The Incorporation of Nucleotides into Amino Acid Transfer Ribonucleic Acid

III. THE INCORPORATION OF URIDYLIC ACID*

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SUMMARY

An enzyme preparation from rabbit muscle catalyzes the incorporation of uridine monophosphate into the terminal nucleotide sequence of yeast amino acid transfer ribonucleic acid. Reactants are UTP, Mg++, and transfer RNA which has been partially digested with snake venom phosphodiesterase. Some characteristics of the incorporation system are presented. Incorporation of UMP into transfer RNA rather than into a different species of RNA was shown by a similar chromatographic profile of the RNA fraction labeled with UMP, or with dTMP, by competition between UMP and CMP for a limited number of sites in the RNA molecule, and by nearest neighbor analysis. The latter indicated that 2 UMP residues cannot be incorporated as neighbors in a manner similar to the subterminal CMP residues in transfer RNA.

The incorporation of uridylic acid (uridine monophosphate) into ribonucleic acid has been described by a number of workers (1-9). In most instances the type of RNA which acted as acceptor was not defined. In experiments done by Hecht et al. (1) with a "pH 5 fraction," it is probable that incorporation was into transfer RNA. Daniel and Littauer (2) showed that UMP was incorporated into an RNA isolated from a pH 5 fraction and demonstrated that cytidine triphosphate inhibited this incorporation when added at the same time as UTP. They also showed that CMP could be incorporated adjacent to UMP. Cannellakis (3) described a terminal UMP incorporation in a rat liver high speed supernatant fraction which might have been similar to the systems noted above. However, Klemperer and Kammen (4) purified the enzyme from this system and found that ribosomal RNA was a better acceptor than transfer RNA. Burton and Smellie (5) described a UMP-incorporating system from Ehrlich ascites cells which was stimulated by RNA isolated from the whole cell, and Strauss and Goldwasser (6) investigated the incorporation of UMP by pigeon liver microsomes. In all of these systems it is possible that UMP was incorporated into a fraction of RNA other than transfer RNA. A fraction with some properties similar to transfer RNA but associated with ribosomes has been described (10). The type of RNA into which UMP was incorporated by two nuclear systems (7, 8) and a crude cytoplasmic system (9) was undefined.

MATERIALS AND METHODS

The preparation of the enzyme fraction from rabbit muscle which catalyzes the incorporation of AMP and CMP into transfer RNA has been described (11). From this preparation the fraction recovered from the DEAE column was precipitated with 56.1 g of ammonium sulfate per 100 ml and isolated by centrifugation. The precipitate was dissolved in 2% bovine serum albumin to give a final concentration of enzyme protein of 10.5 mg per ml, then dialyzed for 18 hours against 2 liters of water at 4°, and stored in small aliquots at -15°. This dialysis step was necessary because the ammonium sulfate in which the enzyme was stored was found to inhibit the incorporation of UMP into RNA (100% inhibition was noted with 0.1 M (NH₄)₂SO₄; inhibition was not noted with CMP incorporation because one-tenth the amount of enzyme was necessary for comparable rates of nucleotide incorporation).

The yeast transfer RNA was obtained from General Biochemicals or was isolated (12) from log phase cultures of Saccharomyces cerevisiae (13). The RNA was pretreated with snake venom phosphodiesterase purified by the acetone procedure described by Williams et al. (14) and the DEAE column procedure described by Razzell (15). The RNA was treated either at 15° (16) or at different temperatures as follows. Fifty milligrams of RNA, an amount of diesterase capable of hydrolyzing 0.31 μmole of calcium-bis(p-nitrophenyl) phosphate per min at pH 8.8, 60 μmols of MgCl₂, and 300 μmols of 2 amino 2 methyl 1,3 propanediol buffer at pH 9.6 were incubated in a volume of 2.2 ml at temperatures ranging from 15° to 27.5° for 90 min. The reaction was cooled to 0° and a mixture of 0.8 M HCl and 0.2 M potassium phosphate buffer, pH 7.4, was added to give a final pH of 7.0. The mixture was heated rapidly over a flame to 100°, held at 100° in a water bath for 2 min, and then cooled slowly. After removal of the precipitate by centrifugation, the super-
The assay and the technique for the nearest neighbor analyses were described in previous publications (11, 16). When the Whatman No. 1 filter paper discs used in the assay were washed with $10^{-4}$ M EDTA and water, and then dried prior to use, the zero time value was lowered.

The RNA which was chromatographed was prepared in an incubation mixture similar to that noted below Fig. 1, but with a total volume of 0.6 to 2.0 ml. For some studies requiring maximum UMP incorporation, the 0 to 20% ethanol fraction of the enzyme was used. After incubation the mixture was cooled to 0°C, and 1.0 mg of carrier RNA, 1 ml of 5% bovine serum albumin, and 8 ml of 0.4 N HClO₄ were added. The precipitate was heated at 100°C for 15 min, centrifuged, and the precipitate was re-extracted with 1 ml of 1 N NaCl at 100°C for 5 min. To the combined supernatant fractions, 2 volumes of ethanol were added, and after 30 min at 0°C, the precipitate was collected by centrifugation, dissolved in distilled water, and chromatographed.

$$\text{Fraction} \quad \text{Specific activity} \quad \text{Relative activity} \quad \text{molecules/hr/mg protein}$$

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<th>Fraction</th>
<th>Specific activity</th>
<th>Relative activity</th>
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<tr>
<td>50-75% (NH₄)₂SO₄</td>
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<td>0-20% ethanol</td>
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<td>DEAE</td>
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The Ecteola cellulose column has been described (29). The methylated albumin column (23) (1.8 x 12 cm) was eluted with a linear gradient with 130 ml each of 0.2 N NaCl-0.05 M sodium phosphate buffer (pH 6.7), and 1.1 N NaCl-0.05 M sodium phosphate buffer (pH 6.7).

**RESULTS**

**Purification of Enzymes**—The enzyme fractions which catalyzed the incorporation of UMP into RNA, and which were used for the following studies, were purified in the same manner as those which catalyzed the incorporation of AMP and CMP (11, 16). In Table I the results of purification of this enzymatic activity are shown. A 146-fold purification was achieved, and the total units recovered represented 34% of the original activity. No activity was found in the 0 to 50% ammonium sulfate fraction, or in the 20 to 50% ethanol fraction. Thus, the behavior of the enzymatic activity responsible for UMP incorporation into RNA was similar to that observed for AMP and CMP incorporation (14). The enzymatic activity of the DEAE fraction was stable when stored either in (NH₄)₂SO₄ or in 2% bovine serum albumin at -15°C.

**Requirements of Reaction**—The results presented in Table II indicated that for UMP incorporation there was an absolute requirement for transfer RNA. For optimal incorporation of UMP into the RNA, prior incubation of the RNA with snake venom phosphodiesterase was necessary. The preparation of commercial yeast RNA, used in these experiments, had the ability to accept some UMP without prior digestion with the phosphodiesterase. This indicated that some of the terminal and subterminal nucleotides were lost in the isolation and preparation of this material; a similar finding has been noted in connection with studies of AMP incorporation (11, 16). Analysis of the terminal nucleotides of the commercial RNA preparation (11) showed the presence of cytidine (48%), adenosine (44%), and uridine (8%). To ensure that the UMP incorporation into RNA was not due to some artefact of degradation, which might have occurred during the preparation of the commercial yeast RNA, transfer RNA was isolated from log phase yeast cells and tested. This material required digestion by phosphodiesterase before it could act as an acceptor for UMP. In Experiment 1, the incorporation was linear at the time of determinations;

1 Ecteola cellulose is epichlorohydrin triethanolamine cellulose.
TABLE II

Requirements for UMSP incorporation

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<th>Experiment and conditions</th>
<th>UMSP incorporation (μmole/mg RNA)</th>
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<td>Phosphodiesterase-treated RNA</td>
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<tr>
<td>Untreated RNA</td>
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<tr>
<td>Phosphodiesterase-treated log phase RNA</td>
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<tr>
<td>Untreated log phase RNA</td>
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<tr>
<td>Phosphodiesterase-treated RNA minus Mg++</td>
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<td>Phosphodiesterase-treated RNA plus ATP</td>
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<td>Phosphodiesterase-treated RNA plus p-hydroxymercuribenzoate</td>
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Experiment 1

No RNA...................... 0.0
Phosphodiesterase-treated RNA........ 4.0
Untreated RNA............ 2.0
Phosphodiesterase-treated log phase RNA........... 3.4
Untreated log phase RNA........ 0.8
Phosphodiesterase-treated RNA minus Mg++........ 0.7
Phosphodiesterase-treated RNA plus ATP........ 4.4
Phosphodiesterase-treated RNA plus p-hydroxymercuribenzoate........ 3.1

Experiment 2

Phosphodiesterase-treated log phase RNA........ 10.4
Untreated log phase RNA........... 0.5
Phosphodiesterase-treated RNA........ 12.4
Untreated RNA............ 5.6

In Table II, Experiment 2, the incorporation had reached a maximum value. Magnesium was required for optimal incorporation. Unlabeled ATP occasionally resulted in a slight stimulation of the rate of UMP incorporation, a stimulation similar to that seen with CMP incorporation (16). Prior incubation of the enzyme with p-hydroxymercuribenzoate did not result in any constant stimulation or inhibition of UMP incorporation. In Table II, the results show some inhibition while in other experiments either no inhibition or a slight stimulation was noted. This is to be contrasted with a significant stimulation of AMP incorporation (16) by p-hydroxymercuribenzoate. The significance of this is unknown.

The possibility was considered that the UTP was contaminated by or converted to CTP and that the radioactivity incorporated came from CMP. This explanation was ruled out by the isolation of UMSP from the RNA at the end of an incubation. After incorporation of 10.4 μmolecules of UMSP per mg of RNA, the RNA was washed repeatedly with acid, neutralized, and degraded with the phosphodiesterase. Ninety-eight per cent of the radioactivity recovered in the four nucleotides after paper electrophoresis was shown to be associated with UMP.

Characteristics of Reaction—Fig. 1 shows the relationship of the time of incubation to the extent of incorporation of UMSP. With this preparation of RNA, approximately 10.5 μmolecules of UMSP were incorporated per mg of RNA in 120 min. In other experiments, up to 20 μmolecules were incorporated per mg. This is less than the estimated 37 μmolecules per mg if 1 UMP residue were incorporated per 50 nucleotide residues. The lower values may be due to variable degradation with phosphodiesterase. A linear relationship between the rate of the reaction and the amount of enzyme added was noted when conditions similar to those noted below Fig. 1 were employed; the time of incubation was 10 min, and the amount of enzyme added per ml of reaction mixture was 62.5 to 500 μg. When 1.0 mg per ml was added, the rate was no longer linear but decreased by 13%. When amounts of RNA varying between 0.25 and 2.0 mg per ml of reaction mixture were added and the reaction was followed to completion, a linear relationship was noted between the amount of RNA added and the amount of UMSP incorporated. Optimum incorporation of UMSP occurred at an elevated pH similar to that observed with AMP and CMP (11, 16). With 2-amino-2-methyl-1,3-propanediol buffer (pH 8.8, 50 mM) incorporation at pH 10.0 was slightly more rapid than at 9.5. With lysine buffer (50 mM) which at pH 10 inhibited the reaction 90% when compared with the propanediol derivative, a broad pH optimum was noted between 9.5 and 10.5 with a maximum at pH 10.0.

Incorporation into Transfer RNA—To demonstrate that the UMSP was incorporated into transfer RNA rather than into some other contaminating RNA fraction, the RNA was isolated after the reaction and chromatographed on an Ecteola cellulose column. The results are shown in Fig. 2. The radioactivity paralleled the main peak of 260 μm-absorbing material, which in previous experiments has been demonstrated to be transfer RNA (22).

A more rigorous analysis of the type of RNA which was labeled was obtained by the chromatography of a mixture of 3H-CMP and UMSP-labeled RNA samples on a methylated albumin column. In Fig. 3, the absorbance values (260 μm) of the UMSP label, and the ratio of UMSP to 3H-CMP are shown. There is no evidence for a single fraction of RNA which is highly labeled by UMSP (10). There is some decrease in the UMSP to 3H-CMP ratio observed in later fractions, but this change is the opposite of that predicted if a fraction similar to the one described by Rosset and Monier (10) were the acceptor for the major portion of the UMSP incorporated.

Further evidence of UMP incorporation into transfer RNA was obtained by determining that UMP and CMP compete for the same sites in the RNA. The results are shown in Fig. 4.
In Section A, the incorporation of UMP or 3H-CMP, in separate reaction mixtures, into the transfer RNA is recorded as a function of time. It is apparent from this experiment that the UMP incorporation was slightly less than the CMP incorporation and that the rates of incorporation were somewhat different. In Section B, the results of an experiment with both UTP and 3H-CTP in the same reaction vessel are shown in which UTP was added at zero time and 3H-CTP was added after 15 min of incubation. The concentration of CTP was approximately 2 times that of the UTP. The results are represented as follows. UMP incorporation is indicated as an absolute value. At this time 3H-CTP was added and after 15 min, 6.1 mpmoles of UMP were incorporated and no additional incorporation of UMP was observed. After a further incubation period of 150 min, 15.4 mpmoles of CMP were incorporated. This value was added to the UMP Incorporation value of 6.1 mpmoles, and the total incorporation of both nucleotides of 21.5 mpmoles was plotted. The total incorporation of the nucleotides in the same reaction mixture was similar to the incorporation of either one alone. The data in Section C represent the results of a similar experiment in which UTP was added at zero time and CTP was added at 30 min. The plots are drawn in the same manner as indicated in Section B and show again that the addition of CTP stopped absolutely the incorporation of UMP, while the final sum of the incorporation of both nucleotides was approximately 20 mpmoles. These experiments provide evidence for a limited number of sites in the RNA acceptor for nucleotide incorporation. Although nearest neighbor data, as described later, were not available for this RNA preparation, it is probable that the incorporation was primarily into the position normally occupied by the CMP adjacent to the terminal AMP. This will be discussed. The reason that the rate of CMP incorporation in this experiment was not much faster relative to UMP as was usually noted is not known; it may be related to the concentration of CTP which was approximately 25 times the $K_m$ of CTP.

**Nucleotides Adjacent to UMP and CMP**—After incorporation of a 5'-nucleoside monophosphate into the RNA, it is possible by alkaline hydrolysis to recover the phosphate in the adjacent nucleotide. This type of analysis of nearest neighbors provides further evidence that UMP was incorporated into the transfer RNA. In these experiments, the RNA used as acceptor
was incubated with a purified snake venom phosphodiesterase and then used with the nucleotide incorporation enzyme fraction and either CTP or UTP. The values obtained with CTP in all the experiments represent levels attained when the incorporation of radioactivity had reached a limiting value. The relationship between the temperature of incubation with phosphodiesterase and the nearest neighbor pattern with CT_{32}P has been discussed previously (15). At a low temperature of incubation (15°), only 1 CMP residue is removed (RNA-X-C) while at higher temperatures (22.5-27.5°), both of the CMP subterminal residues are removed (RNA-X).2

In Table III, the results of the nearest neighbor analyses with CT_{32}P and UT_{32}P are presented. One major conclusion can be drawn from this data: UMP is incorporated in place of CMP in either the inside position or in the outside position next to a CMP residue, but UMP is not incorporated in the outside position when it would be next to a UMP residue; in other words, 2 UMP residues cannot be incorporated adjacent to each other. The data which support the argument that 2 UMP residues cannot be incorporated in vitro, one next to the other, can be found in Table III, Experiments 1 to 5. Both CMP and UMP incorporation in these experiments was followed until maximum values were obtained. The data from Experiment 1 were obtained from an RNA degraded so that both CMP residues were lost. The sum of the CMP incorporated next to AMP, GMP, and UMP equals 6.2 mmoles. If this represents the inside CMP, then an equal amount of CMP (6.2 mmoles) will be incorporated adjacent to these CMP residues. The sum of these values equals 12.4 mmoles. Since a total of 13.2 mmoles of CMP was incorporated, this leaves 0.8 mmole more of CMP incorporated next to the CMP. Of this 0.8 mmole of CMP, possibly 0.4 mmole is in the inside position and 0.4 mmole is incorporated next to it in the outside position. The total of CMP residues incorporated in the inside position would be 6.2 plus 0.4 or 6.6 mmoles. A similar level of UMP residues should be incorporated. Actually a total of 0.2 mmoles of UMP were incorporated and in comparison with CMP, the amounts of UMP incorporated adjacent to CMP and GMP are slightly high and low, respectively. In Experiment 2, the data obtained with an incompletely digested RNA can be analyzed in the same way: 8.9 mmoles of CMP were adjacent to non-CMP residues and, therefore, 8.9 mmoles of CMP were adjacent to these CMP residues. A remainder of 5.0 mmoles of CMP must be adjacent to CMP, and this could represent CMP incorporation primarily into RNA-X-C. The UMP incorporation pattern should show 8.9 mmoles next to AMP, GMP, and UMP, and 5.0 mmoles next to CMP. The observed results are not greatly different from the predicted values. Thus with the RNA lacking 2 CMP residues, one would expect to see ratios of CMP to UMP incorporation of 2:1, and this has been observed many times when maximum incorporation is attained as in Experiment 1.

That UMP can be added to RNA-X-C to form RNA-X-C-U is apparent from the data in Table III, Experiment 5. If primarily the outside CMP residue is removed, then the level of incorporation in vitro of UMP should approach that of CMP. In a few cases, such as Experiments 4 and 5, this has been observed, and it is probable that this was the case in the experiment shown in Fig. 4. A few experiments were performed with U^3_{14}C-UTP, Table IV, which indicated that UMP was incorporated exclusively into a terminal nucleotide position, and upon alkali digestion all the radioactivity incorporated was recovered as uridine. When unlabeled CTP was added to the incubation mixture after the UMP incorporation, 32% of the U^3_{14}C activity was recovered as UMP. This evidence supports the concept that UMP is not incorporated next to UMP and also indicates

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<th>Experiment</th>
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<td>9</td>
<td>7</td>
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2 The abbreviations used are: RNA-XMP-CMP-AMP or RNA-X-C-C-A, the 3'-terminal end of transfer RNA. Degradation with snake venom phosphodiesterase results in species abbreviated RNA-X-C-C, RNA-X-C, and RNA-X. RNA-X-C-C possesses both the "inside" and "outside" subterminal CMP residues while RNA-X-C has only the "inside" subterminal CMP residue.

Table III
Nearest neighbor analysis of CMP_{32}P and UMP_{32}P incorporated into transfer RNA treated with phosphodiesterase at varying temperatures

All reaction mixture volumes were 2.0 ml and contained additions as noted below Fig. 1 with UT_{32}P, 0.250 mM, or CT_{32}P, 0.125 mM. Reactions were followed by the filter paper disc assay until incorporation values ceased to rise. Nearest neighbor analysis was done as noted in "Materials and Methods."
Incorporation of Nucleotides into Amino Acid Transfer RNA. III

Studies of incorporation of UMP-$^{14}$C and AM$^{32}$P

Table IV

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nucleotides in preincubation</th>
<th>Amount incorporated</th>
<th>Nucleotide in incubation</th>
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Fig. 5. The reciprocal initial rate of UMP incorporation into RNA measured as a function of the reciprocal concentration of UTP$^{32}$P in the absence and presence of CTP. Incubation mixtures of 0.2 ml volume contained: 210 μg of the DEAE enzyme fraction, 0.1 mg of phosphodiesterase-treated RNA; MgCl₂, 10 mM; buffer at pH 10, 50 mM; and increasing final concentrations of UTP from 0.08 mM to 0.75 mM. CTP, 0.01 mM, was present in one set of vessels. Incubation was for 12 min at 37°; UMP$^{32}$P incorporated, 5.2; CTP incorporated, 8.8; ATP incorporated, 2.2; UTP$^{32}$P incorporated, 99; results were calculated as millimicromoles per mg of RNA.

that CMP residues can be incorporated next to UMP residues. When ATP was added to the incubation, only a small amount of incorporation next to UMP occurred as indicated by 71% of the radioactivity recovered as AMP.

A further experiment was done to determine whether a terminal AMP could be incorporated next to a UMP residue (Table IV, Experiment 2). RNA was prepared by incubation both with unlabeled UTP and, in a control vessel, with labeled UTP. After maximum incorporation was observed in the control experiment, the unlabeled RNA was re-isolated and used as a substrate for AMP incorporation. The rate of incorporation of AMP$^{32}$P was slow, and the results of a nearest neighbor analysis showed that 71% of the AMP was adjacent to CMP, and 25% was adjacent to UMP. This preliminary experiment indicates that AMP incorporation next to UMP is possible, but very inefficient.

Michaelis Constant for UTP and Effect of Other Nucleoside Triphosphates on Reaction—The Michaelis constant for UTP was determined in a series of experiments under different conditions. Only one $K_m$ was found and the average value for this was $2.6 \times 10^{-4}$ M. The range for six determinations was from 2.1 to $3.3 \times 10^{-4}$ M. This $K_m$ value was observed for UTP regardless of the type of RNA used. A large number of experiments were done with different RNA preparations, (RNA-X and RNA-X-C), different enzyme preparations, different levels of enzyme, varying periods of incubation, and levels of UTP up to 2.0 mM. In view of these extensive experiments, it is reasonable to conclude that only one $K_m$ value exists for UTP in the UMP incorporation reactions into either the inside or outside positions normally occupied by UMP of transfer RNA.

CTP was shown to be a competitive inhibitor of UMP incorporation. A Lineweaver-Burk plot of the incorporation of UMP$^{32}$P in the absence and presence of CTP is indicated in Fig. 5. The $K_m$ for UTP in this reaction was $3.3 \times 10^{-4}$ M while the $K_i$ for CTP was $1.0 \times 10^{-4}$ M. In a second experiment the $K_i$ for CTP was $0.9 \times 10^{-5}$ M. The $K_m$ values determined for CTP under varying conditions range from 0.8 to $5.5 \times 10^{-4}$ M.

UTP is a competitive inhibitor of AMP incorporation. The average of five $K_i$ determinations for UTP was $1.05 \times 10^{-4}$ M, with a range of 0.9 to $1.3 \times 10^{-3}$ M. This $K_i$ value for UTP in the CTP incorporation reaction is about 4 times the $K_m$ value of UTP.

The purine nucleoside triphosphates have very little inhibitory effect on the incorporation of UMP. At UMP concentrations of $1.0 \times 10^{-4}$ M, ATP or GTP at $1 \times 10^{-3}$ M gave 11% and 19% inhibition, respectively.

Rates of Incorporation of UMP and CMP—When maximum velocities were calculated from the reciprocal plots, the $V_{max}$ obtained for UMP incorporation was 475 mpmoles per hour.

D. A. Goldthwait and D. D. Anthony, unpublished observations.
per mg of the DEAE fraction while that obtained from CMP was 4950 mmoles per hour per mg of the DEAE fraction. Thus the rate of incorporation of CMP catalyzed by this preparation is approximately 10 times that of UMP.

**Discussion**

It is of some importance to know whether UMP is incorporated into transfer RNA or into an RNA which is isolated with transfer RNA, but does not have this function. Such an RNA has been identified and may have a 3'-hydroxyl terminal uridylic acid (10). The experiments described here provide evidence that UMP can be incorporated into transfer RNA. The evidence can be summarized as follows. First, for optimum UMP incorporation separate enzymatic sites exist which may have a 3'-hydroxyl terminal uridylic (16). However, the second site or sites which catalyze the incorporation of UMP into transfer RNA. Second, the RNA fraction which accepts UMP has chromatographic properties characteristics of transfer RNA on both Ecteola cellulose and methylated albumin columns and UMP radioactivity parallels the optical density. Third, when the RNA was labeled with both $^{3}H$-CMP and UMP and subjected to chromatography on a methylated albumin column, the ratio of $^{3}H$ to $^{32}P$ did not vary significantly throughout the RNA fraction. Fourth, UMP and CMP were found to compete for a limited number of available sites. With increasing UMP incorporation there was decreasing CMP incorporation, but the sum of the incorporation of both nucleotides remained constant. And finally, the analysis of the nearest neighbor frequencies when UMP or CMP was incorporated into the RNA fractions, provided further support for the hypothesis that both nucleotides were incorporated at the same acceptor sites with the limitation that a second UMP residue was not incorporated next to the first. All of the evidence cited above supports directly and indirectly the idea that UMP incorporation occurs in the transfer RNA fraction. The incorporation of CMP next to UMP, and very inefficient incorporation of AMP next to UMP, was also shown.

The question arises why UMP is not found as a substitute for CMP in the terminal positions of transfer RNA. Since components of the system studied were derived from rabbit and from yeast, the system can only be considered as a model. With this reservation in mind, one can consider two explanations for the absence or restriction of UMP substitutions in vivo. First, the $K_{m}$ for UTP in the incorporation reaction is approximately 20 to 100 times the $K_{m}$ for CTP, and the $K_{i}$ for CTP is about one-twenty-fifth of the $K_{m}$ value for UTP. Second, the rate of incorporation of UMP per mg of enzyme fraction is approximately one-tenth that of CMP. These factors may insure against any significant mistake level in vivo. In the E. coli system, enzyme specificity may exclude completely the incorporation of UMP (24). The decreased ability of the RNA to accept AMP after UMP has been incorporated makes it unlikely that a significant amount of transfer RNA exists with UMP in the terminal positions. This fact plus some sequence knowledge of the 5'-phosphate end of the molecules (25, 26) makes it necessary to conclude that the specificity for the additions must reside with the enzyme sites. Different enzyme sites have been demonstrated for AMP and CMP (16).

The question arises whether the catalytic site responsible for the incorporation of UMP into transfer RNA is the same as the site or sites which catalyze the incorporation of CMP. Evidence favoring this is as follows. The purification of the UMP incorporation activity follows that of the CMP activity, and the $K_{m}$ value for UTP inhibition of UMP incorporation is similar to the $K_{m}$ value for CTP which has been obtained for this enzyme (16). However, the $K_{m}$ value for UTP obtained when either RNA-X or RNA-X-C was used as substrate was only one-fourth the $K_{m}$ value for UTP in the CMP incorporation reaction. This evidence suggests that separate enzymatic sites exist which catalyze the incorporation of CMP and UMP. An enzyme which catalyzes the incorporation of CMP but not that of CMP has been described (4). Further purification of the enzyme preparation used for the experiments discussed in this paper is in progress.

**References**

The Incorporation of Nucleotides into Amino Acid Transfer Ribonucleic Acid: III. THE INCORPORATION OF URIDYLIC ACID
Adolfo Fernandez-Sorensen, Donald D. Anthony, David A. Goldthwait and With the technical assistance of Irene Uktins


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