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Antibody Nucleic Acid Complexes. Immunospecific Retention of N^6-Methyladenosine-Containing Transfer Ribonucleic Acid†

Theodore W. Munns,* M. Kathryn Liszewski, Robert J. Oberst, and Harold F. Sims

ABSTRACT: Antibodies specific for N^6-methyladenosine (m^{6}A) were immobilized on Sepharose and the resulting immunoadsorbent was tested for its ability to retain those *Escherichia coli* tRNAs containing the antigenic hapten, i.e., m^{6}A. Results obtained with [^{32}P]PO_4- and [*methyl*-^{3}H]-methionine-labeled tRNAs indicated that approximately 3 to 5% of the radioactive RNA was retained by the immunoadsorbent. Under identical conditions, but in the presence of m^{6}A (1 mg/mL), less than 0.2% of the radioactivity was retained. Subsequent characterization of the retained tRNA via (a) analysis of *methyl*-^{3}H-labeled, methylated nucleosides, (b) two-dimensional gel electrophoresis, and (c) analysis of the retention of [^{3}H]aminoacyl-tRNA species led to the conclusion that the anti-m^{6}A/Sepharose adsorbent quantitatively and exclusively retained a single tRNA species containing m^{6}A, namely, tRNA^{Val}.

Antibodies that immunospecifically recognize a variety of modified constituents present in nucleic acids have been characterized (Erlanger & Beiser, 1964; Karol & Tanenbaum, 1967; Levine et al., 1971; Sawicki et al., 1971, 1976; Hacker et al., 1972). Recent utilization of such antibody preparations, e.g., anti-m$_2$6A antibodies, has confirmed the presence or absence of N^6,N^6-dimethyladenosine (m$_2$6A)[1] residues in the 23S rRNA species of Kasugamycin-sensitive and -resistant strains of bacteria (Politz & Glitz, 1977), while anti-5-methylcytidine (m^{5}C) antibodies have been used to study the distribution and arrangement of 5-methylcytosine residues in human chromosomes (Lubit et al., 1976). Other investigations have employed antibodies which specifically recognize inosine and the Y nucleoside to isolate those tRNAs containing the corresponding antigenic hapten (Inouye et al., 1973; Fuchs et al., 1974).

In view of the above and other recent findings which indicate that mammalian RNAs (tRNA, rRNA, mRNA, low molecular weight nuclear RNA, and heterogeneous nuclear RNA) possess a variety of methylated nucleosides (Perry & Kelley, 1974; Desrosiers et al., 1974, 1975; Shatkin, 1976; Ro-Choi & Henning, 1977; Weinberg & Penman, 1968), we became interested in examining the possibility that antibodies specific for such minor constituents could be employed as a means of isolating and characterizing nucleic acid populations on the basis of their composition of methylated nucleosides. Our attention has been focused upon characterizing antibodies that specifically recognize N^6-methyladenosine (m^{6}A) and 7-methylguanosine (m^{7}G). Recently, we have demonstrated that immunoadsorbents containing the above antibodies can quantitatively and exclusively retain mono- and oligonucleotides possessing m^{6}A and m^{7}G (Munns et al., 1977a,b). The results described herein demonstrate the ability of anti-m^{6}A immunoadsorbents to retain a specific tRNA population containing m^{6}A, namely, tRNA^{Val} from *Escherichia coli*.

Experimental Procedures

All radioactive isotopes employed in this investigation were obtained from Amersham/Searle. They included carrier-free [^{32}P]PO_4, [*methyl*-^{3}H]methionine (8 to 10 Ci/mmol), [2,3-^{3}H]valine (36 Ci/mmol), [4,5-^{3}H]leucine (58 Ci/mmol), and [2,3-^{3}H]phenylalanine. X-ray film (XM-2), for autoradiographic purposes (Figure 3), was obtained from Eastman Kodak; thin-layer chromatographic plates (Anasil GF, 20 × 20 cm, 0.25 mm thickness), for separating methylated nucleosides, were from New England Nuclear; and aminoacyl-tRNA synthetases were from Miles Laboratories. Enzymes used in the digestion of tRNA were obtained from P-L Biochemicals (*Penicillium* nuclease) and from Worthington (alkaline phosphatase). Conditions for enzymatic digestion of tRNA have been described as have the procedures for sep-

† From the Edward A. Doisy Department of Biochemistry, Saint Louis University School of Medicine, St. Louis, Missouri 63104. *Received January 16, 1978.* This investigation was supported by Grant CA 17715, awarded by the National Cancer Institute, Department of Health, Education and Welfare.

[1] Abbreviations used: m^{6}A, N^6-methyladenosine; m$_2$6A, N^6,N^6-dimethyladenosine; m^{1}A, 1-methyladenosine; m^{2}A, 2-methyladenosine; A^m, 2′-*O*-methyladenosine; A, adenosine; m^{7}G, 7-methyladenosine; m^{1}G, 1-methylguanosine; G^m, 2′-*O*-methylguanosine; G, guanosine; m^{5}U, 5-methyluridine; U^m, 2′-*O*-methyluridine; U, uridine; m^{5}C, 5-methylcytidine; C^m, 2′-*O*-methylcytidine; C, cytidine; NaCl–P, phosphate-buffered saline, pH 7.4; NaDodSO$_4$ (SDS in figures), sodium dodecyl sulfate; PCA, perchloric acid; RNase, ribonuclease; anti-m^{6}A/Sepharose, rabbit anti-m^{6}A antibody-coupled Sepharose; BSA, bovine serum albumin.

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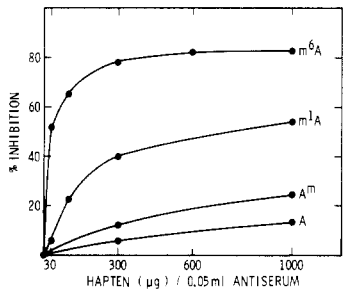

FIGURE 1: Inhibition of the homologous precipitin reaction, i.e., anti-m^{6}A antibody and m^{6}A-BSA, by various nucleosides. Precipitin reactions contained 0.05 mL of antiserum, 15 μg of m^{6}A-BSA, and increasing amounts of nucleoside in a final volume of 1.1 mL in NaCl-P. The incubation and measurement of precipitated protein have been described (Munns et al., 1977a). Values represent the mean of a minimum of two independent assays.

arating methylated nucleosides via thin-layer chromatography (Munns & Sims, 1975a).

Isolation and Characterization of tRNA. Cells (*Escherichia coli* H) growing in early log phase in a medium described by Williams & Dawson (1952) were labeled with either [^{32}P]PO_4 (10 mCi) or [*methyl*-^{3}H]methionine (5 mCi) for 2 h prior to the addition of unlabeled PO_4 or methionine to a final concentration of 10 mM and 2 mM, respectively. The chase aspect of these labeling experiments (1 to 2 h) ensured that the tRNAs labeled under these conditions were mature with respect to containing their full complement of methylated constituents (Munns & Sims, 1975b). Total RNA was extracted from cells at 60 °C with phenol-NaDodSO$_4$ and the tRNA purified by sucrose gradient sedimentation in the presence of NaDodSO$_4$. Preparations of tRNA were characterized via acrylamide gel electrophoresis and, as evaluated from radioactivity profiles, greater than 90% of the radioactive RNA from each preparation migrated within the tRNA band of the gels. The [*methyl*-^{3}H]methionine-labeled tRNA [*methyl*-^{3}H]tRNA was characterized further by determination of the content of *methyl*-^{3}H-labeled methylated nucleosides. The results from these analyses (Table II) revealed that the type and distribution of constituents present resembled those of other bacterial tRNA preparations (Randerath & Randerath, 1973). These analyses also demonstrated that approximately 2% of the [*methyl*-^{3}H]tRNA was identified as [*methyl*-^{3}H]m^{6}A and suggested that only one or several tRNAs possessed this constituent.

Immunochemistry. The production, characterization, and purification of anti-m^{6}A antibodies, and their conjugation to Sepharose have been described previously (Munns et al., 1977a). Immunospecific adsorption of m^{6}A-containing tRNAs was conducted in a manner identical with that reported earlier for mononucleotides (Munns et al., 1977a) and oligonucleotides (Munns et al., 1977b) with the exception that 20 mM $MgCl_2$ (Inouye et al., 1973) was included in the NaCl-P buffer (i.e., NaCl-P; 150 mM NaCl, 10 mM PO_4, pH 7.4, and NaCl-P-Mg, NaCl-P + 20 mM $MgCl_2$). Briefly, 20 to 200 μg of labeled tRNA was incubated with 0.2 mL of immunoadsorbent (wet weight volume) for 30 to 40 min at room temperature (final reaction volume 1.0 to 1.2 mL in NaCl-P-Mg). After this incubation period the adsorbent was quantitatively transferred to a fritted-disc funnel (15 mL) and washed successively with 1.0-mL aliquots of NaCl-P-Mg (4×) and water (1×) prior to the addition of 1 mL of 0.5 M NaCl containing 1% NaDodSO$_4$. This latter treatment (10 min incubation at 24 °C) quantitatively removed immunospecifically retained tRNA from the adsorbent. The tRNA was recovered by ethanol precipitation in the presence of unlabeled carrier tRNA (50 μg) and characterized as described below. A complete description of the conditions associated with adsorption and elution of various tRNA preparations, washing of the anti-m^{6}A/Sepharose, concentration of the tRNA, etc., are described in the legends of the appropriate figures and tables.

The specificity of anti-m^{6}A antibodies was tested by measuring the ability of various nucleoside haptens to inhibit the homologous precipitin reaction, i.e., the precipitation of anti-m^{6}A antibodies with m^{6}A-coupled bovine serum albumin (m^{6}A-BSA). These procedures have been described in detail elsewhere (Kabat, 1961; Inouye et al., 1971; Munns et al., 1977a).

Characterization of Immunospecifically Retained tRNAs. Three distinctly different techniques were utilized to assess the ability of anti-m^{6}A antibodies, coupled to Sepharose, to retain m^{6}A-containing tRNAs. They included two-dimensional gel electrophoresis (Fradin et al., 1975), analysis of the content of *methyl*-^{3}H-labeled methylated constituents (Munns & Simms, 1975a), and immunospecific retention of bacterial tRNAs that had been previously aminoacylated (Yang & Novelli, 1971) with either [2,3-^{3}H]valine, [2,3-^{3}H]phenylalanine, or [4,5-^{3}H]leucine. As determined by the filter-disc method of Santi & Anderson (1974) the nmol of amino acid coupled per mg of unfractionated *Escherichia coli* tRNA were 0.4, 0.4, and 0.6 for phenylalanine, leucine, and valine, respectively. Acylated tRNAs were precipitated with 2 volumes of ethanol and redissolved in acetate buffer (sodium acetate, 10 mM, pH 5.0) just prior to immunospecific adsorption. Experiments employing ^{3}H-labeled, aminoacylated tRNAs and [*methyl*-^{3}H]tRNAs were conducted to assess the extent of deacylation and degradation, respectively, of tRNA during adsorption. These studies consisted of measuring the perchloric acid (PCA)-soluble radioactivity of aliquots (0.1 mL) derived from the incubation mixture (i.e., immunoadsorbent + labeled tRNA). This procedure has been described in detail by Munns & Katzman (1971).

Results

Specificity of Anti-m^{6}A Antibodies. When attempting to isolate immunochemically a RNA species on the basis of its content of methylated nucleosides, one of the major obstacles to be encountered is the specificity of the antibody under consideration. Thus, if an antibody population, e.g., anti-m^{6}A antibodies, cross-reacts with adenosine to any appreciable extent, the likelihood of isolating only those RNA species possessing m^{6}A would be questionable. In view of the above, initial studies were conducted to determine the specificity of anti-m^{6}A antibodies by hapten inhibition techniques. As illustrated in Figure 1, the extent to which increasing amounts of competing hapten can inhibit the homologous precipitin reaction is documented. These results demonstrated that the anti-m^{6}A antibodies employed in the present investigation were specific for m^{6}A with minimal or no cross-reactivity toward adenosine (A). Although there appeared to be some cross-reactivity toward 1-methyladenosine (m^{1}A) and 2′-*O*-methyladenosine (A^m), the quantity of m^{1}A required to achieve a 50% inhibition of the homologous precipitin reaction was 720 μg, compared with 25 μg for m^{6}A. Furthermore, since *Escherichia coli* tRNAs do not contain m^{1}A (Randerath & Randerath, 1973; Munns et al., 1974), this small amount of cross-reactivity was not relevant to the isolation of m^{6}A-containing bacterial tRNAs.

Other haptens (not listed in Figure 1) unable to inhibit the precipitin reaction at concentrations in excess of 1 mg/mL

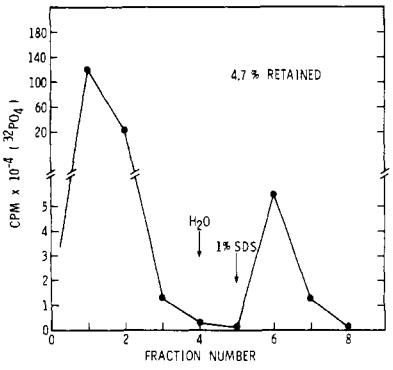

FIGURE 2: A representative radioactive elution profile of [^{32}P]PO_4-labeled tRNA after a 30-min incubation at 24 °C in the presence of anti-m^{6}A/Sepharose. Almost identical profiles were observed with [*methyl*-^{3}H]tRNA (Table I). The quantity of tRNA used per incubation was approximately 25 and 70 μg, respectively, for [^{32}P]PO_4-tRNA and [*methyl*-^{3}H]tRNA.

included uridine (U), cytidine (C), guanosine (G) as well as the methylated constituents present in Table II (except m^{6}A). Somewhat surprising was the finding that m_2^6A was not recognized by anti-m^{6}A antibodies.

Immunospecific Retention of [^{32}P]PO_4- *and methyl-*^{3}*H-Labeled tRNAs.* The ability of anti-m^{6}A antibody, coupled to Sepharose, to retain only those tRNAs possessing m^{6}A was initially tested by examining the radioactive elution profiles of [^{32}P]PO_4- and *methyl*-^{3}H-labeled tRNA following their incubation in the presence of immunoadsorbent. A typical radioactive elution profile is illustrated in Figure 2 and indicated that the bulk of radioactive tRNA (ca. 95%) was eluted with the initial two NaCl-P-Mg washes of the immunoadsorbent while less than 0.2% was removed with the water wash. However, in the presence of $NaDodSO_4$ (0.5 M NaCl, 1% $NaDodSO_4$), an additional 4.7% of the applied radioactivity was eluted from the immunoadsorbent. This latter procedure effectively removed all radioactivity associated with adsorbent. Although the data in Figure 2 represent the results of a single experiment, additional elution profiles of other tRNA preparations revealed that the percentage of radioactivity retained by this adsorption procedure was 4.7, 4.0, and 3.6% for [^{32}P]PO_4-labeled tRNAs and 4.7, 4.5, and 3.9% for [*methyl*-^{3}H]tRNAs (Table I).

To determine whether the retained radioactivity was dependent upon the presence of m^{6}A residues, similar adsorption studies were conducted in the presence of competing hapten, i.e., m^{6}A. These results are presented in Table I and indicated that less than 0.2% of the applied radioactive tRNA was retained by the immunoadsorbent when the adsorption was conducted in the presence of m^{6}A (1.0 mg/mL). These results revealed that the adsorption of radioactive tRNA was immunospecific, rather than nonspecific. The addition of adenosine at a concentration of 1.0 mg/mL had no effect on the immunospecific retention of labeled tRNAs (Table I). This result was expected on the basis of the antibody-specificity data presented in Figure 1.

Characterization of Immunospecifically Retained tRNA. Analysis of methyl-^{3}*H-Labeled, Methylated Nucleoside Constituents.* Presented in Table II is an analysis of the *methyl*-^{3}H-labeled, methylated nucleosides obtained following enzymatic digestion of unfractionated, nonretained, and immunospecifically retained [*methyl*-^{3}H]tRNA. Whereas the distribution and amount of *methyl*-^{3}H-labeled, methylated nucleosides present in unfractionated tRNA resembled those obtained from a variety of other bacterial tRNA preparations

TABLE I: Immunospecific Retention of Labeled tRNAs Utilizing Anti-m^{6}A/Sepharose Adsorbent; Effects of Competing Haptens (m^{6}A and A).[a]

labeled tRNA	Radioact. incubated with adsorbent (cpm × 10^{-4})	Radioact. retained by adsorbent (cpm × 10^{-4})	% retained
[^{32}P]PO_4-labeled tRNA	145	6.84	4.70
[^{32}P]PO_4-labeled tRNA + A	152	4.92	4.60
[^{32}P]PO_4-labeled tRNA + m^{6}A	142	0.12	0.08
[*methyl*-^{3}H]tRNA	52	2.39	4.61
[*methyl*-^{3}H]tRNA + A	52	2.25	4.32
[*methyl*-^{3}H]tRNA + m^{6}A	52	0.10	0.19

[a] Conditions identical with those described in Figure 2 were employed. Where noted, m^{6}A or A at a concentration of 1.0 mg/mL (in NaCl-P-Mg) was added to the incubation mixture just prior to the addition of labeled tRNA. Specific activities for [^{32}P]PO_4- and [*methyl*-^{3}H]methionine-labeled tRNAs were 6×10^4 cpm/μg and 5×10^3 cpm/μg, respectively.

TABLE II: Immunospecific Retention of [*methyl*-^{3}H]tRNA Utilizing Anti-m^{6}A/Sepharose Adsorbent; Distribution of [*methyl*-^{3}H]-Labeled Methylated Nucleosides Present in Unfractionated, Nonretained at Retained [*methyl*-^{3}H]tRNAs.[a]

Methylated nucleosides	[*methyl*-^{3}H]tRNAs[b] Unfractionated	Nonretained	Retained
m^1G	5.9	5.9	<1.0
m^7G	25.2	25.7	20.5
m^2A	8.3	8.4	<1.0
m^6A	1.9	0.2	24.6
m^5U	34.8	35.4	22.4
G^m	11.7	12.0	21.8
A^m	0.8	0.9	<1.0
C^m	1.7	1.8	<1.0
U^m	2.2	2.5	<1.0

[a] Nonretained and retained tRNA preparations were obtained from the initial two NaCl-P-Mg and initial two $NaDodSO_4$ wash fractions, respectively, that were eluted after immunospecific adsorption (Figure 2). The tRNAs were recovered by ethanol precipitation and enzymatically digested to nucleosides that were separated by two-dimensional thin-layer chromatography (see Experimental Procedures). Each value represents the mean of three independent experiments. Greater than 90% of the radioactive nucleosides chromatographed were recovered and identified. [b] Percent [*methyl*-^{3}H]-labeled methylated nucleosides recovered.

(Randerath & Randerath, 1973), the distributions obtained from the nonretained and retained tRNA preparations were significantly different. These differences included (a) the lack of appreciable quantities of m^{6}A in the nonretained fraction and (b) the presence of approximately equimolar quantities of m^{6}A, 7-methylguanosine (m^7G), 5-methyluridine (m^5U), and 2′-*O*-methylguanosine (G^m) in the retained fraction together with the absence of all other methylated nucleosides present in unfractionated tRNA. These results implied that the immunoadsorbent exclusively retained only that population of tRNAs possessing m^{6}A. The almost equimolar quantities of the four methylated nucleosides present in the retained tRNA fraction further suggested the presence of a single tRNA species.

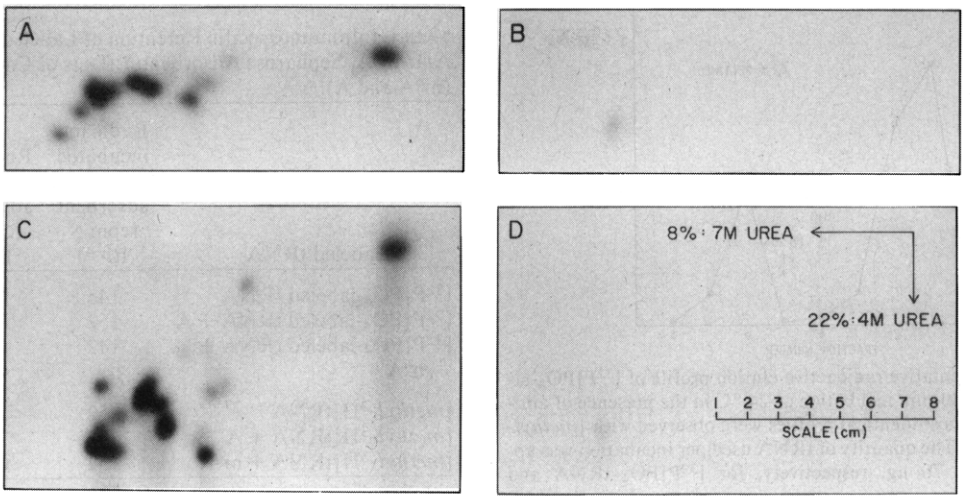

FIGURE 3: Autoradiographs of acrylamide gel slabs (3 mm thickness) after two-dimensional gel electrophoresis of [^{32}P]PO_4-labeled tRNA. The percent acrylamide, urea molarity, and direction of electrophoresis for all gels are as indicated in D. Time of electrophoresis in the first dimension (8% acrylamide, 7 M urea) was 12 h at 500 V (constant) for all gels; in the second dimension (22% acrylamide, 4 M urea) it was 24 h for gels A and B and 72 h for gels C and D (all gels at 200 V, constant). (Gel A) Unfractionated tRNA, 6×10^6 cpm applied. (Gel B) Immunospecifically retained tRNA, 1.5×10^5 cpm applied. (Gel C) Unfractionated tRNA, 8×10^6 cpm applied. (Gel D) Immunospecifically retained tRNA, 8×10^4 cpm applied. Upon completion of electrophoresis, gels were incubated in 7% acetic acid, wrapped in polyethylene bags, and placed directly over x-ray film for 24 h (expose time).

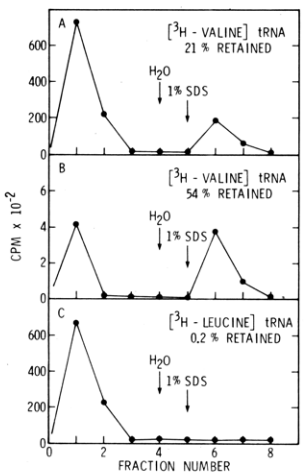

FIGURE 4: Immunospecific retention of aminoacyl-tRNAs. The procedures for aminoacylation of tRNA are described in Experimental Procedures as are those for immunospecific retention (also, see Figure 2). (A) Radioactive elution profile of [2,3-^{3}H]valyl-tRNA after incubation with anti-m^{6}A/Sepharose. (B) Radioactive elution profile of [2,3-^{3}H]valyl-tRNA previously adsorbed to anti-m^{6}A/Sepharose; i.e., the [2,3-^{3}H]-valyl-tRNA eluting with fractions 6 and 7 in A was recovered via ethanol precipitation and reincubated in the presence of the immunoadsorbent. (C) Radioactive elution profile of [4,5-^{3}H]leucyl-tRNA after incubation with anti-m^{6}A/Sepharose. An identical profile (not shown) was obtained with [2,3-^{3}H]phenylalanyl-tRNA.

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was conducted in an attempt to determine (a) the number of tRNA species retained by the immunoadsorbent and (b) if significant degradation of tRNA occurred during the immunospecific adsorption process. Illustrated in Figure 3 are autoradiographs of [^{32}P]PO_4-labeled unfractionated and retained tRNAs. Whereas unfractionated tRNA could be resolved into a number of species depending upon the electrophoretic conditions imposed upon the gel system (Figures 3A and 3C, also see legend), a single, discrete area of radioactivity was observed with the immunospecifically retained tRNA fraction (Figures 3B and 3D). This was especially apparent when electrophoresis was conducted for 72 h in the second dimension (22% acrylamide, 4 M urea). The latter condition increased significantly the resolution of individual tRNA species (compare Figures 3A and 3C).

Recovery studies further revealed that greater than 85% of the immunospecifically retained tRNA applied to gels migrated within these discrete areas of radioactivity. These analyses supported the interpretation that a single tRNA species was adsorbed by the anti-m^{6}A/Sepharose adsorbent and further suggested that ribonuclease activity was minimal or absent during immunospecific adsorption and elution.

The vertical streaks of disperse radioactivity observed primarily in the autoradiographs in Figure 3C were attributed to radiation-induced degradation of RNA, i.e., the disintegration of ^{32}P of phosphodiester bonds. The intensity of these streaks increased significantly as the time of electrophoresis was increased in the second dimension from 24 h (Figure 3A) to 72 h (Figure 3C). The large spot of radioactivity appearing in the upper right-hand corner of autoradiographs A and C was suspected to be 5S RNA on the basis that this species was devoid of *methyl*-^{3}H-labeled, methylated constituents.

Immunospecific Specific Retention of [2,3-^{3}H]Valine tRNA. Of the numerous bacterial tRNAs that have been sequenced (Dayhoff, 1976), only $tRNA^{Val}_1$ has been found to contain an m^{6}A residue (located adjacent to the 3′-end of the anticodon). In view of the above, experiments were designed to determine if the tRNA retained by the anti-m^{6}A/Sepharose adsorbent was a $tRNA^{Val}$ species. Initially, individual preparations of unfractionated *Escherichia coli* H tRNA were aminoacylated with [2,3-^{3}H]valine, [2,3-^{3}H]phenylalanine, or [4,5-^{3}H]leucine and the resulting tRNAs subjected to immunospecific adsorption techniques in a manner identical with that described in the legend of Figure 2. The results of these investigations are presented in Figure 4 and indicated that neither [4,5-^{3}H]leucyl- nor [2,3-^{3}H]phenylalanyl-tRNA was retained by the immunoadsorbent (Figure 4C). However, in regard to [2,3-^{3}H]valyl-tRNA it was observed that approximately 20% of this radioactive species was retained. Furthermore, if the retained portion of this tRNA was recovered and reincubated in the presence of anti-m^{6}A/Sepharose, greater than 50% of the radioactivity was readsorbed. These results led to the conclusions that a significant percentage of the [2,3-^{3}H]valyl-tRNA possessed a m^{6}A residue and that, during

the adsorption process, either the [2,3-^{3}H]valyl-tRNA was undergoing deacylation and/or degradation. Also, it was expected that not all of the [2,3-^{3}H]valyl-tRNA would initially bind to the immunoadsorbent (Figure 4A), since of the three major tRNA^{Val} species (1, 2A, and 2B) only tRNA$^{Val}_1$ possesses an m^{6}A residue (Yaniv & Barrell, 1971; Chase et al., 1974).

To test the possibilities of deacylation and/or degradation, preparations of both [2,3-^{2}H]valyl-tRNA and [*methyl*-^{3}H]-tRNA were incubated in the presence of immunoadsorbent and aliquots withdrawn at selected time intervals for measurement of perchloric acid (PCA)-soluble radioactivity. Whereas the amount of PCA-soluble radioactivity generated in the presence of [2,3-^{3}H]valyl-tRNA would reflect deacylation, that generated with [*methyl*-^{3}H]tRNA was taken as a measure of RNase activity. The results of these investigations are illustrated in Figure 5 and revealed that after a 40 min incubation in the presence of immunoadsorbent approximately 35% of the [2,3-^{3}H]valyl-tRNA became PCA soluble. These results implied that a significant amount of the [2,3-^{3}H]-valyl-tRNA eluting in the initial wash fractions (see Figure 4A and 4B) could be attributed to deacylation. Based upon the findings that (a) 35% of the valyl-tRNA was deacylated during the immunospecific adsorption process and (b) 56% of the initially retained valyl-tRNA could be readsorbed, it was concluded that the adsorption process was 90% efficient (i.e., 35% + 56% = 91%).

The increase in PCA-soluble radioactivity observed with [*methyl*-^{3}H]tRNA was negligible throughout the incubation period (Figure 5) and supported the conclusion derived from autoradiographic data (Figure 3) that nuclease activity was minimal or absent during the immunospecific adsorption process.

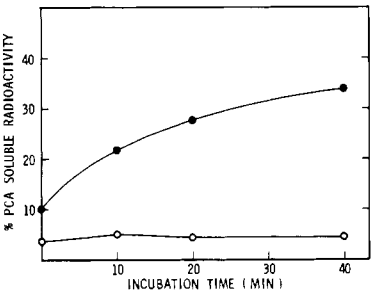

FIGURE 5: The generation of PCA-soluble radioactivity during incubation of [2,3-^{3}H]valyl-tRNA (closed circles) and [*methyl*-^{3}H]tRNA (open circles) with anti-m^{6}A/Sepharose. Conditions of incubation identical with those described in Figures 2 and 4. Values represent the mean of two independent experiments. Measurement of PCA-soluble radioactivity is described in Experimental Procedures. Similar results (not shown) were observed for both [4,5-^{3}H]leucyl-tRNA and [2,3-^{3}H]phenylalanyl-tRNA, i.e., between 25 and 45% deacylation after a 40-min incubation period.

Discussion and Conclusions

Unfractionated bacterial tRNA was chosen for this investigation because, of the numerous species that have been sequenced, only tRNA$^{Val}_1$ contains m^{6}A (Dayhoff, 1976). Furthermore, since m^{6}A is located adjacent to the 3′ end of the anticodon, it seemed likely that the base portion of this nucleoside would be exposed to the surface, rather than buried within the molecule (Kim et al., 1974; Robertus et al., 1974). In addition, the presence of a methyl group in the N^6 position of adenosine is known to destabilize base-pair formation (Griffin et al., 1964), which might otherwise obviate the reaction between hapten and antibody. In view of the above, it was of interest to determine the feasibility of anti-m^{6}A/Sepharose as an immunochemical reagent for the isolation of m^{6}A-containing tRNA species.

The results presented herein demonstrated that anti-m^{6}A antibodies were indeed capable of recognizing an m^{6}A-containing tRNA (Figure 2 and Table I). Furthermore when coupled to Sepharose, the resulting immunoadsorbent quantitatively and exclusively retained a specific tRNA population on the basis of its content of m^{6}A (Table II). Characterization of the immunospecifically retained tRNA population further indicated the presence of a single tRNA species (Figure 3) that was tentatively identified as tRNA^{Val} (Figure 4).

That the tRNA retained by the immunoadsorbent contained approximately equimolar quantities of [*methyl*-^{3}H]m^{6}A, m^{5}U, m^{7}G, and G^m was somewhat surprising in view of sequenced data which indicated that bacterial tRNA$^{Val}_1$ contained only three of the above-listed methylated nucleosides, G^m not being detected previously (Harada et al., 1971; Kimura et al., 1971; Yaniv & Barrell, 1971). Two possibilities for this apparent discrepancy are (a) the difference in the strains of *Escherichia coli* employed, i.e., strains B and H, and (b) the degree of methylation of the purified tRNA$^{Val}_1$ used for sequencing. In regard to the latter, evidence has been provided that approximately 30 min is required for nascent tRNA molecules to acquire their full complement of methylated nucleoside constituents (Munns & Sims, 1975b). Furthermore, whereas the base-methylated constituents appear on precursor tRNA during the early stages of maturation, the bulk of 2′-*O*-ribose methylations occur during the late stages of maturation (Munns & Sims, 1975b). In view of the above and the fact that the doubling time of many *Escherichia coli* is 30 min (enriched media), the possibility exists that the tRNA$^{Val}_1$ used for sequencing was undermethylated with respect to G^m. However, the pulse-chase labeling system used in the present investigation was designed to ensure that the labeled tRNAs were mature with respect to their full complement of methylated constituents.

Based upon these and other investigations (Munns et al., 1977a,b), it has been established that the anti-m^{6}A/Sepharose adsorbent can quantitatively retain mononucleotides, oligonucleotides, and tRNA molecules possessing the corresponding antigenic hapten, i.e., m^{6}A. It remains to be demonstrated that larger RNA molecules containing m^{6}A can be isolated via these or related immunochemical techniques. However, preliminary studies in our laboratory have indicated that the 28S rRNA species, containing a single m^{6}A residue (Maden & Salim, 1974), as well as various mRNA preparations, containing internal m^{6}A residues (Perry & Kelley, 1974; Wei & Moss, 1977) can be detected and isolated by immunospecific adsorption techniques. Thus, the feasibility of this immunochemical approach for the isolation and characterization of nucleic acid appears to be promising. Although the present data demonstrate only one fractionation scheme, the versatility of this approach, in theory, is limited only by the quantity and types of modified constituents present in nucleic acids. In this regard, mammalian tRNAs, rRNAs, mRNAs, low molecular weight nuclear RNA, heterogeneous nuclear RNA, and various viral RNAs seem particularly well suited for analysis.

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