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Inhibition of the biosynthesis of *N*-acetylneuraminic acid by metal ions and selenium *in vitro**

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In liver homogenate the biosynthesis of *N*-acetylneuraminic acid using *N*-acetylglucosamine as precursor can be followed stepwise by applying different chromatographic procedures. In this cell-free system 16 metal ions (Zn^{2+}, Mn^{2+}, La^{3+}, Co^{2+}, Cu^{2+}, Hg^{2+}, VO_3^-, Pb^{2+}, Ce^{3+}, Cd^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Sn^{2+}, Cs^+ and Li^+) and the selenium compounds, selenium(IV) oxide and sodium selenite, have been checked with respect to their ability to influence a single or possibly several steps of the biosynthesis of *N*-acetylneuraminic acid. It could be shown that the following enzymes are sensitive to these metal ions (usually applied at a concentration of 1 mmol l^{-1}): *N*-acetylglucosamine kinase (inhibited by Zn^{2+} and vandate), UDP-*N*-acetylglucosamine-2′-epimerase (inhibited by Zn^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, VO_3^-, Pb^{2+}, Cd^{2+}, Fe^{3+}, Cs^+, Li^+, selenium(IV) oxide and selenite), and *N*-acetylmannosamine kinase (inhibited by Zn^{2+}, Cu^{2+}, Cd^{2+} and Co^{2+}). Dose dependent measurements have shown that Zn^{2+}, Cu^{2+} and selenite are more efficient inhibitors of UDP-*N*-acetylglucosamine-2′-epimerase than vanadate. As for the *N*-acetylmannosamine kinase inhibition, a decreasing inhibitory effect exists in the following order Zn^{2+}, Cd^{2+}, Co^{2+} and Cu^{2+}. In contrast, La^{3+}, Al^{3+} and Mn^{2+} (1 mmol l^{-1}) did not interfere with the biosynthesis of *N*-acetylneuraminic acid. Thus, the conclusion that the inhibitory effect of the metal ions investigated cannot be regarded as simply unspecific is justified.

Keywords: *N*-acetylneuraminic acid biosynthesis, metal ions, selenium, rat liver, inhibition of enzymes

Introduction

The biosynthesis of *N*-acetylneuraminic acid is well established, especially due to studies performed by the groups of Roseman and Warren (for review see Warren & Felsenfeld 1962, Corfield & Schauer 1982). In Figure 1 the metabolic pathway is represented which involves 10 different enzymatic reactions. Co-substrate of most enzymes is either ATP, UTP or finally CTP, leading to CMP-*N*-acetylneuraminic acid, an unusual sugar nucleotide. The singularity of this biosynthetic pathway in carbohydrate biochemistry can be deduced from two reactions: (i) the direct formation of *N*-acetylmannosamine from UDP-*N*-acetylglucosamine, which comprises 2′-epimerization as well as splitting off of UDP, and (ii) the condensation of *N*-acetylmannosamine-6-phosphate with phosphoenolpyruvate leading to *N*-acetylneuraminic acid-9-phosphate. *N*-Acetylneuraminic acid is involved in a variety of biological processes including antigenicity, cellular adhesion, transport, stability of glycoproteins, receptor function, synaptic transmission, the action of some hormones and hemostasis. With regard to the diversity of biological processes in which *N*-acetylneuraminic acid is involved, it is likely that impairment of its biosynthesis may lead to marked consequences. In previous studies it could be shown that the biosynthesis of *N*-acetylneuraminic acid can be inhibited by some sugar analogs such as *N*-propionyl-D-glucosamine or *N*-dimethyl-D-mannosamine (Grünholz *et al.* 1981, Bauer *et al.* 1983, Reutter & Bauer 1986). In this paper different metal ions, together with selenium, have been checked as potential inhibitors of one or several enzymes involved in the biosynthesis of *N*-acetylneuraminic acid. Furthermore, some attempts are made to explain the mechanism of inhibition *in vitro*.

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*Dedicated to Professor Theodor Günther on the occasion of his 60th birthday.

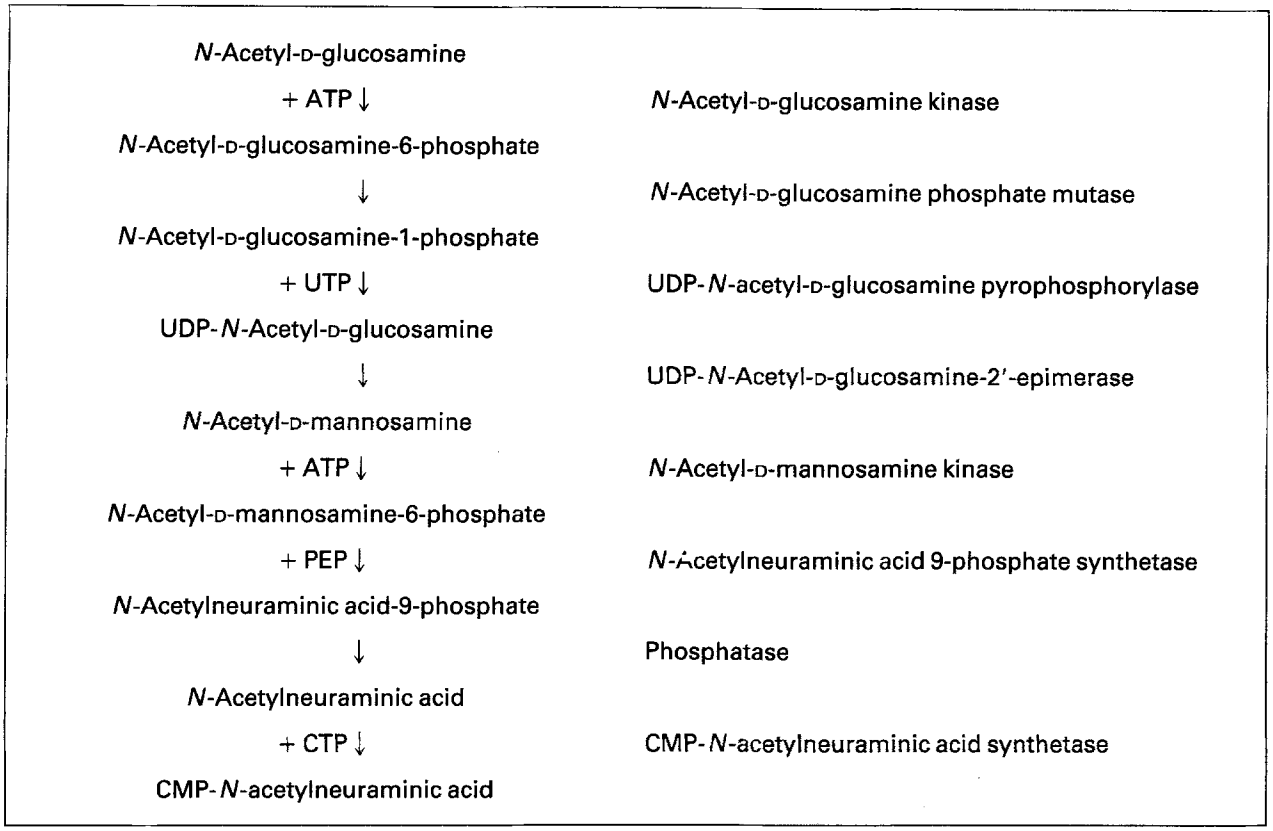

Figure 1. Biosynthetic pathway of CMP-*N*-acetylneuraminic acid from the precursor *N*-acetylglucosamine. The respective enzymes involved are: *N*-acetylglucosamine kinase (2.7.1.59), *N*-acetylglucosamine-6-phosphate mutase (2.7.5.2), UDP-*N*-acetylglucosamine pyrophosphorylase (2.7.7.23), UDP-*N*-acetylglucosamine-2'-epimerase (5.1.3.14), *N*-acetylmannosamine kinase (2.7.1.60), *N*-acetylneuraminic acid 9-phosphate synthase (4.1.3.20), *N*-acetylneuraminic acid 9-phosphate phosphatase (3.1.3.29) and CMP-*N*-acetylneuraminic acid synthase (2.7.7.43).

Materials and methods

Preparation of the homogenate

Wistar rats, weighing 170–200 g each, were perfused with 40 ml of 0.15 mol l^{-1} NaCl under light ether anesthesia. Livers were then removed, squeezed through a sieve and the tissue was mixed with 2.5 volumes of chilled buffer, containing 0.2 mol l^{-1} Tris–HCl, 75 mmol l^{-1} nicotinamide and 2 mmol l^{-1} $MgCl_2$ (pH 7.4). Final homogenization was achieved with a dounce type homogenizer (10 strokes with the loosely fitting pestle) and the homogenate thus obtained was centrifuged for 10 min at 48000 × *g* at 4 °C. The protein concentration was 25 mg ml^{-1}.

Freezing of homogenates should be avoided, because this would lead to a substantial loss of enzyme activity.

Enzyme assay

The enzyme assays contained in a final volume of 350 μl: liver supernatant fraction (250 μl), ATP (1.14 mmol l^{-1}), UTP (1.14 mmol l^{-1}), CTP (1.14 mmol l^{-1}), phosphoenolpyruvate (2.28 mmol l^{-1}) and pyruvate kinase (57 mU μl^{-1}). The reaction performed at 37 °C under aerobic conditions was started by adding [^{14}C]*N*-acetyl-D-glucosamine (100 nCi; incubation time: 5 min) or [^{14}C]UDP-*N*-acetyl-D-glucosamine (100 nCi; incubation time: 10 min) or [^{3}H]*N*-acetyl-D-mannosamine (1 μCi; incubation time: 10 min). The reaction was stopped by addition of 500 μl ethanol (97%); the mixture was then heated for 5 min at 65 °C to stimulate the precipitation of proteins, followed by centrifugation at 5000 × *g* for 3 min.

Paperchromatographic separation of the metabolites

Deproteinized samples (usually 50 μl) together with 5 μl of 2% UMP as an internal standard were applied on Whatman 3MM paper and chromatographed for 22 h with *n*-propanol:1 mol l^{-1} sodium acetate, pH 5.0:H_2O (7:1:2) as solvent (Lewin & Wei 1966). Radioactivity of the separated metabolites was determined by counting 0.5–2.0 cm wide stripes in 10 ml liquid scintillation cocktail Quickszint 501 from Zinsser Analytic (Frankfurt, Germany) by using a Packard (Zürich, Switzerland) liquid scintillation counter. Individual metabolites were identified as described previously (Hultsch *et al.* 1972).

Calculation of the amount of metabolites formed

The metabolites were identified according to their R(UMP) values. The relative percentage of the synthesized metabolites (in % cpm) was calculated from the total radioactivity on the chromatogram.

Protein determination

The protein concentration was determined according to Lowry *et al.* (1951).

Chemicals

Enzymes and coenzymes were from Boehringer Mannheim GmbH. *N*-ethylmaleimide and *p*-chloromercuriphenylsulfonic acid were from Sigma (St Louis, MO, USA). Other chemicals, solutes and metal salts of analytical grade were from Merck (Darmstadt, Germany). *N*-Acetyl-D-[1-^{14}C]glucosamine (58.7 mCi mmol^{-1}) and UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (300 mCi mmol^{-1}) were purchased from Amersham-Buchler (Braunschweig, Germany) and *N*-acetyl-D-[6-^{3}H]mannosamine (25 Ci mmol^{-1}) came from American Radiolabeled Chemicals Inc. (St Louis, MO, USA).

Results

Use of N-*acetyl-D-[1-^{14}C]glucosamine as precursor.*

Figure 2(a) represents a typical profil of the *N*-acetyl-D-[1-^{14}C]glucosamine metabolites formed in a liver homogenate system. During the short incubation period of 5 min *N*-acetylglucosamine is phosphorylated to *N*-acetylglucosamine phosphate and then converted to UDP-*N*-acetylglucosamine. Therefore, the activity of both *N*-acetylglucosamine kinase and the UDP-*N*-acetylglucosamine pyrophosphorylase can be checked in a fairly good approximation. *N*-Acetylglucosamine kinase is nearly completely (99%) inhibited by 1 mmol l^{-1} of $ZnCl_2$ or 10 mmol l^{-1} $NaVO_3$ (Table 1). By addition of 1 mmol l^{-1} $CdBr_2$ to the incubation mixture the phosphorylation of *N*-acetyl-D-glucosamine was not impaired, in contrast to the formation of UDP-*N*-acetyl-D-glucosamine (see Figure 2b). In control experiments—to exclude a negative effect of bromide ions—KBr, however, showed no influence on the metabolization of *N*-acetylglucosamine. In similar experiments using SeO_2, Na_2SeO_3 and Pb(II) acetate of the same molarity inhibition was restricted to the formation of UDP-*N*-acetylglucosamine, while the synthesis of *N*-acetylglucosamine phosphate was not affected.

*Use of UDP-*N*-acetyl-D-[U-^{14}C]glucosamine as precursor.*

Figure 3(a) shows the metabolites formed within 10 min after the addition of UDP-[^{14}C]*N*-acetylglucosamine to the liver homogenate. This short incubation period was chosen in order to measure the activity of the UDP-*N*-acetyl-D-glucosamine-2'-epimerase, the key enzyme of the biosynthesis of

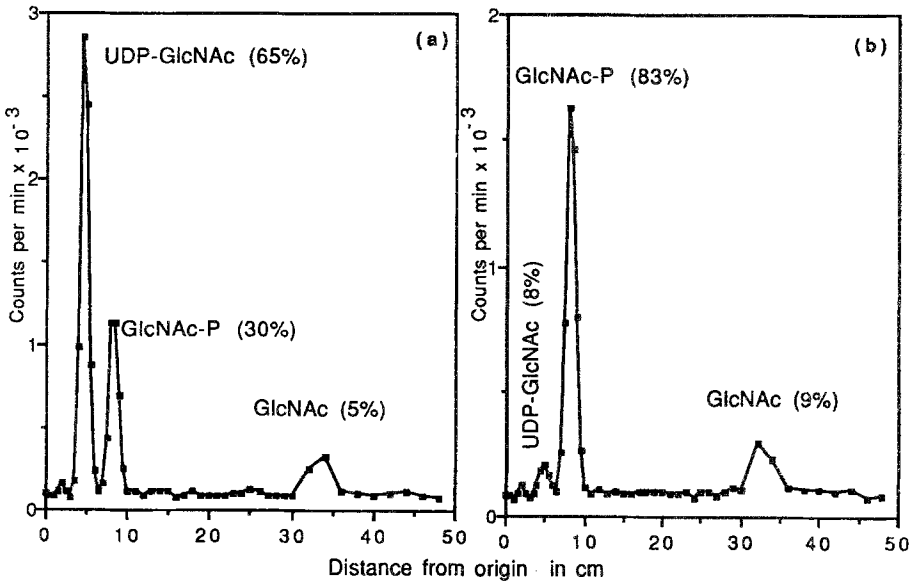

Figure 2. (**a**) *N*-Acetyl-D-glucosamine (GlcNAc) and its metabolites in a liver homogenate. The incubation time was 5 min. The peak of GLcNAc-P is composed of GlcNAc-1-P and GlcNAc-6-P which cannot be separated by the solvent system applied. For further details see Materials and methods. (**b**) Effect of Cd^{2+} (1 mmol l^{-1}) on the metabolization of [^{14}C]*N*-acetyl-D-glucosamine.

Table 1. Inhibition of the *N*-acetylneuraminic acid pathway by metal ions

Precursor	[^{14}C]GlcNAc		[^{14}C]UDP-GlcNAc		[^{3}H]ManNAc	
Incubation time (min)	5		10		10	
Metal ion						
$ZnCl_2$	99	(1)	77	(1)	96	(1)
$NaVO_3$	99	(10)	84	(10)	41	(1)
			–	(1)	19	(1)
$CuSO_4$	–	(1)	93	(1)	81	(1)
Hg^{2+}	ND		93	(1)	19	(1)
SeO_2	+	(1)	82	(1)	13	(1)
Na_2SeO_3	+	(1)	91	(1)	ND	
$CoCl_2$	–	(1)	87	(1)	80	(1)
$CdBr_2$	+	(1)	94	(1)	68	(1)
$Ce_2(SO_4)_3$	–	(1)	ND		10	(1)
$Fe(SO_4)$	–	(1)	34	(1)	16	(1)
$SnCl_2$	–	(1)	8	(1)	+	(1)
$Pb(Ac)_2$	+	(1)	90	(1)	–	(1)
$MnCl_2$	–	(1)	–	(1)	–	(1)
$LaCl_3$	–	(1)	–	(1)	–	(1)
CsCl	–	(1)	54	(1)	–	(1)
Li_3-citrate	–	(1)	47	(1)	–	(1)
$FeCl_3$	–	(1)	54	(1)	–	(1)
$Al(NO_3)_3$	–	(1)	ND		–	(1)

The inhibition is characterized as follows: the inhibition is calculated as percentage of reduction of the precursor formed in relationship to an assay system without any supplements. (+) The formation of a succeeding metabolite is inhibited by more than 50% compared with control. The molarity of the metal ion (in mmol l^{-1}) is given in parenthesis. (–) No inhibition.
ND, not determined.

N-acetylneuraminic acid. From Figure 3(b) it is apparent that $ZnCl_2$ (0.1 mmol l^{-1}) leads to a marked inhibition of enzyme activity (46%). The 2′-epimerase is also affected by other metal ions (final concentration 1 mmol l^{-1}) yet to a different extent: Hg^{2+} (inhibition by 93%), Cu^{2+} (93%), VO_3^- (19%), Co^{2+} (87%), Pb^{2+} (90%), Cd^{2+} (94%), Fe^{3+} (54%), Cs^+ (54%), Li^+ (47%), sodium selenite (91%) and selenate (82%). Figure 5(a) shows the concentration dependency of the inhibition of UDP-*N*-acetylglucosamine-2′-epimerase by Zn^{2+}, Cu^{2+}, selenite and VO_3^- and indicates that Zn^{2+}, Cu^{2+} and selenite are much better inhibitors than VO_3^-.

Use of N-*acetyl-*D*-[6-^{3}H]mannosamine as precursor*

If *N*-acetyl-D-[6-^{3}H]mannosamine instead of labelled *N*-acetyl-D-glucosamine is used as precursor, the last four steps leading to the formation of CMP-*N*-acetylneuraminic acid can be followed. After an incubation time of only 5 min about 70% of *N*-acetylmannosamine was metabolized to its 6-phosphate, neuraminic acid and CMP-neuraminic acid (see Figure 4a). Less than 1% of *N*-acetylneuraminic acid 9-phosphate is formed under these conditions (data not shown). In order to separate the 9-phosphate from CMP-*N*-acetylneuraminic acid the solvent system ethanol:1 mol l^{-1} ammonium acetate, pH 7.5 (2:5) should be used. For further details also see Paladini & Leloir (1952).

Addition of Zn^{2+} (1 mmol l^{-1}) to the incubation mixture inhibited the *N*-acetylmannosamine kinase by 97% (Figure 4b). Apart from Zn^{2+} some other metal ions inhibit the phosphorylation of *N*-acetyl-D-mannosamine quite markedly under the same conditions: Co^{2+} (inhibits by 80%), Cu^{2+} (81%), Cd^{2+} (68%) and VO_3^- (41%). Less effective were Fe^{2+} (16%), Ce^{3+} (10%), selenate (13%) and Hg^{2+} (19%). No appreciable inhibition was observed by the addition of La^{3+}, Pb^{2+}, Cs^+, Li^+, Fe^{3+} or Al^{3+}. These results are summarized in Table 1. The concentration dependency of the inhibition of *N*-acetylmannosamine kinase by the four most potent inhibitors suggests a decreasing inhibitory potency in the order Zn^{2+}, Cd^{2+}, Co^{2+} and Cu^{2+} (see Figure 5b). Zn^{2+} (0.05 mmol l^{-1}) and Co^{2+} (0.1 mmol l^{-1}) led to an inhibition of *N*-acetylmannosamine kinase by 59% and 43%, respectively. The combination of both using the same concentration could not significantly increase this inhibitory effect (63%). However, inhibition of *N*-acetylmannosamine kinase by Zn^{2+} (100 μmol l^{-1}), Cu^{2+} (100 μmol l^{-1}) and Cd^{2+} (100 μmol l^{-1}), could be avoided by addition of 400 nmol of ATP to the assay.

Influence of sulfhydryl reagents on enzymatic activity in vitro

After pretreatment of homogenate at 20 °C for 30 min with 1 mmol l^{-1} *p*-chloromercuriphenylsulfonic acid the enzymatic activity of *N*-acetylglucosamine kinase was reduced by 54%, that of UDP-*N*-acetylglucosamine-2′-epimerase by 30% and that of *N*-acetylmannosamine kinase by 61%. However, performing this experiment with 1 mmol l^{-1} of *N*-ethylmaleimide instead of the mercury derivative did not show any inhibition of the enzymes investigated.

Discussion

In this paper the influence of 16 different metal ions as well as of the selenium compounds has been investigated on the biosynthetic pathway of sialic acid. The main interest was focused on three

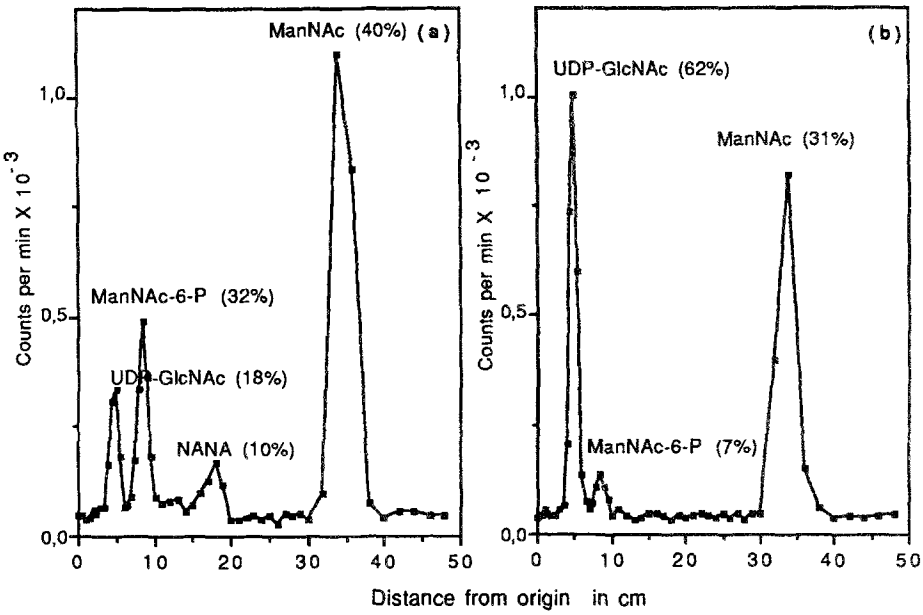

Figure 3. (**a**) UDP-*N*-Acetyl-D-glucosamine and its metabolites in a liver homogenate. The incubation time was 10 min. The separation of the deproteinized supernatants was performed in a solvent system consisting of *n*-propanol:sodium acetate:water. For further details see Materials and methods. (**b**) Inhibition of UDP-*N*-acetyl-D-glucosamine-2′-epimerase activity by Zn^{2+} (0.1 mmol l^{-1}).

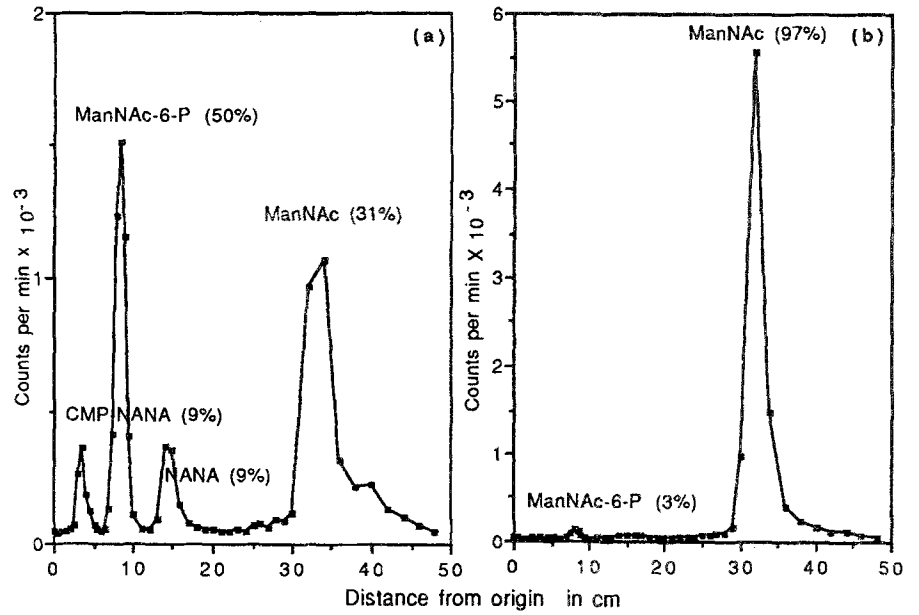

Figure 4. (**a**) *N*-Acetyl-D-mannosamine and its metabolites in a liver homogenate. The incubation time was 10 min. The separation of the deproteinized supernatants was performed in a solvent system consisting of *n*-propanol:sodium acetate:water. For further details see Materials and methods. (**b**) Inhibition of *N*-acetylmannosamine kinase activity by Zn^{2+} (1 mmol l^{-1}).

enzymatic steps, which could easily be checked and quantitated by a simple chromatographic procedure. The enzymes involved are *N*-acetylglucosamine kinase, UDP-*N*-acetylglucosamine-2′-epimerase and *N*-acetylmannosamine kinase. Under the conditions employed, Zn^{2+} was the most potent and universal inhibitor of the activities of all three enzymes, followed by Cu^{2+}, Cd^{2+} and Co^{2+}.

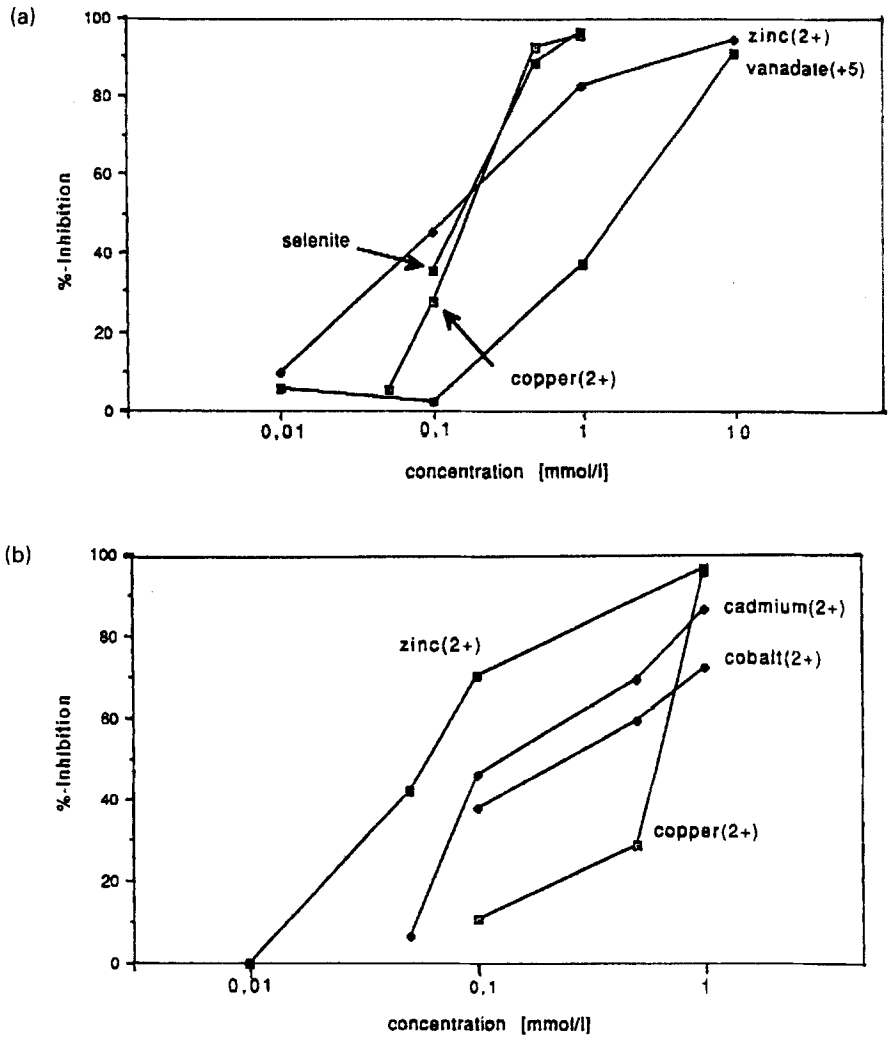

Figure 5. (**a**) UDP-*N*-Acetylglucosamine-2′-epimerase: concentration dependent inhibition by Zn^{2+}, Cu^{2+}, selenite or vanadate, respectively. Incubation time was 10 min. For further details see Materials and methods section and legend to Figure 3. (**b**) Concentration dependent inhibition of *N*-acetylmannosamine kinase by the bivalent cations (Zn^{2+}, Cu^{2+}, Co^{2+} and Cd^{2+}). Incubation time was 10 min. For further details see Materials and methods and legend to Figure 4.

By pretreatment of homogenate with *p*-chloromercuriphenylsulfonic acid the enzyme activities were reduced, revealing the presence of sulfhydryl groups in all three enzymes investigated (Riordan & Vallee 1972). Since *N*-ethylmaleimide did not influence the activity of these enzymes it is concluded that these sulfhydryl groups are not accessible to this reagent under the conditions used.

It is known that some of the ions investigated interact with sulfhydryl-containing compounds. It is noteworthy that *N*-acetylmannosamine kinase is more sensitive towards these metal ions than *N*-acetylglucosamine kinase, suggesting that the former enzyme posses a higher content of cystein or that cystein residues are close to the active center.

However, inhibition may not only be caused by reaction of metal ions with the sulfhydryl groups of an enzyme; it can also be caused by forming an inhibitory active metal–ATP complex which binds in competition with the Mg^{2+}–ATP complex at the enzyme. This could be the case of the inhibition of *N*-acetylmannosamine kinase by Zn^{2+}, Cu^{2+} and Cd^{2+}.

In contrast to the ions described above, selenium can act not only via its anions selenate and selenite, but also by one of its metabolites, i.e. dimethyl selenide (Craig 1986). Furthermore, it is possible that selenium acts as a catalyst on the oxidation of thiol groups of the enzyme followed by a reduced enzymatic activity (Rhead & Schrauzer 1974).

The results obtained are first attempts to reveal the influence of metal ions and selenium compounds on the biosynthesis of *N*-acetylneuraminic acid *in vitro* and are prompting further experiments with isolated enzymes.

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