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51. Morizo Ishidate and Shigeru Tsukagoshi: Reaction of Nitrogen Mustard with the Components of Ribonucleic Acid.

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In 1952, Alexander[1] described that some of the cytotoxic alkylating agents such as nitrogen mustard derivatives esterify the phosphoryl groups of deoxyribonucleic acid (DNA). It has been a common opinion that the attack of these agents on cells is probably orientated to the nuclear constituents of cells, viz. DNA, and that there are at least two kinds of reactive groups in the molecule of polynucleotide which is supposed to be the reactive sites with alkylating agent. The one[2] is the phosphoryl groups and the other[3] is the amino or imino groups in the base component of the nucleotide.

This paper deals with the analysis of the reaction products of N-methyl[^{14}C]-bis(2-chloroethyl)amine hydrochloride(^{14}C-labeled nitrogen mustard or ^{14}C-HN_2) with the components of ribonucleic acid (RNA), purpose of which experiment was to examine, as the preliminary experiment to elucidate the reaction of nitrogen mustard with nucleic acids, how fast and at what particular site of the latter molecules the alkylating agent reacts with them in a buffer solution. Paper chromatography, paper electrophoresis, and autoradiography were used for the analysis of these reaction products.

Experimental and Results

Materials——^{14}C-HN_2 was prepared by the present authors.[4] 3-Adenylic acid (AMP) and cytosine were commercial preparations from Sigma Chemical Co. (U. S. A.). These were kindly supplied by Dr. Y. Miura. Adenine and adenosine were purchased from Wakamoto Pharm. Co. Ltd. Guanine, guanosine, guanylic acid, uracil, uridine, uridylic acid, and cytidine were also purchased from Tokyo Kasei Kogyo Co. Ltd.

Reaction of ^{14}C-Labeled Nitrogen Mustard with the Components of Ribonucleic Acid——To a solution of each of the components of RNA (0.25 μmole/cc., adenine, adenosine, adenylic acid, guanine, guanosine, guanylic acid, uracil, uridine, uridylic acid, cytosine, or cytidine) in 20 cc. of Menzel's buffer (pH 8.9),[5] ^{14}C-HN_2 (0.75 μmole/cc., 6,500 c.p.m./μmole) was added. The mixture was incubated at 37° for various periods of time (1~15 hr.). Then it was kept frozen and lyophilized. The same procedure was carried out for ^{14}C-HN_2 only at the same concentration in order to compare the results. To each of the lyophilized samples, 0.5 cc. of distilled water was added. Approximately 0.01 cc. of this solution was used for spotting on the paper. The concentration of ^{14}C-HN_2 was usually fixed at $7.5\times10^{-4}M$ as described above, but, in some necessary cases, those between 10^{-4} and $10^{-2}M$ were also used. Some purine and pyrimidine bases were difficult to dissolve unless they were warmed with the buffer solution at 60~80° on a water bath. After cooling they were used immediately.

Detection of the Spots on Paper——Purines and pyrimidines were detected by visual observation of light-absorbing spots under ultraviolet ray (Manasulu-Light 2536A) and P was detected by the method of Bandurski and Axelrod.[6] For detection of radioactive spots, autoradiograms with X-ray film were prepared. It took about one to two weeks to get good results of these autoradiograms. Dragendorff reagent was also used for the detection of HN_2.

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Combined Method of Paper Chromatography and Electrophoresis——To find the reaction products from ^{14}C-HN_2 and the components of RNA, the solution of lyophilized samples was spotted on Toyo Roshi No. 51 paper (13×35 cm.), and developed first with the solvent BuOH-AcOH-H_2O (4:2:1)[7] vertically and immediately after drying it was put on the apparatus for electrophoretic analysis (Toyo Roshi Type-C, No. 1297) in a horizontal direction (300 V, 0.9~1.0 mA/cm.) using a borate buffer (pH 9.2). Examples of these results are shown in Fig. 1.

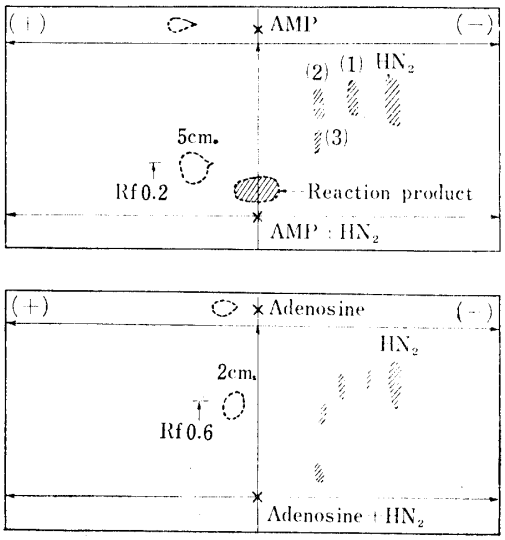

Fig. 1. Combined Method of Paper Chromatography and Electrophoresis for the Reaction Mixture

1st : BuOH-AcOH-H_2O (4:2:1).
2nd : 300 V, 0.9 mA/cm., 3 hr., 25°, borate buffer (pH 9.2).
Reaction : AMP or adenosine (0.25 μmole/cc.) + ^{14}C-HN_2 (0.75 μmole/cc.), 37°, pH 8.9, 2.5 hr., ^{14}C-HN_2 : 6,500 c.p.m./μmole.
(1)~(3) : Transformed or decomposed products of ^{14}C-HN_2.
Spots on the paper chromatograms were detected with the following reagent or methods.
▨ Dragendorff reagent and autoradiography
▩ The method of Bandurski and Axelrod (for nucleotides) and ultraviolet ray

On the contrary, when the same sample as described above was subjected to paper chromatography alone, a few reaction products could be detected, but it was not easy to discriminate the real reaction products on the paper chromatogram from the transformed or decomposed products of ^{14}C-HN_2 itself. Analysis of spots by this combined method of paper chromatography and electrophoresis enables a clear detection of the reaction products in the presence of by-products from the decomposition of ^{14}C-HN_2.

In the cases of purine and pyrimidine bases or nucleosides, no reaction product was found in this condition of experiment.

Two-dimensional Paper Chromatography——The solution of lyophilized samples was spotted on Toyo Roshi No. 51A paper (20×20 cm.) and two-dimensional paper chromatography was carried out, using the solvent BuOH-AcOH-H_2O (4:2:1) as the first developer and the solvent EtOH-$AcONH_4$ (7.5:3)[8] as the second. Examples of these chromatograms are shown in Fig. 2.

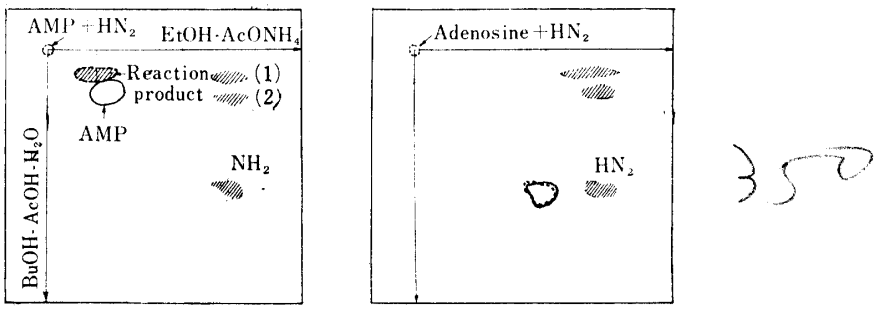

Fig. 2. Two-dimensional Paper Chromatography of the Reaction Mixture
1st : BuOH-AcOH-H_2O (4:2:1).
2nd : EtOH-$AcONH_4$ (7.5:3).
(1)~(2) : Transformed or decomposed products of ^{14}C-HN_2.
Spots were detected as in Fig. 1.

7) T. Kariyone, T. Inoue : Yakugaku Zasshi, **74**, 301 (1954).
8) A. C. Paladini, L. F. Leloir : Biochem. J., **51**, 426 (1952).

In only the case of nucleotides, spots resulting from the formation of reaction products were detected by the method of autoradiography, ultraviolet absorption, and ammonium molybdate reagent.[6] However, no spots were found in the case of purine and pyrimidine bases or nucleosides even at the concentration between 10^{-3} and $10^{-4}M$ of ^{14}C-HN_2.

As for the reaction time, it was found using AMP that clear spots of the reaction products were observed for 2~3 hr. of the reaction time at 10^{-4} to $10^{-3}M$ of ^{14}C-HN_2, but, if the reaction time is much longer (more than 8 hr.), it became somewhat difficult to find clear spots of the reaction products, probably because of the hydrolysis of the reaction products.

Discussion

In this experiment it was shown that phosphoryl groups of AMP, guanylic acid and uridylic acid, mainly reacted with HN_2 in the buffer solution. The nucleosides, adenosine, guanosine, uridine, and cytidine, did not react as were also the bases, adenine, guanine, and cytosine. Though the data in this paper showed only the results for AMP, adenosine, and adenine, similar results were obtained for the other components described above.

So far as known from this experiment, when a higher concentration of ^{14}C-HN_2 was used such as $10^{-2}M$ or more, a certain reaction product was found as shown in Fig. 3. Perhaps the nitrogen in the purine and pyrimidine bases would react with HN_2.

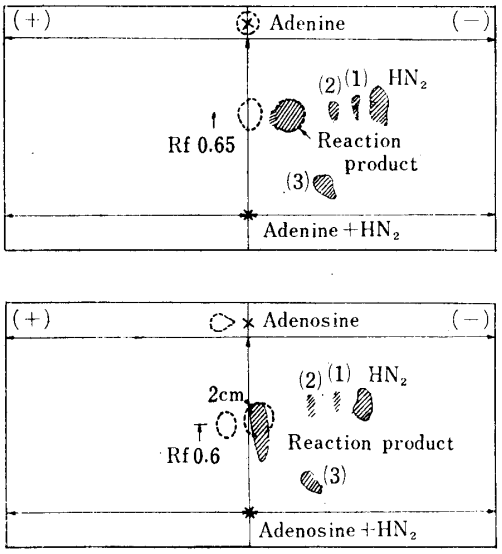

Fig. 3. Examples of the Result at a Higher Concentration of ^{14}C-HN_2.

1st : BuOH-AcOH-H_2O (4:2:1).
2nd : 300 V, 1 mA/cm., 3 hr., 25°, borate buffer (pH 9.2).
Reaction : Adenine or adenosine (0.5 μmole/cc.) + ^{14}C-HN_2 (20 μmole/cc.), 37°, pH 8.9, 2.5 hr, ^{14}C-HN_2 : 6,500 c.p.m./μmole.
(1)~(3) : Transformed or decomposed products of ^{14}C-HN_2.
Spots were detected as in Fig. 1.

It would be concluded, however, that the alkylating reactions on RNA *in vitro* occur with the primary and secondary phosphoryl groups at low concentration of HN_2, but, on the contrary, a far higher concentration seems to be necessary for reaction with the nitrogens of purine and pyrimidine bases. In the present work, the reaction of ^{14}C-HN_2 with only the components of RNA was examined. Therefore, experiments concerning the reaction of this ^{14}C-HN_2 with the components of DNA and with these nucleic acids in the coexistence of proteins are now in progress.

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Summary

Alexander stated that nitrogen mustard derivatives esterify the phosphoryl groups of deoxyribonucleic acid. Therefore, as the preliminary experiment, the present investi-

gation deals with a discussion on the reaction products of ^{14}C-labeled nitrogen mustard with nucleotides (adenylic acid, guanylic acid, and uridylic acid), nucleosides (adenosine, guanosine, uridine, and cytidine), and purine and pyrimidine bases (adenine, guanine, uracil, and cytosine) in a buffer solution. The data of analysis of these reaction mixtures by paper chromatography, electrophoresis, and autoradiography demonstrate that this alkylating agent reacts only with nucleotides, and not with the phosphorus-free components of ribonucleic acid.

The present result seems to support the presumption that the most significant attack of alkylating agents on nucleic acid is the reaction with the phosphoryl groups.

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