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Characterization of Antibodies Specific for N^6-Methyladenosine and for 7-Methylguanosine†

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

ABSTRACT: Antibodies specific for N^6-methyladenosine (m^6A) and for 7-methylguanosine (m^7G) were prepared by immunization of rabbits with nucleoside conjugates of bovine serum albumin (i.e, m^6A-BSA and m^7G-BSA). Specificity of each antibody was assessed by inhibition of the homologous precipitin reaction with various nucleosides. These analyses revealed that the antibodies elicited in response to m^6A-BSA were specific for the N^6-methyl moiety of adenosine with minimal or no cross-reactivity with BSA, adenosine, and guanosine. Although a major fraction of antibodies elicited in response to m^7G-BSA were specific for m^7G, considerable cross-reactivity was observed with BSA. These latter antibodies were removed by affinity chromatography utilizing BSA-Sepharose adsorbent. In similar fashion, antibodies specific for m^6A and m^7G were isolated by immunospecific adsorption to antigen-coupled Sepharose (e.g, m^6A-BSA-Sepharose), eluted, and coupled to Sepharose. The ability of these antibody-coupled adsorbents to retain specific methylated [*methyl*-^{3}H]nucleosides derived from [*methyl*-^{3}H]tRNA digests was assessed. Both the anti-m^7G and anti-m^6A antibody adsorbents quantitatively and exclusively retained 7-[^{3}H]methylguanosine and N^6-[^{3}H]methyladenosine, respectively. The application of these adsorbents to fractionate oligonucleotides and nucleic acids is discussed.

The use of affinity chromatography as a means of fractionating RNA populations on the basis of their content of specific nucleotide sequences or modified nucleotides has been documented. For example, mRNA containing poly(A)[1] has been isolated following chromatography on either poly(dT)-cellulose (Aviv and Leder, 1972) or poly(U)-Sepharose (Lindberg and Persson, 1972). In a somewhat different system, $tRNA^{Arg}$ and yeast $tRNA^{Phe}$ have been purified via affinity-antibody chromatography utilizing antibodies which react immunospecifically with inosine (Inouye et al., 1973) and the Y nucleoside (Fuchs et al., 1974), respectively. While other antibodies which recognize a variety of minor constituents found in RNA have been characterized (Erlanger and Beiser, 1964; Karol and Tanenbaum, 1966; Sawicki et al., 1971; Levine et al., 1971), their application for fractionating RNA populations

† From the Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri, 63104. *Received December 8, 1976.* This investigation was supported by Grant CA 17715, awarded by the National Cancer Institute, Department of Health, Education, and Welfare.

[1] Abbreviations used are: BSA, bovine serum albumin; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; poly(A), poly(adenylic acid); poly(dT)-cellulose, poly(thymidylic acid)-coupled cellulose; poly(U)-Sepharose, poly(uridylic acid)-coupled Sepharose; A, adenosine; m^1A, 1-methyladenosine; m^2A, 2-methyladenosine; m^6A, N^6-methyladenosine; m^6_2A, N^6,N^6-dimethyladenosine; A^m, 2′-*O*-methyladenosine; m^6A^m, 2′-*O*,N^6-dimethyladenosine; m^1I, 1-methylinosine; G, guanosine; G^m, 2′-*O*-methylguanosine; m^1G, 1-methylguanosine; m^7G, 7-methylguanosine; m^2G, N^2-methylguanosine; m^2_2G, N^2,N^2-dimethylguanosine; m^3C, 3-methylcytidine; m^5C, 5-methylcytidine; C^m, 2′-*O*-methylcytidine; m^3U, 3-methyluridine; m^5U, 5-methyluridine; U^m, 2′-*O*-methyluridine; m^7G^*, 2-amino-4-hydroxy-5-(*N*-methylcarboxamido)-6-ribosylaminopyrimidine (i.e., the ring-opened structure of m^7G following treatment with alkali). For spectral data, A_{nm} and E_{nm} are the absorbance (1-cm light path) and molar extinction coefficients, respectively, at a particular wavelength (nm).

has been limited to the examples cited above.

Because of the variety and abundance of methylated nucleosides appearing in tRNA, rRNA, and most recently in mRNA (Munns and Sims, 1975a; Randerath and Randerath, 1973; Desrosiers et al., 1974), we became interested in examining the possibility that antibodies specific for such minor constituents could fractionate oligonucleotides and RNA molecules on the basis of their composition of methylated nucleosides. The research described herein represents our initial findings regarding (a) the conjugation of m^6A and m^7G to BSA, (b) the specificity of the antibodies elicited in response to the above antigens, and (c) the ability of purified anti-m^6A and anti-m^7G antibodies, immobilized on Sepharose, to retain their respective antigenic haptens.

Materials and Methods

Materials. Nucleosides employed in the present investigation included adenosine (A), 1-methyladenosine (m^1A), N^6-methyladenosine (m^6A), N^6N^6-dimethyladenosine (m_2^6A), 2′-*O*-methyladenosine (A^m), guanosine (G), 1-methylguanosine (m^1G), 7-methylguanosine (m^7G), N^2-methylguanosine (m^2G), and 2′-*O*-methylguanosine (G^m) and were purchased from either Sigma Chemical Co. or P-L Biochemicals. Overnight treatment in 6 M NH_4OH resulted in the conversion of m^7G to the ring-opened structure: 2-amino-4-hydroxy-5-(*N*-methylcarboxamido)-6-ribosylaminopyrimidine (Hains et al., 1967). This nucleoside will be designated m^7G^*. Each of the above nucleosides demonstrated a single spot following thin-layer chromatography in three solvent systems (Munns and Sims, 1975a) as well as characteristic absorption spectra (Burton, 1974).

Sepharose 4B and CNBr-activated Sepharose 4B were obtained from Pharmacia, bovine serum albumin (BSA, fraction V) was from Sigma Chemical Co., and [*methyl*-^{3}H]methionine, [2-^{14}C]guanosine, and [2-^{14}C]adenosine were from Amersham Searle.

Conjugation of Haptens. Both m^6A and m^7G were conjugated to BSA via periodate oxidation according to the procedure of Erlanger and Beiser (1964). Briefly, 400 mg of m^7G and 200 mg of m^6A were subjected to periodate oxidation prior to the addition of 200 mg of BSA. After removal of excess periodate with ethylene glycol, the hapten linkages were stabilized using $NaBH_4$. The uncoupled nucleosides were removed by overnight dialysis against water and the BSA conjugates were concentrated by lyophilization. Subsequent spectral analyses of the conjugates were undertaken to ascertain the extent of conjugation as well as the possibility of nucleoside modification. Protein content of the resulting m^7G-BSA and m^6A-BSA conjugates was determined by the method of Lowry et al. (1951) using BSA as the standard.

Immunization. New Zealand White female rabbits were immunized intradermally and subcutaneously in multiple sites with BSA conjugates (2.5 mg/rabbit) emulsified in complete Freund's adjuvant. Identical injections were repeated after 1 week. Booster immunizations were given in the same manner after the initial antibody response had subsided (approximately 4–6 weeks after initial injection). Small amounts of sera were collected 5–7 days thereafter for detection of antibodies by immunoelectrophoresis (Hacker et al., 1970).

The techniques for blood collection (50 mL/rabbit) consisted of swabbing xylene (vasodilator) over the middle auricular artery and injecting a small amount (0.05–0.1 mL) of 1% Xylocaine (Astra Pharmaceuticals) on both sides of the vessel. After several minutes, the artery was punctured with an 18–20-gauge needle and the blood was collected directly into a centrifuge tube. Bleeding was halted either by pressure or by the topical administration of bovine thrombin. The resulting antisera were pooled and processed as described below.

Characterization of Antibodies. Quantitative precipitin and hapten inhibition techniques were conducted as described by Kabat (1961) to ascertain both the amount and specificity of the antibodies elicited in response to m^6A-BSA and m^7G-BSA antigens. These procedures are described in more detail in the legend of Figure 2. Quantitation of precipitable protein formed during the precipitin reactions was determined by the method of Lowry et al. (1951) using bovine γ-globulin as the protein standard and by measurement of the absorbance at 278 nm in 0.5 N NaOH.

Conjugation of Proteins (Antigens and Antibodies) to Sepharose. Preparation of immunospecific adsorbents was conducted according to the procedures described by Axen et al. (1967) and Porath et al. (1967). Antigens coupled to Sepharose included BSA, m^7G-BSA, and m^6A-BSA. These adsorbents were employed as a means of purifying anti-m^7G and anti-m^6A antibodies (as described below) which, in turn, were coupled to Sepharose. Approximately 10 mg of each protein preparation was reacted per g (dry weight) of CNBr-activated Sepharose utilizing bicarbonate buffer (0.2 M $NaHCO_3$, pH 9.0, in 0.5 M NaCl). By this procedure, more than 90% of the protein in each preparation was coupled to Sepharose with the exception of m^7G-BSA which was coupled to the extent of 30%. Upon completion of the coupling reaction, any active sites remaining on Sepharose were neutralized with 1.0 M ethanolamine, pH 8.0. Finally, the Sepharose preparation was washed with acid (0.1 M acetic acid containing 1.0 M NaCl, pH 4.0), with base (0.1 M sodium borate containing 1.0 M NaCl, pH 8.0), with phosphate-buffered saline (150 mM NaCl, 10 mM PO_4, pH 7.4), and stored in phosphate-buffered saline (NaCl-P) at 4 °C.

Purification of Antibodies. Antibodies specific for the nucleoside haptens m^7G and m^6A were purified by immunospecific adsorption on m^7G-BSA-Sepharose and m^6A-BSA-Sepharose, respectively. Because a large fraction of the antibody population elicited in response to m^7G-BSA (but not m^6A-BSA) cross-reacted with BSA (ca. 40%), this population was initially removed by its immunospecific adsorption to BSA-Sepharose prior to the purification of anti-m^7G antibodies via their isolation with m^7G-BSA-Sepharose. Immunospecific adsorption procedures consisted of (a) adding 20 to 30 mL of antisera to an appropriate amount of antigen-coupled Sepharose, (b) incubating the resulting mixture with rotation at 37 °C for 45 min, (c) washing the immunoadsorbent free of unbound serum contaminants with NaCl-P in a fritted-disk funnel, and (d) eluting the retained antibodies by reincubating the immunoadsorbent three successive times in 1.0 M acetic acid (10 mL) for 30 min at 37 °C. Eluted antibodies were dialyzed against water and then coupling buffer (0.2 M $NaHCO_3$, pH 9.0, in 0.5 M NaCl), and conjugated to Sepharose as described above. Homologous precipitin reactions with aliquots of both antisera and purified antibody indicated, respectively, that (a) more than 95% of the specific serum antibody population was adsorbed to and recovered from the immunoadsorbents and (b) a minimum of 98% of the isolated antibodies retained their ability to precipitate antigen (see Table II).

Isolation, Purification, and Characterization of tRNA. The isolation and purification of tRNA from *Escherichia coli* H cells, previously labeled with [*methyl*-^{3}H]methionine, have been described (Munns et al., 1974). [*methyl*-^{3}H]Methio-

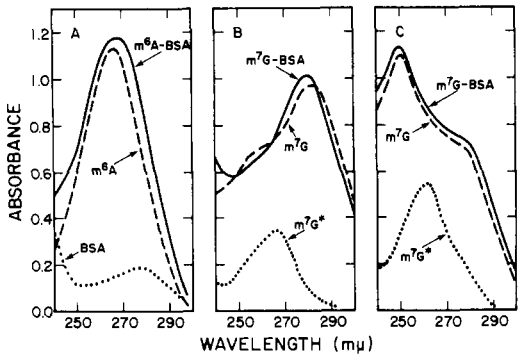

FIGURE 1: Absorbance spectra of nucleosides and nucleoside-BSA conjugates. Concentrations of m^6A, m^6A-BSA, and BSA were 20, 250, and 260 μg/mL in NaCl-P, respectively (inset A). Concentrations of m^7G*, m^7G, and m^7G-BSA were 20, 40, and 150 μg/mL in 0.1 M Tris, pH 9.8 (inset B), and in 1.0 N HCl (inset C), respectively. All spectra in inset C were obtained from preparations which had been heated at 100 °C for 60 min in 1.0 N HCl. Concentrations of m^7G-BSA and m^6A-BSA were based on protein content as described under Materials and Methods section.

nine-labeled tRNA ([*methyl*-^{3}H]tRNA) obtained in this manner possessed a specific activity of 2200 cpm/μg and was judged 98% pure via acrylamide gel electrophoresis (Munns et al., 1974). Analysis of the methylated nucleoside constituents present in [*methyl*-^{3}H]tRNA after enzyme digestion (Penicillium nuclease and alkaline phosphatase) and thin-layer chromatography (Munns and Sims, 1975b) revealed that the type and distribution of methylated [*methyl*-^{3}H]nucleosides closely resembled that of other *E. coli* tRNA preparations (Table III). Hydrolysis of tRNA (50–100 μg) with 50 μg of Penicillium nuclease (P_1 nuclease, P-L Biochemicals) was conducted at 37 °C for 2 h in 10 mM sodium acetate, 0.2 mM EDTA, pH 5.2, and resulted in complete degradation of tRNA to 5′-mononucleotides. The resulting mononucleotides were hydrolyzed to nucleosides with 1–2 units of immobilized bacterial alkaline phosphatase (Worthington Biochemicals) at 37 °C for 2–4 h in 10 mM Tris, 1.0 mM $MgCl_2$, pH 9.0.

Immunospecific Adsorption of Methylated [methyl-^{3}H]Nucleotides. Batchwise adsorption techniques were employed to assess the ability of the two immunoadsorbents to retain specific methylated [*methyl*-^{3}H]nucleotides. Following Penicillium nuclease digestion of 2 to 5 × 10^5 cpm of [*methyl*-^{3}H]tRNA in 0.05 mL, digests were diluted to 1.0 mL with NaCl-P and incubated in the presence of 0.5 mL of the appropriate immunoadsorbent with gentle rotation for 45 min at 37 °C. Upon completion of the incubation step, the adsorbent was transferred to a fritted-disk funnel (15-mL size) and washed at room temperature with aliquots of NaCl-P buffer (6 × 1.0 mL) and water (2 × 1.0 mL) to remove nonretained radioactivity. The radioactivity retained by the immunoadsorbent after the above washings was removed by the addition of 6 M NH_4OH. Upon removal of the NaCl present in the nonretained radioactive fraction via DEAE-cellulose chromatography (Tomlinson and Tener, 1963), both the nonretained and retained fractions were dried under N_2 and treated with alkaline phosphatase, and the resulting [*methyl*-^{3}H] nucleosides were identified by thin-layer chromatographic techniques as described above for tRNA.

Results and Discussion

Characterization of Nucleoside-BSA Conjugates. Spectral analyses of m^6A-BSA and m^7G-BSA were conducted to ascertain (a) the extent to which each nucleoside was conjugated to BSA and (b) whether the purine ring structure of these

TABLE I: Extent of Nucleoside Conjugation to BSA.[a]

Wavelength (nm)	$\epsilon_{nm} \times 10^{-3}$ m^6A	$\epsilon_{nm} \times 10^{-3}$ m^7G	Nucleoside molecules/BSA molecule m^6A-BSA	Nucleoside molecules/BSA molecule m^7G-BSA
250	8.4	4.5	17.4	51.3
260	14.3	5.3	17.1	50.0
270	15.2	5.9	17.1	55.3
280	9.1	7.2	17.7	53.7
290	3.1	6.2	17.6	48.4
Av			17.4	51.7

[a] The extent of conjugation of nucleosides was estimated from a knowledge of molar extinction coefficients (ϵ_{nm}) of nucleosides and BSA as determined by the various spectra presented in Figure 1. Calculations included subtraction of the absorbance due to a given amount of BSA, e.g., from Figure 1A; $A_{270}^{m^6A\text{-}BSA}(1.17) - A_{270}^{BSA}(0.16) = A_{270}^{m^6A}(1.01)$ and determination of the nmol of m^6A bound to 250 μg of BSA (3.85 nmol of BSA). Therefore, $A_{270}^{m^6A}(1.01)/\epsilon_{270}^{m^6A}(15.2 \times 10^3)10^6 = 66$ nmol, and thus provided an estimate of the average number of m^6A residues conjugated to a molecule of BSA (66/3.85 = 17.1). Similar calculations for m^7G were derived from the data presented in Figure 1B, including the ϵ_{nm} listed above.

nucleosides was modified during the conjugation reaction. Figure 1 illustrates typical absorption spectra of the resulting nucleoside-BSA conjugates as well as the nucleosides themselves under various buffer and pH conditions. Only small differences in the spectra were apparent between the coupled and uncoupled nucleosides and these were attributed to the absorption of BSA. The possibility that appreciable quantities of m^7G were modified to the ring-opened structure m^7G* (see Materials and Methods) as a result of the alkaline conditions employed in the conjugation reaction was negated on the basis that the absorbance spectrum of m^7G* was considerably different from either m^7G or m^7G-BSA (Figure 1, insets B and C).

The extent of conjugation of nucleosides to BSA was estimated from the extinction coefficients of nucleosides and nucleoside-BSA conjugates at various wavelengths after the appropriate correction for the absorbance due to BSA. These results are presented in Table I and indicate that an average of 17.4 (m^6A) and 51.7 (m^7G) nucleosides were coupled to each BSA molecule. It is interesting that the conjugation method employed in the present investigation couples the nucleoside derivative to the ϵ-amino group of lysine and that the BSA molecule contains 56 lysine residues (Peters and Hawn, 1967). These data (see Table I) indicated that almost all of the lysine residues of BSA participated in the conjugation of m^7G.

Characterization of Antibodies Elicited in Response to m^6A-BSA and m^7G-BSA. Each of the three rabbits immunized with m^6A-BSA and with m^7G-BSA produced antibodies precipitable with their homologous antigens. Representative precipitin reactions utilizing a constant volume of each pooled antiserum (0.1 mL) and increasing quantities of BSA and BSA conjugates are illustrated in Figure 2. Analysis of these data revealed that the point of maximum antibody precipitation occurred when the amount of homologous antigen was approximately 20 μg. Whereas 40% of the anti-m^7G antibody population cross-reacted with BSA (Figure 2B), similar cross-reactivity was not observed with the anti-m^6A antibody (Figure 2A). Anti-BSA antibodies elicited in response to m^7G-BSA were quantitatively removed by their immu-

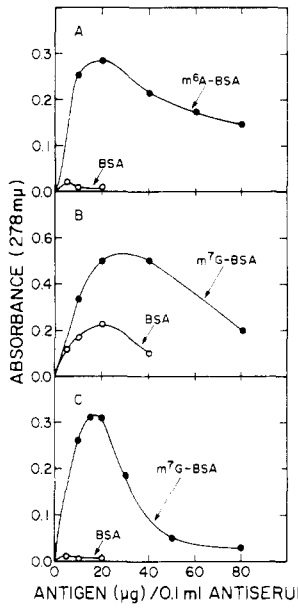

FIGURE 2: Quantitative precipitin reactions of antibodies present in antiserum with homologous antigens and with BSA. Precipitin reactions consisted of 0.1 mL of antiserum plus increasing amounts of antigen in NaCl-P to a final volume of 1.1 mL. The reaction mixture was incubated at 37 °C for 30 min and then overnight at 4 °C prior to centrifugation and three washes of the precipitated antigen–antibody complexes with NaCl-P. Protein content of precipitates was determined by A_{278} measurements and by the method of Lowry et al. (1951). Insets A and B represent the results of precipitin reactions using unadsorbed antisera, while inset C illustrates the precipitin curve generated after adsorption of anti-m^{7}G-BSA antiserum with BSA-Sepharose. Inhibition studies (see Figure 3) with competing haptens were conducted under conditions of maximum antibody precipitation (i.e., 20 μg of antigen protein and 0.1 mL of antiserum, see insets A and C). Values represent the mean of duplicate assays.

TABLE II: Purification of Anti-m^{6}A and Anti-m^{7}G Antibodies Via Affinity Chromatography.[a]

Purification	Total protein (mg/mL)	Precip. antibody[b] (mg/mL)	mg of Precip. antibody/ mg of total protein
Anti-m^{6}A Antibody			
Antiserum	57.2	2.2	3.8
m^{6}A-BSA-Sepharose R[c]	2.3	2.2	95.7
m^{6}A-BSA-Sepharose (NR)	54.7	0	0
Anti-m^{7}G Antibody			
Antiserum	55.1	3.4	6.2
BSA-Sepharose (R)	1.6	1.0	
BSA-Sepharose (NR)	52.6	2.6	4.9
Antiserum − BSA antibodies[d]	52.6	2.6	4.9
m^{7}G-BSA-Sepharose (R)	2.5	2.4	96.0
m^{7}G-BSA-Sepharose (NR)	50.0	0	0

[a] Procedures employed for immunospecific retention and elution of antibodies and protein determination are described under Materials and Methods. [b] Precipitable antibody represents the maximum amount of antibody precipitated with its homologous antigen (i.e., either m^{6}A-BSA or m^{7}G-BSA). [c] R and NR represent the protein in retained and nonretained fractions, respectively, after incubating antiserum with appropriate immunoadsorbent. [d] Antiserum minus BSA antibodies represents that fraction of antiserum *not* retained by BSA-Sepharose.

nospecific adsorption to BSA-coupled Sepharose (Figure 2C and Table II). Estimates of the amount of total anti-m^{6}A and anti-m^{7}G antibodies present under conditions of maximum precipitation (20 μg of antigen) yielded values of approximately 2.2 and 2.5 mg of antibody per mL of antiserum, respectively.

The ability of various haptens to competitively inhibit these precipitin reactions was assessed to ascertain the immunospecificity of each antibody towards its homologous hapten (m^{6}A and m^{7}G) as well as towards other potential cross-reacting nucleosides of similar structure. The degrees to which increasing amounts of hapten inhibited the homologous precipitin reaction under conditions of maximum precipitation are presented in Figure 3. These inhibition curves, based on the micromoles of hapten present per milligram of antibody, revealed that the homologous haptens were the most effective inhibitors, although m^{7}G* was equally effective as m^{7}G in this regard (Figure 3B). Less pronounced inhibitions were observed with 1-methyladenosine (m^{1}A, Figure 3A) and 2′-*O*-methylguanosine (G^m, Figure 3B); yet, little or no inhibition was detected with adenosine (A) or guanosine (G). Because of the low solubility of G, its diphosphate (GDP) was also employed as a competing hapten at higher concentrations.

Included in the legend of Figure 3 are the micromoles of hapten required to inhibit the precipitin reaction by 50%. Although these data only reflect the relative affinities of each antibody towards various haptens, the extreme difference in the concentrations of m^{6}A and A (approximately 1000-fold) required for 50% inhibition indicates a high degree of specificity for the N^6-methyl group of m^{6}A. Similar analysis between m^{7}G (or m^{7}G*) and G revealed preferential specificity toward the N^7-methyl group; yet, this difference was only 20-fold. Other haptens demonstrating only minimal or no inhibition in either assay included m_2^6A, m^{1}G, m^{2}G, as well as both uridine and cytidine and an assortment of methylated pyrimidines (excluding m^{7}G*). Recent experiments with 2′-*O*,N^6-dimethyladenosine (m^{6}A^m) have indicated that this constituent was equally as effective as m^{6}A in these inhibition assays (not shown).

A second factor which influences the amount of hapten required to achieve a particular level of inhibition is the degree of conjugation of the antigen. Since the antigen m^{7}G-BSA contains approximately three times the number of haptens as m^{6}A-BSA (per molecule of BSA, see Table I), it would be expected that more m^{7}G would be required to inhibit its homologous precipitin reaction. Thus, as listed in the legend of Figure 3, approximately three times as much m^{7}G (3.0 μmol) was required to achieve a 50% inhibition than that required by m^{6}A (1.2 μmol).

Purification of Antibodies. Upon completion of the above studies, both anti-m^{6}A and anti-m^{7}G antibodies were purified by immunospecific adsorption to antigen-coupled Sepharose, i.e., m^{6}A-BSA-Sepharose and m^{7}G-BSA-Sepharose. The concentration of both total protein and precipitable antibody was monitored routinely throughout the entire purification procedure, the latter by quantitative precipitin reactions similar to those described in connection with Figure 2. Results from these studies are presented in Table II and indicate that both antibody preparations were quantitatively adsorbed to and eluted from their respective antigen-coupled adsorbents. Data pertaining to the initial immunospecific adsorption of anti-BSA antibodies are also included and were taken in part from that presented in Figure 2B. These procedures resulted in an approximate 20-fold purification of both anti-m^{6}A and anti-m^{7}G antibodies. Equally important was the finding that greater than 90% of the protein in these purified preparations was precipi-

TABLE III: Identification of Methylated [*methyl*-^{3}H]Nucleosides Retained by Immunospecific Adsorption.[a]

Nucleoside	% of Radioactivity in: Unfx tRNA	NR_{fx}	R_{fx}
Anti-m^{6}A-Sepharose			
m^{1}G	5.8	5.8	0
m^{7}G	26.2	27.4	0
m^{2}A	8.2	8.1	0
m^{6}A	2.6	0.0	>98
m^{5}U	34.5	35.6	0
X^m	17.2	18.3	0
Anti-m^{7}G-Sepharose			
m^{1}G	5.8	9.4	0
m^{7}G	26.2	0.0	>98
m^{2}A	8.2	12.7	0
m^{6}A	2.6	3.5	0
m^{5}U	34.5	48.4	0
X^m	17.2	20.6	0

[a] The type and distribution of methylated [*methyl*-^{3}H]constituents present in enzyme digests of unfractionated tRNA (Unfx tRNA), and that in the nonretained (NR_{fx}) and retained (R_{fx}) fractions after immunospecific adsorption (see Figure 4). The percentages listed represent the average of duplicate determinations. X^m reflects the radioactivity incorporated into 2′-*O*-methylnucleosides.

table with homologous antigen (i.e., mg of precipitable antibody/mg of total protein, Table II).

Retention of Specific Mononucleotides Via Affinity-Antibody Chromatography. Once purified, each antibody was coupled to Sepharose and these immunoadsorbents were tested for their ability to retain specific methylated [*methyl*-^{3}H] nucleotide constituents present in *E. coli* H tRNA preparations previously digested with Penicillium ribonuclease. Illustrated in Figure 4 are the radioactive elution profiles derived from each immunoadsorbent after a 45-min incubation in the presence of tRNA digest. Whereas the bulk of radioactivity was eluted during the initial two washes of the immunoadsorbent (nonretained radioactivity), approximately 2.5 and 25% were retained by anti-m^{6}A-Sepharose and anti-m^{7}G-Sepharose, respectively, even after numerous washes in NaCl-P and water. This retained radioactivity was quantitatively removed by incubating the adsorbents at 37 °C in the presence of 6 M NH_4OH for 30 min. Identification of the methylated [*methyl*-^{3}H]constituents in tRNA as well as those eluted in the nonretained and retained fractions is presented in Table III. These results indicated that the monophosphates of m^{6}A and m^{7}G were quantitatively and exclusively retained by the appropriate adsorbents. All other methylated [*methyl*-^{3}H] constituents present in unfractionated tRNA were found in the nonretained fractions. Even A^m and G^m, which cross-reacted to some extent with anti-m^{6}A and anti-m^{7}G antibodies, respectively, were eluted in the nonretained fractions, as were [^{14}C]adenosine and [^{14}C]guanosine (not shown). In all of the above experiments, a minimum of 95% of the radioactivity in tRNA digests was recovered and identified.

Summary and Conclusions

The results presented herein indicate that antibodies specific for m^{6}A and m^{7}G can be elicited in response to m^{6}A-BSA and m^{7}G-BSA and that once purified and immobilized on Sepharose can quantitatively and exclusively retain their corresponding antigenic haptens. The methods employed for hapten retention consisted of batchwise adsorption (i.e., incubation of immunoadsorbent and hapten in NaCl-P for 45 min at 37

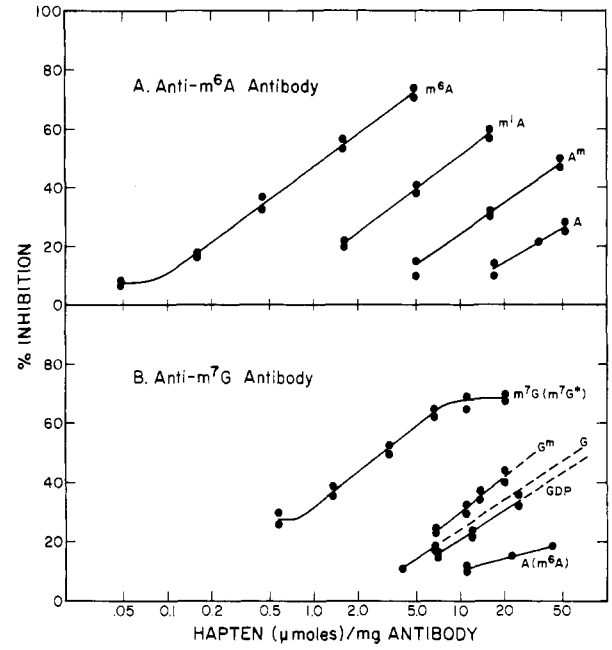

FIGURE 3: Hapten inhibition of quantitative precipitin reactions. Data obtained by incubating increasing amounts of haptens in the homologous precipitin reactions and measuring the decrease in precipitable antibody (anti-m^{6}A antibody, inset A; and BSA-adsorbed anti-m^{7}G antibody, inset B) under conditions of maximum precipitation (see Figure 2). Values reflect duplicate assays as shown. The concentration of each hapten (micromoles) required to achieve a 50% inhibition of the precipitin reactions were 1.2, 9.2, 58, and >1000 for m^{6}A, m^{1}A, A^m, and A, respectively (inset A) and 3.0, 35, >60, and >80 for m^{7}G, G^m, G, and GDP, respectively (inset B).

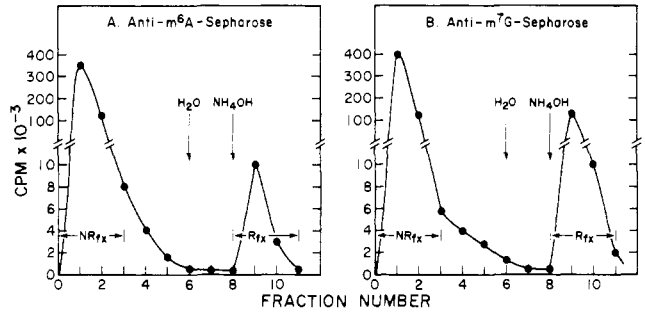

FIGURE 4: Radioactive elution profile of penicillium nuclease-digested [*methyl*-^{3}H]tRNA after incubation in the presence of anti-m^{6}A antibody-coupled Sepharose (A) and anti-m^{7}G antibody-coupled Sepharose (B). Procedures were as described under Materials and Methods. The type and distribution of methylated [*methyl*-^{3}H]constituents present in the nonretained (NR) and retained (R) fractions are presented in Table III.

°C) and elution (i.e., incubation of NaCl-P-washed and water-washed immunoadsorbent with NH_4OH for 30 min at room temperature). Attempts to retain m^{6}A on columns containing immunoadsorbent have proved only partially successful in that [*methyl*-^{3}H]m^{6}A was not quantitatively retained. Nonquantitative retention also was observed during batchwise adsorption with adsorbents containing nonpurified antibodies and thus indicated the importance of antibody purification (Table II).

Although little if any benefit was gained by the employment of these immunoadsorbents to isolate mononucleotides of m^{6}A and m^{7}G as derived from tRNA digests, these procedures did permit a convenient and rapid means of assessing the ability of these antibodies to retain the appropriate hapten. Based on

these results, other studies are now in progress to assess the feasibility of these immunoadsorbents to retain specific oligonucleotides and tRNAs which contain m^6A and m^7G. Preliminary data utilizing anti-m^6A antibody adsorbents and ribonuclease T_1 digest of tRNA as well as with intact tRNAs suggest that specific oligonucleotides and tRNAs possessing m^6A are retained.

Because of the multiplicity of methylated constituents which occur in both bacterial and mammalian tRNA preparations (Munns and Sims, 1975a; Dayhoff and McLaughlin, 1972), the fractionation of tRNAs via affinity chromatography with adsorbents containing antibodies directed towards specific methylated nucleosides may prove useful. Even though both Sawicki et al. (1971) and Inouye et al. (1971) have determined that certain nucleotides located in base-paired regions of nucleic acids do not react with their respective antibodies, examination of previously sequenced tRNA molecules reveals that most methylated constituents are present in unpaired regions of the molecule (Staehelin, 1971; Dayhoff and McLaughlin, 1972). Furthermore, since each species of tRNA contains approximately 6 to 7 (mammalian tRNA) or 2 to 3 (bacterial tRNA) methylated constituents, it is conceivable that the employment of immunospecific adsorbents such as those described herein could result in significant purification of various tRNA species. In this regard, antibodies specific for inosine and the Y nucleotide have been employed successfully to isolate *E. coli* $tRNA^{Arg}$ (Inouye et al., 1973) and yeast $tRNA^{Phe}$ (Fuchs et al., 1974).

The recent findings which demonstrate that mammalian mRNAs contain at their 5′-terminus a methylated cap structure ($m^7G(5')$-ppp$(5')X^m$) as well as internally located m^6A modifications (Adams and Cory, 1975; Wei et al., 1976; Georgieff et al., 1976) suggest other uses for the antibody preparations described above. Although immobilized antibodies may not be capable of retaining intact mRNA, their employment as a means of selecting oligonucleotides containing the appropriate haptens seems quite feasible. Subsequent characterization of these oligonucleotides would provide information regarding the nucleotide sequences adjacent to methylated constituents (Wei et al., 1976) as well as the location of internal m^6A residues relative to the 5′ and 3′ termini of mRNA (Georgieff et al., 1976).

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