THE SELECTIVE DEGRADATION OF PYRIMIDINES IN NUCLEIC ACIDS BY PERMANGANATE OXIDATION.

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In our current studies on the chemical modifications of nucleic acids, we have reported on the selective reaction of semicarbazide derivatives with cytidine residues (Hayatsu and Ukita, 1964 and 1966; Hayatsu et al., 1966; Kikugawa et al., 1967a, b). In this paper we describe a procedure by which pyrimidines in the single stranded region of nucleic acids are degraded selectively. Furthermore, reaction conditions are presented whereby thymidylic acid is exclusively attacked while other deoxyribonucleotides are virtually intact.

This procedure consists of the permanganate oxidation of the nucleic acid under relatively mild reaction conditions of pH 6.7 and 0°C.

Experimental Results. The oxidation reaction was first carried out with major mononucleotides (and nucleosides) in 26.4 m$\underline{M}$ $KMnO_4$, at pH 6.7 and 0°C. In Figure 1, the rate of oxidation as determined by the decrease in ultraviolet absorption is given. It is seen that pyrimidine derivatives were very rapidly oxidized whereas purine nucleotides were essentially stable under these conditions. Paper chromatographic analysis of the reaction mixtures also showed that the disappearance of the originally present pyrimidine derivatives was complete within 10 minutes whereas purine derivatives were recovered unchanged.

When a highly diluted $KMnO_4$ solution was used for the oxidation, selectivity even among the pyrimidines was achieved. Thus, as shown in Figure 2, in 0.4 m$\underline{M}$ $KMnO_4$ thymidylic acid was found to be most sensitive to the

oxidation, while uridylic acid was moderately reactive and cytosine derivatives were only little affected under the conditions employed.

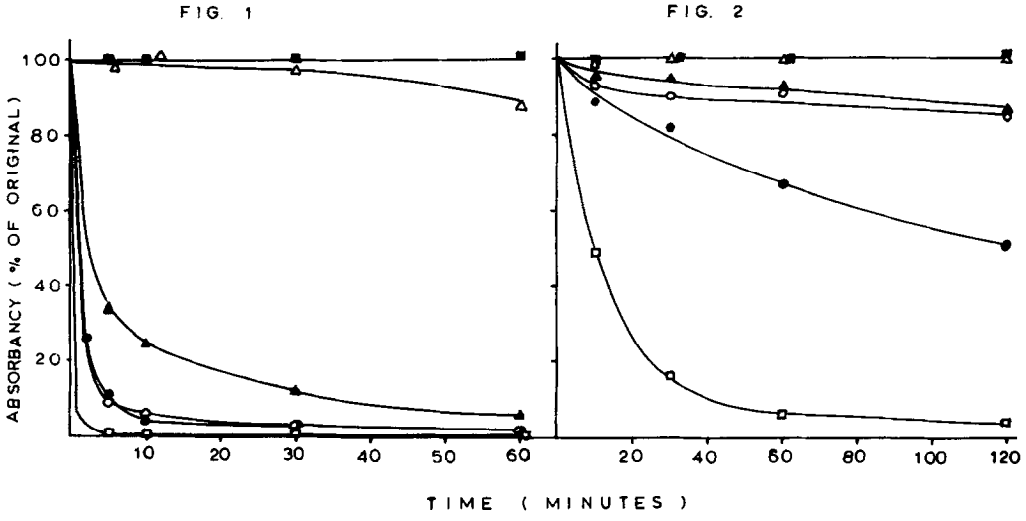

Fig. 1. The oxidation of mononucleotides and nucleosides with 26.4 mM $KMnO_4$. To a 3-ml aqueous solution which contained 50 μmoles of nucleotide (or of nucleoside) and 0.33 M phosphate buffer, pH 6.7, was added 2 ml of chilled 66 mM $KMnO_4$ under ice-cooling. The reaction was allowed to proceed in the ice-bath. The pH stayed constant during the entire reaction period. Aliquot (0.5 ml) was withdrawn from time to time, and mixed with 1 ml of 1 M $NaHSO_3$ to terminate the oxidation. From the resulting colorless solution, 0.1 ml was taken up and diluted with 5 ml of 0.1 M phosphate buffer, pH 6.5, and the diluted solution submitted to the spectrophotometric analysis. The zero-time aliquot was withdrawn from the reaction mixture before the addition of $KMnO_4$. ○, Uridine (260 mμ); ●, uridine 5'-phosphate (260 mμ); ▲, cytidine (270 mμ); □, thymidine 5'-phosphate (260 mμ); △, guanosine 5'-phosphate (260 mμ); ■, adenosine 5'-phosphate (260 mμ).

Fig. 2. The oxidation of mononucleotides with 0.4 mM $KMnO_4$. The 50-ml reaction mixture contained 2.5 μmoles of nucleotide, 0.2 M phosphate buffer, pH 6.7, and 20 μmoles of $KMnO_4$. The reaction was performed at 0°C. The assay method was essentially analogous to that described in Fig. 1. □, Thymidine 5'-phosphate (267 mμ); ●, uridine 5'-phosphate (270 mμ); ○, deoxycytidine 5'-phosphate (270 mμ); ▲, cytidine 5'-phosphate (270 mμ); △, deoxyguanosine 5'-phosphate (260 mμ); ■, deoxyadenosine 5'-phosphate (260 mμ).

In Figure 3, the reaction of calf thymus DNA with 0.8 mM and with 26.4 mM $KMnO_4$ are presented. In 0.8 mM $KMnO_4$ solution, the native,

double-stranded DNA was completely inactive toward the oxidation, whereas the heat-denatured DNA was extensively attacked. Even in 26.4 m$\underline{M}$ $KMnO_4$ solution, the native DNA appeared to be unaffected in the first 30 minutes of the reaction, while the denatured DNA was rapidly oxidized under the identical conditions.

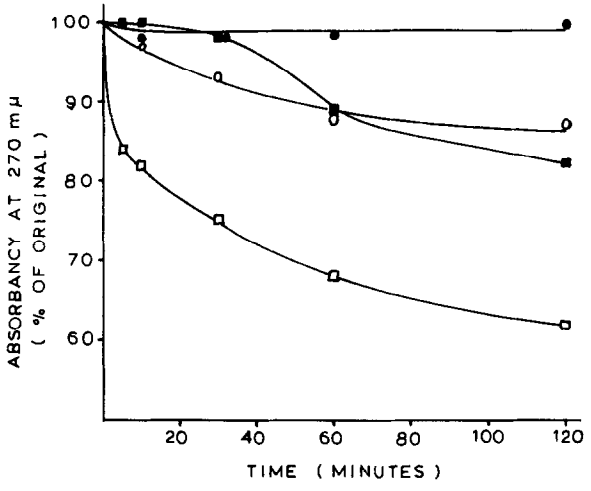

Fig. 3. The permanganate oxidation of DNA. The reaction conditions and the assay method were analogous to those described in Fig.'s 1 and 2. ●, Native DNA in 0.8 m$\underline{M}$ $KMnO_4$; ○, heat-denatured DNA in 0.8 m$\underline{M}$ $KMnO_4$; ■, native DNA in 26.4 m$\underline{M}$ $KMnO_4$; □, heat-denatured DNA in 26.4 m$\underline{M}$ $KMnO_4$.

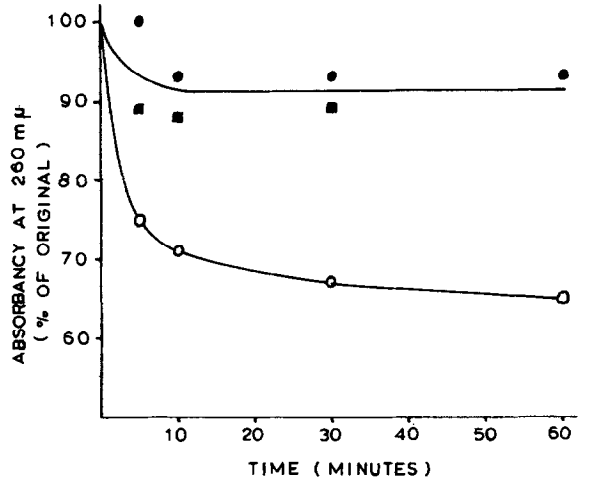

Fig. 4. The oxidation of tRNA with 26.4 m$\underline{M}$ $KMnO_4$. The reaction conditions and the assay method were analogous to those described in Fig. 1. ●, tRNA, experiment No. 1; ■, tRNA, experiment No. 2; ○, alkaline hydrolysate of tRNA.

When yeast tRNA* was oxidized in 26.4 m$\underline{M}$ $KMnO_4$, a limited reaction was observed. As can be seen in Figure 4, the small decrease (7 to 10 %) in the absorbancy at 260 mμ, which took place 10 minutes after the reaction was started, remained unchanged even after 1 hour. The control experiment using the alkaline-hydrolysate of this tRNA exhibited, as expected, an extensive oxidation under the identical conditions. In the experiment (No. 2) presented in Fig. 4, the oxidized tRNA was isolated and the base composition of both of the oxidized and the unoxidized tRNA's were determined. Table 1 shows that the oxidative degradation occurred only in pyrimidines to a limited extent.

Table 1. The base composition of the oxidized tRNA as expressed in molar ratio.

	A	G	U	C
tRNA	100	171	137	134
Oxidized tRNA	100	171	107	110

The tRNA (390 optical density units at 260 mμ) was treated with 5 ml of 26.4 m$\underline{M}$ $KMnO_4$ in 0.2 $\underline{M}$ phosphate buffer, pH 6.7, at 0°C for 30 minutes. The reaction was terminated by addition of 5 ml of 1 $\underline{M}$ $NaHSO_3$. Further working-up procedure included the dialysis against water and repeated precipitation of the tRNA from water-ethanol. The yield of the product was quantitative (350 optical density units). The base composition of the RNA's was determined by hydrolysing the RNA with 0.3 $\underline{N}$ KOH at 37°C for 18 hr followed by separation of the resulting mononucleotide mixture using columns of Dowex 50 (H^+ form) and of Dowex 1 (formate form)(Katz and Comb, 1963).

Discussion. It has been known that the permanganate oxidation of nucleic acids under relatively vigorous conditions gives rise to a complete degradation of guanine residues as well as pyrimidines in them (Jones and Walker, 1963). In the present studies we were able to introduce more

* The tRNA of Baker's yeast was kindly prepared by Mr. K. Takeishi of our laboratory from the crude tRNA according to the procedure of Holley (Holley, 1963; Takeishi et al, 1967). The crude tRNA was a generous gift from Sankyo Co., Ltd., Tokyo.

specificities into this oxidation reaction by employing the conditions much milder than those reported by the previous workers. Thus, in 26.4 m$\underline{M}$ $KMnO_4$ at pH 6.7 and 0°C, as is shown in Fig. 1, pyrimidines could be completely oxidized in 10 minutes whereas purines including guanine are virtually unaffected for at least 30 minutes. The presence of a phospho-monoester group in the substrate does not seem to affect the rate of oxidation.

Interestingly enough, with 0.4 m$\underline{M}$ $KMnO_4$, thymidylic acid is specifically attacked while other deoxyribonucleotides are essentially unaffected (Fig. 2). These reaction conditions, therefore, are of potential use for the selective chemical modification of thymine residues in DNA.

As judged from the results given in Fig.'s 3 and 4, it is highly likely that the oxidation reaction is specific for the single stranded region of nucleic acids. Thus, the native DNA was very little, if any, affected whereas the denatured DNA was extensively oxidized under either of the reaction conditions employed. Of more interest is the limited reaction observed with tRNA. From the results presented in Fig. 4, it appears that only a fraction (20 %) of the total pyrimidine residues in the tRNA is "exposed" to the reagent under the conditions employed. This kind of limited reaction which reflects the high secondary-structure content in tRNA has been often encountered in the oxidative modification of tRNA (Doepner et al., 1966; Burton et al., 1966). From Table 1, it is clear that the observed decrease in the absorbancy of tRNA during the oxidation reaction was actually due to the loss of pyrimidines

The permanganate oxidation of the minor constituents of tRNA will be the subject of a forthcoming paper (Hayatsu, to be published).

The permanganate oxidation reaction presented here has, in principle, a close kinship to the oxidation of nucleic acid with OsO_4 (Burton and Riley, 1966). Thus, both of these agents specifically attack pyrimidines in the single stranded region of nucleic acids. The permanganate oxidation, however, does not give the disadvantage of difficulty in handling which has been encountered in the working-up procedure following the OsO_4-oxidation

of nucleic acids (Burton and Riley, 1966).

The nature of the reaction products remain to be elucidated. Nevertheless, it appears reasonable to postulate that the primary oxidation products derived from the pyrimidine nucleotides are the 5,6-dihydro-5,6-diolpyrimidine derivatives (see, for example, Benn et al., 1960; Burton and Riley, 1966).

The permanganate oxidation is of importance since it is a potential tool for the sequence determination in DNA (Burton and Riley, 1966), and, furthermore, it could provide a useful means in the studies on various functional sites in nucleic acids.

Further work extending the present studies will be reported in the forthcoming papers.

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