Acknowledgment

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Spin-Labeling Studies of Aminoacyl Transfer Ribonucleic Acid*

Peter Schofield, Brian M. Hoffman,† and Alexander Rich

ABSTRACT: Spin-labeling experiments have been carried out in which an organic free radical is linked to *Escherichia coli* Val- or Phe-tRNA by acylation of the α-amino group.

The chemistry of the labeling reaction is studied in detail, and special attention is paid to the specificity of the reaction. Nonspecific labeling of tRNA can be decreased considerably by fractionating the tRNA after the spin-labeling reaction. During this process, a single peak of spin-labeled Val-tRNA and two peaks of Phe-tRNA were recovered. The two species of Phe-tRNA differ by their stability to heat denaturation. A change in mobility of the spin label occurs after ribonuclease treatment and after thermal denaturation. The latter process is reversible, and the spin-melting temperature measured in this process is sensitive to ionic strength. The spin-melting phenomenon is interpreted as indicating two molecular states of tRNA with different activation energies for motion of the spin label at temperatures, respectively, above and below the sharp transition which occurs at the spin-melting temperature.

Spin-labeling is a technique in which a stable organic free radical linked to a macromolecule is used to provide information about the structure and function of the latter. Changes in macromolecular conformation can be observed through their effect on the rotational motion of the label. This technique has been used to study a variety of macromolecules (Stone *et al.*, 1965; Ogawa and McConnell, 1967; Smith, 1968; see also the review by Hamilton and McConnell, 1968), and in particular it has recently been extended to the study of nucleic acids (Smith and Yamane, 1967; Hoffman *et al.*, 1969). As with all methods involving the introduction of chemical probes, interpretation of the data is critically dependent on a knowledge of the site of attachment of the probe. We have applied the spin-labeling technique to study tRNA paying particular attention to the specific labeling of selected sites in a fashion designed to minimize possible interference with the natural state of tRNA.

tRNA contains a relatively high proportion of unusual bases (Miura, 1967) such as N-methylated purines, dihydrouracil, 2- and 4-thiouracil, pseudouridine, and inosine. At first sight, these might seem attractive targets for selective labeling. However, there are very few chemical reagents that will discriminate among these bases to a sufficiently high degree. We have therefore chosen the α-amino group of AA-tRNA as a unique point of attack. This amino group with a pK of 8–9 is strongly basic in contrast to the amino groups and heterocyclic nitrogen atoms of the tRNA bases. Thus a selective chemical attack is possible. An unfractionated mixture of tRNAs can be charged enzymatically with a single amino acid and a spin label can then be attached chemically through an amide linkage to the α-amino group of the aminoacyl ester. The specificity of aminoacylation allows us to investigate only those tRNA species specific for the particular amino acid. This procedure is also less likely to perturb the tRNA structure than labeling one of the

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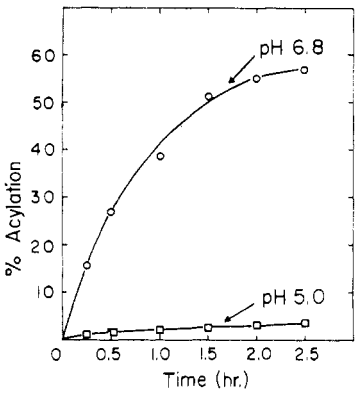


FIGURE 1: Kinetics of reaction of [^{14}C]Val-tRNA with R-*N*-hydroxysuccinimide. Incubation mixtures contained [^{14}C]Val-tRNA (0.5 mg, *ca.* 6000 cpm), R-*N*-hydroxysuccinimide (5 μmoles in 0.1 ml of acetonitrile), and acetate (pH 5) or phosphate (pH 6.8, 10 μM) buffer in a total volume of 0.31 ml. After incubation at 37° for the required period, 40-μl aliquots were transferred to 1 ml of 0.2 M Tris–0.025 M $CuSO_4$ (pH 7.5), incubated for a further 30 min at 37°, then 4 ml of cold 5% trichloroacetic acid was added, and the acid-insoluble radioactivity was collected on Millipore filters and counted in a low-background gas-flow counter.

base residues. The spin-labeled AA-tRNA is then analogous to fMet- or peptidyl-tRNA, which are naturally occurring species *in vivo*.

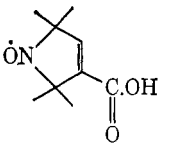
In a previous communication (Hoffman *et al.*, 1969) we reported preliminary results of electron paramagnetic resonance measurements made on spin-labeled Val-tRNA from *Escherichia coli*. In this paper we make a full report of the spin-labeling procedures and present further electron paramagnetic resonance measurements on spin-labeled Phe-tRNA. Upon denaturation Phe-tRNA like the Val-tRNA undergoes an abrupt transition in the molecular motion of the spin label at a temperature which is sensitive to the ionic strength of the medium. During the chromatographic purification of the labeled Phe-tRNA, two separate spin-labeled fractions were isolated which are presumably degenerate members of the set of tRNAs specific for phenylalanine. These two fractions exhibited somewhat different spin immobilization properties.

Materials and Methods

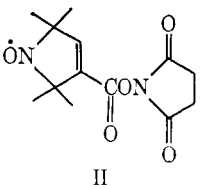
E. coli B tRNA (Schwarz BioResearch) was charged with either [^{14}C]valine or [^{14}C]phenylalanine as described previously (Hoffman *et al.*, 1969). The hydroxysuccinimide ester (II) was prepared by the method of Hoffman *et al.* (1969). Hydroxylapatite was prepared according to Levin (1962). The number of nitroxide residues per tRNA chain was measured by incubating an aliquot of spin-labeled aminoacyl-tRNA with 0.3 N NaOH overnight at 37° and comparing the intensity of the electron paramagnetic resonance signal with standards prepared by incubating for a similar period a mixture of the nitroxide carboxylic acid (I) with 0.3 N NaOH and tRNA.

Poly-L-lysine (Sigma Chemical Co., mol wt 200,000) was spin labeled as follows. A mixture of 2.8 mg of poly-L-lysine, 0.1 mmole of phosphate buffer (pH 6.8), 2.5 moles of the ester (II), and 0.5 ml of acetonitrile in a total volume of 2 ml was shaken at room temperature for 24 hr. The poly-L-lysine was collected by centrifugation, redissolved in 2% potassium acetate buffer (pH 5), and reprecipitated with three volumes of ethanol. After a second redissolution and reprecipitation, the poly-L-lysine was redissolved in 4 ml of 0.01 M sodium acetate (pH 5) and dialyzed exhaustively against the same buffer. Electron paramagnetic resonance measurements were made as described previously (Hoffman *et al.*, 1969).

Chemistry of Labeling



I



II

The spin label chosen for the present study was 2,2,5,5-tetramethyl-3-carboxylpyrrolin-1-oxyl (I) in which the carboxyl group was converted into an active acylating agent by esterification with *N*-hydroxysuccinimide. Recent studies with *N*-hydroxysuccinimide esters have shown that these compounds can selectively acylate the α-amino group of aminoacyl-tRNA (de Groot *et al.*, 1966). We modified the heterogeneous reaction conditions of de Groot *et al.* (1966) by using a mixed organic–aqueous solvent in which both the tRNA and the acylating agent were soluble. In addition, a smaller molar excess of acylating agent was used and the reaction time was reduced to a few hours. Of the organic solvents tested, acetonitrile proved to be most suitable for these purposes. Aqueous solutions of tRNA (5 mg/ml), in the absence of Mg^{2+} were not precipitated by addition of one-half their volume of acetonitrile. In addition the acylating agent II is soluble to at least 14 mg/ml (50 μmoles/ml) in 30% aqueous acetonitrile. Initial acylation studies were performed on Val-tRNA from *E. coli* since the ester bond in this species is relatively stable to hydrolysis (Ishida and Miura, 1965). The extent of acylation of [^{14}C]Val-tRNA at various times was followed by measuring the amount of acid-precipitable radioactivity remaining after dilution of an aliquot of the reaction mixture into a Cu^{2+}-containing Tris buffer. The material was incubated for a period of time sufficient to hydrolyze completely any Val-tRNA. These conditions have previously been shown to be effective in distinguishing between α-*N*-AcVal-tRNA and Val-tRNA (Schofield and Zamecnik, 1968). Results for the acylation of Val-tRNA at two different pH values are shown in Figure 1. The reaction rate is very slow at pH 5 and moderate at pH 6.8. The kinetics of the reaction are complex. Since hydrolysis of the aminoacyl ester bond and of the hydroxysuccinimide ester is also greatly accelerated at higher pH values (Wolfenden, 1963), it is usually advantageous to carry out the acylation reaction at a pH no greater than 6–7. Val-tRNA is particularly stable to hydrolysis under these conditions and has as approximate half-life of 14 hr at pH 6.8 and 37°.

In order to examine the extent to which the nucleic acid bases spin labeled under our conditions, a solution of tRNA (10 mg in 2 ml of 0.05 M phosphate, pH 6.8) which had previously been freed of attached amino acids by incubation

TABLE I: Reaction of R-*N*-Hydroxysuccinimide with Synthetic Polynucleotides.[a]

Polynucleotides	Moles of Nitroxide/Mole of Nucleotide Residues
Poly A	$<10^{-4}$
Poly U	$<10^{-4}$
Poly C	$\sim10^{-3}$
Poly (U-G) (3:1)	$\sim3 \times 10^{-4}$

[a] Solutions of the polynucleotides (4–5 mg) in 1 ml of 0.1 M phosphate buffer (pH 6.8). were mixed with 0.4 ml of an acetonitrile solution of R-*N*-hydroxysuccinimide (14 mg/ml) and kept for 20 hr at 37°. The polynucleotides were then precipitated by addition of 0.2 ml of 1 M sodium acetate (pH 5) and 3 ml of 95% ethanol, redissolved in 1 ml of water and chromatographed on Sephadex G-25. Poly C and poly (U-G) samples were hydrolyzed in 0.3 N NaOH at 37° for 24 hr before determination of the nitroxide and nucleotide content.

TABLE II: Reaction of [^{14}C]-*N*-Acetoxysuccinimide with Stripped tRNA.[a]

Conditions	pH	Moles of [^{14}C]-Acetyl/Mole of tRNA
Heterogeneous	5.0	0.32
Heterogeneous	6.8	0.49
Homogeneous	5.0	0.04
Homogeneous	6.8	0.17

[a] [^{14}C]-*N*-Acetoxysuccinimide was prepared from [1-^{14}C]-acetic acid (0.98 mCi/mmole) as described by de Groot *et al.* (1966). For the *heterogeneous* conditions, a mixture of 200 μl of tRNA (9.23 mg/ml, previously stripped at pH 10.3 for 3 hr at 37°), 20 μl of 1 M acetate (pH 5.0) or phosphate buffer (pH 6.8), and 0.5 ml of dimethylformamide containing 10.7 mg of [^{14}C]-*N*-acetoxysuccinimide was shaken for 22 hr at room temperature, then 2 ml of ethanol was added and the RNA was collected by centrifugation. For the *homogeneous* conditions, a mixture of 1 ml of tRNA (9.23 mg), 50 μl of 1 M acetate (pH 5.0) or phosphate (pH 6.8) buffer and 0.5 ml of acetonitrile containing 3.9 mg of [^{14}C]-*N*-acetoxysuccinimide was incubated for 3 hr at 37° and the RNA was then precipitated with 5 ml of ethanol. All tRNA samples were washed with 95% ethanol, redissolved in 2% sodium acetate buffer (pH 5), and reprecipitated with 2.5 volumes of cold ethanol. A total of four reprecipitations was carried out in this manner. The specific activity of all these samples was determined by treating aliquots with 5% trichloroacetic acid and collecting the acid-insoluble radioactivity on Millipore filters.

at pH 8.0 (Sarin and Zamecnik, 1964) was incubated at 37° for 5 hr with II (14 mg in 1 ml of acetonitrile). It was then freed of excess II by repeated precipitation with ethanol. This sample gave an electron paramagnetic resonance signal and measurement of the number of nitroxide radicals per tRNA chain (see Materials and Methods) gave a value of 4.8% (4.8 moles of nitroxide/100 moles of tRNA). This tRNA was also chromatographed on Sephadex G-25 in the presence of dimethyl sulfoxide to remove any remaining traces of noncovalently bonded nitroxide molecules and the measured radical content after this treatment was 3.8%, not greatly different from the previous value. Since it is rarely possible to charge an unfractionated population of tRNA molecules with a single amino acid to a level of greater than 5%, it was clear that this background reaction of uncharged tRNA constituted a threat to the goal of specific labeling of the α-aminoacyl group and it suggested that a purification step be included after labeling charged tRNA.

In an attempt to locate the site at which this background reaction occurred, the synthetic polyribonucleotides poly rC, poly rU, poly rA, and poly r(U-G) (3:1) were treated with II under the same conditions as above, and were then freed from excess spin label by precipitation with ethanol and chromatography on Sephadex G-25. They were examined for attached spin label and the results appear in Table I. It is clear that, even after prolonged incubation, the extent of reaction is very slight for C and G residues and undetectable for A and U residues. This extent of reaction is insufficient to explain the background labeling described above. Treatment of pseudouridine with II under similar conditions and subsequent examination of the products by thin-layer chromatography in three different solvent systems also failed to reveal any new ultraviolet-absorbing components. The possibility that 4-thiouridylic acid residues might be the site of reaction was tested by oxidizing a stripped tRNA preparation with iodine according to the procedure of Carbon *et al.* (1965) and then carrying this preparation through the standard spin-labeling procedure together with an unoxidized control sample. The level of labeling of the stripped control sample was 4.8% while that of the iodine-oxidized sample was 5.3%. Thus the background reaction appears not to involve 4-(S)U residues. The electron paramagnetic resonance signal given by the stripped tRNA after spin labeling was characteristic of a weakly immobilized nitroxide (Stone *et al.*, 1965) and did not change when the tRNA was treated with pronase. It seems unlikely, therefore, that residual traces of peptidyl-tRNA which had survived the basic stripping procedure could be the cause of the background labeling.

In their study of *N*-acetoxysuccinimide as a specific acetylating agent in an aqueous dimethylformamide heterogeneous system, de Groot *et al.* (1966) used yeast Phe-tRNA as an acyl acceptor and reported the level of acetylation for stripped yeast tRNA to be less than 1 acetyl group/5000 tRNA molecules. There are several possible reasons for this discrepancy with our findings, including differences in reaction conditions, differences in the reactivity of the acylating agent, and possible differences in reactive minor bases in *E. coli* as opposed to yeast tRNA. In order to discriminate among these alternatives, [^{14}C]-*N*-acetoxysuccinimide was prepared and its reaction with stripped *E. coli* tRNA was examined using both the heterogeneous and the homogeneous reaction conditions. These results appear in Table II. The extent of acylation in the heterogeneous system is much greater and

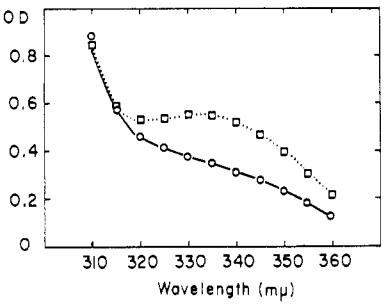


FIGURE 2: Effect of *N*-acetoxysuccinimide on the long-wavelength absorption of tRNA. Ultraviolet spectra were measured on aqueous solutions which contained traces of ethanol and acetate buffer (pH 5). (O——O) tRNA treated with *N*-acetoxysuccinimide under the heterogeneous reaction conditions at pH 6.8, homogeneous reaction conditions (□———□). There was no detectable difference in the long-wavelength absorbance between the samples acylated at either pH 5.0 (or pH 6.8) under the homogeneous conditions and a control sample of untreated tRNA. The long-wavelength spectra of the samples acylated at pH 5.0 or 6.8 under the heterogeneous conditions were virtually superimposable.

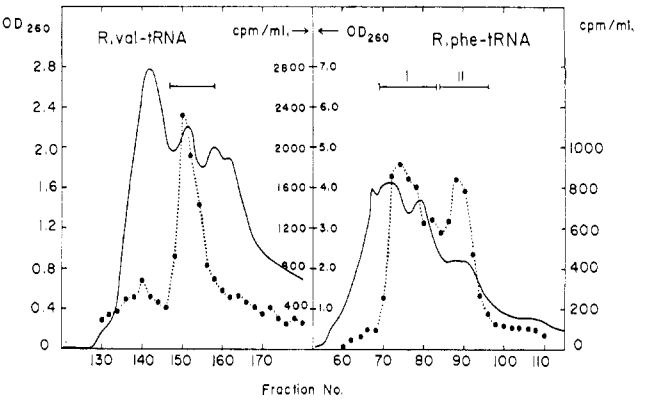


FIGURE 3: Hydroxylapatite chromatography of spin-labeled Val- and Phe-tRNA. (a) R-Val-tRNA: a mixture of [^{14}C]Val-tRNA (25 mg, 62 mμM [^{14}C]valine), phosphate buffer (pH 6.8, 250 μM), and R-*N*-hydroxysuccinimide (42 mg in 2.5 ml of acetonitrile) in a total volume of 8 ml was incubated for 3 hr at 37° and the tRNA was then precipitated with 25 ml of cold 95% ethanol, redissolved in 2.5 ml of 0.08 M phosphate buffer (pH 6.8), and dialyzed against two 250-ml portions of the same buffer. The tRNA was then applied to a column of hydroxylapatite (1.9 × 40 cm), equilibrated at room temperature with 0.08 M phosphate buffer (pH 6.8), and the elution was carried out with a linear gradient of 0.1–0.2 M phosphate (500 + 500 ml) at a flow rate of 21 ml/hr. The fraction volume was 5 ml; aliquots (50 μl) were evaporated to dryness on stainless steel planchets and counted in a low-background gas-flow counter. (b) R-Phe-tRNA spin labeling of [^{14}C]Phe-tRNA was performed as described above for Val-tRNA. A solution of this R-Phe-tRNA (63 mg) in 6.5 ml of 0.08 M phosphate buffer (pH 6.8) was applied to a column of hydroxylapatite (1.9 × 63 cm), equilibrated with 0.08 M phosphate, and water jacketed at 16°. Elution was carried out with a linear gradient of 0.1–0.2 M phosphate buffer (750 + 750 ml) at a flow rate of 38 ml/hr and 7.6-ml fractions were collected. Aliquots (0.1 ml) of these fractions were pipetted into 4 ml of cold 5% trichloroacetic acid and the acid-insoluble radioactivity was determined.

shows a less marked pH dependence than that observed for the homogeneous system. The ultraviolet absorption spectra in the region 300–400 mμ of the *E. coli* tRNA species before and after treatment with the acetylating agent are shown in Figure 2. 4-(S)U residues in tRNA have an absorption maximum at 336 mμ (Lipsett, 1965) and the samples acetylated under the heterogeneous conditions show a modified absorption in this region, whereas those acetylated under the homogeneous conditions are unaltered. This attack upon 4-(S)U residues under the heterogeneous reaction conditions (80% aqueous dimethylformamide) was confirmed by repeating the acylation with a stripped and iodine-oxidized tRNA as substrate. In this case, under the conditions described in the legend to Table II, the level of acetylation at pH 6.8 was reduced from 49 to 32%. It thus appears that, in aqueous dimethylformamide, the reaction of *N*-acetoxysuccinimide with *E. coli* tRNA occurs at least at two distinct sites, one of which is 4-(S)U.[1] The difference in thiobase content between *E. coli* and yeast tRNA therefore explain, at least in part, the discrepancy between the acetylation levels found by us using *E. coli* tRNA and by de Groot *et al.* (1966) using yeast tRNA.

Preparation of Spin-Labeled Val- and Phe-tRNA. Acylation of the α-amino group in AA-tRNA increases the stability of the ester bond to alkaline hydrolysis (Gilbert, 1963). We therefore sought to decrease the proportion of base-labeled tRNA by carrying out a fractionation procedure after the spin-labeling step. Spin-labeled Val-tRNA (R-Val-tRNA, R = nitroxyl) was prepared and chromatographed on hydroxylapatite as described by Pearson and Kelmers (1966). The elution profile is shown in Figure 3. The fractions corresponding to the major radioactive peak were pooled, concentrated under reduced pressure, and dialyzed against 1 mM sodium acetate buffer (pH 5.0). A similar spin-labeling and chromatographic procedure was used to prepare R-Phe-tRNA. In this case two major peaks of radioactivity, fractions I and II, were found (Figure 3), and these were harvested separately. The recovery of acid-insoluble radioactivity from this column was approximately 60%.

Characterization of R-Val- and R-Phe-tRNA. Treatment of spin-labeled AA-tRNA with Cu^{2+}-containing buffers enables us to determine the proportion of total AA-tRNA that carries the spin label on the α-amino group since the ester bond in this species will be stabilized toward Cu^{2+}-catalyzed hydrolysis (Schofield and Zamecnik, 1968). The time course of hydrolysis of the spin-labeled Val-tRNA after chromatography is compared in Figure 4 with that for untreated [^{14}C]Val-tRNA. Evidently 90% of the Val-tRNA molecules in this sample have a blocked α-amino group. The total number of nitroxide molecules per tRNA chain can also be measured (Materials and Methods) and thus we are able to estimate the degree of specificity of spin labeling. The ratio of moles of Cu^{2+}-stable [^{14}C]AA-tRNA: mole of nitroxide is a measure of this specificity. The ideal situation in which the specificity of attack at the α-amino group of AA-tRNA was absolute would correspond to a labeling ratio of 1.0 and a smaller value would imply less specificity of spin labeling. Labeling ratios for the R-Val-tRNA were found to be 1.0, and the ratios for R-Phe-tRNA I and II were both found to be 0.62. The lower ratios found for the two R-Phe-tRNAs relative to R-Val-tRNA probably

[1] The reaction of cytidine with *N*-acetoxysuccinimide has also been observed under these conditions (Chládek *et al.*, 1969).

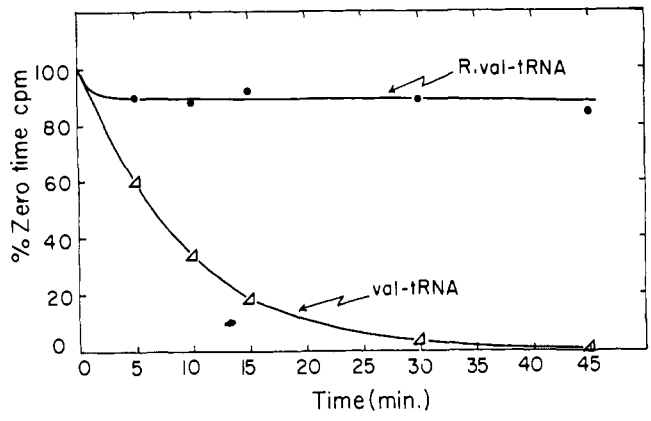


FIGURE 4: Cu^{2+}-catalyzed hydrolysis of R-Val-tRNA and of Val-tRNA. Incubation mixtures contained 0.6 ml of 0.2 M Tris–0.025 M $CuSO_4$ (pH 7.5) and 0.1 ml of a solution of [^{14}C]Val-tRNA (*ca.* 7000 cpm) or 30 μl (*ca.* 1000 cpm) of the pooled and concentrated R-Val-tRNA from fractions 147 to 158 of the hydroxylapatite column. After incubation at 37° for the required period, 100-μl aliquots were pipetted into 4 ml of cold 5% trichloroacetic acid and the acid-insoluble radioactivity was determined.

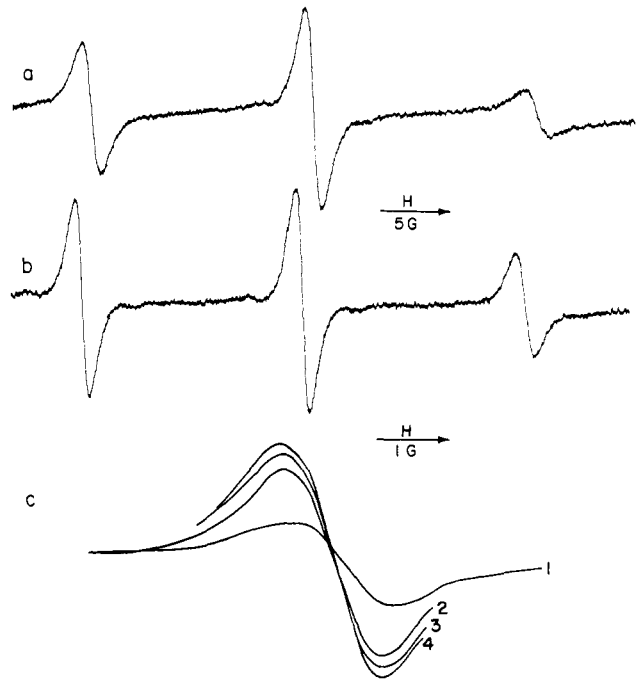


FIGURE 5: The effect of pancreatic RNase on the electron paramagnetic resonance spectra of spin-labeled tRNA (a) is the spectrum of R-Phe-tRNA at 0°, (b) is the same sample at 0° after addition of 8 μg/ml of pancreatic RNase, (c) shows the change of the high-field line of R-Val-tRNA on incubation at 25° with 8 μg/ml of RNase. Successive curves were taken at zero time (1) and at 2 (2), 240 (3) and 1080 (4) min.

reflects the inferior resolution of the hydroxylapatite column observed in this case (Figure 3).

Electron Paramagnetic Resonance Measurements. The stable paramagnetic nitroxide label has a characteristic three-line spectrum when freely tumbling in solution. As the rate of tumbling slows from that in free solution, the three-lines broaden unequally and the spectrum becomes asymmetric because of residual anisotropy in the g and hyperfine tensors (Stone *et al.*, 1965). From a slightly broadened spectrum, characteristic of partial or weak immobilization of the spin label, a rotational correlation time, τ, can be calculated. Following the Debye theory of rotational brownian motion, we first consider an axis through the label at a given instant in time. The direction of this axis will change due to brownian motion. If $\theta(t)$ is defined as the average angle between the initial axis direction and the instantaneous direction at a later time, then τ is defined as the average time required for the label to rotate until

$$\langle {}^1/_2[3\cos^2\theta(t) - 1]\rangle = e^{-1}$$

Thus a large value of τ indicates slow rotation, while rapid tumbling is associated with low values of τ. Changes in macromolecular configuration which influence the surroundings of an attached spin label are reflected in the degree of immobilization of the label. This can be given a quantitative expression in terms of τ. We discuss only the value of τ derived from that term in the standard equations (Stone *et al.*, 1965) which is quadratic in the nuclear spin quantum number as previously described (Hoffman *et al.*, 1969).

Samples for study of melting behavior by electron paramagnetic resonance were prepared by mixing stock solutions of R-AA-tRNA with concentrated solutions of the appropriate reagents and sealing the final solution into a Pyrex capillary tube. With this technique, as little as 25 μl can be used for the determination of a melting curve. Electron paramagnetic resonance melting curves were measured starting at the lowest temperature in order to minimize the danger of AA-tRNA-bond hydrolysis and the resultant release of free spin label. Roughly 10 min was required to perform a measurement at each temperature. After completing a run, cooling the sample to the starting temperature usually reproduced the initial low-temperature spectrum; performing a second or third heating cycle with the sample gave equivalent results. Thus, over the temperature ranges used, changes in the rotational freedom of the label with temperature are reversible and hydrolysis is not a significant factor.

The stock solution of R-Val-tRNA contained 11.0 mg/ml of tRNA, and dilution to 4.4 mg/ml of tRNA caused no change in melting behavior. Furthermore, preincubation of R-Val-tRNA at 60°, either in a previous melting experiment or independently, had no effect on succeeding melting curves carried out on the same sample. Hydroxylapatite chromatography would remove any preexisting tRNA aggregates and these dilution and preincubation experiments appear to eliminate aggregate formation as a factor in the observed melting behavior. Stock solutions of R-Phe-tRNA, fraction I, contained 29 mg of tRNA/ml; stock solutions of fraction II contained 14.7 mg of tRNA/ml.

RNase Digestion of Spin-Labeled AA-tRNA. Treatment of spin-labeled Val- or Phe-tRNA with pancreatic RNase very rapidly produces an increase in the label's rotational freedom. The upper part of Figure 5 shows the effect of RNase on the spectrum of R-Phe-tRNA. Curve a is a spectrum taken at 0° in the absence of RNase. Curve b is taken at 0° immediately after a room temperature sample has been brought to an RNase concentration of c = 8 μg/ml and

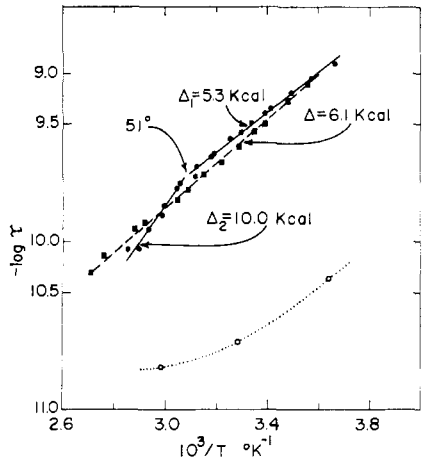


FIGURE 6: A plot of the log τ (correlation time) *vs.* inverse absolute temperature for R-Val-tRNA (●—●), R-poly-L-lysine (■—■), and the unreacted spin-label R-CooH (○·····○). The solutions of R-Val-tRNA and R-COOH both contained 10 mM sodium acetate (pH 5) and 10 mM KCl. The melting curve of R-poly-L-lysine was measured in 10 mM sodium acetate buffer (pH 5).

TABLE III: Spin-Melting Data for R-Phe-tRNA.

Additions[a]	tRNA	Δ_1[b]	Δ_2[b]	T_{sp}^0	T_{OD}^0[c]
None	R-Phe-tRNA I	6.1	10.0	39	
					37.5
None	R-Phe-tRNA II	5.9	8.1	36	
0.01 M KCl	R-Phe-tRNA I	6.2	(7.5)	48	
					49.5
0.01 M KCl	R-Phe-tRNA II	6.0	7.9	38	
0.05 M KCl	R-Phe-tRNA I	6.1		55	
					57.5
	R-Phe-tRNA II	5.6	9.5	43	
0.45 M KCl	R-Phe-tRNA II	5.9		55	

[a] All additions were made to a stock solution containing R-Phe-tRNA in 1 mM sodium acetate–1 mM EDTA (pH 5.0). [b] Values of Δ are estimated to be accurate to within ± 1 kcal/mole. [c] Inflection points in the absorbance–temperature profile of unfractionated tRNA.

rapidly cooled to 0°. The enzyme:substrate ratio in this experiment was approximately 1:2000. The family of curves in Figure 5c shows the progressive change in width of the high-field line for R-Val-tRNA on incubation at 25° with RNase. This line-width changes very rapidly from that characteristic of a weakly immobilized spin label ($\tau \simeq 10^{-9}$ sec) and approaches that observed for the free nitroxide carboxylic acid I ($\tau \simeq 6.10^{-11}$ sec).

Electron Paramagnetic Resonance Melting Curves. As previously described (Hoffman *et al.*, 1969), the variation of τ with temperature for R-AA-tRNA, when expressed as as Arrhenius plot, gives a pattern of two discontinuous straight-line segments

$$\tau_1 = \tau_1^0 e^{\Delta_1/RT} \quad T < T_{sp}$$
$$\tau_2 = \tau_2^0 e^{\Delta_2/RT} \quad T > T_{sp} \qquad (1)$$

This pattern can be characterized by the slopes ($\Delta 1$ and $\Delta 2$) of the straight lines and by the temperature corresponding to the discontinuity, T_{sp}. The values of $\Delta 1$ and $\Delta 2$ do not vary significantly with variations in the ionic strength of the solvent whereas the value of T_{sp} increases as the ionic strength is increased. Spin-melting curves for R-Val-tRNA are shown in Figure 6 where the discontinuous nature of the relationship between ln τ and $1/T$ is clearly evident. In addition, it is clear that there is a large change both in the absolute correlation time and in the response to temperature changes for the free spin label (dotted curve) resulting from its attachment to AA-tRNA.

A sample of poly-L-lysine was also spin labeled with R-*N*-hydroxysuccinimide on its ϵ-amino groups (Materials and Methods). Electron paramagnetic resonance spectra were measured at different temperatures at pH 5 where the molecule is known to assume a random coil configuration (Applequist and Doty, 1962). The ln τ *vs.* $1/T$ plot of this preparation is linear and monotonic over the temperature range studied (dashed line, Figure 6) in sharp contrast to the corresponding plots for R-Val-tRNA. Spin-melting curves of the nonspecifically labeled, stripped tRNA were also measured at pH 7.5. These curves were similar to that seen for spin-labeled poly-L-lysine and showed no break. The discontinuity in the electron paramagnetic resonance melting curves for R-AA-tRNA must therefore be attributed only to those nitroxide molecules attached *via* the amino acid to tRNA. The break temperature could be masked by a large excess of nonspecifically attached spin labels, but if it is observable its value cannot be distorted by them.

Values of Δ and T_{sp} for the two fractions of R-Phe-tRNA separated by hydroxylapatite chromatography are given in Table III. The high-temperature slope, $\Delta 2$, could not be obtained for all salt concentrations because it was necessary to restrict the temperature to less than 55°; hydrolysis was found to be significant above this temperature. The effect of magnesium ion was not tested since T_{sp} was expected to be too high to measure in its presence. T_{sp} for fraction II is consistently lower than that for fraction I when measured under the same ionic conditions. The behavior of fraction I is quite similar to that of R-Val-tRNA, for which similar data of T_{sp} and Δ values have been presented (Hoffman *et al.*, 1969). The effect on the melting curves of R-Val-tRNA of either urea or dimethyl sulfoxide as denaturants has also been published (Hoffman *et al.*, 1969).

Discussion

Prior to the introduction of *N*-hydroxysuccinimide esters, acylation of the α-amino group of AA-tRNA had been carried out with carboxylic acid anhydrides (Haenni and Chapeville, 1966, Simon *et al.*, 1964) or with carboxylic acids activated with a water-soluble carbodiimide (Altunina *et al.*, 1965). There are objections to both of these methods. In their study of the acetylation of Phe-tRNA with acetic anhydride, Haenni and Chapeville (1966) reported that only about 50% of the acetyl groups introduced into the tRNA by this reagent were linked to the phenylalanine α-amino group.

The use of carbodiimides as activating agents for the carboxyl group is open to the objection that acid anhydrides are a frequently observed by-product of these reagents (DeTar and Silverstein, 1966), and, in addition, the carbodiimide itself may react with G and U residues in tRNA (Gilham, 1962). Esters of *N*-hydroxysuccinimide, first introduced by Anderson *et al.* (1964), appear to be selective acylating agents and, under certain conditions, will effect quite specific acylation of the α-amino group in AA-tRNA (de Groot *et al.*, 1966). Our results amply demonstrate, however, that their use must be accompanied by some circumspection. It is clear that both *N*-acetoxysuccinimide and the nitroxide–hydroxysuccinimide ester II can react with a tRNA preparation which has been stripped of attached amino acids. Unpublished experiments (Schofield, 1967) have shown that II is a much less reactive acylating agent than *N*-acetoxysuccinimide, probably because of the steric hindrance offered by the 2,2-*gem*-dimethyl groups to nucleophilic attack on the ester carbonyl group. Our attempts to identify the site(s) of reaction have not been successful, although a number of possibilities have been eliminated. The reaction, at least in the case of the spin-labeling reagent II, appears to involve neither the four major bases, pseudouridine, 4-thiouridine, nor residual peptidyl-tRNA. A possible candidate for this site of attack might be the exocyclic secondary amino group of the minor base 5-methylaminomethyl-2-thiouracil first reported by Carbon *et al.* (1968). This compound is present to the extent of approximately 5 moles/100 moles of tRNA (Carbon *et al.*, 1968), sufficient to account for the observed background spin labeling of stripped tRNA.

If one assumes that these sites are randomly distributed throughout the tRNA population (some preliminary chromatographic results support this idea) then any fractionation procedure which increased the amount of spin-labeled AA-tRNA relative to the total tRNA will also serve to increase the labeling ratio. This is only feasible of course, when the spin-labeled AA-tRNA is stable to the fractionation techniques employed. The hydroxylapatite columns described by Pearson and Kelmers (1966) were chosen for trial since their resolving power is relatively high. The success with R-Val-tRNA was encouraging and a similar column was also used for the purification of R-Phe-tRNA. The ester bond in this latter species is more labile and the column was therefore operated at a lower temperature and with a faster flow rate. The observation of two chromatographically distinct species of R-Phe-tRNA in approximate relative proportions of 1.6:1 was at first somewhat surprising, since in most tRNA fractionation procedures the pattern of Phe acceptance shows one major and one or two minor components (Pearson and Kelmers, 1966). It seems unlikely that this splitting of the Phe-tRNA population into two components is an artifact of the spin-labeling procedure since both components have essentially the same labeling ratio. However, a fractionation method involving DEAE-Sephadex chromatography has been described in which two major species of $tRNA^{Phe}$ were observed in similar relative proportions to those shown in Figure 3 (Nishimura *et al.*, 1967). A related phenomenon has been reported by Stern *et al.* (1969) who found that multiple species of undermethylated *E. coli* $tRNA^{Phe}$ which were not resolved on column chromatography in the absence of aminoacylation could be resolved when charged with phenylalanine. It thus seems that the chromatographic procedures used here have separated two degenerate nitroxide-labeled Phe-tRNAs. That they are different molecules is also suggested by the fact that the stability of the tertiary structure at the aminoacyl end is greater in species I than in species II. Another possible explanation would be the existence of two different tertiary structures for the R-Phe-tRNA species. This behavior is found for $tRNA^{Trp}$ which exhibits chromatographically separable native and denatured forms differing only in their tertiary structure (Gartland and Sueoka, 1966). However, a given sample of either species I or II may be heated and cooled several times in the absence of Mg^{2+} with reproducible results. This fact does not seem to favor a two-structure hypothesis.

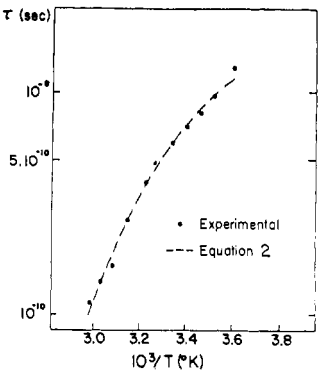


FIGURE 7: Attempt to fit the melting curve of R-Val-tRNA in 1 mM sodium acetate–1 mM EDTA (pH 5) using eq 2 in which $\tau_1^0 = 1.8 \times 10^{-12}$ sec, $\Delta_1 = 1.6$ kcal, $\tau_2^0 = 1.4 \times 10^{-21}$ sec, and $\Delta_2 = 7.8$ kcal/mole.

The data presented here and previously for the melting of R-AA-tRNA (Hoffman *et al.*, 1969) have been interpreted by a model in which the motion of the label is governed by different processes at high and at low temperatures. Furthermore, one process gives way to another at a fairly well-defined spin denaturation temperature. This temperature is strongly dependent on the ionic environment, and for Val- and Phe(I)-tRNA it follows quite closely the ionic strength dependence of the melting temperature, $T_{OD'}$, as measured by the midpoint of the temperature-dependent optical absorption at 280 mμ. However, the spin denaturation temperature, T_{sp}, differs markedly from the absorption melting temperature, T_{OD}, when the tRNA molecule is perturbed by denaturing agents such as urea or dimethyl sulfoxide.

If one chooses to discount the appearance of a discontinuity in slope and frequently in value of τ at T_{sp}, one might postulate two simultaneous, independent processes with different temperature dependencies such as two bond rotations. In such a case, ln τ *vs.* $1/T$ would follow a smooth curve. Figure 7 illustrates an attempt to fit the data for the spin melting of R-Val-tRNA using eq 2 in which the parameters τ_1^0, τ_2^0, Δ_1, and Δ_2 were adjusted by trial and error to give the best fit.

$$\frac{1}{\tau} = \frac{1}{\tau_1} + \frac{1}{\tau_2}, \quad \tau_i = \tau_i^0 e^{\Delta_i/RT} \tag{2}$$

This particular example of a melting curve was chosen because it shows no discontinuity in the value of τ at T_{sp}. Although eq 2 can be made to fit the experimental

data quite well in this instance and over this narrow temperature range, the value of τ_2^0 (1.4×10^{-21} sec) required to give this agreement is physically unreasonable. In contrast, the values of τ and Δ required by equation 1 ($\tau_1^0 = 4 \times 10^{-14}$ sec, $\Delta_1 = 5.5$ kcal, $\tau_2^0 = 5 \times 10^{-17}$ sec, and $\Delta_2 = 9.8$ kcal) are within the range of observed values for simple physical processes (Gutowsky and Holm, 1956; Conti and von Philipsborn, 1967). Values of τ^0 less than 10^{-17} sec have not been reported. Furthermore, if the transition at T_{sp} resulted from a simple physical process, it would not be expected to show a dependence on ionic strength paralleling that of T_{OD}, nor exhibit a marked interaction with Mg^{2+} as contrasted with Na^+ or K^+. The discontinuity in slope and value of τ at T_{sp} also rules out an explanation in terms of a Boltzmann distribution between a most stable state, of energy E_g, and states with different spin immobilization properties at energies $E_g + \Delta_1$ and $E_g + \Delta_2$. If the slopes of the ln τ *vs.* $1/T$ were to be interpreted as energy differences between thermodynamic states, the large energy difference would give vanishingly small Boltzmann populations of the upper states at attainable temperatures. A recent spin-label study in which the label was attached to the sulfhydryl group of Cys-tRNA (Kabat *et al.*, 1970) also showed a temperature dependence of spin mobility similar to that seen with R-Val- and R-Phe-tRNA. This suggests that the transitions observed with these labels are not restricted to the amino moiety of AA-tRNA but may represent the motion of a larger portion of the molecule including part of the RNA chain.

The similarity of values of Δ_1 to activation energies found for spins attached to poly-L-lysine and to stripped tRNA strongly suggests that the dominant motion below T_{sp} is bond rotation, and not a gradual tRNA conformational change. Although we have no similar controls for the high-temperature part of the spin-labeled tRNA melting curve, the approximate constancy of Δ_2 under all conditions where it could be reliably measured suggests that it too is related to a "simple" process. We thus arrive at the outline of a physical model for the thermal denaturation of tRNA in aqueous solution as viewed by electron paramagnetic resonance from the aminoacyl end. There is a transition at T_{sp} which is neither preceded nor followed by *measurable* changes in the movement of the spin label. In both high- and low-temperature forms, the motion of the spin label can reasonably be attributed to a bond rotation of some part of the molecule or equivalent simple processes. The aminoacyl end has a low-temperature conformation which allows motion of the label with an activation energy Δ_1. This conformation does not change measurably until, at a temperature T_{sp}, a change occurs to a high-temperature conformation in which the activation energy for motion of the label is Δ_2. No further change is measurable up to at least 65°.

An obvious model for the above behavior would be a low-temperature form of tRNA in which the final three bases of the aminoacyl end, cytosine, cytosine, and adenine (CCA), form part of a single-stranded stack of bases. The stabilizing forces of this structure would primarily be the base–base interactions. At a critical temperature which is a function of ionic strength, the bases may unstack and lead to a denatured end of the molecule associated with an altered rotational energy barrier for the spin label. It is clear that this model could also be used to describe the abrupt denaturing effects of dimethyl sulfoxide and urea previously found (Hoffman *et al.*, 1969). The spin denaturation temperature, T_{sp}, may be the same as the temperature at which the entire tRNA molecule is disrupted, as measured by changes in absorbance or optical rotation, but it may also be independent of this temperature, depending upon the environment. The evidence for this has already been presented and discussed in the previous spin-label study of tRNA denaturation (Hoffman *et al.*, 1969).

In the absence of detailed information concerning the structure of tRNA, we cannot unambiguously interpret the nature of the transition which occurs when the mobility of the spin label is so abruptly altered. The sharpness of the transition suggests a cooperative process, perhaps one involving a considerable segment of the tRNA molecule. It need not, of course, involve the entire molecule since the spin melting can be separated from the optical density melting, a process which probably involves disruption of the entire tRNA molecule. However, sharp transitions of this type have been noted previously in protein spin labeling (Smith, 1968). Their nature is unknown. Interpretation of the spin transitions in tRNA will be facilitated by further experimentation. The presence of minor bases offers the possibility of placing labels in other selected sites in purified tRNA species (Hara *et al.*, 1970). Studies such as these should provide further insight into the secondary and tertiary structure of the molecule in solution and powerfully complement structural studies carried out on tRNA crystals.

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Preparation and Characterization of Monodisperse, Cross-Linked Low Molecular Weight Deoxyribonucleic Acid*

Robert J. Cohen† and Donald M. Crothers

ABSTRACT: Fractional precipitation in an aqueous buffer–isopropyl alcohol mixture was used to fractionate calf thymus DNA according to molecular weight below about 2×10^6 daltons and a modification of this technique led to a convenient molecular weight determination. Interstrand cross-linking *via* mitomycin C, inducing reversible melting, can be accomplished down to 1.0×10^5 daltons. The T_m's and melting breadths agree qualitatively with the theoretical description of the helix–coil transition of low molecular weight DNA. Hg(II)–DNA Cs_2SO_4 density gradient ultracentrifugation led to fractionation of small molecules by base composition but did not appreciably sharpen the melting transition of the calf thymus DNA. We conclude from this latter observation that there is considerable deviation from random base sequence in sections of calf thymus DNA of 10^5 molecular weight.

The study of the kinetics and mechanism of the helix–coil transition of low molecular weight DNA (R. J. Cohen and D. M. Crothers, paper in preparation) necessitated the development of some new techniques felt to be of interest to the nucleic acid biochemist. The proposed fractional precipitation of DNA in an isopropyl alcohol–BPES[1] buffer is believed to be the first adequate molecular weight fractionation for short DNA molecules. One might mention that solubility differences in mixed solvents have long been used to fractionate synthetic polymers according to molecular weight.

Interstrand cross-linking of DNA *via* mitomycin C has heretofore been only reported for large DNA (>10^7 daltons) (Iyer and Szybalski, 1963, 1964; Szybalski and Iyer, 1964a,b; Summers and Szybalski, 1967). Mitomycin C was chosen for preparative cross-linking for two reasons: (1) among the various reagents, mitomycin C seems to produce the most thermostable links (Szybalski and Iyer, 1964a,b; Iyer and Szybalski, 1964), and (2) compared with other alkylating substances, a greater proportion of bound mitomycin is in the form of interstrand cross-link, about one-fifth to one-tenth (Szybalski and Iyer, 1964a,b). The helix–coil transition of mammalian DNA is considerably broader than that expected of an individual molecule. Marmur and Doty (1962) have found that the T_m's of various DNAs increases linearly with G + C content. So this broadening was thought to arise from the sum of the transitions of fairly large regions of differing base composition, each exhibiting a different temperature profile. It follows that for sheared DNA, there could appear a series of small molecules of varying G + C content each with a rather sharp melting transition.

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[1] Abbreviations used are: BPE, 6 mM Na_2HPO_4–2 mM NaH_2PO_4–1 mM Na_2EDTA; BPES, also has 0.179 M NaCl; HMP, 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4 adjusted to an appropriate pH by 1 M NaOH. T_m is the midpoint of the helix–coil transition using temperature as the disrupting parameter.