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Differences in the Methylated Base Composition of Valyl and Leucyl Soluble Ribonucleic Acids of *Escherichia coli*

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Received May 18, 1964

The base compositions of soluble ribonucleic acids (s-RNA) of *Escherichia coli* that accept valine and leucine have been examined. To label specifically the methylated bases, the s-RNA was isolated from a methionine auxotroph cultured on methionine with radioactive tritium in the methyl group. The s-RNA was enzymatically charged with either valine or leucine and treated with an amino acid *N*-carboxyanhydride, which preferentially reacts with the charged species. The s-RNA precipitated with the resulting insoluble polypeptide was analyzed for major and minor bases. Although no interpretable differences were found in the contents of major bases, different patterns of minor bases were associated with the two species of s-RNA. Thymine, 6-methylaminopurine, 6-dimethylaminopurine, and possibly 2-*N*,*N*-dimethyl-6-hydroxypurine were found in the valine-specific s-RNA; thymine, 1-methylamino-6-hydroxypurine, and a dimethylpurine were present in the leucine-specific s-RNA.

The structures of s-RNA's have been the subject of intensive investigations since their discovery as intermediates in protein synthesis (Hoagland *et al.*, 1957; Hoagland *et al.*, 1958). Detailed structural analyses of purified s-RNA's are required for an understanding of how each species of s-RNA accepts a specific activated amino acid and transfers it to a ribosome-bound peptide. Differences either in the over-all base composition or in sequences of nucleotides could identify the sites on s-RNA molecules responsible for their specific enzymatic reactivities. Important contributions have been made by several authors who have reported many differences between highly purified species of yeast s-RNA (Stephenson and Zamecnik, 1961; Holley *et al.*, 1961, 1963; Zamecnik, 1962; Madison *et al.*, 1963; Cantoni *et al.*, 1963; Ingram and Sjöquist, 1963; Doctor *et al.*, 1963), as well as certain common sequences (Zamir *et al.*, 1964). Berg *et al.* (1962) have also reported differences in the terminal nucleotide sequences of leucine- and isoleucine-specific s-RNA's isolated from *Escherichia coli*. This paper documents significant differences in the methylated base composition of valyl and leucyl s-RNA's isolated from *E. coli*.

Several methods have been reported for the isolation of specific amino acid–acceptor s-RNA's using techniques such as countercurrent distribution (Zachau *et al.*, 1961; Apgar *et al.*, 1962), column chromatography (Ofengand *et al.*, 1961; Hartmann and Coy, 1961; Nishiyama *et al.*, 1961; Sueoka and Yamane, 1962; Tanaka *et al.*, 1962), and periodate treatment (Zamecnik *et al.*, 1960; Von Portatius *et al.*, 1961). A method for the isolation of specific s-RNA's using amino acid *N*-carboxyanhydrides has been described previously (Zachau, 1962; Simon *et al.*, 1962, 1963, 1964; Mehler and Bank, 1963). In this method, a mixture of s-RNA's is charged enzymatically with the amino acid of the s-RNA to be isolated; the free amino group of this amino acid serves as the initiator for the polymerization of an *N*-carboxyanhydride resulting in the preferential precipitation of the charged species of s-RNA as an ester of an insoluble polypeptide.

In the experiments described here specific *E. coli* s-RNA's were precipitated as γ-benzyl-L-glutamate polymers. These precipitates were then hydrolyzed with alkali and the resulting nucleotides were separated by column chromatography. For the detection of the methylated bases known to be present in s-RNA (Davis and Allen, 1957; Littlefield and Dunn, 1958; Smith and Dunn, 1959) we specifically labeled these bases in a methionine auxotroph grown on tritiated methionine.

Experimental

Materials.—s-RNA from *E. coli*, strain B, was obtained commercially from General Biochemicals. Its amino acid acceptance was 0.340 for valine and 0.388 for leucine measured as mμmoles of amino acids per μmole of nucleotide (Berg *et al.*, 1961). Amino acids were measured using commercial radioactive compounds with specific activities of 1–200 μcuries per μmole; nucleotide concentrations were determined as optical density at 260 mμ in a Cary spectrophotometer. Tritiated s-RNA was prepared from the *E. coli* K_{12}-W6 mutant as described previously by Peterkofsky *et al.* (1964) using commercial methyl-labeled tritiated methionine with a specific activity of 27 μc per μmole. Its amino acid acceptance was comparable to that of

the commercial unlabeled s-RNA. Its specific activity was 0.43 μc per μmole of nucleotide.

Amino acid–activating enzymes were prepared by the method of Bergmann *et al.* (1961). The enzymes were desalted with Sephadex G-25. Bacterial alkaline phosphomonoesterase was obtained from Worthington Biochemical Corp.

γ-Benzyl-L-glutamate *N*-carboxyanhydride was prepared by the method of Blout and Karlson (1956) and had a sharp melting point at 92–94°. Dr. G. H. Hitchings of Burroughs Wellcome and Co., Inc., kindly provided us with samples of 1-methylguanine, 2-methylguanine, and 2-methyladenine; the other methylated bases were obtained commercially.[1]

Preparation of Specific s-RNA Precipitates.—Amino acids were charged on s-RNA in 50 ml of a solution containing 6.25 mmoles of potassium cacodylate buffer at *p*H 7.0, 50 μmoles of ATP, 125 μmoles of $MgCl_2$, and 25 μmoles of 2-mercaptoethanol, 2.8 μmoles of tritiated s-RNA containing 1.2×10^6 cpm (as measured in a Packard Tri-Carb scintillation counter), 100 μmoles of unlabeled s-RNA, 5–6 μmoles of cold amino acid, and enough purified activating enzyme to insure maximal charging. To determine the optimal concentration of enzyme necessary, an aliquot of the tritiated labeled and unlabeled s-RNA mixture was charged with ^{14}C-radioactive amino acid just prior to the larger incubation. Incubation was at 37° for 90 minutes.

The charged s-RNA was precipitated at 0° by the addition of 15 ml of 5 M NaCl and 2 volumes of ethanol, washed three times with 20 ml of a 0.5 M NaCl–67% ethanol mixture, and then dissolved in 5.5 ml of water. One ml of 1.0 M potassium phosphate buffer at *p*H 6.8 and 2.0 ml of purified dioxane were added and the mixture was stirred at 0°. γ-Benzyl-L-glutamate *N*-carboxyanhydride (200 mg dissolved in 1.25 ml of dioxane) was added rapidly and mixing was continued for 45 minutes. At this time 25 ml of water was added and the precipitate was recovered by centrifugation. The precipitate was washed three times with 20–30 ml of water to insure removal of any water-soluble material.

The controls for these experiments were carried out identically except for the omission of the cold amino acid during the charging incubation. The amount of activating enzyme added and the procedures for the isolation of the s-RNA and the subsequent reaction with anhydride were the same for both the reacted material and the controls.

Alkaline Hydrolysis and Column Chromatography.—In all experiments, the specific precipitates were suspended in a mixture of 7.5 ml of dioxane and 5.0 ml of water, and 0.6 ml of 10 M KOH was added. The mixture was incubated at 37° for 18 hours. Clearing of the suspension owing to hydrolysis of the γ-benzyl groups was noted after 5–10 minutes of incubation with alkali. The solution was neutralized to *p*H 8.0 by the addition of 6 M perchloric acid, the mixture was centrifuged, the precipitated $KClO_4$ was discarded, and the supernatant solution was evaporated to dryness using a rotary evaporator to remove the dioxane. The residue was dissolved in 10 ml of water.

A modification of the Dowex 1 X-8, <400 mesh column in the formate form as described by Cantoni *et al.* (1962) was used. The solution was placed on a column 0.25 $cm^2 \times 8$ cm and the nucleosides and nucleotides were eluted consecutively with 25 ml of water, 150 ml of a linear gradient from 0.1–0.2 M formic acid, 200 ml of a linear gradient from 0.2–1.0 M formic acid, and 100 ml of 1.0 M formic acid. Two-ml fractions were collected and the optical density at 260 mμ was determined before counting the radioactivity of the entire sample dissolved in 10 ml of the solution described by Bray (1960) in a Packard Tri-Carb scintillation counter. Figure 1 shows the pattern of optical density and tritium counts corresponding to the major and minor bases, respectively, obtained on a sample of unfractionated s-RNA after alkaline hydrolysis. The treatment with *N*-carboxyanhydride did not alter the chromatographic behavior of the nucleotides, but an optical density peak that had an ultraviolet spectrum identical with that of benzyl alcohol was eluted with the water wash. The recovery of both tritium counts and optical density varied from 80 to >95%.

Identification of Major and Minor Components.—The major bases were identified by their characteristic ultraviolet spectra in acid and alkali. The methylated nucleotide peaks were pooled as indicated in Figure 1, lyophilized to dryness, and then treated with either HCl, perchloric acid, or phosphomonoesterase, periodate, and alkali as described by Littlefield and Dunn (1958) to degrade the compounds to the base level.

The products of the various degradation procedures were then chromatographed using ascending chromatography on paper in the following solvent systems: (1) 2-propanol (136 ml), 11.6 N HCl (35.2 ml), and water to 200 ml (Wyatt, 1951); (2) 2-propanol (140 ml), water (60 ml), with NH_3 in the vapor phase (Markham and Smith, 1949); (3) 1-butanol (154 ml), water (26 ml), and 98% formic acid (20 ml) (Markham and Smith, 1952); (4) 1-butanol (176 ml), water (28 ml), with NH_3 in the vapor phase (Markham and Smith, 1952). The R_F values of the unknown tritiated bases were compared with those of the standard compounds. Cochromatography of unknown tritiated compounds with standards having identifiable optical density was also employed. A Vanguard chromatogram scanner was employed to determine radioactivity, while an ultra-

TABLE I
ANALYSIS OF LEUCYL AND VALYL S-RNA PRECIPITATES[a]

Precipitate	Tritium (% pptd)	Optical Density (% pptd)
Leucine-treated (expt I)	4.1	4.2
Control	2.1	2.5
Difference (leucine-specific s-RNA)	2.0 (48.8%)	1.7 (40.5%)
Leucine-treated (expt II)	7.7	10.0
Control	5.1	6.7
Difference (leucine-specific s-RNA)	2.6 (33.8%)	3.3 (33.0%)
Valine-treated (expt I)	3.9	5.1
Control	2.1	2.5
Difference (valine-specific s-RNA)	1.8 (46.0%)	2.6 (51.0%)
Valine-treated (expt II)	4.4	3.5
Control	1.5	1.6
Difference (valine-specific s-RNA)	2.9 (64.7%)	1.9 (54.7%)

[a] Leucyl and valyl s-RNA's were precipitated with γ-benzyl-L-glutamate *N*-carboxyanhydride as described under Methods. The percentage precipitated represents the percentage of the initial tritium and optical density in the s-RNA mixture used that were rendered insoluble by the reaction with anhydride. The percentage difference between the control and amino acid–treated material is in parenthesis.

[1] 2-Methylamino-6-hydroxypurine and 2-*N*,*N*-dimethyl-6-hydroxypurine will be designated as 2-methylguanine and 2-dimethylguanine, while 6-methylamino- and 6-dimethylaminopurines will be called 6-methyladenine and 6-dimethyladenine.

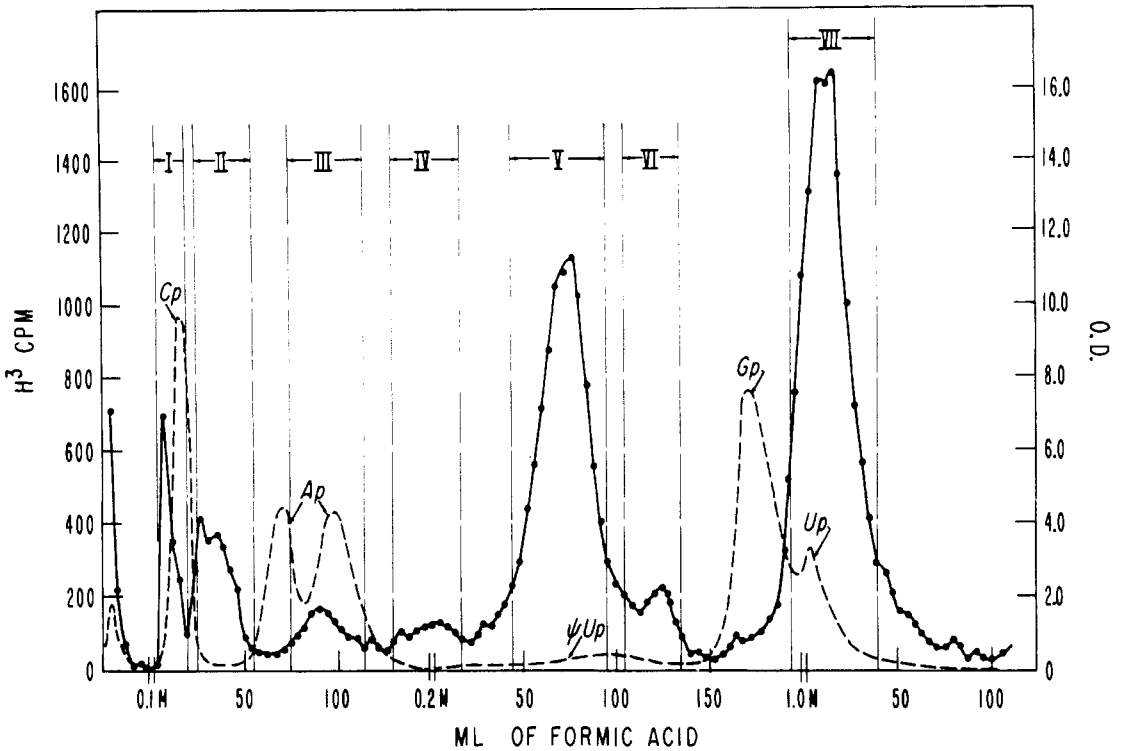

FIG. 1.—Chromatography of an alkaline digest of a mixture of unfractionated s-RNA's, tritiated and unlabeled, on Dowex 1. Details of the procedure are described under Methods. Two-ml samples were collected, the optical density at 260 mμ was determined, and an aliquot was assayed for radioactivity. The solid lines represent the tritium counts corresponding to the methylated bases; the broken lines represent the optical density corresponding to the major bases. The Roman numerals and vertical lines indicate the fractions that were pooled for identification of the methylated bases shown in Table III. Cp = cytidylic acid, Ap = adenylic acid, ψUp = pseudouridylic acid, Gp = guanylic acid, and Up = uridylic acid.

violet lamp was used to visualize ultraviolet-absorbing spots. The pattern of elution of the methylated bases described by Cantoni *et al.* (1962) was valuable in directing the chromatographic identifications.

RESULTS

Table I describes the composition of the precipitates obtained by reacting γ-benzylglutamate *N*-carboxyanhydride and s-RNA charged with either valine or leucine. In each experiment a parallel reaction was carried out with uncharged s-RNA. Variations in the amount of RNA precipitated in the blank reactions reflect differences in the amounts of enzyme included, since the enzyme preparations used were capable of charging small amounts of unidentified amino acids on s-RNA. In the second leucine experiment the blank reaction was greater since more leucine-activating enzyme was used to charge the s-RNA; deterioration of the enzymatic activity of the enzyme preparation necessitated this. In each experiment the counts and optical density obtained in the precipitate with charged s-RNA were approximately twice those of the blank reaction except for the second leucine experiment. The differences shown in the table are attributed to the specific s-RNA precipitated as a result of reaction of the *N*-carboxyanhydride with bound amino acid.

Base Analysis of Unfractionated E. coli s-RNA.—Duplicate samples of tritium-labeled s-RNA mixed with unlabeled s-RNA were hydrolyzed and chromatographed as described under Methods. The major base composition given in Table II was obtained by planimetry of the curves of optical density. The results given are the average of the two determinations. Similarly, the methylated bases were determined as the sum of the radioactivity of individual peaks, and are reported as average values in Table III. Neither 2-methylguanine nor 7-methylguanine was found. The values are comparable to the published data (Dunn *et al.*, 1960; Cantoni *et al.*, 1962).

TABLE II
MAJOR BASE COMPOSITION OF UNFRACTIONATED *E. Coli* s-RNA[a]

Nucleotide	Identified Nucleotides (moles/100 moles)
Adenylic acid	21.9
Cytidylic acid	29.4
Guanylic acid	27.1
Uridylic acid	17.9
Pseudouridylic acid	3.7

[a] A mixture of tritiated and unlabeled s-RNA was subjected to alkaline hydrolysis and chromatographed as described under Methods. The areas under the major base peaks of two experiments (one of which is shown in Figure 1) were measured by planimetry. The results are expressed as the averages of the two experiments.

Analyses of Amino Acid–Specific Precipitates.—Experiments resulting in the precipitation of leucine-specific and valine-specific s-RNA's were performed as described under Methods. The major base ratios of alkaline hydrolysates of these precipitates obtained by Dowex chromatography are presented in Table IV. No significant differences from the control precipitate material are seen except for that of the uridylic acid values in leucyl s-RNA. However, the contamination of the uridylic acid peak with guanylic acid (see Figure 1) makes this measurement difficult to evaluate. In

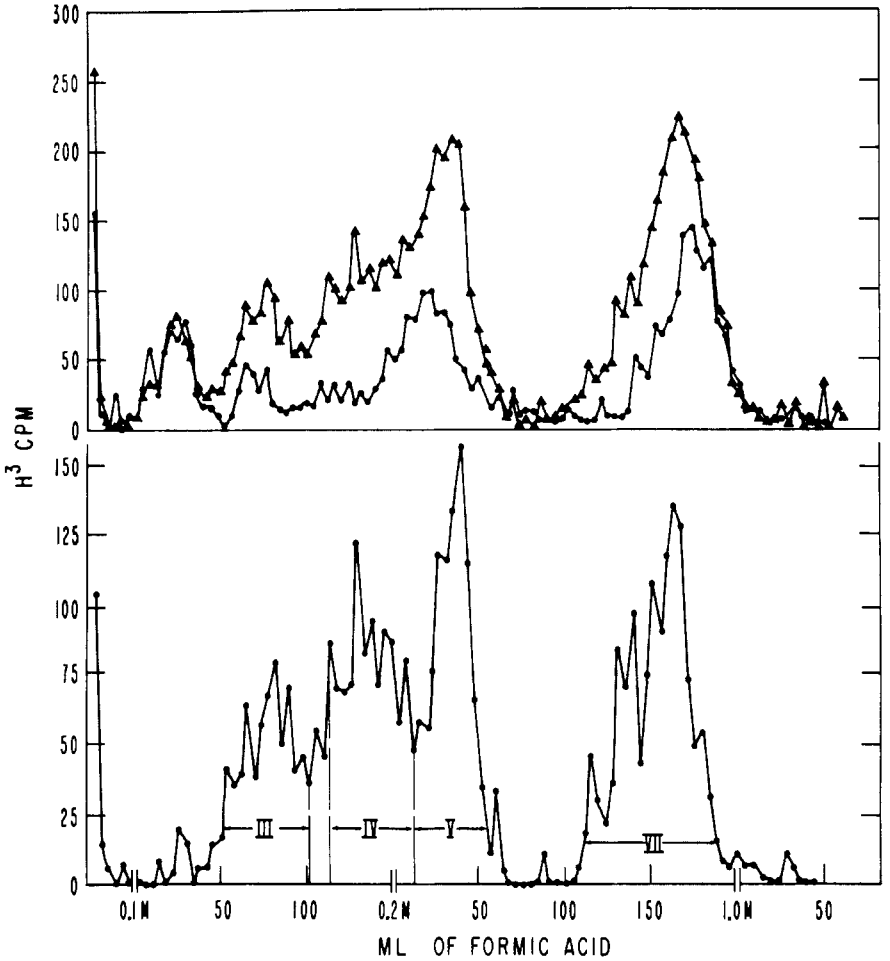

FIG. 2.—Chromatography of the alkaline digests of valine-reacted and control precipitates on Dowex 1. The experimental details are described in the text. Two-ml fractions were collected and the entire sample was assayed for radioactivity. In the upper portion of the figure, the distribution of tritium counts obtained with the valine-reacted precipitate is shown with solid triangles (▲); the control (without amino acid) is represented by solid circles (●). The lower portion of the figure plots the difference in tritium counts between the two curves in the upper portion. This difference represents counts due to reaction with valine. The peaks are identified by Roman numerals.

TABLE III
METHYLATED BASE COMPOSITION OF *E. Coli* s-RNA (UNFRACTIONATED)[a]

Peak	Bases Identified	Identified Nucleotide (moles/100 moles)
I	5-Methylcytosine	0.07
II	2-Methyladenine	0.13
III	6-Methyladenine	0.10
IV	6-Dimethyladenine	0.04
V	6-Dimethyladenine plus 6-dimethyl-guanine	0.28
VI	1-Methylguanine	0.09
VII	Thymine	0.88

[a] The identifications of the methylated bases were done on the same materials and using the same methods as described in the legend of Table II. The results are the averages of two determinations. The moles of methylated base per 100 moles of identified nucleotide were calculated from the known specific activities of the tritiated methionine and s-RNA.

comparing Table II with Table IV, it is seen that all the anhydride-reacted precipitates show higher uridylic and lower adenylic acid values than unfractionated s-RNA. This may reflect a preferential "blank" reaction between pyrimidine bases and the anhydride.

TABLE IV
MAJOR BASE COMPOSITION OF ANHYDRIDE-PRECIPITATED s-RNA'S[a]

	Cp	Ap	Gp	Up
	(moles/100 moles of identifiable nucleotide)			
Leucine				
Control	25.3	16.8	27.0	30.9
Reacted	31.7	19.9	28.9	20.5
Valine				
Control	28.9	16.5	30.3	24.3
Reacted	24.7	17.7	31.5	26.1

[a] Leucine- and valine-charged s-RNA's and controls for each were reacted with γ-benzyl-L-glutamate *N*-carboxy-anhydride. Precipitates were washed and subjected to alkaline hydrolysis and chromatography on Dowex 1 as described under Methods. The optical density in the major base peaks was measured by planimetry. The values are the averages of two experiments.

The methylated base pattern of a representative experiment using valine-reacted and control precipitates is shown in Figure 2. In the lower portion of the figure is plotted the valine-specific material, i.e., the difference between the control and valine-treated patterns. The distribution of the tritium counts is different here from both the control and unfractionated material; it is concentrated in peaks III, IV, V, and VII.

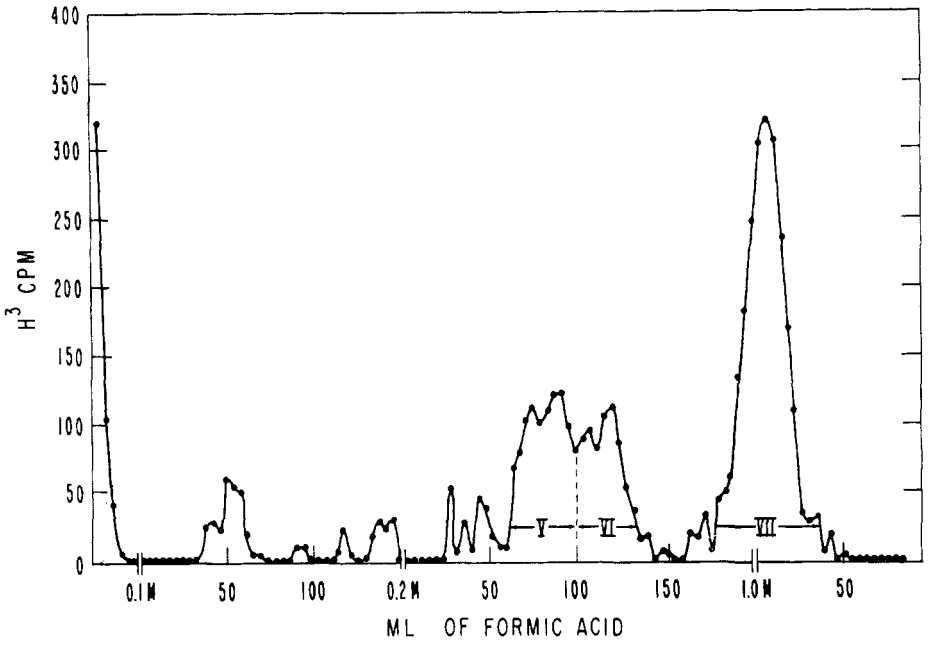

FIG. 3.—Chromatography of the alkaline digests of leucine-reacted and control precipitates on Dowex 1. The results are expressed as the difference in tritium counts between a hydrolysate of leucine-reacted and control material entirely comparable to the lower portion of Fig. 2.

Thus, valyl s-RNA contains 6-methyladenine, 6-dimethyladenine, thymine, and perhaps 2-dimethylguanine.

In Figure 3 the tritium distribution from a typical experiment using leucine-reacted and control precipitates is plotted. Here again the data represent the difference between control and leucine-reacted material. In contrast to the valine-specific s-RNA, leucine-specific s-RNA has the methylated bases located in peaks V, VI, and VII, and questionably in II. Therefore, leucyl s-RNA has 1-methylguanine and thymine as well as either 6-dimethyladenine or 2-dimethylguanine as its methylated base complement. The small peak II above control may represent a small amount of 2-methylaminopurine. The uncertainty about the identity of dimethylpurine in the specific leucyl and valyl s-RNA's results from the facts that both nucleotides are eluted together and that the small amount of dimethylpurine must be determined above a significant blank, which includes both compounds.

DISCUSSION AND CONCLUSIONS

The method described here, while it does not give a highly purified product, is limited only by the magnitude of precipitation of specific material to that of a control without added amino acid. With valyl and leucyl s-RNA's, the ratio of specific to nonspecific precipitation is favorable and the method is successfully employed. Other s-RNA's present in smaller amounts might give less favorable ratios and make analysis more difficult.

The analyses of individual s-RNA's in our experiments indicate very specific associations of certain methylated bases with acceptor activity for a given amino acid. Thus, valyl s-RNA of *E. coli* contains thymine, 6-methyladenine, 6-dimethyladenine, and perhaps 2-dimethylguanine. An estimate of the amounts of these bases indicates that one thymine residue may be present in each molecule of valyl s-RNA. If one assumes one thymine molecule per chain, then 6-methyladenine accounts for 0.33 mole per chain and the dimethylpurines 0.74. Similarly, leucyl s-RNA contains approximately 1 mole of thymine, 0.38 mole of 1-methylguanine, and 0.18 mole of dimethylpurine.

These findings suggest that, in addition to thymine, valyl s-RNA may contain only methylated adenines while leucyl s-RNA contains only methylated guanines. A possible explanation for this is that, while a single uracil residue in both s-RNA's is always methylated to yield thymine, only a single adenine in valyl s-RNA and a guanine in leucyl s-RNA are capable of being methylated. The nature and extent of this methylation might be controlled by differences in the nucleotide sequences of s-RNA molecules accepting the same amino acid or by competition between different available methylating enzymes for a single purine substrate. The result in either event would be a population of specific s-RNA molecules whose reactive purines were either unmethylated, monomethylated, or dimethylated. An alternative interpretation of the data is that certain valyl and leucyl s-RNA chains contain all of the methylated bases present in that species, while other chains contain none. The possible relationship between these differences in the distribution of the methylated bases and the "degenerate" forms of s-RNA accepting a single amino acid is also interesting, regardless of the mechanism of methylation. Separation of the known degenerate forms of leucyl s-RNA (Weisblum *et al.*, 1962, Keller and Anthony, 1963) and their minor base analysis could clarify these problems.

The function of the methylated bases in s-RNA remains unknown. They do not appear to be required for amino acid–acceptor activity (Starr, 1963). However, the findings of significant differences in the distribution of these bases in specific s-RNA's from the same species support the suggestion (Zamecnik, 1962) that they may be involved in the transfer of amino acids from s-RNA to ribosome-bound polypeptides.

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Studies on Acid Deoxyribonuclease. I. Kinetics of the Initial Degradation of Deoxyribonucleic Acid by Acid Deoxyribonuclease*

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Received May 25, 1964

The mechanism of degradation of deoxyribonucleic acid from different sources (chicken erythrocytes, calf thymus, and *Escherichia coli*) by acid deoxyribonuclease preparations from chicken erythrocytes, calf thymus, calf spleen, and hog spleen has been investigated by following the molecular-weight decrease by light scattering. Native deoxyribonucleic acid is degraded according to both "single-hit" and "double-hit" kinetics. The "single-hit" degradation appears to take place through the simultaneous splitting of both strands at the same level. The "double-hit" degradation, which resembles the degradation by pancreatic deoxyribonuclease, becomes evident only after a time lag. Heat-denatured deoxyribonucleic acid is attacked essentially according to the "double-hit" mechanism.

Work carried out in this laboratory in 1960 (Bernardi *et al.*, 1960, 1961; Sadron, 1961) showed that DNA from calf thymus ($M_w = 6.5 \times 10^6$; $s_{20,w} = 20.0$ S) or chicken erythrocytes ($M_w = 8.0 \times 10^6$; $s_{20,w} = 27.5$ S) can be degraded with crude enzyme preparations from chicken erythrocytes.[1] The enzymic activity showed a *p*H optimum close to 5.5 and was strongly inhibited by Mg^{2+}. No appreciable amounts of dialyzable nucleotides were formed during the digestion. The degraded DNA from both sources so formed appeared to consist of particles having a molecular weight of $M_w = (5.5 \pm 0.5) \times 10^5$. The light-scattering data were compatible with a solution of stiff rods having a weight per unit length $M/L = 200 \pm 20$ daltons/A. The sedimentation coefficient $s_{20,w}$ was equal to 5.8 S, and the distribution function of the sedimentation coefficients was very narrow.

In further work (Bernardi and Sadron, 1961), the crude enzyme preparation from chicken erythrocytes was purified 200 times according to a procedure reported in the present paper, and used at concentrations about

* This work has been aided by a grant (UR-E9-[10,60]-80) from the U. S. Department of Agriculture.

[1] Abbreviations used in this work: M_w, weight-average molecular weight; M_n, number-average molecular weight.