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A REVERSIBLE OXIDATIVE INACTIVATION OF SPECIFIC TRANSFER RNA SPECIES

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The exposure of transfer RNA (sRNA) from either *E. coli* or rabbit liver to solutions of dilute iodine-KI (pH 7.0) results in an extremely rapid partial inactivation of acceptor ability for several amino acids. Two of the interesting aspects of this finding are that, in every case, a residual amount of acceptor ability remains entirely resistant to iodine inactivation, and that the inactivated fraction can be reactivated by exposure to reducing agents. It is the purpose of this communication to show that certain of the sRNA molecules specific for a particular amino acid contain an easily oxidized functional group, and that these sRNA species are only active in the reduced state. Evidence will also be presented to show that a 2-thiopyrimidine nucleotide, easily oxidized by iodine, is present in hydrolysates of *E. coli* sRNA. This represents the first evidence for the existence of sulfur-containing bases, synthesized *de novo* from inorganic sulfate, in normal bacterial sRNA. (See *Note added in proof.*)

Materials and Methods.—*E. coli* (strain B) sRNA was isolated and purified as described previously,[1] except that the crude sRNA was purified by elution from DEAE-cellulose.[2] Rabbit liver sRNA was prepared by the method of Brunngraber.[3] Labeled amino acids were purchased from Schwarz BioResearch, Inc., and sodium S^{35}-sulfate was obtained from Abbott Laboratories, Oak Ridge.

The preparation of a partially purified activating enzyme from *E. coli* and the assay of sRNA samples for total amino acid acceptor ability have been described.[4] An activating enzyme preparation from rabbit liver was prepared by the method used by Holley *et al.*[2] for rat liver. All enzymes were stored under liquid nitrogen.

Iodine treatment of sRNA: To a solution of sRNA (5 mg/ml) in 0.01 *M* Tris-HCl (pH 7.0) was added an equal volume of 1.0 mM iodine in 0.5% KI-0.01 *M* Tris-HCl (pH 7.0). After standing at 0° for 15–20 min, the sRNA was precipitated by the addition of 1/10 vol 20% potassium acetate (pH 5.0) and 2 vol of ethanol. The RNA was isolated by centrifugation and freed from iodine by repeated precipitations from 1 *M* sodium acetate (pH 5.0) with ethanol. In most cases the RNA was then dialyzed overnight against 2000 ml of 0.01 *M* Tris-HCl (pH 8.0), although this resulted in little change in amino acid acceptor ability. The time of exposure of the sRNA to the iodine is not critical; samples treated for 5 min give values for acceptor ability

very similar to those treated for 60 min. Control samples were always prepared by treatment of sRNA under the same conditions in the absence of iodine.

Quantitative determination of iodine destroyed by sRNA: (1) *By direct titration:* The reaction of iodine with *E. coli* sRNA is sufficiently rapid to allow direct titration of the RNA with accurately standardized iodine solutions (0.5–1.0 mM I_2-0.5% KI) in 0.01 *M* Tris-HCl (pH 7.0) using a starch indicator. Best results were obtained by titrating 2–5 mg sRNA in 200 μl buffer plus 50 μl 1% starch to a pale blue color that persists for at least 15 sec, using a microburet capable of being read to the nearest μl (Manostat Corp., New York). (2) *By back titration:* Solutions of sRNA (2–10 mg) in 0.6 ml of 0.01 *M* Tris-HCl (pH 7.0) were allowed to stand 30 min at 0° with an excess of dilute iodine-KI present. Blanks were set up in a similar manner without RNA. Residual iodine was then determined by the addition of a known quantity of standard arsenite solution plus 0.2 ml saturated $NaHCO_3$, followed by back titration of the excess arsenite with standardized iodine-KI solution to a starch end-point.

Preparation of S^{35}-labeled sRNA: *E. coli* (strain B) was inoculated into a sterile basal salts medium consisting of the following (in 1000 ml): NH_4Cl, 2 gm; NaCl, 5 gm; KCl, 370 mg; tap water, 300 ml; Na_2HPO_4, 120 mg; KH_2PO_4, 60 mg; $FeCl_3$, 0.8 mg; glucose, 2 gm; Tris-HCl, pH 7.2, 40mm oles; $MgSO_4$, 0.1 mmole; and $Na_2S^{35}O_4$, 2.5 mc (carrier-free). After incubation at 37° with constant agitation for 16–18 hr, the cells were harvested by centrifugation, washed, and the sRNA isolated by the phenol method.[1] At least three phenol extractions were used to ensure thorough removal of protein. The crude sRNA was stripped of amino acids by incubation in 0.1 *M* glycine (pH 10.3) for 30 min at 37°, and purified by chromatography over DEAE-cellulose.[2]

Results.—Partial inactivation of acceptor ability by iodine: The treatment of sRNA with agents capable of reacting grossly with any one of the major nucleotides results finally in complete destruction of amino acid acceptor ability. For example, both bromination[5, 6] and nitrous acid deamination[4, 7] of sRNA will completely de-

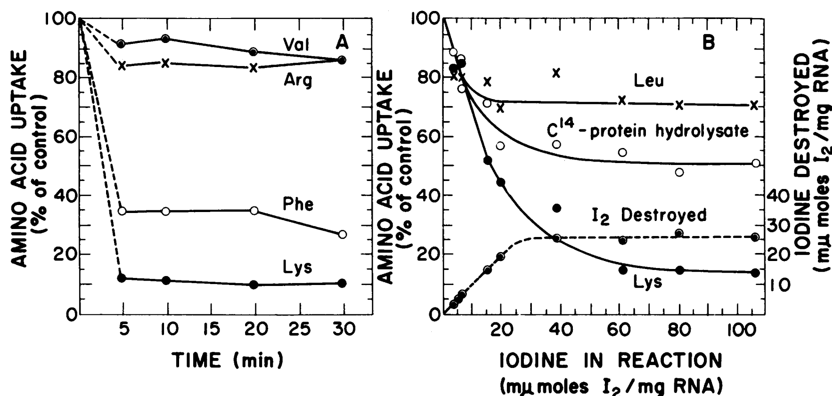

FIG. 1.—Inactivation of amino acid acceptor ability of *E. coli* sRNA by iodine as a function of time (*A*) and of iodine concentration (*B*).

(*A*) The iodine treatment of sRNA was carried out using 1.0 mM I_2-0.5% KI as described in *Materials and Methods*, except that aliquots were withdrawn at 5, 10, 20, and 30 min, precipitated, washed, and dialyzed as described. Control samples were carried through the same procedure without the addition of iodine. Assays for amino acid uptake of the samples were carried out as described elsewhere,[4] using C^{14}-L-lysine (20.8 μc/μmole), C^{14}-L-phenylalanine (22.4 μc/μmole), C^{14}-L-arginine (9.3 μc/μmole), and C^{14}-L-valine (8.8 μc/μmole).

(*B*) *E. coli* sRNA (5 mg/ml) was treated with various concentrations of I_2-KI in 0.01 *M* Tris (pH 7.0) at 0° for 30 min. Aliquots were then withdrawn and the RNA was precipitated and washed in the usual manner. The amount of iodine destroyed in the various reactions was determined on the remaining reaction mixtures by the back-titration method described in *Materials and Methods*. At concentrations of iodine of 20 mμmoles I_2/mg RNA and below, all of the iodine was destroyed by the RNA, as evidenced by adding a starch indicator. Assays for amino acid acceptance ability were carried out as described,[4] using C^{14}-L-leucine (20 μc/μmole), C^{14}-L-lysine (20 μc/μmole), and a C^{14}-algal protein hydrolysate (1.48 mc/mg).

stroy acceptor ability if time is allowed for reaction to occur at every sensitive site. However, the exposure of sRNA to dilute iodine solution at neutral pH follows an entirely different course. As shown in Figure 1*A*, the acceptor ability of the sRNA falls rapidly to some value characteristic of the amino acid in question, and then appears to be resistant to further inactivation by the reagent. Although the data shown in Figure 1*A* do not show the kinetics of the inactivation process before 5 min, the reaction is apparently extremely rapid, and, in fact, is complete in less than 1 min at 0°.

Two possible interpretations of this type of inactivation process are that we are dealing with an equilibrium process and that an increase in the iodine concentration should drive the reaction further to completion, or that the population of sRNA molecules specific for any amino acid is heterogeneous and consists of molecules containing an iodine-sensitive group and others lacking such a group. The first possibility is easily eliminated by demonstrating that the iodine-resistant fraction of any particular acceptor is not decreased by an increase in the iodine concentration above that at which the amount of acceptor ability begins to level off (Fig. 1*B*). In fact, in other experiments, the iodine level has been increased to 10 μmoles I_2 per mg RNA without affecting the activity of the resistant fraction. Evidence in favor of the second possibility will be presented below.

Note that the iodine destroyed by the RNA levels off at approximately 25 mμmoles I_2 per mg sRNA, as determined by the titration methods described above. This value has proved to be extremely constant and has varied only from 22–28 mμmoles I_2/mg RNA for several batches of *E. coli* sRNA. It is significant that the reduction in acceptor ability of the sRNA levels off at about the same point (20–30 mμmoles I_2/mg RNA), providing evidence that the reaction measured by iodine destruction is the same as that causing the loss in acceptor ability.

Assuming a molecular weight for *E. coli* sRNA of 27,000,[8] the stoichiometry of the iodine reaction amounts to 0.6–0.7 moles I_2 destroyed per mole sRNA. Since approximately one half of the total acceptor ability is inactivated by the iodine reaction (Fig. 1*B*), this value is in agreement with the concept that only those sRNA molecules capable of reacting with a single molecule of iodine are inactivated by the reagent.

Nature of the iodine-sRNA reaction: It might be argued that the iodine is reacting not with the RNA itself, but only with traces of protein associated with the preparations. The data presented in Table 1 render this possibility unlikely since (1) the amount of iodine destroyed by sRNA is not reduced by exhaustive phenol extractions of the material, (2) the iodine reactivity of ribosomal RNA is quite low even though protein contamination is similar to the sRNA samples, and (3) all of the iodine-reactive material is rendered dialyzable by 0.3 *M* KOH at 37°, conditions which easily hydrolyze polyribonucleotides but which do not hydrolyze proteins. The fact that the iodine reactivity is not destroyed by alkaline hydrolysis, but is rendered dialyzable, is important and has been made use of in attempts to isolate this material (see below).

When sRNA is treated with I_2^{131}-KI^{131} solution, only minute amounts of I^{131} appear associated with the RNA after the usual precipitation and dialysis procedures are carried out. This bound I^{131} amounts to 1.0–1.2 mμmoles I_2/mg sRNA or about 0.03 moles I_2/mole RNA, a small percentage of the total I_2 which reacts with the

TABLE 1

IODINE REACTION WITH VARIOUS RNA SAMPLES

RNA	Mμmoles I_2/mg RNA
E. coli sRNA (2 × phenol extracted)	23 ± 1
E. coli sRNA (6 × phenol extracted)	25 ± 2
E. coli sRNA (neutralized KOH hydrolysate)	27 ± 2
E. coli sRNA (dialyzed KOH hydrolysate)	0
Yeast sRNA	8.7 ± 0.2
Rabbit liver sRNA	10 ± 1
E. coli ribosomal RNA	1.7 ± 0.1
Yeast ribosomal RNA	<1
I_2^{131}-binding by *E. coli* sRNA	1.1 ± 0.1

The RNA samples were titrated with I_2-KI solution in 0.01 *M* Tris-HCl, pH 7.0, as described in *Materials and Methods*. Each sample had a minimum of two phenol extractions unless specified otherwise. The sRNA preparations were purified by gradient elution from DEAE-cellulose.[2] The iodine-binding ability of sRNA was measured by treating sRNA (5 mg/ml) with 1.67 mM I_2–5.76 mM KI–0.01 *M* Tris (pH 7.0) containing 300 μc of I^{131}. After 30 min at 0° the RNA was isolated and washed by multiple precipitations in the usual manner. Dialysis at 4° against 0.02 *M* Tris (pH 8.0)–0.002 *M* $MgCl_2$ for 21 hr did not remove the bound I^{131} although 80% of the radioactivity was released from the RNA after 16 hr at 37° in 0.1 *M* Tris (pH 8.9).

RNA as determined by chemical analysis. Although this bound iodine remains associated with the sRNA during DEAE-cellulose chromatography, it is readily released from the RNA in 0.1 *M* Tris (pH 8.9) at 37°. These facts make it extremely unlikely that we are dealing primarily with an iodination reaction to form a carbon-iodine bond. Other workers[9] have shown that iodine-KI solutions will not iodinate the four common ribonucleotides at neutral pH.

The data presented in Table 2 show that the inactivation can be reversed by treatment of the sRNA with reducing agents such as sodium thiosulfate or reduced glutathione. Note that dialysis at pH 10.3 or incubation at pH 8.9, conditions which easily remove the bound iodine, will not restore the acceptor ability. We are apparently dealing with an oxidative process which can be reversed by treatment of the oxidized sRNA with an appropriate reducing agent. These data are strongly suggestive of the oxidation of a mercapto function to form a disulfide, a reaction which is known to occur rapidly with iodine and to be reversed by thiosulfate and reduced mercapto compounds.

Fractionation studies on the oxidized sRNA: Sueoka and Yamane[10] have shown that the fractionation of labeled aminoacyl-sRNA on methylated albumin-Kiesel-

TABLE 2

EFFECT OF VARIOUS TREATMENTS ON THE AMINO ACID ACCEPTOR ABILITY OF *E. coli* sRNA

	C^{14}-Lysine (cpm/mg RNA)			C^{14}-Phenylalanine (cpm/mg RNA)		
	Normal	Oxidized	%	Normal	Oxidized	%
None (control)	1500	260	17	1150	280	24
Dialysis versus 0.01 *M* Tris, pH 7.0	1150	265	23	930	300	32
Dialysis versus 0.2 *M* glycine, pH 10.3	1265	320	25	995	360	36
0.2 *M* glycine, pH 10.3, 37°, 1 hr	1230	250	20	1000	270	27
0.1 *M* sodium thiosulfate	1230	1000	81	1015	1030	100
0.1 *N* sodium arsenite	1275	275	22	1090	290	27
0.1 *M* L-cysteine	1240	290	23	1080	840	78
0.1 *M* reduced glutathione	1380	880	64	1010	875	87

Oxidized sRNA was prepared by the iodine treatment described in *Materials and Methods*. Iodine was carefully removed by multiple precipitations of the sRNA from 1 *M* sodium acetate (pH 5.0). Dialysis experiments were for 16 hr at 4°. The control and oxidized samples of sRNA were exposed to the various reducing agents at a concentration of 5 mg RNA per ml at 0° for 1 hr, and then washed by multiple precipitations in the usual manner. Assays for C^{14}-amino acid uptake were as described previously.[4] The values reported represent maximal uptake of the amino acid and are the averages of three determinations. The C^{14}-amino acids were used at a specific activity of 20 μc/μmole.

guhr (MAK) columns[11] is capable of resolving multiple acceptors for several of the amino acids. We have made use of this technique to demonstrate that oxidation of sRNA with iodine results in selective inactivation of at least one of a group of acceptors specific for a particular amino acid (Fig. 2).

For example, H^3-lysine was charged onto a sample of normal rabbit liver sRNA and then mixed with a sample of C^{14}-lysyl-sRNA, prepared from iodine-treated sRNA (rabbit). When applied to the MAK column and eluted with a linear gradient in NaCl,[10] the acid-insoluble tritium counts appeared as two well-resolved peaks followed by a third smaller component (Fig. 2*A*). However, the second of the two large peaks was completely absent in the C^{14}-lysyl-sRNA, prepared from oxidized material. A similar fractionation of a sample of C^{14}-lysyl-sRNA (rabbit), prepared from sRNA that had been iodine-treated and then *reactivated* by sodium thiosulfate, again showed both main components (Fig. 2*B*). It is apparent that the lysine-specific sRNA from rabbit liver consists of at least three components, only one of which can be reversibly inactivated by iodine.

Isolation of a 2-thiopyrimidine derivative from E. coli sRNA hydrolysates: It was pointed out above that alkaline hydrolysates of *E. coli* sRNA are capable of reacting with as much iodine as is the intact RNA (Table 1). Fractionation of the hydrolysate over Dowex-1 (formate), followed by direct I_2 titration of the material in each of the peaks, reveals only one peak (labeled *3* in Fig. 3) capable of reacting with iodine. This peak invariably appears between the adenosine and CMP peaks in Dowex-1 (formate) separations of *E. coli* sRNA hydrolysates and accounts

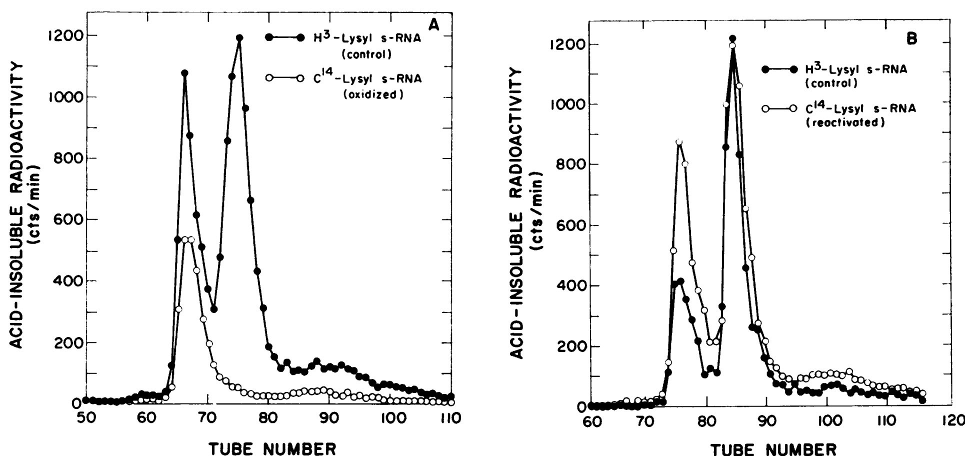

FIG. 2.—Fractionation of mixtures of H^3-aminoacyl-sRNA (normal) and C^{14}-aminoacyl-sRNA (oxidized) on MAK columns; (*A*) H^3-L-lysyl-sRNA (normal) and C^{14}-L-lysyl-sRNA (oxidized), rabbit liver; (*B*) H^3-L-lysyl-sRNA (normal) and C^{14}-L-lysyl-sRNA (oxidized, then reduced with thiosulfate), rabbit liver. MAK columns were prepared and fractionations carried out as described by Sueoka *et al.*,[10] except that the column size was 1.8 × 17 cm, 2-ml fractions were collected, and a shallower linear gradient was employed, using 150 ml 0.2 *M* NaCl, 0.05 *M* phosphate (pH 6.7) in the mixer, and 150 ml 1.0 *M* NaCl, 0.05 *M* phosphate (pH 6.7) in the reservoir. After adding 1 mg carrier RNA to each tube, an equal volume of cold 10% TCA was added, and the precipitates were collected on membrane filters and counted as previously described.[10]

Oxidized sRNA was prepared as described in *Materials and Methods*. A portion of this material was reactivated by 0.1 *M* sodium thiosulfate as described in the legend to Table 2. The washed and dialyzed RNA samples were charged with a labeled amino acid in the presence of the other 19 C^{12}-amino acids as described by Sueoka *et al.*[10] The labeled amino acids had the following specific activities: C^{14}-L-lysine, 166 μc/μmole; H^3-L-lysine, 200 μc/μmole.

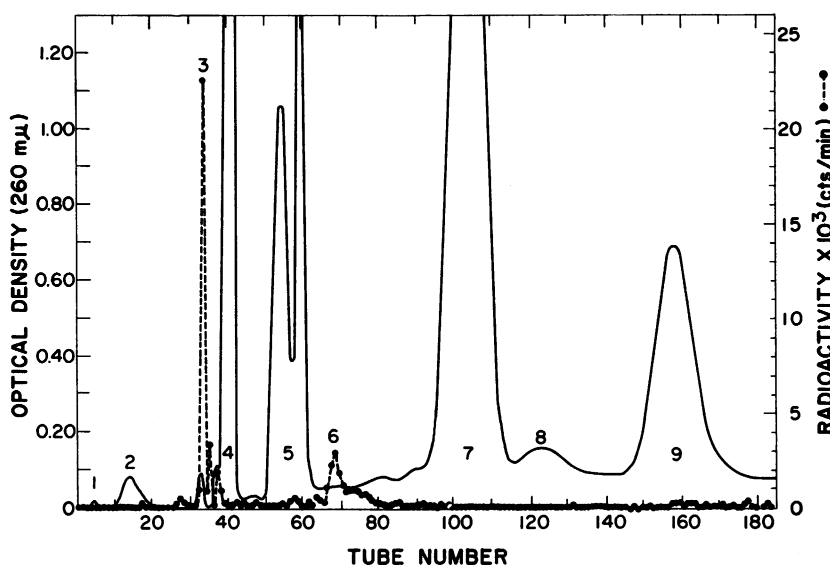

FIG. 3.—Dowex-1 (formate) chromatography of an alkaline hydrolysate of S^{35}-labeled sRNA (*E. coli*). The growth of *E. coli* (strain B) in minimal media containing $Na_2S^{35}O_4$ and isolation of the sRNA are described under *Materials and Methods*. The sRNA (10 mg) was hydrolyzed in 0.3 *M* KOH at 37° for 18 hr, neutralized to pH 8 with Dowex-50 (H+), and applied to a column of Dowex-1 (formate) (0.8 × 30 cm). Details of the elution schedule have been described.[7] Aliquots (100 μl) of each fraction (4 ml) were removed, applied to Whatman 3MM paper disks, dried, and counted by the scintillation method. The radioactivity in peak *3* (24,400 cpm) amounted to 5.6% of the total S^{35}-radioactivity applied to the column (435,000 cpm). An additional 101,000 cpm (23%) was recovered by stripping the column with 2 *N* HCl. The various peaks were identified as (*1*) cytidine, (*2*) adenosine, (*3*) unknown (see text), (*4*) 2′(3′)-CMP, (*5*) 2′(3′)-AMP, (*6*) unknown, (*7*) 2′(3′)-GMP, (*8*) 2′(3′)-pseudo-UMP, and (*9*) 2′(3′)-UMP.

for approximately 5–10 per cent of the iodine-reacting ability of the total hydrolysate. We have not been able to recover the remaining 90–95 per cent of iodine-reacting ability from Dowex-1 columns, although in other experiments (not shown) this is recoverable from DEAE-cellulose columns. Note that peak *3* contains S^{35} when the sRNA is obtained from *E. coli* grown in basal salts media containing $Na_2S^{35}O_4$, although the radioactivity again amounts to only 5 per cent of the total radioactivity in the hydrolysate (Fig. 3).

The material comprising peak *3* consists of a mixture of components, easily separable by paper chromatography using an *n*-butanol-NH_3-H_2O solvent system in which nucleotides move with a rate much slower than do nucleosides.[12] Under these conditions, the S^{35}-labeled compound remains at or very near the origin, while the contaminating materials (apparently nucleosides) move well away from the origin. All of the iodine-reacting ability of peak *3* remains associated with the labeled compound as determined by direct iodine titration of the substances eluted from the paper with water.

Although the structures of the compounds present in Dowex peak *3* are still under active investigation, it is apparent from the UV spectra of the labeled component that we are dealing with a 1-substituted-2-thiouracil derivative (Fig. 4). Note the marked similarity of the absorption spectra of the unknown substance (Fig. 4*B*) to that of 2-thiouridine (Fig. 4*A*). This type of ultraviolet-absorption picture has previously been shown to be typical of compounds of this structural

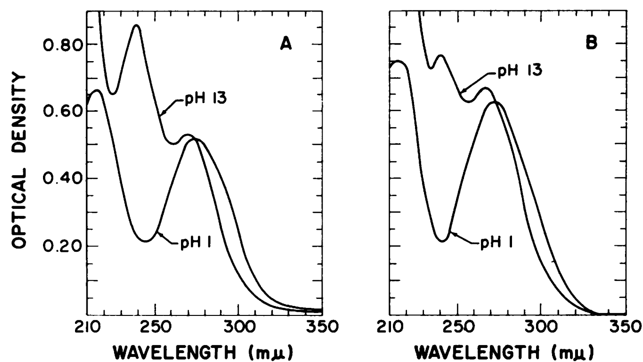

FIG. 4.—Ultraviolet-absorption spectra of (*A*) 2-thiouridine, and (*B*) the unknown material from peak *3* (Fig. 3) in 0.1 *M* HCl and 0.1 *M* NaOH. The experiment shown in Figure 3 was repeated on a larger scale (500 mg sRNA) and the material contained in peak *3* was applied to Whatman 3MM paper and chromatographed for 36 hr using an *n*-butanol-NH_3-H_2O system.[12] Under these conditions, the S^{35}-labeled material remains at the origin. The paper was eluted with water and the spectra were taken on a Cary recording spectrophotometer. The 2-thiouridine was a gift from Dr. G. Shaw, Bradford Institute of Technology, England.

type.[13] Despite the obvious similarities in the absorption spectra, the unknown is definitely neither 2-thio-UMP nor 5-methyl-2-thio-UMP.

Discussion.—The heterogeneity of the sRNA population specific for any particular amino acid has been studied by several groups of workers.[10] For example, the leucine-specific sRNA from *E. coli* has been separated into five components by countercurrent distribution[14] and by partition chromatography on Sephadex columns.[15] These findings have been interpreted as a reflection of the degeneracy of the genetic code, each codon requiring a different sRNA molecule.[16] Our results constitute further direct evidence for the heterogeneity of the various acceptors, in that only a fraction of the sRNA molecules appear to be sensitive to oxidative inactivation.

It has been proposed that certain species of sRNA could act as *modulators* of protein synthesis at the ribosomal translation level.[17, 18] Sueoka and Kano-Sueoka[19] have also recently proposed that an *in vivo* modification of an sRNA molecule could shut off certain genes which require that particular sRNA for translation. This *adaptor modification hypothesis*[19] requires that a mechanism exist within the cell which would be capable of rapidly activating and inactivating specific sRNA molecules. The reversible oxidative inactivation of acceptor ability described here would meet this requirement, although we have presented no evidence that an enzymatic oxidative inactivation of specific acceptors can occur. It is apparent, however, that enzymatic systems capable of reversibly inactivating specific codon translators could exert an exact control over protein synthesis at the ribosomal level. The mild conditions under which the iodine inactivation occurs suggest that a biological counterpart might eventually be found.

The oxidative inactivation of one of a group of amino acid-specific acceptors (Fig. 2) offers a powerful tool for the study of the specificity of sRNA in protein synthesis. For example, the cell-free synthesis of a protein of known amino acid sequence, such as hemoglobin, using sRNA preparations deleted of one lysine acceptor by the iodine method (Fig. 2*A*), could give information on which acceptor is needed to place lysine in each of its positions in the polypeptide chain. Recent

efforts to gain this type of information have been based upon the laborious separation of multicomponent sRNA mixtures by countercurrent distribution,[20] and are complicated by the facile enzymatic interchange of a labeled amino acid from one sRNA species to another.[21]

Although 2-thio-UMP has been shown to be present in *E. coli* (strain K12) grown in the presence of added 2-thiouracil,[22] sulfur-containing nucleotides have not been reported to be present in normal RNA. (See *Note added in proof*). It is of interest, therefore, that the sRNA of *E. coli* contains a 2-thiopyrimidine nucleotide as a normal constituent, and that this nucleotide is synthesized *de novo* from sulfate. We cannot state with certainty whether any or all of the inactivation caused by iodine treatment of sRNA is due to oxidation of this particular nucleotide. The fact that this material only accounts for 5–10 per cent of the total iodine-reactivity of the sRNA makes it apparent that this is only part of the total picture.

Note added in proof: A recent report substantiates our finding of thiopyrimidine bases in *E. coli* sRNA [see Lipsett, M. L., *Federation Proc.*, **24**, 216 (1965)].

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