, 5150.
Viale, G. L., Restelli, A. F., and Viale, E. (1967), *Tumori 53*, 533.
Wainfan, E., Srinivasan, P. R., and Borek, E. (1965), *Biochemistry 4*, 2845.
Wainfan, E., Srinivasan, P. R., and Borek, E. (1966), *J. Mol. Biol. 22*, 349.
Yang, S. S., and Sanadi, D. R. (1969), *J. Biol. Chem. 244*, 5081.

Levels of 5,6-Dihydrouridine in Relaxed and Chloramphenicol Transfer Ribonucleic Acid*

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ABSTRACT: Levels of the minor nucleosides dihydrouridine, pseudouridine, and ribothymidine in relaxed and chloramphenicol transfer ribonucleic acid of *Escherichia coli* and *Salmonella typhimurium* were determined. The levels were determined by labeling ribonucleic acid for several minutes at various times after uncoupling of ribonucleic acid and protein synthesis and determining radioactivity in nucleosides after bidimensional thin-layer chromatography. In agreement with the literature, pseudouridine and ribothymidine are present at normal levels in relaxed and chloramphenicol transfer ribonucleic acid. However, progressively less label enters dihydrouridine. Preparations of transfer ribonucleic acid, labeled for about 15 min after 2 hr of ribonucleic acid synthesis without protein synthesis, have only one-half of the normal level of labeled dihydrouridine. The data probably indicate that transfer ribonucleic acid deficient in dihydrouridine is synthesized. Essentially none of the uridine moieties of transfer ribonucleic acid destined to become dihydrouridine, pseudouridine, or ribothymidine are derived from cytidine moieties.

In bacteria, RNA synthesis will occur in the absence of protein synthesis under special experimental conditions. Withholding a required amino acid from an *Escherichia coli* strain carrying the relaxed allele of the RNA control gene leads to net RNA synthesis without protein synthesis (Borek *et al.*, 1955; Stent and Brenner, 1961). Also, a high concentration of chloramphenicol (200 μg/ml) effectively stops protein synthesis while permitting RNA synthesis with or without required amino acids. In *E. coli*, rRNA[1] synthesized under either of these conditions is undermethylated (Dubin and Gunalp, 1967; Gordon and Boman, 1964; Sypherd, 1968) and rRNA synthesized in the presence of chloramphenicol was reported to be deficient in pseudouridine (Dubin and Gunalp, 1967). The rRNA so synthesized is considered to be precursor rRNA.

The methylated base and pseudouridine levels of tRNA synthesized during treatment of *E. coli* with chloramphenicol are essentially normal, according to Dubin and Gunalp (1967). Data of Mandel and Borek (1963) indicate that RNA synthesized in an RC^{rel} strain of *E. coli* under relaxation conditions has normal levels of methylated constituents and pseudouridine. Their studies were done on total RNA but probably reflect the levels of minor nucleosides in tRNA since these levels are higher in tRNA than rRNA.

Thus, it appears that *E. coli* tRNA, in contrast to rRNA, is unaffected by uncoupling of RNA and protein synthesis, whether this is accomplished by use of an RC^{rel} strain or treatment with chloramphenicol. However, data reported by Mandel and Borek (1963) for pseudouridine levels and by Dubin and Gunalp (1967) for pseudouridine and methylated nucleoside levels were obtained by labeling RNA for short or long periods immediately after chloramphenicol treatment or imposing relaxation conditions. Since that procedure tends to mask effects on minor nucleoside levels developing only after conditions are well established, we reinvestigated relaxed and chloramphenicol tRNA by labeling for relatively short periods at various times after initiation of the particular condition of RNA synthesis.

This report concerns only the minor nucleosides dihydrouridine, pseudouridine, and ribothymidine. The biochemistry of pseudouridine (Ginsberg and Davis, 1968; Goldwasser and Heinrikson, 1966; Kusama *et al.*, 1966) and of the methylation of RNA (Borek and Srinivasan, 1966) has been under study for several years. However, dihydrouridine was first reported as a naturally occurring constituent of RNA ($tRNA^{Ala}$ from yeast) by Madison and Holley (1965).

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[1] Abbreviations used are: rRNA, high molecular weight rRNA; 5S RNA, low molecular weight ribosomal RNA; RC^{rel}, relaxed allele of the RNA control gene; RC^{str}, stringent allele of the RNA control gene; Hrd, dihydrouridine.

It now appears to occur in most tRNA molecules. Also dihydrouridine (Huang and Bonner, 1965) and ribodihydrothymidine (Jacobson and Bonner, 1968) occur in chromosomal RNA of higher organisms. The mode of biosynthesis of dihydropyrimidines occurring in RNA is unknown and is an area of current research.

Our results confirm the finding that ribothymidine and pseudouridine levels in relaxed and chloramphenicol tRNA are normal. However, the dihydrouridine level in relaxed and chloramphenicol tRNA is significantly lower, especially at later times after uncoupling of RNA synthesis from protein synthesis.

Materials

General. Lysozyme (muramidase, twice crystallized) was obtained from Worthington Biochemical Corp. [2-^{14}C]Uracil (specific activities varied from 51 to 59 mCi per mmole) and [2-^{14}C]cytidine (23 mCi/mmole) were obtained from Schwarz BioResearch. [6-^{3}H]Uracil (8.9 Ci/mmole) was obtained from New England Nuclear Corp. Chloramphenicol was donated by Parke, Davis and Co.

Bacteria and Media. E. coli Hfr Cavalli, requiring methionine and uracil, and CP79, requiring arginine, histidine, leucine, threonine, and thiamine, were furnished by Dr. James D. Friesen and have the relaxed allele of the RNA control gene. *E. coli* 15T$^-$ (strain 555-7), requiring arginine, methionine, tryptophan, and thymine, was furnished by Dr. K. G. Lark. *Salmonella typhimurium* Nic-13 was furnished by Dr. A. Eisenstark and requires methionine, tryptophan, tyrosine, nicotinic acid, and thiamine. *S. typhimurium* DL38 (parent organism of DP45) and DP45 were furnished by Dr. J. Ingraham and Dr. Jan Neuhard. Both lack cytidine deaminase activity, and DP45 lacks cytidine triphosphate synthetase activity as well (Neuhard and Ingraham, 1968). Thus, DP45 requires cytidine; cytosine, uracil, or uridine do not replace cytidine. Bacteria were grown with aeration in M9 glucose–salts medium (Anderson, 1946) supplemented with growth requirements (minimal medium) at 37°. Required supplements were in the medium at these levels in micrograms per milliliter: arginine, 40; histidine, leucine, or tyrosine, 20; methionine, 30; threonine, 50; tryptophan, 14; cytidine or uracil, 20; thymine, 4; nicotinic acid or thiamine, 2. Growth of bacterial cultures was measured in 10-mm internal diameter tubes at 450 nm with a Coleman Junior II spectrophotometer. Under these conditions an A_{450} reading of 0.10 corresponds to 2–3 $\times$ 10^8 cells/ml depending on the strain.

Generation times of the *E. coli* strains in minimal media were: 15T$^-$, 40 min; CP79, 55 min; and Hfr Cavalli, 55 min. For the *S. typhimurium* strains, generation times were: DL38, 48 min: DP45, 40–60 min; and Nic-13, 48 min.

Methods

General Outline of Method. In a typical experiment, a required amino acid was withdrawn from an exponential phase culture of a RC^{rel} strain or chloramphenicol (200 μg/ml) was added to allow RNA synthesis in the absence of protein synthesis. Labeled uracil was added to portions of culture for a given period at various times after uncoupling of RNA and protein synthesis. After a chase period with unlabeled uracil, cells were harvested by centrifugation at 5° for 10 min at 4000*g*. The pellet was washed with cold 0.9% saline and stored at −20°.

All cultures were in exponential growth at less than 3 $\times$ 10^8 cells/ml when experiments were initiated. When it was necessary to remove a required compound from the medium for labeling or relaxation of RNA synthesis, cells were rapidly collected by filtration on nitrocellulose membranes (Type B-6, Carl Schleicher & Schuell Co.) and washed well with M9 medium minus glucose at 37°. Cells were resuspended in appropriate medium at 37°. Filtration and resuspension of the cells were accomplished in 2 min or less. Growth ceased immediately after removal of a required amino acid or addition of chloramphenicol, which was always used at a concentration of 200 μg/ml.

The volume of culture used in a labeling experiment varied from 25 to 200 ml. When less than 100 ml of culture was involved, carrier cells were mixed with experimental cells during the saline wash or carrier tRNA was added at the time of extraction. A labeling period was always followed by a chase period with unlabeled uracil to reduce contamination of tRNA preparations with labeled mRNA.

Cells in exponential phase of growth or which had been relaxed for RNA synthesis 60 min or less incorporated about 70% or more of the available label. Cells which had been relaxed for RNA synthesis for longer periods incorporated correspondingly less label but not less than 30% after 120-min relaxation.

Preparation of tRNA. tRNA was prepared from experimental cells by a modified method of direct phenol extraction (von Ehrenstein and Lipmann, 1961). Cells were suspended in 1.0 ml of 40 mM Tris-HCl (pH 7.4) containing 4 mM magnesium acetate and 0.1% bentonite (Tris buffer). Then 1.0 ml of redistilled phenol, saturated with buffer, was immediately added, and the mixture was stirred and maintained at 0–5° for 10 min. All subsequent steps were done at 0–5° unless otherwise indicated. The phases were separated by centrifugation and the aqueous phase was removed. After washing the phenol phase with 0.5 ml of buffer, the aqueous phases were combined. RNA was precipitated from the combined aqueous phases by the addition of 0.1 volume of 20% potassium acetate and 2.5 volumes of 95% ethanol. Precipitation was allowed to proceed for at least 1 hr at −20°. After collection by centrifugation the precipitate was dissolved in Tris buffer and reprecipitated as above. The recovered precipitate was dissolved in 0.15 ml of water, 0.05 ml of 8 M LiCl was added, and after 30 min at 0° the precipitate of rRNA was removed by centrifugation. tRNA was precipitated from the supernatant solution with 3 volumes of 95% ethanol. The recovered precipitate was reprecipitated twice from 0.5 ml of 40 mM potassium acetate buffer (pH 5.0) containing 4 mM magnesium acetate, washed with 1 ml of ethanol–ether (50:50), and finally with ether. tRNA was dissolved in water and stored at −20°. A similar method using LiCl to remove rRNA was recently reported in detail with supporting data by Avital and Elson (1969).

Digestion of tRNA. tRNA was enzymically digested to nucleosides as previously described (Jacobson *et al.*, 1968), except that dihydrouridine was added to the digestion mixture.

Chromatography of Nucleosides from tRNA. Preparation of thin layers of microcrystalline cellulose and thin-layer

TABLE I: Nucleoside Labeling Ratios of Relaxed and Chloramphenicol tRNA from *E. coli* Hfr Cavalli.[a]

Conditions of Labeling	Labeling Period (min)	Nucleoside Labeling Ratios (cpm/cpm)					No. of Analyses
		Urd/Cyd	Thd/Urd	ψrd/Thd	Hrd/Thd	ψrd/Hrd	
Exponential	30	0.50	0.071	1.7	1.7	1.0	13
− Methionine	0–6	0.55	0.019			1.1	2
− Methionine	23–29	0.88	0.009			1.2	2
− Methionine	42–48	1.10	0.008			1.4	2
− Methionine	80–86	1.57	0.006			1.6	2
− Methionine	120–130	2.08	0.002			1.5	2
+ Chloramphenicol	63–73	1.36	0.060	1.7	1.1	1.5	7
+ Chloramphenicol	123–133	2.01	0.053	1.7	0.9	1.7	6

[a] A culture of *E. coli* Hfr Cavalli was shifted to minimal medium minus uracil and methionine. A portion of the deficient culture was immediately mixed with [^{14}C]uracil to give 0.08 μCi/ml for the 0–6-min labeling period. The remaining culture was adjusted to 20 μg of uracil/ml. For each subsequent labeling period, a portion of the methionine-starved cells was shifted to minimal medium minus methionine and uracil plus 0.08 μCi/ml of [^{14}C]uracil. Exponential phase cells were labeled for 30 min with 0.8 μCi/ml of [^{3}H]uracil. For labeling during chloramphenicol treatment, a portion of treated culture was shifted just prior to a labeling period to minimal medium minus uracil plus chloramphenicol and 0.08 μCi/ml of [^{14}C]uracil. Following a labeling period cells were harvested after a 20-min chase with 20 μg/ml of uracil. tRNA was prepared and analyzed as in Methods.

chromatography were done as previously described (Jacobson and Hedgcoth, 1970). Nucleosides, except dihydrouridine, were located on plates by examining them in short-wave ultraviolet light. Initially, the position of dihydrouridine was located by the method of Fink *et al.* (1956). However, for chromatograms of experimental material other nucleosides were used as reference points and the dihydrouridine region was marked accordingly.

The areas of cellulose containing labeled nucleosides were scraped into scintillation vials, 5 ml of toluene-base scintillation fluid was added, and radioactivity was determined in a Beckman LS-200B liquid scintillation spectrometer. Scintillation fluid contained 5 g of Packard Permablend II (98% 2,5-diphenyloxazole and 2% 1,4-bis-2-(5-phenyloxazolyl)benzene) per l. of toluene.

Results

The labeling ratios of minor nucleoside to minor nucleoside are very reproducible for labeled tRNA preparations of a given organism and condition of labeling. However, ratios of minor nucleoside to uridine (and cytidine) vary somewhat in different preparations of labeled tRNA, indicating variable contamination with small amounts of labeled RNA other than tRNA. Thus, the ratios of minor nucleoside to minor nucleoside were used as the most critical determinant of changes in composition of these minor nucleosides in tRNA synthesized under a defined condition.

Nucleoside Labeling in Relaxed and Chloramphenicol tRNA. To study the dihydrouridine, pseudouridine, and ribothymidine content of tRNA synthesized in the absence of protein synthesis, RNA of *E. coli* Hfr Cavalli (RC^{rel}) was labeled for brief intervals during a 130-min period after withdrawal of methionine. An outline of the experiment and ratios of labeled nucleosides from tRNA are given in Table I. Because methionine provides the methyl group for ribothymidine, labeled uracil was not converted into ribothymidine residues in tRNA in the methionine-deficient culture. During starvation for methionine the pseudouridine:dihydrouridine ratio of Hfr Cavalli tRNA increased.

To compare the pseudouridine and dihydrouridine levels with the level of ribothymidine, tRNA was obtained from a culture treated with chloramphenicol with methionine in the medium. The pseudouridine:dihydrouridine ratio also increased in tRNA with increasing time of treatment with chloramphenicol (Table I). Also, the dihydrouridine:ribothymidine ratio decreased to about a value of 1. Further, the pseudouridine:ribothymidine ratio was the same as the value for exponentially labeled tRNA. Thus, there appeared to be a decrease in the amount of dihydrouridine in tRNA at later stages during treatment with chloramphenicol.

The ratio of labeled uridine to labeled cytidine from tRNA also increased with time of starvation of the culture for methionine. The changing ratio of major nucleosides could possibly be explained as the result of increasing contamination of tRNA preparations with RNA of high uridine content. However, pseudouridine:uridine ratios did not change appreciably from exponential phase values, whereas contamination of tRNA with RNA of higher uridine content would depress this ratio. A more reasonable explanation is based on the increasing size of the CTP pool relative to the UTP pool in relaxed cells (Edlin and Neuhard, 1967) with concomitant changes in specific activities of the pools of precursors.

Additional experiments using the same approach were done with *E. coli* strains CP79 and 15T$^-$. Leucine was withheld from a culture of CP79 (RC^{rel}), and a culture of 15T$^-$ was treated with chloramphenicol. Data of Table II indicate that the dihydrouridine level in late relaxed tRNA from CP79 and chloramphenicol tRNA from 15T$^-$ was lower than the exponential phase level. The decrease in dihydrouridine level is about 40% in late tRNA which would amount to a change from about 2 mole % to 1.2 mole %.

An increase in the uridine:cytidine labeling ratio was also

TABLE II: Nucleoside Labeling Ratios of Relaxed tRNA from *E. coli* CP79 and Chloramphenicol tRNA from *E. coli* 15T⁻.[a]

Strain	Condition of Labeling	Labeling Period (min)	Nucleoside Labeling Ratios (cpm/cpm)				No. of Analyses
			Urd/Cyd	Thd/Urd	ψrd/Thd	Hrd/Thd	
CP79	Exponential	40	0.47	0.083	1.7	1.6	2
CP79	− Leucine	0–7	0.52	0.078	1.8	1.7	4
CP79	− Leucine	42–49	0.87	0.076	1.6	1.4	1
CP79	− Leucine	108–118	2.09	0.075	1.6	1.3	1
CP79	− Leucine	130–140	2.32	0.057	1.7	1.1	4
CP79	− Leucine	140–155	2.79	0.067	1.6	1.1	1
15T⁻	Exponential	60	0.47	0.068	1.9	1.9	8
15T⁻	+ Chloramphenicol	70–85	1.25	0.072	1.8	1.1	2
15T⁻	+ Chloramphenicol	100–115	1.63	0.061	1.8	0.9	2

[a] A culture of *E. coli* CP79 was shifted to minimal medium minus leucine. At the indicated times, a portion of the leucine-deficient culture was labeled with 0.08 μCi/ml of [^{14}C]uracil. Exponential phase cells were labeled for 40 min with 0.2 μCi/ml of [^{3}H]uracil. A portion of a chloramphenicol-treated culture of *E. coli* 15T⁻ was labeled in 0.05 μCi/ml of [^{14}C]uracil at each indicated time after addition of chloramphenicol. Exponential phase cells were labeled for 60 min with 0.04 μCi/ml of [^{14}C]uracil. Following a labeling period cells were harvested after a 20-min chase with 20 μg/ml of uracil. tRNA was prepared and analyzed as described in Methods. Data for the 0–7-min labeling period for CP79 are from two experiments. Other data are from single experiments.

TABLE III: Nucleoside Labeling Ratios of Chloramphenicol tRNA from *S. typhimurium* Nic-13.[a]

Conditions of Labeling	Labeling Period (min)	Nucleoside Labeling Ratios (cpm/cpm)				No. of Analyses	No. of Expt
		Urd/Cyd	Thd/Urd	ψrd/Thd	Hrd/Thd		
Exponential	20	0.53	0.061	1.6	1.8	3	1
+ Chloramphenicol	60–80	1.10	0.066	1.7	1.1	4	2

[a] A culture of *S. typhimurium* Nic-13 was shifted to minimal medium with 2 μg/ml of uracil, 5 μg/ml of adenosine, and chloramphenicol, but without tryptophan and tyrosine. At each indicated time, a portion of the treated culture was labeled in 0.1 μCi/ml of [^{14}C]uracil. Following a labeling period, cells were harvested after a 20-min chase with 20 μg/ml of uracil. tRNA was prepared and analyzed as described in Methods.

observed for relaxed and chloramphenicol tRNA of CP79 and 15T⁻.

As shown in Table III the dihydrouridine level in late chloramphenicol tRNA of *S. typhimurium* Nic-13 was much less than normal and about the same as the ribothymidine level, indicating that observations of decreased dihydrouridine levels were not peculiar to the strains of *E. coli* used. As observed for *E. coli* strains, the uridine:cytidine ratio increased with time of chloramphenicol treatment.

Comparison of tRNA Obtained by Direct Phenol Extraction and Total Extraction. tRNA for the experiments of Tables I, II, and III was obtained by direct phenol extraction of whole cells. If dihydrouridine-rich tRNA synthesized late in relaxation of RNA synthesis is not extracted by the direct extraction method, subsequent analysis of labeled nucleosides in tRNA could give results compatible with data presented here. To test this point, total RNA of *E. coli* CP79, labeled after 2-hr RNA synthesis in the absence of leucine, was obtained using lysozyme and freeze-thaw cycles and fractionated on a column of Sephadex G-75 into rRNA, 5S RNA, and tRNA (Robins and Raacke, 1968). A comparison of ratios of labeled nucleosides from tRNA prepared by extraction of total RNA and by direct extraction revealed that the level of labeled dihydrouridine was identical for the tRNA preparations and the same as the 130–140-min point of Table II.

Labeled nucleoside ratios were obtained for 5S RNA and rRNA from the preparation of total RNA of CP79. The apparent dihydrouridine deficiency in tRNA was not accounted for in the preparations of 5S RNA and rRNA.

Uridine as the Precursor of Dihydrouridine. The apparent decrease in the dihydrouridine level of relaxed and chloramphenicol tRNA was accompanied by an increase in the uridine:cytidine labeling ratio. This observation would explain the decrease in labeled dihydrouridine in relaxed and chloramphenicol tRNA if cytidine residues in tRNA were precursors of dihydrouridine. Since *S. typhimurium* DP45 requires cytidine for growth, and uracil does not replace cytidine,

TABLE IV: Labeling of *S. typhimurium* DP45 tRNA with [^{14}C]-Uracil and [^{14}C]Cytidine.[a]

Nucleoside	Source of Label (cpm/cpm)	
	[^{14}C]Uracil	[^{14}C]Cytidine
Dihydrouridine	1,320	1,818
Pseudouridine	1,273	1,614
Ribothymidine	714	980
Uridine	10,171	16,768
Cytidine	298	117,762

[a] A culture of *S. typhimurium* DP45 was shifted to minimal medium minus cytidine. Half of the culture was mixed with [^{14}C]cytidine and cytidine to give final concentrations of 0.02 μCi/ml and 1.0 μg/ml. After 40 min, additional cytidine was added to bring the total added cytidine to 2 μg/ml. After 60-min labeling, the cytidine concentration was adjusted to 20 μg/ml, and cells were harvested 20 min later. The other half of the culture was labeled in 0.02 μCi/ml of [^{14}C]uracil and 20 μg/ml of cytidine. Following a 10-min labeling period cells were harvested after a 30-min chase with 2 μg/ml of uracil. tRNA was prepared and analyzed as described in Methods.

DP45 tRNA labeled with [^{14}C]cytidine or [^{14}C]uracil provided a means for deciding between uridine or cytidine as the precursor to dihydrouridine in tRNA.

The distribution of radioactivity among the various nucleosides of DP45 tRNA is shown in Table IV. Although DP45 (and its parent organism, DL38) incorporated only about 0.4% of the exogenous uracil which *E. coli* Hfr Cavalli incorporated under similar conditions, enough [^{14}C]uracil entered tRNA to determine that the various nucleoside labeling ratios were normal: Hrd:Urd, 0.129; Hrd:Thd, 1.85; Hrd:ψrd, 1.04. These results could not have been obtained if cytidine were the precursor of dihydrouridine in tRNA.

Further, in the experiment with [^{14}C]cytidine the labeling ratios (Hrd:Urd, 0.108; Hrd:Thd, 1.85; Hrd:ψrd, 1.13) are again in accord with uridine as the precursor of dihydrouridine. The relatively large amount of radioactivity (15%) in uridine and minor nucleosides probably arose by an endogenous formation of uridine from a cytidine derivative. Two points support that hypothesis. (1) Exogenous uridine was used almost as poorly as uracil by DP45; on a mole basis, DP45 incorporated only 1% as much uridine as *E. coli* Hfr Cavalli did uracil. (2) A cytidine derivative rather than cytidine is implicated because cytidine deaminase is nonfunctional in DP45 (Neuhard and Ingraham, 1968).

To ensure that *S. typhimurium* DP45 was not abnormal with respect to labeling of RNA by [^{14}C]uracil, nucleoside labeling ratios were obtained for tRNA of DL38. It is clear from Table V that the nucleosides of tRNA from DL38 and DP45 were labeled like nucleosides of tRNA from the other organisms used in this study.

Stability of tRNA Dihydrouridine under Relaxed Conditions. Since information on the stability of dihydrouridine residues in tRNA *in vivo* under various metabolic conditions was not available, an experiment was done to determine its stability during RNA synthesis in the absence of protein synthesis. RNA of *E. coli* Hfr Cavalli was labeled with [^{3}H]uracil during exponential growth. Then the culture was starved for methionine. Portions of culture were removed at various times after withdrawal of methionine, and nucleoside labeling ratios of tRNA were determined. It is clear from Table VI that there was no preferential loss of dihydrouridine from the exponentially labeled tRNA.

Data in Table VI also indicate that tRNA synthesized in exponential phase was stable during methionine starvation. Appreciable degradation of tRNA and reutilization of nucleotides would have affected minor nucleoside ratios involving ribothymidine. The recovery of ^{3}H-labeled tRNA from

TABLE V: Ratios of Labeled Nucleosides of tRNA from *S. typhimurium* DL38 and DP45.[a]

Strain	Nucleoside Labeling Ratios (cpm/cpm)					
	Urd/Cyd	ψrd/Urd	Hrd/Urd	Thd/Urd	ψrd/Hrd	ψrd/Thd
DL38	0.80	0.102	0.100	0.058	1.0	1.8
DP45	34.1	0.125	0.129	0.070	1.0	1.8

[a] Cultures of *S. typhimurium* DL38 and DP45 were labeled with 0.33 μCi/ml of [^{14}C]uracil for 60 min. Then after a 20-min chase with 20 μg/ml of uracil, cells were harvested. tRNA was prepared and analyzed as described in Methods.

TABLE VI: Effect of Relaxation Conditions on Nucleoside Labeling Ratios of Hfr Cavalli tRNA Labeled During Exponential Growth.[a]

Time of Harvest after Removal of Methionine (min)	Nucleoside Labeling Ratios (cpm/cpm)					
	Urd/Cyd	Thd/Urd	ψrd/Thd	Hrd/Thd	ψrd/Hrd	No. of Analyses
0	0.50	0.071	1.7	1.7	1.0	13
26	0.54	0.067	1.9	1.7	1.1	2
67	0.50	0.070	1.5	1.7	0.9	2
131	0.47	0.072	1.6	1.6	1.0	4

[a] A culture of *E. coli* Hfr Cavalli in exponential growth with an A_{450} of 0.035 was labeled for 30 min in minimal medium with 0.8 μCi/ml of [^{3}H]uracil and with sufficient uracil (2 μg/ml) for growth to an A_{450} of 0.15. Then uracil was added to a concentration of 20 μg/ml, and after 20 min the culture was shifted to minimal medium minus methionine. A portion of the cells were harvested at each of the indicated times. tRNA was prepared and analyzed as described in Methods.

exponential cells was about 37% and that from cells after 131 min in medium lacking methionine was 35%.

Evidence against the Possibility of the Open-Ring Form of Dihydrouridine in Relaxed and Chloramphenicol tRNA. Dihydrouridine is stable under the conditions of enzymic digestion and thin-layer chromatography used in this study. However, if it existed *in vivo* as the open-ring form in tRNA, *i.e.*, the *N*-ribosyl-3-ureidopropionate derivative in the RNA polymer, *N*-ribosyl-3-ureidopropionic acid, or an oligonucleotide containing *N*-ribosyl-3-ureidopropionic acid would be released during enzymic digestion of tRNA *in vitro*. That possibility was explored after determining the mobilities of *N*-ribosyl-3-ureidopropionic acid, prepared by treating dihydrouridine with alkali, in the thin-layer chromatographic system. *N*-Ribosyl-3-ureidopropionic acid has R_F values of 0.55 and 1.17, relative to ribothymidine, in solvent systems 1 and 2, respectively, and thus is well separated from other nucleosides.

Two preparations of *S. typhimurium* Nic-13 tRNA labeled with [^{14}C]uracil were obtained essentially as described in Table III. One was labeled during exponential growth; the other was labeled from 60 to 80 min after addition of chloramphenicol. tRNA was enzymically digested to nucleosides and chromatographed bidimensionally as for other experiments in this study.

Nucleoside labeling ratios of the respective preparations were the same as those reported in Table III. The radioactivity of the region of the chromatogram corresponding to the position where *N*-ribosyl-3-ureidopropionic acid would be found was determined to be 2% (35 to 45 cpm) of the pseudouridine radioactivity for both the exponentially labeled tRNA and tRNA labeled during chloramphenicol treatment. The value of 2% is obviously too low since the dihydrouridine to be accounted for amounted to 30–40% of the pseudouridine value. Further, autoradiograms of the thin-layer chromatograms were identical, and all radioactivity was found in positions occupied by the major and minor pyrimidine nucleosides. Oligonucleotides containing dihydrouridine were also eliminated as a possibility because oligonucleotides are well resolved from nucleosides in the bidimensional development. Additional data indicate that over 50% of the open-ring form remains open during chromatography.

Discussion

The increase in uridine:cytidine labeling ratios in relaxed and chloramphenicol tRNA probably reflects the relative changes which occur between UTP and CTP pools of cells with metabolic stresses. Edlin and Neuhard (1967) reported data on nucleoside triphosphate pool sizes of *E. coli* CP79 indicating that a decrease occurs in the UTP:CTP pool ratio from about 1.76 at the time of relaxation of RNA synthesis to about 0.85, 90 min later. Data indicating that there is a considerable lag between the time label enters the UTP pool and the time it enters the CTP pool are also available (Edlin and Neuhard, 1967; Irr and Gallant, 1969). Analogous changes in UTP and CTP pools of chloramphenicol-treated cells probably occur because such changes occur in several cases of metabolic stress.

It is well established that ribothymidine in RNA is formed by methylation of polynucleotide uridine (Fleissner and Borek, 1962). The available evidence indicates that pseudouridine is formed at the polynucleotide level (Ginsberg and Davis, 1968; Kusama *et al.*, 1966; Weiss and Legault-Demare, 1965), probably by rearrangement of uridine moieties to pseudouridine. In contrast, there is no available literature on the biosynthesis of dihydrouridine either in tRNA or chromosomal RNA. It is assumed that dihydrouridine is also formed at the polynucleotide level by reduction of specific uridine moieties.

The changes reported here in the relative distribution of exogenous labeled uracil between uridine and cytidine moieties of tRNA would cause no difficulty if studies concerned only minor nucleosides derived from either uridine or cytidine. Dihydrouridine, pseudouridine, and ribothymidine in tRNA were originally considered as deriving from uridine at the polynucleotide level, thus establishing a basis for using minor nucleoside ratios as a determinant for changes in one or more of the minor nucleoside levels. Because less labeled uracil enters cytidine of relaxed and chloramphenicol RNA concomitant with less labeled dihydrouridine being found (Tables I, II, and III), it was necessary to determine whether cytidine is a precursor of uridine destined to become dihydrouridine. Experiments with *S. typhimurium* DP45, a cytidine-requiring strain, furnished evidence (Table IV) that dihydrouridine is derived essentially solely from uridine.

We considered the possibility that the lower level of dihydrouridine in relaxed and chloramphenicol tRNA is an artifact due to an abnormal state of dihydrouridine in such RNA. The conditions of enzymic digestion and thin-layer chromatography do not alter dihydrouridine, *e.g.*, by conversion into its open-ring form, *N*-ribosyl-3-ureidopropionic acid. If, in relaxed or chloramphenicol tRNA, dihydrouridine were in the form of the polynucleotide derivative of *N*-ribosyl-3-ureidopropionic acid, the open-ring form would have been detected by autoradiography of the chromatogram; however, it was not found.

The data of Table VI indicate that the tRNA synthesized in cells in exponential growth is stable when the cells are subsequently subjected to chloramphenicol treatment or relaxation conditions, and suggest that the lower level of dihydrouridine results from a lack of synthesis rather than a loss of dihydrouridine. The decreased ability to make dihydrouridine in tRNA is quite likely a result of protein degradation or inactivation and a lack of resynthesis of the protein.

The tRNA deficient in dihydrouridine may be a precursor tRNA. Waters (1969) reported that leucine and phenylalanine tRNAs from chloramphenicol-treated *E. coli* have chromatographic profiles on reversed-phase columns differing from normal profiles. He suggested the possibility of precursor tRNA in the preparations of chloramphenicol tRNA.

The observations may explain, in part at least, the lag in resumption of growth during recovery by cells from relaxation or chloramphenicol treatment. tRNA deficient in dihydrouridine could function improperly and thus compete with normal tRNA.

Studies are in progress to determine the ability of cells to recover their potential for dihydrouridine synthesis and if tRNA is degraded during this period. The studies may provide insight into the biosynthesis and function of dihydrouridine in tRNA especially if the tRNA deficient in dihydrouridine

represents a precursor to tRNA.

Added in Proof

Results of preliminary experiments indicate that the level of 4-thiouridine in chloramphenicol tRNA of *E. coli* is 30–60% of the exponential phase level.

References

Anderson, E. H. (1946), *Proc. Nat. Acad. Sci. U. S. 32*, 120.

Avital, S., and Elson, D. (1969), *Biochim. Biophys. Acta 179*, 297.

Borek, E., Ryan, A., and Rockenback, J. (1955), *J. Bacteriol. 69*, 460.

Borek, E., and Srinivasan, P. R. (1966), *Annu. Rev. Biochem. 35*, 275.

Dubin, D. T., and Gunalp, A. (1967), *Biochim. Biophys. Acta 134*, 106.

Edlin, G., and Neuhard, J. (1967), *J. Mol. Biol. 24*, 225.

Fink, R. M., Cline, R. E., McGaughey, C., and Fink, K. (1956), *Anal. Chem. 28*, 4.

Fleissner, E., and Borek, E. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1199.

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

Goldwasser, E., and Heinrikson, R. L. (1966), *Progr. Nucl. Acid Res. Mol. Biol. 5*, 399.

Gordon, J., and Boman, H. G. (1964), *J. Mol. Biol. 9*, 638.

Huang, R. C. C., and Bonner, J. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 960.

Irr, J., and Gallant, J. (1969), *J. Biol. Chem. 244*, 2233.

Jacobson, R. A., and Bonner, J. (1968), *Biochem. Biophys. Res. Commun. 33*, 716.

Jacobson, M., and Hedgcoth, C. (1970), *Anal. Biochem.* (in press).

Jacobson, M., O'Brien, J. F., and Hedgcoth, C. (1968), *Anal. Biochem. 25*, 363.

Kusama, K., Prescott, D. M., Froholm, L. O., and Cohn, W. E. (1966), *J. Biol. Chem. 241*, 4086.

Madison, J. T., and Holley, R. W. (1965), *Biochem. Biophys. Res. Commun. 18*, 153.

Mandel, L. R., and Borek, E. (1963), *Biochemistry 2*,560.

Neuhard, J., and Ingraham, J. (1968), *J. Bacteriol. 95*, 2431.

Robins, H. I., and Raacke, I. D. (1968), *Biochem. Biophys. Res. Commun. 33*, 240.

Stent, G. S., and Brenner, S. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 2005.

Sypherd, P. S. (1968), *J. Bacteriol. 95*, 1844.

von Ehrenstein, G., and Lipmann, F. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 941.

Waters, L. C. (1969), *Biochem. Biophys. Res. Commun. 37*, 296.

Weiss, S. B., and Legault-Demare, J. (1965), *Science 149*, 429.