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# Chemical Modification of Yeast Alanine Transfer Ribonucleic Acid with a Radioactive Carbodiimide*

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ABSTRACT: 1-Cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide [$^{14}$C]methiodide reacts with the base in inosinic acid and dihydrouridylic acid.

In yeast alanine transfer ribonucleic acid, one of the first sites of chemical attack by the carbodiimide is the inosinic acid of the anticodon.

1-Cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide metho-*p*-toluenesulfonate (CMCMT)[1] was first shown by Gilham and coworkers to form addition products with guanylic acid, uridylic acid, pseudouridylic acid, and thymidylic acid but not with adenylic acid or cytidylic acid (Gilham, 1962; Ho and Gilham, 1967). The addition products were found to be resistant to enzymatic cleavage by pancreatic ribonuclease, which normally cleaves ribonucleotides at the 3′-phosphoryl bond of pyrimidines, or by ribonuclease $T_1$, which cleaves at the 3′-phosphoryl bond of G and I (Ho and Gilham, 1967). In addition the carbodiimide reaction was found to be reversible under conditions of mild alkali (Gilham, 1962).

Other workers in the field have used 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide methiodide (CMCMI) or CMCMT to investigate the structural properties of nucleic acids. Augusti-Tocco and Brown (1965) used [$^{14}$C]CMCMI to investigate the extent of nonhydrogen-bonded or randomly coiled regions in various polynucleotides. Knorre and his coworkers (Knorre *et al.*, 1966; Drevitch *et al.*, 1966) also investigated the reaction of [$^{14}$C]CMCMT with tRNA and DNA and reported the effect of this reaction on the ability of yeast to accept amino acids (Girshovich *et al.*, 1966).

In a previous paper we reported the reaction of [$^{14}$C]-CMCMI with $tRNA^{Ala}$ purified from yeast (Brostoff and Ingram, 1967). It was demonstrated that [$^{14}$C]CMCMI attaches to mononucleotides in the sequences ψpGp, UpCp, and UpUpIpGpCp of that molecule, but does not form addition products with the expected bases in the sequence TpψpCpGpApUp. Further evidence will now be presented for the reaction of this reagent with the mononucleotide Ip, and with Ip in the proposed anticodon of the $tRNA^{Ala}$ molecule. Preliminary evidence will also be presented which suggests that the mononucleotide diHUp may also react with CMCMI.

## Materials and Methods

*Mononucleotides and tRNA.* Mononucleotides were obtained from Calbiochem, Inc. (Los Angeles, Calif.). Yeast tRNA was obtained from the General Biochemicals Corp. (Chagrin Falls, Ohio). $tRNA^{Ala}$ was purified from the commercial yeast tRNA by two successive countercurrent distributions (Apgar *et al.*, 1962; Armstrong *et al.*, 1964).

*Preparation of* [$^{14}$C]*CMCMI.* [$^{14}$C]$CH_3I$ (1.5 mCi, 128 mg, New England Nuclear Corp., Boston, Mass.) was added to excess (1–2ml) 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]-carbodiimide (redistilled, Aldrich Chemical Co.) and the mixture was allowed to stand in the dark for at least 15 hr at room temperature in a glass-stoppered tube. The precipitate formed was taken up in chloroform and filtered. The chloroform was removed from the filtrate by evaporation, and the residue was taken up in acetone and again filtered. Anhydrous ether (two volumes) was added to the filtrate and crystals were allowed to form at room temperature. The white crystals were collected, washed with ether, and dried. The yield was 60% and the purity was comparable with commercially available CMCMT (Aldrich Chemical Co.) as determined by infrared spectrophotometry. After electrophoresis of [$^{14}$C]CMCMI in pH 1.9 buffer, more than 99% of the radioactivity present on the paper was located in

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[1] Abbreviations used are: CMCMT, 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide metho-*p*-toluenesulfonate; CMCMI, 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide methiodide; DiHU, 5,6-dihydrouridine; DiMeG, $N^2$-dimethylguanosine; MeG, 1-methylguanosine; MeI, 1-methylinosine; Ψ, pseudouridine; T, ribothymidine; U*, a mixture of uridine and dihydrouridine.

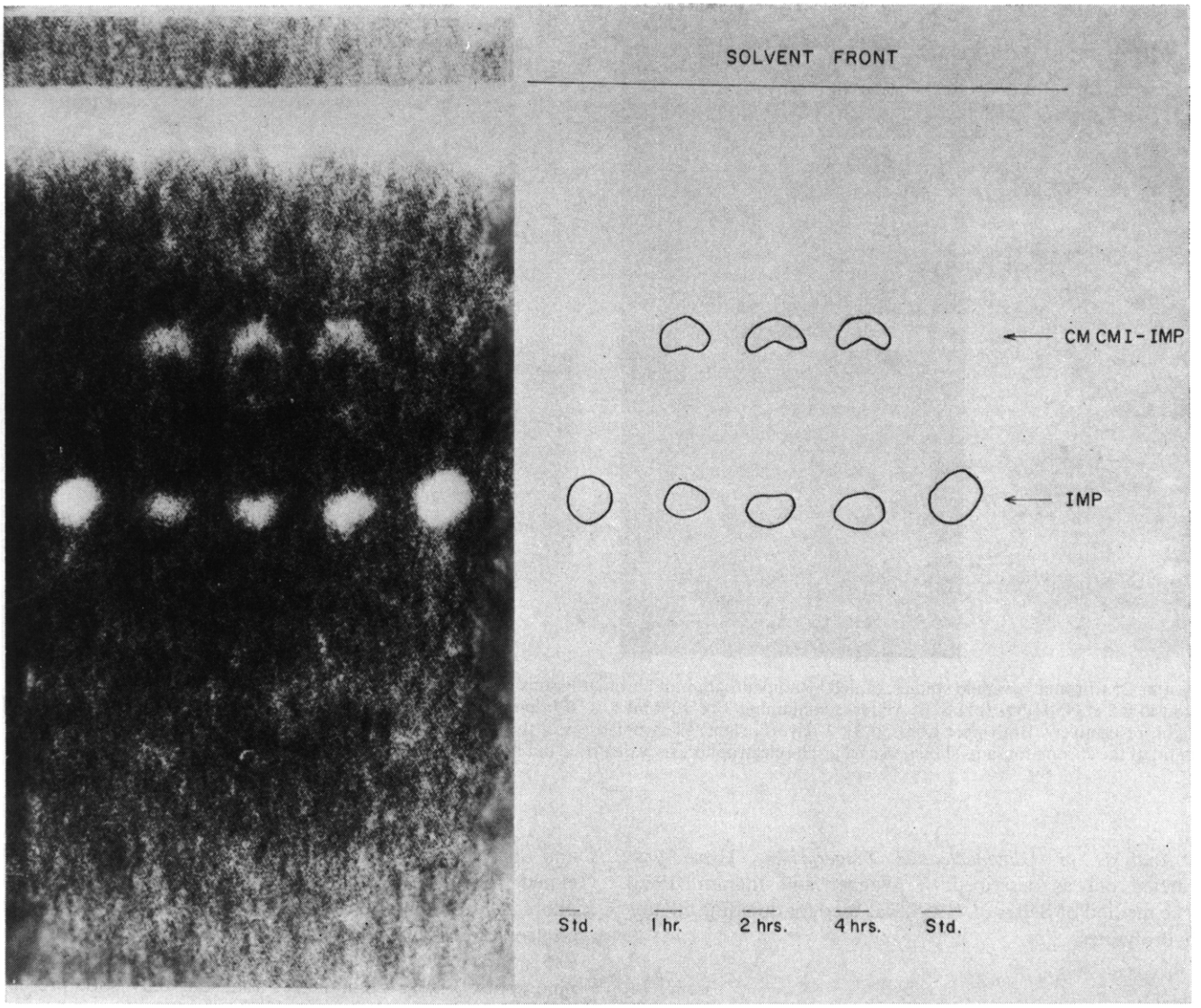


FIGURE 1: Ascending chromatography of IMP after incubation with CMCMI at 38° in 0.2 M $Na_2HPO_4$ at pH 8.0. A contact print showing the ultraviolet-absorbing spots is shown at the left, and a tracing of these spots is shown at the right. Samples (5 $\mu$l) were subjected to chromatography after 1-, 2-, and 4-hr incubation.

one spot. This [$^{14}$C]CMCMI spot had the same electrophoretic mobility as cytidine, a molecule of comparable size and charge. The specific radioactivity of the [$^{14}$C]CMCMI was $10^7$ dpm/mg.

Nonradioactive CMCMI was made similarly.

*Reaction of Nucleotides with [$^{14}$C]CMCMI.* Inosine 5′-phosphate (IMP) (2.0 mg/ml) or dihydrouridylic acid (2.0 mg/ml, a mixture of the 2′- and 3′-phosphates) was incubated with [$^{14}$C]CMCMI (20 mg/ml, a 10:1 molar excess) at 38° in 0.2 M $Na_2HPO_4$ (pH 8.0). Aliquots were withdrawn at various times, spotted on Whatman No. 3MM paper, and subjected to ascending chromatography in a solvent containing equal volumes of *t*-butyl alcohol and pH 1.9 buffer (Ingram and Pierce, 1962).

*Reaction of tRNA with [$^{14}$C]CMCMI.* The reaction was carried out in the following way: [$^{14}$C]CMCMI (25 mg/ml) and tRNA (2.5 mg/ml) were reacted in 0.01 M borate (pH 8.0) and 0.02 M $MgCl_2$ for 60 sec at 38°. The reaction was stopped by precipitating the tRNA with two volumes of cold 95% ethanol.

*Digestion of tRNA$^{Ala}$.* This was generally carried out 1 mg at a time in 100 $\mu$l of 0.1 M borate buffer (pH 7.5) containing 12.5 $\mu$g of pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N. J. ) and/or 12.5 $\mu$g of takadiastase ($T_1$) ribonuclease (Calbiochem, Inc., Los Angeles, Calif.). The solution was incubated for 90 min at 38° and evaporated on Parafilm in a vacuum desiccator. The residue was dissolved in 10 $\mu$l of water in a constriction pipet and subjected to fingerprinting.

*Fingerprinting Procedure.* Method D of Armstrong *et al.* (1964) was used with the following modifications. Electrophoresis was carried out in pH 1.9 buffer (Ingram and Pierce, 1962) and was followed by ascending chromatography in a solvent containing equal volumes of the electrophoretic buffer and *t*-butyl alcohol. For radioautography Ansco Nonscreen X-ray film was used.

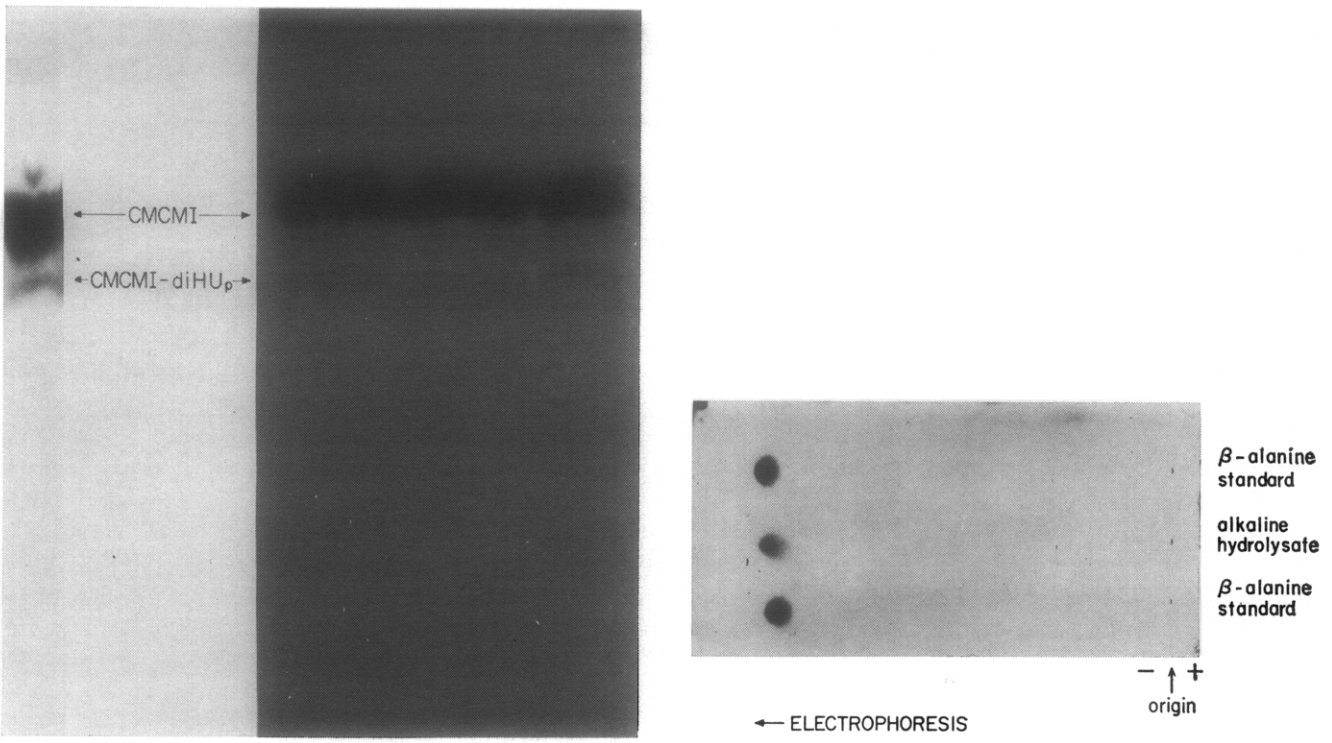


FIGURE 2: Chromatographic studies. (a, left) Radioautogram of chromatograms of a mixture of diHUp and [$^{14}$C]CMCMI after 2-hr incubation in 0.2 M $Na_2HPO_4$ (pH 8.0). Aliquots containing $3 \times 10^7$ and $1 \times 10^7$ dpm were spotted on the chromatograms shown at the left and right, respectively. Both were exposed for 1 day. (b, right) Electrophoresis of the alkaline hydrolysate of the material eluted from the lower band of the chromatograms shown in part a. The electrophoresis was carried out in pH 1.9 acetate–formate buffer.

*Analysis of Oligonucleotide Fingerprints.* These were carried out as described by Wagner and Ingram (1966). The method of Bell *et al.* (1964) was used for desalting alkaline hydrolysates.

## Results

It was of interest to learn whether inosinic acid and dihydrouridylic acid, both minor bases in $tRNA^{Ala}$, formed addition products with [$^{14}$C]CMCMI or with CMCMI. Dihydrouridylic acid appears in three different locations in the molecule and inosinic acid is located in the proposed anticodon site.

Figure 1 indicates that CMCMI forms addition products with IMP. The addition products were eluted from the paper and subjected to electrophoresis in pH 1.9 buffer. They moved as a single spot toward the negative terminal. The addition products were also submitted to alkaline hydrolysis (0.33 N KOH for 16 hr at 38°). Inosinic acid was recovered demonstrating the reversibility of the addition reaction with IMP.

The reaction of diHUp with [$^{14}$C]CMCMI was more difficult to ascertain because diHUp, unlike most nucleotides, is not visible under ultraviolet light. Aliquots of the reaction mixture (2-hr incubation) were subjected to ascending chromatography followed by radioautography of the resulting chromatogram. As can be seen in Figure 2a, a weak band of radioactivity appears below the [$^{14}$C]CMCMI. The presence of diHUp in this band was demonstrated by its conversion into β-alanine in alkali according to the method of Magrath and Shaw (1967). The alkaline hydrolysate of the material eluted from the radioactive band was submitted to electrophoresis with β-alanine standards in pH 1.9 buffer. The electrophoretogram was stained with ninhydrin solution (0.5% in acetone) and heated for 30 min to locate the β-alanine spots. Figure 2b indicates that the only spot present in the alkaline digest coincided with the β-alanine standards. This indicates that the radioactive band below the [$^{14}$C]-CMCMI in Figure 2a contains both diHUp and CMCMI and suggests that this band could be [$^{14}$C]CMCMI-diHUp.

In an effort to locate the bases in the alanine tRNA molecule that are most susceptible to CMCMI, the following experiment was done. Samples of [$^{14}$C]CMCMI–alanine tRNA that had been reacted for 60 sec at 38° and which contained 1.7 moles of [$^{14}$C]CMCMI/mole of tRNA was digested simultaneously with $T_1$ and pancreatic ribonucleases and fingerprinted. Figure 3 shows the radioautogram of this fingerprint, and Figure 4 shows a tracing of the radioautogram. In addition to A and B, which are also found after 24-hr reaction (see Brostoff and Ingram, 1967), three other spots were present. The material at Z from several fingerprints was combined and submitted to alkaline hydrolysis followed by electrophoresis. The hydrolysate contained equimolar concentrations of Ip and Gp as determined by ultraviolet spectral analysis. Thus Z contains IpGp (the Ip being modified by the reagent) since Ip is present in $tRNA^{Ala}$ only in the sequence IpGpCp (Figure 5), the proposed anticodon. This sequence is one of the first to react with CMCMI, since Z is present as a major spot after 60-sec reaction (Figures 3 and 4) whereas it is only present as a minor

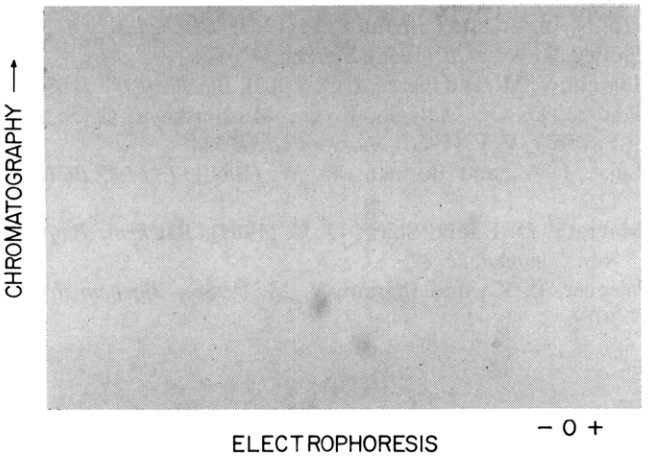


FIGURE 3: Radioautogram (4-day exposure) of the fingerprint of the $T_1$ and pancreatic ribonuclease digestion of [$^{14}$C]CMCMI–tRNA$^{Ala}$ (1.7 moles of [$^{14}$C]CMCMI/mole of tRNA) formed during incubation for 60 sec at 38°. The sample applied contained $2 \times 10^5$ dpm.

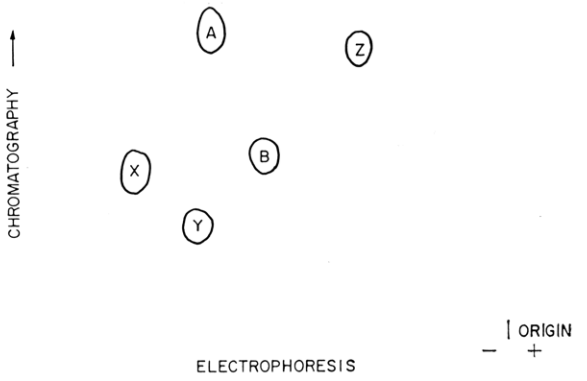


FIGURE 4: Tracing of the radioactive spots shown in Figure 3.

spot after 24-hr reaction (Brostoff and Ingram, 1967). The material in spots X and Y contained amounts of radioactivity comparable with Z, but although traces of Cp, Gp, and Up were found, no meaningful information about sequence could be deduced.

## Discussion

Figure 5 shows the now well known cloverleaf structure of yeast alanine tRNA first proposed by Holley *et al.* (1965). It is evident, from our experiments that the CMCMI attaches to bases in the lower loop which contains the sequences UpUpIpGpCp and ψpGp and specifically reacts with the Ip and ψp (Brostoff and Ingram, 1967) residues of these sequences. The sequence IpGp is one of the first to react and is more susceptible to CMCMI than ψpGp since reacted ψpGp was not detected in the fingerprint of the digest of alanine tRNA that had been reacted for 60 sec at 38°.

The material at spot A (Figure 4) was reported previously to contain the oligonucleotide UpCp (Brostoff and Ingram, 1967). In an experiment with a sample of alanine tRNA that had been extensively reacted with [$^{14}$C]CMCMI, the location of some, at least, of that UpCp was traced to the sequence ApGpU$^*_{\psi}$Cp. It was shown that after digestion of this sample with pancreatic ribonuclease alone, most of the radioactivity remained at the origin when the digest was fingerprinted. When this origin material was digested with $T_1$ ribonuclease and again fingerprinted, the UpCp appeared at its usual location (Brostoff and Ingram, 1967). Only the UpCp from the sequence ApGpU$^*_{\psi}$Cp would have remained at the origin after digestion with pancreatic ribonuclease alone since it would have been part of the nonanucleotide GpGpGpApGpApGpU$^*_{\psi}$Cp.

The sequence ApGpU$^*_{\psi}$Cp has been proposed as a nonhydrogen-bonded fourth loop when tRNA is arranged in the cloverleaf structure. This idea is supported by the finding that the Up residue of this sequence reacts with CMCMI.

The results indicate that two sequence must be available

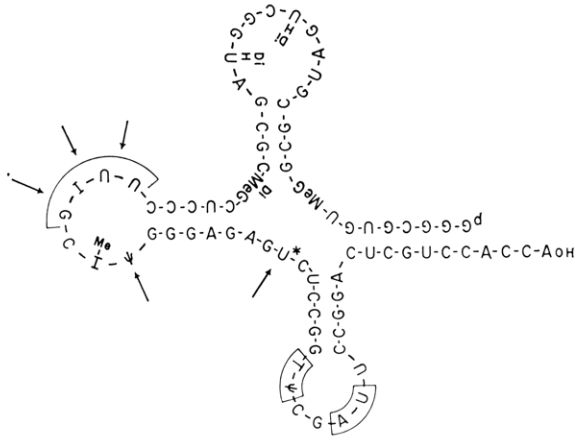


FIGURE 5: The sequence of yeast tRNA$^{Ala}$ arranged in the cloverleaf structure proposed by Holley *et al.* (1965). The arrows point to the regions of the sequence which react with [$^{14}$C]CMCMI. The bases enclosed in boxes do not react.

for reaction with CMCMI in any model of the secondary or tertiary structure of alanine tRNA. These sequences are UpUpIpGpCpMeIpψpGp and ApGpU$^*_{\psi}$Cp. The cloverleaf structure of tRNA$^{Ala}$ (Figure 5), in which the nonhydrogen-bonded bases are represented by loops, is inadequate by itself to explain the pattern of reaction of tRNA$^{Ala}$ with CMCMI. The observation that the sequence Tpψ pCpGp-ApUp does not react with CMCMI (Brostoff and Ingram, 1967) indicates that this sequence is not available as indicated by the cloverleaf model. This sequence could actually be hydrogen bonded, could be involved in base stacking or could be buried within the conformation of the molecule. A folded cloverleaf model such as proposed by the optical rotatory dispersion studies of Cantor *et al.* (1966) or the X-ray diffraction studies of Lake and Beeman (1968) would be more consistent with the pattern of reaction of yeast tRNA$^{Ala}$ with CMCMI.

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# Ribonucleic Acid Sulfurtransferase from *Bacillus subtilis* W168. Sulfuration with β-Mercaptopyruvate and Properties of the Enzyme System*

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ABSTRACT: A ribonucleic acid (RNA) sulfurtransferase activity has been isolated from *Bacillus subtilis* W168 and purified some 150-fold; the procedures for enzyme isolation and purification are described. In this system, both cysteine and β-mercaptopyruvate function as sulfur donors; however, β-mercaptopyruvate is far more efficient in this capacity and exhibits a $K_m$ 200–300 times smaller than the $K_m$ for cysteine. Studies on the properties of the partially purified enzyme show that sulfurtransferase activity has an absolute requirement for adenosine triphosphate (other nucleotides are inactive), a divalent metal ion, and transfer RNA as the sulfur acceptor. Although other cellular species of RNA and synthetic ribohomopolymers do not accept sulfur in this system, denatured deoxyribonucleic acid prepared from various sources appears to function as a good sulfur acceptor in the *in vitro* reaction.

Several reports from this and another laboratory have described the catalytic transfer of cysteine sulfur to tRNA by cell-free bacterial extracts (Hayward and Weiss, 1966; Hayward *et al.*, 1966; Lipsett and Peterkofsky, 1966; Lipsett *et al.*, 1967). This activity, designated as RNA sulfurtransferase, requires enzyme, magnesium ion, ATP, cysteine as the sulfur source, and tRNA as the sulfur acceptor. A survey of several different microorganisms indicated that crude extracts from *Bacillus subtilis* W168 contained a relatively high level of sulfurtransferase activity and that partial purification of this enzyme activity was possible.

While studying the effects of various sulfur-containing compounds on this enzyme activity *in vitro*, it was observed that the transfer of radioactive sulfur from [$^{35}$S]cysteine to tRNA was markedly suppressed in the presence of β-mercaptopyruvate. Kinetic data indicated that this thiol keto acid behaved like a competitive inhibitor of the sulfurtransferase reaction; further studies with [$^{35}$S]β-mercaptopyruvate indicated that it was a very efficient sulfur donor for tRNA sulfuration, more so than with cysteine. In this communication, we wish to describe the preparation of the sulfurtransferase enzyme from *B. subtilis* W168 and its properties and to examine the nature of the sulfuration activity when either cysteine or β-mercaptopyruvate serves as the sulfur precursor.

## Methods

*B. subtilis* W168 was purchased from Grain Processing Corp., Muscatine, Iowa, and stored at −85°. Extracts prepared from cells that had been frozen for 1 year or more still maintained most of their activity.

[$^{35}$S]Cystine (crystalline) was purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y., and converted into [$^{35}$S]cysteine by the following treatment. The labeled compound was dissolved in 0.02 M mercaptoethanol, adjusted

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