180 °, Trägergas: N_2, Durchflußgeschwindigkeit: 30 ml/min).

Enzymatische Hydrolyse von I——70 mg I wurden in 10 ml Acetat-Pufferlösung (pH 5.2) mit 60 mg β-Glukosidase 24 h bei 37° gerührt. Nach üblicher Aufarbeitung wurden 27 mg farbloses Öl von $[\alpha]_D^{20}$ +245.0° (c=1.10, $CHCl_3$) erhalten. MS m/e 208.1464 (M^+ für $C_{13}H_{20}O_2$, Ber. 208.1463), 152.0840 ($C_9H_{12}O_2$, 11%), 108.0583 (C_7H_8O, 100%). UV λ_{max}^{MeOH} nm (log ε): 238 (4.20), 283 (3.30). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 2960, 1655, 1630. 975, PMR (60 MHz, $CDCl_3$) δ: 0.98 (3H, s), 1.03 (3H, s), 1.28 (3H, d J=7 Hz, H-10), 1.94 (3H, d, J=0.5 Hz, H-13), 2.14 (1H, br.s, H-2), 2.27 (1H, br.s, H-2), 2.53 (1H, d, J=8 Hz, H-6), 4.32 (1H, m, H-9), 5.35 (1H, dd, J=16 Hz und 8 Hz, H-7), 5.68 (1H, dd, J=16 Hz und 5 Hz, H-8), 5.89 (1H, br.s, H-4). ^{13}C-NMR (C_5D_5N) δ: 198.0 (s, C-3), 161.8 (s, C-5), 140.7 (d, C-8), 125.9 (d, C-4), 125.4 (d, C-7), 67.5 (d, C-9), 55.5 (d, C-6), 48.0 (t, C-2), 36.1 (s, C-1), 27.8 (q, C-10), 27.0 (q, C-13), 24.5 (q, C-11), 23.2 (q, C-12).

Oxydation von II——14 mg II in 3 ml Äther wurden mit 0.1 ml der Lösung von 1 g Natriumbichromat und 1.36 g H_2SO_4 in 5 ml H_2O versetzt, dann 1 h bei Raumtemp. gerührt, darauf mit H_2O versetzt. Nach Extraktion mit Äther, Waschen, Trocknen und Eindampfen wurde der Rückstand an Kieselgel (15 g) mit $CHCl_3$ chromatographiert. Es wurde aus Äther/Hexan kristallisiert, wobei 5 mg farblose Würfel vom Schmp. 72° und $[\alpha]_D^{20}$ +335° (c=0.21, $CHCl_3$) erhalten wurden. UV λ_{max}^{MeOH} nm (log ε): 236 (4.33). IR λ_{max}^{KBr} cm^{-1}: 2950, 1675, 1625, 1435, 1375, 1355, 1320, 1260, 990, 910, 885, 845. MS m/e 206 (M^+), 150, 109, 108 (100%), 107, 79, 77, 43, 41, 39. PMR (60 MHz, $CDCl_3$) δ: 1.02 (3H, s), 1.09 (3H, s), 1.90 (3H, d, J=0.5 Hz, H-13), 2.28 (5H, br.s, H-10 und H-2), 2.68 (1H, d, J=8 Hz, H-6), 5.98 (1H, s, H-4), 6.10 (1H, d, J=16 Hz, H-8), 6.65 (1H, dd, J=16 Hz und 8 Hz, H-7).

Isolierung von I aus *Dennstaedtia wilfordii*——Die bei der Kieselgel-Säulenchromatographie mit $CHCl_3$/MeOH (90: 10~80: 20) eluierten Fraktionen (siehe experimentellen Teil von (7)) wurden weiter durch Säulenchromatographie (60 g Al_2O_3 Woelm (neutral), $CHCl_3$ bei zunehmendem MeOH-Zusatz) und anschließende mehrfache präparative DC (Kieselgel, $CH_3COOC_2H_5$/MeOH, 15: 1) aufgetrennt. 1.2 kg oberirdische Teile ergaben 100 mg I.

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Degradation of Nucleic Acids with Ozone. I. Degradation of Nucleobases, Ribonucleosides and Ribonucleoside-5′-monophosphates

Kozo Ishizaki,[a] Nariko Shinriki,*,[a] Akira Ikehata,[a] and Tohru Ueda[b]

Government Industrial Development Laboratory, Hokkaido,[a] Tsukisamu-Higashi 2-17, Toyohira-ku, Sapporo, 061-01, Japan and Faculty of Pharmaceutical Sciences, Hokkaido University,[b] Kita-ku, Sapporo, 060, Japan

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The degradation of nucleobases, ribonucleosides and ribonucleotides with ozone was examined as a model of the action of ozone on nucleic acids. The degradation rates of nucleobases were in the following order: G,T>U>C>A. Nucleosides and nucleotides were degraded in the same order as in the corresponding nucleobases. The ribose moiety was degraded more slowly than the base portion in nucleosides and nucleotides except in the cases of adenosine and AMP. The degradation of D-ribose and D-ribose-5-phosphoric acid (R5P) was also examined, and it was found that R5P resisted the attack of ozone.

Inorganic phosphate was liberated after the degradation of the ribose moiety.

Keywords——ozone; degradation; oxidation; ozonization; nucleobases; ribonucleosides; ribonucleotides; D-ribose; D-ribose-5-phosphoric acid

Recently the effectiveness of ozone for inactivating viruses in drinking water has been attracting attention.[1] In addition, many studies on the mutagenic effects of ozone on bacteria and microorganisms are available.[2] While it is apparent that virus inactivation and mutation are directly related to the chemical changes of RNA or DNA of viruses, few studies have been made on the mode of action of ozone on nucleic acids and their constituents.[3] As the first step towards clarify the mechanism of virus inactivation by ozone, the degradation of nucleobases, ribonucleosides and ribonucleotides with ozone in aqueous solution was investigated in the present work. A preliminary communication was published by us in 1978.[4]

Materials and Methods

Materials——Nucleobases, ribonucleosides, ribonucleoside-5′-monophosphates, D-ribose and D-ribose-5-phosphoric acid (R5P) were obtained from Sigma Chemical Co.

Ozonization——Sample solutions were treated with ozone-oxygen gas mixture (gas flow rate, 330 ml/min; ozone content, 2 mg/l) in a 200 ml glass reactor equipped with a gas diffuser and a sampling tap. The starting concentration of the sample was 1 mM except for the case of guanine (0.32 mM). The solutions were buffered with 0.05 M H_3BO_3–NaOH solution (pH 7.8). All reactions were carried out at room temperature (22°).

Analysis——A Hitachi model 635 high performance liquid chromatograph (HPLC) with a UV detector was employed for the analysis. Nucleobases and ribonucleosides were determined at 264 nm by using a cation-exchange column (Hitachi Custom Ion Exchange Resin #2618) with 0.2 M CH_3COONa at a flow rate of 0.8 ml/min as the eluent. Ribonucleotides were determined at 264 nm by using an anion-exchange column (Hitachi Custon Ion Exchange Resin #2632) with 0.1 M NaCl–0.005 N HCl at a flow rate of 1 ml/min as the eluent. The column temperature was kept at 50°.

Sugars were determined colorimetrically by the orcinol method.[5] It should be noted that the chromogen produced from R5P by this method had a higher absorbance than that produced from D-ribose (1.36 times at 660 nm). When ribonucleotides were treated with ozone, the residual sugar reacting with orcinol–H_2SO_4 was considered to be R5P and not D-ribose, since the liberation of inorganic phosphate occurred only after the destruction of the ribose moiety.[6] Therefore, standard R5P solutions were used to prepare the calibration curve of sugar content in these cases. Sugars determined by the orcinol method are abbreviated as $R_{(orcinol)}$.

Inorganic phosphate was determined colorimetrically by the ammonium molybdate–stannous chloride method.[7]

Results and Discussion

Ozonization of Nucleobases

The time courses of degradation of uracil (U), cytosine (C), guanine (G), adenine (A) and thymine (T) by ozonization are shown in Fig. 1. G and T were degraded much faster than the others. The product peaks which appeared on HPLC (monitored at 264 nm) at the half-life time of the degradation of the parent material are listed in Table I. Ozone was reported to attack initially the 5,6-double bond in the pyrimidine nucleus and the 4,5-double bond in the purine nucleus to give UV-transparent degradation products.[8]

Ozonization of D-Ribose and R5P

R5P was degraded more slowly than D-ribose (Fig. 1). For instance; 7% and 14% of R5P was degraded after ozonization for 60 min and 120 min, respectively, while 53% and 80% of D-ribose was degraded after the same ozonization periods. In addition, inorganic phosphate liberated from R5P amounted to 2% and 5% of the total phosphate after ozonization for 60 min and 120 min, respectively.

Ozonization of Ribonucleosides and Ribonucleotides

The degradation of uridine (Urd), cytidine (Cyd), guanosine (Guo), adenosine (Ado), UMP, CMP, GMP and AMP with ozone was examined. Among them, the time courses of the degrada-

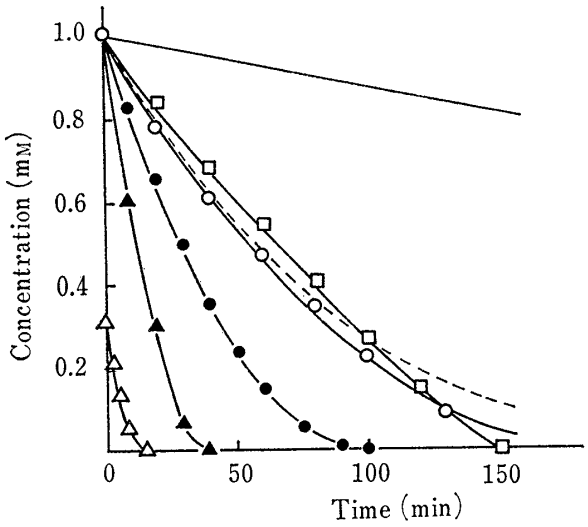


Fig. 1. Degradation of Nucleobases, D-Ribose and D-Ribose-5-phosphoric Acid with Ozone

—□—, adenine; —○—, cytosine; —●—, uracil; —▲—, thymine; —△—, guanine (starting concn., 0.32 mM); ——, D-ribose-5-phosphoric acid; ——, D-ribose.

tion of UMP, GMP and AMP are shown in Fig. 2. Degradation patterns of Urd and CMP were similar to that of UMP, except that Urd or CMP disappeared completely after 110 min and the degradation of the ribose moiety started after 20 min or 60 min, respectively. The degradation pattern of Cyd was akin to that of GMP, but the degradation of Cyd was completed after 150 min and the half-life time of the degradation of the ribose moiety was 110 min. Degradation patterns of Guo and Ado were almost superimposable on those of GMP and AMP, respectively.

In the case of UMP, neither U nor Urd was detected on HPLC throughout the ozonization (Table I). The sum of the concentration of the residual UMP and $R_{(orcinol)}$ retained at 1 mM within 40 min (as shown by the dashed line), which means that the degradation of the ribose moiety did not occur. The liberation of inorganic phosphate from UMP was not observed within 60 min. Consequently, the primary product within 40 min should be pRU′ (p stands for phosphate, R stands for the ribose moiety, and the prime indicates the portion partially degraded to various extents by ozone), which became detectable by the orcinol method. The loss of conjugation involving the 5,6-double bond in the base moiety seems to labilize the glycosyl linkage of pRU′ to the acidic treatment involved in the orcinol method.

Table I. Retention Times (min) of Nucleobases, Ribonucleosides, Ribonuleotides and Their Ozonization Products on HPLC[a]

Nucleobases[b]	Products of each nucleobase[b]	Nucleosides[b]	Products of each nucleoside[b]	Nucleotides[c]	Products of each nucleotide[c]
U 4.0		Urd {2.2[d] 2.6		UMP 5.8	
C 5.6	1.6, 2.9	Cyd 3.6		CMP 1.5	
G 10.0	1.5, 2.0, 2.6	Guo 6.2	1.5, 2.0	GMP 11.2	
A 8.8	1.4	Ado 5.5	1.4, 2.1, 3.4, 8.8	AMP 2.1	1.4[e]
T 6.2	1.4				

a) Retention times (t_R) of products appearing at the half-life time of degradation of the parent material are shown. Taking the starting height of the parent material as 100, the t_R of peaks exceeding 2% are given. Peaks below 2% were neglected.
b) Applied to a cation-exchange column.
c) Applied to an anion-exchange column.
d) Uridine-borate complex formed in borate buffer.
e) Contained adenine as judged by using a cation-exchange column.

In the ozonization of GMP (pRG), no G, Guo or other UV-absorbing material was detected and the phosphate was not liberated within 30 min. The guanine moiety in GMP was degraded much more quickly than the ribose moiety (as is clear from the difference between the dashed line and circles). The total sugar content decreased from the beginning (as shown by the dashed line), which means that pRG′ was degraded quickly to produce pR′G′. It should be noted that, in contrast with the case of R5P, the destruction of the phosphoribosyl moiety of pRG to give pR′G′ was rapid.

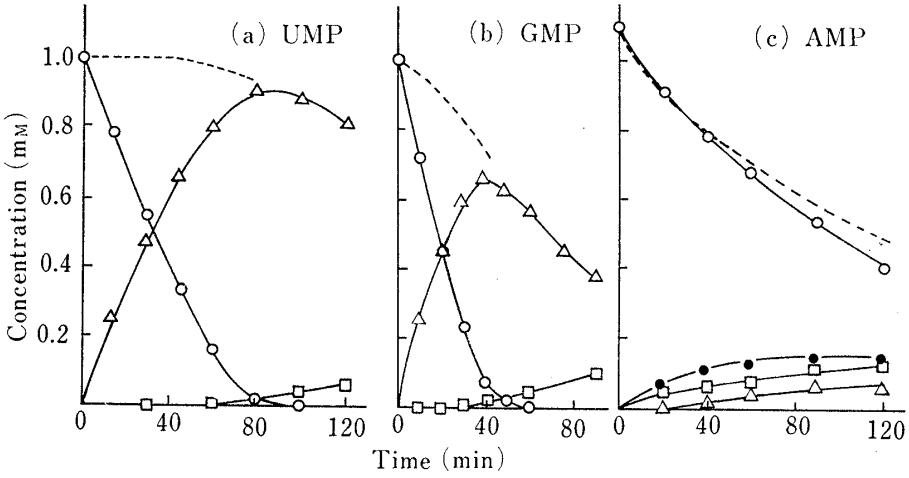
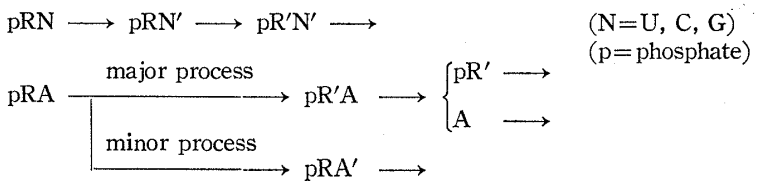


Fig. 2. Degradation of UMP, GMP and AMP with Ozone

—○—, residual nucleotide ([pRN]); —△—, ribosyl moiety other than that of nucleotide ([pRN']); -----, total sugar ([pRN]+[pRN']); —□—, PO_4^{3-}; —●—, adenine.

The degradation of AMP (Ado) was quite different from that of the other nucleosides and nucleotides (Fig. 2-c). The base, adenine, was detected as the reaction started (as shown by solid circles). The degradation rate of AMP (Ado) coincided with that of the ribose moiety within 30 min. This suggests that, in AMP (Ado), the ribose moiety is the target of the initial attack by ozone. Such degradation of the ribose moiety would labilize the N-glycosyl bond of pR'A (R'A) to release adenine. Since the product reacting with orcinol-H_2SO_4 was detected after 30 min, though at a low level, the degradation of the adenine moiety of AMP (Ado) did occur as a minor process. The liberation of inorganic phosphate occurred to a substantial extent from the initial stage, in contrast to the cases of UMP, CMP or GMP. This result is in accord with the report that liberation of inorganic phosphate from AMP due to the oxidation of the ribose moiety was observed upon irradiation of ribonucleotides with X-rays.[6]

Consequently, the following processes can be postulated for the main degradation of ribonucleotides.



The relative reactivity of the base moiety in nucleosides and nucleotides is in the following order: G(T)>U>C>A. The present findings suggest that the site of initial attack of ozone may be the guanine moiety in RNA. As the next study of this series, ozonization of RNA and DNA is being carried out, and the results will be reported elsewhere.

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Determination of Sulthiame in Plasma by High-Performance Liquid Chromatography

Tsuneo Sadanaga,[a] Kozo Hikida,[a] Katsunobu Tameto,[a] Michio Nakanishi,[a] Yoshikazu Matsushima,[b] and Yosuke Ohkura*,[b]

Research Laboratories, Yoshitomi Pharmaceutical Ind. Co., Ltd.,[a] Yoshitomi-cho, Fukuoka, 871, Japan and Faculty of Pharmaceutical Sciences, Kyushu University 62,[b] Maidashi, Higashi-ku, Fukuoka, 812, Japan

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A simple high-performance liquid chromatographic method for the determination of sulthiame, N-(4′-sulfamoylphenyl)-1,4-butanesultam, in plasma is described. The method permits the accurate determination of the drug in plasma at concentrations as low as 150 ng/ml and is suitable for monitoring the drug in the therapeutic dose range and for investigation of the bioavailabilities of preparations of the drug.

Keywords——sulthiame; high-performance liquid chromatography; UV detection; plasma; drug monitoring; precise assay

Sulthiame, N-(4′-sulfamoylphenyl)-1,4-butanesultam, is an antiepileptic agent.[1] Several pharmaceutical preparations containing sulthiame have been used clinically. The therapeutic effects of the preparations may be related to its concentration in plasma. Thus, for comparison of the bioavailabilities of the preparations and for drug monitoring during therapy, a rapid and sensitive method of assay was required.

We reported assay methods for psychotropic and other drugs in plasma by means of high-performance liquid chromatography (HPLC).[2,3] HPLC was also found to be effective for the analysis of sulthiame, as described in the present paper. Several methods have been proposed for the determination of sulthiame in biomedical samples, *i.e.* UV spectrometry after separation by thin-layer chromatography,[4] gas-liquid chromatography with[5] or without derivatization,[6] and reversed phase HPLC.[7,8] However, the present method is very sensitive, reliable, and suitable for routine drug assay. Unfailing analytical results have always been obtained, since the method was introduced in our laboratories.

Experimental

Materials——Sulthiame and its preparations were the products of Bayer AG. Aminopyrine was obtained from Ebisu Pharm. Ind. Co. All solvents and chemicals were of reagent grade.