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BIOSYNTHESIS OF RESPIRATORY TRACT MUCINS

III. METABOLISM OF AMINOSUGARS BY TRACHEAL MUCOSAL EXTRACTS*

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SUMMARY

1. The activities of enzymes involved in the metabolism of aminosugars were measured in a particle-free enzyme preparation obtained from bovine tracheal mucosa.

2. This crude extract was capable of catalyzing the series of reactions involving *N*-acetylmannosamine and leading to the formation of cytidine monophospho-sialic acid, the immediate precursor of the sialic acid residues of mucin glycoproteins.

3. Uridine diphospho-*N*-acetylglucosamine 2'-epimerase, the enzyme that catalyzes the *de novo* synthesis of *N*-acetylmannosamine, and cytidine monophospho-sialic acid synthetase were further purified for kinetic studies.

INTRODUCTION

The epithelial surface of the mammalian respiratory tract is protected by a mucous secretion whose major macromolecular components are glycoproteins[1]. This secretion is produced by the epithelial goblet cells and the tracheobronchial mucous glands found in the cartilage-containing airways[2]. Little is known about the enzyme activities of these mucus-producing structures. The information that is available has been mainly derived from histochemical studies. While the presence of several hydrolytic enzymes has been reported in bronchial epithelium[3–5], knowledge of biosynthetic enzymes is minimal[6,7].

N-Acetylhexosamines and sialic acids are important constituents of the oligosaccharide side chains of tracheobronchial mucin glycoproteins[8]. CMP-*N*-acetylneuraminic acid (CMP-NANA), the immediate precursor of sialic acid in glycoproteins, is formed by a series of reactions from UDP-*N*-acetylglucosamine (UDP-GlcNAc). A

Abbreviations: GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; NANA, *N*-acetylneuraminic acid; PEP, phosphoenolypyruvate.

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soluble enzyme system has been prepared from bovine tracheal mucosal scrapings that is able to phosphorylate *N*-acetylhexosamines, and catalyze the condensation of *N*-acetylmannosamine 6-phosphate (ManNAc-6-*P*) with phosphoenolypyruvate (PEP) to form sialic acids. UDP-GlcNAc 2'-epimerase and CMP-NANA synthetase were also shown to be present in these extracts.

This report describes these various investigations in which the activities of enzymes involved in the utilization of aminosugars were measured in tracheobronchial tissue extracts. The regulation of tracheal L-glutamine:D-fructose-6-phosphate aminotransferase (EC 2.6.1.16), a key enzyme in the synthesis of glucosamine, has recently been described[6]. This work is part of a broader study concerned with the biosynthesis of respiratory tract mucins[6,7,9] that hopefully will provide insight into the pathogenesis of obstructive lung disease states.

MATERIALS AND METHODS

Chemicals

Glucosamine, mannosamine, GlcNAc, ManNAc, $NADP^+$ and dithiothreitol were purchased from Sigma Chemical Co. UDP-GlcNAc and PEP were obtained from Boehringer Mannheim Corp. ATP, CTP and uridine from P-L Biochemicals, and NANA from Pfanstiehl Laboratories. [1-^{14}C]Acetic anhydride and UDP-*N*-acetyl[1-^{14}C]-glucosamine was purchased from New England Nuclear Corp. *N*-Acetyl[1-^{14}C]-mannosamine and N-acetyl[1-^{14}C]glucosamine were prepared by the N-acetylation of the free hexosamines with [1-^{14}C]acetic anhydride. DEAE-cellulose (Whatman DE-32) was obtained from Reeve Angel and precycled as described by the manufacturers. Enzyme grade $(NH_4)_2SO_4$ and polymyxin sulfate were purchased from Mann Research Laboratories, and calcium phosphate gel from Nutritional Biochemicals. All other chemicals were of the highest commercial quality available.

Particle-free tracheal mucosal enzyme preparations

All operations were conducted at 4 °C. The mucosal linings of fresh bovine tracheas, obtained from a local slaughter house and kept on ice until used, were removed by scraping with a scalpel. For the preparation of enzymes involved in the phosphorylation of *N*-acetylhexosamines, the formation of NANA and CMP-NANA, scrapings were homogenized in 2 vol. of ice-cold water in a Dounce homogenizer. When UDP-GlcNAc 2'-epimerase was the enzyme under study, the scrapings were homogenized with 2 vol. of ice-cold 5 mM potassium phosphate buffer pH 7.5, containing 2 mM EDTA and 0.5 mM uridine in a Waring blender for 1 min. The homogenates were centrifuged for 1 h at 105 000 × *g*. The clear supernatants were used as the enzyme preparations. UDP-GlcNAc 2'-epimerase was further purified through the $(NH_4)_2SO_4$ state essentially by the method described for purification of the liver enzyme[6,11]. All solutions used in the fractionation procedure contained 2 mM EDTA and 0.5 mM uridine. Prior to use, the $(NH_4)_2SO_4$ precipitated enzyme was taken up in a small volume of phosphate buffer containing 0.1 mM dithiothreitol (pH 7.5) and dialyzed for 2 h against the same buffer.

Assay of UDP-N-acetylglucosamine 2'-epimerase

The standard assay mixture was as follows: 0.5 μmole of UDP-GlcNAc; 50

μmoles of Tris–HCl buffer (pH 7.5); 12.5 μmoles $MgSO_4$ and 0.1 ml of the mucosal supernatant fraction in a total volume of 0.25 ml. Incubation was carried out at 37 °C for 20 min and the reaction was stopped by boiling for 2 min. After centrifugation, aliquots of the supernatant were used for the colorimetric determination of ManNAc[11]. Controls consisted of incubation mixtures lacking the supernatant fraction, or containing heat-inactivated supernatant. Kinetic studies with the purified form of this enzyme were carried out using a radioactive assay. The incubation mixtures contained in a volume of 0.1 ml: 200 mM Tris–HCl buffer (pH 7.5); 50 mM $MgSO_4$; 2 mM UDP-*N*-acetyl[1-^{14}C]glucosamine (0.125 mCi/mmole) and the enzyme to be added. Formation of labeled ManNAc was monitored as previously described[10,12]. Radioactivity was estimated with a liquid scintillation counter. Controls consisted of incubation mixtures containing heat-inactivated enzyme.

Phosphorylation of N-acetylhexosamines

The incubation mixtures contained the following components in a final volume of 0.2 ml: 30 μmoles Tris–HCl buffer (pH 7.5), 5 μmoles $MgCl_2$, 1 μmole ATP, 0.25 μmole of GlcNAc or ManNAc and 0.1 ml of the mucosal supernatant.

Assay 1. This method is based on the specific removal of phosphorylated sugars by the addition of $ZnSO_4$ and $Ba(OH)_2$ after enzyme incubation according to the procedure of Somogyi[13]. After incubation for 30 min at 37 °C the reaction was stopped by adding 0.3 ml of 5% $ZnSO_4$ followed by 0.3 ml of 0.15 M $Ba(OH)_2$. After centrifugation to remove protein and esterified *N*-acetylhexosamines an aliquot of the supernatant was taken for the estimation of free *N*-acetylhexosamine. Disappearance of *N*-acetylhexosamine corresponded to the amount phosphorylated. Standards and complete but unincubated mixtures were also assayed in the same way.

Assay 2. As the presence of GlcNAc 2-epimerase[14] in tracheal extracts could result in the formation of GlcNAc-6-*P* from ManNAc, a radioactive assay was used to measure ManNAc-6-*P* formation in the presence of GlcNAc-6-*P*. In this method the rate of phosphorylation was determined with the aid of *N*-acetyl[1-^{14}C]mannosamine as substrate. After incubation the reaction was stopped by heating in a boiling water bath for 2 min. An aliquot of the supernatant was subjected to high voltage paper electrophoresis in 0.05 M phosphate buffer (pH 7.5) at 40 V/cm for 20 min in order to separate the *N*-acetylhexosamines from their phosphate esters[12]. The *N*-acetylhexosamine phosphates were eluted from the paper and dephosphorylated with potato phosphatase[14]. The resulting *N*-acetylhexosamines were then separated by high voltage paper electrophoresis in 0.05 M sodium tetraborate (pH 9.5) at 60 V/cm for 30 min[12,14], and the *N*-acetyl[1-^{14}C]mannosamine determined by liquid scintillation techniques.

Formation of sialic acids

The system contained in a final volume of 2 ml: 150 μmoles Tris–HCl buffer (pH 7.6); 5 μmoles ATP: 4 μmoles PEP; 0.1 μmole $NADP^+$; 30 μmoles $MgCl_2$; 2.5 μmoles of either ManNAc or GlcNAc and 1.5 ml of the mucosal supernatant fraction. Incubation was for 2 h at 37 °C, and aliquots (0.5 ml) were removed at 0, 60 and 120 min. The reaction was terminated by boiling and the sialic acids measured by the thiobarbituric acid method of Warren[15]. The reaction was essentially linear over the 2-h time period.

Assay of CMP-NANA synthetase

Enzyme activity was assayed by measuring the formation of CMP-NANA according to the procedure of Kean and Roseman[16]. CMP-NANA was determined in the presence of NANA by the method of Warren[15] after reduction with sodium borohydride. The standard incubation mixture consisted of 40 μmoles Tris buffer (pH 9.0); 4 μmoles $MgCl_2$; 1 μmole NANA; 1 μmole CTP and 0.05 ml of the mucosal supernatant in a final volume of 0.2 ml. After 30 min at 37 °C the mixture was treated with 3 mg of sodium borohydride in 0.03 ml of cold water. Tubes were shaken well and allowed to stand at room temperature for 15 min. In order to destroy the excess borohydride, 0.04 ml of acetone was added to the mixtures which were shaken and allowed to stand for an additional 15 min, and then analyzed by the thiobarbituric method[15]. Controls consisted of mixtures lacking CTP or containing heat-inactivated supernatant.

Analytical techniques

Protein was determined by the method of Lowry *et al.*[17] using crystalline bovine serum albumin as a standard. Sialic acids were estimated by the thiobarbituric acid assay of Warren[15]. *N*-Acetylhexosamines were measured by a modification[18] of the Morgan–Elson procedure.

Enzyme units

1 unit of enzyme was defined as the amount catalyzing the formation of 1 nmole of product per h.

RESULTS

UDP-GlcNAc 2'-epimerase

This enzyme, responsible for the formation of ManNAc, the first step in the series of reactions leading to the synthesis of sialic acid, was shown to be present in the 105 000 × *g* supernatant fraction of fresh tracheal mucosal scrapings. Although activity was low, approx. 10% of that found in rat liver[19,20], formation of ManNAc by the tracheal supernatants was linear with time and proportional to protein concentration. The product of the reaction which passed through a Dowex-1 (formate) column was indistinguishable from authentic ManNAc by paper chromatography and high-voltage electrophoresis[12].

While the enzyme was extremely unstable, it was purified approx. 10-fold

TABLE I

PURIFICATION OF UDP-GlcNAc 2'-EPIMERASE FROM TRACHEAL MUCOSAL SCRAPINGS

Enzyme units are calculated as nmoles of product formed per h under the standard assay conditions.

Fraction	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor*
Crude extract	804	924	54	100	1
Polymyxin supernatant	625	906	72	98	1.3
0–40% $(NH_4)_2SO_4$	96	1020	534	110	10

(Table I) using 0.5 mM uridine and 0.1 mM dithiothreitol as stabilizing agents. Precipitation and dialysis of the $(NH_4)_2SO_4$ precipitated enzyme with buffers containing dithiothreitol allowed activity to be retained for several days. As shown in Table I, the relatively low activity found in the tracheal supernatant is retained in the $(NH_4)_2SO_4$ precipitate as compared to the purified liver enzyme[10]. Kinetic experiments were carried out with this partially purified $(NH_4)_2SO_4$ precipitated enzyme. The effect of substrate concentration on rate of reaction is shown in Fig. 1. The apparent K_m was calculated to 0.2 mM. The pH dependence of the enzyme was studied using a series of Tris–maleate buffers covering the pH range 6.2–8.5. The tracheal epimerase exhibited a double pH optima of about 7.1 and 8.2 (Fig. 2). These values compare favorably with the K_m and pH optima of the rat liver enzyme[10].

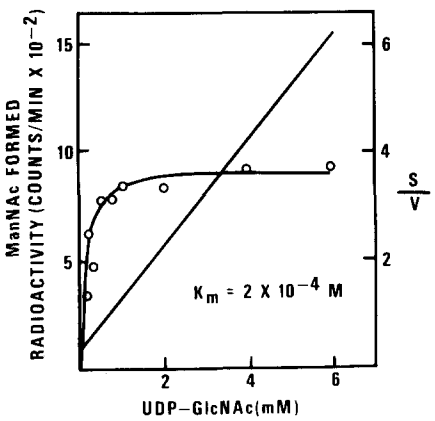

Fig. 1. Effect of substrate concentration on the reaction rate of UDP-GlcNAc 2′-epimerase. The enzyme was assayed under standard conditions except that UDP-GlcNAc was added at the concentrations indicated. K_m was calculated from a computer-generated linear regression analysis of the data.

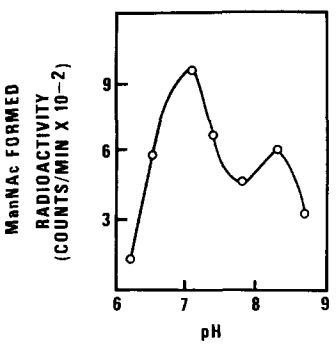

Fig. 2. UDP-GlcNAc 2′-epimerase activity as a function of pH. Composition of the incubation mixtures and assay conditions were the same as described in the text except that each vessel contained 50 μmoles of Tris–maleate buffer at the indicated pH.

Phosphorylation of N-acetylhexosamines

The next reaction in the biosynthesis of sialic acid is the phosphorylation of ManNAc. Both GlcNAc and ManNAc were phosphorylated by tracheal mucosal supernatants in the presence of ATP (Table II). The Somogyi reagents[13] were used to precipitate protein and phosphates after the incubation, and the residual unchanged *N*-acetylhexosamine was measured colorimetrically[18]. The values reported in Table II indicate that tracheal mucosal extracts are capable of phosphorylating *N*-acetylhexosamines at a rate approx. 50% of that of liver preparations[19,20]. It is possible, however, because of the presence of GlcNAc 2-epimerase[14] and GlcNAc kinase in tracheobronchial tissues, GlcNAc-6-*P* might be the end product rather than ManNAc-6-*P*. The assay procedure used in the studies reported in Table II only measures the amount of unchanged *N*-acetylhexosamines remaining at the end of the incubation. It has been reported that extracts prepared from tracheal tissues do not contain ManNAc kinase, while GlcNAc 2-epimerase and GlcNAc kinase were especially active

TABLE II

PHOSPHORYLATION OF *N*-ACETYLHEXOSAMINES BY A PARTICLE FREE PREPARATION OF BOVINE TRACHEAL MUCOSA

Experimental conditions were as described in the text. Units are expressed as nmoles of substrate phosphorylated per h. Results are given as means ± S.D. with number of measurements shown in parentheses.

Substrate	*Activity (units/mg protein)*
N-Acetylglucosamine	192 ± 12 (4)
N-Acetylmannosamine	60 ± 5 (6)

in lung and tracheal mucosa[14]. The presence of these two enzymes results in the formation of GlcNAc-6-*P* from ManNAc.

In a detailed search for ManNAc kinase in tracheal tissue, high-voltage paper electrophoresis and *N*-acetyl[1-^{14}C]mannosamine were used to measure ManNAc-6-*P* formation in the presence of GlcNAc-6-*P*. Fig. 3 demonstrates that while ManNAc is readily converted to GlcNAc-6-*P* by the trachea, sufficient ManNAc-6-*P* is formed to account for the formation of sialic acid from ManNAc (Table III). The enzyme responsible for the formation of ManNAc-6-*P* was exceedingly labile, activity being lost when tracheal scrapings were homogenized too vigorously. This lability of the tracheal enzyme is presumably the reason that extracts of a number of tissues, capable of synthesizing sialic acid containing polymers, were reported to have no detectable ManNAc kinase in the soluble protein fraction[14].

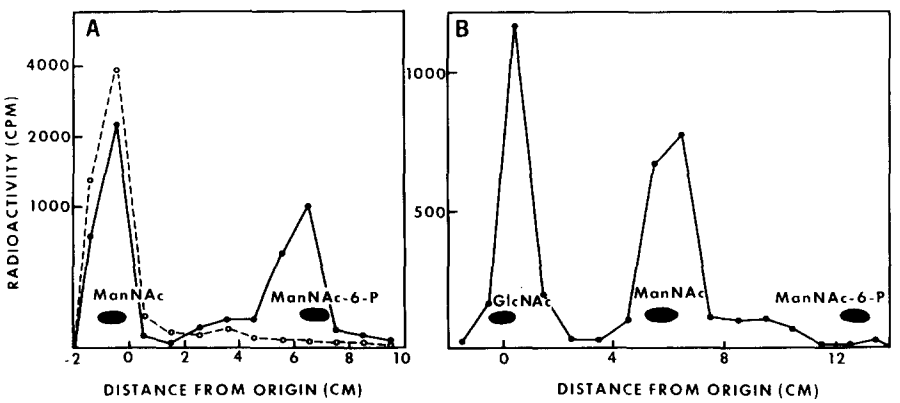

Fig. 3. Assay for the formation of ManNAc-6-*P*. A. Separation of *N*-acetylhexosamines and their phosphate esters by electrophoresis in 0.05 M phosphate buffer (pH 7.5), at 40 V/cm. An aliquot of the incubation mixture was streaked across a Whatman No. 3MM paper and subjected to electrophoresis for 20 min. – – –, radioactivity at 0 time; ———, following 30 min incubation at 37 °C with tracheal extract. B. Separation of GlcNAc from ManNAc by electrophoresis using a 0.05 M borate buffer, (pH 9.5), at 60 V/cm. The phosphorylated sugars isolated in A were eluted from the paper, lyophilized and then dephosphorylated with potato phosphatase[14]. Suitable aliquots were subjected to electrophoresis for 30 min. Radioactive areas were detected by sectioning a strip of the electrophoretogram and measuring radioactivity with a liquid scintillation counter. The shaded areas represent the distance moved by standards in the two systems.

TABLE III

FORMATION OF SIALIC ACIDS BY A PARTICLE FREE PREPARATION FROM BOVINE TRACHEAL MUCOSA

Values are given in nmoles of sialic acid formed per mg protein per h ± S.D. with number of experiments in parentheses.

Substrate	*Sialic acid formed*
N-Acetylmannosamine	10.4 ± 0.4 (4)
N-Acetylglucosamine	3.1 ± 0.2 (3)

Formation of sialic acid

The mucosal supernatant was shown to be active in catalyzing the condensation of PEP with ManNAc in the presence of ATP. Sialic acid was also formed from GlcNAc by this preparation. These results are shown in Table III. The product of the reaction was positively identified as NANA by chromatographic and electrophoretic techniques. The formation of NANA from GlcNAc presumably occurred through conversion to ManNAc *via* GlcNAc 2-epimerase[14].

CMP-NANA synthetase

This enzyme, which is responsible for the nucleotide activation reaction to form CMP-NANA, the immediate precursor of sialic acid residues of glycoproteins, was shown to be present in the 105 000 × *g* supernatant fraction (Table IV). The specific

TABLE IV

PURIFICATION OF CMP-NANA SYNTHETASE FROM TRACHEAL MUCOSAL SCRAPINGS

Enzyme units are given as nmoles of CMP-NANA formed per h under the standard assay conditions.

Fraction	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification factor*
Crude extract	253	10 200	40	100	1
DEAE-cellulose eluate	16	6 400	400	63	10
Calcium phosphate gel eluate	2.4	2 700	1120	26.5	28

activity of the crude extract is 10–15% of that found in submaxillary gland extracts[16], the richest source of the enzyme, and approx. 25% of that of rat liver[16,20]. The enzyme was purified approx. 30-fold in good yield following the procedure described by Kean and Roseman[16] for the submaxillary gland enzyme. Activity was proportional to protein concentration and was linear up to 1 h. Optimum enzyme activity was observed at a pH between 8.5 and 9.5.

DISCUSSION

The *de novo* synthesis of the sialic acid residues of mucous glycoproteins is a complex process involving the formation of UDP-GlcNAc from glucose, the conversion of UDP-GlcNAc to CMP-NANA and the transfer of sialic acid from CMP-NANA to acceptor glycoproteins. L-Glutamine:D-fructose-6-phosphate aminotransferase,

the key regulatory enzyme of the initial phase, and sialyltransferase the enzyme that catalyzes the final step were recently studied in respiratory tract tissues[9,21].

The enzymes involved in the reactions from UDP-GlcNAc to CMP-NANA were the main object of the present investigation. The results show similar enzyme systems are present in tracheobronchial tissue for the metabolism of hexosamines as in other mammalian tissues. The activities of enzymes in rat liver have been reported to be of the order of 120–250 (refs. 10, 11, 19, 20), 140–280 (refs. 14, 19, 20), and 150–190 (refs. 16, 20) units per mg protein for UDP-GlcNAc 2′-epimerase, ManNAc kinase and CMP-NANA synthetase, respectively. The activities of these enzymes in tracheal mucosal extracts were found to be 25–50% of the values reported above. While ManNAc kinase appeared to be the rate-limiting step, sufficient ManNAc-6-*P* is formed (Fig. 3) to allow for sialic acid synthesis (Table III). This demonstration of ManNAc kinase in tracheal tissue extracts together with the observation of Kent and Draper[22] that intestinal mucosal extracts can also form sialic acid from ManNAc and GlcNAc, is of particular importance when viewed in relation to the results of Kundig *et al.*[14]. These workers stated that a number of tissues did not form ManNAc-6-*P* from ManNAc but rather formed GlcNAc-6-*P*. This observation seems unusual since the tissues synthesize glycoproteins containing sialic acid. These results are all the more paradoxical since these tissues, in particular lung, trachea and intestine, were shown to contain other sialic acid biosynthetic enzymes[14]. The present results establish that tracheal tissue actively phosphorylates ManNAc to give ManNAc-6-*P*. While ManNAc is indeed rapidly converted by tracheal extracts to GlcNAc-6-*P*, *via* GlcNAc 2-epimerase and GlcNAc kinase as reported by Kundig *et al.*[14], sufficient ManNAc-6-*P* is formed to allow for the formation of sialic acid shown in Table III.

The formation of sialic acid from GlcNAc, while extremely low, may nevertheless be important in tracheal tissue. This observation indicates synthesis of ManNAc-6-*P* from GlcNAc *via* GlcNAc 2-epimerase[23] does occur despite the efficient formation of GlcNAc-6-*P* by GlcNAc kinase (Table II). Inhibition of this kinase by UDP-GlcNAc[24] could shift the equilibrium of the epimerase towards ManNAc allowing for more efficient synthesis of sialic acid from GlcNAc.

The histopathological characteristics of human chronic bronchitis can be induced in animals[25,26]. As hypersecretion as well as rheological changes in the mucus are associated with chronic obstructive lung diseases, it becomes important to consider the levels of the sialic acid enzymes in the respiratory tract of such diseased animals. The recent observation[19] that the activities of UDP-GlcNAc 2′-epimerase and ManNAc kinase are significantly different in the livers of normal and diabetic rats emphasizes the importance of these studies in disease states. Although the differences in enzyme activities were not large[19], most of the pathology of diabetes and also chronic bronchitis results from affliction with the disease over a long period of time. Thus, cumulative effects of small differences cannot be ignored. Current investigations are directed towards studies of these enzymes in animal models of chronic bronchitis.

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