SUBSTRATES AND INHIBITORS OF NEURAMINIDASES

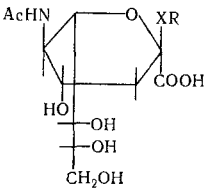
COMMUNICATION 1. SYNTHESES OF O-, S-, AND N-KETOSIDES OF N-ACETYL-D-NEURAMINIC ACID*

I. M. Privalova and A. Ya. Khorlin

UDC 542.978+542.91+547.455

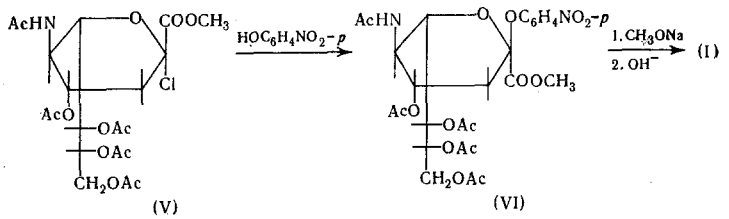
The specific activity of numerous sialo-containing oligo- and polysaccharides, glycolipids, glycopeptides, and mixed biopolymers, which perform important biological functions in living cells, is determined by the presence of terminal neuraminic acid residues and is lost as a result of their elimination by neuraminidases which specifically cleave α-ketoside bonds of sialic acids [1-3]. Neuraminidases have been detected in animal tissues [4-6], in pathogenic microorganisms [7], in myxoviruses [7], and in particular, in various strains of influenza virus [8]. The important biological role played by these enzymes, both in the normal state and in pathology, has been established. Therefore investigations directed toward determining the mechanism of the enzyme–substrate interaction of neuraminidases, toward a study of the limits of their specificity, and toward a search for specific inhibitors and activators of neuraminidases of various origin, are taking on vital importance.

In this communication we describe the synthesis of 2-O-p-nitrophenyl-N-acetyl-α-D-neuraminic acid (I), 2-deoxy-2-methylthio-N-acetyl-α-D-neuraminic acid (II), 2-deoxy-2-p-nitrophenylthio-N-acetyl-α-D-neuraminic acid (III), and 2-deoxy-2-p-nitrophenylamino-N-acetyl-α-D-neuraminic acid (IV); the interaction of these neuraminosides with neuraminidase of the cholera vibrio was studied, data characterizing their interaction with neuraminidases of influenza viruses were obtained.



(I) R=p-$O_2NC_6H_4$—, X=O (III) R=—$O_2NC_6H_4$—, X=S
(II) R=CH_3, X=S (IV) R=$O_2NC_6H_4$—, X=NH

The O-ketoside (I) was synthesized by glycosylation of p-nitrophenol with the methyl ester of acetochloroneuraminic acid (V), followed by removal of the protective groups in the acetate of the methyl ester of the ketoside (VI) formed:



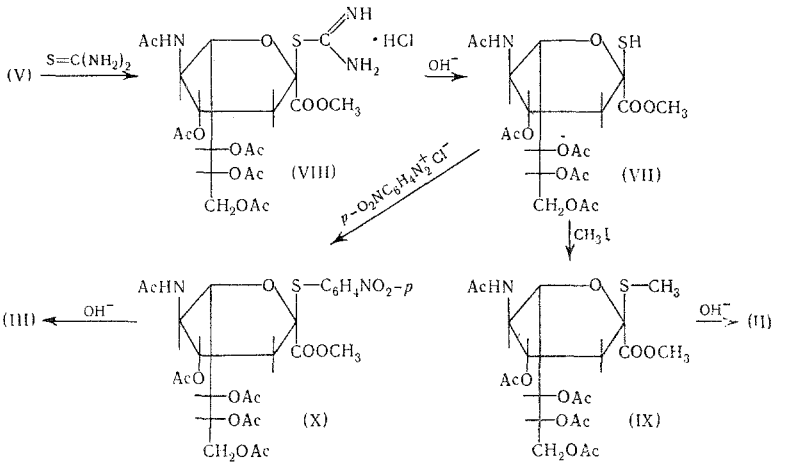
(V) (VI)

*The article is published on the basis of a resolution of the Conference of Editors-in-Chief of the Journals of the Academy of Sciences of the USSR from July 12, 1962, as the dissertation work of I. M. Privalova.

Institute of the Chemistry of Natural Compounds, Academy of Sciences of the USSR. Translated from Izvestiya Akademii Nauk SSSR, Seriya Khimicheskaya, No. 12, pp. 2785-2792, December, 1969. Original article submitted April 16, 1969.

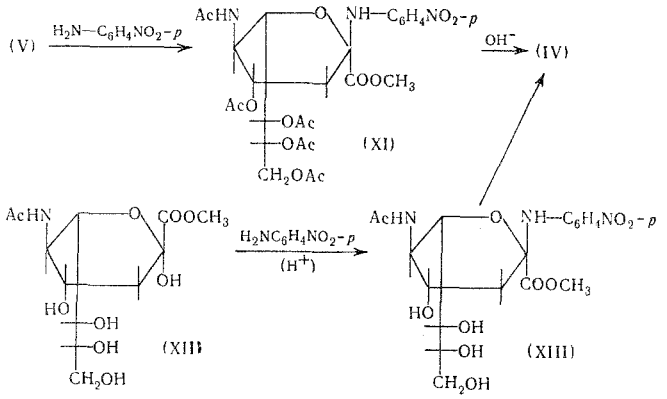
Glycosylation of p-nitrophenol was conducted in chloroform at room temperature in the presence of Ag_2CO_3 as an HCl acceptor and Drierite. These reaction conditions, approaching the usual conditions of the Königs–Knorr synthesis, differed from those described earlier for the synthesis of O-neuraminosides [9], in which the step of glycosylation was accomplished by alcoholysis of acetochloroneuraminic acid in a medium of the aglycone component. Therefore the results that we obtained indicate the theorectical possibility of the synthesis of neuraminosides with a structurally complex aglycone, in particular, oligosaccharides with a terminal residue of neuraminic acid.

As the key compound in the syntheses of S-ketosides (II) and (III) we used the methyl ester of 2-deoxy-4,7,8,9-tetra-O-acetyl-2-thio-N-acetyl-α-D-neuraminic acid (VII), which was produced by the action of thiourea on the chloride (V), followed by hydrolysis of the thiuronium salt (VIII)



Conversion of the thiol (VII) to methyl esters of acetates of thioketosides (IX) and (X) was achieved by alkylation or arylation of (VII). Removal of the protective groups in (IX) and (X) leads to the S-ketosides (II) and (III) sought. The high yields at all stages of synthesis of the S-ketosides (II) and (III) indicate the preparative convenience of the general means of production of thioglycosides (X) as applied to the synthesis of thioneuraminosides.

The synthesis of the N-ketoside (IV) was conducted in two ways. In the first place, by the action of p-nitroaniline on the chloride (V), followed by elimination of the protective group in the methyl ester of the acetate (XI). In the second place, by the interaction of the methyl ester of N-acetyl-D-neuraminic acid (XII) with p-nitroaniline, followed by saponification of the methyl ester of the α-N-ketoside (XIII) formed to the N-neuraminoside (IV)



The structure of the O-, S-, and N-ketosides (I)-(IV) obtained was confirmed not only by the data of their elemental analysis, but also as follows. As a result of the acid hydrolysis of the ketosides (I)-(IV), N-acetyl-D-neuraminic acid and the corresponding aglycone, identified by paper chromatography or thin-layer chromatography on silica gel, are formed. The values of the molecular rotation of the ketosides (I)-(IV) indicate an α-configuration of the ketoside bond in these compounds. Moreover, in the case of O-p-nitrophenylneuraminoside (I), the α-configuration of the ketoside bond is confirmed by hydrolytic cleavage

TABLE 1. Constants of Inhibition (K_I) of the Enzymatic Hydrolysis of the O-Ketoside (I) by Cholera Vibrio Neuraminidase for N-Acetylneuraminic Acid and Its Derivatives

Compound	$K_I \cdot 10^3$, M
N-Acetyl-D-neuraminic acid	4.88
2-Deoxy-2-p-nitrophenylamino-N-acetyl-α-D-neuraminic acid (IV)	2.34
2-Deoxy-2-methylthio-N-acetyl-α-D-neuraminic acid (II)	0.84
2-Deoxy-2-p-nitrophenylthio-N-acetyl-α-D-neuraminic acid (III)	0.23

of it by neuraminidases of cholera vibrio and influenza virus. The S- and N-ketosides (II)-(IV) are practically not cleaved by the neuraminidase of the cholera vibrio and are only slowly hydrolyzed in the presence of the neuraminidase of influenza virus. And yet, the S- and N-ketosides (II)-(IV) are active competitive inhibitors of neuraminidases (see below), which, taking into consideration the rigid requirements of specificity of glycosidases with respect to the configuration of the glycoside center, should be considered as supplementary evidence of an α-configuration of the neuraminoside bond in compounds (II)-(IV).

Usually preparations of neuraminyllactoses are used as a standard natural substrate in the study of the activity of neuraminidases [11]. The method of kinetic measurements in this case, based on a quantitative determination of the neuraminic acid eliminated, is marked by complexity and laboriousness [12]. Recently a number of synthetic substrates of neuraminidases were proposed [9], of which only O-phenyl-N-acetyl-α-D-neuraminoside permits the course of enzymatic hydrolysis to be followed according to the phenol formed [13]. Since the methods of quantitative determination of p-nitrophenol [14] are simpler than the determination of phenol [15], the 2-O-nitrophenyl-N-acetyl-α-D-neuraminoside (I) that we synthesized is an extremely convenient synthetic substrate in kinetic investigations of neuraminosidases. At a substrate concentration of $1.17 \cdot 10^{-3}$ M, the rate of enzymatic hydrolysis, determined by the amount of p-nitrophenols formed in 30 min of incubation, is proportional to the concentration of the enzyme (0.1-0.5 mg/ml) within sufficiently wide limits. The dependence of the enzymatic hydrolysis of the O-ketoside (I) on the time at a constant concentration of the enzyme is presented in Fig. 1 for various substrate concentrations.

The dependence of the initial rate of enzymatic hydrolysis, determined by extrapolation to zero time according to the Newton–Gregory method [16], entirely corresponds to the conditions of Michaelis–Menten equilibrium kinetics, and presented graphically according to Lineweaver–Burk [17], permits a determination of the values $K_m = 1.67 \cdot 10^{-3}$ M and $V_m = 5.3 \cdot 10^{-5}$ mole/liter·min for the O-ketoside (I) (Fig. 2).

The use of enzymatic hydrolysis of the O-ketoside (I) as a background reaction for studying the inhibiting effect of S- and N-ketosides (II)-(IV) and of neuraminic acid itself permitted indentification of the competitive nature of the inhibiting action of these compounds in all cases (see Fig. 2) and a determination of the values of the inhibition constants for them (Table 1).

These values were close to the value of K_m for the O-ketoside (I) or less than it, and therefore we can consider the constants of binding of the inhibitors close to the constant of binding of the substrate (I).

An explanation for the differences in the catalytic action of cholera vibrio neuraminidase on O-neuraminoside (I), on the one hand, and on S-neuraminosides (II) and (III), on the other, should apparently be sought in the relatively high nucleophilicity of the proton–donor catalytic group of the enzyme, which participates in the reaction of coupled acid–base catalysis. The possibility remains that such a group in neuraminidase, inert with respect to the S-neuraminoside bonds, as in the case of β-galactosidase of E. coli, strain ML309, which does not cleave β-thiogalactosides [18], is the S–H-group of the cysteine residue. Thus, the data obtained in this communication show that p-nitrophenyl-N-acetyl-α-D-neuraminoside (I), synthesized according to the Königs–Knorr reaction, is a convenient and readily available substrate in kinetic investigations of neuraminidases. The synthesized S- and N-ketosides (II)-(IV) represent competitive inhibitors of the enzymatic hydrolysis of the O-ketoside (I). Since the S- and N-ketosides (II)-(IV) are distinguished by stability to the hydrolytic action of various neuraminidases, their behavior in the enzymatic reaction can serve as a supplementary characteristic of the specific activity of neuraminidases of various origins.

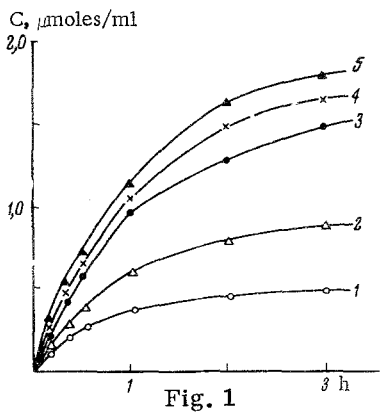


Fig. 1

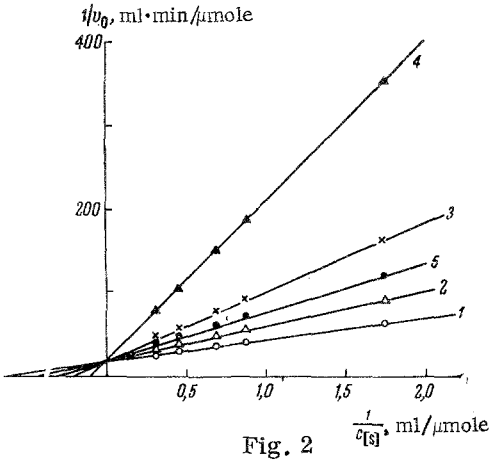


Fig. 2

Fig. 1. Hydrolytic cleavage of the O-ketoside (I) by neuraminidase of the cholera vibrio ([E] = 0.3 mg/ml) in 0.1 M acetate buffer (pH 5.6), containing 0.9% NaCl and 0.1% $CaCl_2$ at 37°. Concentration of the substrate (I) (M): 1) $0.58 \cdot 10^{-3}$; 2) $1.17 \cdot 10^{-3}$; 3) $1.75 \cdot 10^{-3}$; 4) $2.3 \cdot 10^{-3}$; 5) $3.5 \cdot 10^{-3}$.

Fig. 2. Dependence of the initial rate of enzymatic hydrolysis of the O-ketoside (I) on its concentration in the absence (1) and in the presence of an inhibitor ([I] = $1.1 \cdot 10^{-3}$ M): 2) N-acetyl-D-neuraminic acid; 3) S-ketoside (II); 4) S-ketoside (III); 5) N-ketoside (IV). For conditions of hydrolysis see Fig. 1.

EXPERIMENTAL

Descending paper chromatography on Whatman No. 3 chromatographic paper in the solvent system (by volume): n-propanol–0.1 N HCl–n-butanol, 2 : 1 : 1 (A), was used. Chromatography in a thin fixed layer of silica gel (KSK, 150-200 mesh) in the systems chloroform–methanol, 92:8 (B), n-propanol–water, 7 : 3 (C), and ether (D) was conducted. The spots were detected with aniline phthalate, with Svennerholm's reagent, with an ammonia solution of silver nitrate, and with sodium methylate in methanol. The melting point (corrected) was determined on a Kofler block. The solvents were evaporated under vacuum at a temperature no higher than 35°.

Methyl Ester of N-Acetyl-D-neuraminic Acid (XII). The ester was produced according to the method of [19], yield 95%, mp 179-180° (from methanol–ether), $[\alpha]_D^{20}$ –26.0° (C 1; water), R_f 0.62 (C). According to the literature data: mp 179-180°, $[\alpha]_D^{20}$ –28° (C 1; water).

Methyl Ester of Acetochloroneuraminic Acid (V). (V) was produced according to [19], yield 90%; $[\alpha]_D^{20}$ –65.6° (C 1; $CHCl_3$), R_f 0.67 (B). According to the literature data: $[\alpha]_D^{20}$ –63° (C 1; $CHCl_3$).

Methyl Ester of 2-Deoxy-2-p-nitrophenyl-4,7,8,9-tetra-O-acetyl-N-acetyl-α-D-neuraminic Acid (VI). To a suspension of 250 mg p-nitrophenol, 100 mg freshly prepared Ag_2CO_3, and 500 mg Drierite in 3 ml abs. $CHCl_3$ we added 200 mg of the methyl ester of acetochloroneuraminic acid (V), mixed for 3 h at room temperature, diluted to 10 ml with $CHCl_3$, and filtered through celite. The filtrate was concentrated to a small volume, and the residue chromatographed on silica gel (column 2 × 18 cm). p-Nitrophenol was eluted with ether, the product (VI) was eluted with a mixture of $CHCl_3$–methanol, 1 : 1. The eluate, containing pure (VI), was evaporated and the residue dried at 40° (1 mm). The yield of (VI) was 124 mg (51%), colorless powder, chromatographically homogeneous, R_f 0.36 (D), 0.53 (B), $[\alpha]_D^{20}$ +4.2° (C 1.05; methanol). Found: C 50.88; H 5.78; N 4.38%. $C_{26}H_{32}N_2O_{15}$. Calculated: C 50.98; H 5.27; N 4.57%.

Methyl Ester of 2-Deoxy-2-methylthio-4,7,8,9-tetra-O-acetyl-N-acetyl-α-D-neuraminic Acid (IX). To a suspension of 100 mg thiourea in 5 ml anhydrous acetone we added 500 mg of the freshly prepared methyl ester of acetochloroneuraminic acid (V) and boiled for 30 min. The solution obtained was left overnight at 0°; the precipitate of thiourea formed (R_f 0.21, B, detection with $AgNO_3$) was filtered off, the filtrate evaporated, 10 ml of $CHCl_3$ added to the residue, the precipitate of thiourea again filtered off, and the mother liquor evaporated to dryness. We obtained 509 mg (88.5%) (VIII), R_f 0.58 (B). To a solution of 500 mg of the thiuronium salt (VIII) in 5 ml of acetone we added 3 ml of a saturated aqueous solution of K_2CO_3; after 20 min 4.5 ml of CH_3I was added. The mixture was mixed for 2.5 h at 20°, poured out into 20 ml of ice water, mixed for 30 min, and extracted with $CHCl_3$. The extracts were dried with K_2CO_3 and

TABLE 2. Ketosides of N-Acetyl-α-D-neuraminic Acid

Compound	Yield, %	Found, %			Empirical formula	Calculated, %			$[\alpha]_D$ (C 1; methanol)	$[M]_D$	R_f (B)
		C	H	N		C	H	N			
2-O-p-Nitrophenyl-N-acetyl-α-D-neuraminic acid (I)	92	47.70	4.96	6.38	$C_{17}H_{22}N_2O_{11}$	47.44	5.11	6.51	−1.33°	−5.76°	0.64
2-Deoxy-2-p-nitrophenylamino-N-acetyl-α-D-neuraminic acid (IV)	89	48.00	5.26	9.82	$C_{17}H_{23}N_3O_{10}$	47.55	5.40	9.79	0°	0°	0.66
2-Deoxy-2-methylthio-N-acetyl-α-D-neuraminic acid (II)	90	42.49	6.69	3.81	$C_{12}H_{21}NO_8S$	42.48	6.24	4.15	+ 1.6°	+ 5.41°	0.60
2-Deoxy-2-p-nitrophenylthio-N-acetyl-α-D-neuraminic acid (III)	75	45.24	4.71	6.09	$C_{17}H_{22}N_2O_{10}S$	45.73	4.97	6.28	+ 1.5°	+ 6.7°	0.55
2-O-Methyl-N-acetyl-α-D-neuraminic acid	–	–	–	–	$C_{12}H_{21}NO_9$	–	–	–	−1° [19]	−3.23°	–
2-O-Methyl-N-acetyl-β-D-neuraminic acid	–	–	–	–	$C_{12}H_{21}NO_9$	–	–	–	−46° [19]	−148.5°	–

evaporated. The residue was dissolved in methanol, decolorized with charcoal, and evaporated. After drying under vacuum we obtained 224 mg (44%) (IX), colorless powder, chromatographically homogeneous, R_f 0.83 (B), $[\alpha]_D^{20}$ +13.1° (C 1.54; $CHCl_3$). Found: C 48.15; H 5.96; N 6.57%. $C_{21}H_{31}NO_{12}S$. Calculated: C 48.36; H 5.97; N 6.15%.

Methyl Ester of 2-Deoxy-2-p-nitrophenylthio-4,7,8,9-tetra-O-acetyl-N-acetyl-α-D-neuraminic Acid (X). A saturated aqueous solution of K_2CO_3 was added to a solution of 880 mg of the thiuronium salt (VIII) in 5 ml of water, the mixture mixed for 1 h at 20°, deionized with Amberlite IR-120 (H^+ form), and evaporated. A solution of p-nitrophenyldiazonium chloride (from 0.46 g of p-nitroaniline) was added to a solution of 760 mg of the residue in 5 ml of methanol with vigorous mixing and cooling of the reaction mass to −10°; after 20 min it was poured out into 30 ml of ice water. The filtrate was extracted with $CHCl_3$ (3 × 30 ml), dried with K_2CO_3, decolorized with charcoal, and evaporated. The residue was chromatographed on a column (2 × 20 cm) with silica gel, eluted with mixtures of $CHCl_3$ → methanol, and the fractions giving a positive color with Ehrlich's reagent were combined and evaporated. We obtained 420 mg (38.6%) (X) in the form of a colorless powder, R_f 0.4 (D), $[\alpha]_D^{20}$ + 5.4° (C 1; MeOH). Found: C 50.09; H 4.87; N 4.90; S 4.61%. $C_{26}H_{32}N_2O_{14}S$. Calculated: C 49.67; H 5.09; N 4.44; S 5.08%.

Methyl Ester of 2-p-Nitrophenylamino-4,7,8,9-tetra-O-acetyl-N-acetyl-α-D-neuraminic Acid (XI). To a suspension of 1.7 g p-nitroaniline and 2.4 g freshly prepared Ag_2CO_3 in a mixture of 5 ml abs. $CHCl_3$ and 5 ml abs. ether we added 1 g of the methyl ester of acetochloroneuraminic acid (V), mixing for 24 h at room temperature. The residue was filtered, the filtrate evaporated, 5 ml of a 1 : 1 methanol–water mixture added, and the mixture left overnight at 0°. On the following day the precipitate of p-nitroaniline was filtered off, the filtrate evaporated, the residue chromatographed on a column (2 × 18 cm) with neutral Al_2O_3 (activity IV), and eluted first with $CHCl_3$, then with a mixture of $CHCl_3$–methanol, 1 : 1. We obtained 510 mg (42%) of (XI) in the form of a light-yellow powder, R_f 0.72 (B), $[\alpha]_D^{20}$ −5.1° (C 0.9; methanol). Found: C 50.92; H 5.12; N 6.77%. $C_{26}H_{33}N_3O_{14}$. Calculated: C 51.06; H 5.43; N 6.87%.

2-Deoxy-2-p-Nitrophenylamino-N-acetyl-α-D-neuraminic Acid (IV). To a solution of 500 mg N-acetyl-D-neuraminic acid and 500 mg p-nitroaniline in 10 ml of abs. methanol we added one drop of conc. HCl and left the mixture overnight at 0°. On the following day the mixture was evaporated, a solution of the residue in 1 ml of methanol chromatographed on a column (2 × 18 cm) with Dowex-1 ($HCOO^-$ form), eluted first with 100 ml of methanol, and then with 250 ml of a mixture of methanol and 0.3 N HCOOH, 1 : 1, collecting the fractions giving a positive color with Ehrlich's reagent, and evaporated. We obtained 420 mg (61%) of (IV), yellowish powder, R_f 0.13 (B), 0.64 (C), $[\alpha]_D^{20}$ −1.1° (C 1; methanol). The product was identical with the N-ketoside obtained in the deionization and subsequent saponification of (XI).

Saponification of (VI, (IX), (X), and (XI). A 0.001 M portion of (VI), (IX), (X), or (XI) was dissolved in 5 ml of abs. methanol, 1.5 ml of 0.1 N CH_3ONa in methanol was added, and the mixture kept for 3 h at

room temperature. Then 5 ml of water was added, and 1 N NaOH dropwise to pH 9. On the following day the mixture was neutralized with dry Amberlite IR-120 (H^+ form), the resin was filtered off, thoroughly washed with methanol, the combined filtrates decolorized with charcoal and evaporated, and the residue dried at 40° (0.8 mm) over P_2O_5. The results of the saponification are given in Table 2.

Acid Hydrolysis of the Ketosides (I), (II), (III), and (IV). A solution of 20 mg of the ketoside (I)-(IV) in 3 ml of 0.1 N H_2SO_4 was heated for 1 h at 80°, neutralized with a saturated solution of $Ba(OH)_2$, and the residue of $BaSO_4$ filtered off and washed with water. The filtrate was deionized with Amberlite IR-120 (H^+ form); only N-acetyl-D-neuraminic acid, R_f 0.4 (A), 0.17 (C), and the corresponding aglycone component: $NO_2C_6H_4OH$, R_f 0.77 (D) from (I), $O_2NC_6H_4SH$, R_f 0.9 (D) from (III), and $NO_2C_6H_4NH_2$, R_f 0.75 (D) from (IV) were identified chromatographically.

Enzymatic Hydrolysis of the Ketosides (I), (II), (III), and (IV). A preparation of neuraminidase from a filtrate of cholera vibrio (N. V. Philips–Duphar Company, Holland) and influenza virus, strain A2 (Smolensk, 72-67) was used. Enzymatic hydrolysis was conducted in 0.1 M acetate buffer at pH 5.6 [20], containing 0.9% NaCl and 0.1% $CaCl_2$, at 37°. The p-nitrophenol eliminated was determined as follows: after definite periods of time 0.1 ml of the incubation mixture was collected, the enzymatic reaction was stopped by adding 2.9 ml of borate buffer at pH 9.8 [20], and the optical density of the solution obtained at 400 nm was determined on a spectrophotometer.

CONCLUSIONS

1. The synthesis of 2-O-p-nitrophenyl-N-acetyl-α-D-neuraminic acid, 2-deoxy-2-methylthio-N-acetyl-α-D-neuraminic acid, 2-deoxy-p-nitrophenylthio-N-acetyl-α-D-neuraminic acid, and 2-deoxy-2-p-nitrophenylamino-N-acetyl-α-D-neuraminic acid was described, and their behavior in enzymatic hydrolysis by neuraminidases of cholera vibrio and influenza virus was studied.

2. 2-O-p-Nitrophenyl-N-acetyl-α-D-neuraminoside is a convenient synthetic substrate in kinetic investigations of neuraminidases. N- and S-ketosides are distinguished by their stability to neuraminidases from various sources.

LITERATURE CITED

1. W. Gielen, Naturwissenschaften, 55, 104 (1968).
2. R. B. Kemp, Nature, 218, 1255 (1968).
3. A. Gottschalk, The Enzymes, 4, 461 (1966).
4. L. Warren and C. W. Spearing, Biochem. Biophys. Res. Commun., 3, 489 (1960).
5. K. Sandhoff and H. Jatzkewitz, Biochim et Biophys. Acta, 152, 136 (1968).
6. H. Tuppy and P. Palese, Hoppe–Seylers Z. Phys. Chem., 349, 1169 (1968).
7. J. T. Cassidy, G. W. Jourdian, and S. Roseman, Methods in Enzymol., 8, 680 (1966).
8. M. E. Rafelson, S. Gold, and I. Priede, Methods in Enzymol., 8, 677 (1966).
9. P. Meindl and H. Tuppy, Monatsh. Chem., 96, 802 (1967).
10. M. Cerny and Y. Pacak, Collect. Czechoslov. Chem. Commun., 24, 2566 (1959).
11. M. Schneir, R. Y. Winzler, and M. E. Rafelson, Biochem. Prepar., 9, 1 (1962).
12. D. Aminoff, Biochem. J., 81, 384 (1961).
13. P. Meindl and H. Tuppy, Monatsh. Chem., 98, 53 (1967).
14. J. Boroach, D. H. Leaback, and P. G. Walker, Biochem. J., 78, 111 (1961).
15. S. P. Colowick and N. O. Kaplan, Methods in Enzymol., 3, 448 (1957).
16. J. D. Algranat, Biochem. et Biophys. Acta, 73, 152 (1963).
17. H. Lineweaver and D. Burk, J. Amer. Chem. Soc., 56, 658 (1934).
18. K. Wallenfels and O. P. Malhotra, The Enzymes, 4, 409 (1960).
19. R. Kuhn, P. Lutz, and D. L. MacDonald, Chem. Ber., 99, 611 (1966).
20. S. P. Colowick and N. O. Kaplan, Methods in Enzymol., 1, 140, 146 (1955).