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Kethoxal—A Potentially Useful Reagent for the Determination of Nucleotide Sequences in Single-Stranded Regions of Transfer Ribonucleic Acid*

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ABSTRACT: β-Ethoxy-α-ketobutyraldehyde (kethoxal) is believed to react specifically with guanylic acid residues in ribonucleic acid (RNA). Evidence is presented that residues present in single-stranded regions of transfer ribonucleic acid (tRNA) react much more rapidly with kethoxal than those present in double-stranded regions. Extensive treatment with kethoxal does not cause substantial irreversible damage to tRNA. Pancreatic ribonuclease digests of kethoxal-treated RNA may be analyzed by a two-dimensional mapping procedure yielding information about nucleotide sequences containing modified guanylic acid residues.

It is well established that involvement of tRNA nucleotides in secondary structure decreases their reactivity toward chemical reagents (Penniston and Doty, 1963; Marciello and Zubay, 1964; Kisselev *et al.*, 1964; Augusti-Tocco and Brown, 1965; Hayatsu and Ukita, 1966; Knorre *et al.*, 1966). The results of these studies have been interpreted as showing that only residues which occur in nonhydrogen-bonded "loops" remain susceptible to reaction with the appropriate reagents. However, this knowledge has not yet been generally applied to the identification of specific nucleotide sequences present in looped regions of purified tRNAs. In this report, it will be shown that β-ethoxy-α-ketobutyraldehyde (kethoxal) shows considerable promise as a tool for this purpose.

It was shown by Staehelin (1959) that kethoxal reacts specifically with guanylic acid among the four major

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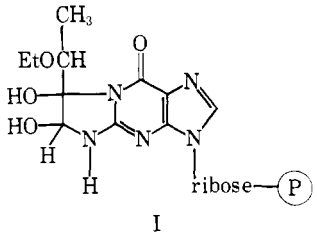
ribonucleotides. Staehelin suggested that the product has structure I. This structure is supported by the



I

recent studies of Shapiro and Hachmann (1966) on the structure of the analogous guanosine–glyoxal adduct. The kethoxal–guanylic acid adduct is stable in weakly acidic solutions, but is quite susceptible to base-catalyzed decomposition (Staehelin, 1959; Shapiro and Hachmann, 1966).

Three conditions must be fulfilled if kethoxal is to be useful as a tool for labeling looped regions of tRNA. (1) Guanylic acid residues located in double-stranded regions must be resistant to attack by the reagent. (2) Treatment with kethoxal must not cause degradation of RNA. (3) It must be possible to degrade kethoxalated RNA with appropriate nucleases and to separate the resulting oligonucleotides without undue loss of the label.

Methods

RNA. Unfractionated tRNA prepared from New Zealand Brewer's yeast was kindly provided by Dr. P. L. Bergquist.

Kethoxal. A sample of unlabeled kethoxal was kindly provided by Dr. G. E. Underwood, The Upjohn Co., Kalamazoo, Mich. ^{3}H-labeled kethoxal was synthesized essentially according to the method of Tiffany *et al.* (1957). This method involves selenium dioxide oxidation of crotonaldehyde in the presence of ethanol. [2-^{3}H]Ethanol of sp act. 0.35 mc/mmole (New England Nuclear Corp.) was used as the labeled precursor. The quantities of all reagents were scaled down tenfold from those used by Tiffany *et al.* The product was analyzed by thin layer chromatography on silica gel (Eastman Chromagram) in benzene–chloroform–ethyl acetate (2:2:1, v/v). Spots were located by spraying the chromatograms with DNP reagent or by eluting strips with ethanol and counting the eluates in a liquid scintillation counter. Our [^{3}H]-kethoxal and the Upjohn sample behaved identically on thin layer chromatography; on spraying with DNP each gave two yellow spots with R_F values of 0.0 and 0.7. Eighty per cent of the radioactivity in the tritium-labeled sample was associated with the spot whose R_F was 0.7; the remainder stayed at the origin. We suspect that the spot at the origin was due to irreversible adsorption of a portion of the kethoxal to the silica gel.

The specific activity of the labeled kethoxal was checked by allowing it to react with guanylic acid, separating the adduct from excess kethoxal and unreacted guanylic acid by paper electrophoresis at pH 1.9, and analyzing the eluate from the adduct spot by ultraviolet spectrophotometry and liquid scintillation counting. The millimolar extinction coefficient for the kethoxal–guanylic acid adduct at 252 mμ and pH 5.0 was taken as 13.7, the same as for guanylic acid (Staehelin, 1959). The specific activity of the adduct as determined in this way was 0.35 mc/mmole, as expected. The [^{3}H]kethoxal was stored as a 0.25 M aqueous solution at 4°; it was stable for at least 6 months. However, a red precipitate of selenium gradually coated the walls of the container.

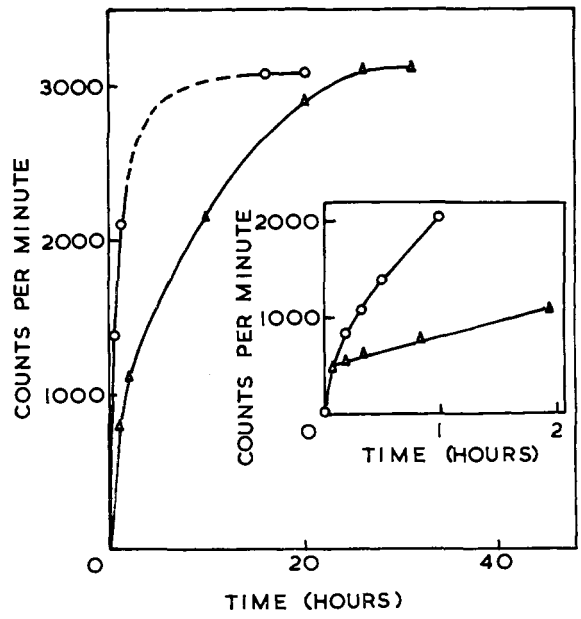


FIGURE 1: Reaction of [^{3}H]kethoxal with tRNA at pH 5.0, 37°. Reaction mixtures contained (per milliliter of final volume) 3.2 mg of tRNA, 2.4 mg of bentonite, 0.1 ml of 1 M sodium acetate (pH 5.0), 40 μmoles of [^{3}H]kethoxal, plus 10μ moles of EDTA (O———O) or $MgCl_2$ (Δ—Δ). At appropriate intervals, duplicate 10-μl aliquots were removed, added to 1 ml of ice-cold water, and precipitated with 1 ml of cold 10% TCA. The precipitates were Millipore filtered, washed, dried, and counted (see text). Each point represents the average of the duplicate determinations; deviations between duplicate samples were usually less than 5%. The zero-time value of 150 cpm has been subtracted from all points. Saturation of all guanines with kethoxal corresponds to about 3100 cpm in this experiment. The insert shows the initial phase of the reaction.

Kinetics of the Reaction between Guanylic Acid and Kethoxal. A Cary Model 15 spectrophotometer equipped with a thermostated cell compartment was used to follow the spectral change at 270 mμ which accompanies this reaction (Staehelin, 1959). To correct for slow changes in absorbance observed when kethoxal is incubated alone, the absorbance was recorded against a blank containing all the components of the reaction mixture except guanylic acid.

Reaction of [^{3}H]Kethoxal and tRNA. In preliminary

experiments, uptake of [^{3}H]kethoxal by tRNA was followed by chromatographing 50-μl aliquots of reaction mixtures on 0.8 × 25 cm columns of Sephadex G-50 with 0.05 M ammonium formate as eluent. The labeled RNA appeared in the effluent immediately after the void volume and was well separated from excess reagent. For isolating larger amounts of labeled RNA (up to 10 mg) the RNA was precipitated with ethanol, washed with 70% ethanol–0.1 M sodium acetate (pH 5.0), and redissolved in 0.2–0.5 ml of water before Sephadex chromatography.

Aliquots of fractions from Sephadex columns were counted by liquid scintillation after dissolving them in a scintillation fluid consisting of ten volumes of 0.3% PPO[1]–0.03% dimethyl-POPOP in toluene plus three volumes of 2-methoxyethanol. Counting efficiencies, as determined by a channels ratio method, were normally between 14 and 18%.

For the experiment of Figure 1, [^{3}H]kethoxal bound to tRNA was determined by precipitation in cold 5% TCA followed by filtration and washing on Millipore filters. The filters were dried for 30 min in a vacuum desiccator over P_2O_5 and KOH, immersed in scintillation fluid, and counted. The counting efficiency, determined by precipitating and counting [^{3}H]uridine-labeled rRNA of known specific activity, was 18%. This method gave results which were in good agreement with those obtained by the more laborious Sephadex procedure if tRNA concentrations were kept below 100 μg/ml during TCA precipitation.

Sedimentation Constants. These were determined with a Spinco Model E ultracentrifuge equipped with ultraviolet optics.

Pancreatic Ribonuclease Digestions. Digestion mixtures contained (per milliliter of final volume) 2 mg of kethoxalated tRNA, 0.20 ml of 1 M ammonium acetate adjusted to pH 6.5 with acetic acid, 20 μl of 0.1 M EDTA (pH 7.0), and 40 μg of pancreatic RNase (Worthington, recrystallized). After 4-hr incubation at 23°, digestion mixtures were lyophilized in a desiccator over P_2O_5 and KOH.

Fractionation of Oligonucleotides. The two-dimensional mapping system of Bergquist and Scott (1964) was used. Spots and blanks from each map were eluted in 0.5% formic acid. The eluates were dried *in vacuo* and then dissolved in 200 μl of water. Fifty microliters of each eluate was taken for determination of ^{3}H and the remainder was analyzed by ultraviolet spectrophotometry. Kethoxal was removed from oligonucleotides before spectral data were obtained with a Zeiss PMQ-II spectrophotometer equipped with a microcell attachment.

Although ultraviolet spectral data can furnish tentative identification of some of the spots, further information is clearly desirable. For this purpose, spots from maps were submitted to alkaline hydrolysis. The hydrolysates were desalted on small columns of CM-Sephadex (M. H. Vaughan, personal communication, 1966) and submitted to paper electrophoresis in 20% acetic acid (Litt and Ingram, 1964) or to thin layer electrophoresis in 0.05 M ammonium formate (pH 3.45) (Bergquist, 1965).

Some of the spots from the maps of kethoxalated tRNA digests were suspected of containing more than one oligonucleotide. In such cases, eluates were treated with ammonia to remove kethoxal and then rechromatographed in 1-propanol–water–concentrated NH_4OH (55:35:10, v/v) (Armstrong *et al.*, 1964). Spots on these chromatograms were identified by their ultraviolet spectra at pH 1 (Stanley and Bock, 1965) and by their mobilities relative to a 3′(2′)-UMP marker.

Assays of Amino Acid Acceptor Activity. These were performed using a crude amino acid–tRNA ligase preparation from yeast according to Scott (1967). Assay conditions were adjusted to give maximal incorporation of amino acids into normal tRNA.

Results and Discussion

Reaction of Guanylic Acid with Kethoxal. The reaction follows pseudo-first-order kinetics. Rate constants at several temperatures and in the presence and absence of Mg^{2+} are given in Table I. It was found that replacement of EDTA by Mg^{2+} (at 38.5°) caused a 30% reduction in the rate constant.

Integrity of tRNA Treated with Kethoxal. Ultraviolet-absorbing material soluble in cold 5% TCA was gradually generated when kethoxal was incubated with tRNA at pH 5.0 and 37°. Initially, this was taken as an indication of RNA degradation. It was then discovered that the incorporation of bentonite into the reaction

TABLE I: Pseudo-First-Order Rate Constants for the Reaction of Kethoxal with Guanylic Acid.[a]

Run	Temp (°C)	EDTA	Mg^{2+}	k (min^{-1})
1	37.0	+	−	0.138
2	21.5	+	−	0.025
3	38.5	+	−	0.162
4	38.5	−	+	0.115

[a] Reaction mixtures contained (per milliliter of final volume) 0.10 ml of 1 M sodium acetate adjusted to pH 5.0 with acetic acid, 0.10 ml of 0.1 M EDTA (pH 7.0) or 0.1 M $MgCl_2$, 0.010 ml of 4.5 M kethoxal, and 31 mμmoles of 3′(2′)-guanylic acid.

[1] Abbreviations used: PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene; TCA, trichloroacetic acid; 3′(2′)-UMP, 3′(2′)-uridine monophosphate. Abbreviations for oligonucleotides used in Table IV are similar to those of Rushizky and Knight (1960); where the sequence has not been determined, the letters are enclosed in brackets. The symbol G′ represents the kethoxal derivative of guanylic acid.

mixture greatly decreased the rate of production of this material. The bentonite had to be present in the reaction mixture; pretreatment of the kethoxal with bentonite followed by removal of the bentonite by centrifugation did not affect the rate of apparent degradation of the tRNA by kethoxal. These effects were observed with the Upjohn preparation as well as with our own ^{3}H-labeled material.

More recently, we discovered that the ultraviolet spectrum of the acid-soluble material produced in kethoxal–tRNA reaction mixtures has no peak but increases monotonically with decreasing wavelength from 300 to 230 mμ. It may be that this phenomenon is a result of the gradual coagulation of the colloidal selenium which invariably contaminates kethoxal preparations (Tiffany *et al.*, 1957). Bentonite may act by adsorbing some of the partially coagulated selenium. These speculations are supported by the observation that when an RNA–kethoxal reaction mixture was kept at 37° in the presence of bentonite for 2 weeks, a reddish-brown precipitate, presumably consisting of selenium and bentonite, had settled to the bottom of the tube.

The problem of RNA degradation by kethoxal was investigated further by incubating unlabeled kethoxal with a sample of [^{3}H]uracil-labeled RNA (kindly provided by Mr. J. D. Watson of the University of Auckland). After 20-hr incubation at pH 5.0 and 37°, there was no significant change in the amount of acid-precipitable radioactivity (Table II). It was also found that bentonite is not required to prevent degradation as assessed by this method.

TABLE II: Effect of Kethoxal on [^{3}H]Uracil-Labeled rRNA.[a]

Tube	Cpm	
1	276[b]	308[c]
2	274[b]	327[c]

[a] Incubation mixtures contained (in 0.10 ml of final volume) 0.45 A_{260} unit (7150 dpm) of RNA, 10 μl of 1 M sodium acetate (pH 5.0), and 4.5 μmoles of kethoxal. Tube 2 also contained 0.2 mg of bentonite. Aliquots of 25 μl taken at zero time and after 20-hr incubation at 37° were diluted to 0.5 ml with water and precipitated with 0.5 ml of cold 10% TCA. The suspensions were filtered through Millipore filters and the filters were dried and counted (see text). [b] At zero time. [c] After 20 hr at 37°.

A sensitive test of the integrity of the kethoxal-treated tRNA would be furnished by measuring the amino acid acceptor ability of tRNA which had first been extensively treated with kethoxal and which had then been stripped of kethoxal by exposure to weakly alkaline pH. tRNA which had been [^{3}H]kethoxalated to the extent of 1 mole/mole of guanine was incubated overnight in 0.1 M Tris-chloride at pH 7.6 and 37°. The RNA was precipitated with ethanol and washed once with 70% ethanol–0.1 M sodium acetate (pH 5.0). This procedure removed 95% of the tritium from the tRNA. The acceptor activity of the tRNA for three amino acids was then compared with that of nonkethoxalated tRNA which had been similarly incubated and precipitated. The results (Table III) show that the stripped tRNA regained about one-half of its normal acceptor activity. In other experiments, we have been unable to remove the 5% of the tritium which resists overnight incubation at pH 7.6 by the use of more extreme conditions (*i.e.*, 48-hr incubation at room temperature and pH 10.5). Nor have we been able to restore the acceptor activity of fully kethoxalated tRNA completely to its normal value. However, the experiments described above indicate that kethoxal does not cause substantial degree of damage to the integrity of the primary structure of tRNA.

TABLE III: Amino Acid Acceptor Activity of Kethoxalated and Stripped tRNA.

		Acceptor Act. (mμmoles/mg of RNA)	
Amino Acid	Normal tRNA	Kethoxalated tRNA	Stripped tRNA
Serine	3.7	0	2.3
Glycine	0.91	—	0.39
Lysine	0.83	—	0.41

Physical Properties of Kethoxalated and Stripped tRNA. Sedimentation constants of fully kethoxalated tRNA (containing 1 mole of kethoxal/mole of guanine), untreated tRNA, and tRNA which had been stripped of kethoxal by overnight incubation in 0.1 M Tris (pH 7.6) as described above were determined in a solvent consisting of 0.1 M sodium cacodylate–10^{-3} M EDTA (pH 6.5). Hypsochromicities of these tRNA samples were determined by hydrolyzing aliquots of known

TABLE IV: Some Physical Properties of Kethoxalated and Stripped tRNA.

	$s^0_{20,w}$ (S)	% Hypsochromicity
Untreated tRNA	3.6 ± 0.1	29
Kethoxalated tRNA	2.6 ± 0.1	18
Stripped tRNA	3.3 ± 0.1	24

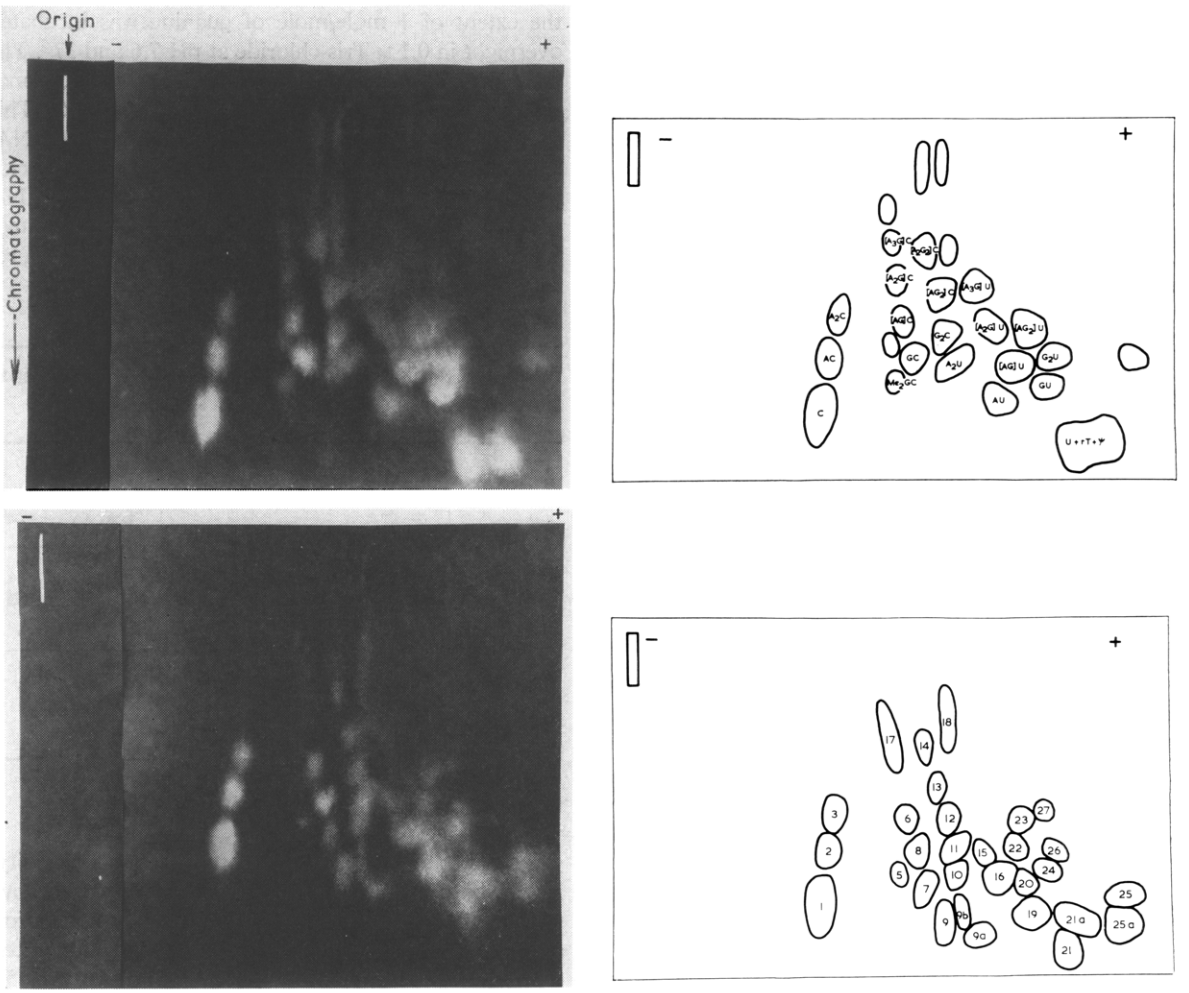


FIGURE 2: Two-dimensional maps of pancreatic RNase digests of tRNA. (a) (upper left) Untreated tRNA, ultraviolet contact print. Approximately 1.5 mg of digest in 0.2 ml of water was wet-loaded on a 2-in. line, 3 in. from the short end of an 18 × 22.5 in. sheet of Whatman No. 1 paper. Electrophoresis was in 0.2 M formic acid–10^{-4} M EDTA adjusted to pH 2.45 with NH_4OH. For descending chromatography the system 1-butanol–0.08 M formic acid–isoamyl alcohol (50:50:2, v/v) was used. (b) (upper right) Tracing of two-dimensional map (see a). The spots were identified by comparison of their positions with those of spots on reference maps (Bergquist and Scott, 1964; P. L. Bergquist, personal communication, 1967). (c) (lower left) [^{3}H]Kethoxalated tRNA. Other conditions were the same as in Figure 2a. (d) (lower right) Tracing of map of Figure 2c.

absorbance in 0.3 M NaOH for 14 hr at 37°. Hydrolysates were neutralized to pH 7.6 before determination of absorbance. The results of these studies are shown in Table IV.

The decrease in sedimentation constant coupled with the decrease in hypsochromicity brought about by extensive kethoxalation suggests that the modified tRNA has lost much of its secondary structure as a result of the blocking of the guanine hydrogen-bonding sites. As in the case of amino acid acceptor activity, removal of 95% of the kethoxal brings about only partial restoration of the normal properties of the tRNA.

Kinetics of the Reaction between tRNA and Kethoxal. The time course of the reaction of kethoxal with tRNA at pH 5.0 and 37° in the presence and absence of Mg^{2+} is shown in Figure 1. In both cases, an initial rapid reaction of about 15–20% of the guanines is followed by a slower phase in which the remaining guanines become kethoxalated. Mg^{2+} has little effect on the rapid phase of the reaction; the rates of this phase are about what could be expected for free guanylic acid. Mg^{2+} does, however, decrease the rate of the slow phase by about a factor of five relative to the rate in the presence of EDTA. We postulate that the guanines which react in the rapid phase are in single-stranded regions; the reaction rate of the remaining guanines is controlled by the fraction of the time the guanines in the double-stranded regions are accessible due to "breathing" or temporary opening of these regions. By effectively neutralizing the negative charge on the polynucleotide backbone, Mg^{2+} decreases the rate of opening of double-helical segments and hence slows the rate of kethoxalation. This interpretation is

suggested by the work of Englander and Englander (1965) who found a similar effect of Mg^{2+} on the rate of tritium exchange of tRNA.

It is possible that kethoxal acts as a denaturant in much the same way as formaldehyde (Haselkorn and Doty, 1961). This does not seem likely because we find that 0.045 M kethoxal (the same concentration as used in our kinetic studies) has no effect on the thermal dissociation of the double-stranded poly A–poly U complex.

Enzymatic Digestion and Fractionation of Labeled Oligonucleotides. By allowing the reaction to proceed for a sufficient time, it is possible to label all the guanines in tRNA with kethoxal. tRNA which had been labeled in this way to the extent of 1 mole of kethoxal/mole of guanine was used to develop methods for analysis of oligonucleotides from enzymatic digests. Pancreatic RNase seemed to be the most suitable enzyme for these degradative studies as it does not release guanylic acid as a mononucleotide and hence should give more information than T_1 RNase about guanine-containing sequences.

Contact prints of two-dimensional maps of pancreatic RNase digests of normal and kethoxalated tRNA are shown in Figure 2. Characterizations of some of the radioactive spots on the map of the kethoxalated RNA digest are given in Table V.

TABLE V: Characterization of Spots on the Map of Kethoxalated tRNA.

Spot	Nucleotide Composition[a] C	A	G	U	Sp Act. (mc/mmole)	Identity of Spot
7	7.2	6.9	8.3	—	0.36	(AG′)C
9	51	6	56	—	0.37	G′C
9[a]	8.8	—	19	—	0.59	G'_2C
19	—	6.8	6.7	6.4	0.36	(AG′)U
21		*b*			*b*	G′U, G'_2U, G'_3U[c]

[a] Millimicromoles eluted after paper or thin layer electrophoresis of alkaline hydrolysate. [b] Not determined. [c] Oligonucleotides detected after rechromatography of spot in 1-propanol–H_2O–concentrated NH_4OH (see text).

A generalization which may be drawn from a comparison of maps of normal and kethoxalated RNA is that kethoxalation of an oligonucleotide causes a slight increase in its electrophoretic mobility and a considerable increase in its R_F value. The latter effect is responsible for the accumulation of oligonucleotides in the region of spot 21 (Figure 2c,d). However, several spots containing pure kethoxalated oligonucleotides (such as G′C, (AG′)C, and (AG′)U) are present. These spots give the expected base ratios after alkaline hydrolysis and have specific activities close to the expected value of 0.35 mc/mmole of guanine (Table V).

It is evident that some of the kethoxal has been removed from the oligonucleotides during the process of digestion and fractionation. Thus, weak spots corresponding to nonkethoxalated oligonucleotides containing guanine appear on the maps of kethoxalated tRNA. The relative amounts of corresponding kethoxalated and normal oligonucleotides indicate that approximately 30–35% of the kethoxal has been removed. The loss of kethoxal probably occurs during the RNase digestion as the kethoxal–guanylic acid adduct is stable in the solvents employed for the mapping procedure. Perhaps this loss could be minimized by performing RNase digestions at a lower pH.

Conclusions

We have shown that tRNA may be fully labeled with kethoxal without causing substantial damage to the integrity of its primary structure. The kinetics of labeling, especially in the presence of Mg^{2+}, suggest that single-stranded regions are preferentially labeled during the early phases of the reaction. We would predict that regions of tRNA molecules most susceptible to nuclease attack would also be the earliest regions to become labeled with kethoxal. If such molecules were labeled to the extent of 0.10–0.15 mole/mole of guanine, one would expect that alterations in two-dimensional maps of pancreatic RNase digests would involve only a small fraction of the oligonucleotides and hence would be relatively easy to observe. We are currently attempting to carry out studies of this sort on purified yeast tRNAs of known nucleotide sequence.

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Doju Yoshikami and John Ullman contributed to the early phases of this work. We are indebted to Dr. Peter Bergquist for helpful discussions and to Miss Louise Fisher for technical assistance.

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Interaction of Heparin with the Plasma Proteins in Relation to Its Antithrombin Activity*

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ABSTRACT: The distribution of heparin among the plasma proteins has been studied by gel filtration, anion-exchange chromatography, and electrophoresis. Protein-bound heparin appears predominantly among components in the low molecular weight group of proteins isolated from Sephadex G-200. The plasma cofactor responsible for the antithrombin activity of heparin is identified in these fractions. Electrophoretic studies indicated that albumin and fibrinogen do not form stable complexes with heparin at the normal physiological level of pH. γ-Globulin and thrombin form complexes with heparin, the latter with a reduction in its activity. The antithrombin action of heparin, however, is weak in comparison with the activity of heparin with cofactor. Cofactor combines with heparin to form an active complex identified in gel filtration eluates of heparinized defibrinated plasma. The antithrombin activity of this complex is eliminated by the addition of protamine. Chromatographically the plasma cofactor is characterized by a molecular size similar to albumin and an isoelectric point similar to γ-globulin. In electrophoretic studies the peak of cofactor activity appeared in the α-2-globulins, although the activity was distributed through the α- and β-globulins. Evidence from the staining of electrophoretograms of plasma fractions suggests that cofactor may be a glycoprotein.

A number of authors have conducted investigations into the reaction of heparin with proteins and complex bases (Fischer, 1931; Fischer and Astrup, 1935; Jacques, 1943; Gorter and Nanninga, 1952a,b). The important conclusion to be drawn from these studies is that the mode of action is one of salt formation, that the reactions are reversible, dissociation taking place in accordance with the laws of mass action. While this in itself is valuable information, the studies have been conducted largely with components which are not found in the blood plasma. Therefore the information is relevant to the anticoagulant action only in so far as these physicochemical principles may have any implication.

It has long been realized that a component of the plasma is a necessary requirement for the inhibitory action of heparin in blood coagulation. The early studies of Howell and Holt (1918), Quick (1938), and Ziff and Chargaff (1940) indicated that the required "cofactor" was either albumin or a component closely related to albumin. Ferguson (1940) demonstrated that crystalline albumin had no antithrombin activity with heparin, and Chargaff *et al.* (1941) conducting electrophoretic studies found a component in globulin fractions to have such activity.

On the basis of the physicochemical principles established by Fischer and Astrup (1935) it might be expected that heparin would combine with a number of plasma proteins. However, in the reports available on this subject discrepancies exist and the relationship of the findings to the anticoagulant action of heparin is not indicated. Chargaff *et al.* (1941) studied the effect of heparin on the plasma proteins by electrophoresis and

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