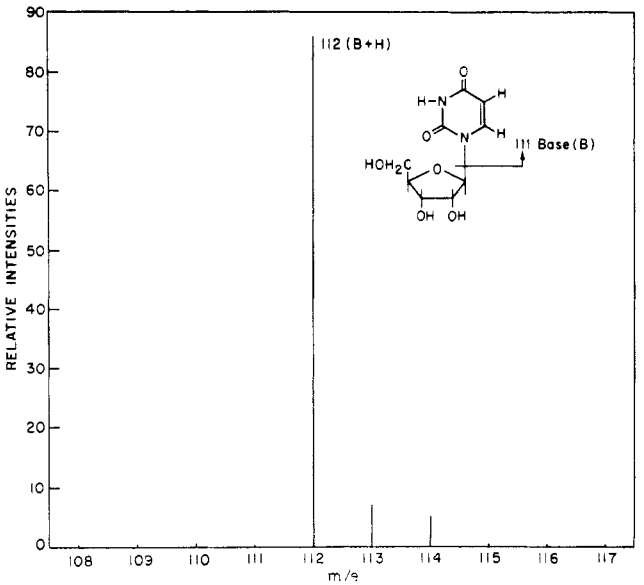

FIG. 4.—Portion of mass spectrum of uridine isolated from s-RNA of cells grown on methionine-methyl-D_3.

may also apply to other substitution reactions which take place at the 5 position of pyrimidine nucleotides, such as the reactions catalyzed by thymidylate synthetase and deoxycytidylate hydroxymethylase. Certainly no similar mechanism can be involved in the alkylation of the double bond of oleic acid in the enzymatic formation of tuberculostearic acid.

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We are indebted to Mr. Malcolm Smart for assistance in the operation of the analytical ultracentrifuge and to Dr. R. Sundararajan, who assayed the s-RNA samples for amino acid-acceptor activity. Dr. Albert Chung provided aid and encouragement on several occasions. We are grateful to Drs. Klaus Biemann, E. J. Corey, Fred Kahan, and Bianca Tchoubar for helpful discussions during the course of these experiments.

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Pseudouridylic Kinase Activity in *Escherichia coli**

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An enzyme fraction that catalyzes the synthesis of the pseudouridine diphosphate and pseudouridine triphosphate from pseudouridylic acid has been isolated from *Escherichia coli*. Separation of pseudouridylic from uridylic kinase activity was not observed. The rate of the kinase reaction with pseudouridylic acid was 1–2% of that with uridylic acid.

Pseudouridylic acid (5-ribosyluridylic acid) is an important component of transfer RNA (Davis *et al.*, 1959; Dunn, 1959; Otaka *et al.*, 1959; Dunn *et al.*, 1960), but the mechanism of its incorporation into RNA is still unknown. We have previously shown that pseudouridine (ψU) chemically phosphorylated in its 5′ position is converted to its nucleoside triphosphate by an enzyme system present in partially purified yeast extracts (Goldberg and Rabinowitz, 1961a). Further-

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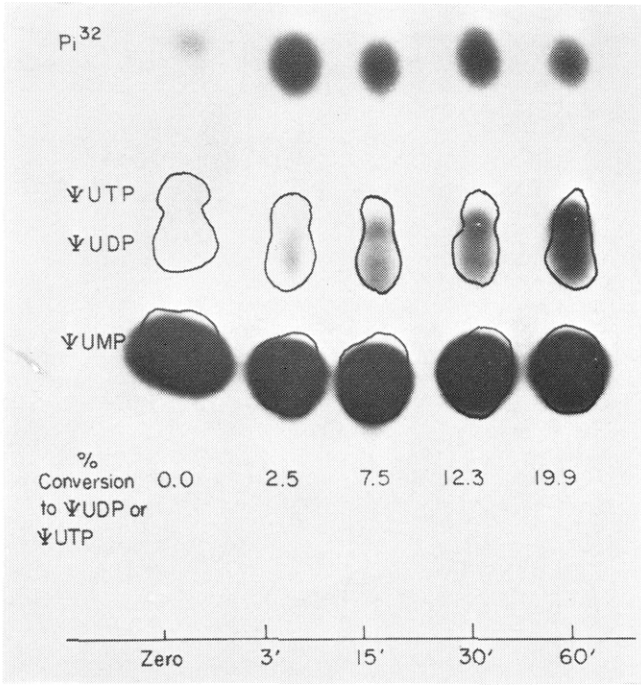

FIG. 1.—Time course of *E. coli* ψUMP kinase reaction. The 0.05-ml reaction mixtures were as described in the text. Enzyme (160 μg) eluted from the hydroxyapatite column was added to each reaction. Aliquots contained 4.5×10^4 cpm ^{32}P were subjected to electrophoresis in 0.05 M phosphate–5×10^{-3} M EDTA, *p*H 7.5. Radioautography was performed using Kodak "No Screen" film.

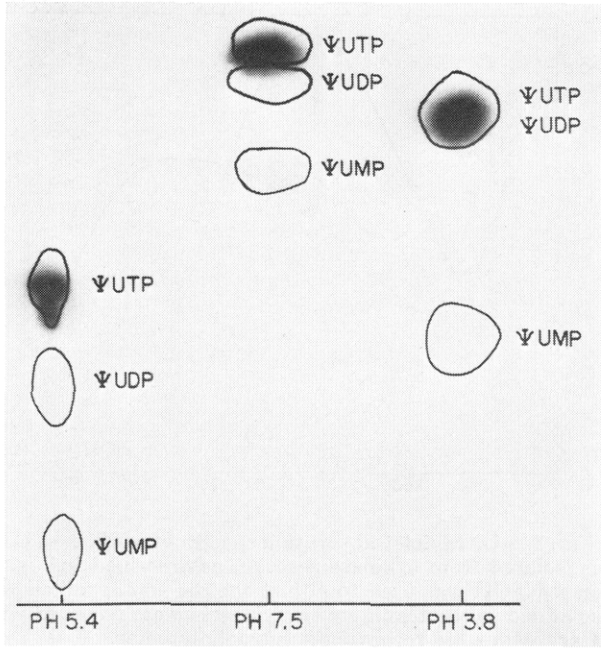

FIG. 2.—Electrophoresis of [^{32}P]ψUTP synthesized by *E. coli* kinase. The kinase reaction was run as described but scaled for a 10-ml reaction. The products were separated in DEAE-cellulose using a linear gradient from 0.02 to 0.25 M triethylammonium carbonate. The ψUDP and ψUTP peaks were rechromatographed on DEAE-cellulose. Electrophoresis was performed at *p*H 3.8, 5.4, and 7.5 for 18 hours at 250 v.

more, ψUTP replaces UTP as the fourth required nucleoside triphosphate in the DNA-dependent RNA polymerase obtained from HeLa cells (Goldberg and Rabinowitz, 1961b), and from *Escherichia coli* (Kahan and Hurwitz, 1962; Goldberg and Rabinowitz, 1963). However, when both ψUTP and UTP are present in the reaction, pseudouridylic acid is preferentially incorporated into RNA sites neighboring purine, whereas uridylic acid favors positions near pyrimidine nucleotides (Goldberg and Rabinowitz, 1961b, 1963). This is the only information suggesting differential incorporation of pseudouridylic acid into RNA.

Other data suggest, however, that analogous to methylation of RNA and DNA (Fleissner and Borek, 1962; Gold *et al.*, 1963), pseudouridylic acid may be synthesized on RNA by intramolecular rearrangement. The experiments of Robbins and Hammond (1962), in which pseudouridylic acid isolated from transfer RNA synthesized from uridine, labeled either in the pyrimidine ring or in the ribose, demonstrated specific activities similar to those of uridylic and cytidylic acids, suggest this mechanism. Furthermore, Kahan and Hurwitz (1962) could demonstrate no enzymes in *E. coli* capable of converting ψUMP to ψUTP. This resembles the case of ribothymidylic acid (Kahan and Hurwitz, 1962) and its analog bromuridylic acid (Goldberg and Rabinowitz, 1963), for which a kinase could not be demonstrated. Subsequently ribothymidylic acid was shown to be formed by methylation of the uridylic acid in transfer RNA (Fleissner and Borek, 1962; Gold *et al.*, 1963).

This paper will show that pseudouridylic acid kinase activity is present in extracts of *E. coli B*, but that the activity is 1–2% of that found for uridylic acid. The enzymatic activities have not been separated from each other. The reason that this activity has not previously been detected is probably that the presence of an active nucleoside-5′-phosphomonoesterase successfully competes for substrate ψUMP.

METHODS

Pseudouridine was isolated from urine as previously described (Goldberg and Rabinowitz, 1963), converted to its isopropylidine derivative (Chambers *et al.*, 1957), and phosphorylated with cyanoethylphosphate (^{32}P) by the method of Tener (1961). The nucleoside monophosphate was isolated by ion-exchange chromatography on DEAE-cellulose. [^{32}P]UMP was prepared similarly starting with isopropylidine uridine (Aldrich Chemical Co.).

Electrophoreses were performed using a Durrum-type apparatus in 0.025 M sodium citrate buffer, *p*H 3.8 or 5.4; or in 0.05 M phosphate–0.005 M EDTA, *p*H 7.5. Descending paper chromatography was performed on Whatman 3MM paper. *System I:* ethanol–0.5 M ammonium acetate, *p*H 3.8 (5:2, v/v); *system II:* isobutyric acid–0.5 M NH_4OH (7:3, v/v).

Enzyme Assay.—The 0.05-ml assay mixture contained 0.05 M glycine buffer, *p*H 7.5, 0.01 M $MgCl_2$, 0.004 M phosphoenolpyruvate, 0.1 μg pyruvate kinase (Calbiochem-Boehringer), and enzyme protein. Incubation was at 37° for 20–60 minutes. After the reaction was stopped in a boiling-water bath for 2 minutes, an aliquot of the supernatant fluid was subjected to electrophoresis in the phosphate-EDTA *p*H 7.5 system. UMP, UDP, and UTP were added as markers in the electrophoresis. There is no detectable difference in the mobilities of the uridine and pseudouridine compounds in this system; hence the uridine compounds may be used as markers in the ψUMP assay. The mobility of inorganic phosphate is greater than that of di- and triphosphates, which run close together and well ahead of UMP. The radioactive spots were counted on the paper with a Geiger-Muller tube.

Enzyme Preparation.—*E. coli B* was grown in an inorganic salt medium M 56 (Wiesmeyer and Cohn, 1960) and harvested in the exponential growth phase. Cultures were washed with buffer A (0.01 M Tris, *p*H

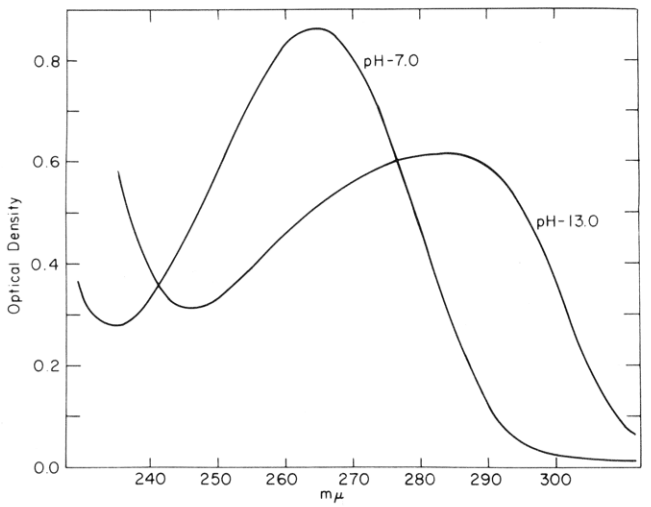

FIG. 3.—Ultraviolet-absorption spectra of ψUTP. ψUTP was isolated from a kinase reaction as described in Fig. 2. Ten N NaOH was used to adjust the *p*H to 13; alkali was also added to the blank cuvet. Spectra were recorded with a Cary Model 14 recording spectrophotometer.

7.9–0.01 M $MgCl_2$–10^{-4} M EDTA) and either used immediately or frozen at −20° for future use. The cells (50–100 g) were ground with 3–4 times their weight of alumina A 301 (Merck) and then extracted with 2 volumes of buffer A. Centrifugation for 20 minutes at 10,000 *g* and for 40 minutes at 20,000 *g* removed cell debris and the alumina. The clear yellow fluid was diluted with solution A to contain about 12.0 mg/ml protein, and mercaptoethanol was added to a final concentration of 0.01 M. One-twentieth volume of 10% streptomycin (w/v) was added, and the precipitate was separated by centrifugation at 23,000 *g* for 10 minutes and discarded. One-tenth volume of 1% protamine sulfate (w/v) was added to the supernatant fluid and the precipitate that formed was also discarded. $(NH_4)_2SO_4$ (Mann, enzyme grade) was then added to 75% saturation. After 30 minutes in the cold, the mixture was centrifuged for 30 minutes at 23,000 *g*. The precipitate was dissolved in a minimal amount of buffer A containing 0.01 M mercaptoethanol, and dialyzed against 0.02 M Tris, *p*H 7.5–0.01 M mercaptoethanol for 18 hours. After application of the dialyzed enzyme to a 30 × 3-cm DEAE-cellulose column equilibrated with buffer A, the column was washed with Buffer A–0.01 M mercaptoethanol until the initial inactive protein peak was eluted (400 ml). Gradient elution was then performed using a 500-ml mixing chamber containing buffer A–0.01 M β-mercaptoethanol, and an upper chamber containing a solution of 0.4 M KCl in buffer A–0.01 M β-mercaptoethanol. The eluate was collected in 5-ml fractions. A broad protein peak was eluted. The fractions collected up to half-way on the peak were combined. This contained the enzyme activity. Nucleoside-5′-phosphomonoesterase activity appeared at the peak and the descending limb, and was well separated from the kinase. The enzyme was then concentrated by absorption on a 3 × 1.2–cm hydroxyapatite column that had been equilibrated with phosphate buffer, *p*H .5,a and washed with water. The enzymatic activity w7s eluted in concentrated form with 0.5 M phosphate buffer, *p*H 7.5, and appeared in 1–5 ml of the eluate. The enzyme was assayed in the presence of the phosphate buffer, thus minimizing a small Pi[^{32}P]ATP exchange which was present in some enzyme preparations.

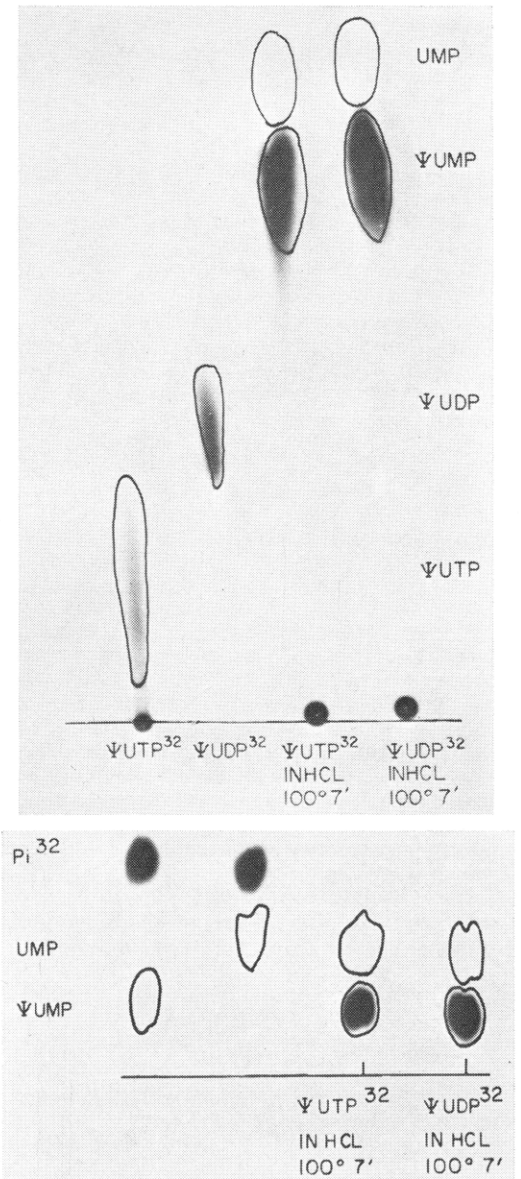

FIG. 4.—Acid hydrolysis of ψUDP and ψUTP synthesized by *E. coli* kinase. [^{32}P]ψUDP and [^{32}P]ψUTP, isolated on DEAE-cellulose as described in Fig. 2, were hydrolyzed in 1 N HCl for 7 minutes. The HCl was removed by evaporation *in vacuo* and the products were chromatographed in system I, marker ψUMP and UMP being added (upper). The labeled product of acid hydrolysis ran with the ψUMP. The products were eluted and chromatographed in system II (lower). The radioactivity had mobility identical to that of authentic ψUMP.

RESULTS

E. coli extracts contain considerable nucleoside-5′-phosphomonoesterase activity, making it difficult to demonstrate enzymatic conversion of ψUMP to ψUTP until it has been removed by enzyme fractionation. With the partially purified enzyme, the formation of ψUDP and ψUTP in yields up to 20% could be readily demonstrated.

The time course of formation of ψUDP and ψUTP is seen in Figure 1. Residual 5′-phosphomonoesterase activity is indicated by the ^{32}Pi formed, which moves ahead of the ψUTP and ψUDP. After incubation of 10 ml of reaction mixture with the enzyme, the products were chromatographed on a DEAE-cellulose (carbonate) column, triethylammonium carbonate being used for elution (Goldberg and Rabinowitz, 1961a).

The ψUDP and ψUTP peaks from the first columns were combined, evaporated to dryness *in vacuo* repeatedly to remove triethylamine carbonate, and rechromatographed in the same manner.

Electrophoresis of the isolated [^{32}P]ψUTP at *p*H 3.8, 5.4, and 7.5 is seen in Figure 2. At all *p*H values the mobility is identical with UTP marker. Furthermore, the isolated ψUTP is active in replacing UTP as the fourth required nucleoside triphosphate in RNA synthesis by the *E. coli* RNA polymerase (Chamberlin and Berg, 1962).

Since the reactions did not proceed to completion, it was essential to prove that the nucleoside triphosphate synthesized was ψUTP and not contaminating UTP. The ultraviolet-absorption spectrum of the labeled nucleoside triphosphate isolated by DEAE-cellulose chromatography is seen in Figure 3, and displays the alkaline bathochromic shift characteristic of pseudouridine-containing nucleotides. A small contamination with ATP is evidenced by the 280/260 ratio of 1.35 rather than the 1.5 at *p*H 13 (Goldberg and Rabinowitz, 1961a), and by the shoulder at 260 mμ.

Acid hydrolysis of the products ψUTP and ψUDP yields compounds which by paper chromatography in systems I and II have the mobility of authentic ψUMP (Figure 4).

The relative activity of the kinase preparations using UMP and ψUMP as substrates is seen in Table I.

TABLE I
RELATIVE ACTIVITY OF KINASE PREPARATIONS USING UMP AND ψ UMP AS SUBSTRATES[a]

Source	Substrate	Enzyme (μg)	Conversion (%)	Incubation Time (min)	Ratio of Activity UMP/ψUMP
E. coli	UMP	8	25	20	79
	ψUMP	160	19	60	
Yeast	UMP	1.6	13	20	61
	ψUMP	160	21	20	

[a] Assays were performed as described under Methods. To calculate the ratio of activity of UMP/ψUMP it was assumed that the reaction rates were linearly related to enzyme concentration and to time of incubation.

With enzymes derived from either *E. coli* or from brewers' yeast, the activity with ψUMP is approximately 1–2% of that with UMP. Furthermore, the significant accumulation of ψUDP in the presence of an active pyruvate kinase system indicates a very poor activity with this system. The K_m values in these crude enzyme systems of UMP and ψUMP are both about 1×10^{-4} M; therefore differences in substrate affinity cannot explain the greatly differing reaction rates with the 2 nucleotides.

No evidence was obtained for separation of activities with UMP and ψUMP in any of the enzyme fractions.

DISCUSSION

It has been shown that pseudouridylic acid kinase activity is present in *E. coli*. Therefore its previously supposed absence cannot be taken as evidence that ψUMP is not incorporated into transfer RNA from a nucleoside triphosphate precursor. Heinrickson and Goldwasser (1964) have demonstrated the formation of ψUMP from uracil and ribose-1-PO_4 in *Tetrahymena pyriformis* but this activity has not been demonstrated in any other organism or tissue. There have been reports of isolation of diribosyluridine derivatives which might serve as intermediates in the conversion of uridylic acid into their pseudouridine counterparts (Lis and Lis, 1962; Pollack and Arnstein, 1962), but their actual participation in such reactions has not been conclusively demonstrated. Robbin's kinetic data suggest intramolecular rearrangement; this could occur on the mononucleotide as well as on the polynucleotide level. It would seem that the method of incorporation of ψUMP into s-RNA is still to be defined.

As in the uridylic transfer enzymes (Rabinowitz and Goldberg, 1961) and in glycogen synthetase (Rabinowitz and Goldberg, 1963), the activity of uridylic kinase is much lower when pseudouridylic acid is substituted for its uridylic acid counterpart. This is also true in the synthesis of polypseudouridylic acid by polynucleotide phosphorylase (Sasse *et al.*, 1963) where it may be related to the additional hydrogen-bonding site in the pseudouridine nucleotides.

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