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THE USE OF HIGH-VOLTAGE PAPER ELECTROPHORESIS IN STUDIES OF THE BIOSYNTHESIS OF MUCIN GLYCOPROTEINS

DANIEL B. ELLIS

Department of Biochemistry, Smith Kline & French Laboratories, 1500 Spring Garden Street, Philadelphia, Pa. 19101 (U.S.A.)

SUMMARY

The mammalian respiratory tract produces a secretion in which the major macromolecular components are mucin-type glycoproteins. High-voltage paper electrophoresis has proved a valuable research tool in studies of the formation of these glycoproteins. Separations of hexoses, amino sugars, oligosaccharides, sugar nucleotides and mucin glycoproteins are rapidly and easily obtained by paper electrophoresis at voltages of 50 to 100 V/cm. The use of high-voltage paper electrophoresis to follow amino sugar interconversions and the transfer of glycosyl units to mucin acceptors is described.

INTRODUCTION

The separation of small molecules by high-voltage paper electrophoresis (HVPE) is usually considered to be primarily of interest in clinical biochemistry[1] and several clinical applications of high-voltage and thin-layer electrophoresis were described earlier in this symposium by CLOTTEN[2]. However, the advantages offered by HVPE also make the technique an exceedingly useful research tool, and we have used it extensively in glycoprotein biosynthesis studies that are part of a basic research program on chronic obstructive lung disaeses.

We are interested in the biosynthesis of mucin-type glycoproteins because they are the major macromolecular component of respiratory tract secretions. These mucins have structural patterns characteristic of other "epithelial glycoproteins" and are composed of neutral, sialo- and sulfomucins[3]. The respiratory glycoproteins are reported to be made up of galactose and N-acetylhexosamine residues attached to a polypeptide core[3]. Superimposed on this basic oligosaccharide structure are fucose, sialic acid and/or sulfate groups[3,4]. These mucin-type glycoproteins provide the necessary visco-elasticity to allow the tracheobronchial secretion to perform its vital physiological role. Pathological conditions of the respiratory tract, however, are usually characterized by a hypersecretion of mucus with altered physicochemical properties. This hypersecretion is either the primary finding or is a major contributing factor in such respiratory diseases as chronic bronchitis, asthma, and emphysema[5].

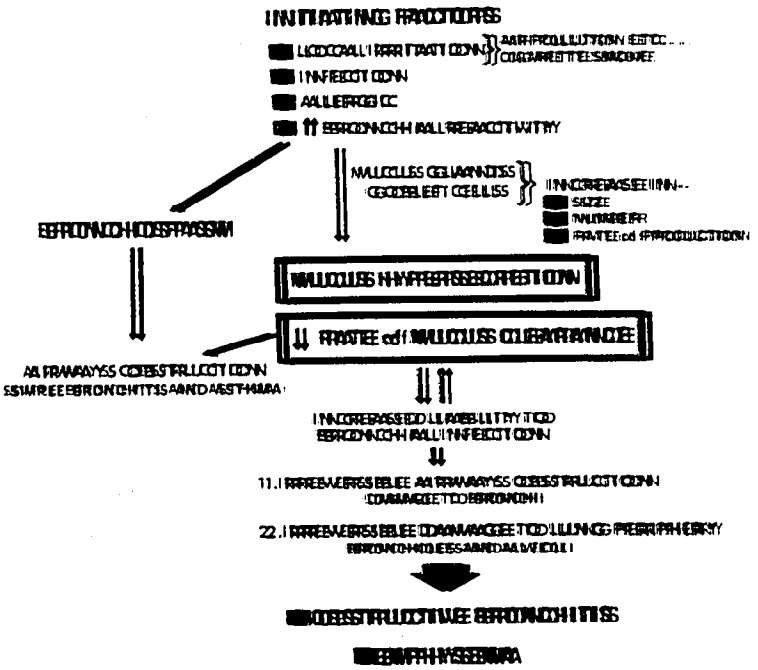

Fig. 1. Schematic outline of the development of bronchitis.

A diagrammatic scheme of what is generally considered to be the natural history of bronchitis is outlined in Fig. 1. Mucus hypersecretion is the central point of this scheme and our approach to the study of chronic obstructive lung disease is based on the thesis that the abnormality in mucus secretion plays a primary role in the

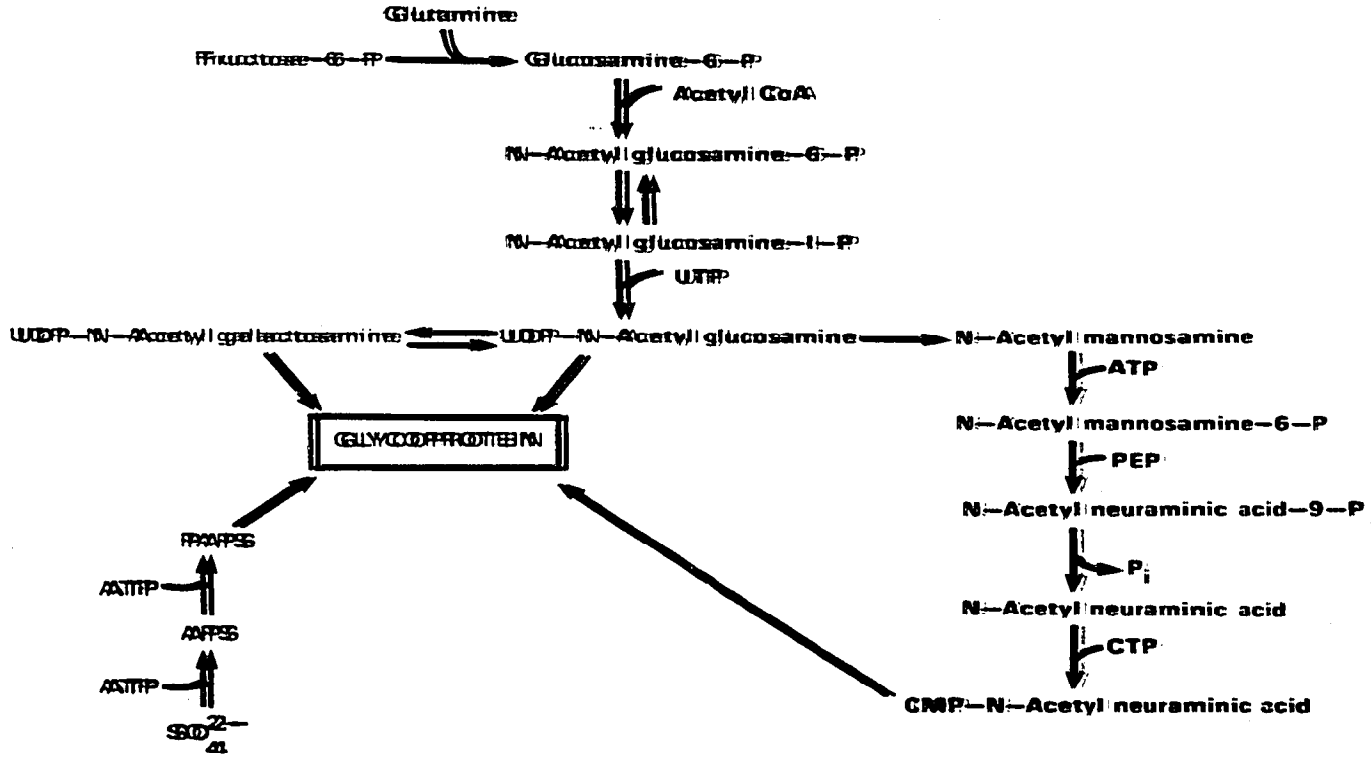

Fig. 2. Pathways of glycoprotein biosynthesis.

TABLE I

BUFFER SYSTEMS FOR HVPE OF CARBOHYDRATES

Compounds were detected as follows: free hexosamines with an alkaline silver nitrate reagent, N-acyl derivatives by yellow fluorescence after spraying with 0.5 *N* alcoholic NaOH and heating at 100° for 5 min (ref. 7), neutral sugars were detected with a *p*-aminohippuric acid spray[8], and sugar phosphates and nucleotides were visualized by fluorescence under UV following treatment with an alcoholic quinine sulfate reagent[9].

Buffer	*Separation of*
0.05 *M* borate, pH 9.5	Hexoses, hexosamines acetylhexosamines
1% borate, pH 9.0	Mucins, sugar nucleotides
0.05 *M* phosphate, pH 7.5	Sugars, sugar phosphates
0.08 *M* pyridine–0.04 *M* acetic acid, pH 6.5	Sugars, sugar phosphates

overall pathogenesis and progression of the disease. This work is aimed at an understanding of the biochemical pathways involved in the formation of mucin glycoproteins by the respiratory tract.

The synthesis of the oligosaccharide units of glycoproteins results from the conversion of glucose to the individual monosaccharides, and the activation of these monosaccharides to the corresponding nucleotide sugar, followed by polymerization[6]. An outline of the pathways involved in the biosynthesis of the monosaccharides and their subsequent activation and incorporation into glycoproteins is shown in Fig. 2. As sulfomucins appear to be an integral component of respiratory secretions[3], the formation of activated sulfate from inorganic sulfate is also included in this figure. While little is presently known, concerning the biosynthesis of sulfated glycoproteins, it is believed that sulfate is transferred from phosphoadenosine phosphosulfate (PAPS) to a glycoprotein in a manner similar to the sulfation of mucopolysaccharides. The main enzyme systems we are investigating in respiratory tissues are shown in Fig. 2 and include the pathways for the biosynthesis of sialic acid and the transfer of individual monosaccharides to the glycoprotein core. Major use has been made of HVPE during the course of these studies and in the preparation and purification of essential radioactively labeled substrates.

TABLE II

ELECTROPHORETIC BEHAVIOR OF N-ACETYL AND FREE HEXOSAMINES

Whatman No. 3MM paper, 0.05 *M* borate, pH 9.5, 30 min at 60 V/cm. The compounds were visualized as described in Table I.

Compound	*Migration to anode (cm)*
Galactosamine	2.0
Glucosamine	0
Mannosamine	9.9
N-Acetylgalactosamine	3.6
N-Acetylglucosamine	0.2
N-Acetylmannosamine	6.3
Galactose	12.5

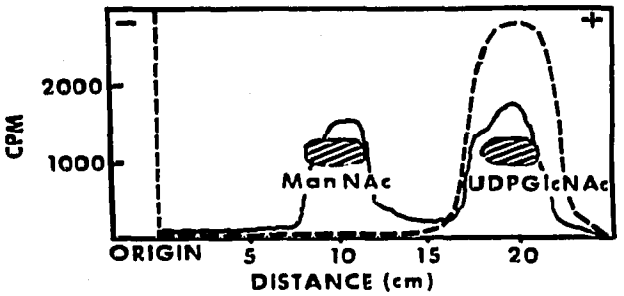

Fig. 3. Separation of ManNAc and UDP-GlcNAc by HVPE using a 0.05 *M* borate buffer, pH 9.5, at 60 V/cm. Aliquots of the epimerase incubation mixture were subjected to electrophoresis for 55 min. Areas corresponding to standards are cross-hatched. Radioactive areas were detected by strip scanning and then quantitated by sectioning the paper and measuring radioactivity with a liquid scintillation counter. — – – —, Radioactivity at 0 time; ————, following 30-min incubation at 37° with UDP-GlcNAc 2-epimerase.

EXPERIMENTAL AND DISCUSSION

Several typical buffer systems that we have employed most frequently are listed in Table I. An application of each system in our research is described below. The electrophoretic behavior of several amino sugars in one of these systems, a borate buffer, at 60 V/cm is shown in Table II. Galactose is also included in this table to show its rate of migration in this buffer system compared to the amino sugars.

We are particularly interested in UDP-N-acetylglucosamine-2-epimerase, the enzyme responsible for the formation of N-acetylmannosamine (ManNAc), the first step in the series of reactions leading to the synthesis of sialic acid nucleotides, essential precursors for glycoprotein synthesis (see Fig. 2). This enzyme has been shown to be subject to feedback inhibition[10], and hence it is important to the possible regulation of glycoprotein biosynthesis. HVPE is used to separate the product of the reaction, ManNAc, from unreacted UDP-N-acetylglucosamine (UDP-GlcNAc). Typical separations are shown in Fig. 3. The use of HVPE and radioactive UDP-GlcNAc allows for a simpler assay of the enzyme as compared to standard chromatographic and colorimetric assays[10,11]. Using this technique we have carried out extensive studies on the regulation of this enzyme[12].

The next reaction in the biosynthesis of sialic acid is the phosphorylation of ManNAc (Fig. 2). During studies of the distribution of ManNAc-kinase in mammalian tissues, it was reported that extracts prepared from tracheal tissues did not contain this enzyme[13]. In addition, in all tissues tested, the kinase was accompanied by GlcNAc-2-epimerase and by GlcNAc-kinase. The latter two enzymes were found to be especially active in tissues, particularly lung and tracheal mucosa, in which ManNAc-kinase was not detected[13]. The presence of these two enzymes results in the formation of N-acetylglucosamine-6-phosphate from ManNAc.

In a detailed search for ManNAc-kinase in tracheal tissue, we have made extensive use of HVPE to measure ManNAc phosphate formation in the presence of GlcNAc phosphate. The rate of phosphorylation is determined with the aid of [^{14}C]-ManNAc as substrate. N-Acetyl-hexosamines are separated from their phosphate esters using either a pyridine–acetate or phosphate buffer system, as shown in Fig. 4. The N-acetyl-hexosamine phosphate esters are isolated and then dephosphorylated

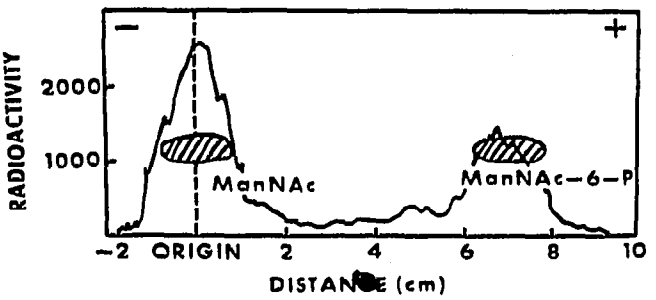

Fig. 4. Separation of N-acetylhexosamines and their phosphate esters. HVPE in 0.05 *M* phosphate, pH 7.5, at 40 V/cm for 20 min. Radioactive areas were detected as described in Fig. 3.

with potato phosphatase[13]. The resulting ^{14}C-labeled N-acetylhexosamines are then separated, using the borate system described in Table II. Using these techniques, we have been able to demonstrate the presence of significant ManNAc-kinase activity in tracheal tissue[14], eliminating the need to postulate the existence of an alternative pathway for the synthesis of ManNAc-6-phosphate in this tissue. While ManNAc is rapidly converted to GlcNAc-6-phosphate by the trachea, sufficient ManNAc-6-phosphate is formed to allow for sialic acid synthesis[14].

HVPE has also proved to be an exceedingly useful tool in studies of the transfer of glycosyl units to mucin acceptors by tracheal enzymes. In general, the assembly of the carbohydrate units of glycoproteins involves a series of glycosyl transferases (termed a multiglycosyl transferase system[15]) that transfer individual monosaccharides to growing oligosaccharide side chains. As part of our program on the biosynthesis of respiratory mucins, we are studying the glycosyl transferases of the canine tracheobronchial tree in normal and diseased animals.

In order to study the individual transferase enzymes and their receptor specificity in tracheal tissue, it is essential that well-characterized acceptor glycoproteins be used. As purified glycoproteins prepared from respiratory mucus are not available, we have used such purified acceptors as desialized sheep and pig submaxillary mucins and sialidase-treated fetuin. Using such acceptors, various tracheal transferases can be characterized. A typical reaction of a monosaccharide glycosyl transferase, in this case CMP-sialic acid:glycoprotein sialyl transferase, is

CMP-sialic acid + GalNAc-protein → sialic acid-GalNAc-protein + CMP

Sialic acid is transferred from CMP-sialic acid to a desialized acceptor, resulting in the formation of a sialic acid (α, 2:6) N-acetylgalactosamine (GalNAc) linkage. While this specifically depicts ovine submaxillary sialyltransferase, a similar reaction involving

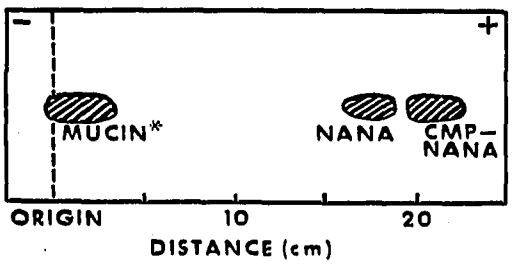

Fig. 5. Assay for sialyltransferase. 1% sodium tetraborate, pH 9.0, 90 V/cm for 30 min.

the transfer of sialic acid to a GalNAc-protein acceptor has been demonstrated in the respiratory tract[16].

HVPE greatly simplifies the assay for these transferases. Enzyme preparations are incubated with radioactively labeled CMP-sialic acid (CMP-NANA) in the presence and absence of acceptor. Product formation is followed by subjecting aliquots of the reaction mixture to electrophoresis, as shown in Fig. 5. The excess substrate, CMP-NANA, and its degradation products, primarily sialic acid, migrate rapidly from the origin. Protein-bound radioactivity, representing sialic acid transferred to the glycoprotein acceptor and depicted in Fig. 5 as mucin*, remains near the origin; this section of the paper is cut out and counted in a liquid scintillation counter. Transfer of sialic acid to small molecular weight acceptors to give such products as sialyllactose, which moves only a short distance from the origin, can also be studied using this system[15].

Another of the multiglycosyltransferase enzymes that have been studied in tracheal tissue is UDP-galactose:glycoprotein galactosyl transferase[17]. This enzyme catalyses the transfer of galactose to a GalNAc-protein acceptor to give a galactosyl-GalNAc linkage. The transfer of GalNAc to a polypeptide acceptor has also been studied using labeled UDP-GalNAc and ovine submaxillary mucin previously treated with sialidase and N-acetylgalactosidase[16].

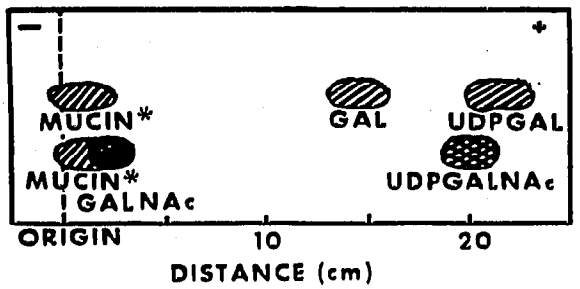

Fig. 6. Assay for galactosyltransferase. 1% sodium tetraborate, pH 9.0, 90 V/cm for 30 min.

These transferases are assayed in a similar manner to the sialyltransferase using a borate system, as shown in Fig. 6. In the galactosyltransferase assay protein-bound radioactivity remains near the origin while galactose and excess UDP-galactose migrate away from the origin. In the assay for N-acetylgalactosaminyl transferase, however, free GalNAc remains near the origin in this system (Fig. 6). In this case, the electrophoresis paper is dried and the origin washed with 80% ethanol by descending chromatography to remove any free GalNAc formed during the incubation. The material now remaining at the origin represents protein bound [^{14}C]GalNAc and is counted as previously described.

These HVPE assays for glycosyltransferases are much more rapid than procedures involving acid precipitation and repeated washings of labeled glycoproteins. In addition, the assay can be carried out in very small reaction volumes conserving valuable acceptors and substrates. The transfer to small acceptors such as monosaccharides can also be followed by HVPE. Such studies are extremely useful in characterizing specific linkages in the oligosaccharide side chains and also in mixed enzyme experiments. Detailed enzyme studies of the multiglycosyltransferase system

TABLE III

ELECTROPHORETIC BEHAVIOR OF NUCLEOTIDES AND ACTIVATED SULFATE

0.025 *M* sodium citrate, pH 5.8, 30 min at 70 V/cm. The compounds were detected by radioactivity or UV absorbance.

Compound	*Migration to anode (cm)*
AMP	5
ATP	11
PAPS	14
SO_4^{2-}	23

of canine respiratory tissue are being carried out by BAKER and coworkers[16,17] using these analytical procedures.

As sulfate groups appear to be superimposed on the basic oligosaccharide side chains of respiratory tract mucins[3,4], we are investigating the incorporation of [^{35}S]-sulfate into tracheal mucins[18]. The biosynthesis of PAPS and its transfer to acceptors are being studied in tracheal tissue extracts. HVPE is again being used in this research. The electrophoretic behavior of "activated sulfate" (PAPS) in a citrate buffer system is shown in Table III. As PAPS is formed by a two-step condensation of ATP with inorganic sulfate, the nucleotides AMP and ATP are also included in this table.

CONCLUSIONS

The studies described above demonstrate the usefulness of HVPE in studies of the biosynthesis of mucin glycoproteins. The use of HVPE greatly simplifies several enzyme assay procedures that are laborious and time-consuming using conventional chromatographic, colorimetric and acid-precipitating techniques. It is hoped that these relatively simple applications of HVPE will encourage further use of HVPE in biochemical research laboratories.

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