Abstract.—The circular dichroism arising from 4-thiouridine residues in E. coli transfer RNA has been studied under conditions in which the secondary and tertiary structure of the macromolecule is either intact or totally disrupted. Studies on both unfractonated tRNA and on highly purified species of methionine-, valine-, and lysine-specific tRNA suggest that the circular dichroism of the 4-thiouridine residue is highly sensitive to its local environment in the macromolecule and reveal interesting differences between these purified species. A new chromophore, resembling 4-thiouridine in some respects but showing distinctive chemical and optical properties, has been detected. The implications of these results on model-building studies of tRNA are discussed.

Transfer RNA (tRNA) from E. coli has been shown to contain 4-thiouracil among the minor bases. The wavelength of maximum absorption of this residue in the high polymer lies at 336 mλ, just beyond the absorption envelope of the major and most minor constituents. This property permits one to study the absorption characteristics and, as shown below, the circular dichroic properties of this residue without undue contribution from the remainder of the macromolecule. Thus, the 4-thiouridine (4TU) residue may be used in the manner of a "reporter" molecule.⁵ There has appeared recently a report on the nuclear magnetic resonance and optical rotatory dispersion properties of synthetic poly-nucleotides containing 4TU.⁶ We wish here to present the results of a study on tRNA from E. coli B including in addition to unfractionated tRNA, purified tRNA₃¹², tRNA₃¹¹, tRNA₃¹⁰, and tRNA₃⁹ and to report evidence on an as yet unidentified chromophore which absorbs in the same region as 4TU.

Methods.—The tRNA was purchased from Schwarz BioResearch and the purified species were obtained as described by Schofield.⁷ The isolation techniques involved chromatography on DEAE-Sephadex⁸ or benzoylated DEAE-cellulose⁹ followed by rechromatography on reverse-phase⁸ or hydroxyapatite columns.⁹ The maximum amino acid acceptance level for tRNA₃¹² and tRNA₃¹¹ was at least 1400 μmoles/ODU.⁵ The purity of all these species was also checked by two-dimensional thin-layer chromatography of T1 RNase digests and revealed no gross contamination by species having a markedly different base sequence.⁶ The absorption spectra were obtained at room temperature with a Cary model 11 spectrophotometer and the CD spectra with a Cary model 6001 circular dichroism attachment for the model 60 spectropolarimeter. Unless otherwise stated, the solvent was 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0 (SSC). Dimethyl sulfoxide (Me₂SO), spectrophotometric grade, was purchased from Fisher Scientific Company and used without further purification. Iodine oxidation was carried out as described by Carbon et al.¹⁰

For the recording of CD spectra the concentration of tRNA was adjusted to yield an absorbance of 0.5 to 1.5 in the region of interest. No dependence of ellipticity on absorbance was noted in this range. In some cases where treatment caused a marked reduction in the A₃₂₅₀, the A₉₅₀ was adjusted to approximately 40 for spectra in the region...
310–400 m\(\mu\). Absorption and CD spectra of the solvent and the solvent plus sample were obtained in the same cuvette and the data reduced by point-by-point subtraction. The residue concentration was calculated from \(A_{336 \, m\mu}\) in SSC using \(\epsilon_{336} = 7.43 \times 10^4\), which was obtained by independent measurements of total phosphorous\(^{11}\) and absorbance for the mixed tRNA. CD curves are expressed as \(\Delta \epsilon (\epsilon_L - \epsilon_R)\) per residue.

Results and Discussion.—As seen in Figure 1, unfractionated tRNA shows a well-defined absorption maximum at \(\lambda 336 \, m\mu\) as well as a positive CD band of low intensity but of essentially the same shape as the absorbance band. This latter band is distorted by the presence of an overlapping negative band which has an apparent extremum near 295 m\(\mu\).\(^{11}\) If the \(\Delta \epsilon\) calculation is based on the absorbance at 336 m\(\mu\) (\(\epsilon_{336} = 15,000\) estimated from the hypochromism data below and the value for 4-thiouridylic acid\(^{11}\)) then the band is in fact moderately strong (\(\Delta \epsilon_{\text{max}} = +3.6\)). On oxidation with triiodide the absorption band centered at 336 m\(\mu\) is replaced by an extended weak “toe” to the main absorption band. The CD band is altered in shape in such a way as to suggest that the original band centered at 336 m\(\mu\) has been replaced by a positive band at shorter wavelengths, which is in turn obscured by the overlapping negative band at 295 m\(\mu\). This interpretation is supported by the CD spectrum of the oxidized RNA in 80 per cent Me\(\text{SO}\) (Fig. 1) in which the solvent 295 band is abolished and a positive shoulder is revealed with an inflection at approximately 320 m\(\mu\).

The effect of Me\(\text{SO}\) on the absorption and CD bands is illustrated in Figures 1 and 2. Considering first Figure 2, one observes that, as has been found by others,\(^{14}-\text{16}\) Me\(\text{SO}\) leads to a loss of the secondary and tertiary structure as evidenced by a hyperchromicity of the 336 m\(\mu\) absorption band and a concomitant decrease in the CD at the same wavelength. The hypochromicity and CD are largely, but not completely, restored upon dilution of the 80 per cent Me\(\text{SO}\)
solution back to 40 per cent Me₂SO by addition of the aqueous solvent. Also illustrated in Figure 2 is the extent of the change in the CD measured at the wavelength of the positive band centered (in SSC) near 265 μm. It should be noted in this figure that the CD band centered at 265 μm remains positive over this range of Me₂SO concentration while that at 336 μm changes from positive to negative with increasing Me₂SO concentration. In Figure 1 this change in the CD spectrum in passing from 0 to 80 per cent Me₂SO is illustrated. In addition to inversion of the sign of the CD curve, one notes that the negative band which produced a sharp downturning of the other CD spectra at the short wavelength end has apparently vanished and the curve passes through 0 at approximately 313 μm. There is a blue shift in both the absorption and CD maxima. Similar changes in the absorption spectra have been reported for heat-denatured tRNA.¹,² Essentially the same effect on the CD spectrum was obtained with 80 per cent (v/v) dimethyl formamide and 80 per cent (v/v) ethylene glycol. The total hyperchromicity at λ₃₃₅ μm of the tRNA in these solvents relative to SSC was 1.35 and 1.39, respectively. We shall consider the interpretation of the change in sign of the CD spectra in these solvents later.

Lipsett³ has shown that the 336 μm band demonstrates a hyperchromic profile on heating which differs somewhat from that for the 260 μm absorption band. In this study we find (Fig. 2) a close correspondence between the hyperchromicity at 336 μm induced by Me₂SO and the reduction in dichroism both at λₘₐₓ in the 265 μm region and the 336 μm region. A further point relative to Figure 1 is the loss in the presence of 80 per cent Me₂SO of the negative band centered at ca. 295 μm. This is only suggested by the data in the figure, but we have studied this latter band in some detail and these data will be presented in a separate communication.

Considering now the optical data on the group of methionine acceptor tRNA’s, it appears that there are two sets of absorption data: one containing tRNA₉⁷ and tRNA₉³ and the second containing tRNA₉² and tRNA₉³. These are distinguished by: (1) the λₘₐₓ of the second set occurs at a longer wavelength than that of the first set (339 vs. 336 μm) and (2) there is a distinct shoulder on the spectra of the second set which appears to result from an unresolved band centered at approximately 355 μm. Furthermore, it was found that the chromophore of the second set differed by the absence of a hypochromic shift of Aₘₐₓ with increasing pH which is shown by the members of the first set and which
previously had been demonstrated by Lipsett\textsuperscript{2} for the 4TU residue of unfraccionated tRNA. It was also found that the spectrum of the second set was unaffected by treatment with sodium thiosulfate or dithiothreitol.

These observations led us to examine the sensitivity of the chromophores of these four species to triiodide oxidation. The results are presented in Figure 3. While the chromophores of the species of the second set are essentially resistant to the action of triiodide, there is, in the first set, a substantial loss of absorbance at 336 m\textmu which is, however, not as great as that seen in unfraccionated tRNA. The magnitude of the effect on tRNA\textsuperscript{met} is approximately 1/2 that in tRNA\textsuperscript{met} which is itself approximately 1/2 that for unfraccionated tRNA. The spectra of these oxidized tRNA’s show faint shoulders at the wavelengths of the peak and inflection seen in the spectra of triiodide-resistant species. It is possible that this residual absorbance represents a small amount of cross contamination resulting from an incomplete separation of the tRNA\textsubscript{f,1,2} and the tRNA\textsubscript{m,1,2} sub-species.

The CD spectra of three of the tRNA\textsuperscript{met} species are striking on two counts: (1) the diversity in shape and magnitude and (2) the negative sign which is opposite to that of unfraccionated tRNA (Fig. 1).

A study of the CD band of tRNA\textsubscript{f1} in SSC suggests that it could be the sum of a band similar to that seen in unfraccionated tRNA in the presence of 80 per cent Me\textsubscript{2}SO and the overlapping and stronger negative band centered at ca. 295 m\textmu. This interpretation is supported by the CD spectrum of this species in the presence of 80 per cent Me\textsubscript{2}SO (Fig. 3A). The fact that the CD spectrum of tRNA\textsubscript{f1} in the presence and absence of Me\textsubscript{2}SO is essentially unchanged together with the similarity of these spectra to that of unfraccionated tRNA in 80 per cent Me\textsubscript{2}SO leads us to suggest that the chromophore is in a substantially different environment in the tRNA\textsubscript{f1} than it is in the unfraccionated tRNA or, as we shall see below, in the tRNA\textsuperscript{val}. Further, we suggest that in this species of tRNA the motion of the chromophore is relatively unrestricted. An alternative statement would be that the chromophore is restricted, but the restricted conformation is the same as that which the chromophore assumes in 80 per cent Me\textsubscript{2}SO in the macromolecule. The recent paper by Scheit and Saenger\textsuperscript{4} is in-

![CD spectra of tRNA met species](image-url)
teresting in this regard. They suggest differences in the sign and magnitude of the Cotton effect related to 4TU in single- and double-stranded synthetic polymers containing 4TU which are consistent with our conclusions relative to the difference in order between the 4TU region in tRNA\textsuperscript{met}\textsubscript{f1} and tRNA\textsuperscript{val}. Unfortunately, the tRNA\textsuperscript{met}\textsubscript{m1} was in insufficient quantity to obtain reliable CD spectra. However, it is clear that the dichroism is neither of a strong negative or positive character.

The CD spectrum of tRNA\textsuperscript{met}\textsubscript{f2} is striking in its magnitude in SSC and, furthermore, in the persistence of a relatively strong band in the presence of 80 per cent Me\textsubscript{2}SO (Fig. 3B). This latter fact suggests that the chromophore is in some manner hindered in its rotation about the C\textsubscript{1}—N\textsubscript{1} bond, while the decrease in dichroism in the presence of Me\textsubscript{2}SO suggests further that the chromophore is in a more ordered portion of the tRNA molecule.

The CD spectrum of tRNA\textsuperscript{met}\textsubscript{m2} closely resembles that of tRNA\textsuperscript{met}\textsubscript{f2} except for the fact that it is less intense and also that there is a plainly discernible positive band, some portion of which appears between the negative bands centered at ca. 340 m\textsubscript{u} and 295 m\textsubscript{u}. In this connection it should be noted that the absorption spectrum for tRNA\textsuperscript{met}\textsubscript{m2} has a higher specific absorbance at 310 m\textsubscript{u} than does tRNA\textsuperscript{met}\textsubscript{f2} suggesting an additional absorption band in this region which may give rise to the positive CD band.

Turning to the tRNA\textsuperscript{val}, one notes in the absorption spectrum a rather higher A\textsubscript{max}/A\textsubscript{min} (1.63) than for unfractionated tRNA (1.19) or tRNA\textsuperscript{met}\textsubscript{m1} (1.36) and there is, furthermore, no evidence of a shoulder in the vicinity of 355 m\textsubscript{u}. The CD band is positive and more intense than that for unfractionated tRNA even when the latter is corrected for the smaller absorbance at 336 m\textsubscript{u} relative to that at 260 m\textsubscript{u}. The response of this band to 80 per cent Me\textsubscript{2}SO closely follows that of the unfractionated tRNA. The interpretation would then be as given below for the unfractionated tRNA.

The absorption spectrum of tRNA\textsuperscript{by\textsubscript{s}} (Fig. 4) closely resembles that of the unfractionated tRNA: the maximum/minimum ratios are essentially identical (1.21 vs. 1.19) and the ratio of A\textsubscript{336 m\textsubscript{u}}/A\textsubscript{260 m\textsubscript{u}} indicates the presence of one 4TU per molecule. The amino acid acceptance is quickly abolished by triiodide oxidation and restored by treatment with reducing agents. The CD spectrum presents a shape unlike that seen in any of the previous spectra. This spectrum might be interpreted as a result of three overlapping curves: First (on the long wavelength side) there is a weak negative band such as that seen in the tRNA\textsuperscript{met}\textsubscript{f1}, this is overlapped by a following positive band of somewhat greater intensity (see also tRNA\textsuperscript{met}\textsubscript{m2} Fig. 3C) which is in turn overlapped by the still stronger negative band centered around 295 m\textsubscript{u}.

For the purpose of further discussion of these results let us assume that the major contribution to the absorption band centered at 336 m\textsubscript{u} in unfractionated tRNA and in all the purified species examined except tRNA\textsuperscript{met}\textsubscript{f1,m2} is by 4TU. In all of these, the position of the maximum absorbance, the sensitivity to triiodide oxidation (reversible with reducing agents) and the behavior at elevated pH support this assumption. Furthermore, this residue has been identified by others in tRNA\textsuperscript{met}\textsubscript{f1}, tRNA\textsuperscript{met}\textsubscript{m1} and tRNA\textsuperscript{val}.\textsuperscript{19}
An interpretation of the CD spectrum in the region of this absorption band must consider some of the factors which might influence the shape and magnitude of the CD spectrum: 

1. the interaction of one 4TU with a second 4TU leading to exciton splitting of the CD band, 

2. interactions of 4TU with nearest neighbors other than itself and

3. an interaction of 4-thiouracil with the ribose ring to which it is attached.

From the absorption data, one estimates there to be approximately one 4TU per molecule in the purified species and somewhat less (on the average) in the unfractionated tRNA. This agrees with the data of Lipsett and the known primary structures. We have found no concentration dependence of the CD spectrum nor evidence of dimerization of the tRNA at the concentrations used. Therefore, 4TU interaction with itself, between, or within the molecules, is not likely to be a factor in these cases.

With regard to the second factor listed above, equations relating the rotational strength due to interaction of electronic transitions in closely situated (nearest neighbor) molecules contain a term in the denominator which is the difference between the squared frequencies of the interacting transitions. Thus, for a frequency separation such as that between the long wave transition of 4TU and the nearest \( \pi-\pi^* \) transition of the major bases, one would expect in this case little circular dichroism arising from the nearest neighbor interaction. The lack of interaction between the longwave transition of dihydropyridine in DPNH (340 mp) with the 259 mp transition of the adenosine residue in the same molecule has been noted by Miles et al. We have considered the possibility of an interaction between the 335 mp band of 4TU and the 295 mp band of tRNA. Since the origin of this latter band remains unexplained we cannot assess the likelihood of such an interaction. We must then consider the interaction between the 4TU and the ribose to which it is attached. The CD spectra of 4TU and 4-thiouridylic acid have been published recently. We have also measured the CD spectra of 4TU in water, 0.15 \( M \) KF, 0.01 \( M \) Na2S2O4, and in 80 per cent Me2SO in water. We find the sign of the CD in all of these solvents to be the same (+) as found by Samejima et al. for Ap4TU and Gp4TU in which the sign of the Cotton effect is positive and there is evidence of base interaction in the form of hypochromicity and a bathochromic shift of the 4TU absorption band. The optical rotary dispersion curve shows no indication of exciton splitting. Unfortunately no data are given for the effect of temperature on the optical rotary dispersion of these dinucleoside monophosphates. These results lead us to suggest that there

![Fig. 4.—The effect of Me2SO on the absorption and CD spectra of purified tRNA and tRNA. Panel A, curves 1 and 2—tRNA in SSC; curve 3—ditto in 80% aqueous Me2SO. Panel B, curves 1 and 2—tRNA in SSC.](image-url)
is an effect of the high polymer environment on the CD properties of the 4TU residue, and that interpretation of the data presented for the various RNA's must, at present, be made within the set of high polymers. Thus the clearest conclusion is that the conformation of 4TU in tRNA\textsuperscript{met}\textsubscript{F} must be substantially different from that in tRNA\textsuperscript{val}, or, for that matter, from the 4TU residues in the bulk of the unfractionated tRNA. The sensitivity of the CD of 4TU to environment is well illustrated by the results of Samejima et al.\textsuperscript{24} who have demonstrated that the sign of the CD of 4-thiouridylic acid goes from positive to negative on binding to ribonuclease. These observations are particularly intriguing because the 4-thiouridine residue in both tRNA\textsuperscript{met}\textsubscript{F} and tRNA\textsuperscript{val} occupies the same position (no. 8 from the 5' end) in the primary structure with G on either side in the former,\textsuperscript{17} and U and A in positions 7 and 9, respectively in the latter.\textsuperscript{19} In the cloverleaf structures drawn for these species, residue number 8 is the first non-paired base from the 5'-terminus encountered after leaving the stem of the cloverleaf. The CD data, taken together with the unusual chromatographic properties of tRNA\textsuperscript{met}\textsubscript{F} as well as the unusual dependence of absorbance at 336 m\textmu on ionic strength\textsuperscript{26} would suggest that the secondary (and/or tertiary) structure of tRNA\textsuperscript{met}\textsubscript{F} must be quite different from what would be expected from the "cloverleaf" model as usually written.

The properties of the unknown chromophore responsible for the absorption band in the vicinity of 340 m\textmu in tRNA\textsuperscript{met}\textsubscript{F2,m2} suggested to us that the molecule possessed a 4-thione group which was by some means prevented from ionizing at elevated pH and could not be as easily oxidized as 4-thiouracil. It seemed reasonable to consider a 3-substituted 4-thiopyrimidine. We synthesized 1,3-dimethyl-4-thiouracil, 1,3-dimethyl-2,4-dithiopyrimidine, and 1,3-dimethyl-4-thiothymine\textsuperscript{27} and examined the effects of elevated pH and triiodide treatment on the ultraviolet absorption of these compounds. All of them showed little or no change in the ultraviolet absorption spectrum at elevated pH or on treatment with triiodide. Of these three compounds only 1,3-dimethyl-2,4-dithiopyrimidine yielded a long wavelength absorption band resembling in shape and position the spectrum of tRNA\textsuperscript{met}\textsubscript{F2,m2}.

We also considered the possibility that the unknown chromophore might be a photochemical product resulting from the exposure of tRNA\textsuperscript{met}\textsubscript{F1,m1} to long wavelength ultraviolet radiation from the fluorescent laboratory lighting. A sample of tRNA\textsuperscript{met} was dissolved in SSC and irradiated in Pyrex vessel with light from a medium pressure Hg lamp. This treatment resulted in a decrease in the absorbance in the 336 m\textmu region without a significant change in the shape of the absorption or CD band. Hence we think it unlikely that the unknown chromophore is a photochemical product arising during the fractionation of the tRNA. However, considering the results of Uziel\textsuperscript{28} and Yaniv (personal communication) the possibility remains that this chromophore is an accidental in vitro derivative of 4TU.

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Commission (Contract AT(30-1)-2643). This is publication 1360 of the Harvard Cancer Commission.

5 Schofield, P., manuscript in preparation.
22 Ibid., 91, 831 (1969).