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INHIBITION OF *in vitro* BIOSYNTHESIS OF *N*-ACETYLNEURAMINIC ACID BY *N*-ACYL- AND *N*-ALKYL-2-AMINO-2-DEOXYHEXOSES

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ABSTRACT

The biosynthesis of *N*-acetylneuraminic acid is markedly inhibited by 2-deoxy-2-propionamido-D-glucose (GlcNProp) and to a much lesser extent by 2-deoxy-2-propionamido-D-mannose (ManNProp), but not by 2-deoxy-2-propionamido-D-galactose and *N*-methylated derivatives of 2-amino-2-deoxy-D-glucose. 2-Deoxy-2-trimethylamino-D-glucose is a weak inhibitor of 2-acetamido-2-deoxy-D-mannose metabolism. When incubated in a cell-free system from rat liver, GlcNProp gives the 6-phosphate, which is converted into *N*-propionylneuraminic acid. Evidence is presented which shows that it is the metabolites GlcNProp-6-P and ManNProp-6-P which are the competitive inhibitors, and not GlcNProp itself.

INTRODUCTION

Tumour cells show such characteristic changes in social behavior as unlimited growth, de-differentiation, invasiveness, and altered antigenicity. Changes in cell-surface properties and composition occur after oncogenic transformation[1], many of which involve the carbohydrate moieties of glycoconjugates[2–5]. Modification of the amount and structure of plasma membrane carbohydrates may be correlated with cell behavior. Also, the structural and functional differences of carbohydrates on normal and tumour cell surfaces could serve as a means of selective chemotherapy[6]. Modification of *N*-acetylneuraminic acid (NeuAc) seems to be most promising for this purpose, because of its terminal position and negative charge[7].

Enzymic removal of NeuAc by neuraminidase has already been attempted as a

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tumour treatment[8], but has encountered severe problems due to limited access to the substrate, antigenicity of the enzyme preparations, and the transitory nature of the effect, since cell-surface NeuAc is restored quickly[9]. Therefore, inhibition of NeuAc synthesis might be more advantageous.

We now describe the inhibitory effect of some hexosamine (HexN) derivatives on NeuAc synthesis in a cell-free system of rat liver. The biosynthesis of *N*-propionylneuraminic acid (NeuProp), which is not known to occur naturally, is reported.

MATERIALS AND METHODS

Chemicals. — 2-Amino-2-deoxy-D-galactose (GalN) and the resins for chromatography were obtained from C. Roth OHG (Karlsruhe, Germany), 2-amino-2-deoxy-D-mannose (ManN) and *N*-acetylneuraminic acid aldolase (EC 4.1.3.3) from the Sigma Chemical Co. (St. Louis, MO., U.S.A.), 1-^{14}C-GlcN and 1-^{14}C-ManN from the Radiochemical Centre (Amersham, England), enzymes and coenzymes from Boehringer Mannheim GmbH (Mannheim, Germany), and all other chemicals (reagent grade) from E. Merck AG (Darmstadt, Germany).

Synthesis of HexN derivatives. — GlcNProp, ManNProp, and GalNProp were synthesised by *N*-propionylation of the respective amino sugars (free bases) in a manner analogous to the *N*-acetylation procedure described[10] for GlcN. GlcNProp-6-P was made from GlcN-6-phosphate and propionic anhydride in a manner analogous to that used for the synthesis GlcNAc-6-phosphate[11]. The synthesis of *N*-methyl (GlcNMe) and *N*,*N*-dimethyl ($GlcNMe_2$) derivatives of GlcN was performed by the method of Černý *et al.*[12], using methylamine or dimethylamine, respectively, and 1,6:2,3-dianhydro-4-*O*-benzyl-β-D-mannopyranose. The iodide of 2-deoxy-2-trimethylammonio-D-glucose was prepared from $GlcNMe_2$ by the action of methyl iodide in acetonitrile[13].

The synthesis of 1-^{14}C-labelled GlcNMe and $GlcNMe_2$ was based on the procedure of Kuehl *et al.*[14]. A solution of 0.5 mCi of 1-^{14}C-GlcN · HCl (32 mg, 0.14 mmol) in water (0.4 ml) was treated with M sodium hydroxide (0.15 ml) and with a solution of methyl sulfate (20 mg, 0.16 mmol) in methanol (0.15 ml). The mixture was shaken for 25 min at ambient temperature and then chromatographed on Whatman No. 3 paper (4 developments with 1-butanol–ethanol–water, 52:33:15). The appropriate zones were eluted with aqueous 0.3% hydrochloric acid, and the solutions were concentrated to dryness under diminished pressure to give the hydrochlorides of GlcN (105 μCi), GlcNMe (118 μCi), and $GlcNMe_2$ (99.6 μCi). GlcNMe, after re-chromatography, had an activity of 61.7 μCi.

Preparation of the cell-free system[15]. — Female Wistar rats (150–220 g), fed a commercial diet (Altromin R, Lage, Germany) and water *ad libitum*, were killed by exsanguination. The livers were chilled in ice-cold medium [0.1M Tris–HCl (pH 7.4), 75mM nicotinamide, 2mM $MgCl_2$] and squeezed through a sieve in order to remove connective tissue. Each gram of squeezed material was diluted with 2.5 ml of medium. After homogenisation in a Potter–Elvehjem-type homogeniser, with 6 strokes by

hand, and centrifugation at 700*g* for 2 min in a Sorvall RC2-B-type centrifuge, the supernatant solutions, substrates, and coenzymes were placed in 1.5-ml cups (Eppendorf Gerätebau, Hamburg, Germany) and incubated in a metabolic shaker (Braun, Melsungen, Germany) at 37°. The reaction was stopped either by heating at 100° for 90 s or by adding the same volume of ethanol and incubating at 40° for 6 min. After centrifugation, the supernatant solution was analysed by p.c.

Chromatography and identification of labelled metabolites. — Chromatography on Whatman No. 3 paper involved *A*, 1-propanol–water–M sodium acetate[16] (pH 5.0, 7:2:1); *B*, ethanol–M ammonium acetate[17] (pH 7.5, 5:2); *C*, ethanol–M ammonium acetate[17] (pH 3.8, 5:2); and *D*, 1-butanol–pyridine–water[18] (6:4:3).

HexNAc-1-phosphates and sugar nucleotides were hydrolysed with 0.1M HCl at 100° for 15 min; the acyl groups were split off by hydrolysis in M HCl at 100° for 60 min. Enzymic hydrolysis of the HexNAc-6-phosphates was performed with alkaline phosphatase (EC 3.1.3.1). NeuAc was cleaved by *N*-acetylneuraminic acid aldolase[19] (EC 4.1.3.3).

Protein determination. — In order to extract the lipids, an aliquot (100 µl) of each homogenate was mixed with 1,4-dioxane (1.5 ml), and the protein content of the precipitate was determined by the biuret method[20] with bovine serum albumin as the standard.

Determination of radioactivity. — Radioactivity was measured in a Packard

TABLE I

IDENTIFICATION OF THE METABOLITES OF [^{14}C]-GlcNProp

Peak	R_{UMP}[a]	*Hydrolysis conditions*	R_{UMP} *after hydrolysis*[a]	*Substance(s) identified*
I	0.6	Alkaline phosphatase	0.6 + (2.1)	UDP-GlcN, UDP-GlcNAc, GlcN-P, NeuAc-9-P
II	1.2	Alkaline phosphatase	3.9	GlcNAc-P
III	1.7	Alkaline phosphatase	4.6	
		0.1M HCl, 15 min, 100°	1.7 + 0.6	GlcNProp-P
IV	3.2	M HCl, 60 min, 100°	3.2	
		0.1M HCl, 15 min, 100°	3.2	GlcN
V	3.9	M HCl, 60 min, 100°	3.2	
		0.1M HCl, 15 min, 100°	3.9	GlcNAc
VI	4.6	M HCl, 60 min, 100°	3.2	
		0.1M HCl, 15 min, 100°	(3.2) + 4.6	GlcNProp
GlcN	3.2	M HCl, 60 min, 100°	3.2	
		0.1M HCl, 15 min, 100°	3.2	
GlcNAc	3.9	M HCl, 60 min, 100°	3.2	
		0.1M HCl, 15 min, 100°	3.9	
GlcNProp	4.6	M HCl, 60 min, 100°	3.2	
		0.1M HCl, 15 min, 100°	(3.2) + 4.6	

[a]P.c. (solvent *A*).

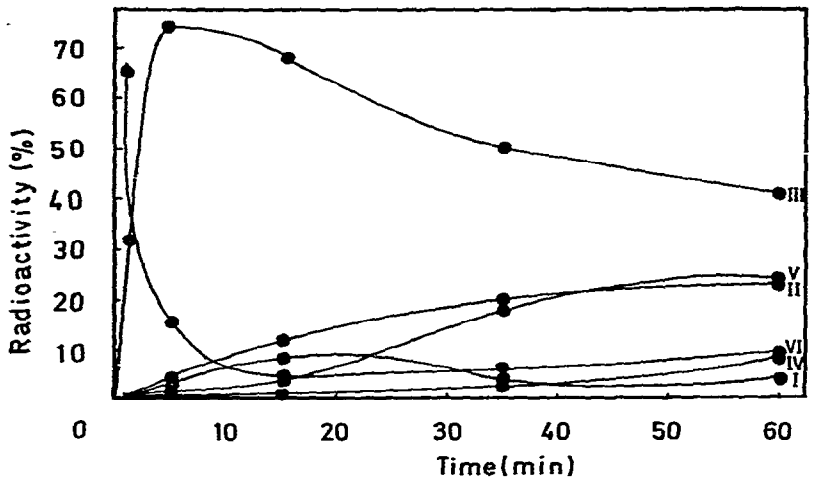

Fig. 1. Formation of metabolites of [^{14}C]-GlcNProp in the 700-*g* supernatant of rat-liver homogenate. The incubation mixture (2.1 ml) contained mM ATP, 2mM PEP, mM UTP, protein (55 mg), and [^{14}C]-GlcNProp (0.5 μCi, 58 Ci/mol).

Tri-carb scintillation spectrometer, using Bray's scintillator[21] for aqueous solutions. Paper strips from chromatograms were counted in a toluene scintillator.

RESULTS

Metabolism of HexNProp in a liver cell-free extract. — After incubation of ^{14}C-Glc-NProp in a cell-free system of rat liver, p.c. (solvent *A*) revealed at least six components (I–VI), each of which was eluted and submitted to enzymic and acid hydrolysis. The results are shown in Table I and Fig. 1. GlcNProp was, in part, enzymically

TABLE II

[^{14}C]-GlcNProp METABOLISM[a] IN THE 700-*g* SUPERNATANT AND IN THE SEPHADEX G-25 FILTRATE OF THE 30,000-*g* SUPERNATANT

	700-g Supernatant	*30,000-g Sephadex filtrate*
GlcNProp	9.4	23.4
GlcNProp-6-P	37.9	55.2
GlcN	7.2	0.5
GlcNAc	20.9	1.0
GlcNAc-6-P	21.0	2.9
UDP-amino sugar (+ GlcN-1-P)	3.6	17.0

[a]The incubation mixture (0.435 ml) contained 2mM ATP, 2mM PEP, 2mM UTP, [^{14}C]-GlcNProp (58 Ci/mol) 240,000 d.p.m., and protein [3.8 mg (700-*g* supernatant) and 2 mg (Sephadex filtrate)]. Incubation for 60 min at 37°.

TABLE III

METABOLITES[a] OF [^{14}C]-GlcNProp AND [^{14}C]-ManNProp IN THE 30,000-*g* SUPERNATANT

Peak	*Substances identified*	*Percentage of total radioactivity after incubation with*	
		GlcNProp	*ManNProp*
I	UDP-amino sugar, HexN-P, NeuAc-9-P, CMP-NeuAc	3.6	0.3
II	Hex-NAc-P	12.8	1.1
III	HexNProp-P	59.9	6.1
IV	NeuProp	0.9	3.5
V	HexN	2.2	0.3
VI	HexNAc	16.6	1.0
VII	HexNProp	11.0	87.7

[a]The incubation mixture (1.47 ml) contained 1.3mM ATP, 2.6mM PEP, 1.3mM UTP, [^{14}C]-GlcNProp (58 Ci/mol) 240,000 d.p.m., [^{14}C]-ManNProp (220 Ci/mol) 290,000 d.p.m., and protein (85 mg). Incubation for 70 min at 37°.

depropionylated and subsequently acetylated, yielding GlcNAc and its metabolites. GlcNProp was stable in heat-inactivated liver homogenate.

Fig. 1 shows that GlcNProp (VI) was almost completely phosphorylated (→ III) within 15 min. After 5 min, 75% of the radioactivity could be identified as GlcNProp-6-P, so that a turnover rate of 1.8 nmol/mg of protein/min can be calculated (the incubation mixture contained 20mM ATP, 10mM ^{14}C-GlcNProp, and 9.0 mg of protein in a final volume of 0.3 ml).

The influence of substances of low molecular weight (*e.g.*, nucleotides, acetyl-CoA) present in the 700-*g* supernatant of rat-liver homogenate was eliminated by incubating GlcNProp with the Sephadex G-25 filtrate of the 30,000-*g* supernatant. Comparative data are shown in Table II. As expected, acetyl derivatives of GlcN were synthesised to a lesser extent, but the depropionylase activity was also markedly diminished.

Table III shows the distribution of radioactivity after incubation of ^{14}C-GlcNProp and ^{14}C-ManNProp with the 30,000-*g* supernatant. Although GlcNProp was almost completely metabolised within 70 min, only 12.3% of the ManNProp was converted into metabolites, due to slower depropionylation and phosphorylation.

Identification of N-*propionylneuraminic acid.* — After incubation of ^{14}C-ManNProp in the cell-free system, a substance was formed (Table III, peak IV) which was present to a lesser extent among the metabolites of ^{14}C-GlcNProp. In p.c. (solvent *A*), it migrated faster than NeuAc (R_{UMP} 2.4). The ethanol-soluble supernatant of the incubation mixture was subjected to p.c. (solvent *B*). Metabolite IV migrated like NeuAc; after 20 h, it was 2 cm from the origin. It was eluted, re-chromatographed (p.c., solvent *A*), and incubated with NeuAc aldolase (p.c., solvent B), to give only one product (p.c., solvent *B*) with R_{GlcNAc} 1.2 (HexNProp), suggesting

TABLE IV

INFLUENCE OF *N*-METHYLATED GlcN ON [^{14}C]-ManNAc METABOLISM[a]

Metabolites of [^{14}C]-*ManNAc (percentage of total radioactivity)*	*Inhibitor*							
	After incubation for 15 min with [^{14}C]-*ManNAc*				*After incubation for 60 min with* [^{14}C]-*ManNAc*			
	GlcNMe	*GlcN(Me)$_2$*	*GlcN(Me)$_3$*	*No inhibitor*	*GlcNMe*	*GlcN(Me)$_2$*	*GlcN(Me)$_3$*	*No inhibitor*
ManNAc	14.1	12.3	21.8	12.9	19.3	15.5	27.7	18.1
ManNAc-6-P	50.2	48.3	50.5	48.1	10.7	10.8	13.2	10.0
NeuAc	14.5	13.1	15.1	13.4	45.1	41.1	40.8	43.0
NANA-9-P + CMP-NeuAc	21.2	26.3	12.2	25.5	24.8	32.6	18.3	28.9

[a]The incubation mixture (1.5 ml) contained 1.3mM ATP, 2.6mM PEP, 1.3mM CTP, 13mM GlcNMe, [^{14}C]-ManNAc (220 Ci/mol) 190,000 d.p.m., and protein (84 mg).

TABLE V

INFLUENCE OF HexNProp ON [^{14}C]-HexNAc METABOLISM[a]

Metabolites of [^{14}C]-GlcNAc and [^{14}C]-ManNAc (percentage of total radioactivity)	*Inhibitor*							
	[^{14}C]-GlcNAc substrate				*[^{14}C]-ManNProp substrate*			
	GlcNProp	*ManNProp*	*GalNProp*	*No inhibitor*	*GlcNProp*	*ManNProp*	*GalNProp*	*No inhibitor*
HexNAc	81.6	27.0	8.4	6.4	82.5	33.3	14.4	13.5
HexNAc-6-P	14.4	56.5	64.7	61.9	10.6	47.8	49.2	46.9
NeuAc-9-P	0.4	2.0	4.0	4.7	1.7	4.3	6.4	8.6
NeuAc	0.7	1.7	4.0	5.7	3.8	9.7	18.5	18.5
NeuAc + UDP-GlcNAc	2.9	12.8	19.3	21.3	1.5[b]	5.1[b]	11.5[b]	12.3[b]

[a]The incubation mixture (1.3 ml) contained 1.6mM ATP, 3.2mM PEP, 1.6mM CTP, 16mM HexNProp, [^{14}C]-ManNAc (220 Ci/mol) 150,000 d.p.m., [^{14}C]-GlcNAc (47 Ci/mol) 190,000 d.p.m., and protein (78 mg). Incubation for 15 min at 37°. [b]CMP-NeuAc only.

TABLE VI

INFLUENCE OF GlcNProp-6-P ON [^{14}C]-ManNAc METABOLISM

Metabolites of [^{14}C]-ManNAc (percentage of total radioactivity)	*GlcNProp-6-P inhibitor*	*GlcNProp inhibitor*	*No inhibitor*
ManNAc	97.4	83.1	12.8
ManNAc-6-P	1.0	10.3	43.3
NeuAc	1.6	5.3	36.5
NeuAc-9-P + CMP-NeuAc	0.01	1.2	7.4

[a]The incubation mixtures (1 ml) contained 1.9mM ATP, 3.9mM PEP, 19.3mM GlcNProp or GlcNProp-6-P, respectively, [^{14}C]-ManNAc (220 Ci/mol) 150,000 d.p.m., and protein (22 mg). Incubation for 15 min.

that the parent substance was NeuProp which was cleaved into ManNProp and pyruvate. The low substrate-specificity of the enzyme was confirmed by the finding[19] that it cleaves NeuAc and *N*-glycolylneuraminic acid.

Inhibition of NeuAc-biosynthesis by hexosamine derivatives. — $GlcN(Me)_3^+I^-$ was a weak inhibitor of ManNAc metabolism, whereas GlcNMe and $GlcNMe_2$ had no effect (Table IV). GlcNProp and, to a lesser degree, ManNProp strongly inhibited NeuAc synthesis *in vitro* by decreasing the extent of phosphorylation of ManNAc and GlcNAc (Table V), whereas GalNProp did not affect their metabolism. The synthesis of NeuAc from ManNAc-6-P and NeuAc-9-P was not influenced (data not shown). The most potent inhibitor of NeuAc synthesis from ManNAc was GlcNProp-6-P (Table VI).

Inhibition of the HexNAc phosphorylation was not due to the trapping of ATP by the formation of *N*-propionylhexosamine phosphates, because incubation

TABLE VII

INFLUENCE OF ADP ON THE METABOLISM OF [^{14}C]-ManNAc

Metabolites of [^{14}C]-ManNAc (percentage of total radioactivity)	*ADP inhibitor*	*No inhibitor*
ManNAc	23.1	19.2
ManNAc-6-P	69.7	57.8
NeuAc	0.7	12.4
NeuAc-9-P + CMP-NeuAc	5.5	10.6

[a]The incubation mixture (0.535 ml) contained 3.7mM ATP, 7.4mM PEP, 18.7mM ADP, [^{14}C]-ManNAc (220 Ci/mol) 150,000 d.p.m., and protein (7.3 mg). Incubation for 15 min.

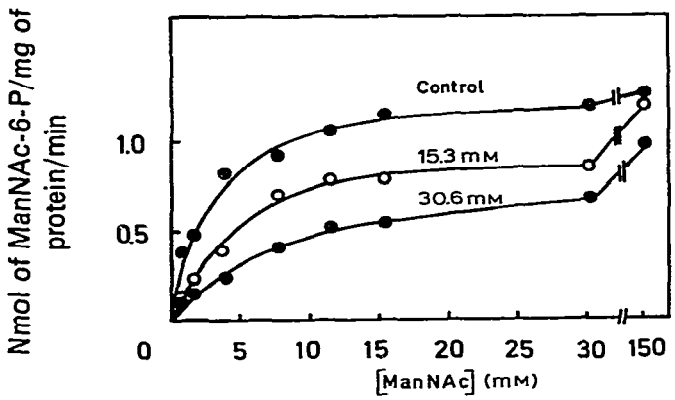

Fig. 2. ManNAc saturation-curve in the presence of GlcNProp. The incubation mixture (0.655 ml) contained 30.5mM ATP and protein (10.1 mg). Incubation for 8 min at 37°.

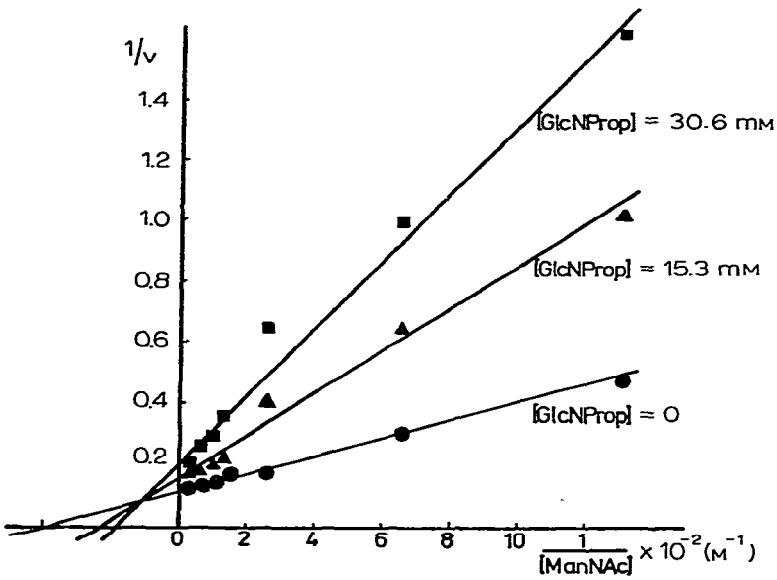

Fig. 3. Lineweaver–Burk plot for data in Fig. 2.

with increasing concentrations of ATP does not enhance the metabolism of ManNAc in the presence of *N*-propionylhexosamines. ADP, which results from phosphorylation of *N*-propionylhexosamines, inhibits several kinases in a competitive way with respect to ATP and in a more complex way with respect to the other substrate[22]. The phosphorylation of ManNAc remained unchanged after incubation with ADP. However, NeuAc synthesis was diminished, probably due to inhibition of the formation of NeuAc-9-P from ManNAc-6-phosphate (Table VII). Free propionic acid, which was split from *N*-propionylhexosamines, did not influence the metabolism of ^{14}C-labelled HexNAc. GlcN, also formed, might have the specific radioactivity of

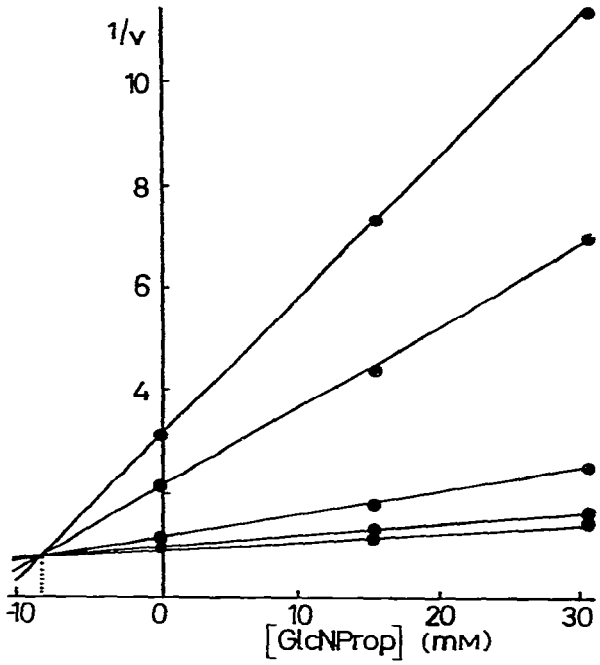

Fig. 4. Dixon plot for data in Fig. 3.

the metabolites of ^{14}C-HexNAc, thus imitating an inhibition. However, incubation of ^{14}C-ManNAc with 8.7mM GlcN showed no difference in the amount of ManNAc metabolites as compared to controls.

Fig. 2 shows the substrate (ManNAc) saturation curve in the presence of two inhibitor (GlcNProp) concentrations and illustrates that the inhibitory effect could be almost, but not completely, reversed by excess of ManNAc. There was no deviation from the Michaelis–Menten relationship, as evident from the Lineweaver–Burk

TABLE VIII

INFLENCE OF HexNAc ON THE METABOLISM OF [^{14}C]-GlcNProp IN THE 700-*g* SUPERNATANT

Metabolites of [^{14}C]-GlcNProp (percentage of total radioactivity)	*GlcNAc inhibitor*	*ManNAc inhibitor*	*No inhibitor*
GlcNProp	98.2	97.0	80.8
GlcNProp-6-P	0.2	1.2	17.1
GlcN	0.4	0.5	0.5
GlcNAc	1.2	1.1	1.0
GlcNAc-6-P	0.0	0.1	0.5

[a]The incubation mixture (0.355 ml) contained 28mM ATP, 28mM HexNAc, and [^{14}C]-GlcNProp (58 Ci/mol) 120,000 d.p.m. Incubation for 5 min at 37°.

plot in Fig. 3, from which a K_m value of 2.5mM was calculated for ManNAc-kinase in the 700-*g* supernatant of rat-liver homogenate.

To determine the type of inhibition and the K_i value, the Michaelis–Menten equation was transformed[23] into a linear relationship with $1/v$ and the inhibitor concentration as variables and with the substrate concentration constant (Fig. 4). The common intersection point indicated a mainly competitive mechanism of inhibition with a K_i value of 8.3mM. Thus, the affinities of the enzyme for the inhibitor and the substrate were in the same range. The competitive type of inhibition was confirmed not only because the phosphorylation of HexNAc was inhibited by GlcNProp, but also because the metabolism of GlcNProp was diminished by HexNAc (Table VIII).

DISCUSSION

A cell-free system of rat liver was used to study the influence of HexN derivatives on the synthesis of NeuAc. Among the substances examined, GlcNProp and, to a lesser extent, ManNProp were potent inhibitors of NeuAc synthesis from GlcNAc or ManNAc. The phosphorylation of these precursors was inhibited by a mainly competitive mechanism; there was no indication of allosteric regulation. The most potent inhibitor was GlcNProp-6-P, synthesised from GlcNProp in the cell-free system. ManNProp was phosphorylated to a lesser extent, which may account for its weaker inhibitory effect. Among the *N*-methylated GlcN derivatives, only GlcNMe$_3^+$I$^-$ was a weak inhibitor of ManNAc metabolism. Schultz and Mora[24] reported a comparable inhibitory effect of 2-deoxy-2-(2-fluoroacetamido)-D-glucopyranose on the accumulation of UDP-GlcNAc and CMP-NeuAc in extracts from cultured, mouse-embryo cells. When a HexNProp was applied to a cell-free system of rat liver, not only was an inhibition of NeuAc synthesis achieved, but also the synthesis of NeuProp was observed which is not known to occur naturally. NeuProp has been chemically synthesised[25].

Inhibition of NeuAc biosynthesis and modification of its structure may be of value in the following situations. (*1*) A decrease in the amount of sialic acid or its modification might reveal its function in glycoconjugates or in cell structures by altering their qualities.

(*2*) Plasma membranes of neoplastic cells, and particularly the carbohydrate moieties of their glycoconjugates, appear to have structural and functional differences which can be utilised for chemotherapeutic attack. These differences are, in part, due to an alteration in the amount and metabolism of NeuAc[26,27]. Thus, a reduction in the degree of side-chain *O*-acylation of sialic acid for malignant colorectal tumours has recently been postulated, which may be of diagnostic value[28]. The altered social behavior of transformed cells might be the result of "over-protection" of specific recognition sites by *N*-acetylneuraminic acid[29]. Moreover, removal of NeuAc or alteration of its structure may unmask cell-surface antigens or lead to new antigenic determinants, thus enhancing cellular immunogenicity. Thus, it is conceivable that

NeuAc metabolism can be a site of chemotherapeutic intervention, as is already attempted by tumour treatment with neuraminidase. Furthermore, lack of adequate substrate after administration of inhibitors of NeuAc synthesis may block cell replication in tumours.

(*3*) The function of some receptors for infectious agents, *e.g.*, myxoviruses, cholera toxin, and tetanus toxin, is linked to their NeuAc content. Prevention of these infections seems to be possible by introduction of a modified sialic acid into these receptors. For *N*-acetyl-2,3-didehydro-2-deoxyneuraminic acid[30], which occurs in saliva, an influence on caries and periodontitis has been suggested[31].

Preliminary results have shown that GlcNProp taken up by rat liver is metabolised as in the cell-free system and that NeuAc metabolism is also affected *in vivo*.

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