Studies on the Thionucleotides in Transfer Ribonucleic Acid. Addition of *N*-Ethylmaleimide and Formation of Mixed Disulfides with Thiol Compounds*

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ABSTRACT: *N*-Ethylmaleimide reacts selectively and quantitatively with 4-thio-2′(3′)-uridylate or with the 4-thiouracil residues in *Escherichia coli* transfer ribonucleic acid at pH values above neutrality. This reagent does not react with the 2-thiopyrimidine residues or other bases in transfer ribonucleic acid. Yeast transfer ribonucleic acid does not react, in line with previous observations on the lack of 4-thiouracil in transfer ribonucleic acid from this organism. The use of [^{14}C]*N*-ethylmaleimide in this reaction gives a value of 1 4-thiouracil/125 nucleotides in unfractionated *E. coli* transfer ribonucleic acid. This method could also be used for the selective labeling of 4-thiouracil residues in oligonucleotides. Saturation of transfer ribonucleic acid with *N*-ethylmaleimide causes only a 10–12% reduction in total amino acid acceptor capacity, whereas iodine oxidation of transfer ribonucleic acid results in a 30% reduction. Samples of transfer ribonucleic acid fully saturated with *N*-ethylmaleimide, are not protected against the oxidative inactivation of amino acid acceptor capacity, indicating that most of this inactivation is due to disulfide-bond formation involving 2-thiopyrimidines. Treatment of transfer ribonucleic acid in the presence of 2-mercaptoethanol or L-cysteine with an excess of aqueous iodine results in the formation of mixed disulfides (transfer ribonucleic acid-S-S-R).

Previous work has shown that *Escherichia coli* tRNA contains various sulfur-containing bases as normal minor constituents of the tRNA. These include a relatively large quantity of 4-thiouracil residues (1/100–150 nucleotides) (Lipsett, 1965), along with a smaller quantity of 2-thiocytosine and 5-methylaminomethyl-2-thiouracil residues (Carbon *et al.*, 1968). Recently, the presence of methyl 2-thiouridine-5-acetate in enzymatic digests of yeast tRNA was reported (Baczynskyj *et al.*, 1968). The occurrence of thio bases in *Bacillus subtilis* tRNA has been described by Goehler and Doi (1968).

The mild oxidation of *E. coli* tRNA with dilute aqueous iodine solutions leads to a rapid loss in the acceptor ability for various amino acids, a condition which is readily reversed by reduction of the oxidized tRNA with sodium thiosulfate or reduced glutathione (Carbon *et al.*, 1965). This loss of the ability of oxidized tRNA to react normally in the reactions catalyzed by the amino acid activating enzymes has been ascribed to the formation of intramolecular disulfide bonds in the tRNA, presumably resulting in a change in molecular conformation (Carbon *et al.*, 1965; Lipsett, 1967). However, in work reported recently (Lipsett, 1966; Lipsett and Doctor, 1967) a purified $tRNA^{Tyr}$, containing two 4-thiouracil residues per polynucleotide chain, readily formed an intramolecular disulfide upon iodine oxidation, without affecting the ability of the molecule to accept L-tyrosine.

We have now investigated the reactivity of the thio groups in *E. coli* tRNA toward the sulfhydryl reagent, NEM.[1] In addition, the mixed disulfides between the thio bases in tRNA and either 2-mercaptoethanol or L-cysteine have been prepared. The results of amino acid acceptor studies on these derivatives indicate that the blocking of the free 4-thiouracil groups in tRNA as the NEM derivative (Scheme I) does not necessarily lead to a loss in reactivity toward the activating enzymes. Moreover, samples in which essentially all of the 4-thiouracil residues have been combined with NEM still display the usual loss in amino acid acceptor ability when subjected to oxidation with iodine. Since the 2-thiopyrimidines do not react with NEM, it is likely that the oxidative inactivation of certain tRNA species is largely due to disulfide-bond formation involving 2-thiocytosine and/or 5-methylaminomethyl-2-thiouracil residues. The use of [^{14}C]NEM in this reaction offers a convenient method for the specific labeling of the 4-thiouracil bases in oligonucleotides or tRNA.

Materials and Methods

tRNA from *E. coli* was isolated by the method of Zubay (1962) or purchased from the General Biochemicals Co. Yeast tRNA was obtained from the same commercial source. Rabbit liver tRNA was isolated from frozen livers by the method of Brunngraber (1962). [^{14}C]-

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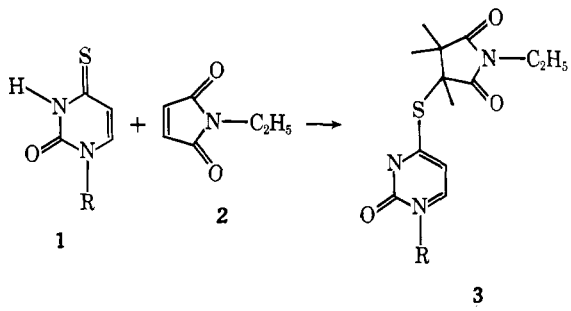
[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: NEM, *N*-ethylmaleimide; 4-thioUMP, 4-thio-2′(3′)-uridylate; NEM-tRNA, tRNA saturated at all the available 4-thiouracil sites with NEM.

Scheme I



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Amino acids, [^{14}C]NEM, and [^{35}S]cystine were obtained from Schwarz BioResearch, Inc. Sterile sodium[^{35}S] sulfate solution was a product of Abbott Laboratories. 4-ThioUMP was isolated from an alkaline hydrolysate of *E. coli* tRNA (Lipsett, 1965). Crude activating enzymes from *E. coli* were prepared as described previously (Carbon *et al.*, 1966). Venom phosphodiesterase (0.3 potency unit/mg) and bacterial alkaline phosphatase (35 units/mg) were products of Worthington Biochemical Corp.

Electrophoresis was carried out on either Whatman No. 3MM paper or on cellulose thin-layer sheets (Eastman Chromagram with fluorescent indicator), using either 0.4 M triethylammonium acetate buffer (pH 3.5) or 0.05 M ammonium carbonate buffer (pH 8.5). Paper electrophoresis was at 20 V/cm for 3 hr and thin-layer electrophoresis at 10 V/cm for 2–3 hr. Ultraviolet spectra were taken with a Cary Model 14 recording spectrophotometer.

Preparation of tRNA Hydrolysates. Alkaline hydrolysis of tRNA was carried out in 0.3 M NaOH at a concentration of 5 mg of RNA/ml at 37° for 18 hr. The hydrolysate was neutralized to pH 7 by the careful addition of Dowex 50 (H^+) before subjecting it to electrophoresis. Enzymatic hydrolysis of tRNA to the 5′-mononucleotides was carried out at 0.5–1.0 mg/ml of tRNA in ammonium bicarbonate buffer (pH 8.9) in the presence of 150 μg/ml of venom phosphodiesterase at 37° for 18 hr. The mixture was freed of ammonium bicarbonate by repeated evaporation to dryness *in vacuo*. Hydrolysis of tRNA to the nucleosides was carried out in a similar manner with the addition of 20 μg/ml of bacterial alkaline phosphatase to the above reaction mixture.

Treatment of tRNA or 4-ThioUMP with NEM. 4-ThioUMP (0.042 mM) was treated with a 3.3 mM solution of NEM in 0.05 M Tris-Cl buffer (pH 7.8) at 37°. The reaction was followed by observation of the ultraviolet spectra at various time intervals using a 3.3 mM solution of NEM in the same buffer as a blank (see Figure 1A). The loss of the 335-mμ absorption peak in *E. coli* tRNA was followed in a similar reaction mixture containing 29 A_{260} units/ml of tRNA and 5 mM NEM. At this concentration the tRNA has an absorbance at 330 mμ of 0.45 (read against the NEM blank).

The effect of pH on the rate of binding of NEM to tRNA was studied in reaction mixtures containing 0.5 mg/ml of tRNA in either 0.05 M sodium phosphate buffer (pH 6.0) or 0.05 M Tris-Cl buffers of pH 7.6 or 8.4. The solutions contained 0.4 μmole/ml of [^{14}C]NEM (approximately 4.5×10^6 cpm per μmole). Reactions were carried out at 37°. At the specified time intervals, aliquot samples (100 μl) were withdrawn, added to 5 ml of cold 1 M HCl, and filtered with suction through glass fiber disks (Whatman GF/C, 24 mm). The disks were washed in turn with five portions of 1 M HCl (5 ml each) and finally with ethanol. After drying under heat lamps, the radioactivity remaining on the disks was counted by the scintillation method. Control samples taken at zero time or incubated in the absence of RNA retained no more than 15–20 cpm with this method. Because of the rapid decomposition of NEM in aqueous solutions above pH 7, this method did not saturate the available reactive sites of the tRNA.

tRNA was saturated with NEM residues by the following method. A solution of the appropriate tRNA (1 mg/ml) in 0.05 M Tris-Cl buffer (pH 8.4) containing 4.0 μmoles/ml of [^{14}C]NEM (1.87×10^5 cpm per μmole) was kept at 37°. At intervals of 2 hr, samples were removed for measurement of radioactivity bound to the tRNA as described above. An additional 4.0 μmoles/ml of [^{14}C]NEM and Tris-Cl (pH 8.4) were added after each 2-hr interval to replace the NEM lost by hydrolysis to *N*-ethylmaleamic acid (Gregory, 1955). Isolation of [^{14}C]NEM–tRNA was carried out by adding one-tenth volume of 20% potassium acetate and two volumes of cold ethanol to the reaction mixture. The precipitate was isolated by centrifugation and freed of unreacted NEM and hydrolysis products by repeated precipitations from 0.2 M potassium acetate buffer (pH 5) with two volumes of ethanol. The pellet was washed with ethanol, then with ether, and dried *in vacuo*. The tRNA was then dissolved in a small volume of water and stored frozen at −20°.

Hydrolysates of ^{35}S-labeled tRNA were treated with NEM using the multiple addition method described above to fully saturate the available reactive SH groups, except that a 0.1 M ammonium bicarbonate buffer (pH 8.3) was used, and the [^{35}S]nucleotides or nucleosides were present at a concentration of 23 A_{260} units/ml. After 8 hr at 37°, with additions of NEM every 2 hr (see above), the mixtures were evaporated to dryness repeatedly *in vacuo* to remove ammonium bicarbonate. The residue was taken up in water, acidified to pH 3–4 with acetic acid, and extracted thoroughly with 1-butanol to remove NEM and *N*-ethylmaleamic acid. The resulting solution was evaporated and subjected to electrophoresis on cellulose thin-layer plates.

Iodine Oxidation of tRNA in the Presence of Mercapto Compounds. Oxidation of tRNA with aqueous I_2–KI solutions was carried out as described previously (Carbon *et al.*, 1965). Reaction mixtures for the oxidation in the presence of 2-mercaptoethanol or L-cysteine consisted of *E. coli* tRNA (2.5 mg/ml) in 0.01 M Tris-Cl buffer (pH 7.0) containing either 2.5 mM 2-mercaptoethanol or 2.5 mM L-cysteine. After mixing, the solution was made 2.5–3.0 mM in iodine by adding the appropriate amount of 10 mM I_2 in 5% KI solution. The iodine solution was always added last, and the final solution was checked to make certain that an excess of iodine was present (by the brown iodine color or by use of a starch

indicator). After incubation at 0° for 30 min, the tRNA was isolated by the usual ethanol precipitation method and washed repeatedly to free it of unreacted iodine. The ultraviolet spectra of these samples were routinely checked to make certain that the 4-thiouracil absorption peak at 330 mμ had disappeared. The 330-mμ peak could be restored in these samples by incubation at room temperature with 0.1 M sodium thiosulfate for 15–20 min.

Amino Acid Acceptance of tRNA Samples. The various tRNA samples were assayed for amino acid acceptor ability in reaction mixtures (250-μl total volume) containing 25 μmoles of Tris-Cl (pH 7.0), 1.25 μmoles of $MgCl_2$, 0.5 μmole of ATP, 0.5–1.0 A_{260} unit of tRNA, the appropriate [^{14}C]amino acid (5–10 mμmoles; 20–50 μCi/μmole), and 10 μl of a dialyzed protamine-treated 100,000*g* supernatent from *E. coli* (Carbon *et al.*, 1966). After 30 min at 37°, the tRNA was precipitated by the addition of 5 ml of cold 1 M HCl. The reaction mixtures were filtered through 24-mm glass fiber disks (Whatman GF/C) in a special stainless steel holder, washed with five portions (5 ml each) of cold 1 M HCl, followed by 10 ml of ethanol. The disks were dried under heat lamps and counted by the scintillation method. Care was taken to ensure that samples were loaded to completion by running the appropriate time curves in preliminary studies. Acceptance measurements were routinely carried out at two different levels of tRNA to ensure that the loading response was directly proportional to tRNA concentration. Sulfhydryl reagents, such as 2-mercaptoethanol, were not added to these assays, to avoid any possible reduction of the disulfide bonds in oxidized tRNA.

Results

Reaction of NEM with 4-Thiouridylate and tRNA. Previous work has shown that the oxidation of 4-thiouridine to the symmetrical disulfide leads to a loss in the intensity of the ultraviolet absorption peak near 330 mμ, with a shift in the absorption maximum to approximately 311 mμ (Fox *et al.*, 1959; Lipsett, 1966). The loss of the 335-mμ absorption peak can be used as a measure of the extent of disulfide formation in intact *E. coli* tRNA, as this peak is readily discernible in the absorption spectra of native tRNA, but disappears upon treatment of the tRNA with aqueous iodine solutions (Lipsett, 1966; Lipsett and Doctor, 1967). Subsequent reduction of disulfide tRNA with aqueous sodium thiosulfate causes a gradual reappearance of the 335-mμ absorption maximum. This spectral change is presumably a result of a shift of structure of the 1-substituted 4-thiouracil from the thione form (**1**) to the substituted thiol (or thioether) form (as in **3**) (Scheme I), and should therefore be usable to detect the reaction of the 4-thiouracil residues with reagents such as NEM (**2**) or acrylonitrile, which alkylate the sulfur atom.

NEM is known to react rapidly and quantitatively with aliphatic mercapto groups (Friedman *et al.*, 1949), and has been widely used to block reactive sulfhydryl groups in various proteins (Cecil, 1963). The availability of [^{14}C]NEM has made it possible to selectively label

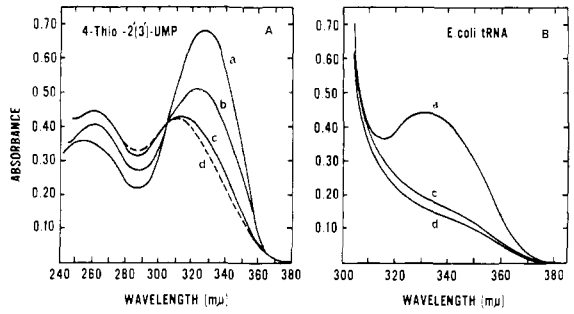


FIGURE 1: Effect of incubation with NEM at pH 7.8 on the ultraviolet absorption spectra of (A) 4-thio-2′(3′)-uridylic acid and (B) *E. coli* tRNA. The reactions were carried out as described in Materials and Methods. The spectra were determined at zero time (a), after 1 hr at 37° (b), and after 3 hr at 37° (c). After 3-hr incubation with NEM, the reaction mixtures were brought to 0.1 M in sodium thiosulfate, incubated an additional hour at 37°, and the spectra were determined (d).

such molecules at the reactive cysteine sites (Kielley and Barnett, 1961). The use of this reagent with *E. coli* tRNA is of some interest, since it offers a way to determine the effect of alkylation of a single minor base (4-thiouracil) on the various enzymatic reactions of tRNA. Furthermore, the use of [^{14}C]NEM should enable one to selectively label those chains or oligonucleotides containing 4-thiouracil residues.

Samples of 4-thioUMP were isolated from alkaline hydrolysates of *E. coli* tRNA by chromatography over DEAE-cellulose (Lipsett, 1965). The characteristic ultraviolet absorption spectrum of this compound was not altered by treatment with aqueous solutions of NEM (3.3 mM) at pH values below 7 at room temperature for several hours. However, the use of buffers of slightly alkaline pH at 37° resulted in a moderately rapid addition of NEM to the 4-thiouridylate, as evidenced by an irreversible loss of the absorption peak at 328 mμ. For example, at pH 7.8 in 0.05 M Tris-Cl buffer containing 3.3 mM NEM and 0.042 mM 4-thioUMP, the reaction appeared to proceed to completion in 3 hr at 37° (see Figure 1A). Because of the high ultraviolet absorbance of NEM solutions of this concentration, it is necessary to run the spectra *vs.* reagent blanks containing an equivalent concentration of the reagent. Although the shift in the ultraviolet spectra observed upon NEM treatment of 4-thioUMP is similar to that observed during disulfide formation, it is *not* reversed by aqueous sodium thiosulfate (Figure 1A, curve d), conditions which readily restore the 328-mμ peak in oxidized samples. A sample of 2-thiouridine[2] (Shaw *et al.*, 1958) would not react with NEM under the same reaction conditions. Similarly, Ofengand (1967) has reported that 4-thiouridine reacts readily with acrylonitrile, while 2-thiouridine is inert to this reagent.

A similar treatment of *E. coli* tRNA (1.4 mg/ml) with NEM at pH 7.8 resulted in a loss of the small absorp-

[2] We are grateful to Dr. Mitsuji Sano, Daiichi Seikyau Co., Tokyo, Japan, and Dr. G. Shaw, Bradford Institute of Technology, Bradford, England, for samples of synthetic 2-thiouridine.

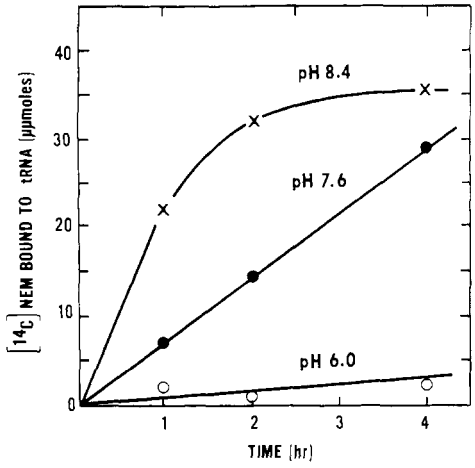


FIGURE 2: Effect of pH on the addition of [^{14}C]NEM to *E. coli* tRNA at 37°. The reactions were carried out as described in Materials and Methods.

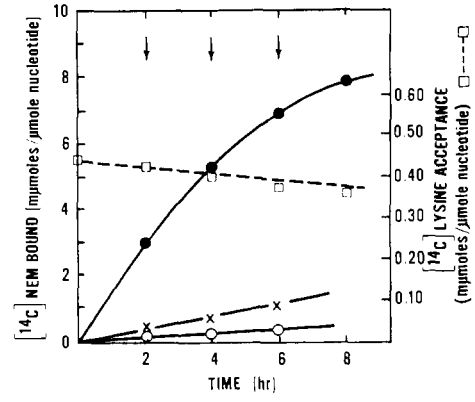


FIGURE 3: Saturation of tRNA from various sources with [^{14}C]NEM at pH 8.4, demonstrating the lack of any effect upon L-[^{14}C]lysine-acceptor capacity of *E. coli* tRNA. The reactions were carried out as described in Materials and Methods, with fresh additions of [^{14}C]NEM at the times indicated by the arrows. The tRNA was from *E. coli* (●), rabbit liver (×), and yeast (○). Samples of *E. coli* tRNA were isolated at the indicated times from a similar reaction mixture using unlabeled NEM, and assayed for the capacity to accept L-[^{14}C]lysine (see Materials and Methods), as shown by the dotted line (□).

tion peak at 335 mμ, presumably due to reaction of NEM with the 4-thiouracil residues in the RNA (Figure 1B). This reaction appeared to be complete after 4 hr at 37°, although it is not possible to determine by this spectral method if quantitative addition of NEM to the 4-thiouracil residues has occurred. Spectral studies of this type, however, indicate that the level of unreacted 4-thiouracil residues in NEM-treated tRNA must be quite small, and probably amounts to less than 5% of the total. Samples of NEM-treated tRNA did not recover the 335-mμ absorption peak when treated with sodium thiosulfate solutions at 0°, conditions which readily reduced disulfide tRNA (Figure 1B, curve c).

In order to obtain additional evidence for the reaction of NEM with intact tRNA and to obtain an accurate measure of the amount bound to the polynucleotide chains, reactions were carried out in the presence of [^{14}C]NEM. Preliminary experiments (Figure 2) indicated that, although little or no [^{14}C]NEM became bound to *E. coli* tRNA at pH 6.0, significant quantities were bound at pH values above 7, in agreement with the spectral studies described above. As shown in Figure 2, incubation of *E. coli* tRNA with [^{14}C]NEM at pH 7.6 results in a progressive conversion of the radioactivity into an acid-insoluble form which cannot be removed by repeated washings of the tRNA with 1 N HCl or ethanol. The reaction at pH 8.4 is significantly faster, although it soon stops due to the rapid destruction of unreacted [^{14}C]NEM at alkaline pH values (Gregory, 1955). The addition of fresh reagent after 2 hr causes a new burst of incorporation of [^{14}C]NEM into the tRNA (see below). The reactive species in the addition of 4-thiouracil to the double bond of NEM is apparently the sulfide anion, which has a pK of 8.2 (Lipsett, 1965).

At pH values which are alkaline enough to give a reasonably rapid addition of NEM to 4-thiouracil, the base-catalyzed hydrolysis of NEM to form *N*-ethylmaleamic acid becomes a serious competing reaction. We therefore turned to a procedure in which the reaction was carried out at pH 8.4, and additional [^{14}C]NEM was added to the reaction mixture at 2-hr intervals until the reactive sites on the tRNA were completely saturated. The results of an experiment of this type, using tRNA samples isolated from *E. coli*, yeast, and rabbit liver, are shown in Figure 3. Addition of [^{14}C]NEM to *E. coli* tRNA appears to cease when approximately 8 mμmoles of NEM/μmole of nucleotide have become bound. This is equivalent to 1 molecule of NEM bound/125 nucleotide bases in the tRNA, a figure in excellent agreement with the amount of 4-thiouracil in *E. coli* tRNA (approximately 1 in 140 bases; see Lipsett, 1965). Note that yeast tRNA, which is free of 4-thiouracil residues, does not react with [^{14}C]NEM, indicating that the reaction is quite specific for 4-thiouracil residues.

The relatively small addition of [^{14}C]NEM to rabbit liver tRNA (Figure 3) is of unknown significance. Iodine oxidation studies carried out on tRNA isolated from the liver of various species have indicated that certain amino acid acceptors, in particular, lysine and glutamic acid, are partially inactivated by the oxidation and reactivated by subsequent reduction (Carbon *et al.*, 1965; Carbon and Hung, 1966). Although this can be taken as presumptive evidence for the presence of thio bases in certain liver tRNA species, no direct evidence has been obtained. Attempts to label rat liver tRNA *in vivo* by the injection of [^{35}S]cystine into animals with actively regenerating livers have been unsuccessful (J. Carbon and H. David, unpublished data).

Additional evidence for the reaction of NEM with 4-thiouracil derivatives was obtained by making use of electrophoresis on cellulose thin-layer plates to detect substitution of the rather acidic SH group. As shown in Figure 4A, the [^{35}S]4-thioUMP ion has an electrophoretic mobility approximately 1.20 times greater than 5'-UMP at pH 8.5, due to the increased negative charge contributed by the sulfur atom at this pH. After treatment with NEM, however, the resulting product moves slightly slower than 5'-UMP (R_{pU} 0.84) at pH 8.5 as

would be predicted. Similarly, the electrophoretic mobility of 4-thiouridine at pH 8.5 (R_{Urd} 3.1) is reduced to R_{Urd} 0.49 after NEM addition (Figure 4B). The use of ^{35}S to localize these compounds on the plates eliminates the possibility that hydrolytic loss of the sulfur has occurred, a reaction to which 4-thiouracil is especially prone (Lipsett, 1965).

Several attempts were made to isolate the NEM addition product of 4-thiouridylate from hydrolysates of NEM-treated tRNA. These experiments were carried out both with [^{14}C]NEM plus unlabeled tRNA and with unlabeled NEM plus [^{35}S]tRNA. Hydrolysates were prepared using alkaline cleavage of the phosphodiester bonds (0.3 M KOH at 37° for 18 hr) and enzymatically with snake venom phosphodiesterase (Nihei and Cantoni, 1963). In all cases, however, the hydrolysis conditions were sufficiently drastic to destroy the labile NEM addition product. The experiments using [^{35}S]tRNA indicated that a large percentage (40–50%) of the sulfur was lost from the 4-thiouracil residues during the hydrolysis of NEM–tRNA, with the remainder appearing as free 4-thiouridylate. The exact pathway of the decomposition of NEM–4-thiouridylate has not been ascertained; however a simple base-catalyzed elimination to re-form free NEM and 4-thiouridylate would appear to be involved, at least in part. The acrylonitrile adduct of 4-thiouridine is apparently also quite labile in alkaline solution (Ofengand, 1967). Although [^{14}C]NEM could conceivably be used to detect 4-thiouracil residues in oligonucleotide fragments of tRNA, it is probable that the labeled NEM would not be retained during subsequent hydrolytic procedures.

Amino Acid Acceptance Studies. It was clearly of interest to determine the effect of the NEM alkylation on the amino acid acceptor properties of the tRNA. Although 4-thiouracil apparently occurs in several of the amino acid specific tRNAs in *E. coli*, the function of this minor base has not been elucidated. If this base plays an important role in the interaction occurring between various tRNAs and the activating enzymes, then addition of the bulky NEM group should interfere markedly with the enzymatic loading of the tRNA.

In preliminary experiments, samples of tRNA substituted with various amounts of NEM were assayed for the ability to accept [^{14}C]lysine (Figure 3). Enzymatic charging of this amino acid was investigated because of the marked susceptibility of $tRNA^{Lys}$ to inactivation by iodine oxidation (Carbon *et al.*, 1965), presumably an indication of the presence of important thio groups in this tRNA molecule. Surprisingly, however, $tRNA^{Lys}$ appeared to be quite resistant to inactivation by NEM, even under conditions where the tRNA was completely saturated with NEM residues (Figure 3). As shown in Table I, although the capacity of NEM–tRNA for the acceptance of [^{14}C]lysine was at least 92% that of control samples, iodine oxidation results in almost complete inactivation of the $tRNA^{Lys}$. Furthermore, prior treatment of the tRNA with NEM does not protect the samples against subsequent iodine oxidation. Apparently the thio groups in $tRNA^{Lys}$ do not react with NEM, a possible indication that this tRNA contains 2-thiopyrimidines (unreactive toward NEM). Similarly,

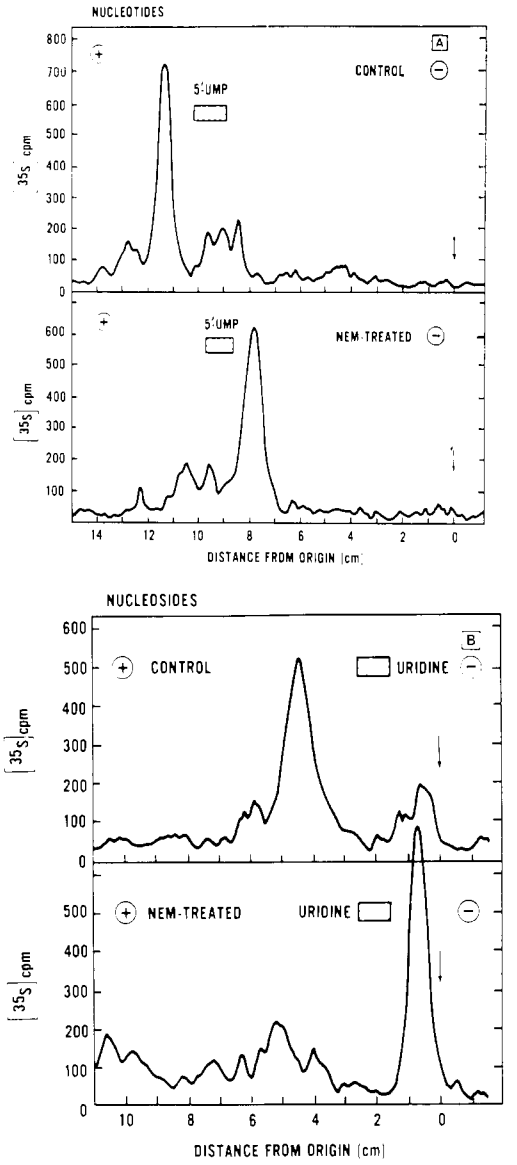


FIGURE 4: Effect of NEM addition on the electrophoretic mobility at pH 8.5 of (A) [^{35}S]4-thioUMP and (B) [^{35}S]4-thiouridine. Hydrolysates of [^{35}S]tRNA were prepared and treated with NEM as described in Materials and Methods. Electrophoresis was carried out on cellulose thin-layer plates at 10 V/cm for 2 hr. After drying, the plates were scanned for radioactivity on a Packard strip scanner.

$tRNA^{Glu}$ is not affected by the NEM treatment, but is completely inactivated by oxidation with iodine. Again, saturation with NEM does not protect this tRNA against subsequent oxidative inactivation (Table I).

The use of a mixture of 19 [^{14}C]amino acids in the amino acid acceptor assays revealed that NEM-saturated tRNA accepted about 10–12% less of the labeled mixture than did untreated samples. In contrast, iodine-oxidized tRNA was inactivated to a much greater extent (27% loss in acceptor ability). Presaturation with NEM again did not protect the tRNA from subsequent oxidative inactivation (Table I). If one makes the logical assumption that the NEM-substituted 4-thiouracil residues in tRNA would not react with iodine to form disulfides, then we must conclude that most of the inac-

TABLE I: Effect of NEM Treatment on Normal and Iodine-Oxidized *E. coli* tRNA.

[^{14}C]Amino Acid	Control	[^{14}C]Amino Acid Acceptance (mμmoles/10 A_{260} units)		
		NEM Treated, 8 hr	I_2 Treated	NEM Treated, Then I_2 Treated[a]
L-Lysine	0.39	0.36	0.04	0.04
L-Glutamic acid	0.13	0.14	<0.01	<0.01
L-Serine	0.49	0.30	0.11	0.11
L-Phenylalanine	0.47	0.29	0.11	0.13
L-Proline	0.41	0.26	0.26	0.26
L-Amino acid mixture[b]	5.80	5.09	4.24	4.11

[a] The tRNA was first saturated with NEM as described in Materials and Methods, isolated, and then oxidized with aqueous iodine. [b] The [^{14}C]amino acid mixture consisted of the usual 19 amino acids (without glutamine or asparagine) at concentrations ranging from 0.10 to 0.13 μmole per ml each. The specific activities of these amino acids ranged between 20 and 40 mCi/mmole.

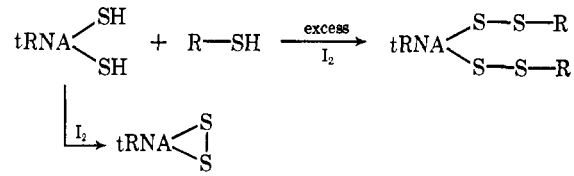
tivation occurring upon iodine treatment of tRNA is due to disulfide formation involving the 2-thiopyrimidine residues.

Other amino acid specific tRNAs, known to be sensitive to oxidative inactivation, were tested for amino acid acceptor ability after saturation with NEM. As shown in Table I, the acceptors for L-serine and L-phenylalanine were partially inactivated by the NEM treatment, but not to the same extent as after iodine oxidation. Note that the NEM and iodine effects are *not* additive, indicating that, in these cases, a portion of the oxidative inactivation is due to disulfide bonds involving 4-thiouracil residues. We have observed one case, that of $tRNA^{Pro}$, in which the extent of inactivation observed in NEM-saturated tRNA is identical with that shown by oxidized samples (see Table I), and, again, the effects are not additive.

Samples of NEM-saturated tRNA were checked after enzymatic loading with amino acids to be certain that the NEM was not released from the tRNA during the course of the aminoacylation reaction. Samples of [^{14}C]-NEM–tRNA did not lose any acid-precipitable radioactivity during the enzymatic reaction. Furthermore, NEM–tRNA, when reisolated from such reaction mixtures, did not show the characteristic absorption peak at 335 mμ due to free 4-thiouracil residues.

tRNA Containing Mixed Disulfides with 2-Mercaptoethanol or L-*Cysteine.* The iodine oxidation of tRNA containing thio bases in the presence of limited quantities of other SH compounds could result in the formation of mixed disulfides, rather than the usual intramolecular disulfide bonds (Lipsett and Doctor, 1967).

Solutions of *E. coli* tRNA in Tris buffer at pH 7.0 in the presence of 2.5 mM 2-mercaptoethanol or L-cysteine were oxidized with an excess of aqueous iodine solution. Care was taken to ensure that a large excess of iodine was present as evidenced by the typical brown iodine color and by the starch test. Samples of tRNA isolated from such reaction mixtures were completely free of the



usual 335-mμ absorption peak, indicating that complete oxidation of the 4-thiouracil residues had occurred (Figure 5). In contrast to the usual iodine-oxidized preparations of tRNA, however, the samples oxidized in the presence of 2-mercaptoethanol or L-cysteine were almost completely normal with regard to their ability to accept L-[^{14}C]lysine or L-[^{14}C]glutamic acid (Table II). It should be pointed out that treatment of tRNA with 2-mercaptoethanol or L-cysteine solutions *after* the iodine oxidation has already been carried out is not generally effective in reversing the inactivation of $tRNA^{Lys}$, although 0.1 M sodium thiosulfate or reduced glutathione readily bring about a reductive reactivation (Carbon *et al.*, 1965).

An investigation of the ultraviolet absorption spectra of various samples of iodine-oxidized tRNA was carried out both before and after exposure to the normal enzymatic loading reaction mixture in order to determine if reduction of the disulfide bonds in tRNA occurs during the course of the enzymatic aminoacylation. As shown in Figure 5, iodine-oxidized tRNA did not recover the 335-mμ absorption peak (curve c) when exposed to the loading reaction mixture plus enzyme at 37° for 10 min (see Materials and Methods section for a description of the usual aminoacylation reaction mixture). Apparently, intramolecular disulfide bonds involving 4-thiouracil residues are not affected by the presence of the *E. coli* 100,000*g* supernatant used as a source of activating enzymes in these experiments. In contrast to these results, however, the tRNA samples oxidized in the presence of 2-mercaptoethanol or cysteine regained

TABLE II: Amino Acid Acceptance of tRNA Samples Oxidized with Aqueous Iodine in the Presence of Mercapto Compounds.

tRNA	[^{14}C]Amino Acid Acceptance (mμmoles/10 A_{260} units)	
	L-Lysine	L-Glutamic Acid
Control	0.43	0.28
I_2 oxidized, no R-SH	0.11	0.02
I_2 oxidized in 2.5 mM 2-mercaptoethanol[a]	0.42	0.24
I_2 oxidized in 2.5 mM L-cysteine[a]	0.33	0.25

[a] See Materials and Methods for a description of the reaction conditions. The tRNA samples were routinely checked for loss of the absorption peaks at 335 mμ before assaying for [^{14}C]amino acid acceptor capacity.

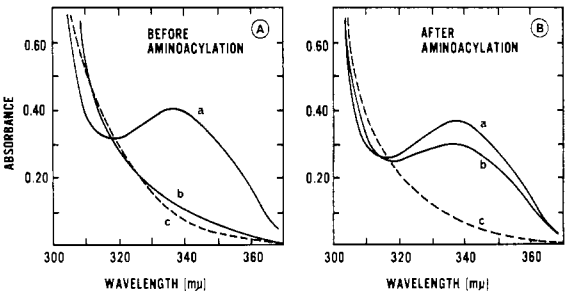


FIGURE 5: Ultraviolet absorption spectra above 300 mμ of normal and various oxidized samples of *E. coli* tRNA, shown before (A) and after (B) exposure to the usual enzymatic aminoacylation reaction. tRNA samples were untreated (curve a), oxidized with 3.0 mM iodine in the presence of 2.5 mM 2-mercaptoethanol (curve b), or oxidized with 3.0 mM iodine (curve c), as described in Materials and Methods. The tRNA was incubated in the usual aminoacylation reaction mixture at 37° for 10 min, precipitated by adding two volumes of ethanol, and recovered by centrifugation. The spectra were determined on aqueous solutions with an absorbance at 260 mμ of 35–40.

a large percentage of the 335-mμ absorption peak during the course of enzymatic loading (Figure 5, curve b).

These tRNA samples were perfectly stable in the reaction medium containing all of the components except the 100,000*g* supernatant fraction. Apparently, a rapid transfer of the bound mercapto compounds to protein SH groups occurs, thus freeing the 4-thiouracil residues in the tRNA. We cannot be certain, however, that a similar reduction of mixed disulfides involving the 2-thiopyrimidine residues occurs during enzymatic aminoacylation of tRNA samples previously oxidized in the presence of mercapto compounds. It is possible that these samples give normal amino acid acceptance values simply because of a rapid loss of the bound 2-mercaptoethanol or L-cysteine residues during the incubation at 37° in the presence of protein.

Discussion

The availability of a reagent capable of reacting selectively with only one of the many minor bases occurring in tRNA clearly is of some utility in studies of the relationship of tRNA structure to biological function. Previous work on the effect of chemical modifications of tRNA on its reactivity in various reactions has often suffered from the disadvantage that a relatively nonspecific alteration of bases had occurred, and, as a result, interpretations were somewhat ambiguous (for example, see the review by Miura, 1967). The use of a reagent, such as NEM, which apparently reacts only with 4-thiouracil in tRNA, could eventually give valuable information on the role of this minor base in tRNA chemistry.

Previous estimates of the amount of 4-thiouracil residues in unfractionated *E. coli* tRNA were determined by actual isolation of 4-thioUMP from tRNA hydrolysates. This procedure gave a value of 1 4-thiouracil residue/140 nucleotides (Lipsett, 1965). Our estimate of 1 4-thiouracil/125 nucleotides, as determined by saturation of the tRNA with [^{14}C]NEM, is in good agreement with this figure. Results of iodine oxidation studies, and isolation of the disulfide of 4-thioUMP from hydrolysates of such oxidized tRNA, suggest that the RNA chains containing 4-thiouracil contain at least two residues of this base (Lipsett, 1967). (Dimers resulting from the formation of intermolecular disulfide bonds have not been found in iodine-oxidized tRNA (J. Carbon, unpublished data)). Assuming an average chain length of 80 nucleotides/tRNA chain and the presence of at least 2 residues of 4-thiouracil in those chains containing this base, one can calculate that 32% of the tRNA chains could contain 4-thiouracil, although this figure might be appreciably lower if several tRNAs carried a high concentration of this thio base.

The effect of saturation of the tRNA with NEM on enzymatic aminoacylation, using a mixture of 19 [^{14}C]-amino acids, does not appear to be very large (10–12% reduction in total acceptor ability of the tRNA). Although addition of NEM to the tRNA apparently does inactivate certain tRNA species (see Table I), the inactivation is not nearly as great as is seen after iodine oxidation. Furthermore, tRNA samples that are fully saturated with NEM and whose 4-thiouracil residues are thus unavailable for disulfide-bond formation still are inactivated by iodine oxidation, and to exactly the same extent as is normal tRNA. We take this to mean that much of the loss in amino acid acceptor capacity shown by oxidized tRNA is due to disulfide-bond formation involving the thio bases unreactive to NEM, such as 2-thiocytosine and 2-thio-5-methylaminomethyluracil. It should be pointed out in this regard that a purified $tRNA^{Tyr}$, containing two residues of 4-thiouracil, is not inactivated when converted into the intramolecular disulfide form by iodine oxidation (Lipsett, 1966; Lipsett and Doctor, 1967).

The specificity of the reaction of [^{14}C]NEM with the 4-thiouracil residues is apparent from the complete lack

of reactivity with yeast tRNA, known to be free of 4-thiouracil (Figure 3). We can therefore be quite certain that NEM does not react with any of the four common nucleotides, or with minor bases such as pseudouracil, dihydrouracil, or the methylated bases. The small incorporation of [^{14}C]NEM observed using tRNA from rabbit liver is still of unknown significance.

It now appears that the iodine oxidation of *E. coli* tRNA in the presence of various low molecular weight SH compounds results in the formation of mixed disulfides with the thio bases in the tRNA. It seems quite likely that the use of isotopically labeled SH compounds (such as [^{35}S]- or [^{14}C]cysteine) in this reaction could serve as a method for the sensitive detection of thio groups in RNA samples from other sources. This method, in combination with the use of [^{14}C]NEM, should permit the microdetection of thionucleotides in the oligonucleotides obtained during the course of tRNA sequence determination studies.

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