A NEW CHEMICAL PROBE FOR SINGLE-STRANDED RNA

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Summary: The reaction of cytidine with sodium bisulfite and methylamine gives transamination to N^4-methylcytidine and deamination to uridine. At pH 7.4, however, transamination takes place exclusively. This reaction was applied to yeast RNA (7 days, 37°) and afforded the specific conversion of 54% of the cytidine residues to N^4-methylcytidine residues. The same procedure converted up to 95% of the cytidines of polycytidylic acid to N^4-methylcytidines. A complex of polycytidylic acid with polyinosinic acid was, however, totally resistant to the reaction. Because of its cytosine specificity, single-strand specificity, possibility of use of radioactive methylamine, mild conditions, and ease of analysis, this reaction seems ideally suited as a chemical probe for single-stranded regions in RNA.

There is considerable interest in the determination of the higher structure of transfer, ribosomal, and viral RNA molecules.[1,2] Chemical reagents have been employed to distinguish single-stranded, exposed, nucleotides from those which are in helical complexes, or otherwise protected from reaction. The following properties are considered highly desirable in a reagent used for this purpose: high selectivity for a specific nucleotide (in tRNA this can be a minor component; however, reagents of this type will not be useful with other RNA's and are not considered here); high selectivity for a single-stranded region, ease of analysis of the reaction, and preservation of the conformation of the RNA molecule during the reaction. The reagents used as single-stranded probes include nitrous acid,[1] monoperphthalic acid,[1] kethoxal,[1] water-soluble carbodiimide,[1] methoxyamine,[2] N-bromosuccinimide,[3] and sodium bisulfite.[4-7] All of the above show some degree of single-strand specificity. Kethoxal, methoxyamine and sodium bisulfite are specific for one of the major nucleotides of RNA. In each case, however, a considerable amount of labor is needed to analyze and interpret the results of the reaction. Chemical modification alters the

characteristic elution pattern from a DEAE-cellulose column (or the two-dimensional electrophoretogram) produced by an enzymatic hydrolysate of the RNA. These alterations are caused by a change in the charge of the oligonucleotide fragment and by the inhibition of enzymatic cleavage. These changes in pattern are, in fact, used to follow the progress of the reaction. However the modified fragments must be isolated purified, and identified, which involves much effort.

We wish to describe a new procedure which may prove helpful in studies of RNA conformation. It involves the reaction of cytosine with methylamine, at pH 7.4, catalyzed by sodium bisulfite, to give N^4-methylcytosine.[8] It has the following desirable properties: (1) It affects only cytosine, of the major RNA components (uracil also reacts with bisulfite under the conditions described, but this can be reversed by a pH 9 treatment after the reaction[6]) (2) only one product is produced in the reaction (methoxyamine is cytosine-specific but gives two products) (3) it is completely single-strand specific (4) it allows for the rapid determination of the identity of the reactive cytosines within the RNA.

We had previously reported that when cytosine derivatives were allowed to react with amines in the presence of bisulfite, transamination took place. This process was inevitably accompanied by a variable amount of deamination, to a uracil derivative.[8] We now made an intensive study of the reaction of methylamine with cytidine. The progress of the reaction was followed by two-dimensional thin-layer chromatography in 1-butanol-conc. NH_4OH-H_2O (86:1:14)(solvent A) and in 2-propanol: H_2O (80:20)(solventB). The products were eluted and determined spectrophotometrically. The pH was found to be the most important factor controlling the course of the reaction. At pH 6 or below (1.5 M methylammonium chloride, 1.2 M $NaHSO_3$, 0.1 M phosphate buffer, 37°, 48 hours) cytidine was converted completely to uridine. Under the same conditions, but at pH 7.5, a mixture of 2 parts of N^4-methylcytidine to 1 part of unreacted cytidine was obtained. The rate of reaction could be increased by raising the temperature, the methylamine concentration, or the bisulfite concentration.

Table 1. ANALYSIS OF RNA MODIFIED BY TRANSAMINATION

	Nucleotide Composition of RNA (%)				
Amine Used	Ap	Gp	Up	Cp	C*p
None (unmodified RNA)	20.2	27.1	34.7	17.8	
Methylamine	20.4	26.0	35.2	8.5	9.9
Aniline	19.5	26.1	35.6	11.6	7.2

Each reaction was run in duplicate. The results agreed to within 1%. The abbreviations Ap, Gp, Up, Cp, and C*p refer to the 2' - (3') - phosphates of adenosine, guanosine, uridine, cytidine, and N^4 - substituted cytidine respectively.

The reaction was now applied to yeast RNA (Schwarz/Mann, Orangeburg, N. Y.). The conditions used were 10 mg RNA, 2.4 M sodium bisulfite, 2.85 M methylamine, 37°, 0.1 M phosphate buffer, pH 7.5, 168 hours. The workup of the reaction mixture and determination of the composition of the RNA were performed by methods previously described.[4] The peak observed in the position of cytidine 2'-(3')-phosphate was demonstrated by thin layer chromatography in 2-propanol-conc. NH_4OH-H_2O (60:30:10) to consist of two components. One was cytidine 2'-(3') phosphate. The other was identified as N^4-methylcytidine 2'-(3') phosphate. It was converted by alkaline phosphatase to a nucleoside identical in its chromatographic and spectroscopic properties with N^4-methylcytidine.

The results of the nucleotide analysis of the unmodified and modified RNA samples are summarized in Table I. It can be seen that a specific conversion of 54% of the cytosine residues to N^4-methylcytosines had taken place. An attempt was made to demonstrate the

generality of the reaction by the use of another amine, aniline. The reaction mixture was 1 M in aniline, and 0.4 M in sodium bisulfite in 40% aqueous ethanol, pH 7.2. The reaction was run at 37° for 14 days, neutralized, extracted with ether three times to remove excess aniline, and worked up in the same manner as the methylamine reaction. A new peak was eluted between guanylic acid and uridylic acid. It was converted by alkaline phosphatase to a product identical with N^4-phenylcytidine in its spectroscopic and chromatographic properties. The nucleotide composition of the aniline reaction is included in Table I. It can be seen that a 38% conversion of cytidylic acid to N^4-phenylcytidilic acid had taken place in a specific manner.

In order to test the conformational specificity of the reaction, studies were undertaken with poly rC and poly rI·poly rC. The conditions used were 2.2 M $CH_3NH_2 \cdot HCl$, 1.8 M $NaHSO_3$, 0.15 M Na_2HPO_4 buffer, pH 7.4,38°, 15 days. The polynucleotide was purified by chromatography on Sephadex G-25 and digested to nucleosides by a combination of venom phosphodiesterase and alkaline phosphatase. The reaction mixture was analyzed by thin-layer chromatography in solvents A and B and in 2-propanol:conc.HCl:H_2O (65:16.7: 18.3). These conditions converted 49% of the cytosine residues of polycytidylic acid to N^4-methylcytosine residues. The cytosine residues of the poly rI. poly rC complex were unchanged under the same conditions (2% or more of transamination would have been detected in our system). The reaction thus had complete specificity for the single-stranded polycytidylic acid. By the use of more vigorous conditions (51.5°, 18 days) it was possible to convert 95% of the cytosine residues of polycytidylic acid to N^4-methylcytosines. Poly-N^4-methylcytidylic acid has been of interest for physical studies.[9] It was prepared by a route involving the synthesis and polymerization of N^4 - methylcytidine diphosphate.[10,11] The transamination route offers a simpler alternative

route to this polymer and to other N^4 - substituted polycytidylic acids.

If this procedure were to be applied to an RNA of well-defined secondary structure, and the modified RNA were analyzed by enzymatic hydrolysis followed by DEAE - cellulose chromatography or 2-D electrophoresis, little or no change in the characteristic elution curve or electrophoratogram would be expected. The methyl group added does not block the action of pancreatic ribonuclease[11] and has only a small effect on the acid dissociation of cytidine. If, however, radioactive methylamine were used (both ^3H and ^{14}C methylamine are readily available commercially), each peak or spot from a ribonuclease digest would contain an amount of radioactivity directly proportional to the reactivity of the cytosine within it. In one experiment, $^{14}CH_3NH_3^+Cl^-$ was used in the bisulfite-catalyzed transamination of polycytidylic acid. One portion of the reaction mixture was hydrolyzed and analyzed in the usual manner. The amount of transamination indicated was 10%. Another portion was dialyzed against phosphate buffer, concentrated, dissolved in Aquasol liquid scintillation counting medium and counted on a Beckmann L5-233 scintillation counter. The radioactivity incorporated corresponded to 13% transamination which agreed reasonably with the other method. When a polycytidylic-polyinosinic acid complex was allowed to react under the same conditions, the counts incorporated were no greater than the normal background of counts.

This reaction is, of course, slower than those normally employed for the modification of biological macromolecules. It is questionable whether a tRNA molecule, for example, would maintain its integrity for one week in solution, even with reaction conditions that are close to physiological ones. This handicap can be overcome,

however, by using sufficiently radioactive methylamine. If the amount of tRNA commonly employed [6,7] (2mg) were used, with ^{14}C - methylamine of an activity of 1.5 mc/mmole, (a 1 to 10 dilution of commercially available material) then a 1% reaction of 1 cytosine residue of the tRNA would result in the incorporation of several thousand cpm into the relevant peak or spot of the chromatogram. Experiments to demonstrate the applicability of this procedure are in progress.

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