Specific spin-labeling of transfer ribonucleic acid molecules.

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Received 8 September 1975

ABSTRACT

The spin labels anhydride (ASL), bromoacetamide (BSL) and carbodiimide (CSL) were used to label selectively tRNA^{Glu}, tRNA^{fMet} and tRNA^{Phe} from E.coli. The preparation and characterization of the sites of labeling of eight new spin-labeled tRNAs are described. The sites of labeling are: s²U* using ASL, BSL and CSL and tRNA^{Glu}; s⁴U using ASL and BSL on tRNA^{fMet} and tRNA^{Phe}; U-37 with CSL on tRNA^{fMet}; U-33 with CSL on tRNA^{Phe}. The rare base X at position 47 of tRNA^{Phe} has been acylated with a spin-labeled N-hydroxysuccinimide (HSL). The 3' end of unfractionated tRNA molecules has been chemically modified to a morpholino spin-labeled analogue (MSL). Their respective e.s.r. spectra are reported and discussed.

INTRODUCTION

In the past five years, the spin-labeling technique has been explored to study the conformational and functional properties of transfer RNA (tRNA) molecules. Rich and coworkers (1,2) have spin-labeled the α -amino group of Val-tRNA^{Val} and Phe-tRNA^{Phe} from E.coli and the sulphhydryl group of Cys-tRNA^{Cys} (3) to study the temperature-induced conformational perturbations of the CCA end of these tRNA molecules. Latter, the group of Nishimura (4) succeeded in spin-labeling the s⁴U residue located in the region between hU stem and CCA stem of various E.coli tRNAs. In a previous communication (5) we reported the selective labeling of the rare base s²U* at the wobble position of the anticodon of E.coli tRNA^{Glu} using a spin label anhydride (ASL).

Abbreviations used: ASL, anhydride spin label; BSL, bromoacetamide spin label; CSL, carbodiimide spin label; HSL, N-hydroxysuccinimide spin label; MSL, morpholino spin label; s⁴U, 4-thiouridine; s²U*, 2-thio-5(N-methylaminomethyl) uridine; X, 3-(3-amino-3-carboxy-n-propyl) uridine.

These spin label experiments provided valuable information about the motional freedom of the nitroxide moiety at or near the site of labeling. It would be extremely useful to prepare other labeled-tRNA molecules with spin labels located at other strategic positions of the molecule. It is exactly the goal that we had in mind when we started this project. The changes in spin mobility reflecting the behavior of a particular portion of the molecule of such tRNA derivatives would be of great value to monitor the structure-function relations in tRNA molecules.

We present here the detailed preparation and characterization of eight new spin-labeled tRNAs using the four spin labels shown below:

We have also labeled the 3'CCA end of unfractionated tRNA by carrying out the following transformations (6) of the terminal adenosine ribose ring:

Some of the specific problems encountered in preparing such tRNA derivatives and the future potential usefulness of these molecules are discussed.

EXPERIMENTAL

Materials

Unfractionated tRNA from E.coli B was purchased from Plenum Scientific Research Inc. (Hackensack, N.J.). Purified tRNA from E.coli K-12 (tRNA^{fMet}-lot.15-290, activity 100%; tRNA^{Glu}-lot.15-291, activity 78%; tRNA^{Phe}-lot.15-292, activity 69%) were kindly provided by Dr. A. Kelmers of the Oak Ridge National Laboratory.

3-(2-bromoacetamido)-2,2,5,5-tetramethyl pyrrolidine-1-oxyl (BSL) and 3-carboxy-2,2,5,5-tetramethyl pyrrolidine-1-oxyl anhydride with ethyl hydrogen carbonate (ASL) were obtained from Syva Corp. (Palo Alto, Calif.). 4-amino-2,2,6,6-tetramethyl piperidine-1-oxyl was prepared from 4-acetamido-2,2,6,6-tetramethyl piperidine-1-oxyl (Frinton Labs, S. Vineland, N.J.) according to Rosantzev (7). 1-(N-methyl-2-morpholinoethyl)-3-(2,2,6,6-tetramethyl piperidine-1-oxyl) carbodiimide p-toluenesulfonate (CSL) was synthesized following the procedure of Kumarev and Knorre (8). The N-hydroxysuccinimide ester of 3-carboxy-2,2,5,5 tetramethyl pyrrolidine-1-oxyl (HSL) was prepared according to the procedure of Anderson and Fölsch (9).

DEAE-Sephadex A-25 was obtained from Pharmacia Chemicals (Montreal, Canada); Dowex 1-X2 (Cl⁻) resin was a product of Bio-Rad Labs. (Richmond, Calif.); DEAE-cellulose was purchased from Schwarz-Mann (Orangeburg, N.Y.). RNase T₁ was a product of Calbiochem. (San Diego, Calif.). RNase T₂ and pancreatic RNase A were obtained from Sigma (St-Louis, Miss.).

Buffer solutions were made by dissolving the appropriate chemicals of the purest form available in doubly distilled deionized water.

Instrumentation

The EPR spectra were recorded on a Bruker 414S spectrometer operating at 9.5 GHz and equipped with a rectangular cavity. The microwave power was kept below 5 mW. The samples were taken into a small aqueous flat cell (Scanlon Co.). Absorbances were measured on a Perkin-Elmer Coleman 55 spectrometer.

Spin-labeling of tRNA

a) using ASL and BSL

The general procedure reported by Hara et al. (4) has been followed. A solution of 5.6 mg of spin label in 0.3 ml of methanol was added to a solution of 5.0 mg of tRNA in 5.0 ml of a buffer solution containing 5 mM Na_2CO_3 and 90 mM NaHCO_3 (pH 8.9). The resulting mixture was stirred for 5 hrs at 25°. The solution was then neutralized by addition of 1.0 M NaOAc (pH 5.0). The spin-labeled tRNA was recovered by precipitation from cold ethanol and finally taken up in 0.25 ml of a 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM MgCl_2 . The tRNA solutions were stored frozen at -20° and attempts to separate the labeled from the unlabeled tRNA molecules have been so far unsuccessful.

b) using CSL

A solution of 5.0 mg of tRNA and 20 mg of the spin label in 1.0 ml of a 20 mM Tris-HCl buffer (pH 8.3) containing 10 mM MgCl_2 was incubated at 37° for 17 hrs. (The reaction was also performed in 50 mM Tris-HCl (pH 8.3) without MgCl_2 and gave the same result.) The resulting solution was then neutralized with 0.2 M NaOAc (pH 5.0) and the spin-labeled tRNA was recovered and stored as above.

c) using MSL

According to the procedure of I. Smith and B. Malchey (private communication), 100 mg of unfractionated E.coli tRNA was dissolved in 20 ml of a 1.0 M NaOAc buffer (pH 5.0) containing 20 mM NaIO_4 . The solution was kept at 4° for 2 hrs. After neutralization with 4.0 M NaOAc, the tRNA was precipitated from cold ethanol. The precipitate was then dissolved in 10 ml of a 0.2 M Na_2CO_3 buffer (pH 9.5) containing 10% DMSO and 160 mg of 4-amino-2,2,6,6-tetramethyl piperidine-1-oxyl was added. The resulting mixture was stirred at 0° for 90 min. Then 0.75 ml of a 0.6 M NaBH_4 solution was added and the solution was stirred for 30 min., after which another 0.75 ml of the NaBH_4 solution was added and the solution was stirred for an additional 30 min. The mixture was then exhaustively dialysed against doubly distilled deionized water and the spin-labeled tRNA was recovered and stored as above.

d) using HSL

According to the procedure of Gillam et al (10) 20 mg of purified tRNA^{Phe} (E. coli) was dissolved in 2.5 ml of a 50 mM NaOAc buffer (pH 4.5) containing 10 mM MgCl₂. A solution of 60 mg of HSL in 0.5 ml of freshly distilled dioxane was added. The pH was then quickly adjusted to pH 8.0 with 1.0 N NaOH and the resulting solution was stirred at room temperature for 17 hrs. After neutralization with glacial acetic acid, the spin-labeled tRNA^{Phe} was recovered and stored as above.

Conditions for enzymatic digestions (11)

All incubations were conducted at 37°. For pancreatic RNase A digestions, 20 O.D. of tRNA and 70 µg of enzyme were dissolved in 0.5 ml of a 20 mM Tris-HCl buffer (pH 7.5) and were incubated for 16 hrs. For RNase T₁ digestions, the mixture consisted of 20 O.D. of tRNA and 50 units of enzyme in 0.5 ml of a 50 mM Tris-HCl buffer (pH 7.5) and was incubated for 18 hrs. For RNase T₂ digestions, 20 O.D. of tRNA were incubated for 6 hrs. with 2.5 units of enzyme in 0.1 ml of a 50 mM KH₂PO₄ buffer (pH 4.7).

Chromatography of tRNA digests

RNase T₁ and/or pancreatic RNase A digests of tRNA were chromatographed on DEAE-Sephadex A-25 in 7 M urea according to Rushizky et al. (12). In a typical experiment, the digest was applied by means of a peristaltic pump to a DEAE-Sephadex A-25 column (120 x 0.5 cm) preequilibrated with a 20 mM Tris-HCl buffer (pH 7.5) containing 7 M urea and 0.14 M NaCl. Elution was carried out with a linear gradient obtained with 500 ml each of 0.14 M NaCl and 0.70 M NaCl in the presence of 20 mM Tris-HCl (pH 7.5) and 7 M urea. The flow rate was approximately 10 ml/hr and fractions of 5.2 ml were collected.

RNase T₂ digests of tRNA were chromatographed on Dowex 1-X2 (CHO₂⁻) according to Offengand et al. (13). In a typical procedure, the digest was applied by means of a peristaltic pump to a Dowex 1-X2 (CHO₂⁻) column (30 x 0.5cm) preequilibrated with a 10 mM NH₄CHO₂ buffer (pH 3.7). Elution of s²U* was performed with a linear gradient established with 100 ml each of 10 mM and 40 mM NH₄CHO₂ (pH 3.7). The flow rate was maintained at 10 ml/hr and 3.5 ml fractions were collected.

Desalting of chromatographic fractions

Using the procedure reported recently (14), mono-, di- and trinucleotides were desalted by means of Dowex 1-X2 (HCO_3^-) resin and 2.0 M NH_4HCO_3 . For higher oligonucleotides, DEAE-cellulose was used instead of Dowex. Solutions containing salt free oligonucleotides were then concentrated in a rotary evaporator.

Thin layer chromatography

Analysis of oligonucleotides obtained from column chromatography were performed using a two-dimensional thin layer chromatographic system (11). Oligonucleotides were first hydrolysed by incubation at 37° for 6 hrs with 1.0 N NaOH (total volume 0.5ml). Commercial cellulose plates (Eastman #6065) were used. The plate was eluted in the first dimension with isobutyric acid-0.5 M NH_4OH (5:3) and with isopropyl alcohol-conc. $\text{HCl-H}_2\text{O}$ (70:15:15) in the second. The U.V. absorbing spots were eluted from the plate with water.

RESULTS

Using the procedures described in the Experimental Section, E.coli $\text{tRNA}^{\text{fMet}}$, tRNA^{Phe} and tRNA^{Glu} were successfully spin-labeled respectively with ASL, BSL and CSL. In addition, E.coli tRNA^{Phe} was coupled to HSL and the 3' end terminal adenosine of unfractionated E.coli tRNA was transformed into MSL. All those chemical modifications were conducted in conditions such that the conformational integrity of the different tRNA species is assumed to be preserved. The EPR spectra obtained from these eleven spin-labeled tRNA molecules are depicted in Figure 1 and are typical of molecules carrying only one spin label (1-5).

In order to be able to draw local conformational data from these spectra, it is obvious that the exact location of the spin label on each tRNA molecule has to be known.

a) Characterization of ASL-, BSL- and CSL- $\text{tRNA}^{\text{fMet}}$

According to Hara *et al.* (4), BSL reacts specifically with the thio nucleotide s^4U of E.coli tRNAs and the disappearance of the s^4U -characteristic absorbance at 330 nm is an indication of its modification. The elution patterns obtained from column chromatography of the RNase T_1 digests of the three spin-labeled $\text{tRNA}^{\text{fMet}}$ as well as that of the control native

Fig. 1 EPR spectra of spin-labeled tRNAs at $\sim 22^{\circ}$ in 0.02 M Tris-HCl (pH 7.5) containing 10 mM MgCl_2 .

tRNA^{fMet} are illustrated on Figure 2.

As seen in the figure, both ASL- and BSL-tRNA^{fMet} give the same elution profile in which there is no absorbance at 330 nm under peak 4, the relative intensity of which is strongly decreased as compared to same peak in the control pattern. On the other hand, a large increase in relative intensity is apparent in peak 1b whose fractions, after desalting, yielded an EPR spectrum representative of a spin-labeled oligonucleotide (5). Analysis of this tRNA fragment by two-dimensional T.L.C. on a cellulose plate afforded the sequence GGGG⁴U in both cases. Consequently the residue s⁴U-8 is the site of labeling in both ASL- and BSL-tRNA^{fMet}.

The CSL-tRNA^{fMet} digest gives an elution profile (Fig. 2c) that is almost identical to that of the control except for the

Fig. 2 Elution patterns of RNase T_1 digest on DEAE-Sephadex A-25. A) native tRNA^{fMet}; B) ASL-, BSL-tRNA^{fMet}; C) CSL-tRNA^{fMet}. The arrow indicates the peak containing the spin-labeled material.

Fig. 3 Elution patterns of RNase T_1 digest on DEAE-Sephadex A-25. A) native tRNA^{Phe}; B) ASL-, BSL-tRNA^{Phe}. The arrow indicates the peak containing the spin-labeled material.

loss in relative intensity that is apparent in peak 11 and which is accompanied by an increase of about the same order of magnitude in relative intensity in peak 8. After desalting, the material obtained from peak 8 gave an EPR spectrum related to a spin-labeled oligonucleotide (5). Analysis of the material recovered from peak 11 of the control elution yielded the fragment $C_m 2UCAUAACCCG$ of the anticodon region of $tRNA^{fMet}$ (15). Such an analysis performed on the paramagnetic material obtained from peak 8 showed also the presence of that same anticodon fragment. Chang's results on the chemical modification of $tRNA^{fMet}$ (16) showed that there are two nucleotides in that macromolecule that are extensively reactive toward a carbodiimide reagent, namely U-37 in the anticodon and U-47 in the miniloop. Accordingly, we conclude from the above mentioned results that U-37 in the anticodon is the location of CSL in $tRNA^{fMet}$. No sign of labeling on U-47 has been found.

b) Characterization of ASL-, BSL- and CSL- $tRNA^{Phe}$

The elution patterns afforded by chromatography of the RNase T_1 digests of both ASL- and BSL- $tRNA^{Phe}$ along with that of the control native $tRNA^{Phe}$ are shown in Figure 3.

The two derivatized $tRNA^{Phe}$ yield the same profile in which absorbance at 330 nm is absent as in the case of ASL- and BSL- $tRNA^{fMet}$. The argumentation used then still holds here and it is concluded that s^4U-8 is the site of labeling in both ASL- and BSL- $tRNA^{Phe}$.

The case is however different with CSL- $tRNA^{Phe}$. Figure 4 shows the elution profile of a pancreatic RNase digest of the spin-labeled $tRNA^{Phe}$ together with that of the control native $tRNA^{Phe}$.

It is seen in the former that the almost complete disappearance of peak 6 coincides with an equivalent increase in relative intensity of peak 2. After desalting, the material recovered from peak 2 gave an EPR spectrum consistent with a spin-labeled oligonucleotide (5). Analysis of the paramagnetic fragment yielded the sequence $UGAAA^*A\psi$ located in the anticodon region of $tRNA^{Phe}$ (17). However, considering the normal mode of action of pancreatic RNase (18), this fragment should have been $GAAA^*A\psi$. The presence of an uridine (U-33) at the 5' end of this fragment can only

Fig. 4 Elution patterns of pancreatic RNase A digest on DEAE-Sephadex A-25. A) native tRNA^{Phe}; B) CSL-tRNA^{Phe}. The arrow indicates the peak containing the spin-labeled material.

be explained if this residue is the site of labeling since it is known that pancreatic RNase does not operate on a carbodiimide-modified nucleotide (19). Thus, on the basis of all present data, U-33 in the anticodon appears to be the site of labeling of CSL on tRNA^{Phe}.

c) Characterization of BSL- and CSL-tRNA^{Glu}

Both BSL- and CSL-tRNA^{Glu} pancreatic RNase digests give an elution profile that is identical to that of control native tRNA^{Glu}. Figure 5 shows this profile and the peak under which an EPR signal is found.

However, analysis of the material contained in this peak did not provide results significant enough to arrive unambiguously at the site of labeling. These elution profiles show nevertheless that only one site of labeling is involved.

Since previous spin-labeling experiments on tRNA^{Glu} showed that the odd nucleotide s²U*-35 was much reactive toward ASL (5), we proceeded to isolate this residue from both BSL- and CSL-

Fig. 5 Elution pattern of pancreatic RNase A digest on DEAE-Sephadex A-25 for native, BSL and CSL-tRNA^{Glu}. The arrow indicates the peak containing the spin-labeled material.

tRNA^{Glu} as well as from native tRNA^{Glu}. This was done by complete digestion of the three tRNA involved with RNase T₂ and chromatography of these digests on Dowex, according to Carbon *et al.* (20). The material recovered from both BSL- and CSL-tRNA^{Glu} gave an EPR spectrum that is typical of a spin-labeled nucleotide (5), and the material isolated from native tRNA^{Glu} yielded a U.V. spectrum in accordance with the one reported for s²U* (20). This authentic s²U* was then divided in two parts that were respectively spin-labeled with BSL and CSL using the experimental conditions in which was labeled the whole tRNA molecule. Both BSL- and CSL-s²U* were isolated by chromatography on Dowex (20). The U.V. spectra of these spin-labeled s²U* and of the paramagnetic material isolated respectively from BSL- and CSL-tRNA^{Glu} are presented on Figure 6.

Fig. 6 U.V. spectra (water, pH 3.7) A) (—) authentic s²U*; (---) CSL-s²U*; (...) BSL-s²U*; B) paramagnetic material isolated from (---) CSL-tRNA^{Glu}; (...) BSL-tRNA^{Glu}. Absorbance at 200 nm is set arbitrarily to zero.

As seen on the figure, each set of spectra is in accordance inter se and we conclude from these results that s^2U^{*-35} is the site of location of both BSL and CSL on $tRNA^{Glu}$. The Figure also suggests that BSL reacts with the thio group of s^2U^* while CSL prefers the amino group.

d) Characterization of HSL- $tRNA^{Phe}$

According to Friedman et al. (21), the odd nucleotide X-47 is easily acylated with the N-hydroxysuccinimide ester of carboxylic acids. It is also known that the trinucleotide m^7GXC located in the miniloop of $tRNA^{Phe}$ is resistant to either RNase T_1 and pancreatic RNase (17). Accordingly a combined RNase T_1 and pancreatic RNase digest of HSL- $tRNA^{Phe}$ yielded after chromatography an elution profile consisting of only four peaks.

The third one gave, after desalting, an EPR spectrum identical to those reported for a spin-labeled oligonucleotide (5).

Since such a combined digestion is expected to afford only one trinucleotide and since the peak considered to contain trinucleotides in the elution profile provides a paramagnetic material, we infer that X-47 is the location of HSL on $tRNA^{Phe}$.

e) Characterization of MSL-E.coli tRNA

The transformation used to prepared MSL-E.coli tRNA can only take place on the 3'end terminal adenosine since it alone bears a free, 1,2-diol group. On the other hand, this residue is the only nucleoside that is present in a pancreatic RNase digest of a tRNA molecule and consequently it is eluted with the void volume of the column. Accordingly, the pancreatic RNase digest of MSL-E.coli tRNA was chromatographed on DEAE-Sephadex and the fractions contained under the very first peak were combined and exhaustively extracted with chloroform. The organic solution, once concentrated gave an EPR spectrum related to a spin-labeled nucleotide (5). We then conclude from this that the 3'end terminal adenosine was indeed transformed into its morpholino derivatives MSL.

DISCUSSION

Table 1 summarizes the results and gives the location of labeling and the nature of the attachment of the labels as based on previous results (4-6,10,16). For all the elution patterns, only

one peak contains the paramagnetic material, the analysis of which showing that in all cases only one position on the tRNA molecule carries the label, and this in spite of the fact that other groups are also susceptible to react. This behavior might reflect the fact that the high selectivity of the reactions is directly a consequence of the particular reaction conditions. Of course, what is most important for further studies is the exact location of the label rather than the nature of its attachment. Furthermore, all our spin-labeled tRNAs proved to be stable up to several months at -10°C as no detectable changes occurred in their respective EPR spectra. Estimation of the yield of the reactions using ASL, CSL and HSL by spin concentration (5) gives a lower limit of 12 to 35% of labeling, depending on the tRNA molecules and spin labels used. Alternatively, the loss of absorbance at 330 nm in Figs 2 and 3 suggests that for BSL the yield of the reactions are very high. Moreover, carbodiimide reactions with exposed residues usually go very well (16). With MSL, the reaction is nearly quantitative.

TABLE I: Results of Spin-labeling of the tRNA Molecules		
tRNA and labels	Site of labeling	τ_c (nsec)
tRNA ^{Glu}		
ASL	NH side chain of s^2U^* ; anticodon loop	1.65 (5)
BSL	S of s^2U^* ; anticodon loop	2.10
CSL	NH side chain of s^2U^* ; anticodon loop	0.35
tRNA ^{fMet}		
ASL	on s^4U ; between 5'end and hU stems	1.38
BSL	S of s^4U ; between 5'end and hU stems	2.56
CSL	on U-37; anticodon loop	0.30
tRNA ^{Phe}		
ASL	on s^4U ; between 5'end and hU stems	2.34
BSL	S of s^4U ; between 5' end and hU stems	2.40
CSL	on U-33; anticodon loop	0.36
HSL	side chain of rare base X-47; miniloop	1.68
Unfractionated tRNA		
MSL	on modified A at the 3'end	1.28

The shape of these EPR spectra (Fig. 1) reflects the mobility of the spin labels at their respective site of attachment. Assuming that a good correlation exists between the degree of immobilization of the spin label and the space occupied by the label and that the native conformation of the tRNA molecule is still preserved in the presence of the spin label attached to the nucleotide residue, the following observations can be made. The EPR spectra of spin-labeled tRNAs indicate only partial constraint of the spin label; the degree of constraint is expressed by their respective rotational correlation time (τ_c) as indicated in Table I. None of the spectra are strongly immobilized. However, our spectra of BSL-tRNA^{fMet} and BSL-tRNA^{Phe} are appreciably more immobilized than those published by Nishimura and coworkers (4). This could be a consequence of the presence of Mg^{+2} ions present in our preparations imposing a greater interaction of the label with some parts of the tRNA molecule.

In the case of CSL-tRNAs, the spectra are very mobile and corresponding τ_c values are small. This could be attributed to the bulkyness of the label which forces it to be oriented away from the backbone of the tRNA molecule. The label MSL is directly attached to the 3'-CCA end of the tRNA molecules and the shape of the EPR spectrum indicates that this region is quite flexible. Furthermore, the small τ_c value for HSL-tRNA^{Phe} suggests that the miniloop region is more accessible to the solvent than the region surrounding the s⁴U residue.

Since we have not as yet succeeded in separating pure labeled tRNA from unlabeled tRNA in the reaction mixture, amino acid acceptance activities are of little significance at this point. However, we have done the amino acid acceptance activity on our mixture of ASL-tRNA^{Glu} and BSL-tRNA^{Phe} and found that only the latter remains fully active as already shown by Nishimura's work (4). Nevertheless we are confident, however, that other spin-labeled tRNAs will retain most of their original biological activities. Yang and Söll (22) have shown for instance that a variety of fluorescent-labeled tRNAs, labeled at position similar to our spin labels, retain from 10 to 30% of aminoacyl activity.

Fig. 7 summarizes schematically the location of the site of labeling of each spin label used in this study. This diagram speaks by itself. Such a distribution of spin labels at these

very strategic regions of the tRNA molecules emphasizes further work, on the local effects of Mg^{+2} ions and various enzymes and factors responsible for the structure and function of the tRNA molecules. Therefore, the spin label method should be a useful tool to study conformational transitions in tRNA molecules in solution since they bear direct relevance to the molecule's biological activity. In this respect, a detailed study of the thermal denaturation of these spin-labeled tRNAs are presented in the following paper (24).

Fig. 7 The different sites of spin-labeling on the three tRNAs using the static model deduced by Rich (23) from his X-ray work.

Acknowledgements. This research was supported by a grant from the National Research Council of Canada (NRCC). One of us (M.C.) acknowledges the NRCC for financial support in the form of a post-graduate scholarship. We would also like to thank the technical assistance of Miss L. Ayotte.

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