SELECTIVE MODIFICATION OF CYTIDINE RESIDUE IN RIBONUCLEIC ACID BY SEMICARBAZIDE.

Hikoya Hayatsu and Tyunosin Ukita

Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo.

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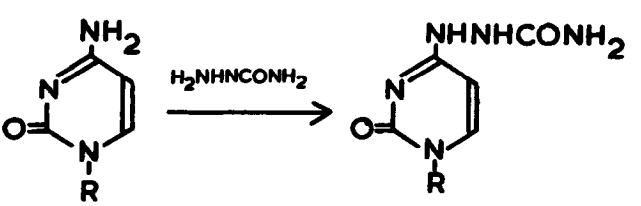
Some search has been made to find reagents to selectively modify the structure of nucleic acids (e.g., see Kochetkov et al., 1963, Verwoerd and Zillig, 1963). We have observed that semicarbazide (SC) affords a specific reaction with cytidine residue in RNA under certain mild conditions.

Each of the four RNA nucleosides, namely, adenosine, guanosine, uridine and cytidine, was treated in 1M SC at pH 4.2, 6.4 and 9.4 at 37°. The reaction was followed by paper chromatography (solvent mixture: isopropanol-ammonia-water, 7:1:2) and by ultraviolet absorption. Reaction was observed only with cytidine at pH 4.2. None of other reaction mixtures showed any change even after 125 hrs at 37° and additional 260 hrs at room temperature. In the reaction of cytidine at pH 4.2, the spot caused by cytidine at Rf 0.48 gradually faded with



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R=CH_3(a), RIBOSE(b), RIBOSE-PHOSPHATE(c)

reaction time and a new spot of Rf 0.27 appeared and grew more intense. After 125 hrs, only the latter was observed on the chromatogram. The ultraviolet absorption maxima shifted to longer wave lengths (5~7 mμ) with a simultaneous increase in absorbancy (A) (Fig. 1). Behavior of deoxycytidine in 2M SC was similar to that of cytidine. Following paragraphs describe the above reaction in detail and its application to the modification of RNA.

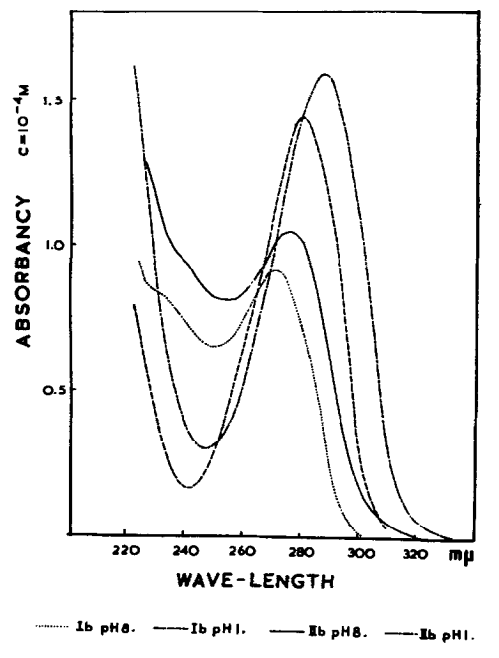

Fig. 1. Ultraviolet absorption spectra of cytidine (Ib) and SC-nucleoside (IIb). The spectra of IIb were determined by diluting the 48 hrs-reaction mixture of Ib and SC with appropriate buffer solutions.

Reaction rate and pH-effect. A solution of 40 μmoles cytidine in 2 ml. 2M SC and containing an equivalent amount of acetic acid which fixed the solution at pH 4.2, was kept at 37 ± 0.2°. A 0.1 ml. aliquot was withdrawn at different time intervals and diluted with 0.2M Tris-buffer, pH 8.0, to 25 ml. and the A at 290 mμ was determined. The results are given in Fig. 2. The reaction was shown to follow the pseudo-first order rate. The

rate constant was approximately $2 \cdot 10^{-5}$ l/mole/sec ($t_{1/2} \simeq 4.5$ hrs). The rate was found proportional to the concentration of cytidine and of SC. Therefore, the reaction was believed to be bimolecular. At a lower temperature, 4.6~5.3°, the half-time increased to 50~60 hrs. At 63°, it diminished to 1 hr. The effect of pH on the reaction rate was determined by a similar procedure; the optimum pH was 4.2. At pH 5.0, the rate was approximately half of that at pH 4.2. At pH 7, no reaction occurred.

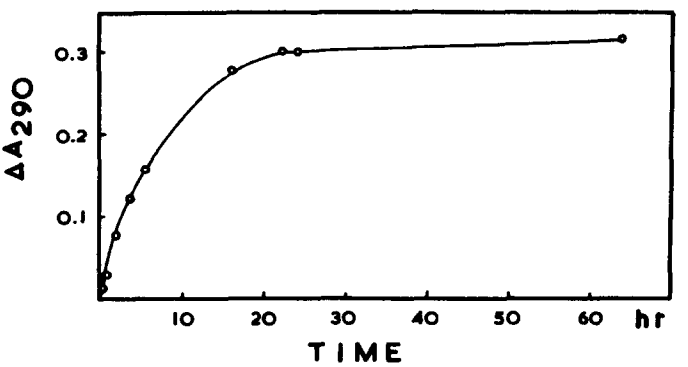

Fig. 2. Increase of A at 290 mμ as a function of time. Initial A_{290} was 0.104.

Structure of the reaction product. 1-Methylcytosine (240 mg.)(Ia) was dissolved in 3 ml. of 2.5M SC at pH 4.2 and kept at 37° for 159 hrs. Fine crystals which precipitated were collected and recrystallized from water to colorless prisms, m.p. 162~3° (decomp.). Yield, 318 mg. (83 %). Anal. Calcd. for $C_6H_9O_2N_5 \cdot H_2O$: C, 35.82; H, 5.52; N, 34.81. Found: C, 35.55; H, 5.65; N, 34.95. This compound was identical with 4-semicarbazino-1-methyl-pyrimidone (IIa) which was synthesized from 4-thio-1-methyluracil (Fox et al., 1959) and SC. The difference in ultraviolet absorption spectra of Ia ($\lambda_{max}^{pH\ 7}$274 mμ(ε, 8150), $\lambda_{max}^{pH\ 1}$283(12300)(Fox and Shugar, 1952)) and IIa ($\lambda_{max}^{pH\ 7}$278(8950), $\lambda_{max}^{pH\ 1}$289(14500)) was quite similar to that of cytidine and its product. Therefore,

the product derived from cytidine is believed to have a semicarbazino-structure, IIb. On a paper chromatogram, compound IIb consumed periodate and yielded a yellow color with Ehrlich's reagent*. On treatment with acid or alkali, IIa and IIb gave 1-methyluracil and uridine respectively. The pK_a of IIb, determined spectrophotometrically, was 2.5, while that of cytidine as a control was found to be 4.1.

Reaction of SC with mononucleotides. A solution of cytidine-2'(3')-phosphate (200 mg.)(Ic) in 4.5 ml. of 2.67M SC (pH 4.2) was incubated at 37° for 48 hrs. The pH was adjusted to 7.4 by the addition of 1.4 g. of $Ba(OH)_2$. A trace amount of insoluble material was removed by centrifugation and the Ba-salt of the modified nucleotide (IIc) was precipitated by the addition of 2 vols. of ethanol. The Ba-salt thus produced was washed in turn with 66 % ethanol, absolute ethanol and ether. The dried material weighed 262 mg. A portion of the sample was analysed by Dowex-1 (Cl^-) resin column chromatography (Fig. 3). The chromatography showed that the starting-material content in the product was less than 0.5 % of the semicarbazino-nucleotide, IIc.

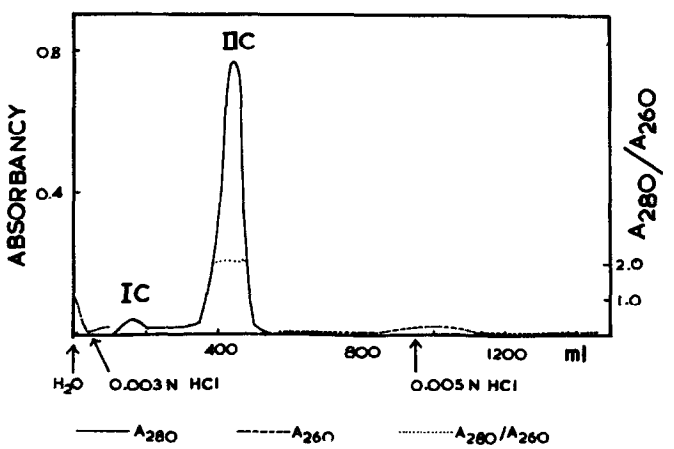

Fig. 3. Chromatographic analysis of SC-nucleotide (IIc). 3.93 mg. of Ba-salt of IIc was applied on Dowex-1, X-2 (Cl^-), 10.5 cm x 0.83cm^2 column. Flow rate, 1.0 ml./min.

* It was reported that urea derivatives gave yellow color with this reagent (Fink et al., 1956).

Reaction of SC with RNA. 200 mg. yeast RNA* , which was purified by alcohol-precipitation and dialysis, was treated in 6 ml. of 2.35M SC (pH 4.2) at 37° for 96 hrs. The pH was adjusted to 7~8 with 2N NaOH and the mixture was dialysed against 0.2M NaCl. Alcohol-precipitation followed by washing with ethanol and ether gave 178 mg. of SC-RNA. The alkaline hydrolysate of modified RNA was analysed by ion-exchange column chromatography (Fig. 4). The cytidine-2'(3')-phosphate content was reduced to 7 % of that in the starting material**. The semicarbazino-nucleotide fraction was eluted at the same position of that of adenosine-3'-phosphate. During the alkaline treatment of SC-RNA, some of the semicarbazino-nucleotides were presumed to be converted to uridine-nucleotides since it was found that on treating IIc with 0.3N KOH at 37° for 20 hrs. some 30 % of IIc was converted to uridine-2'(3')-phosphate. When SC-RNA was hydrolysed with bovine pancreatic RNase-A (Hirs et al., 1953) and the resulting mono-nucleotides were separated in ion-exchange column, SC-nucleoside-3'-phosphate, uridine-3'-phosphate and a trace amount of cytidine-3'-phosphate were obtained. The results indicated that the ester of modified cytidine-3'-phosphate is the substrate for RNase. The semicarbazino-nucleotide IIc was converted to the 2',3'-cyclic phosphate with dicyclohexyl carbodiimide. The cyclic nucleotide thus obtained was found hydrolysable by RNase-A. The rate of the hydrolysis was approximately equal to that of uridine-2',3'-cyclic phosphate.

*Sigma Chemical Co.

**The result suggests either that the rate of the reaction is reduced because cytidine occurs as a bound constituent of RNA and is unavailable or that several cytidine residues in the RNA are protected from the attack of the reagent by their specific configuration in the RNA molecule.

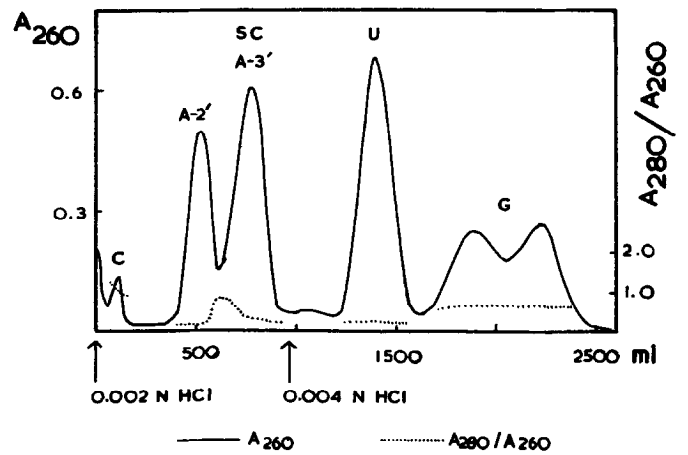

Fig. 4. Column chromatography of alkaline hydrolysate of SC-RNA. SC-RNA (25 mg.) was treated in 1.2 ml. of 0.3N KOH for 21 hrs at 37°. Hydrolysate was neutralised with $HClO_4$ and precipitate was removed. Supernatant was applied on Dowex-1 (Cl^-), 11.0 cm x 0.83cm^2 column. Flow rate, 1.0 ml./min.

The method for the modification of cytidine which could be used readily for the study of nucleic acids is described here. Further investigations along this line are now in progress.

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