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Radioimmunoassays for the modified nucleosides N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]-L-threonine and 2-methylthioadenosine

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ABSTRACT

Radioimmunoassays were established for the modified nucleosides N-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]-L-threonine, t^6A, and 2-methylthioadenosine, ms^2A. The assays depended on the production of antisera specific for t^6A and ms^2A that have not been previously reported. The nitrocellulose membrane filtration and saturated ammonium sulfate RIA techniques were compared for efficiency. Various radioactive antigens were employed to establish which type of antigen would give the best binding. The tritium post-labeling procedure of Randerath and Randerath was used to obtain labeled nucleosides of high enough specific activity to be useful for RIAs when the labeled nucleoside was not available commercially. The specificity of the antibodies toward nucleosides and purified tRNAs is reported. Although the titer of the t^6A antiserum was low, the specificity was very sharp. An interesting finding was that threonine, a major structural component of the side-chain modification of t^6A, was completely ineffective as an inhibitor.

INTRODUCTION

As part of our continuing interest in antibodies directed toward those modified nucleosides that occur in tRNA, antibodies to t^6A and ms^2A were investigated. This is the first reported preparation of antibodies against these compounds; however, antibodies against several other modified nucleosides have been made. Considering only naturally occurring adenosine modifications, antibodies have been reported for N^6-methyladenosine (1), N^6-(Δ^2-isopentenyl)adenosine, i^6A (2-4), and N^6,N^6-dimethyladenosine(5).

The t^6A and ms^2A modifications are of particular interest because of their importance in tRNA studies. The t^6A modification occurs in the anticodon loop of tRNAs that respond to codons beginning with A. The ms^2A modification occurs primarily as part of the N^6-(Δ^2-isopentenyl)-2-methylthioadenosine, ms^2i^6A, moiety in the anticodon loop of tRNAs that respond to codons beginning with U. There is also evidence that the ms^2A modification is important in development in Bacillus subtilis (6). An inter-

esting mutant strain of Escherichia coli exists that is deficient in the ms^2i^6A modification (7,8). Antibodies specific for i^6A were instrumental in the identification of this deficiency (8). This E. coli strain was also used as a model system to demonstrate the effectiveness of i^6A antibodies in screening for mutants deficient in the i^6A modification in tRNA (Vold and Nolen, in preparation). In this paper, the specificity of the antibodies to t^6A and ms^2A are investigated and a radioimmunoassay (RIA) system suitable for similar application with these antisera is described. Levine et al. (9) have employed RIAs, using antibodies specific for naturally occurring nucleosides, to determine the levels of these nucleosides in the sera of patients with malignancies. Such applications demonstrate the potential usefulness of RIA systems employing naturally occurring modified nucleosides.

MATERIALS AND METHODS

Antibody preparation

Immunogens were prepared by conjugation of the nucleoside to BSA via the method of Erlanger and Beiser(10). Antibodies were made in rabbits; a total of 5 mg conjugate (mixed with Complete Freund's Adjuvant) per rabbit was introduced in three injections into the hind legs on Days 0, 14, and 21. The animals were bled on Day 28. Immunization, animal care, and sera collection were performed by Antibodies Incorporated, Davis, California. Their standard protocol was used and was not necessarily optimal. Post-immune serum was precipitated twice at 0°C with 50% saturated ammonium sulfate (SAS), pH 7.0, and then dialyzed against 0.14 M NaCl, 10 mM sodium phosphate, pH 7.0 (PBS).

Preparation of radioactively labeled antigens

t^6A and i^6A were custom-tritiated using tritium gas substitution by Moravek Biochemicals, City of Industry, California [i^6A, which is listed as 6(γ,γ-dimethylallylamino)purine riboside-8-[^3H], 6 Ci/mmole; t^6A, listed as N-(Nebularin-6-ylcarbamyl)-L-threonine[^3H(G)], 0.7 Ci/mmole]. The ms^2A lost its sulfur group in the exchange process, however, and could not be labeled by that procedure. Therefore, the ms^2A was labeled in our laboratory using the tritium postlabeling method of Randerath and Randerath (11); a specific activity of 1.7 Ci/mmole was obtained. The potassium boro[^3H]hydride was not diluted and was used at a specific activity of 4.5 Ci/mmole. The "prime" designation after a nucleoside abbreviation,

such as ms^2A', refers to the compound produced by periodate oxidation and borohydride reduction in which the ribose ring is opened--the trialcohol.

The $[^3H]i^6A$ made by Moravek Biochemicals was checked for purity by co-chromatography with a nonradioactive t^6A standard on two-dimensional TLC. The $[^3H]t^6A$ was 93% pure after one year of storage. The $[^3H]ms^2A'$ made in our laboratory had deteriorated considerably, however, after 8 months of storage. Repurification was accomplished on Whatman No. 1 chromatography paper using 10% ethanol in one dimension; ms^2A' had an R_f of 0.59 and the one major contaminant had an R_f of 0.89. The contaminant showed no binding to the antibodies to ms^2A.

A multivalent, radioactively labeled hapten was made for t^6A by conjugating the stock $[^3H]t^6A$ radioisotope to HSA, using the Erlanger and Beiser procedure(10). The resulting components were chromatographed over a column of BioGel P-2. Radioactivity in the eluates was measured before and after precipitation with 10% trichloroacetic acid (TCA). The conjugate was located by the fractions containing radioactivity after TCA precipitation. Pooled conjugate fractions were dialyzed against water. The mononucleoside fraction was used as a source of $[^3H]t^6A'$.

Radioimmunoassay using saturated ammonium sulfate (SAS)

This method is based on the difference in solubility in 50% SAS between the antigen-antibody complex and the labeled antigen (12). SAS was made in water and stored at 4°C. Reaction volumes were 300 µl, and the total protein content was normalized using carrier normal rabbit immunoglobulins--typically, 500 µg of total protein/assay. Reactions containing antiserum, carrier protein, ^3H-antigen, and--when appropriate--the nonradioactive inhibitor were incubated for 1 h at 37°C in borate-saline buffer: 75 mM NaCl, 0.1 M boric acid, 25 mM sodium tetraborate, pH 8.3. After the reactions were cooled on ice, an equal volume of cold SAS was added. The solution was mixed thoroughly, and the incubation was continued for 30 min at 0°C. At the end of the 30-min incubation, the suspensions were centrifuged for 15 min at 4°C and 9,400 × g and the supernatant fluid was removed by aspiration. The pellet was resuspended in 200 µl of ice-cold 50% SAS in borate-saline buffer, mixed, recentrifuged, and drained as before. The washed pellets were then resuspended in 200 µl of borate-saline buffer; 175 µl was withdrawn and added to 5 ml of Aquasol scintillation fluid. Radioactivity was measured in a scintillation spectrophotometer.

Radioimmunoassay using the membrane binding technique

The principle behind the nitrocellulose membrane filtration technique is discussed by Van Vunakis and Levine (13). The reaction mixture contained antiserum and ^{3}H-antigen in Tris-NaCl buffer (10 mM Tris, pH 7.2, 140 mM NaCl), with a final volume of 250 µl. Reactions were incubated for 30 min at 37°C followed by 30 min at 0°C. Samples of 200 µl were filtered on prewetted Millipore membrane filters (HAMK, 22 mm, 0.45 µ) and washed with 3 x 2 ml of cold Tris-NaCl buffer. Since the partially purified immunoglobulins did not bind the antigen nonspecifically after the washing procedure, the protein content of each reaction was not normalized. Filters were dried and the radioactivity was measured by scintillation spectroscopy.

RESULTS AND DISCUSSION

Comparison of radioimmunoassay techniques

As mentioned in the introduction, the establishment of an RIA technique for $t^{6}A$ and $ms^{2}A$ measurement would have important applications in measuring those nucleosides in physiologic fluids and in screening procedures for detecting mutants deficient in specific nucleoside modification. The membrane binding assay procedure using [^{3}H]$i^{6}A$ was very useful for antibodies produced against a $pi^{6}A$-BSA conjugate. However, the antibodies produced against $ms^{2}A$-BSA and $t^{6}A$-BSA had lower titers. Therefore, alternative methods for optimizing their RIA procedures were investigated.

Results obtained with the membrane-binding and SAS techniques were compared. Figure 1 shows the amount of radioactivity from [^{3}H]$t^{6}A$ or [^{3}H]$ms^{2}A'$ retained by the filters as a function of the number of washes using the membrane-binding technique. Each wash was performed by suction of 2 ml of cold RIA buffer through the filter after the antibody-antigen complex had been applied to the membrane. Incubation temperatures were varied in the $t^{6}A$ tests. The standard procedure employing an incubation for 30 min at 37°C followed by incubation at 0°C for 30 min gave the highest binding. Incubation at only 0°C or only 37°C resulted in lower binding. A control using an equivalent amount of normal rabbit immunoglobulin protein is also represented. For the $t^{6}A$ antiserum, the washing procedure resulted in a considerable loss of radioactivity in the antigen-antibody complex (bound radioactivity with antiserum minus the nonspecific bound material in the control). This is probably due to a dissociation of

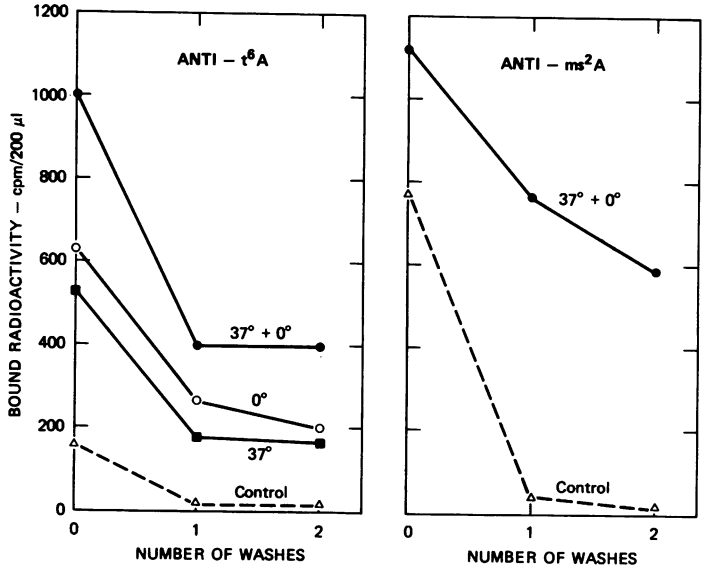


Figure 1. Effect of washing procedure on [^{3}H]t^{6}A or [^{3}H]ms^{2}A' using the membrane-binding technique. t^{6}A assays contained 4 pmoles of [^{3}H]t^{6}A and 400 µg of either antiserum protein or normal rabbit γ-globulins. ms^{2}A assays contained 4 pmoles of [^{3}H]ms^{2}A' and 100 µg of either antiserum protein or normal rabbit γ-globulin. Incubation temperatures before filtration are shown on the graph. Assays were incubated for 30 min at each temperature. Cpm values are not corrected for nonspecific adsorption.

the complex since the filters should not be overloaded by the 400 µg of protein used in the assay. Van Vunakis and Levine (13) estimated that a 25 mm-diameter filter can retain about 700 µg of serum proteins before its binding capacity is exceeded.

Results of SAS assays for the t^{6}A and ms^{2}A antisera are given in Figure 2. Incubation times for the SAS assay were 1 h at 37°C followed by 30 min at 0°C. Incubation of the ms^{2}A assays for 1 h at 37°C followed by an overnight incubation at 4°C resulted in a 12% increase in binding; thus, incubation at 0°C for 30 min seemed to be sufficient. To compare the efficiency of each technique and the effect of washing on the amount of antigen bound, the radioactivity measured for each assay had to be corrected (a) to represent total assay volume and (b) for a difference in counting efficiency between filters counted in Omnifluor-toluene and liquid samples counted in Aquasol. Amounts of antibody protein giving about 30-50% antigen-antibody binding were chosen since that is the range

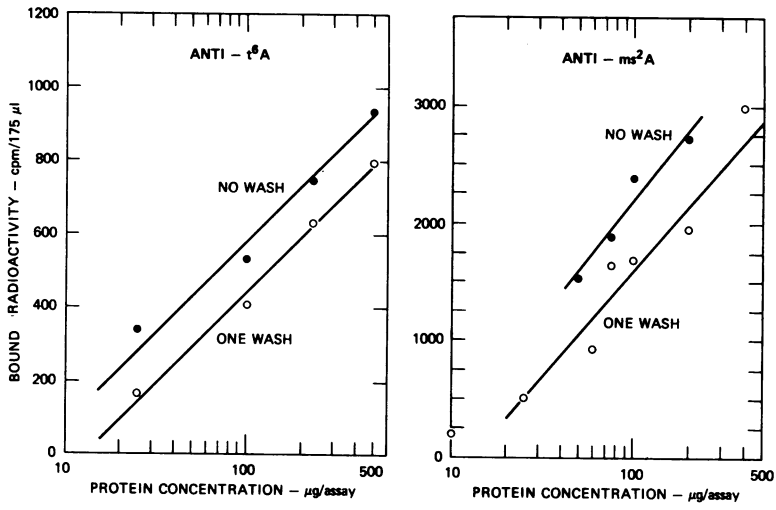


Figure 2. Effect of washing procedure on [^{3}H]t^{6}A or [^{3}H]ms^{2}A′ using the SAS technique. Each reaction contained 4 pmoles of tritiated antigen and varying amounts of antiserum protein, all normalized to a total protein concentration of 500 µg with normal rabbit γ-globulin. Cpm are not corrected for nonspecific adsorption.

useful for competitive inhibition binding studies. Corrected values for the amount of antigen bound are given in Table 1. One wash with either technique is sufficient to reduce the nonspecific binding to very low values. As mentioned before, however, washing the t^{6}A antibody-antigen complex seemed to result in a dissociation of the complex, and the best binding was achieved when the filters were not washed. Not washing the filters is undesirable with the ms^{2}A antigen-antibody system, however, since the [^{3}H]ms^{2}A′ showed considerable nonspecific binding to the filters. Although there is not a great deal of difference between the two techniques, the SAS method seemed preferable because of the low nonspecific binding and stability of the complexes to the washing procedure. The SAS technique also has the advantage of accommodating larger amounts of protein than the membrane binding technique does, which is useful when comparing saturation curves for a variety of antisera with different titers.

Evaluation of best radioactive antigen for use in RIA and results obtained with acid-treated antiserum

Some antibody preparations recognize part of the structure forming the covalent link between the hapten and carrier. Thus, both Humayun and

Table 1

EFFICIENCY OF RIA TECHNIQUES

Antibody System	Number of Washes	Membrane Binding			SAS		
		pmoles bound		%[a]	pmoles bound		%
		+ Ab	+ NRγG		+ Ab	+ NRγG	
t^6A	0	2.5	0.4	53	1.4	0.2	30
4 pmoles Ag	1	1.0	0	25	1.2	0	30
400 μg Ab	2	1.0	0	25	-	-	
ms^2A	0	1.9	1.3	15	1.7	0.3	35
4 pmoles Ag	1	1.2	0.1	28	1.3	0	33
100 μg Ab	2	1.0	0.1	23	-	-	

[a] % = $\frac{\text{pmoles bound in presence of Ab minus non-specific control binding}}{\text{total Ag added to reaction}}$

Jacob (3) and Levine et al. (9) reported that antibodies elicited by nucleoside-protein conjugates made by the Erlanger and Beiser procedure were much more reactive when either the periodate-oxidized form of the nucleoside was used (3) or when the nucleosides to be measured were converted to their ε-aminocaproate derivatives (9). An increase in binding energy can thus be contributed by part of the carrier, although B. D. Stoller (personal communication) observed that oxidized and reduced 7-methylguanosine was only slightly more effective as a competitor in a RIA when the hapten was attached to ε-aminocaproic acid. In addition, the use of a multivalent antigen has been effective in some cases (13).

To check antigen preference in our system, three radioactively labeled antigens were used with the antiserum for t^6A: the nucleoside, $[^3H]t^6A$; the tritiated trialcohol derivative obtained by periodate oxidation and borohydride reduction, $[^3H]t^6A'$; and the multivalent antigen $[^3H]t^6A$-HSA. Two techniques--the membrane-binding assay and the SAS assay--were compared. The results are shown in Figure 3. In addition to using antisera that had been precipitated with ammonium sulfate and dialyzed (untreated

immunoglobulin fraction), the same immunoglobulin fraction was further treated with 1 N acetic acid and dialyzed to ensure dissociation of any nucleosides that might have been present in the serum as antibody-antigen complexes. The results with the acid-treated sera are also shown in Figure 3.

Although the [^{3}H]t^{6}A nucleoside seemed to be a better antigen than the [^{3}H]t^{6}A′ in the membrane-binding assay, the difference was not great, and no difference between the two was evident using the SAS assay method. The multivalent conjugate was less effective than either the nucleoside or the trialcohol. Therefore, either the [^{3}H]t^{6}A nucleoside or the [^{3}H]t^{6}A′ are reasonable antigens for the RIA. No great benefit seems to be gained by acid dissociation of the antiserum. Although the acid-treated serum was more effective with either antigen in the membrane-

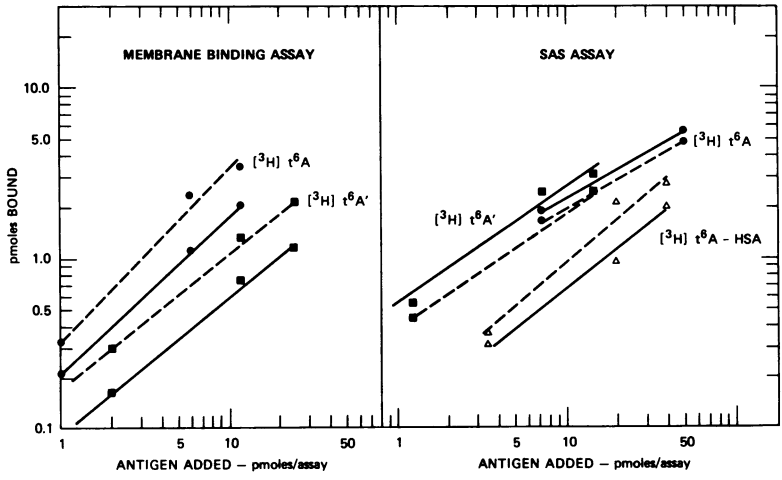


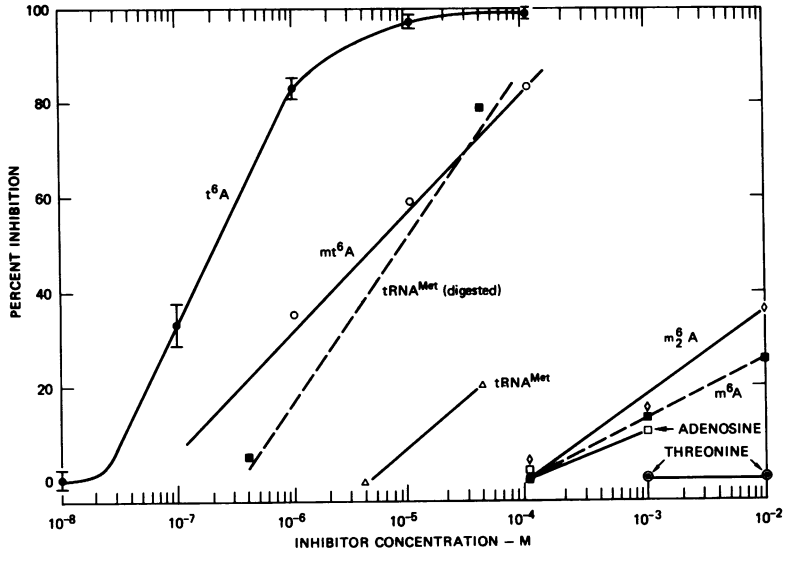
Figure 3. Antigen preference and effect of acid dissociation treatment of anti-t^{6}A serum assayed by both the membrane-binding and SAS techniques. Standard procedures using two washes for the membrane-binding assay and one wash for the SAS assay were used; 400 μg of antiserum protein was used in each assay for either technique. The antigens were prepared as described in Materials and Methods. Acid-treated antisera were made 1 N in acetic acid, dialyzed for 30 min against 1 N acetic acid at room temperature, and then dialyzed against PBS in the cold for 24 h, with two changes of buffer. After dialysis, the suspension was centrifuged to remove a precipitate, and the supernatant fluid was used as "acid-treated antiserum."
The following symbols are used: [^{3}H]t^{6}A (●); [^{3}H]t^{6}A′ (■); [^{3}H]t^{6}A-HSA (△); a solid line = untreated γ-globulin fraction, and a dashed line = acid-treated antiserum.

binding assay, it was not particularly better in the SAS assay.

Specificity of antisera for nucleosides and tRNA

Specificity was determined for each type of antiserum by the hapten inhibition technique. Nonradioactive nucleosides or tRNAs were used as competitive inhibitors for the binding of ^{3}H-antigen using the SAS technique. Although the titer of the antibodies was not as high as that obtained by the hapten i^{6}A, nevertheless the antibodies formed were very specific. Antisera used in this study were precipitated with ammonium sulfate and dialyzed, but were not purified by any type of adsorption technique or affinity chromatography. The titer, therefore, could be improved by addition of an affinity column procedure, although for the t^{6}A antiserum, the specificity was sufficient without this extra step.

The results for the t^{6}A system are shown in Figure 4. The antibody for t^{6}A was very specific. Only t^{6}A, mt^{6}A, and a purified tRNA that contains t^{6}A acted as inhibitors. Adenosine, m^{6}A, and m$_{2}^{6}$A were equally inactive as inhibitors and caused about a 30% inhibition at a concentration



Figute 4. Competitive inhibition experiments with anti-t^{6}A serum. Binding was measured in the SAS assay system using 5 pmoles of [^{3}H]t^{6}A and 500 µg of antiserum protein per assay. The mean and standard deviation for assays conducted on three different days are given for the inhibitor t^{6}A. Abbreviations not already noted in the text are: mt^{6}A, N[(9-β-D-ribofuranosylpurin-6-yl)-N-methylcarbamoyl]-L-threonine; m^{6}A, N^{6}-methyladenosine; m$_{2}^{6}$A, N^{6}-N^{6}-dimethyladenosine.

of 10^{-2} M, whereas t^6A caused a 30% inhibition at a concentration of about 10^{-7} M. Since t^6A and mt^6A are so closely related structurally, it is interesting that about 10 times more mt^6A was necessary to achieve the same amount of inhibition. It is also interesting that threonine, which is the major structural component of the side chain of t^6A did not inhibit the antibody even at a concentration of 10^{-2} M. As we had observed with the anti-i^6A and tRNA containing ms^2i^6A (4), a tRNA containing the appropriate nucleoside is recognized by the antibody, but the ability of the tRNA to act as an inhibitor is greatly increased when it is digested with ribonuclease to nucleotides. The digested material did not inhibit as well as the t^6A, probably because it is not structurally identical to the tritiated antigen.

The results for the ms^2A system are shown in Figure 5. Although ms^2A and nucleosides or tRNA containing the ms^2A group were effective inhibitors, adenosine was a more active inhibitor with the ms^2A antiserum than it was with the t^6A antiserum. The base, adenine, with a methyl group in the 2 position, was a relatively good inhibitor, effective enough to cause a possible problem in RIA systems with digests of unpurified tRNA used as a source of inhibitors. The free nucleoside, ms^2i^6A, was a less effective inhibitor than might have been anticipated; this was also true

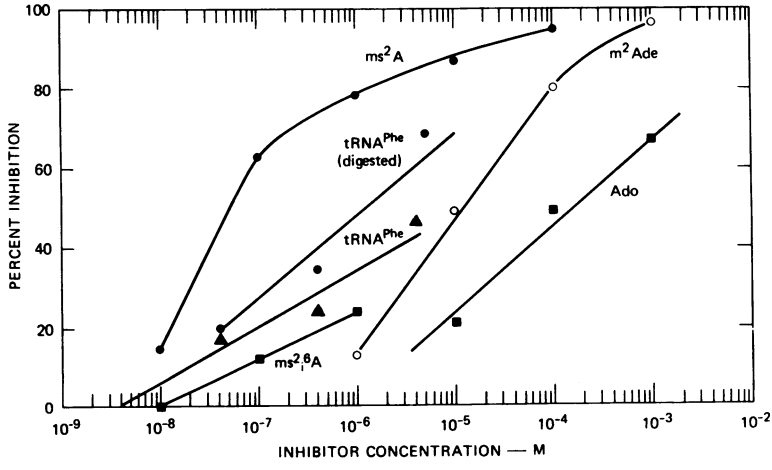


Figure 5. Competitive inhibition experiments with anti-ms^2A serum. Binding was measured in the SAS system using 5 pmoles of $[^3H]ms^2A'$, 50 µg of antiserum protein, and 350 µg of normal rabbit γ-globulin per assay. Abbreviations not already noted in the text are: Ado, adenosine; m^2Ade, 2-methyladenine.

of the $tRNA^{Phe}$, which contains ms^2i^6A. As with other anti-nucleoside sera that we have tested, digesting the tRNA aids its ability to inhibit the RIA.

CONCLUSIONS

1. Both the membrane-binding and the SAS techniques are effective as RIAs; however, the SAS technique is preferable because of low non-specific binding, stability of the complexes to the washing procedure, and the potential of accommodating larger amounts of protein.

2. Either a nucleoside labeled with tritum by gas substitution or a nucleoside trialcohol obtained by periodate oxidation and reduction with potassium boro[^{3}H]hydride can be used as an antigen. The latter procedure is particularly useful to obtain high specific activities with nucleosides unstable to the standard commercial procedures or with nucleosides available in amounts too small to subject to commercial labeling protocols. The multivalent hapten composed of a tritium-labeled nucleoside-protein conjugate was no more effective than the free nucleosides.

3. tRNAs containing a modified nucleoside are much more effective as inhibitors when digested with ribonuclease.

4. Threonine, adenosine, m^6A, and m^6_2A did not cross-react to a significant extent with the anti-t^6A serum. Threonine, a component of the side chain modification, was completely ineffective as an inhibitor of binding of t^6A to anti-t^6A.

5. t^6A used as a hapten can elicit an antiserum of very high specificity, although in our hands the titer was low. The hapten ms^2A gave an antiserum of higher titer, but lower specificity. The specificity of the t^6A antiserum after ammonium sulfate precipitation and dialysis was high enough so that further purification by affinity chromatography was unnecessary. The titer was not improved by dissociation with acid. In working with modified nucleosides that are not commercially available, it is important to know that sera with low titers can be very specific and usable in RIAs.

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