PURIFICATION AND CHARACTERIZATION OF A SIALIDASE INHIBITOR, SIASTATIN, PRODUCED BY *STREPTOMYCES*

HAMAO UMEZAWA, TAKAAKI AOYAGI, TADAZUMI KOMIYAMA, HAJIME MORISHIMA, MASA HAMADA and TOMIO TAKEUCHI

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo, Japan

(Received for publication June 17, 1974)

Siastatins A and B were isolated as part of a program designed to find *Streptomyces*-produced inhibitors of sialidase from *Clostridium perfringens*. Siastatin A was more effective than was siastatin B in the inhibition of sialidases prepared from *Cl. perfringens* and chicken chorioallantoic membrane. However, siastatin B was a stronger inhibitor of sialidases prepared from streptomyces and rat organs than was siastatin A. Siastatin B also inhibited β-glucuronidase and N-acetyl-β-D-glucosaminidase, two enzymes unaffected by siastatin A.

Sialidase (N-acetyl-neuraminate glycohydrolase EC 3.2.1.18) is a glycosidase, hydrolysing sialic acid to yield terminal carbohydrate chains of glycoproteins or glycolipids[1,2]. This enzyme is widely distributed among microorganisms and animal tissues, but its biological function is still open to question. It has been observed that the biological activity of human-luteinizing hormone is markedly reduced when its terminal sialic acid residue is removed either enzymatically or chemically[3]. It is also known that desialoglycoprotein rapidly disappears from circulating blood during passage through the liver[4,5]. Additional reports have suggested roles for sialidase in various biological functions such as immune response[6,7], oncogenesis[8], metastasis of tumor[9], sperm penetration to rabbit ova[10] and viral infection[11~13].

As reported in this paper, sialidases, depending on source, differ in specificity to inhibitors. It was suggested also that inhibitors that are specific to individual sialocompounds might be useful in the analysis of the physiological role of this enzyme. Thus, we searched for new sialidase inhibitors in culture filtrates of microorganisms. In this communication we describe the isolation, purification and characterization of the inhibitors, siastatins A and B.

Materials and Methods

The source of siastatins. A strain of streptomyces (MB695-A4) found to produce sialidase inhibitors was classified as *Streptomyces verticillus* var. *quintum*. This organism was grown with vigorous shaking at 27°C in liquid medium of the following composition: 1.0 % potato starch, 1.0 % glucose, 0.75 % meat extract, 0.75 % Polypeptone, 0.3 % NaCl, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.0007 % $CuSO_4 \cdot 5H_2O$, 0.0001 % $FeSO_4 \cdot 7H_2O$, 0.0008 % $MnCl_2 \cdot 4H_2O$, 0.0002 % $ZnSO_4 \cdot 7H_2O$. Production of inhibitor reached a plateau after 3-day cultivation.

Enzymes. Sialidases of *Clostridium perfringens* and *Streptomyces* were purified by the methods described by CASSIDY *et al.*[14] and KUNIMOTO *et al.*[15] Sialidases of rat mammary gland, brain and liver were prepared by the method of TAHA *et al.*[16] from the appropriate organs of 12-days-lactating rats. Sialidase of *Vibrio cholerae* (500 units per ml) and β-glucuronidase (70,000~100,000 units per g) of bovine liver were purchased from General Biochemicals,

U.S.A. and Nutritional Biochem. Co., U.S.A., respectively. N-Acetyl-β-D-glucosaminidase from equine kidney was prepared by the method of SEYAMA[17]. The A/Aichi/2/68 (H3N2) strain of influenza virus and the SATO strain of Newcastle disease virus (NDV) were propagated in embryonated eggs. Viruses in the chorioallantoic fluids were concentrated by differential centrifugation and purified by 10~50 % linear sucrose gradient centrifugation[18~20]. The virus particles thus obtained were employed to test the action of inhibitors against viral sialidases.

Substrates. Bovine sialyllactose (BSL) was prepared from cow colostrum according to the method of KUHN *et al.*[21] Phenolphthalein glucuronic acid, 4-nitrophenyl-N-acetyl-β-D-glucosaminide, bovine albumin and fetuin were purchased from Sigma Chemical Co., Ltd., U.S.A., BDH Chemical Ltd., England, Daiichi Pure Chemicals Co., Ltd., Japan and Gland Island Biological Co., U.S.A., respectively.

Materials employed for extraction and purification. Amberlite IR-120, Dowex 1×2 and Dowex 50W×2 were purchased from Rohm and Haas Co., and Dow Chemical Co., U.S.A., and activated carbon, silica gel (Wakogel C-200) and microcrystalline cellulose (Avicel) were obtained from Wako Junyaku and Asahi Kasei Co., Japan, respectively.

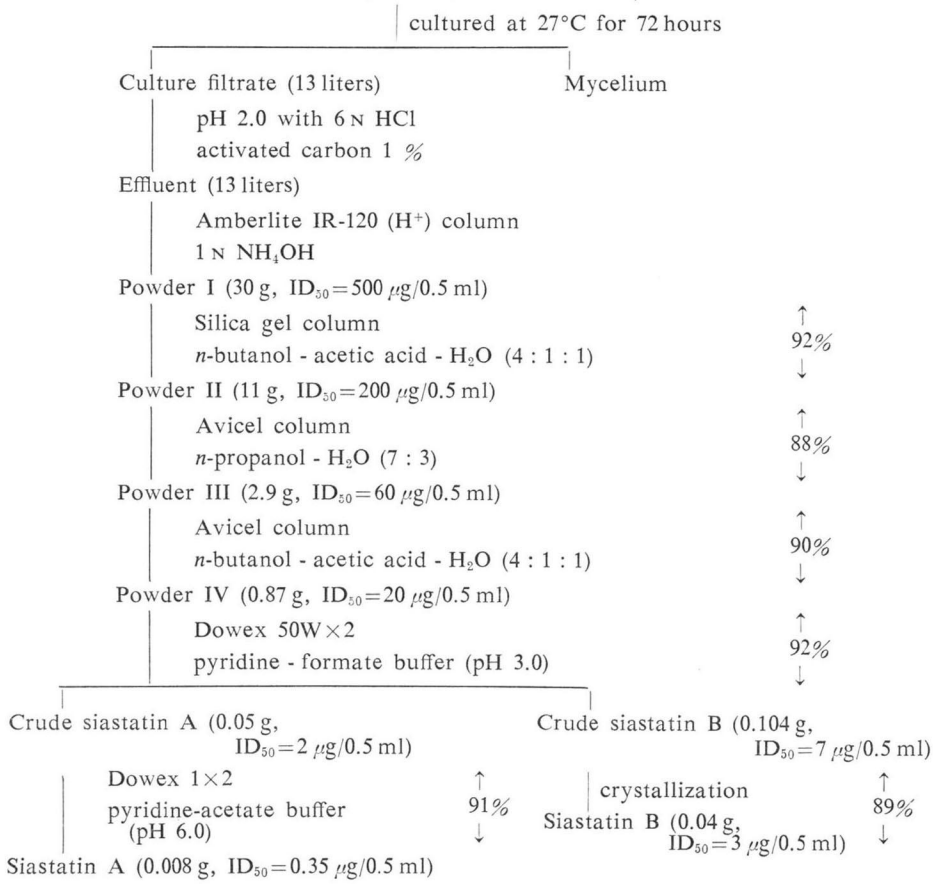
Determination of enzymatic activity. Unless otherwise noted, the sialidase prepared from *Cl. perfringens* was used for determination of concentrations of siastatins. The activities of sialidases with the exception of the enzyme from *V. cholerae* were measured by the following method: A reaction mixture consisting of 0.1 ml of BSL (0.16 μ mole), 0.25 ml of 0.1 M sodium phosphate buffer (pH 6.0), 0.1 ml of distilled water with or without the test material was incubated for 3 minutes at 37°C and then 0.05 ml of enzyme preparation containing 0.8 enzyme unit was added. After 30 minutes at 37°C, the released N-acetylneuraminic acid (NANA) was measured by the method described previously[20,22,23]. In the case of sialidase from *V. cholerae*, a reaction mixture containing 0.05 ml of fetuin (450 μg), 0.25 ml of 0.1 M sodium phosphate buffer (pH 6.0), 0.05 ml of calcium chloride (0.5 μ moles), 0.05 ml of bovine albumin (100 μg), and 0.05 ml of distilled water with or without a test material was incubated for 3 minutes at 37°C and then 0.05 ml of enzyme solution was added. After 30 minutes at 37°C, the released NANA was measured by the method described above. The inhibition of β-glucuronidase was determined as follows: A reaction mixture consisting of 0.01 ml of 0.01 M phenolphthalein-glucuronic acid, 0.1 ml of 0.2 M sodium acetate (pH 5.0), 0.07 ml of distilled water with or without the test material was incubated for 3 minutes at 37°C and then 0.02 ml of β-glucuronidase (5 mg/ml in the same buffer) was added. After 1 hour at 37°C, the reaction was terminated by the addition of 0.5 M glycine-NaOH buffer (pH 10.5) and the reaction mixture centrifuged at 3,000 rpm for 5 minutes. The concentration of released phenolphthalein in the supernatant was measured at 550 nm. In the case of N-acetyl-β-D-glucosaminidase, a reaction mixture consisting of 0.05 ml of 0.025 M phenyl-N-acetyl-β-D-glucosaminide, 0.3 ml of 0.1 M sodium citrate (pH 4.5), 0.1 ml of distilled water with or without the test material was incubated for 3 minutes at 37°C and then 0.05 ml of enzyme solution was added. After 30 minutes at 37°C, the reaction was terminated by the addition of reagents A and B as described by ASP[24]. The extent of the reaction was measured at 510 nm.

Results

Purification of Siastatins

The purification procedures with step-wise yields are shown in Fig. 1. After 72-hour incubation at 27°C, the cultured broth was separated from the mycelium by centrifugation. The pH of the supernatant fraction was adjusted to pH 2.0 with 6 N HCl and then activated carbon (150 g) was added with stirring. After 30 minutes at room temperature, the carbon was separated by filtration. The filtrate was passed through a column of Amberlite IR-120 (5.5×37 cm). The adsorbed siastatins were eluted with 1 N NH_4OH, and the active eluate was evaporated under reduced pressure (powder I). Powder I thus obtained was subjected to

Fig. 1. Isolation and purification of siastatins



silica gel column chromatography (5.5×37 cm), using *n*-butanol - acetic acid - water (4 : 1 : 1) as the solvent. The active fraction was evaporated under reduced pressure (powder II). Powder II was purified through Avicel column (4×60 cm), using *n*-propanol - water (7 : 3) as the solvent and active fractions were evaporated to dryness (powder III). Powder III was subjected again to Avicel column (4×60 cm) using *n*-butanol - acetic acid - water (4 : 1 : 1) as the solvent and active fractions were dried by evaporation (powder IV). Powder IV was dissolved in 30 ml of 1 % (v/v) formic acid aqueous solution and subjected to Dowex 50W×2 (pyridine form, 200~400 mesh, 3×30 cm) column chromatography with a linear gradient between 1.5 % pyridine-formic acid buffer (v/v, pH 3.0, 300 ml) and 2.5 % pyridine-formic acid buffer (v/v, pH 3.0, 300 ml). As shown in Fig. 2, two active peaks were obtained. The active agent in the first peak

Fig. 2. Elution pattern of siastatins on Dowex 50W×2 column chromatography (Activity in inhibiting *Clostridium* sialidase)

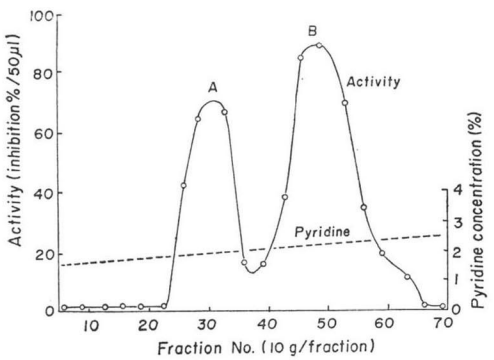
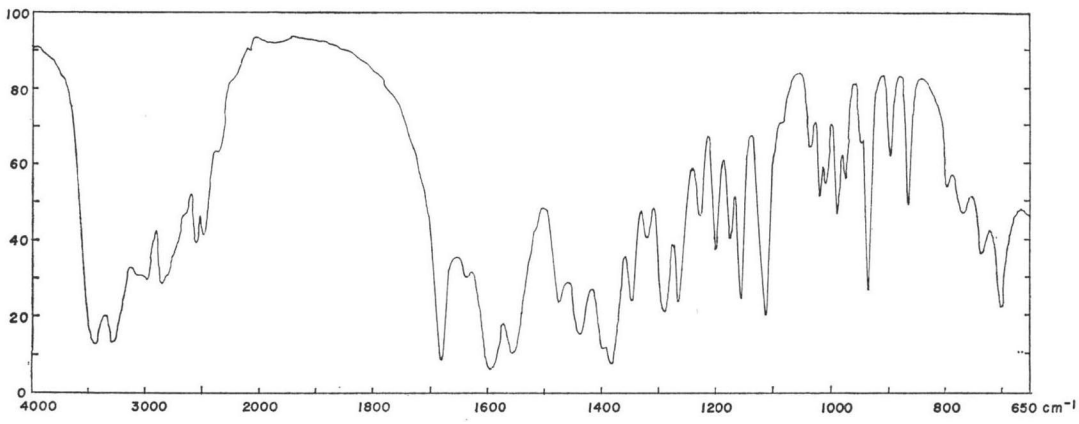


Fig. 3. Infrared absorption spectrum of siastatin B (KBr)



was named siastatin A and that in the second peak was named siastatin B. Active fractions from each peak were pooled and dried. Further purification of siastatin A was carried out on Dowex 1×2 (acetate form, 200~400 mesh, 15×20 cm) column using a linear gradient between 8 % pyridine-acetic acid buffer (v/v, pH 6.0, 300 ml) and 20 % pyridine-acetic acid buffer (v/v, pH 6.0, 300 ml). Active fractions so obtained were pooled and dried. Siastatin B was dissolved in a small amount of water and crystallized by adding methanol dropwise. Colorless needle crystals of siastatin B were obtained.

Chemical Properties

Properties of siastatin B were as follows: m.p. 137°C (dec.); $[\alpha]_D^{22}+57.2°$ (*c* 1.0, H_2O); pKá 3.27, 6.86 (titration equivalent 240); no maximum at 210~400 nm with the end absorption. The IR spectrum is shown in Fig. 3. The elemental analysis was as follows: calcd. for $C_8H_{14}N_2O_5 \cdot H_2O$: C 40.67, H 6.83, N 11.86, O 40.46; found: C 40.77, H 6.88, N 11.48, O 40.86. Siastatin B gave a positive reaction for ninhydrin, nitroprusside-acetaldehyde and RYDON-SMITH. The inhibitor is soluble in water and insoluble in methanol, ethanol, butanol, acetone, ethylacetate, chloroform, ethylether, petroleum ether, benzene, hexane, acetic acid and pyridine. Siastatins A and B gave the following Rf values on silica gel thin-layer chromatography: 0.16 and 0.16 with *n*-butanol - acetic acid - water (4 : 1 : 1), 0.23 and 0.23 with ethyl acetate - pyridine - acetic acid - water (5 : 5 : 1 : 3). Siastatins A and B moved to the cathode in formic acid - acetic acid - water (25 : 75 : 900, pH 1.9) on high voltage paper electrophoresis at 3,500 V for 15 minutes with an Rm value of 0.72 and 0.89 respectively, referring to L-alanine as 1.0. The structure of siastatin B was determined as 2 (S/R)-acetamido-3 (S/R), 4 (R/S)-dihydroxypiperidine-5 (R/S)-carboxylic acid. The structure study will be reported in the next paper. Chemical studies on siastatin A are now in progress.

Inhibitory Effect of Siastatins on Sialidases

The inhibitory effects of siastatins A and B varied with enzyme source and with inhibitor as summarized in Table 1. Siastatin A was found to be much the more potent inhibitor against the sialidases from *Cl. perfringens* and chorioallantoic membrane with no other demonstrable activity other than that from the Streptomyces. In contrast siastatin B, while

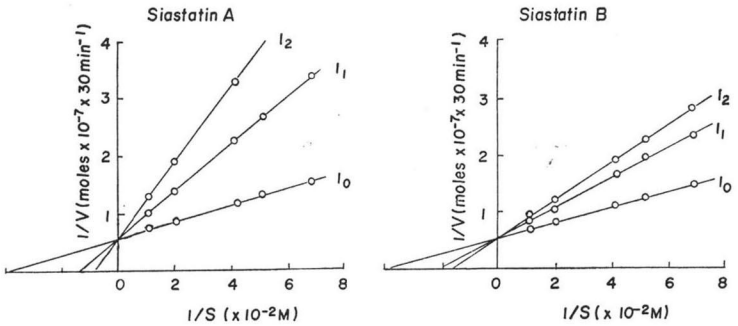
Table 1. Effect of siastatins A and B against sialidases, β-glucuronidase and N-acetyl-β-D-glucosaminidase

Enzymes		ID_{50} (μg/0.5 ml)	
		Siastatin A	Siastatin B
Sialidases	*Cl. perfringens*	0.35	3
	Streptomyces	360	10
	V. cholerae	>250	>250
	Rat mammary gland	>500	110
	〃 brain	>500	400
	〃 liver	>500	170
	Chorioallantoic membrane	1.7	55
	Inf. Aichi[1)]	>250	>250
	NDV Sato[2)]	>250	>250
Others	β-Glucuronidase	>250	4
	N-Acetyl-β-D-glucosaminidase	>250	18

1) A/Aichi/2/68 (H3N2) strain of influenza virus
2) Sato strain of Newcastle disease virus

Fig. 4. Competitive inhibition of the sialidase from *Cl. perfringens* by siastatins A and B

Substrate was BSL and the sialidase concentration was 75 ng of protein. The concentrations of siastatin A: I_0, no inhibitor; I_1, 0.5 μg; I_2, 1 μg. The concentration of siastatin B: I_0, no inhibitor; I_1, 1.7×10^{-5} M; I_2, 2.5×10^{-5} M. The determination of the inhibitory activity was carried out as described in the text. However, in this case the reaction system contained 100 μg of bovine albumin to stabilize the purified enzyme and the reaction time was 30 minutes at 37°C.



much less inhibitory to the two first enzyme preparations, demonstrated activity against a broader range of sialidases, albeit at a lower order of activity. Neither siastatin A nor siastatin B inhibited sialidases from the two viruses and *V. cholerae*.

Table 2. Km and Ki values of siastatin B on sialidases of *Clostridium perfringens* and *Streptomyces*

Enzymes	$Ki\times10^{-5}$ M	$Km\times10^{-3}$ M
Cl. perfringens	1.7	2.5
Streptomyces	4.3	0.57

Kinetic studies were carried out on the effects of siastatins A and B on sialidases from *Cl. perfringens* and *Streptomyces*. LINEWEAVER-BURK plots of the results are shown in Fig. 4. The action of both inhibitors was in competition with the substrate (BSL). *Km* and *Ki* values are given in Table 2.

The enzymatic activities of β-glucuronidase and N-acetyl-β-D-glucosaminidase were measured by colorimetry using phenolphthalein glucuronic acid and 4-nitrophenyl-N-acetyl-β-D-glucosaminide as substrates, respectively. As reported in Table 1, siastatin B strongly inhibited both enzymes but siastatin A showed no inhibition even at the concentration of 500 μg/ml. These results further support the more specific action of siastatin A.

Biological Activities of Siastatin B

Siastatins A and B at 100 μg/ml demonstrated no inhibition of the growth of Gram-positive and Gram-negative bacteria in a nutrient medium. Both siastatins A and B had relatively low toxicity. Intravenous injection of 125 mg/kg of either siastatin was not lethal to mice.

Discussion

To date only a limited number of aromatic compounds have been demonstrated to have an inhibitory effect on viral sialidase[25~27]. 2-Desoxy-2, 3-dehydro-N-acyl-neuraminic acid, the only one example of a non-aromatic compound possessing this property, has a *Ki* of 1×10^{-5} M for the sialidase from *V. cholerae*[28]. The mode of inhibition of the last compound is substrate-competitive.

The structure of siastatin B differs from those of previously reported inhibitory compounds while the structure of siastatin A is not yet known. The difference in the inhibitory properties of the two siastatins, undoubtedly a function of differences in chemical structures, are of interests. With respect to the sialidases prepared from *Cl. perfringens* and chorioallantoic membrane, siastatin A is more effective (ID_{50}= 0.35 μg/0.5 ml and 1.7 μg/0.5 ml) than is siastatin B (ID_{50}= 3 μg/0.5 ml and 55 μg/0.5 ml). In contrast, when *Streptomyces* and mammalian sialidases are employed, the comparative inhibitory effects are reversed with siastatin B being more potent than siastatin A (Table 1). Furthermore, β-glucuronidase and N-acetyl-β-D-glucosaminidase are also inhibited by siastatin B while siastatin A had no effect on these enzymes. These and other differences in the inhibitory effects of siastatins A and B may be useful in discriminating amongst various types of sialidases. The chemical structure of siastatin B and its mechanism of action against mammalian sialidase will be reported in following papers.

References

1) GOTTSHALK, K.: The chemistry and biology of sialic acid and related substances. pp. 75~87, Cambridge Univ. Press, London, 1960
2) ROSEMAN, S.: The synthesis of complex carbohydrates of multiglycosyl-transferase systems and their potential function in intercellular adhesion. Chem. Phys. Lipids 5: 270~297, 1970
3) DUFAU, M. L.; K. J. CATT & T. TSURUHARA: Retention of *in vitro* biological activities by desialylated human luteinizing hormone and chorionic gonadotropin. Biochem. Biophys. Res. Commun. 44: 1022~1029, 1971
4) NELSESTUEN, G. L. & J. W. SUTTIE: Properties of asialo and aglycoprothrombin. Biochem. Biophys. Res. Commun. 45: 198~203, 1971
5) MORELL, A. G.; G. GREGORIADIS & I. H. SCHEINBERG: The role of sialic acid in determining the survival of glycoprotein in the circulation. J. Biol. Chem. 246: 1461~1467, 1971
6) SIMONS, R. L. & A. RIOS: Differential effect of neuraminidase on the immunogenicity of viral associated and private antigens of mammary carcinomas. J. Immunol. 111: 1820~1825, 1973
7) ROTT, R.; H. BECHT & M. ORLICH: The significance of influenza virus neuraminidase in immunity. J. Gen. Virol. 22: 35~41, 1974
8) BEKESI, J. G.; G. ST-ARNEAULT & J. F. HOLLAND: Increase of leukemia L1210 immunogenicity by *Vibrio cholerae* neuraminidase treatment. Cancer Res. 31: 2130~2132, 1971

9) GASIC, G. & T. GASIC: Removal of sialic acid from the cell coat in tumor cells and vascular endothelium, and its effects on metastasis. Proc. Nat. Acad. Sci. U.S.A. 48: 1172~1177, 1962
10) SOUPART, P. S. & T. H. CLEWE: Sperm penetration of rabbit zona pellucida inhibited by treatment of ova with neuraminidase. Fert. Steril. 16: 677~689, 1965
11) ACKERMANN, W. W.; N. ISHIDA & H. F. MASSAB: Growth characteristic of influenza virus concerning the binding of virus by host cells. J. Exptl. Med. 102: 545~554, 1955
12) HAFF, R. F. & R. C. STEWART: Role of sialic acid receptors in absorption of influenza virus to chick embryo cells. J. Immunol. 94: 842~851, 1965
13) LIPKIND, M. A. & I. V. TSVETKOVA: Studies on the role of myxovirus neuraminidase in virus-cell receptor interaction by means of direct determination of sialic acid split from cells. II. Establishment of the method on myxovirus-chick embryo cell monolayer system. Arch. Ges. Virusforsch. 29: 370~380,1970
14) CASSIDY, J. T.; G. W. JOURDIAN & S. ROSEMAN: The sialic acids. VI. Purification and properties of sialidase from *Clostridium perfringens*. J. Biol. Chem. 240: 3501~3506, 1965
15) KUNIMOTO, S.; T. AOYAGI, T. TAKEUCHI & H. UMEZAWA: Purification and characterization of streptomyces sialidases. J. Bact. in press
16) TAHA, B. H & R. CARUBELLI: Mammalian neuraminidase: Intracellular distribution and changes of enzyme activity during lactation. Arch. Biochem. Biophys. 119: 55~61, 1967
17) SEYAMA, Y. & T. YAMAKAWA: Multiple components of β-N-acetylhexosaminidase from equine kidney: Their action on glycolipids and allied oligosaccharides. J. Biochem. 75: 495~507, 1974
18) NOLL, H.; T. AOYAGI & J. ORLANDO: The structural relationship of sialidase to the influenza virus surface. Virology 18: 154~157, 1962
19) NEROME, K.; M. KUMAGAI & T. AOYAGI: Effect of panosialin on myxoviruses. Arch. Ges. Virusforsch. 39: 353~359, 1972
20) AOYAGI, T.; J. SUZUKI, K. NEROME, R. NISHIZAWA, T. TAKEUCHI & H. UMEZAWA: Sialic acid residues exposed on mammalian cell surface: The effect of adsorption of denatured virus particles. Biochem. Biophys. Res. Commun. 57: 271~278, 1974
21) KUHN, R. & R. BROSSMER: Über O-Acetyl-lactaminsäure-lactose aus Kuhn-colostrum und ihre Spaltbarkeit durch Influenza-Virus. Chem. Ber. 89: 2013~2019, 1956
22) AOYAGI, T.; M. YAGISAWA, M. KUMAGAI, M. HAMADA, Y. OKAMI, T. TAKEUCHI & H. UMEZAWA: An enzyme inhibitor, panosialin, produced by *Streptomyces*. I. Biological activity, isolation and characterization of panosialin. J. Antibiotics 24: 860~869, 1971
23) WARREN, L.: The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234: 1971~1975, 1959
24) ASP, N. G.: Improved method for the assay of phenylglycosidase activity with a 4-aminoantipyrine reagent. Anal. Biochem. 40: 281~286, 1971
25) EDMOND, J. D.; R. G. JOHNSON, D. KIDD, H. J. RYLANCE & R. G. SOMMERVILLE: The inhibition of neuraminidase and antiviral action. Brit. J. Pharmac. Chemother. 27: 415~426, 1966
26) TUTE, M. S.; K. W. BRAMMER, B. B. KAYE & R. W. BROADBENT: The inhibition of viral neuraminidase by 1-phenoxymethyl-3,4-dihydroisoquinolines. I. Steric effects. J. Med. Chem. 13: 44~48, 1970
27) HASKELL, T. H.; F. E. PETERSON, D. WATSON, N. R. PLESSAS & T. CULBERTSON: Neuraminidase inhibition and viral chemotherapy. J. Med. Chem. 13: 697~704, 1970
28) MINDL, V. P. & H. TUPPY: Kompetitive Hemmung der Vibrio-cholerae-neuraminidase durch 2-Desoxy-2,3-dehydro-N-acyl-neuraminsäuren. Hoppe-Seyler's Z. Physiol. Chem. 350: 1088~1092, 1969