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STUDIES ON THE CHEMICAL MODIFICATIONS OF NUCLEIC ACIDS. THE PERMANGANATE OXIDATION OF THYMINE.

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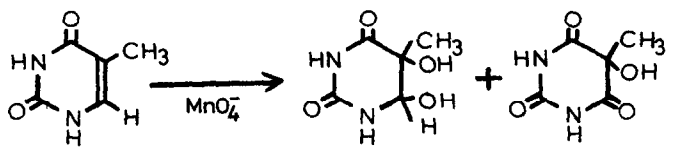
In our previous communication, we have reported that pyrimidine residues, especially thymine residues, in nucleic acids can be selectively oxidized with permanganate under mild reaction conditions of pH 6.7 and 0° (1). This new method of chemical modification of nucleic acids is potentially useful in the studies of correlation between structures and functions of biologically active nucleic acids as well as in the studies of primary structures of them. Bearing these objectives in mind, we thought it is essential to know the nature of the reaction occurring in nucleic acid components under the reaction conditions employed.

Earlier work by Jones and coworkers (2) has shown that oxidation of thymine with potassium permanganate at pH 7 or 9 and 37° for 19 hr gives cis-thymine glycol as a primary product, and that, in the course of this reaction, this glycol is hydrolysed and oxidized further to give urea, acetol, pyruvaldehyde, pyruvic acid and formic acid.

In the present paper, it is shown that oxidation of thymine with potassium permanganate for a short period at pH 7 greatly reduced the complexity of the reaction, giving rise mainly to two products; cis-thymine glycol (diol A) and 5-hydroxy-5-methylbarbituric acid (acid B), a compound isolated for the first time as the product of permanganate oxidation of thymine. Indications are also given that acid B, in addition to diol A, is a primary product in this reaction.



Thymine Diol A Acid B

Experiments For setting of the reaction conditions to be employed for our present purpose, we first followed the decrease in absorption at 260 mμ of thymine under the oxidation conditions analogous to those reported in the previous paper (1). From this experiment we found that under the reaction conditions described below the absorption at 260 mμ reached a minimum value in 2 min.

Thus, the oxidation reaction was carried out with 14.3 mM thymine (1.0 g.) in 20 mM potassium permanganate solution at pH 6.8-7.1 (in phosphate buffer) and 15-17°. After 2 min, sodium bisulfite solution was added to the reaction mixture to terminate the oxidation. Manganese dioxide which precipitated was removed by centrifugation and the supernatant solution concentrated under reduced pressure at below 30°. By extracting with acetone, the reaction products were transferred into the acetone phase*, most of the inorganic salts being removed as a semi-solid. The products were then fractionated by use of cellulose column chromatography (eluting solvent; n-butanol-water, 86:14, v/v). Detection of the products in each fraction of the column chromatography was carried out by measuring dry weight after the fraction was evaporated to dryness. Only two major peaks were detected: one was diol A and the other acid B**.

Acid B, which was eluted in the first peak, was obtained in colorless crystals (recrystallized from ethanol) as a free acid. m.p. 226-227°. Yield, 0.51 g. (40 %). Anal. Calcd. for $C_5H_6N_2O_4$: C, 37.98; H, 3.83; N, 17.72. Found: C, 38.12; H, 3.72; N, 17.87. Diol A, which was subsequently eluted from the column, was recrystallized from ethanol-water to colorless crystals. m.p. 215-216° (decomp.). Yield, 0.45 g. (35.5 %). Anal. Calcd. for $C_5H_8N_2O_4$: C, 37.50; H, 5.04; N, 17.50. Found: C, 37.75; H, 5.36; N, 17.52.

Diol A was assigned to be cis-thymine glycol (5,6-dihydroxy-5,6-dihydrothymine). This assignment was based on (a) melting point (the reported m.p. is 214-216° (decomp.)(2)), (b) the mode of periodate consumption (2, 3), (c) IR spectra (4), (d) NMR spectra (5, 6), and (e) Rf values in paper chromatography (3, 7). In all of these terms, the characteristics found for diol A were in good coincidence with those of cis-thymine glycol.

5-Hydroxy-5-methylbarbituric acid was synthesized from diethyl methylmalonate and urea according to Doumas and Biggs (8). This authentic 5-hydroxy-5-methylbarbituric acid

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* In a separate experiment using radioactive thymine, it was found that more than 95 % of the total radioactivity used was present in this acetone phase.

** Upon paper chromatographic analysis of the total reaction mixture, neither thymine nor urea was detectable.

(m.p. 225-226°) was indistinguishable from acid B obtained as above from the oxidation mixture of thymine : namely, a mixed m.p. gave no depression; IR spectra were superimposable; and Rf values in several paper chromatographic systems were identical.

In order to gain deeper and confirmative knowledge of the product distribution in this oxidation reaction, 2-^{14}C-thymine was oxidized with permanganate in an identical manner as in the case of non-radioactive thymine, and, after the work-up, the acetone extract which contained the products was examined by means of paper chromatography (solvent system; n-butanol-water, 86:14, v/v). The percentage distribution of radioactivity on the paper chromatogram was found to be as follows: acid B (Rf, 0.30), 41.8 %; diol A (Rf, 0.20), 41.6 %; and another spot (Rf, 0.04), 13.9 %*. Practically no radioactivity was located in areas other than these.

From these experiments it was concluded that, under the reaction conditions employed, thymine was completely oxidized to give mainly diol A and acid B in 1 to 1 ratio.

Next, diol A was treated with potassium permanganate under the reaction conditions as described before. Paper chromatographic analysis indicated that diol A was resistant to the permanganate oxidation and virtually no acid B was produced in this reaction mixture. It is evident, therefore, that diol A is not an intermediate in the formation of acid B from thymine. The same is true, of course, for acid B, because treatment of acid B with potassium permanganate gave no diol A as expected. Hence, both diol A and acid B are produced from thymine simultaneously via separate routes, that is, acid B as well as diol A is a primary product in this reaction.

Of additional note in these paper chromatographic studies is that immediately after the alkali spray, both diol A and acid B, especially the latter compound, strongly absorbed UV light, and this greatly facilitated the detection of these compounds**.

Discussions It should be pointed out that the production of cis-diol A and acid B through the oxidation of 5,6-double bond of thymine bears a close similarity to the permanganate oxidation of olefins. Thus, it has been established that permanganate oxidation of olefins in

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* Nature of the third product (or products), which was (or were) formed in a small but distinct amount and which hardly travelled in the paper chromatography, has not yet been clarified. Incidentally, the Rf values of thymine and urea, as determined by applying the standard samples on the paper alongside the reaction mixture, were 0.50 and 0.25, respectively.

** The ureido compounds that were produced on the paper after this exposure to alkali gave characteristic coloration with p-dimethylaminobenzaldehyde reagent (9).

neutral solution gives rise to both cis-diol and ketol, and that the ketol is not obtainable from the diol on treatment of the latter with permanganate (10, 11). This suggests that the mechanism of permanganate oxidation of thymine is similar to that of the oxidation of olefins (12).

Recently Jones and coworkers were able to isolate thymidine glycol, a diol A-type nucleoside, from the complex mixture of permanganate-oxidized thymidine (6). It is interesting whether or not an acid B-type nucleoside is produced from thymidine under our reaction conditions.

In the light of the data that have been presented in our previous work (1), the oxidation conditions employed in our present investigation would allow predominant degradation of thymine residues in nucleic acids. It is to be mentioned, however, that certain minor nucleosides such as thiouridine (13) and N^6-isopentenyl adenosine (14) are oxidized with potassium permanganate as rapidly as (or even more rapidly than) thymine nucleosides.

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