Biochem. J. (1984) **223**, 323–328
Printed in Great Britain

Subcellular localization and tissue distribution of sialic acid-forming enzymes

N-Acetylneuraminate-9-phosphate synthase and *N*-acetylneuraminate 9-phosphatase

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(*Received 26 March 1984/Accepted 2 July 1984*)

The activities of *N*-acetylneuraminate 9-phosphate synthase and *N*-acetylneuraminate 9-phosphatase, the two enzymes involved in the final steps of the biosynthetic pathway of *N*-acetylneuraminic acid, were measured with the substrates *N*-acetyl[^{14}C]mannosamine 6-phosphate and *N*-acetyl[^{14}C]neuraminic acid 9-phosphate respectively. Subcellular localization studies in rat liver indicated that both enzymes are localized in the cytosolic fraction after homogenization in sucrose medium. To test the possibility of misinterpretation due to the hydrolysis of *N*-acetylneuraminic acid 9-phosphate by non-specific phosphatases, the hydrolysis of various phosphate esters by the cytosolic fraction was tested. Only *p*-nitrophenyl phosphate was hydrolysed; however, competition studies with *N*-acetylneuraminic acid 9-phosphate and *p*-nitrophenyl phosphate indicated that two different enzymes were involved and that no competition existed between the two substrates. In various other rat tissues *N*-acetylneuraminate-9-phosphate synthase and *N*-acetylneuraminate 9-phosphatase activities were detected, suggesting that *N*-acetylmannosamine 6-phosphate is a general precursor for *N*-acetylneuraminic acid biosynthesis in all the tissues studied.

The generally accepted pathway of NeuAc biosynthesis starting from UDP-GlcNAc is completed in four steps:

$$\text{UDP-GlcNAc} \xrightarrow{1} \text{ManNAc} \xrightarrow{2} \text{ManNAc-6-}P \xrightarrow{3} \text{NeuAc-9-}P \xrightarrow{4} \text{NeuAc}$$

(Corfield & Schauer, 1982). Recently we have reported that the first two enzymes in the pathway, UDP-GlcNAc 2-epimerase (UDP-2-acetamido-2-deoxy-D-glucose 2-epimerase, EC 5.1.3.14) and ManNAc kinase (ATP:2-acetamido-2-deoxy-D-mannose 6-phosphotransferase, EC 2.7.1.60) are found in the cytosolic fraction after differential centrifugation of rat liver homogenate in sucrose medium (Van Rinsum *et al.*, 1983). In addition, other experiments have indicated that newly synthesized NeuAc is channelled to the activating enzyme CMP-NeuAc synthase (Ferwerda *et al.*, 1983). Towards an understanding of the cellular organization of CMP-NeuAc synthesis and the interrelationship between the NeuAc-synthesizing enzymes and the nuclear localized activating enzyme, we determined the subcellular localization of the final two enzymes for NeuAc biosynthesis, NeuAc-9-*P* synthase [*N*-acetylneuraminate-9-phosphate pyruvate-lyase (pyruvate phosphorylating), EC 4.1.3.20] and NeuAc 9-phosphatase (*N*-acetylneuraminate-9-phosphate phosphohydrolase, EC 3.1.3.29) in rat liver by using radioactive substrates. In earlier investigations NeuAc synthesis was measured colorimetrically in crude tissue fractions with ManNAc-6-*P* as substrate, and no distinction was made between the products NeuAc-9-*P* and NeuAc (Joseph & Bachhawat, 1964; Watson *et al.*, 1966). NeuAc-9-*P* formation

Abbreviations used: GlcN, glucosamine; ManN, mannosamine; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; NeuAc, *N*-acetylneuraminic acid; *P*, phosphate; Teb, triethylammonium bicarbonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

has only been detected with purified enzymes from liver and salivary glands (Warren & Felsenfeld, 1961*b*, 1962; Roseman *et al.*, 1961; Watson *et al.*, 1966). In the present paper we report the presence of NeuAc-9-*P* synthase activity in various non-glycoprotein-secreting tissues and also the activity of the specific phosphatase NeuAc 9-phosphatase, which has only been detected in rat liver (Warren & Felsenfeld, 1962) and in human erythrocyte lysates (Jourdian *et al.*, 1964; Watson *et al.*, 1966).

Materials and methods

The following materials were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.: *N*-acetylmannosamine, ATP (disodium salt) and acid phosphatase from potatoes. Hexokinase (yeast), phosphoenolpyruvate, NAD^+, $NADP^+$, *p*-nitrophenyl phosphate and alkaline phosphatase (calf intestine) were obtained from Boehringer Mannheim G.m.b.H., Mannheim, Germany. *N*-Acetyl-D-[U-^{14}C]mannosamine (sp. radioactivity 254Ci/mol) was a product of The Radiochemical Centre, Amersham, Bucks., U.K., and its contamination with *N*-acetyl-D-[U-^{14}C]glucosamine was checked by high-voltage electrophoresis. We only used batches that contained 1% or less GlcNAc. *N*-Acetyl[4-^{14}C]neuraminic acid (sp. radioactivity 4Ci/mol) was obtained from New England Nuclear, Boston, MA, U.S.A. Dowex AG1 (X8; 100–200 mesh; Cl^- form) was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A., and was converted into the formate or the HCO_3^- form as recommended by the manufacturer. All other chemicals were obtained from commercial sources and were of analytical grade.

Adult male rats (Wistar strain), fed *ad libitum* and weighing 175–200g, were used. They were killed by decapitation, always occurring between 09:00 and 10:00h.

Preparation of the substrates

[^{14}C]ManNAc-6-*P* was prepared from [^{14}C]ManNAc (sp. radioactivity 0.1Ci/mol) in succession by de-*N*-acetylation in 1M-HCl at 80°C for 2h, neutralization with KOH, phosphorylation with hexokinase (Jourdian & Roseman, 1962) and re-*N*-acetylation (Distler *et al.*, 1958). The reaction mixture was applied to a Dowex AG1 (X8; 100–200 mesh; formate form) column (23.0cm × 0.9cm) followed by 30ml of water and a wash of 30ml of 0.5M-formic acid. [^{14}C]ManNAc-6-*P* was eluted with 30ml of 4M-formic acid and was analysed by high-voltage electrophoresis before and after treatment with potato acid phosphatase in 0.1M-sodium acetate buffer, pH4.8. The yield of [^{14}C]ManNAc-6-*P* varied between 40 and 60%. The batches contained variable amounts of impurities, which were detected by high-voltage electrophoresis and which were responsible for a high background activity in our enzyme assays. Batches containing considerable amounts of impurities were purified additionally on a Dowex AG1 (X8; 100–200 mesh; HCO_3^- form) column (100cm × 0.5cm), eluted with a linear concentration gradient of 0.005M- to 0.35M-Teb buffer, pH7.8. [^{14}C]ManNAc-6-*P* was eluted at 0.075–0.100M-Teb buffer. We only used [^{14}C]ManNAc-6-*P* batches that contained less than 5% impurities.

[^{14}C]NeuAc-9-*P* was prepared enzymically from [^{14}C]ManNAc. The composition of the incubation mixture (25ml) was the same as described from the NeuAc-9-*P* synthase assay (see below), only with 2mM-[^{14}C]ManNAc (sp. radioactivity 0.05Ci/mol) as substrate. The enzyme source was a 10800000*g*-min supernatant of a 30% (w/v) rat liver homogenate in water. The mixture was incubated at 37°C for 2h and the reaction was stopped by heating at 100°C for 3min. The mixture was centrifuged, the pellet was washed three times with water and the combined supernatants were dialysed against 5mM-Teb buffer, pH7.8. The diffusate was applied to a Dowex AG1 (X8; 100–200 mesh; HCO_3^- form) column (100cm × 0.5cm) and the products were eluted with a linear concentration gradient of 0.005M- to 0.35M-Teb buffer, pH7.8. [^{14}C]NeuAc-9-*P* was eluted at 0.25–0.35M-Teb buffer; the yield was 13–15%. The purity of [^{14}C]NeuAc-9-*P* was checked by the thiobarbituric acid assay (Warren, 1959) before and after treatment with potato acid phosphatase. The dephosphorylated product was co-eluted with NeuAc from a Dowex AG1 (X8; 100–200 mesh; HCO_3^- form) column (9.0cm × 0.5cm) and had the R_F value of NeuAc after high-voltage electrophoresis.

Preparation of subcellular rat liver fractions and rat tissue fractions

The subcellular rat liver fractions and the soluble cell fractions of various rat tissues were prepared as described previously (Van Rinsum *et al.*, 1983), except that homogenization was always performed with a Potter–Elvehjem homogenizer with a clearance of 0.25mm.

Enzyme assays

The incubation system NeuAc-9-*P* synthase was a modification of the system described by Warren & Glick (1966) and consisted of 0.1M-Tris/acetic acid buffer, pH7.6, 2mM-[^{14}C]ManNAc-6-*P* (sp. radioactivity 0.1Ci/mol), 8mM-phosphoenolpyruvate, 2mM-ATP, 0.1mM-NAD^+, 0.1mM-$NADP^+$, 12mM-magnesium acetate, 25mM-NaF and 100μl of tissue fraction (2–4mg of protein) in a total volume of 250μl. In some assays NaF was omitted (see the Results section). Control

incubation mixtures lacked the tissue fraction. The mixtures were incubated at 37°C for 1h and the reaction was stopped by heating at 100°C for 3min. Separation of substrate and products was achieved on Dowex AG1 (X8; 100–200 mesh; HCO_3^- form) column (4.0cm × 0.5cm) eluted with various concentrations of Teb buffer, pH7.8, as described previously (Van Rinsum *et al.*, 1983). NeuAc-9-*P* synthase activity was calculated from the radioactivity (d.p.m.) measured in the NeuAc-9-*P* fraction (eluted with 1000mM-Teb buffer), or from the radioactivity (d.p.m.) measured in the NeuAc-9-*P* + NeuAc fraction (eluted with 40mM-Teb buffer) when no NaF had been added to the incubation mixture.

The incubation system for NeuAc-9-phosphatase consisted of 0.1M-Tris/HCl, pH7.2, 2mM-[^{14}C]NeuAc-9-*P* (sp. radioactivity 0.05Ci/mol) and 20μl of tissue fraction (0.4–0.8mg of protein) in a total volume of 100μl. Control incubation mixtures lacked the tissue fraction. The mixtures were incubated at 37°C for 30min and the reaction was stopped by heating at 100°C for 3min. Separation of substrate and product was achieved as described for the NeuAc-9-*P* synthase assay. NeuAc 9-phosphatase activity was calculated from the radioactivity (d.p.m.) measured in the NeuAc fraction eluted with 40mM-Teb buffer. No other products were formed under these assay conditions.

The dephosphorylating activity in tissue fractions towards Glc-6-*P*, β-glycerophosphate and *p*-nitrophenyl phosphate was determined under the same conditions as used for the determination of NeuAc 9-phosphatase activity only with 2mM-Glc-6-*P*, 2mM-β-glycerophosphate or 5mM-*p*-nitrophenyl phosphate as substrate. The dephosphorylation of Glc-6-*P* and β-glycerophosphate was stopped by adding 200μl of 10% (w/v) trichloroacetic acid. The mixture was centrifuged and the supernatant was assayed for P_i by the method of Ames (1966).

The dephosphorylation of *p*-nitrophenyl phosphate was stopped by adding 2ml of 0.02M-NaOH. The phosphatase activity was calculated from the absorbance at 405nm of the total reaction mixture.

One unit of enzyme activity was defined as the amount of protein catalysing the formation of 1μmol of product/min at 37°C.

Analytical methods

High-voltage electrophoresis was performed in a 26mM-sodium tetraborate solution, pH9, at 5–10°C on Whatman 3MM paper strips, wet with a 26mM-sodium tetraborate solution, pH8, containing 5mM-$MgCl_2$, with 50V/cm for 90min and with Bromophenol Blue as a marker. Radioactivity on the strips was counted as described previously (Van Rinsum *et al.*, 1983). Under these conditions the following R_F values were found: GlcNAc and GlcN, 0.02; ManN, 0.25; ManNAc, 0.50; NeuAc, 0.70; NeuAc-9-*P*, GlcNAc-6-*P*, GlcN-6-*P* and ManN-6-*P*, 0.96; ManNAc-6-*P* and GlcNAc-1-*P*, 1.04.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin solutions in 0.25M-sucrose as standards. Radioactivity was determined with a Berthold liquid-scintillation counter (BF 8000), equipped with an automatic external standard for calucation of the d.p.m. The scintillation fluid used was Maxifluor A (Baker Chemicals BV, Deventer, The Netherlands).

Results

Subcellular localization

Subcellular rat liver fractions were prepared by differential centrifugation. According to the distribution of biochemical markers, fraction 1 consisted mainly of nuclei, fraction 2 contained mitochondria with some plasma-membrane and nuclear contamination, fraction 3 consisted of lysosomes and some rough endoplasmic reticulum, fraction 4 contained rough and smooth endoplasmic reticulum, and fraction 5 was free of subcellular particles and represented the soluble cell fraction (Van Rinsum *et al.*, 1983). The NeuAc-9-*P* synthase and NeuAc 9-phosphatase activities were predominantly found in the soluble cell fraction, as shown in Fig. 1.

The NeuAc-9-*P* synthase activity was determined in the presence of NaF to protect the product and the substrate against specific and non-specific phosphatase activities.

To investigate the effect of NaF on NeuAc-9-*P* synthase we tested liver soluble cell fraction with increasing concentrations of [^{14}C]ManNAc-6-*P* in the presence and in the absence of NaF (results not shown). In the presence of NaF no free NeuAc could be detected, whereas in the absence of NaF almost all NeuAc-9-*P* was converted into NeuAc. From reciprocal plots the K_m values for ManNAc-6-*P* were determined to be 2.1×10^{-4}M and 2.5×10^{-4}M in the presence and in the absence of NaF respectively. However, NaF inhibited the NeuAc-9-*P* formation (see under 'Tissue distribution' below).

The dephosphorylation of NeuAc-9-*P* measured in the liver soluble cell fraction (Fig. 1) could have been due to non-specific phosphatase activity. Therefore we tested the dephosphorylation of Glc-6-*P*, β-glycerophosphate and *p*-nitrophenyl phosphate under the same experimental conditions as for NeuAc 9-phosphatase (Table 1). It appeared that fractions that exhibited dephosphorylating activity towards Glc-6-*P* and β-glycerophosphate dephosphorylated NeuAc-9-*P* hardly or not at all.

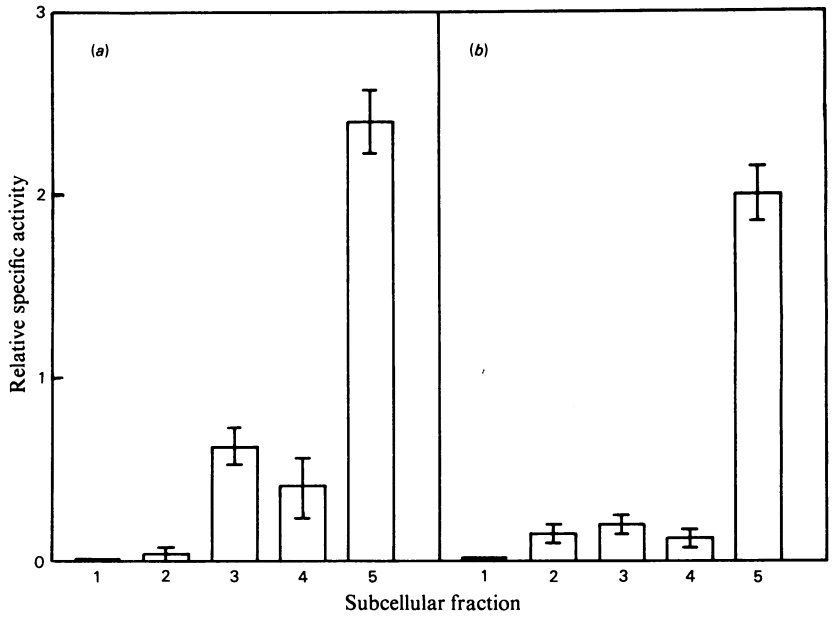

Fig. 1. *Subcellular distribution of NeuAc-9-P synthase (a) and NeuAc 9-phosphatase (b) in rat liver*
For the preparation of the cellular subfractions and the enzyme assays see the Materials and methods section. Enzyme activities are given as specific activities relative to the cell-free homogenate, and the values are expressed as means ± S.E.M. (represented by bars) for three separate experiments. The fractions were: 1, nuclei; 2, mitochondria; 3, lysosomes and rough endoplasmic reticulum; 4, microsomal fraction; 5, soluble cell fraction. The means of the total enzyme activity recovered from the homogenates were 130% and 64% for NeuAc-9-*P* synthase and NeuAc 9-phosphatase respectively.

Table 1. *Dephosphorylating activity in rat liver cellular subfractions towards some phosphate esters under identical experimental conditions*
Cellular subfractions were prepared and enzyme activities were determined as described in the Materials and methods section. Enzyme activities are given as specific activities of the fractions relative to the homogenate. The values are expressed as means ± S.E.M. for three different experiments. The fractions were: 1, nuclei; 2, mitochondria; 3, lysosomes and rough endoplasmic reticulum; 4, microsomal fraction; 5, soluble cell fraction.

Substrate	Relative specific activity				
Subcellular fraction ...	1	2	3	4	5
[^{14}C]NeuAc-9-*P*	0	0.09 ± 0.01	0.14 ± 0.03	0.16 ± 0.03	1.9 ± 0.1
Glc-6-*P*	0.8 ± 0.5	0.5 ± 0.1	2.8 ± 0.1	3.0 ± 0.1	0
β-Glycerophosphate	0.4 ± 0.4	0.8 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	0
p-Nitrophenyl phosphate	0.2 ± 0.1	0.7 ± 0.1	1.8 ± 0.2	1.2 ± 0.1	0.9 ± 0.1

The liver soluble cell fraction did not dephosphorylate Glc-6-*P* or β-glycerophosphate, but dephosphorylated only *p*-nitrophenyl phosphate in addition to NeuAc-9-*P*. Other phosphate esters, such as ATP, ADP, CTP, CMP, phosphoenolpyruvate, GlcN-6-*P*, GlcNAc-1-*P* and ManNAc-6-*P*, were not dephosphorylated by the liver soluble cell fraction or only to a very small extent (results not shown).

To examine whether NeuAc-9-*P* and *p*-nitrophenyl phosphate were dephosphorylated by two different enzymes or by one single enzyme acting on both substrates, competition studies were carried out with liver soluble cell fraction. In this fraction the determined K_m values for NeuAc-9-*P* and *p*-nitrophenyl phosphate were 1.4×10^{-3} M and 1.1×10^{-3} M respectively. From these data it is possible to calculate to what extent dephosphorylation of NeuAc-9-*P* and *p*-nitrophenyl phosphate can be expected when both substrates are dephosphorylated simultaneously either in the case where one enzyme is acting or in the case of two enzymes

Table 2. *Theoretical and experimental dephosphorylating activity of rat liver soluble cell fraction towards [^{14}C]NeuAc-9-P and p-nitrophenyl phosphate*

Liver soluble cell fraction was incubated simultaneously with 1.6mM-[^{14}C]NeuAc-9-*P* and 1.6mM-*p*-nitrophenyl phosphate (Expt. I) or 3.2mM-[^{14}C]NeuAc-9-*P* and 3.2mM-*p*-nitrophenyl phosphate (Expt. II). The incubation conditions were as described in the Materials and methods section. In the experiments the dephosphorylation of both substrates was measured by determination of the products NeuAc and *p*-nitrophenol formed. For NeuAc-9-*P* and *p*-nitrophenyl phosphate the K_m values were 1.4×10^{-3}M and 1.1×10^{-3}M respectively and the $V_{max.}$ values were 14.2 and 23.7munits/mg of protein respectively. The theoretical dephosphorylation was calculated from these data for two independent phosphatases (*a*) and for one single phosphatase acting on both substrates (*b*) according to the method of Dixon & Webb (1958).

		Product formed (munits/mg of protein)		
		Theoretical		
Expt. no.	Substrate	(*a*)	(*b*)	Experimental
I	[^{14}C]NeuAc-9-*P*	7.6	4.5	7.3
	p-Nitrophenyl phosphate	14.0	9.6	18.3
II	[^{14}C]NeuAc-9-*P*	9.9	5.2	8.4
	p-Nitrophenyl phosphate	17.6	11.1	23.5

Table 3. *NeuAc-9-P synthase and phosphatase activities in the soluble cell fraction of various rat tissues*

NeuAc-9-*P* synthase activity was measured in the presence and in the absence of NaF. For purposes of comparison with NeuAc 9-phosphatase activity, the non-specific phosphatase activity was measured with *p*-nitrophenyl phosphate. For experimental details see the Materials and methods section. Enzyme activities are given in munits/mg of protein and are expressed as means ± S.E.M. for three different experiments. Abbreviation: N.D., not determined.

	NeuAc-*P* synthase		Phosphatase	
Tissue	+ NaF	− NaF	[^{14}C]NeuAc-9-*P*	*p*-Nitrophenyl phosphate
Liver	0.47 ± 0.06	0.63 ± 0.03	7.9 ± 1.0	14.7 ± 3.2
Kidney	0.43 ± 0.06	0.62 ± 0.07	4.2 ± 0.6	22.5 ± 4.1
Spleen	0.52 ± 0.06	N.D.	8.1 ± 0.9	26.5 ± 3.6
Brain	0.40 ± 0.05	0.63 ± 0.04	7.9 ± 1.6	18.6 ± 1.2
Lung	0.18 ± 0.05	N.D.	1.4 ± 0.2	11.5 ± 0.9
Muscle	0.02 ± 0.01	N.D.	3.5 ± 1.1	7.9 ± 2.0
Erythrocytes	0.09 ± 0.03	N.D.	1.2 ± 0.1	13.7 ± 1.7
Intestinal mucosa	0.29 ± 0.05	0.84 ± 0.08	5.1 ± 0.2	14.0 ± 0.3
Salivary gland	1.70 ± 0.39	N.D.	5.4 ± 0.7	14.3 ± 1.0
Pancreas	0.18 ± 0.03	N.D.	1.6 ± 0.4	9.6 ± 1.6
Thymus	0.32 ± 0.04	N.D.	N.D.	N.D.

(Dixon & Webb, 1958). The experimental results are very similar to the theoretical values calculated for two independent enzymes (Table 2).

Tissue distribution

The distribution of NeuAc-9-*P* synthase and NeuAc 9-phosphatase in various rat tissues is presented in Table 3. Both enzymes appeared to be detectable in all tissues tested. As was reported for liver, NaF also inhibited the NeuAc-9-*P* formation in kidney, brain and intestinal mucosa. To measure non-specific phosphatase activity in the soluble cell fractions of the various tissues, these fractions were incubated with Glc-6-*P*, β-glycerophosphate and *p*-nitrophenyl phosphate. As was found for the liver soluble cell fraction, the soluble cell fractions of the various tissues dephosphorylated *p*-nitrophenyl phosphate (Table 3) but not Glc-6-*P* and β-glycerophosphate (results not shown).

Discussion

Previously NeuAc-9-*P* synthase and NeuAc 9-phosphatase activities have been assayed simultaneously in crude tissue fractions by the determination of NeuAc formation (Warren & Felsenfeld, 1961*a*; Joseph & Bachhawat, 1964; Watson *et al.*, 1966; Kikuchi *et al.*, 1971; Okamoto & Akamatsu, 1980). We assayed NeuAc-9-*P* synthase in the presence of NaF, thus inhibiting NeuAc 9-phosphatase and non-specific phosphatases. However, NaF suppressed NeuAc-9-*P* formation, by extents varying from 25 to 65% (Table 3). We do not know

whether this inhibition is caused by a direct effect on the synthase or by inhibition of removal of phosphate from phosphoenolpyruvate during the synthase reaction, or simply by product inhibition. The K_m values of the synthase for ManNAc-6-*P*, 2.1×10^{-4}–2.5×10^{-4} M, are similar to those reported by Warren & Felsenfeld (1962) and Joseph & Bachhawat (1964). The activity of the synthase found in the soluble cell fraction of rat liver, measured in the absence of NaF (Table 3), agrees with the activities found by Kikuchi *et al.* (1971) and Okamoto & Akamatsu (1980) in liver, but the activities of the synthase in other tissues are lower than reported by Watson *et al.* (1966).

In our subcellular-localization studies with rat liver we found the highest NeuAc 9-phosphatase activity in the soluble cell fraction (Fig. 1), and no phosphatase activity towards other phosphate esters was detected except towards *p*-nitrophenyl phosphate (Tables 1 and 3). In this fraction NeuAc-9-*P* and *p*-nitrophenyl phosphate were dephosphorylated by two different enzymes each specific for its own substrate (Table 2). This agrees with the finding by Jourdian *et al.* (1964), who reported that partially purified NeuAc 9-phosphatase is unable to hydrolyse *p*-nitrophenyl phosphate. The K_m value of NeuAc 9-phosphatase for NeuAc-9-*P*, 1.4×10^{-3} M, is the same as reported by Jourdian *et al.* (1964). So it is concluded that the values obtained for NeuAc 9-phosphatase activity in liver soluble cell fraction are reliable. For soluble cell fractions of other tissues no correlation appeared to exist between dephosphorylating activity towards NeuAc-9-*P* and *p*-nitrophenyl phosphate (Table 3). So it seems likely that true NeuAc 9-phosphatase activities were measured in the soluble cell fractions of all tissues. The presence of NeuAc-9-*P* synthase, NeuAc 9-phosphatase (Fig. 1) and other NeuAc-precursor-forming enzymes (Van Rinsum *et al.*, 1983) in the liver soluble cell fraction indicates that NeuAc biosynthesis is a cytosolic event.

Our tissue-distribution studies confirm the view that in all tissues the second part of the synthesis of NeuAc is catalysed by the same set of enzymes. However, the activity of the first two enzymes in the synthetic pathway, UDP-GlcNAc 2-epimerase and ManNAc kinase, were below the detectable level in non-glycoprotein-secreting tissues (Van Rinsum *et al.*, 1983). These results were not due to the presence of inhibitors, because the activities of these enzymes in the liver soluble cell fraction were not affected by the addition of soluble cell fractions of non-glycoprotein-secreting tissues (J. Van Rinsum, W. Van Dijk, G. J. M. Hooghwinkel & W. Ferwerda, unpublished work). ManNAc-6-*P* might be synthesized in these tissues by direct epimerization of GlcNAc-6-*P*, although the enzyme involved in this reaction has been detected only in bacteria (Ghosh & Roseman, 1965). Another possibility is that the synthesis rate of ManNAc-6-*P* is low in non-secreting tissues and hardly measurable *in vitro*.

We thank Mrs. C. A. R. L. Govers for her technical assistance, and we are indebted to Dr. A. P. Corfield (Department of Medicine, University of Bristol, Bristol, U.K.) for critical reading of the manuscript.

References

Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118

Corfield, A. P. & Schauer, R. (1982) in *Sialic Acids: Chemistry, Metabolism and Function* (*Cell Biology Monographs*, vol. 10) (Schauer, R., ed.), pp. 195–261, Springer-Verlag, Vienna

Distler, J. J., Merrick, J. M. & Roseman, S. (1958) *J. Biol. Chem.* **230**, 497–509

Dixon, M. & Webb, E. C. (1958) *Enzymes*, 1st edn., pp. 91–96, Longmans, London

Ferwerda, W., Blok, C. M. & Van Rinsum, J. (1983) *Biochem. J.* **216**, 87–92

Ghosh, S. & Roseman, S. (1965) *J. Biol. Chem.* **240**, 1525–1530

Joseph, R. & Bachhawat, B. K. (1964) *J. Neurochem.* **11**, 517–526

Jourdian, G. W. & Roseman, S. (1962) *Biochem. Prep.* **9**, 44–47

Jourdian, G. W., Swanson, A. L., Watson, D. & Roseman, S. (1964) *J. Biol. Chem.* **239**, PC2714–PC2716

Kikuchi, K., Kikuchi, H. & Tsuiki, S. (1971) *Biochim. Biophys. Acta* **252**, 357–368

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Okamoto, Y. & Akamatsu, N. (1980) *Biochem. J.* **188**, 905–911

Roseman, S., Jourdian, G. W., Watson, D. & Rood, R. (1961) *Proc. Natl. Acad. Sci. U.S.A.* **47**, 958–961

Van Rinsum, J., Van Dijk, W., Hooghwinkel, G. J. M. & Ferwerda, W. (1983) *Biochem. J.* **210**, 21–28

Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975

Warren, L. & Felsenfeld, H. (1961*a*) *Biochem. Biophys. Res. Commun.* **4**, 232–235

Warren, L. & Felsenfeld, H. (1961*b*) *Biochem. Biophys. Res. Commun.* **5**, 185–190

Warren, L. & Felsenfeld, H. (1962) *J. Biol. Chem.* **237**, 1421–1431

Warren, L. & Glick, M. C. (1966) *Methods Enzymol.* **8**, 131–133

Watson, D. R., Jourdian, G. W. & Roseman, S. (1966) *J. Biol. Chem.* **241**, 5627–5636