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Antibodies to N^6-(Δ^2-isopentenyl) adenosine and its nucleotide: interaction with purified tRNAs and with bases, nucleosides and nucleotides of the isopentenyladenosine family

Dave S.Milstone, Barbara S.Vold, Dohn G.Glitz[+] and Nadene Shutt[+]

Biomedical Research Department, SRI International, 333 Ravenswood Ave., Menlo Park, CA 94025, and [+]Department of Biological Chemistry, UCLA School of Medicine, University of California, Los Angeles, CA 90024, USA

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ABSTRACT

The interaction of antibodies directed toward N^6-(Δ^2-isopentenyl)adenosine, i^6Ado, or its nucleotide with related bases, nucleosides, nucleotides and purified tRNAs is described. The selectivity of the antibody preparation was tested in inhibition experiments utilizing a sensitive radioimmunoassay to quantitate the binding of [^{3}H]i^6Ado to the antibody. Purified tRNAs containing various modified nucleosides adjacent to the 3'-end of the anticodon were tested to provide information about the selectivity of the antibody preparation toward nucleotides in this position of the tRNA chain. Antibodies directed against the nucleotide hapten were used to purify tRNAs which contain i^6Ado and to quantitate the amount of that nucleotide. The same order of selectivity was expressed whether the nucleotides were free or in a tRNA molecule. Interaction of the antibody with compounds from the i^6Ado family demonstrated dominance of the hydrophobic isopentenyl group and the importance of positional differences of modifications.

INTRODUCTION

In various experimental animals, antibodies have been induced which were directed against several types of RNA components, RNAs or ribonucleoproteins (1,2). With the exception of the ribosome, in each instance the RNA or component was coupled to a natural protein or synthetic protein-like carrier. In this paper, we consider the usefulness of antibodies made against a nucleotide or nucleoside hapten covalently bound to a protein conjugate in applications involving the occurrence of that hapten in a transfer ribonucleic acid molecule. This part of the research was done primarily to establish conditions whereby the amount of i^6Ado in tRNA could be quantitated, and to see what types of compounds might interfere with the assay. Also, it has not been clearly established whether the order of specificity for cross-reaction with closely related modified nucleosides or nucleotides will be the same where the nucleoside is free or in a tRNA. Accordingly, a series of bases, nucleosides, and nucleotides were also tested for their ability to inhibit the interaction of the antigen with the antibody preparation.

It is known that antibodies made against a modified nucleotide (or nucleo-

side)-protein conjugate will recognize that nucleoside moiety when it occurs in an RNA molecule. For example, antibodies made toward modified nucleosides have been used to purify tRNA (3,4), to purify oligonucleotides containing certain modifications (5) and to map the location of N^6, N^6-dimethyladenosine in the small subunit of the E. coli ribosome (6). However, in one case where the comparison between the ability of the free nucleoside and the tRNA containing that nucleoside were compared (7), antibody binding of the tRNA was less efficient based on the absolute amount of the relevant nucleoside. This raises the question of whether antibody to nucleoside or nucleotide will bind the component or a derivative or related nucleotide in a polymerized form such as tRNA as efficiently as is found with the free component.

We have chosen nucleotides of the isopentenyl adenosine [N^6-(Δ^2-isopentenyl)adenosine, abbreviated i^6Ado]family for these studies. Antibodies toward i^6Ado have been described previously (7,8). The i^6Ado group seems to provide a particularly good antigenic determinant (9). The i^6Ado group in a tRNA molecule only occurs in a single position--in the anticodon loop immediately adjacent to the last nucleotide of the anticodon or the first position of the codon. The i^6Ado is a particularly interesting nucleoside not only because it is a hypermodified component occurring in the anticodon loop of tRNA but also because as a free molecule it has been found to have an effect on cell growth of plant (10) and animal cells (see discussion in reference 7). The nucleoside, i^6Ado, has been found in the tRNA of bacteria, yeast, animals, and plants. In some bacteria, it is further modified to 2-methylthio-i^6Ado (ms^2i^6Ado) and in higher plants and some plant-associated bacteria the isopentenyl side chain is hydroxylated to give N^6-(4-hydroxy-3-methylbut-2-enyl) adenosine (iO^6Ado) or ribosylzeatin (11). As monomers, members of this nucleoside family are cytokinins and stimulate plant growth and differentiation (10). Secondary substitutes or modifications can have a drastic effect on biological activity. For instance, the 2-methylthio group in ms^2i^6Ado makes it significantly less active than i^6Ado as a cytokinin (12). In tyrosine tRNA from E. coli, the presence of ms2i^6Ado instead of i^6Ado significantly increased its codon-induced ribosome binding ability (13). It was of interest, therefore, to see what effect secondary substituents or modifications would have on antibody recognition.

MATERIALS AND METHODS

General

Any unspecified chemicals were of reagent grade, or of the best available commercial grade. Bovine serum albumin used for nucleotide conjugation was

Pentex Fraction V from Miles Laboratories. N^6-(Δ^2-isopentenyl)adenosine (i^6Ado) and its 5' nucleotide (pi^6Ado) were purchased from Sigma Biochemicals and P-L Biochemicals. Tritiated i^6Ado was prepared by Moravek Biochemicals, 15302 E. Proctor Ave., City of Industry, CA, 91745. Tritium-labeled nucleoside trialcohol was prepared by oxidation of 15 μmoles of i^6Ado with an equivalent amount of $NaIO_4$ for 20 min in the dark (total volume 0.3 ml), followed by reduction with 25 mCi of potassium boro[^{3}H]hydride (Amersham) of specific activity 500 mCi/mmole. After standing at room temperature overnight, excess borohydride was destroyed by acidification and the labeled nucleoside was purified by gel filtration on a 0.8 by 35 cm column of Sephadex G10 using water as the eluant.

Isopentenyladenine, kinetin, kinetin riboside, N^6-mono and dimethyladenosine, 1-methyladenosine, and ethenoadenosine were purchased from Sigma Biochemicals. Mono- and dimethyladenine, adenine, adenosine, AMP and poly(A) were purchased from Calbiochem, and puromycin was from U.S. Biochemicals. N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]-L-threonine, t^6Ado, was a gift from G. B. Chheda. Compounds from the i^6Ado family (Table II) were provided by Nelson J. Leonard. Filters used for the binding assay were of mixed cellulosenitrate-celluloseacetate composition (Millipore Corporation). Toluene-omnifluor was prepared by dissolving 15.2 gm of omnifluor (New England Nuclear) in 8 pints of AR Grade toluene (J. T. Baker). Scintillation cocktail 3a70B was purchased from Research Products International, Elk Grove Village, Illinois.

Preparation of immunogens

Nucleotide was conjugated to BSA using the method of Erlanger and Beiser (14) except that the conjugate was dialyzed for 28 hours against a total of 16 ℓ of distilled water and was subsequently lyophilized. Spectrophotometric analysis indicated that 20 moles of pi^6Ado had been conjugated to each mole of BSA.

Conjugation of the nucleoside, i^6Ado, to BSA followed the same procedures, except that molar equivalents of nucleoside and periodate were used, and the ethylene glycol addition was omitted. In three separate preparations, 18, 21, and 24 moles of i^6Ado were conjugated to each mole of BSA.

Transfer RNA

Crude tRNA from rat or rabbit liver was isolated as described by Roe (15) using tissues purchased from Pel Freeze. Mixed wheat germ tRNA was isolated by phenol extraction (16). Yeast tRNA was purchased from Sigma Biochemicals. *Bacillus subtilis* tRNA was prepared as previously described (17). *Escherichia coli* $tRNA^{Phe}_1$, $tRNA^{Phe}_2$, $tRNA^{Met}_2$, and $tRNA^{Arg}_1$ were provided by G. David Novelli.

One sample of $tRNA^{Phe}$ was purchased from Boehringer Mannheim. Wheat germ $tRNA^{Phe}$ was supplied by Bernard Dudock. Undermethylated E. coli $tRNA^{Phe}$, prepared as described by Isham and Stulberg (18), was a gift from Melvin P. Stulberg and Lee Shugart.

RNase Digestion

18 nmole of E. coli $tRNA^{Phe}_1$ was digested with 20 units of RNase T2 (Sigma) in 200 µl of 0.05 M ammonium acetate pH 4.5, and 1 mM EDTA at 37°C for 4 hours.

Heat Denaturation

E. coli $tRNA^{Phe}_1$ was denatured by heating for 10 min in an 80-85°C water bath and immediately transfered to an ice-water bath. This preparation was used within 10 min in the inhibition assay.

Immunization

Procedures used in the production of antisera to nucleosides and nucleotides have been described previously (19). In the case of pi^6Ado immunization, animal care and sera collection were also performed by Antibodies Incorporated of Davis, California.

Membrane Binding Assay

The procedure is a modification of that reported by Humayun and Jacob (20). All filtrations were performed on a Millipore sampling manifold. Dry filters (Millipore, type HAWP or HAMK, 24 mm, 0.45 µm pore size) were placed on the manifold and wetted by filtering 2 ml of room-temperature buffer (10 mM tris-Cl, pH 7.2, 0.14 M NaCl) approximately 1 min before sample filtration.

Each assay contained up to 7.5 µg control-serum protein or column-purified antiserum protein and 5×10^{-8} M $[^3H]i^6Ado$ in buffer (250 µl total volume). $[^3H]i^6Ado$ was added last. Samples were incubated at 37°C for 30 min and transfered to an ice-water bath for 30 min. Two hundred-µl aliquots were filtered quickly (0.5 to 1.0 sec) and the filters were washed three times with 2 ml of ice-cold buffer. Filters were dried under a heat lamp and transfered to scintillation vials. Radioactivity was measured with a Beckman Liquid Scintillation System after addition of toluene-omnifluor scintillation fluid or scintillation cocktail 3a70B.

Unfractionated anti-pi^6Ado serum bound 5.4 nmole of i^6Ado per ml serum in this assay, and a typical preparation of anti-i^6Ado serum bound 4.2 nmole i^6Ado per ml serum. Activity calculations were made assuming that all of the protein-bound i^6Ado in the filtered aliquots was retained by the membrane filters. This assumption is supported by the observations of Yarus and Berg (21) that up to 50 µg of bovine serum albumin is quantitatively retained by cellulose nitrate filters.

Antigen and antibody saturation curves were determined using a constant amount of antiserum protein (7.5 μg) and up to 4 x 10^{-7} M [^{3}H]i^{6}Ado in a total volume of 250 μl of buffer, and by varying antiserum protein with a constant level of ligand (5 x 10^{-8} M).

Inhibition of binding assays

Assay mixtures contained a constant amount of antiserum protein and [^{3}H] i^{6}Ado, plus varying quantities of inhibitors, in a total volume of 250 μl. The antiserum was added last, and samples were then treated as in the binding assay. Antigen saturation curves were used to determine the optimum [^{3}H]i^{6}Ado concentration for measuring inhibition. The concentrations chosen gave good binding while still being in the linear portion of the saturation curve. In assays of anti-pi^{6}Ado, 7.5 μg of crude or column purified antiserum protein were used with 2 x 10^{-8} M [^{3}H]i^{6}Ado; assays of anti-i^{6}Ado used 5 μg of purified globulin fraction with 5 x 10^{-8} M [^{3}H]i^{6}Ado. In one series of experiments, [^{3}H]i^{6}Ado was replaced by tritiated isopentenyl adenosine trialcohol. Separation of free and antibody-bound ligand was also sometimes accomplished using dextran-coated charcoal (22); 0.5 ml of a cold suspension of 5 mg/ml Norit A plus 1 mg/ml Dextran T70 in buffer was added to each assay mixture. After 2 min at 0^0 samples were centrifuged for 5 min at 1000 xg, 375 μl of each supernatant was removed by pipette, and [^{3}H] was measured in 10 ml of 3a70B scintillation cocktail.

Fractionation of tRNA with anti-i^{6}Ado-Sepharose

Anti-i^{6}Ado globulins were prepared from serum by precipitation from 40% saturated ammonium sulfate, followed in most instances by gel filtration on Ultrogel ACA 22 (L.K.B. Instruments), or by passage through a small column of DEAE cellulose layered over carboxymethylcellulose (23). Globulins from 10 ml of serum were mixed with 3 gms of washed cyanogen bromide activated Sepharose 4B (Pharmacia), and the suspension adjusted to pH 8.5 with Na_2CO_3 solution. The suspension was kept overnight in the refrigerator, washed with Tris-NaCl buffer (0.02 M Tris-HCl, pH 7.5; 0.14 M NaCl), M ethanolamine, pH 8, and again with Tris-NaCl buffer.

Chromatography columns of ca 5 ml were poured using 6 ml disposable syringes as tubes. The tRNA sample was applied in 10 ml of Tris-NaCl buffer at a flow rate of ca 0.2 ml/min. The column was then washed with buffer until the absorbance of the effluent at 260 nm was equivalent to that of the eluting buffer. Bound tRNA was eluted with 10 ml of 10% pyridine in Tris-NaCl buffer, pH 7.5. RNA in the bound fraction and the unbound preparation (usually the first 20 to 25 ml of eluant from the column) were precipitated with 2.5 vol.

ethanol overnight at -20°C.

Assay of amino acid acceptance

Bacterial aminoacyl-tRNA synthetases were prepared from Bacillus subtilis 168 trp C2 according to Vold (24). Rat liver aminoacyl-tRNA synthetases were prepared from Pel-Freeze livers as described by Yang and Novelli (25). Tritiated amino acids were purchased from New England Nuclear Corp. Aminoacylation reactions were carried out as described by Vold and Minatogawa (26).

RESULTS

Comparison of binding of [^{3}H]i^{6}Ado using increasing amounts of control-serum and antiserum protein in conditions of hapten excess showed that the antiserum bound significantly greater amounts of [^{3}H]i^{6}Ado than did the control serum ($P < 0.001$) although the control serum exhibited a very small but significant ($P < 0.05$) binding capacity.

Inhibition of binding of i^{6}Ado to anti-i^{6}Ado or anti-pi^{6}Ado antisera by various nucleosides, nucleotides and polyadenylic acid

Figure 1 shows the inhibition of binding of [^{3}H]i^{6}Ado to anti-pi^{6}Ado serum by i^{6}Ado and by related mononucleosides, nucleotides and polyadenylic acid, poly(A). The hapten used for immunization (pi^{6}Ado) was the best inhibitor. To obtain equivalent inhibition with i^{6}Ado, a 100-fold greater concentration was

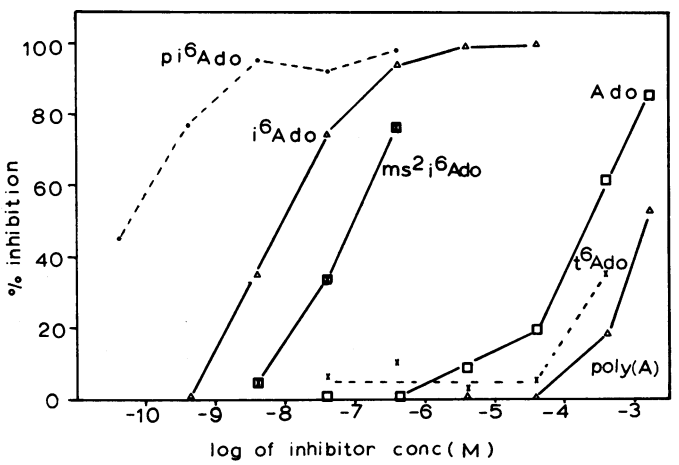

Figure 1. Inhibition of binding of [^{3}H]i^{6}Ado to an antibody preparation elicited with the nucleotide, pi^{6}Ado, as hapten. Non-radioactive nucleosides, nucleotides, and polyadenosine were used as competitive inhibitors. t^{6}Ado designates N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]-L-threonine. See text for other abbreviations.

required, and with ms^2i^6Ado a 1,000-fold greater concentration was required. Using the same criteria, adenosine (Ado) was seen to be approximately 5 x 10^6-fold less effective as an inhibitor than was pi^6Ado but 10-fold more effective than polyadenylic acid. The molar concentration of poly(A) is given in terms of adenosine residues. t^6Ado was less effective as an inhibitor than was Ado.

Figure 2 shows the inhibition of $[^3H]i^6Ado$ binding to anti-i^6Ado serum; the results are qualitatively similar to those shown previously. Again the hapten used in immunization, i^6Ado, is the best inhibitor. But this antibody preparation is less influenced by the sugar or its substitution. The nucleotide pi^6Ado is nearly as effective an inhibitor as i^6Ado over much of the the inhibition curve, although a change in slope at higher concentrations suggests that a population of lower affinity antibodies interacts less well with the phosphorylated form. Adenosine is about 3 x 10^5-fold less effective an inhibitor than i^6Ado, but 100-fold better than poly(A) (expressed as AMP).

The assay used above could poorly represent the binding properties of the whole sera by selecting only a population of antibodies which strongly bind intact nucleosides. However, essentially identical selectivity was seen if the radioactive ligand was a $[^3H]i^6Ado$ trialcohol, formed from nucleoside by oxidation with periodate followed by reduction with tritiated borohydride (27). Selective binding of only part of the antibody population to the Millipore

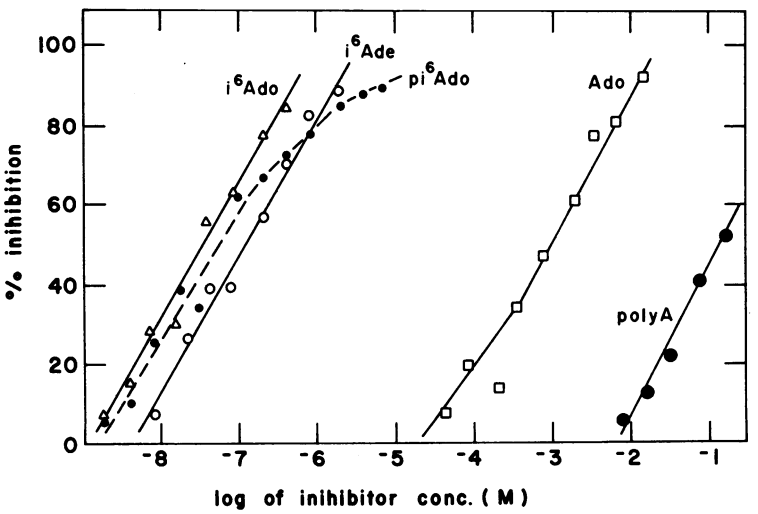

Figure 2. Inhibition of binding of $[^3H]i^6Ado$ to an antibody preparation elicited with the nucleoside, i^6Ado, as hapten. Non-radioactive bases, nucleosides, or nucleotides were used as competitive inhibitors. See text for abbreviations.

filters is also improbable; dextran-coated-charcoal separation of free from antibody-bound ligand also gave results like those shown in Figure 2.

Structural elements recognized by antibodies to i^6Ado and pi^6Ado

To further delineate the elements of nucleoside structure recognized by the antibody preparations, a variety of inhibitors were examined in the filter binding assay. The results are summarized in Table I; data shown in Figures 1 and 2 are included for comparison.

The absolute affinity of the anti-pi^6Ado antibodies for the nucleotide is seen to be very high; nearly 10^3-fold higher levels of nucleotide are required to 50% inhibit binding of i^6Ado by the anti-nucleoside antibody preparation. Thus, the phosphate plays an important role in the recognition process of the anti-pi^6Ado antibodies. The higher affinity of the anti-nucleotide antibody is also apparent with relatively poor inhibitors such as poly(A) and adenosine.

A dominant effect of the modification at N^6 is seen in comparison of the results with anti-i^6Ado antibodies. Both the free base, i^6Ade, and its nucleotide are nearly comparable with the nucleoside, indicating a minor role of

TABLE I Inhibitor concentration necessary to produce a 50% inhibition of the binding of [^{3}H]i^6Ado by antibody preparations.

Inhibitor	Concentration (M)	
	anti-i^6Ado	anti-pi^6Ado
i^6Ado	3.5×10^{-8}	1×10^{-8}
pi^6Ado	4.0×10^{-8}	4×10^{-11}
i^6Ade	1.3×10^{-7}	
Kinetin riboside	6×10^{-7}	
Kinetin	1.5×10^{-6}	
Ado	1×10^{-3}	2×10^{-4}
Puromycin	3×10^{-3}	
$m^6{}_2$Ado	3×10^{-3}	
m^6Ado	4×10^{-3}	
m^1Ado	4×10^{-3}	
1,N^6ethenoAdo	1×10^{-2}	
Ade	2×10^{-2}	
$m^6{}_2$Ade	3×10^{-2}	
Poly(A)	1×10^{-1}	1×10^{-3}
AMP	5×10^{-1}	

sugar or sugar phosphate in the recognition process. Kinetin (N^6-furfuryladenine) or its nucleoside are the only compounds tested which gave inhibition which approaches that shown by the isopentenyl derivatives. The poor inhibition shown by dimethyladenosine and its derivatives and by N^6-monomethyladenosine is somewhat surprising, since these substitutions are relatively small and hydrophobic and were expected to partially resemble the isopentenyl group.

Because of their greater affinity for the hapten used in immunization, antibodies to the nucleotide were thought to be more likely to be of value in studies with tRNA. Further studies of binding inhibition were performed in order to assess potential reactivity with tRNA containing nucleotide components closely related to isopentenyladenosine.

Table 2 summarizes inhibition data with a number of compounds in the isopentenyladenosine family.

Substitution of the methylthiol group at position 2 is common in bacterial tRNA, and results in only about a 10-fold increase in the concentration needed to halve binding of [^{3}H]i^{6}Ado. The presence of a hydroxyl group on the isopentenylamino substituent results in a larger effect; the cis or trans isomers were more than 200-fold less effective as inhibitors than is pi^{6}Ado. Both the cis or trans isomers are found as free cytokinins or in

TABLE II Effect on antibody recognition of the position of various groups on the adenosine residue: inhibitor concentration necessary to produce a 50% inhibition of the binding of [^{3}H]i^{6}Ado by anti-pi^{6}Ado

Adenosine Derivative		Concentration (M)
Substituent at Position 6	Substituent at Position 2	
Δ^2isopentenyl amino	H	8×10^{-9}
Δ^2isopentenyl amino	CH_3S	8×10^{-8}
4-hydroxy-cis-2-isopentenyl amino	H	1.7×10^{-6}
4-hydroxy-trans-2-isopentenyl amino	H	2.5×10^{-6}
phenyl-CH_2S	Δ^2isopentenyl amino	5×10^{-6}
NH_2	CH_3S	3×10^{-5}
NH_2	H	2×10^{-4}
threonyl carbonyl amino	H	ca 10^{-3}

the tRNAs of certain plants and plant associated bacteria (11). Switching the position of the isopentenyl group from the N^6 to the 2 position of the adenosine residue also results in a significant loss in binding ability, which demonstrates the importance of position in antibody recognition. Also, if the N^6 position is occupied with a threonyl carbonyl amino group, antibody recognition is even less effective than if the N^6 position is occupied with an amino group. Therefore, the hydrophobic versus hydrophilic properties of the substituents make a more important contribution than the size of the group or similarity of sidechain complexity.

Inhibition of binding of [^{3}H]i^{6}Ado to anti-pi^{6}Ado by purified tRNAs containing different nucleosides adjacent to the 3'-end of the anticodon

Figure 3 shows the inhibition of binding of [^{3}H]i^{6}Ado to anti-pi^{6}Ado antiserum by purified tRNAs. The nucleosides listed in the figure and discussed in the text below are those which occur in the position adjacent to the 3'-end of the tRNA anticodon.

Normally methylated E. coli $tRNA^{Phe}$ contains ms^{2}i^{6}Ado. We used two preparations: $tRNA^{Phe}_1$ and $tRNA^{Phe}_2$. Undermethylated E. coli $tRNA^{Phe}$ presum-

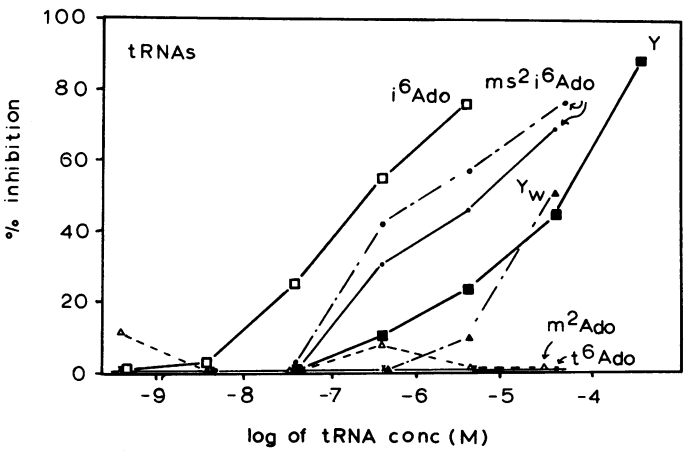

Figure 3. Selectivity of the anti-pi^{6}Ado antibody preparation toward purified tRNAs with different modified nucleosides adjacent to the 3'-end of the anticodon. The tRNAs which were used are described in the text. The nucleoside abbreviations in the figure designate those nucleoside residues which occur in the position adjacent to the 3'-end of the tRNA anticodon. Selectivity was measured as the ability of a compound to inhibit the binding of the [^{3}H]i^{6}Ado.

ably contains the i^6Ado group but lacks thiomethylation in position 2 of the adenosine residue (18).

Yeast $tRNA^{Phe}$ contains the nucleoside designated as Y. The base component of Y is tricyclic and different from i^6Ade (28). Wheat germ $tRNA^{Phe}$ contains the nucleoside Yw (29). The structure of Yw has not been determined but its UV and fluorescence spectra are similar to those of Y from Yeast $tRNA^{Phe}$ (30). Yw also has hydrophobic properties similar to those of Y but the free compound does not co-chromatograph with Y (31). E. coli $tRNA^{Arg}_1$ contains 2-methyladenosine (m^2Ado). E. coli $tRNA^{Met}_2$ contains t^6Ado. The structures of i^6Ado, ms^2i^6Ado, t^6Ado, and Y have been presented by McCloskey and Nishimura (26).

Undermethylated $tRNA^{Phe}$ (i^6Ado) is the best native tRNA inhibitor. E. coli $tRNA^{Phe}_1$ and $tRNA^{Phe}_2$ (ms^2i^6Ado) give approximately 10-fold less effective inhibition. This result is similar to the relative inhibition seen with free nucleosides in the same binding assay. tRNA containing Y (Yeast $tRNA^{Phe}$) and its suspected modification Yw (wheat germ $tRNA^{Phe}$) each give about 70- to 100-fold less effective inhibition than does undermethylated E. coli $tRNA^{Phe}$ (i^6Ado). E. coli $tRNA^{Arg}_1$ (m^2Ado) and $tRNA^{Met}_2$ (t^6Ado) do not detectably inhibit binding in this system.

These inhibition experiments were repeated using antiserum made with a different pi^6Ado-BSA-antigen preparation in a different rabbit. In this case, mixed Bacillus subtilis tRNAs containing i^6Ado and ms^2i^6Ado were tested. As in the results shown in Figure 3, E. coli $tRNA^{Arg}_1$ (m^2Ado) and $tRNA^{Met}_2$ (t^6Ado) did not inhibit. Mixed B. subtilis tRNAs (i^6Ado and ms^2i^6Ado) were the best inhibitors followed by E. coli $tRNA^{Phe}_1$ and $tRNA^{Phe}_2$ (ms^2i^6Ado), and Yeast $tRNA^{Phe}$ (Y). In contrast to the results in Figure 3, wheat germ $tRNA^{Phe}$ (Yw) did not detectably inhibit binding. Possibly, Y and Yw interacted with a sub-class of antibodies in the first anti-pi^6Ado antiserum.

Similar but less extensive inhibition studies were performed using antibodies to the nucleoside, i^6Ado, and the results were comparable to those of Figure 1. The best inhibitor tested was rat liver $tRNA^{Ser}$ containing i^6Ado; 50% inhibition of binding occurred at a tRNA level of about 3×10^{-6} M. A commercial sample of E. coli $tRNA^{Phe}$ (ms^2i^6Ado) was a less effective inhibitor, with 50% inhibition occurring at about 4×10^{-5} M. In each case, this is about a 10-fold greater concentration of inhibitor than was needed using antibodies to the nucleotide.

Inhibition assays using native, heat denatured and RNase digested tRNA

Comparisons of results of inhibition assays using free nucleoside (Fig-

ure 1) or a tRNA containing the nucleoside (Figure 3) show the former to be the more effective inhibitors. Similarly, adenosine or AMP are better inhibitors than polyadenylate. The basis of this difference could be the RNA conformation, or some aspect of the structure in a polymerized form. Therefore, purified E. coli $tRNA_1^{Phe}$ was tested as an inhibitor in its native form, heat denatured, or digested with RNase T2. The results are shown in Figure 4. Polynucleotide conformation appears to have a small effect, but the major factor appears to be related to the polymerized structure of the tRNA.

Purification of tRNA species containing i^6Ado or its derivatives by affinity chromatography

Antibodies to i^6Ado were bound to cyanogen bromide activated Sepharose 4B, and used as an adsorbent for purification of tRNA. Table 3 summarizes results of several attempts at tRNA purification. When fractionating several mg of unlabeled tRNA, about 75% of the material was rapidly eluted and recovered from the first approximately 20 ml of effluent. Additional material absorbing at 260 nm was slowly eluted, over a volume of up to 500 ml; this RNA was not characterized or recovered. Tightly bound tRNA was finally eluted with a 10% pyridine buffer, and usually accounted for 2-3% of the initial

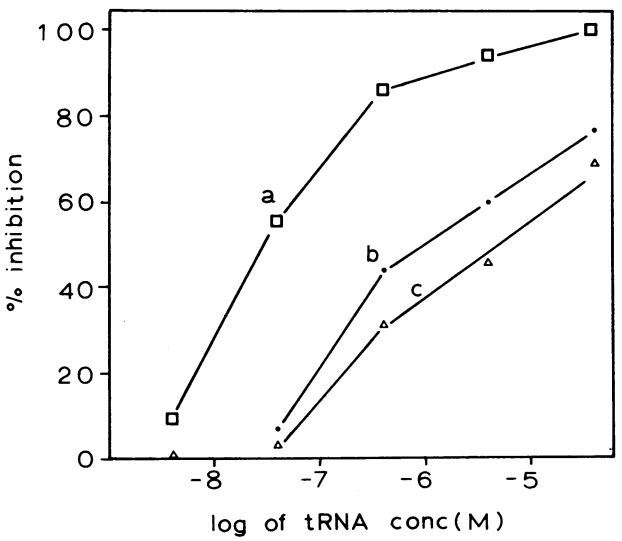

Figure 4. Ability of E. coli $tRNA_1^{Phe}$ to act as an inhibitor of binding of [^{3}H]i^6Ado to the anti-pi^{6}Ado antibody preparation. Nucleotides resulting from ribonuclease digestion (a), the heat denatured intact tRNA (b), or the native tRNA molecule (c) were used as competitive inhibitors.

preparation (except for wheat germ tRNA, when no measurable amount of tRNA was eluted with pyridine). Results using smaller amounts (ca 250 μg) of ^{32}P-labeled tRNA were similar but not identical. Somewhat less tRNA was quickly eluted from the column, and a larger percentage was eluted with pyridine buffer. Repeated use of a single anti-i^{6}Ado-Sepharose column showed no change in the fractionation results. The capacity of the column appeared essentially unchanged after 10 chromatographic runs.

Some of the tRNA fractions obtained above were characterized with respect to biological activity. Table 4 summarizes amino acid acceptance activity of the B. subtilis tRNA preparations; tRNA^{Tyr}, which contains i^{6}Ado or ms^{2}i^{6}Ado, was depleted in the unretarded fraction, and enriched ca 7.5 fold in the bound fractions. In contrast, tRNA^{Lys}, which lacks i^{6}Ado, was essentially unchanged in the unbound fractions and reduced to less than 10% of the tRNA^{Tyr} levels in the antibody-bound fractions.

Assays of amino acid acceptance by rabbit or rat liver tRNA fractions indicated at least comparable enrichment for i^{6}Ado-containing tRNA^{Ser}. Relative to the unfractionated tRNA applied to the columns serine acceptance

TABLE III Fractionation of tRNA by chromatography on anti-i^{6}Ado Sepharose

tRNA Source	Sample Applied A_{260} Units	tRNA Recovered	
		Unbound	Eluted
Rat liver	360	265 (74%)	6.5 (1.8%)
Rabbit liver	320	250 (78%)	7.5 (2.3%)
Yeast	420	335 (80%)	10.0 (2.4%)
Wheat germ	1000	695 (70%)	n.d.[a]
Bacillus subtilis			
native	25	18.6 (74%)	0.71 (2.8%)
heat denatured	25	19.4 (78%)	0.76 (3.0%)
	cts/min		
Escherichia coli ^{32}P	9 x 10^6 cpm	5.35 x 10^6 cpm (60%)	0.59 x 10^6 cpm (6.5%)
Serratia marcescens ^{32}P	13.4 x 10^6	9.2 x 10^6 (69%)	0.76 x 10^6 (5.7%)

[a] n.d., not detectable (< 1 A_{260} unit).

TABLE IV Amino acid acceptance of B. subtilis tRNA fractions.

tRNA Sample	A_{260} Units	Tyrosine		Lysine	
		pmoles bound	pmoles/ A_{260}	pmoles bound	pmoles/ A_{260}
Unheated					
control	2.4	94	39	139	58
unbound	19.0	627	33	1254	66
bound	0.71	210	296	18	25
Heat denatured					
control	2.5	110	44	165	66
unbound	19.5	585	30	1229	63
bound	0.74	243	329	8	11

of antibody-bound rat liver tRNA was increased 13-fold, to 371 pmoles/A_{260}; serine acceptance of bound rabbit liver tRNA was increased 12-fold to 448 pmoles/A_{260} unit. In each unbound tRNA sample, serine accepting ability was reduced to about two-thirds of the original level. Nucleoside analysis of the bound rat liver tRNA also showed a minor-nucleoside composition characteristic of the $tRNA^{Ser}$ species (31), and consistent with at least 70% purity of mixed $tRNA^{Ser}$ (i.e., 70% of expected recoveries of minor nucleosides). Nucleoside analysis should be a more accurate way to estimate purity since the assays for amino acid acceptance were not necessarily done under conditions which maximize acceptance.

Discussion

These experiments permit the quantitative comparison and evaluation of the ability of antibodies, directed against a modified nucleoside or nucleotide hapten, to bind several chemically related nucleic acid components, both free and in a biologically active polynucleotide.

One important conclusion relates to the central role of the hydrophobic isopentenyl group in antigen recognition by either population of antibodies. In the absence of the isopentenyl group (e.g., adenosine, poly(A), $m^6{}_2$Ado, t^6Ado) neither antibody population shows binding properties which would interfere in studies of the i^6Ado in tRNA. In contrast, if the N^6-isopentenyl group is present, even if the nucleoside itself is otherwise modified, a strong and specific antibody interaction occurs. Replacement of the isopen-

tenyl group by the similarly-hydrophobic furfuryl group of kinetin or its riboside or with hydrophobic residue Y gave better inhibition than the addition of a hydrophilic hydroxyl group to the isopentenyl side chain or the substitution of the isopentenyl group in the N^6 position with the hydrophilic threonyl carbonyl amino group.

A second point concerns the previously observed (1,19) importance of the 5'-phosphate group in recognition by anti-nucleotide antibodies. Antibodies directed against the nucleotide show a considerable preference for binding nucleotide, while those directed against the nucleoside are much less influenced by the presence or absence of the charged, hydrophylic phosphate substituent. This observation suggests that either antibody preparation should be capable of interaction with an isopentenyl-related nucleoside residue in a polynucleotide form. However, the greater absolute affinity of the anti-nucleotide antibodies suggests that this preparation might be of more value in studies using intact tRNA.

Several conclusions can be drawn from the results of the tRNA studies. First, antibodies directed against a nucleotide hapten, covalently bound by a protein carrier, can be used to study a tRNA containing the nucleotide. A similar conclusion has been drawn from other studies of nucleotide haptens in tRNA (3,4), ribosomes (6) or other RNA. Quantitative measurement of the nucleotide in intact tRNA is also seen to be possible, although prior nuclease hydrolysis of the tRNA would increase sensitivity (Figure 4). This conclusion is in accord with the i^6Ado assay procedure developed by Kahn et al. (32).

A more important conclusion is that the antibodies can be expected to show the same selectivity for related nucleotides, whether the nucleotides are free or in tRNA. Thus, assays of inhibition of binding using purified nucleoside or nucleotide inhibitors can be used to predict antibody reactivity with tRNA species containing structures related to the nucleotide hapten.

The capacity of agrose-bound antibodies to fractionate tRNA was previously shown (3,4). This work serves to reaffirm the selectivity of antibody for a given nucleotide structure, and to suggest that antibodies (or Fab fragments derived from them) could provide useful probes of the availability of a given nucleotide within a tRNA structure, either free in solution or when complexed with a component of the protein-synthesizing system.

Finally, the assays of inhibition of [^{3}H]i^6Ado binding as used here or by Kahn et al. (33) provide a sensitive means to quantitate the amounts of i^6Ado or related compounds either free or in tRNA. These techniques should

be applicable to the study of nucleosides of the isopentenyladenosine family in a variety of biological materials.

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