# A new reaction useful for chemical cross-linking between nucleic acids and proteins

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Cytosine in nucleic acids can be converted into $N^4$-aminocytosine by treatment with a mixture of hydrazine and bisulfite. The hydrazino group thus formed at position 4 of the pyrimidine ring can be linked to a sulhydryl group in proteins by the use of bromopyruvate as a linker. Successful use of this scheme of chemical cross-linking between nucleic acid and protein was demonstrated in the linking of poly(C) with glutathione, and of RNA with protein in the *E. coli* 30 S ribosomal subunit.

*Chemical cross-linking* *RNA-protein interaction* *Hydrazine-bisulfite* *Cytosine-specific reaction* *Ribosome*

## 1. INTRODUCTION

Chemical cross-linking between nucleic acids and proteins has been used for studying the mutual arrangement of these molecules in biological systems. For example, information on the spacial proximity of RNA and proteins in *E. coli* ribosomes has been obtained by using cross-linking techniques [1,2]. We describe here a new reaction that enables cross-linking between cytosine residues in nucleic acid and SH groups in proteins under mild conditions.

We have shown that cytosine in single strand nucleic acids can be selectively modified by treatment with a mixture of semicarbazide and bisulfite [3,4]. Similarly, a mixture of hydrazine and bisulfite can be used for modification of cytosine in single stranded polynucleotides; the reaction, when carried out under neutral conditions, follows the scheme shown in fig.1 [5–7]. In this reaction, no change is induced in adenine or guanine. Uracil and thymine can undergo addition of bisulfite across the 5,6-double bond of the pyrimidine ring, but the adducts formed can easily revert to the original molecules on standing in aqueous solutions [8–10]. The hydrazino group at position 4 of the product of this reaction, $C^{am}$, can serve as a means to derivatize further the modified nucleic acids. For example, ketones and aldehydes are expected to form hydrazones from this hydrazino group. Bromopyruvate has been used as an SH-blocking agent for proteins [11]. Thus, by use of bromopyruvate as a linker, $C^{am}$ residues generated in poly(C) have been combined with the SH group of glutathione (see fig.2). Furthermore, this reaction scheme has been used for cross-linking between RNA and proteins in the *E. coli* 30 S ribosomal subunit.

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*Abbreviations:* $C^{am}$, $N^4$-aminocytidine; $imC^{am}$, ethylacetimidate-modified $N^4$-aminocytidine (3-methyl-5,6-dihydro-*s*-triazolo[4,3-c]pyrimidine riboside



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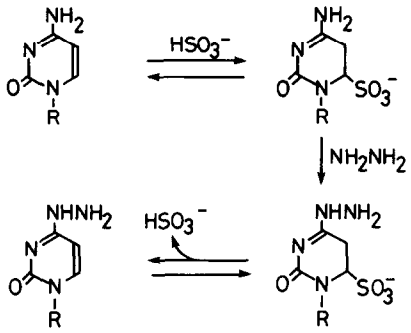


Fig. 1. Reaction of cytidine with hydrazine bisulfite.

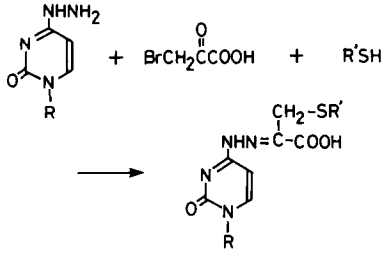


Fig. 2. Scheme for cross-linking.

## 2. EXPERIMENTAL

### 2.1. *Chromatographic solvents*

Three solvents were used: *n*-butanol–acetic acid–water (2:1:1); (2) ethanol–1 M ammonium acetate (pH 7.5) (7:3); (3) isopropanol–ammonia–water (7:1:2).

### 2.2. *Ribosomal subunits*

The 30 S and 50 S ribosomal subunits were prepared from *E. coli* strain PR 13 as in [12]. For $^{35}S$-labeled ribosomes, cells were grown on low-sulfate medium using $H_2{}^{35}SO_4$ (New England Nuclear) as in [13].

### 2.3. *Modification of poly(C) with hydrazine-bisulfite*

A solution of poly(C) (Sigma; 10 mg/ml, in 2 M $NH_2NH_2$–1 M $NaHSO_3$, pH 6.1) was incubated at 37°C for 75 min. The solution was diluted with 14 vols of 0.1 M sodium phosphate buffer (pH 7.6) and dialyzed against this buffer for 24 h. The solution was then dialyzed against 0.05 M NaCl for 12 h, and the polynucleotide was precipitated by addition of excess ethanol.

To estimate the $C^{am}$ content in the poly(C,$C^{am}$), the $C^{am}$ residues were converted first into $imC^{am}$ [14]. This was done by treatment of poly(C,$C^{am}$) with 0.1 M ethyl acetimidate (Eastman) at pH 7.0 and room temperature for 30 min, followed by treatment at pH 4.0 (adjusted with acetic acid) for 30 min. The polymer was then digested with nuclease P1 (Yamasa), and the nucleoside 5′-phosphates (p-C and p-$imC^{am}$) that formed were fractioned by paper chromatography (solvent 1) and quantified by UV absorption ($\epsilon$ of p-$imC^{am}$ at $\lambda_{max}$ 263 nm, and pH 7, 13 500). In the present reaction, the molar ratio of p-C : p-$C^{am}$ thus estimated was 58–42.

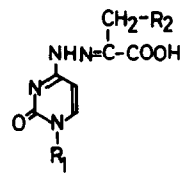
### 2.4. *Linking of poly(C,$C^{am}$) with glutathione*

To a solution of glutathione (123 mg, Wako) in 1 M Tris–HCl (pH 8.0) (1 ml), was added bromopyruvic acid (33 mg, Sigma), the final pH being 8.0. After 10 min of reaction, an aqueous solution of the poly(C,$C^{am}$) that had been prepared as described above (5 $A_{270}$ units in 1 ml) was mixed with this solution, and the mixture was allowed to stand at room temperture for 1 h. Sodium pyruvate (110 mg, Wako) was then added, and the reaction was allowed to proceed for 1 h. The mixture was dialyzed against 0.05 M NaCl for 32 h at 4°C, and the product was isolated by ethanol precipitation.

### 2.5. *Characterization of poly(C,$C^{am}$) linked to glutathione*

The cross-linked material (3.5 $A_{310}$ units) was treated with nuclease P1 (50 $\mu$g) in 0.25 M acetate buffer (80 $\mu$l, pH 5.0) at 50°C for 1 h. The digested material was further treated with *E. coli* alkaline phosphomonoesterase (0.075 units, Sigma) and snake venom phosphodiesterase (20 $\mu$g, Worthington) at pH 8.0 and 37°C for 3 h. The digest was subjected to paper chromatographic analysis (see table 1), giving 3 spots corresponding to cytidine and compound I, and II, respectively. The standard samples of I and II were prepared from cytidine. Only spot II was positive in the ninhydrin test. Furthermore, when the material corresponding to II was treated with 6 N HCl at 100°C for 12 h to hydrolyze the peptide bonds and then chromatographed (solvent 1), it gave a UV-absorbing spot corresponding to uracil (characterized also by spectra, $R_f$ 0.60) and 3 ninhydrin-positive spots ($R_f$ 0.35, 0.25 and 0.13)

Table 1
Properties of pyruvate derivatives of $C^{am}$ and its nucleoside

| Compound number | $R_1$ | $R_2$ | $R_f$ in paper chromatography[a] | | | Paper electrophoresis[b] ($R_{UMP}$) | UV absorption, $\lambda_{max}$ ($\epsilon$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | | pH 1 | pH 7 | pH 13 |
|  | H | H[c] | 0.54 | 0.58 | 0.44 | 0.70 | 313 (24 600) | 297 (19 800) | 310 (12 600) 351 (11 900) |
| | H | glutathione (GS-) | 0.27 | 0.14 | 0.27 | 1.0 | 323 | 305 | 313 |
| I | ribose | H | 0.55 | 0.33 | 0.47 | 0.66 | 322 | 300 | 325 |
| II | ribose | glutathione (GS-) | 0.29 | 0.08 | 0.04 | 0.80 | 328 | 310 | 325 |

[a]See text for solvent systems
[b]Electrophoresis was run for 2.5 h at 200 V, using 0.05 M sodium phosphate buffer, pH 7.0. The mobility is expressed relative to that of uridine 5′-phosphate.
[c]This compound was obtained as a crystalline material in a high yield (91%) from $C^{am}$ and pyruvate: m.p. 243–247°C (dec), analyzed as $C_7H_8O_3N_4 \cdot 5/4\ H_2O$, mass spectrum 196 ($M^+$) and 151 ($M^+$-COOH)

that corresponded to those formed on the same treatment of glutathione. On this HCl treatment, glutamic acid gave the $R_f$ 0.35, glycine $R_f$ 0.25 and cysteine $R_f$ 0.13 spots, respectively.

### 2.6. *Cross-linking of RNA and protein(s) in the 30 S ribosomal subunit*

$^{35}$S-labeled 30 S subunit ($3.5 \times 10^6$ dpm, 26 $A_{260}$ units) suspended in buffer 1 (30 mM $NH_4Cl$, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.8) was treated with 2 M $NH_2NH_2$, 1 M $NaHSO_3$, 10 mM $MgCl_2$ (0.4 ml) at pH 7.0 and 37°C for 30 min. The solution was then dialyzed against buffer 1 at 4°C for 20 h. The solution was mixed with 1 vol. of 20 mM bromopyruvic acid, 10 mM $MgCl_2$, 0.4 M Tris-HCl (pH 8.0) and the mixture was incubated at 37°C. Aliquots were withdrawn at times, cooled in ice, and dialyzed at 4°C with buffer 1 for 12 h and buffer 2 [0.1% sodium dodecyl sulfate, 2 mM EDTA, 6 mM 2-mercaptoethanol, 25 mM Tris-HCl (pH 7.8)] for 12 h. Samples containing about $10^5$ dpm material were fractionated by centrifugation through a 5–20% sucrose density gradient in buffer 2, using a Hitachi RPS 50-2 rotor, at 42 000 rpm and 4°C for 7 h.

To estimate the extent of modification in 16 S RNA during the treatment with hydrazine bisulfite, a separate experiment was done in which a sample of unlabeled 30 S subunit was treated with the reagents as described above, 16 S RNA was isolated by phenol extraction, and the $C^{am}$ content was analyzed as described for poly(C,$C^{am}$). In this case, high-pressure liquid chromatography (with Partisil 10 SAX as the column) was used for the quantification of p-im$C^{am}$.

### 2.7. *Activities of chemically treated 30 S ribosomal subunit*

The poly(U)-mediated poly(Phe) synthesis was carried out in the presence of intact 50 S subunit using [$^{14}$C]Phe (Daiichi Pure Chemicals) as in [12]. With 10 mCi/mmol [$^{14}$C]Phe, 1 $A_{260}$ unit of 30 S (untreated), and 2 $A_{260}$ of 50 S subunits, the radioactivity of poly(Phe) obtained was 5500 cpm. [$^{14}$C]Poly(U) binding to the 30 S subunit was assayed as in [15]. With 0.25 mCi/mmol [$^{14}$C]poly(U) (Miles) and 2 $A_{260}$ untreated 30 S subunit, the radioactivity measured for the bound material was 2118 cpm. The elongation factor-Tu mediated [$^{14}$C]Phe-tRNA binding was measured in the presence of intact 50 S subunit as in [16] with some modification. With 424 mCi/mmol [$^{14}$C]Phe-tRNA, 0.17 $A_{260}$ 30 S (untreated) and 0.37 $A_{260}$ 50 S subunits, the radioactivity observed was 2326 cpm. The crystalline Tu·GDP and [$^{14}$]Phe-tRNA

were generous gifts from Drs Y. Kaziro and M. Tanaka.

## 3. RESULTS AND DISCUSSION

Poly(C) was treated with hydrazine bisulfite to convert about 40% of the cytidine into $C^{am}$ residues. The poly(C,$C^{am}$) was coupled with glutathione that had been freshly treated with bromopyruvate. After the coupling, the polymer was treated with pyruvate to block any unreacted $C^{am}$ residues. The non-dialyzable, ethanol-precipitable product was then collected and digested enzymatically into nucleosides. Paper chromatographic analysis of this material gave three spots that gave $R_f$ values identical to cytidine, compound I, and II, respectively. The paper electrophoretic behavior and the UV absorption spectra of the product II were also identical with those of the standard sample (see table 1). As judged from the absorbance of I and II on the chromatogram, 66% of the $C^{am}$ residues in the polynucleotide were linked to glutathione.

To determine whether this reaction can be used for more complex natural systems, *E. coli* 30 S ribosomal subunit was subjected to this two-step cross-linking reaction. The 30 S subunit is composed of 16 S RNA and 21 proteins, and contains titratable SH groups [17,18]. In the first step, two of the 347 cytosines [19] in 16 S RNA were modified on average. After the second step, i.e. bromopyruvate treatment, the subunit was subjected to sucrose density gradient centrifugation in the presence of sodium dodecyl sulfate and EDTA that would dissociate the proteins from the RNA [20]. If the cross-linking took place as expected, the cross-linked protein should co-sediment with RNA. As fig. 3 shows, certain portions of the protein co-sedimented with RNA. This was observed

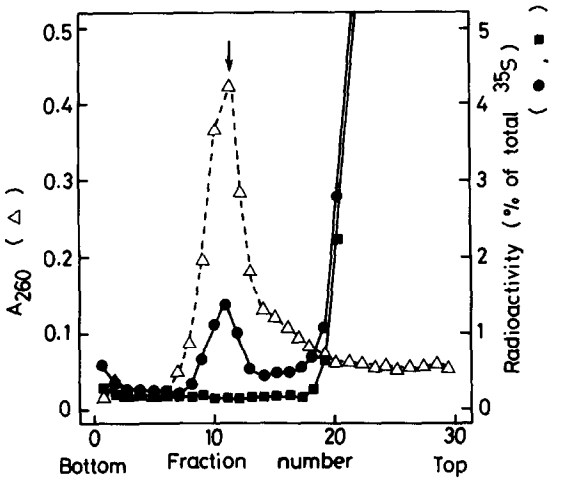



Fig. 3. Sucrose density gradient centrifugation of chemically cross-linked 30 S ribosomal subunit. The centrifugation was done as described in the text. (● and △), Cross-linked 30 S subunit (1st step, 30 min; 2nd step, 60 min); (■), untreated 30 S subunit. The arrow indicates the position where 16 S RNA of untreated subunit sedimented. Samples that had undergone either the 1st step only or the 2nd step only gave results identical to that of the untreated 30 S subunit.

Table 2
Effect of chemical treatment on activities of 30 S ribosomal subunit

| Treatment | Time of treatment (min) | Activity (% of control)[a] | | |
|---|---|---|---|---|
| | | [$^{14}$C]Poly(Phe) synthesis | [$^{14}$C]Poly(U) binding | [$^{14}$C]Phe-tRNA binding |
| Hydrazine bisulfite | 15 | 86 | 79 | 103 |
| | 30 | 68 | 84 | 103 |
| | 60 | 69 | 97 | 107 |
| Hydrazine-bisulfite (30 min) followed by bromopyruvate (60 min) | 11 | 88 | 17 | |

[a]See text for assay methods
For preparing controls, mock treatments (in buffer only) were given to the 30 S subunit

only when the two-step reaction was carried out: when either hydrazine bisulfite or bromopyruvate treatment was omitted, there was no co-sedimentation. Furthermore, the amount of proteins that co-sedimented with the RNA increased until it reached a plateau, as the time of the bromopyruvate treatment increased: the value, [radioactivity (dpm) in the RNA fraction]/[total radioactivity (dpm) loaded in the centrifugation], was 3290/115 600 (2.9%) at 5 min, 5510/134 800 (4.0%) at 30 min, 5690/124 400 (4.6%) at 60 min, and 5570/128 400 (4.5%) at 120 min. These results clearly show that cross-linking between 16 S RNA and protein(s) took place. Determination of the cross-linked regions in the RNA and proteins awaits further investigation.

We have also examined biological activities of the treated 30 S subunit, i.e. polyphenylalanine synthesis, poly(U) binding and Phe-tRNA binding. As shown in table 2, the treatment with hydrazine bisulfite did not cause extensive loss of these activities. This suggests that the topological integrity of the 30 S particles was largely retained at the start of the bromopyruvate treatment. Even after subsequent treatment with bromopyruvate, the poly(U) binding activity was mostly retained, although the protein synthesizing activity, which requires dynamic action of ribosomes, was abolished.

In conclusion, this new reaction for cross-linking nucleic acids with proteins, which can be carried out under mild conditions (pH 6–8, 37°C, 1–2 h), may be useful for studying the mutual arrangement of these polymers in biological systems.

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