PURIFICATION AND CHARACTERIZATION OF GOAT MILK OLIGOSACCHARIDES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND HIGH FIELD PROTON NUCLEAR MAGNETIC SPECTROSCOPY

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **PURIFICATION AND CHARACTERIZATION OF GOAT MILK OLIGOSACCHARIDES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND HIGH FIELD PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY** in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy in Department of Biosciences and Biotechnology, University of Roorkee, Roorkee, is an authentic record of my own work carried out during a period from January 1985 to September 1988, under the supervision of Professor C.B. Sharma.

The matter embodied in this thesis has not been submitted by me for the award of any other Degree.

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This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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PURIFICATION AND CHARACTERIZATION OF GOAT MILK OLIGOSACCHARIDES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND HIGH FIELD PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

ABSTRACT

Five novel neutral oligosaccharides have been purified from goat milk using Bio-Gel P-4 and reverse phase C-18 high performance liquid chromatography. Their structures, as determined by high field ^{1}H-NMR, are :

P-V GlcNAc (β 1,6) Gal (β 1,4) Glc

P-II Gal (β 1,4) GlcNAc (β 1,6) Gal (β 1,4) Glc

P-I Gal (β 1,3) → GlcNAc (β 1,3) Gal (β 1,4) Glc
Gal (β 1,4) →

P-III Gal (β 1,4) GlcNAc (β 1,6) Gal (β 1,4) Glc
↑
Fuc (α 1,3)

P-IV Gal (β 1,3) GlcNAc (β 1,6) Gal (β 1,4) Glc
↑
Fuc (α 1,3)

Structurally, the goat milk oligosaccharides differ markedly from that of the corresponding oligosaccharides from human milk [Dua, V.K. and Bush, C.A. (1983) Anal. Biochem. 133, 1-8]. The oligosaccharide P-I has an interesting structure in the sense that it has two galactose residues linked via β 1 → 3 and β 1 → 4 linkage, respectively, to the same GlcNAc residue at the non-reducing end. The biological functions of these oligosaccharides are unknown. However, they can serve as substrates for various glycosyltransferases involved in the synthesis of complex carbohydrates of milk. They would also be useful in characterization of related structures from glycolipids and glycoproteins.

LIST OF CONTENT

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CHAPTER - I

1.0 INTRODUCTION

Identification of naturally occurring oligosaccharides is a continuous endeavour because a great variety of different molecular types, from simple to highly complex molecules, exists in plant and animal species. In addition to their occurrence in free forms, they also occur in covalently bonded forms to other types of bio-molecules, e.g. lipids (glycolipids) and proteins (glycoproteins). The diverse chemical properties of biomolecules are well demonstrated in nature's use of carbohydrates and the functional groups of simple monosaccharides were extensively utilized to generate an array of compounds including polymers, which greatly enhanced the functional versatility of this class of compounds.

It is generally assumed that the carbohydrate moieties of glycoproteins play important roles in (i) maintainance of protein conformation and solubility (126, 85); (ii) proteolytic processing and stabilization of the polypeptide against uncontrolled proteolysis (9), (iii) intercellular sorting and externalization of glycoproteins (85) and immune response (98, 30), just to name a few. Their importance is highlighted by the findings that the difference of a single sugar residue determines whether a blood group substance glycoprotein has A-type specificity or B-type specificity (13, 9). Studies on various blood group substances isolated from different sources reveal that all of them are derived from a single composite structure and differ only in the fine structure, that is, in the type or position or linkage of some sugar residues (68). This indicates that microheterogeneity is the hallmark of the oligosaccharide part of the glycoproteins or glycolipids. The recent advances in carbohydrate

structural analysis are motivated by a growing understanding of the relevance of glycoproteins to disease. Initially, the carbohydrate structures were determined by the several classical chemical methods available then, such as Smith's degradation, methylation analysis, etc. Enzymatic methods were also used for carbohydrate structural elucidation. But these methods required relatively large amounts of purified substances and are time consuming and cumbersome. Furthermore, the substance was lost or modified during the analysis.

In the later part of seventies, structural determination was accomplished with the help of high resolution ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy (38). This method not only offered a fast and non-destructive technique for carbohydrate structure elucidation, but also revealed the microheterogeneity of the oligosaccharide chain. Meanwhile, High Performance Liquid Chromatography also provided one of the most convenient and excellent method for carbohydrates purification and thus using the combination of HPLC and high resolution ^{1}H-NMR spectroscopy, a number of human milk oligosaccharides have been purified and characterized (41,44). These milk oligosaccharides are used in studies on the acceptor specificity of glycosyl transferases, substrate specificity of glycosidases and the structure of antigenic determinants (74, 70, 73). Apart from the milk oligosaccharides, a large number of oligosaccharides of glycoproteins obtained from various sources have also been purified and characterized. Thus, a library of purified and well-characterized oligosaccharides is available in the literature to be used as reference for identification of purified oligosaccharides of unknown structures isolated from different sources.

Although human milk oligosaccharides have been extensively worked upon, so far studies on milk oligosaccharides of other mammals have not

been conducted. It should be interesting to know if milk oligosaccharides from all mammals have similar structure and if not, what are the differences ? Do they bear some kind of evolutionary pattern ? In the present work, studies have been conducted on oligosaccharides of goat milk which is extensively used by tribal people, particularly in hilly areas. The goat milk is also preferred for infants because of its easy digestibility and relatively lower fat content. Five oligosaccharides, two of them fucose-containing ones, have been purified by HPLC and their structures have been determined by high resolution ^{1}H NMR spectroscopy.

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CHAPTER - 2

LITERATURE REVIEW

2.1 INTRODUCTION

Studies on milk oligosaccharides were prompted by the recognition of the various important biological roles played by the oligosaccharide moieties of glycoproteins. As the milk oligosaccharides were found to have the same structural pattern as those of the oligosaccharide chains of glycoproteins, they became an important source of the compound to understand the structure and functions of the oligosaccharide chains of glycoproteins. Almost all the methodologies used for purification and characterization of milk oligosaccharides have been adopted from those developed for the oligosaccharide moieties of glycoproteins. Therefore, it becomes essential to discuss the biological importance of oligosaccharides of glycoproteins and the methods evolved to purify and characterize them before embarking on a discussion of milk oligosaccharides.

The purpose of this literature review is to give a brief account of the different functions performed by the sugar moieties of glycoproteins and the various techniques used for their purification and characterization with special emphasis on reverse phase High Performance Liquid Chromatography (HPLC) and high field ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy and then review the applications of these techniques for purification and structural elucidation of milk oligosaccharides.

2.2 ROLE OF OLIGOSACCHARIDES IN GLYCOPROTEIN SYNTHESIS

Circular dichroism measurements and spectral analysis of native RNase revealed that the larger oligosaccharides influenced the environment

around several tyrosine side chains to effect a stabilization on the surface structure. X-Ray crystallographic data on the 3-dimensional structure of an intact human immunoglobulin (110) also supported the view that the carbohydrate side chains do play a role in the conformation of glycoproteins. Leavitt et al. (85) demonstrated that although the non-glycosylated proteins of sindbis virus and vesicular stomatitis virus were stable in the infected cell, they were unable to migrate to the cell-surface. The non-glycosylated proteins of both viruses were found to be insoluble in non-ionic detergents owing to aggregation whereas their glycosylated counterparts were soluble (54). These findings indicated that in some cases, the oligosaccharide units of the viral glycoproteins can have a profound effect on the properties of these molecules. Studies on glycoprotein, bovine pancreatic ribonuclease-B, have shown that it is less sensitive to tryptic digestion than the un-glycosylated bovine pancreatic ribonuclease-A (9). Since the two proteins are identical in amino acid sequence (100) and have similar, if not exactly identical, 3-dimensional configuration, the carbohydrate fraction of ribon-uclease-B, might be functioning as a block to proteolysis.

2.3 OLIGOSACCHARIDES AS BLOOD-GROUP DETERMINANTS

The ABO group system was discovered when Landsteiner observed in 1900 that the serum of some individuals agglutinated the red cells of others. Almost 160 blood group antigens have been identified since then which are distributed among 20 or more systems. These antigens are widely distributed throughout the body and occur in three chemical forms :

(i) On the surface of red cells and other cells as glycosphingolipids and also possibly as glycoproteins;

(ii) as oligosaccharides in milk and urine; and

(iii) as O-glycosidically linked oligosaccharides attached to mucins secreted in the gastrointestinal, genitourinary and respiratory tracts.

The ovarian cyst fluids provided relatively large amounts of mucin for structural studies which led to the elucidation of the antigenic determinants responsible for A, B, H, Le^a and Le^b activities. The immunological determinants of the ABH (O), Lewis, MN, P,Ii and possibly the En antigens were found to be oligosaccharides (66, 67, 94, 128). The blood group determinant oligosaccharides for various groups are listed in Table 1.

2.4 ROLE OF OLIGOSACCHARIDES IN ALTERATIONS IN GLYCOPROTEINS OF THE CELL-SURFACE

The transformed cells have been shown to have altered surface membranes as shown by tumor-specific surface antigens (98) and by alterations in the patterns of glycolipids and glycoproteins. Studies in this direction supported the view that the oligosaccharides are located on the exterior surface of cell surface glycoprotein (51, 121). The theoretical suitability of these sugars as specific structures, perhaps receptors, was pointed out by Shen and Ginsburg in 1968 (109), in that four different monosaccharides forming a heterosaccharide can be arranged in more combinations than can be 20 amino acids in a polypeptide of similar size. Transformed or growing cells have consistently been shown to have perbutations in the terminal residues of cell-surface oligosaccharide chains when compared to normal cells. It has also been shown that certain enzymes involved in the addition of more sugar residues to an existing acceptor sequence were either greatly reduced or entirely absent in transformed cells as compared to the normal cells (31). But inspite of all these evidences, it can not be concluded that changes in various oligosaccharides are of primary importance in growth regulation and not secondary consequences

TABLE I : DETERMINANTS FOR BLOOD GROUP ANTIGENS H,A,B,Le^a and Le^b

Specificity	Structure	
	Type 1	Type 2
H	Gal(β-1,3) GlcNAc– ↑ Fuc(α-1,2)	Gal(β-1,4) GlcNAc– ↑ Fuc(α-1,2)
A	GalNAc(α-1,3)Gal(β-1,3)GlcNAc– ↑ Fuc(α-1,2)	GalNAc(α-1,3)Gal(β-1,4)GlcNAc– ↑ Fuc(α-1,2)
B	Gal(α-1,3)Gal(β-1,3)GlcNAc– ↑ Fuc(α-1,2)	Gal(α-1,3)Gal(β-1,4)GlcNAc– ↑ Fuc(α-1,2)
Le^a	Gal(β-1,3)GlcNAc– ↑ Fuc(α-1,4)	–
Le^b	Gal(β-1,3)GlcNAc– ↑ ↑ Fuc(α-1,2) Fuc(α-1,4)	

of a more fundamental change in metabolism.

With the realization of the various important roles played by oligosaccharides in biological systems and their use in studies on the acceptor specificity of glycosyltransferases (22, 63), substrate specificity of glycosidases (3, 131) and as antigenic determinants for both production as well as characterization of polyclonal and monoclonal antibodies (5,19), a need was felt to purify and characterize oligosaccharides.

2.5 ISOLATION, ESTIMATION AND PURIFICATION OF OLIGOSACCHARIDES

Several methods have been used to isolate oligosaccharides of glycoproteins. They include alkaline borohydride treatment (87), hydrazine treatment (6,7,50,104,133) and extensive digestion with proteases of low specificity (114,116). The glycopeptides obtained after protease digestion were separated either by dialysis (114,117) or Sephadex G-25 and G-50 filtration (91) or by ethanol precipitation techniques (106).

In order to estimate the total amount of sugar, classical sugar reagents were used (37,4) such as the phenol-sulphuric acid test which measures the total content of neutral sugars (46). Spiro in 1960 employed descending paper chromatography on Whatman No. 1 in n-butanol-ethanol-water (10:1:2) for quantitative paper chromatography of neutral hexoses (113). Earlier Gardell in 1953 had used column chromatography to separate and quantify neutral sugars (52). He had also described a procedure for the separation of glucosamine and galactosamine on Dowex-50 columns (53). To estimate the number of acetal groups present in the hexosamines and sialic acid residues, Ludowig and Dorfman in 1960 gave a method based on the conversion of the acetyl groups to methyl acetate which was distilled and determined colorimetrically (90).

Fractionation of glycopeptides was essential for characterizing the carbohydrate units. Spiro used ion-exchange chromatography on DEAE-cellulose columns for sialic acid containing glycopeptides (117). Montgomery and Wu in the year 1963 employed preparative zone electrophoresis for the resolution of glycopeptides (93). This technique, just like ion-exchange chromatography, was based on differences in the charge of the glycopeptides due to variations in either the amino acid or sialic acid residues. Gel filtration has also been used to separate glycopeptides differing substantially in molecular weight (91).

Since middle of sixties, there came into wide use, methods for the separation and estimation of neutral sugars and hexosamines that relied on automated ion-exchange chromatography and gas-liquid chromatography and yielded much better sensitivity, resolution and accuracy. A number of automated borate complex anion exchange systems have been reported (97,86,125). The quantity of each sugar in a sample analyzed by borate-complex chromatography was determined by comparing the area under its peak with that of the internal standard, rhamnose. Spiro and Bhoyroo (119) employed borate complex chromatography to separate the reduced di-and tri-saccharides of earthworm cuticle collagen as well as reduced glucuronic acid-mannose disaccharides formed after such treatment of Neries cuticle collagen.

In 1965, Sawardeker (107) showed that it was possible to separate the alditol acetates of mono-saccharides by gas-liquid chromatography. This finding was utilized by several workers to analyze the neutral sugars present in glycoproteins (69, 88). The alditol acetates had an advantage over other derivatives such as trimethylsilyl ethers (102), in that the reduction of the carbonyl group performed during their preparation elimi-

nated the possibility of anomer formation or ring - isomerization, and therefore, resulted in only one peak for each sugar. The quantity of each sugar in sample was determined by comparing the area under its peak to that of the internal 2-deoxyglucose standard. In 1985, Neil Jentoft (64) developed a method for the analysis of carbohydrate composition of glycoproteins and similar glycoconjugates by methanolysis followed by reverse phase chromatography of perbenzoylated methyl glycosides. This methodology had several advantages over other techniques. The major advantage of this procedure was that the molar response factor used were rational and invariant whereas it was not the case with gas chromatographic system. Further, the benzoylated derivatives were found to be stable for several months and peaks could easily be collected from the HPLC for further characterization of the sample.

2.6 PURIFICATION OF OLIGOSACCHARIDES BY HPLC

Inspite of the refinement of the techniques discussed above, several disadvantages and shortcomings appeared in these approaches. Especially, they were highly time and material consuming and had severe limitations in the analysis of mixtures of closely related compounds. The heterogeneity of a single carbohydrate chain of a glycoprotein gives rise to a mixture of partial structures difficult to fractionate. The seventies saw the advent of high performance liquid chromatography technique which was found to be more suitable than the classical approach. Developments in the technology of packings for HPLC columns led to a faster and more selective media for carbohydrate separation. Linden and Lawhead (89) in 1975 used amino bonded silica for HPLC of unsubstituted sugars using refractive index detector. In 1977, Jones et al. (65) also used the same system to separate sugars by HPLC. In 1978, Aizetmuller (1) and in 1979 Wheals

and White (131) used in-situ coating of silica with a polyfunctional amine in the eluent for rapid separation of unsubstituted sugars. Oshima et al. (99) in 1980 showed that polystyrene based anion-exchange resins column was another convenient method for fractionating sugars. Cyano-bonded silica columns have also been used to separate sugars (103). All these columns were used with varying success for oligosaccharide separations. But, each one of them was found to have some shortcomings. Amino-bonded phases were subject to deterioration and fouling (89,65). In-situ modification with amines was able to resolve up to at least DP 8 (DP = degree of polymerization) but long elution times and low solubility in acetonitrile/ water system imposed limitations for higher oligomers.

In 1981, Cheetham and Sirimanne (86) gave a high performance liquid chromatographic separation of carbohydrate oligomers by reverse-phase column. The reverse-phase columns were first discovered by Howard and Martin (62) in liquid chromatography of fatty acids on liquid paraffin and n-octane as stationary phase. Cheetham (15) used a specialist Waters 'Dextropak' catridge, (10 x 1 cm). It was packed with C-18 bonded silica. Water was used as eluent and separation of oligosaccharides upto DP 7 was achieved. It was also shown that the time taken for the complete analysis was far less than that of Bio-gel P-2 column chromatography of the same sample. Another important feature was the separation of oligo-saccharides into their α - and β -anomeric forms on reverse-phase column. The same workers in 1981 were able to fractionate methylglycosides of monosaccharides using 'Dextropak' plastic catridge (10 x 1 cm) with water as eluent (26). It was possible to isolate small amounts of a chosen glycoside by this system. In 1982, Mellis and Baenziger (92) developed a method for separation of reduced neutral oligosaccharides on columns of Micropak AX-5 (Varians) with a mobile phase consisting of acetonitrile/

water and utilizing a solvent program. Starting with an initial composition of acetonitrile/water (65:35, v/v), with a solvent program which increased the proportion of water to acetonitrile at 0.5% per minute, they were able to obtain excellent resolution of oligomers containing 1 to 20 glucose residues in less than 60 minutes. The same workers had earlier been able to reduce the time required for gel permeation chromatography on Bio-gel P-4 but this method did not resolve oligosaccharide species containing more than five to seven sugar residues (95). Earlier studies by several workers (96, 29, 36) had indicated that bonded phase columns bearing amine functional groups could be utilized for the separation of neutral oligosaccharides. However, the range of oligosaccharides which could be fractionated was relatively small or incompletely characterized. Thus, the method of Mellis and Baenziger (92) offered an excellent system for analysis of oligosaccharides with rapidity and resolution not possible earlier. Blumberg et al. (13) gave a reverse phase HPLC system capable of separating oligosaccharides containing N-acetyl amino sugars on the basis of chain length, stereochemistry and anomeric linkage. Nanomolar level detection was accomplished by using far UV-absorbance monitoring. Their results showed that the main mode of retention and fractionation of oligosaccharides containing N-acetyl amino sugar was hydrophobic bonding and reverse-phase chromatography. Alpenfels et al. (2) were able to rapidly fractionate sugar-dansyl hydrazones on a 5μ Radial-Pak-column with a gradient of acetonitrile in 10 mM ammonium sulphate at pH 7 using a Flourescent detector. In 1983, Bergh et al. (8) were able to separate isomeric oligosaccharides that formed part of the complex-type carbohydrate chains of glycoproteins employing a Lichrosorb-NH_2 analytical column (4 x 250 mm) eluted with an isocratic mixture of acetonitrile and

deionized distilled water containing 15 mM potassium phosphate (pH 5.2) followed by a linear gradient, increasing the buffer content by 0.5%/min. The ability to separate isomeric substances renders this technique ideally suitable for studies on the specificity of glycosidases and glycosyltransferases, where it is of extreme importance to discriminate between different types of substitution. Cheetham and Sirimanne (27) conducted HPLC of methanolysis products of various carbohydrates of polysaccharides utilizing a Waters Dextropak reverse phase plastic catridge eluted with distilled water. The ability to monitor the formation and disappearance of methyl glycosides and to collect small amounts of materials for identification or as standards, were the most useful attributes of this system.

Lamblin et al. (84) fractionated sialic acid containing oligosaccharides by HPLC on a 5 μ Lichrosorb-NH_2 column using a linear gradient of 4:1 to 1:1 acetonitrile : water containing 25 mM ammonium hydrogen carbonate, for 70 minutes at room temperature. Hounsell and co-workers (67) suggested that HPLC of oligosaccharides containing acetamido and neutral sugars required more than one column system in the purification of multiple oligosaccharide isomers. Dua et al. (42) successfully applied a combination of normal and reverse phase HPLC with NMR for isolation and characterization of oligosaccharide alditols from ovarian cyst mucin. They observed that water being the weakest solvent available for fractionation of a complex mixture of oligosaccharides, all of which elute rapidly with water in RP, it was difficult to separate such a mixture with this system. Since normal phase HPLC on amino bonded silica operates by an entirely different mechanism, it can fractionate by rechromatography, the unresolved part of the reverse phase chromatogram. Using normal phase in conjunction with reverse phase, these workers were able to isolate and purify 13

different oligosaccharides from a single glycoprotein. In 1985, Dua et al. (43) were able to fractionate the oligosaccharide alditols isolated from an I-active ovarian cyst mucin glycoprotein using Alltech Associates 605 RP 5μ (250 x 4.6 mm) and Perkin Elmer 3μ (100 x 4.6 mm) reverse phase columns eluted with water.

2.7 STRUCTURE DETERMINATION

Several approaches to the study of the sugar sequences and linkages of the mono-saccharide residues present in the carbohydrate units of glycoproteins have been employed. Graded acid hydrolysis was found to be useful in obtaining information with regard to the sugar sequence by measuring the rate of release of the monosaccharide residues under conditions of mild acid hydrolysis (115, 118). A still better approach to this problem was the enzymatic release of monosaccharides by glycosidases (48). Since the glycosidases are very specific and generally release sugars only from the terminal non-reducing end, they give more precise information regarding the sequence of sugars. In 1959, Smith (111) showed that the technique of periodate oxidation followed by sodium borohydride reduction can be used in the degradation of polysaccharides because of the increased acid lability of the acetal bonds of the resulting polyalcohols. These acetal bonds could be splitted by mild acid hydrolysis without breaking the glycosidic bonds of the unoxidized sugars. Spiro (116) successfully applied this technique of periodate oxidation, sodium borohydride reduction and mild acid hydrolysis to a glycoprotein 'fetuin' to bring about the degradation of carbohydrate units and obtained information of the sequence and linkages of the sugars of 'fetuin'. Hakomori (56) gave the Methylation method which provided important structural information about the linkages

of sugar residues and the extent of branching in the carbohydrate units of glycopetides. The identification of the O-methyl derivatives of the neutral sugars gives information about their position in the oligosaccharide chain. The method involves the methylation of all free hydroxyl groups, followed by acid hydrolysis which cleaves glycosidic linkages but does not disturbs the methyl ether linkage. In the product, free hydroxyls mark the position of the linkages in the starting material. Methylation in conjunction with gas liquid chromatography and mass spectrometry of the alditol acetates of the sugars provided an excellent method for separation and identification of neutral sugars (10,11,12). The alditol acetates were obtained by reducing the neutral sugars obtained after acid hydrolysis of methylated glycoprotein or glycopeptide with sodium borohydride followed by acetylation with acetic anhydride in pyridine.

2.8 DETERMINATION OF OLIGOSACCHARIDE STRUCTURES BY ^{1}H-NMR SPECTROSCOPY

In the year 1977, Vliegenthart and co-workers (38) introduced the application of high resolution proton NMR spectroscopy as a new approach for the structure elucidation of underivatized carbohydrate chain obtained from glycoprotein. This technique had several advantages over the techniques which were in use earlier. Firstly, the spectrum could be used as an identity card to compare carbohydrate chains obtained from different sources. Secondly, the concept of structural reporter groups was developed to interpret the NMR spectrum, i.e. the chemical shifts and coupling constants of protons resonating at clearly distinguishable positions in the spectrum bear the essential information to assign the primary structure (124). Therefore, information on the anomeric linkages as well as on the sequence of sugars and their linkage position, could be obtained. Since carbohydrate chains of glycoproteins are usually rather complex structures,

the investigation was carried out on relatively simple part of the molecule followed by the study of compounds of gradually increasing complexity. Furthermore, the relative intensities of the signals in the NMR spectrum can be used as markers for the purity of the compounds. Vliegenthart and co-workers (120, 39) studied the structures of various oligosaccharides obtained from different sources using high field nuclear magnetic spectroscopy. Fournet et al. (49) determined the primary structures of 16 asialocarbohydrate units derived from human plasma α_1-acid glycoprotein by 360 MHz ^{1}H-NMR spectroscopy and permethylation analysis. They observed that using ^{1}H-NMR spectroscopic method, the determination of oligosaccharide structures of glycoproteins can be completed within 30 minutes and with as little as 2 mg of a homogeneous oligosaccharide, as compared to the conventional chemical and enzymatic techniques where larger amounts of material and above all an enormous amount of time would be required. Almost simultaneously, Dorland et al. (40) determined the type of branching in complex asparagine-linked glycan chains of glycoproteins employing 360 MHz ^{1}H-NMR spectroscopy. The NMR data obtained by them were so characteristic that they allowed a rapid and reliable recognition of the bi-, tri- and tetra-antennary class of complex asparagine-bound glycan chains. The use of 500 MHz NMR spectrometer in 1980 greatly helped in elucidating the N-acetyllactosamine type asparagine bound carbohydrate chains of glycoproteins (122). The increased resolution of 500 MHz strong magnetic field provided significant differences in the line widths of the anomeric signals which turned out to be of great value in the spectral interpretation. Bush et al. (20) in the same year were able to study the conformation of the glycopeptide linkage in asparagine-linked glycoproteins by employing ^{1}H-NMR spectroscopy in conjunction with circular dichroism studies. Carver and Grey (23) conducted a detailed analysis of the ^{1}H-NMR spectral para-

meters for the anomeric and C-2 hydrogen resonance of 63 different glycopeptides and oligosaccharides of known structure which revealed a general method for the determination of primary structure of glycopeptides for most of the known classes of structures. A particular aspect of this analysis was the use of the C1-H chemical shift versus the corresponding value of C2-H which can be correlated with specific structural microenvironments. They suggested that this correlation arises from the existence of well-defined three dimensional structures for the sequence of hexoses making up the microenvironment. Carver et al. (24) determined the structures of four glycopeptides purified by Dowex and Bio-gel P-2 chromatography from Pronase-digests of hen-ovalbumin using 360 MHz spectroscopy. Herlant-Peers et al. (59) and Van Halbeek et al. (123) also determined structures of oligosaccharides isolated from new born meconiums and Hog-submaxillary gland mucin glycoproteins respectively, employing methylation analysis in conjunction with mass spectrometry and ^{1}H-NMR spectroscopy. IN 1983, Brisson and Carver (16,17) described the solution conformation of asparagine-linked oligosaccharides (16) as well as oligomannosides (17) using ^{1}H-NMR spectroscopy. They also determined the sequences and branching patterns in four classes of N-linked oligosaccharides through the use of Nuclear Overhauser Effect (18), a technique which was developed by Richarz and Wuthrich (105) for observing individual multiplets in proton NMR spectra of biological macromolecules. This technique offered a rapid and non-destructive approach to the elucidation of sequences in carbohydrate chains, in contrast to enzymatic and chemical methods which were highly time-consuming and destructive. ^{1}H-NMR studies have also proved to be useful in studies on the immunochemistry of the Lewis blood group systems (34) as well as in conformational studies of antigenic oligosaccharides (14). In 1986, Dua et al. (45) characterized oligosaccharide

alditols from ovarian cyst mucin glycoproteins using HPLC and high field ^{1}H-NMR spectroscopy. They observed that in exception to earlier proposals that nuclear overhauser effect on irradiation of the anomeric proton should always be observed at the proton attached to the aglycone carbon (14,76), for the linkage of GalNAc (1 → 3) Gal, nuclear overhauser effect on irradiation of the α-anomeric proton resonance is observed not at H-3, but at H-4 of galactose, indicating that complex oligosaccharides may adopt conformations in which the aglycone proton is not the one nearest to the anomeric proton.

2.9 MILK OLIGOSACCHARIDES

Studies on milk oligosaccharides started in the fifties by Kuhn and co-workers, who isolated several oligosaccharides from human milk (78-83). Kobata et al. (70-75) demonstrated in the late sixties that the oligosaccharides present in individual samples of milk can vary with the ABO or Lewis blood type of the donor, as the enzymes involved in their synthesis were also responsible for the formation of the structural determinants of these blood types (55,70,71,75). Milk oligosaccharides also found extensive use as models in studies on the acceptor specificities of glycosyltransferases, the substrate specificities of glycosidases and the structures of antigenic determinants (70,73,74). In 1969, Kobata et al. (72) gave a method involving Sephadexgel filtration and paper chromatography for the separation and determination of the known oligosaccharides in individual samples of milk. Monosaccharides obtained from these oligosaccharides after hydrolysis, were determined by gas chromatography of their tri-methyl silyl derivatives. Kobata and Ginsburg(74,75) isolated and characterized Lacto-N-hexaose (74) and Lacto-N-neohexaose (75) using Sephadex gel filtration and paper chromatography in conjunction with

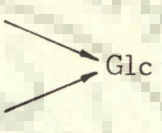
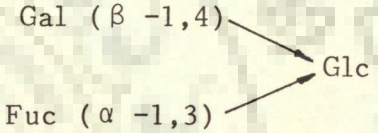
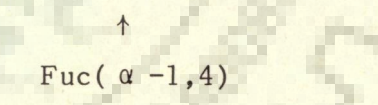
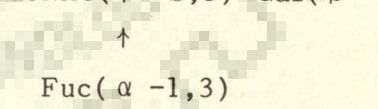
enzymatic digestion. The other techniques used by them included methylation, gas chromatography and X-Ray diffraction Yamashita et al. (132) isolated and characterized two nonasaccharides, monofucosyllacto N-octaose and monofucosyllacto N-neooctaose by sequential enzymic degradation and quantitative methylation analysis. In 1970s, high performance liquid chromatography and high field NMR spectroscopic techniques were developed which were found to be better suited for purification and characterization of oligosaccharides. Cheetham and Dube (28) showed that reverse phase HPLC was better suited for fractionation of human milk oligosaccharides. They observed that in reverse phase separation of milk oligosaccharides, structure predominated over molecular weight in the interactions with C-18 stationary phase. The branched oligosaccharides eluted the earliest leading to the tentative conclusion that linear molecules interact most with C-18 stationary phase.

Dua and Bush (41) were able to fractionate and identify human milk oligosaccharides using reverse phase HPLC and ^{1}H-NMR spectroscopy. For HPLC, they used a C-18 reverse phase Alltech Associates column (250 x 4.6 mm). Elution was accomplished with water and oligosaccharides were monitored by UV absorbance at 202 nm at which the amide group of the oligosaccharides provided a good chromophore for UV detection at low level. ^{1}H-NMR spectroscopic analysis was conducted on a 300 MHz NMR spectrometer and acetone was used as an internal standard. Interpretation of the NMR spectra was done using the method developed by Vliegenthart and co-workers (123). In the same year, (1983) Egge et al. (47) separated several fucose-containing oligosaccharides from human milk by high performance thin layer chromatography (HPTLC), column chromatography on 'lactobeads', reverse phase HPLC of oligosaccharides obtained after borohydride reduction and peracetylation. The oligosaccharides were

identified by fast-atom bombardment (FAB) - mass spectrometry. Dabrowski et al. (35) conducted ^{1}H-NMR studies of peracetylated derivatives of the oligosaccharides isolated from human milk. An advantage of using peracetylated derivatives was the property of the acetate groups to act as 'Shift-substituent', thus disentangling the over-lapping signals. In 1984, Prieto and Smith (101) isolated and characterized a sialyloligosaccharide from human milk using anti-oligosaccharide antibodies. Bush et al. (21) in the same year fractionated and characterized human milk oligosaccharides by reverse phase chromatography and ^{13}C-NMR spectroscopy (21). One year later, Dua et al. (44) characterized Lacto-N-hexaose and two fucosylated derivatives from human milk by normal phase HPLC on amino-bonded silica column and ^{1}H-NMR spectroscopy. Recently, in 1987, Smith and co-workers (112) have isolated a novel sialyl-fucopentaose from human milk, whose presence is not dependent on expression of the secretor or Lewis fucosyltransferases, by affinity chromatography on a column of immobilized Ricinus communis lectin. They characterized this oligosaccharide by exoglycosidase digestions, binding by specific anticarbohydrate antibodies and analysis of the ^{3}H labelled glucitol derivative obtained after permethylation and hydrolysis.

Although a lot of human milk oligosaccharides have been purified and characterized (Table II), it is not known whether similar structure are present in milk from other mammals. Since goat milk is commonly used by inhabitants of hilly areas in India and is preferred for infants also because of its easy digestibility than cow or buffalo milk, it was considered worthwhile to analyse the goat milk oligosaccharides by reverse phase HPLC and NMR studies and compare them with those found in human milk. In addition, the characterized oligosaccharides would serve as reference compounds for characterization of similar oligosaccharides from other biological sources.

TABLE II : OLIGOSACCHARIDES OF HUMAN MILK

TRIVIAL NAME	STRUCTURE
2' - Fucosyllactose[a]	Fuc (α -1,2) Gal (β -1,4) Glc
3' - Fucosyllactose[b]	
Lactodifucotetraose[c]	
Lacto-N-tetraose[d]	Gal(β -1,3) GlcNAc(β -1,3) Gal(β -1,4) Glc
Lacto-N-neotetraose[e]	Gal(β -1,4) GlcNAc(β -1,3) Gal(β -1,4) Glc
Lacto-N-fucopentaose-I(LNF-I)[f]	Fuc(α -1,2) Gal(β -1,3) GlcNAc(β -1,3) Gal(β -1,4)Glc
Lacto-N-fucopentaose-II(LNF-II)[g]	
Lacto-N-fucopentaose-III(LNF-III)[h]	

(Contd...)

(Contd...Table II)

TRIVIAL NAME	STRUCTURE
Lacto-N-difucohexaose-I[i]	Fuc(α -1,2) Gal(β -1,3) GlcNAc(β -1,3) Gal(β -1,4)Glc ↑ Fuc(α -1,4)
Lacto-N-difucohexaose-II[j]	Gal(β -1,3) GlcNAc(β -1,3) Gal(β -1,4)Glc ↑ ↑ Fuc(α -1,4) Fuc(α -1,3)
3' - Sialyllactose[k]	NANA (α -2,3) Gal(β -1,4)Glc
6' - Sialyllactose[l]	NANA (α -2,6) Gal(β -1,4)Glc
LS - tetrasaccharide a[m]	NANA (α -2,3) Gal(β -1,3) GlcNAc(β -1,3) Gal(β -1,4)Glc
LS - tetrasaccharide b[n]	NANA (α -2,6) Gal (β -1,3) → GlcNAc(β -1,3) Gal(β -1,4)Glc
LS - tetrasaccharide C[o]	NANA (α -2,6) Gal(β -1,4) GlcNAc(β -1,3) Gal(β -1,4)Glc
Disialyllacto-N-tetraose[p]	NANA (α -2,3) Gal(β -1,3) → GlcNAc(β-1,3) Gal(β-1,4)Glc ↑ NANA (α-2,6) →
Lacto-N-hexaose[q]	Gal(β -1,4) GlcNAc(β -1,6) → Gal(β -1,4)Glc Gal(β -1,3) GlcNAc(β -1,3) →
Lacto-N-neohexaose[r]	Gal(β -1,4) GlcNAc(β -1,6) → Gal(β -1,4)Glc Gal(β -1,4) GlcNAc(β -1,3) →

(Contd....)

(Contd... Table II)

TRIVIAL NAME	STRUCTURE
S - 5[s]	NANA (α -2,6) Gal(β -1,4) GlcNAc(β -1,6) → Gal(β -1,4)Glc ← Gal(β -1,3 and 4) GlcNAc(β -1,3)
S - 6[t]	Fucosyl S-5
N-2, (Monofuco lacto-N-hexaose)[u]	Fuc(α -1,3) ↓ Gal(β -1,4) GlcNAc(β -1,6) → Gal(β -1,4)Glc ← Gal(β -1,3) GlcNAc(β -1,3)
N-2, (Monofuco lacto-N-neohexaose)[v]	Fuc(α -1,3) ↓ Gal(β -1,4) GlcNAc(β -1,6) → Gal(β -1,4)Glc ← Gal(β -1,4) GlcNAc(β -1,3)
N-3, (Difuco lacto-N-hexaose)[w]	Fuc (α -1,3) ↓ Gal(β -1,4) GlcNAc(β -1,6) → Gal(β -1,4)Glc ← Gal(β -1,3) GlcNAc(β -1,3) ↑ Fuc(α -1,4)

(Contd....)

(Contd.... Table II)

TRIVIAL NAME	STRUCTURE
N-3, (Difuco lacto-N-neohexaose)[x]	Fuc(α -1,3) ↓ Gal(β -1,4) GlcNAc(β -1,6) → Gal(β -1,4)Glc Gal(β -1,4) GlcNAc(β -1,3) ↗ ↑ Fuc(α -1,4)
Fucosyl LS-tetrasaccharide C[y]	NANA (α -2,6) Gal(β -1,4) GlcNAc(β -1,3) Gal(β -1,4)Glc ↑ Fuc(α -1,3)
Monofucosyl lacto-N-octaose[z]	Fuc(α -1,3) ↓ Gal(β -1,4) GlcNAc(β -1,3) Gal(β -1,4) GlcNAc(β -1,6) ↑ Gal(β -1,3) GlcNAc(β -1,3) Gal(β -1,4)Glc
Monofucosyl lacto-N-neo-octaose[z]	Fuc(α -1,3) ↓ Gal(β -1,3) GlcNAc(β -1,3) Gal(β -1,4) GlcNAc(β -1,6) ↑ Gal(β -1,4) GlcNAc(β -1,3) Gal(β -1,4)Glc

[a-x] Kobata, A. Isolation of Oligosaccharides from human milk in, 'Methods in Enzymology' (ed. Ginsburg, V.). 28. 262-271.

[y] see reference (120).

[z] see reference (140).

CHAPTER - 3

MATERIALS AND METHODS

3.1 MATERIALS

Fresh goat milk was procured from a local dairy and kept in ice until processed. Bio-Gel P-4 (200-400 mesh size, exclusion limit, 4000 daltons), was purchased from Bio-Rad (U.S.A.). HPLC grade acetonitrile and methanol were purchased from Spectrochem (India). High purity D_2O was obtained from Merck, Sharp and Dohme Co. (U.S.A). All other chemicals were analytical reagent grade from standard commercial companies.

3.2 PRELIMINARY TREATMENT OF MILK AND PREPARATION OF CRUDE MILK OLIGOSACCHARIDE FRACTION

Unless stated otherwise, all operations were performed at 0-4°C. The preliminary processing of the goat milk was done essentially as described by Kobata and Ginsburg (74) with slight modifications. One liter goat milk was chilled in ice for 1 to 2 hr. and then centrifuged at low speed (500xg) for 15-20 min at 4°C in 250 ml Pyrex glass tubes. A thick layer of solidified lipid was formed on top of the fluid. It was removed by filtration through a loosely packed glass wool column (2 x 10 cm) which was precooled to 2°C. To the clear filtrate enough amount of ethanol was added dropwise with continuous stirring to a final ethanol concentration of 68 percent and the mixture was left overnight at 4°C. The precipitate, mostly proteins, thus formed, was removed by centrifugation for 20 min at 1000xg. The supernatant containing milk oligosaccharides was separated from the residue by decantation. The pellet was washed 2x with 100 ml of 68% ethanol and the washings were pooled with the first alcohol supernatant. The combined alcohol soluble supernatant fluid was evaporated to a syrup under reduced pressure

in rotary thin film flash evaporator. The syrup containing milk oligasaccharides was redissolved in double glass distilled water and any suspended matter was removed by centrifugation as above. Finally, the supernatant was lyophilized, dissolved in minimal volume of sterilized water and stored at -20°C until used. This fraction will be referred to as crude milk oligosaccharides. The total carbohydrate content was determined by the phenol-sulfuric acid method (46). An aliquot (50 µl) was diluted with water to 1 ml, followed by addition with stirring of 1 ml of 5% phenol and 5 ml of concentrated H_2SO_4. The reaction mixture was immediately mixed, incubated at room temperature for 30 min and optical density was measured at 490 nm. Total sugar content were estimated with the help of a glucose standard plot prepared concurrently.

3.3 PURIFICATION OF MILK OLIGOSACCHARIDES

3.3.1 Bio-Gel P-4 Chromatography

The crude oligosaccharide solution obtained after the preliminary treatment of milk described above was subjected to Bio-Gel P-4 liquid column chromatography. Bio-Gel P-4 (15 g) was suspended in glass double distilled water (1 liter) and allowed to hydrate for four hours at room temperature. After the hydration was complete, the supernatant and fine particles were decanted off. The gel was resuspended in 500 ml water, allowed to settle down and fines, once again, removed by decantation along with the supernatant. The gel was then suspended in 250 ml water and the slurry was poured gently into the glass column (1.5 x 60 cm). The column was packed as per standard procedure and washed with 1 liter water. The void volume of the column was determined using bovine serum albumin (BSA) and found to be approximately 19.5 ml.

After the P-4 column caliberation, the sugar sample (1 ml; ca equal to 250 mg glucose) was loaded on to the column. Glass double distilled water was used as eluent at a flow rate of 0.25 ml/min. Fractions (0.75 ml) were collected. Aliquots (50 µl) were drawn from each fraction and analysed for carbohydrate content by the phenol-sulfuric acid method (46). Carbohydrate-containing fractions under various peaks were pooled separately, lyophilized and then analysed by HPLC.

3.3.2 High Performance Liquid Chromatography (HPLC)

3.3.2.1 Reverse Phase HPLC

The Bio-Gel P-4 lyophilized fractions were dissolved in minimal volume of HPLC grade water and subjected to reverse phase HPLC. The reverse phase mode refers to a non-polar stationary phase and a polar mobile phase. The former consists of a non-polar ligand bonded to the surface of microporous silica particles such as octadecyl (C-18), octyl (C-8) etc. and the latter (mobile phase) usually consists of methanol, acetonitrile or tetrahydrofuran diluted with water, etc. Retention in reversed-phased separation is based on solute polarity. More polar molecules elute first and less polar ones elute later, the polar molecules interact more strongly with the more polar mobile phase than less polar solutes, which interact only weakly with the non-polar stationary phase. The reverse phase HPLC was carried out on a Shimadzu model LC-4A liquid chromatograph fitted with a microprocessor controlled solvent delivery unit, detectors (uv; fluorescence and refractive index), column oven and a Du-Pont Zorbax C-18 reverse phase column (4.6 x 250 mm, packed with 5µ spherical silica particles coated with a molecular fur of covalently bonded C-18 alkyl groups . The sugar sample (10-20 µg,20µl) was injected manually with the help of a microsyringe. HPLC grade water was used as the mobile phase at a flow rate of 1 ml per min. The effluent

was monitored by uv absorbance at 202 nm. The amide group of the N-acetylamino sugars acts as a good chromophore at 202 nm. The column performance was routinely checked by running the N-acetylglucosamine, as standard reference, on it.

3.3.2.2 Preparative HPLC

Preparative chromatography was carried out on the same column by collecting the fractions manually at the detector outlet. Multiple runs were made in order to collect sufficient amount of each sugar peak resolved on the C-18 column. The purity of each sugar sample was checked on the same column and each sugar sample was repeatedly chromatographed on the same column until a purity level of over 95% was achieved. About 2 mg sample of purified oligosaccharides were collected this way.

3.3.2.3 Normal Phase HPLC

The sugar peaks purified on the reverse phase C-18 column were then checked for contamination by any cross peak by normal phase chromatography using a Du-Pont Zorbax - NH_2 column (5 μ diameter, and 4.6 x 250mm) as the polar stationary phase and a mixture of acetonitrile and water (60:40, v/v) as the mobile phase. The flow rate was maintained at 1 ml for min and the elution of sugars was monitored by uv absorbance at 202 nm. Since the mechanism of retention in normal phase chromatography is displacement, the retention is based on the competition between the solute molecules and mobile phase molecules for active sites on the stationary phase surface. In other words, retention is related to the relative energies of interaction with the surface for the solute and mobile phase.

3.3.2.4 Calculation of Relative Retention Time (k') of Oligosaccharides

The k' values were calculated by dividing the retention time of the sugar (t_r) by the column dead time (t_o), i.e.,

$$k' = \frac{t_r}{t_o}$$

where, the retention time, t_r, is the time from injection of the sample until the peak elutes from the column, and the column dead time, t_o, is the time taken by non-retained compound to pass through the column and was measured from sample injection until the first disturbance in the baseline.

3.3.2.5 Carbohydrate Composition of Oligosaccharides by HPLC

Carbohydrate analysis was accomplished by HPLC of the benzoylated methyl glycosides of oligosaccharides using Jentoft's method (105). Methanolic HCl (0.2 ml) was added to approximately 1 μ mol of each sugar sample and heated at 80°C for 8 hours. The samples were then cooled to room temperature and 0.1 ml of 80% v/v t-butanol in methanol was added to each and dried under a stream of N_2. The hexosamines were reactylated by dissolving the sample in 0.1 ml dry methanol followed by addition of 40 μl pyridine and 40 μl of acetic anhydride and incubating the samples for 1 hour at room temperature. The samples were dried under a stream of N_2. 40 μl of toluene was added and samples were redried and dissolved in $CH_3CN:H_2O$ (90:10), v/v. Sample (10 μl) was analysed on a 25 cm, 5 μ Zorbax C-18 column. The column was eluted with 90% acetonitrile at a flow rate of 0.5 ml/min. The retention times of sugars in acid hydrolysate were compared with those of standard samples.

3.4 PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPIC ANALYSIS

Proton NMR spectroscopic experiments were carried out in part on a 400 MHz Brukers NMR spectrometer at the Regional Sophisticated Instrument Centre, Central Drug Research Institute, Lucknow and a 270 MHz Brukers NMR spectrometer at the Sophisticated Instrument Facility, Indian Institute of Science, Bangalore, India.

3.4.1 Preparation of Sample

Prior to recording the spectra, the samples were dissolved in D_2O and then repeatedly exchanged with D_2O at room temperature followed by lyophilization. The samples were then dissolved in 0.3 ml high purity D_2O in a 5 mm NMR tube.

3.4.2 Recording of the Spectra

Typically 4K to 8K spectral data points were collected over a 3-KHz spectral width. All the spectra were recorded at room temperature (22°C) except for those recorded to observe the α-Fuc[3] H-5 resonance at 77°C. Spin Difference Decoupling Spectroscopy (SDDS) experiments were also conducted at same temperature. The SDDS results were obtained by subtracting control from the irradiated spectrum and the observed chemical shifts reported relative to internal sodium 4, 4-dimethyl-4-silapentane-1 sulfonate (DSS) using acetone as internal standard (δ = 2.225 ppm downfield from DSS).

NOE experiments were conducted at 70°C to overcome effects of unfavourable rotational correlation time (45). The pulse sequence for NOE experiments utilized a 3s pre-irradiation followed by a 90° observation pulse and acquisition with the irradiation off. The observed chemical shifts are reported relative to internal DSS using acetone as an internal standard

(δ = 2.225 ppm downfield from DSS).

3.4.3 Interpretation of the Proton NMR Spectra

The interpretation of the ^1H NMR spectra was done using the method introduced by Vliegenthart and co-workers (124) for the structure elucidation of underivatized carbohydrate chains obtained from glycoproteins. The SDDS has been successfully applied for recognizing the individual resonances in the conglomerate of unresolved signals in the spectra of oligosaccharides (32-34, 57-58). SDDS gave a reasonable balance between the measuring time invested and the spectral information obtained.

3.4.4 Determination of Sugar Composition and Anomeric Configuration of the Sugar Ring

The number of sugar residues was determined by integrating the H-1 signals in the anomeric region of the spectrum. The number of amino sugars and fucose units was obtained by the integrals of the methyl singlets of the acetamido groups and the doublets of the C-6 methyl groups, respectively. The anomeric configuration of the sugar unit followed from its $J_{1,2}$ coupling constant, a value of 3-4 Hz indicating an α-isomer and that of 7-9 Hz, a β-isomer. The possible confusions between β-glucose, β-galactose, and β N-acetylglucosamine, α-glucose and α-fucose were unambiguously excluded by establishing the connectivities with the corresponding H-2 resonances, which differed characteristically, by SDDS. The occurence of galactose residue was also confirmed by its easily recognizable H-4 signal. As a consequence of this combined approach, both the sugar components of the oligosaccharide chain and their anomeric structure were determined.

3.4.5 Determination of the Site of Glycosidic Linkage

For determining the site of glycosidic linkage, advantage was taken of the fact that whenever substitution occurs at a saccharide ring by another sugar unit, it induces chemical shift changes in both of them, the largest occuring at the site of glycosidic linkage and vicinal to it (33), for example, glycosylation of a terminal β-galactose residue by α-galactose at site 4 leaves the δ(H-2) of the former practically unaffected whereas the glycosylation at site 3, i.e., vicinal to 2, induces a downfield shift δ(H-2) = 0.10. These two alternative substitution patterns were thus unambiguously distinguished. Since a subsequent elongation of the oligosaccharide chain has no further effect on chemical shifts of already glycosylated rings, these patterns were obviously recognizable in all higher members of the series. The same information was obtained when the aglycon effect of the substituted sugar residue on the substituting one was considered, for example, a sugar residue linked to site 3 of another sugar residue differed from that linked to site 4, in chemical shifts of its H-1, H-2, H-3 and H-5 resonances. Chemical shift analogies of the reference oligosaccharides were also used to establish the site of glycosidic linkage (Tables III, IV, V). NOE was also used in some cases to establish the site of glycosidic linkages.

3.4.6 Sequence Determination

The sequence of the sugar residues in the oligosaccharide chain was established by using the structural regularities of the various oligosaccharides of the series. Chemical shift analogies of the reference oligosaccharides (Tables III, IV, V) characterized earlier by ^{1}H NMR spectroscopy were also used in some cases to determine the sequence of the sugar residues.

3.5 ENZYMATIC DIGESTION

Fucosidase digestion was used to assist in the oligosaccharide structure determination. 2 mg of the compound was dissolved in 1 ml of 1.0 mM sodium acetate buffer (pH 5.0) and 200 µl of bovine kidney α-fucosidase (sigma co) (equivalent to 1 unit of enzyme) was added to it. The reaction mixture was incubated at 37°C for 16 hours. This was followed by the addition of an additional unit of α-fucosidase and the reaction mixture was incubated for 16 hours at 37°C. The products were assayed by C-18 reverse phase HPLC using water as eluent at a flow rate of 1 ml/min.

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TABLE III : ^{1}H NMR SHIFTS OF SOME REFERENCE HUMAN MILK OLIGOSACCHARIDES[a,b]

Chemical shifts are reported relative to internal (DSS) using acetone as the internal standard (δ = 2.216 ppm downfield from DSS)

Residue		Oligosaccharide		
		Lactose	LNT	LNneoT
α-Glc	H_1	5.214	5.209	5.208
β-Glc	H_1	4.654	4.652	4.651
	H_2	3.277	3.267	3.266
β-Gal^{4}	H_1	4.440	4.427	4.424
	H_4	-	4.151	4.154
β-Gal^{3,3}	H_1	-	4.427	-
β-Gal^{4,3}	H_1	-	-	4.470
β-GlcNAc^{3}	H_1	-	4.712	4.680

[a] Probe temperature was 22°C except for LNneoT (6°C)

[b] Data from reference (41).

TABLE IV : ^{1}H NMR CHEMICAL SHIFTS OF SOME REFERENCE HUMAN MILK OLIGOSACCHARIDES[a]

Chemical shifts are reported relative to internal (DSS) using acetone as the internal standard (δ =2.216 ppm downfield from DSS)

Residue		Oligosaccharide			
		LNF-I	LNF-II	LNF-III	LND-I
α-Glc	H_1	5.208	5.210	5.210	5.209
β-Glc	H_1	4.680	4.654	4.642	4.600
	H_2	3.266	3.275	3.272	3.274
β-Gal^4	H_1	4.411	4.425	4.429	4.415
	H_4	4.130	4.149	4.150	4.129
β-Gal^{3,3}	H_1	4.620	4.493	-	4.415
β-Gal^{4,3}	H_1	-	-	4.455	-
β-GlcNAc^3	H_1	4.60	4.684	4.668	4.660
α-Fuc(1 → 2)	H_1	5.179	-	-	5.147
	H_5	4.282	-	-	4.335
	H_6	1.226	-	-	1.246
α-Fuc(1 → 3)	H_1	-	-	5.122	-
	H_6	-	-	1.167	-
α-Fuc(1 → 4)	H_1	-	5.020	-	5.019
	H_5	-	4.867	-	4.850
	H_6	-	1.172	-	1.190

[a] Data from reference (41).

TABLE V : ^{1}H NMR SHIFTS OF SOME REFERENCE HUMAN MILK OLIGOSACCHARIDES[a,b]

Chemical shifts are reported relative to internal (DSS) using acetone as the internal standard (δ = 2.225 ppm downfield from DSS)

Residue		Oligosaccharide		
		LNH	Monofuco LNH	Difuco LNH
α-Glc	H_1	5.221	5.218	5.213
	H_2	3.589	3.585	3.585
β-Glc	H_1	4.637	4.639	4.629
	H_2	3.290	3.287	3.284
	H_3	3.635	3.629	3.632
$\beta\text{-Gal}^{4}$	H_1	4.431	4.424	4.424
	H_2	3.573	3.583	3.546
	H_3	3.732	3.705	3.695
	H_4	4.143	4.147	4.145
$\beta\text{-Gal}^{4,6}$	H_1	4.472	4.450	4.453
	H_2	3.510	3.527	3.489
	H_3	3.673	-	3.620
$\beta\text{-Gal}^{3,3}$	H_1	4.446	4.440	4.505
	H_2	3.513	3.512	3.496
	H_3	-	-	3.627

(Contd.....)

(Contd....) Table V

β-GlcNAC[3]	H_1	4.780	4.712	4.692
	H_2	3.880	3.881	3.937
	H_3	-	-	4.077
	H_4	-	-	3.749
	N-Acetyl	2.026	2.024	2.030
β-GlcNAc[6]	H_1	4.66	4.670	4.663
	H_2	3.741	3.901	3.913
	H_3	-	-	3.706
	N-Acetyl	2.057	2.050	2.050
α-Fuc[3]	H_1	-	5.105	5.098
	H_2	-	3.680	3.683
	H_4	-	3.900	3.910
	H_5	-	4.866	4.873
	H_6	-	1.170	1.170
α-Fuc[4]	H_1	-	-	5.025
	H_2	-	-	3.785
	H_4	-	-	3.880
	H_5	-	-	4.915
	H_6	-	-	1.178

[a] Probe temperature was 24°C

[b] Data from reference (44).

CHAPTER - IV

RESULTS

4.1 PURIFICATION OF GOAT MILK OLIGOSACCHARIDES

4.1.1 Bio-Gel P-4 Chromatography

Figure 1 shows the elution profile of goat milk oligosaccharides from the Bio-Gel P-4 column. A substantial amount of sugar was eluted in the void volume along with the Bovine Serum Albumin (BSA) standard indicating that the carbohydrate peak was a high molecular weight polymer. Apart from this peak, four more distinct carbohydrate-containing peaks, designated as A,B,C and D were obtained. The k' values and the approximate molecular size of the oligosaccharides that are expected to be present in each peak are given in Table VI. The peak D which corresponds to disaccharide, was found to contain mainly lactose. Peak C (frac. nos. 57-65), peak B (frac. nos. 51-54) and peak A (frac. nos. 32-36) fall in the k' range 2.2-2.5, 1.9-2.1 and 1.3-1.4, respectively. These results suggest that peak C would contain tri- to penta-saccharides whereas peak B would contain hexa- to nona-saccharides. Peak A consists of relatively large oligosaccharides. Judging from the peak area, peak C is the most dominant fraction, accounting for about 60 percent of the total sugar eluted after the void volume followed by peak B and peak A. Thus, besides lactose, small oligosaccharides namely tri- to hexasaccharides seem to be present in much higher concentration than that of the higher oligosaccharides in the goat milk. Perhaps the former are the enzymic degradation products of the larger oligosaccharides. Fractions under peak C (57-65) were pooled and analysed by HPLC.

Fig. 1 - Bio-Gel P-4 chromatography of goat milk oligosaccharides. One ml sample (ca equal to 250 mg glucose) was loaded on the Bio-Gel column (1.5 x 60 cm) and oligosaccharides were eluted with water at a flow rate of 0.25 ml/min. Fractions (0.75 ml) were collected. Aliquots (50 µl) from each fraction were used for carbohydrate assay by phenol-sulfuric acid method (46) as described in the text.

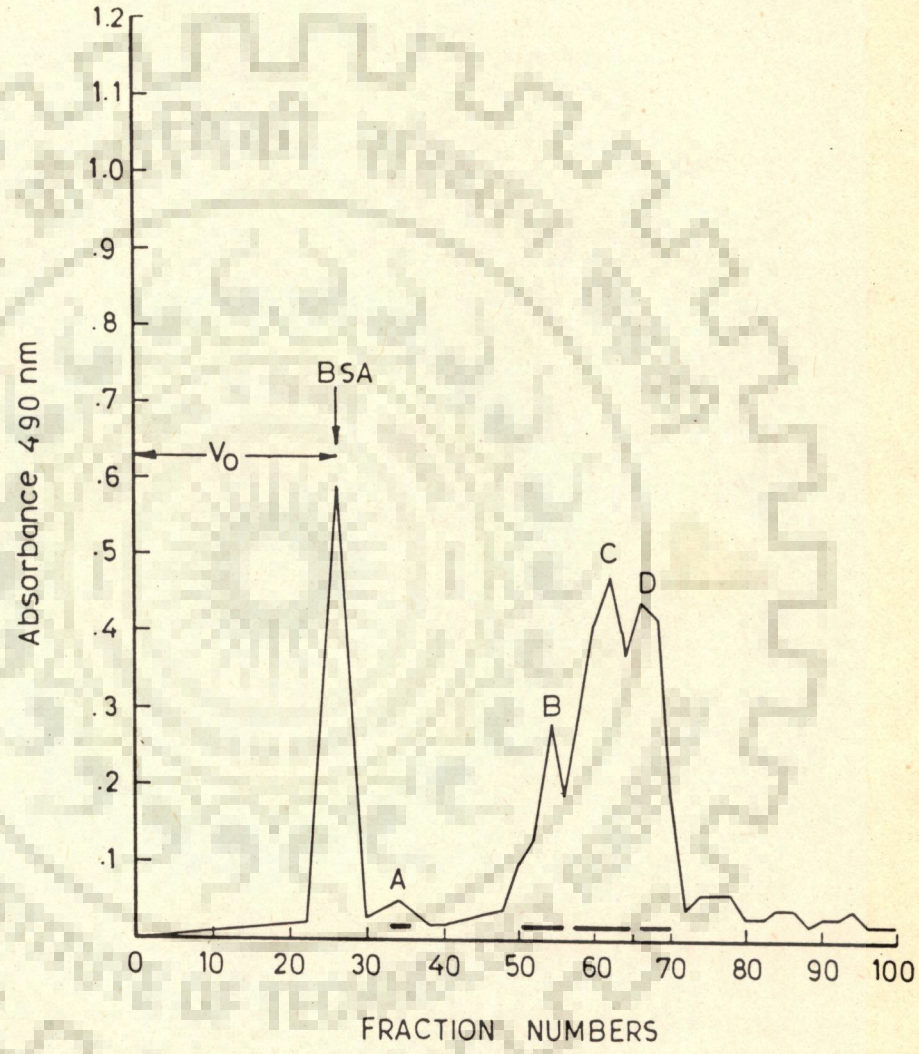

1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
Absorbance 490 nm
BSA
V_O
A
B
C
D
0
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBERS

TABLE VI - k' VALUES AND APPROXIMATE MOLECULAR SIZE OF VARIOUS OLIGOSACCHARIDE FRACTIONS FROM Bio-Gel P-4 COLUMN

Fraction	k' (ml)	Approximate Molecular Size of Oligosaccharide[a] (Number of monosaccharides)
A	1.3 - 1.4	16 - 19
B	1.9 - 2.1	6 - 9
C	2.2 - 2.5	3 - 5
D	2.5 - 2.7	2 - 3

a - Yamashita, K., Mizuochi, T. and Kobata, A. (1982) Analysis of oligosaccharides by gel filtration In,'Methods in Enzymology' (Ginsburg, V.ed.) Vol. 83, pp. 105-126.

4.1.2 Purification of Peak C oligosaccharides by HPLC

4.1.2.1 Reverse phase HPLC

The HPLC of peak C was conducted on a Shimadju LC-4A liquid chromatograph fitted with a Du-Pont Zorbax C-18 column, a UV detector and a Shimadju C-R2AX data processor system, using HPLC grade water as eluent at a flow rate of 1 ml/minute. The results are shown in Figure. 2. As can be seen, fraction 'C' was clearly resolved into five distinct oligosaccharide peaks, designated as P-I, P-II, P-III, P-IV and P-V having k' values 1.3, 2.2, 2.9, 4.2 and 5.5 respectively (Table VII). The k' values, obtained by dividing the elution volume of each peak by the void volume, were sufficiently different from each other indicating that the separation was quite good. Surprisingly, however, the reducing terminal monosaccharide residues of all the five sugars were not resolved into their α-and β-anomeric forms as has been generally observed for reducing sugars in reverse phase chromatography (41, 13, 28). This might be due to the unfavourable mutarotation time of the sugar (13), since a partial resolution into anomeric forms was achieved when the chromatographic analysis was carried out at a lower temperature of about 7-8°C. Fig. 3 and 4 show the partial resolution of the trisaccharide P-V and tetrasaccharide P-II, respectively, into their α - and β -anomers on the reverse phase C-18 column eluted with water at a flow rate of 1 ml/min. The most likely reason for such a phenomenon is that at low temperature, the mutarotation time of the sugar residue at the reducing terminal is decreased, thereby leading to a partial separation into anomeric forms.

Preparative chromatography for all the five peaks, obtained on the reverse column, was carried out on the same column (Du-Pont Zorbax C-18) as described in Material and Methods and each peak was purified up

Fig. 2 - Reverse phase HPLC of the Bio-Gel P-4 fraction C. Ten µl sample was injected on a Zorbax C-18 column and eluted with water at a flow rate of 1 ml/min. The elution was monitored by UV absorption at 202 nm. Five oligosaccharide fractions, P-I, P-II, P-III, P-IV and P-V were resolved.

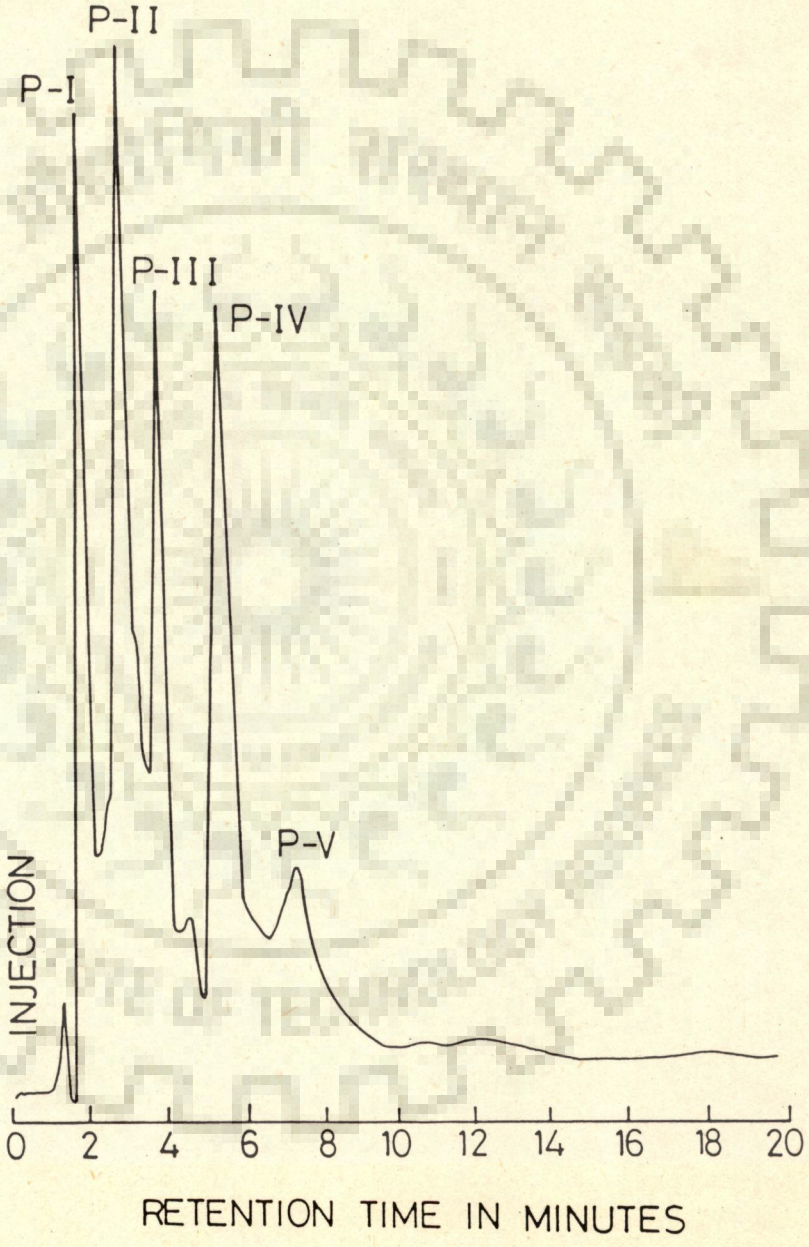

P-II
P-I
P-III
P-IV
P-V
INJECTION
0
2
4
6
8
10
12
14
16
18
20
RETENTION TIME IN MINUTES

TABLE VII - REVERSE PHASE HPLC VALUES OF GOAT MILK OLIGOSACCHARIDES

Oligosaccharide	Retention time (t_r)	Relative retention *(k')
P-I	1.8	1.3
P-II	3.0	2.2
P-III	4.0	2.9
P-IV	5.8	4.2
P-V	7.5	5.5

Chromatographic conditions

Column	Du-Pont Zorbax C-18
Solvent	Water
Flow	1 ml/min

* Values have been obtained by dividing retention time (t_r) by the column dead time (t_o)

Fig. 3 - Reverse phase HPLC of the trisaccharide P-V purified on reverse phase C-18 at low temperature. The HPLC was carried out at 7-8°C on a Zorbax C-18 column using water as the mobile phase at a flow rate of 1 ml/min. The elution was monitored by UV absorption at 202 nm. The α-and β-anomers of the reducing terminal monosaccharide are partially resolved.

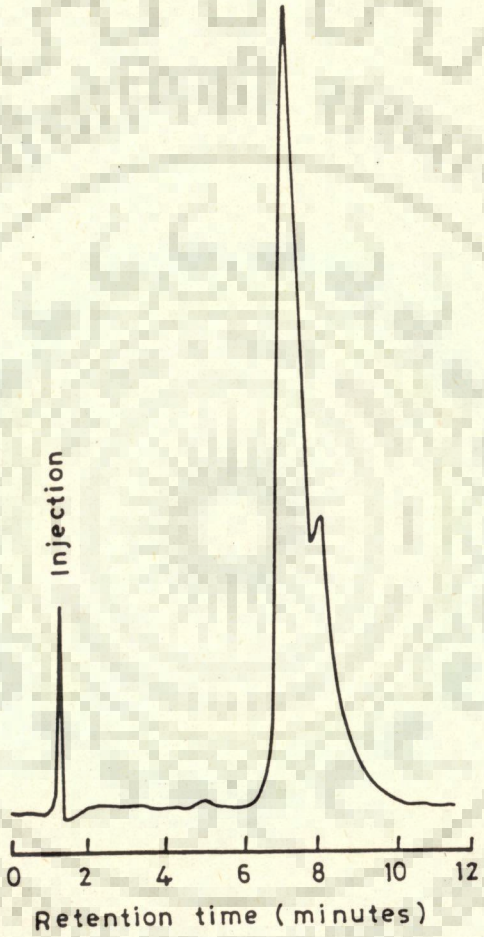
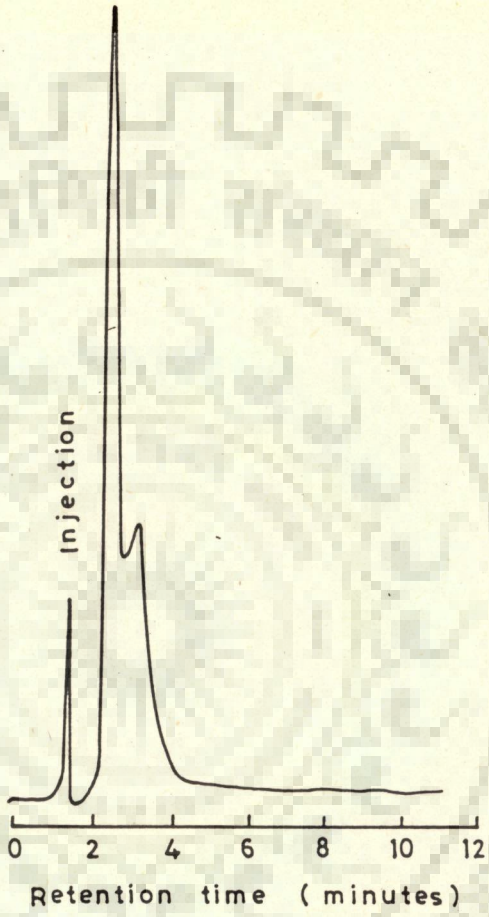

Injection
0
2
4
6
8
10
12
Retention time (minutes)

Fig. 4 - Reverse phase HPLC of the tetrasaccharide P-II purified on reverse phase C-18 column at low temperature. The HPLC was carried out at 7-8^{o}C on a Zorbax C-18 column using water as the mobile phase at a flow rate of 1 ml/min. The elution was monitored by UV absorption at 202 nm. The α-and β-anomers of the reducing terminal sugar are partially resolved.


Injection
0
2
4
6
8
10
12
Retention time (minutes)

to a purity level of 95% or above by repeated chromatography on the same column. Figures 5(a) and 5(b) show the chromatograms of compound P-I, obtained during the various stages of purification. Fig. 5(a) shows the chromatogram obtained after the first preparative run, yielding only 86% pure compound and P-II was present as the minor contaminant at this stage. The rechromatography on the same column improved the purity level to 97% (Fig. 5b), suitable for NMR studies. Subsequent runs did not improve the purity level any further.

Figure 6(a-c) show the chromatograms obtained after various steps of purification of compound P-II. The compound P-II was only 70% pure after the first preparative run with P-I and P-III as impurities. The purity improved to 88% after the second preparative run and finally, the third preparative run yielded 95% pure compound. Similarly, the compound P-III also was found to be only 70% pure after the first preparative run containing compound P-II as the major impurity (Fig. 7a). The second run enhanced the purity level to 82% (Fig. 7b) while 97% pure compound was obtained after the third and final preparative run (Fig. 7c). The purity levels of compound P-IV obtained after the first, second and third preparative runs are shown in Fig. 8(a-c). The compound was purified up to a level of 98%. The compound P-V was purified upto 97% after the second preparative run (Fig. 9 a-b). Hence, it was possible to purify five oligosaccharides upto 95% or above by reverse phase C-18 HPLC using water as the mobile phase from the goat milk.

4.1.2.2 Normal phase HPLC

The purified oligosaccharide fractions from the C-18 column were checked for contamination by any cross peak on the normal phase Du-Pont

Fig. 5 - Reverse phase chromatograms of the compound P-I during various steps of purification. HPLC was carried out on a Zorbax C-18 column using water as the mobile phase at a flow rate of 1 ml/min. The elution was monitored by UV absorption at 202 nm and the fractions were collected manually :

(a) after the first preparative run (86% pure) and
(b) after the second preparative run (97% pure)

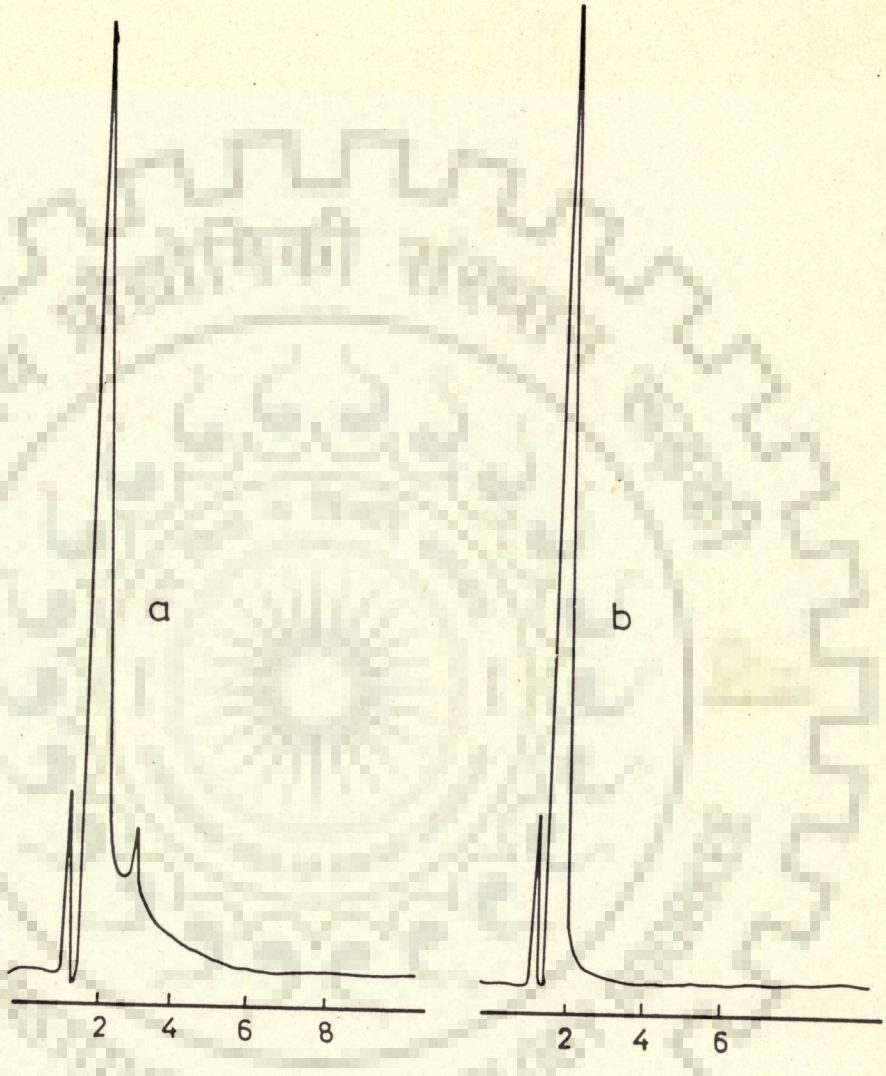

a
b
2
4
6
8
2
4
6

Fig. 6 - Reverse phase chromatograms of the compound P-II during various steps of purifications. HPLC was carried out on a Zorbax C-18 column using water as the mobile phase at a flow rate of 1 ml/min. The elution was monitored by UV absorption at 202 nm. Fractions were manually collected :

(a) after the first preparative run (70% pure);
(b) after the second preparative run (88% pure) and
(c) after the third preparative run (95% pure).

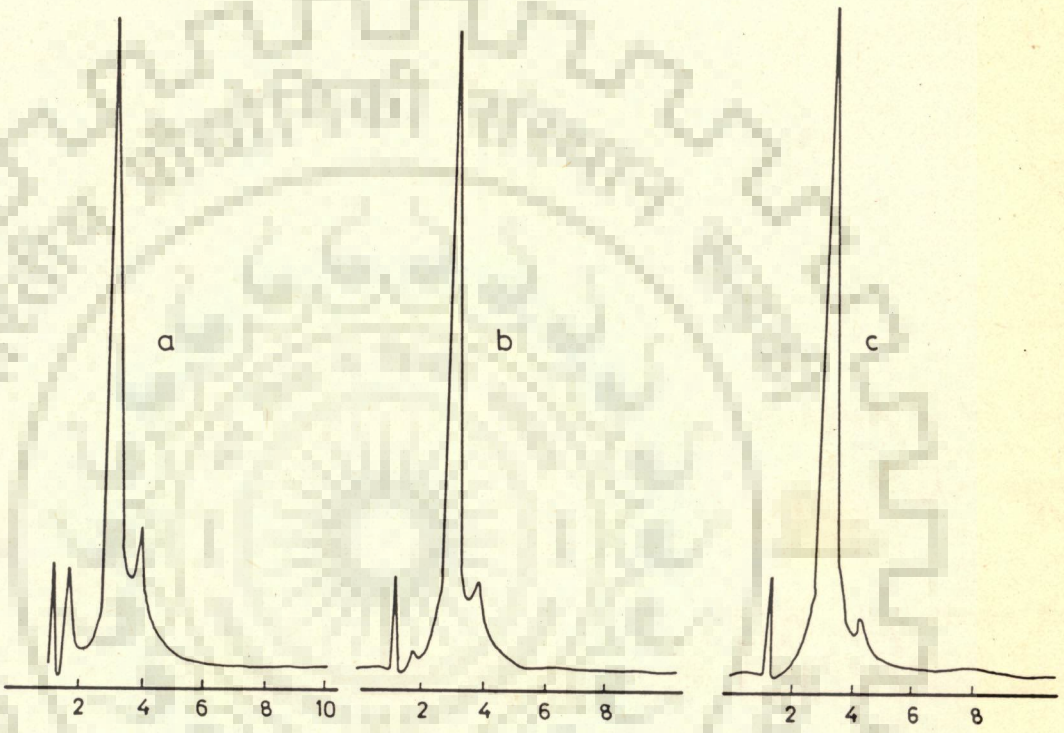

a
b
c
2
4
6
8
10
2
4
6
8
2
4
6
8

Fig. 7 - Reverse phase chromatograms of the compound P-III during various steps of purification. HPLC was carried out on a Zorbax C-18 column using water as the mobile phase at a flow rate of 1 ml/min. The elution was monitored by UV absorbance at 202 nm and fractions were manually collected :

(a) after the first preparative run (70% pure);
(b) after the second preparative run (82% pure) and
(c) after the third preparative run (97% pure).

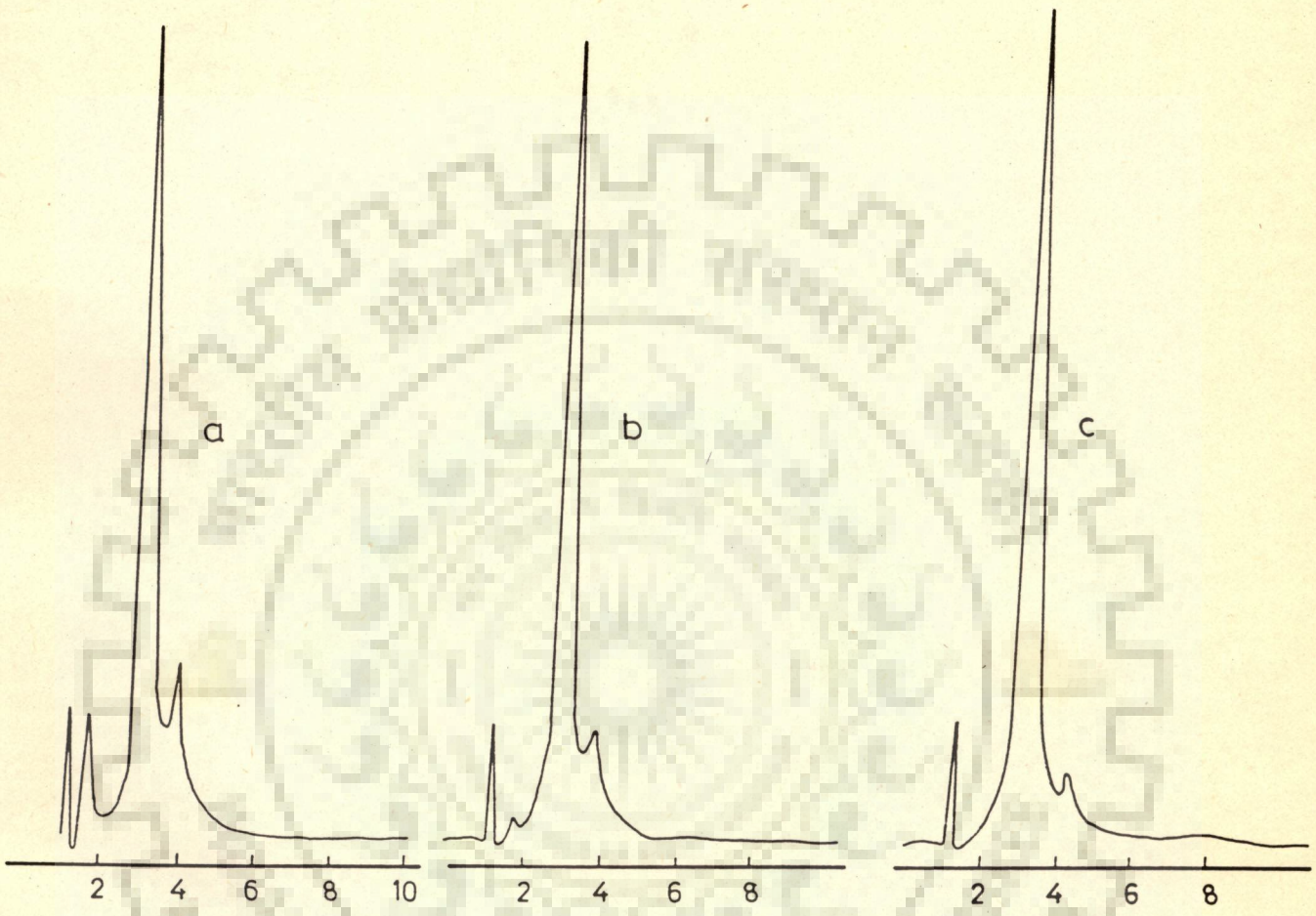

a
b
c
2
4
6
8
10
2
4
6
8
2
4
6
8

Fig. 8 - Reverse phase chromatograms of the compound P-IV during various steps of purification. HPLC was carried out on a Zorbax C-18 column using water as the mobile phase at a flow rate of 1 ml/min. The elution was monitored by UV absorbance at 202 nm. Fractions were collected manually.

(a) after the first preparative run (80% pure);
(b) after the second preparative run (86% pure) and
(c) after the third preparative run (98% pure).

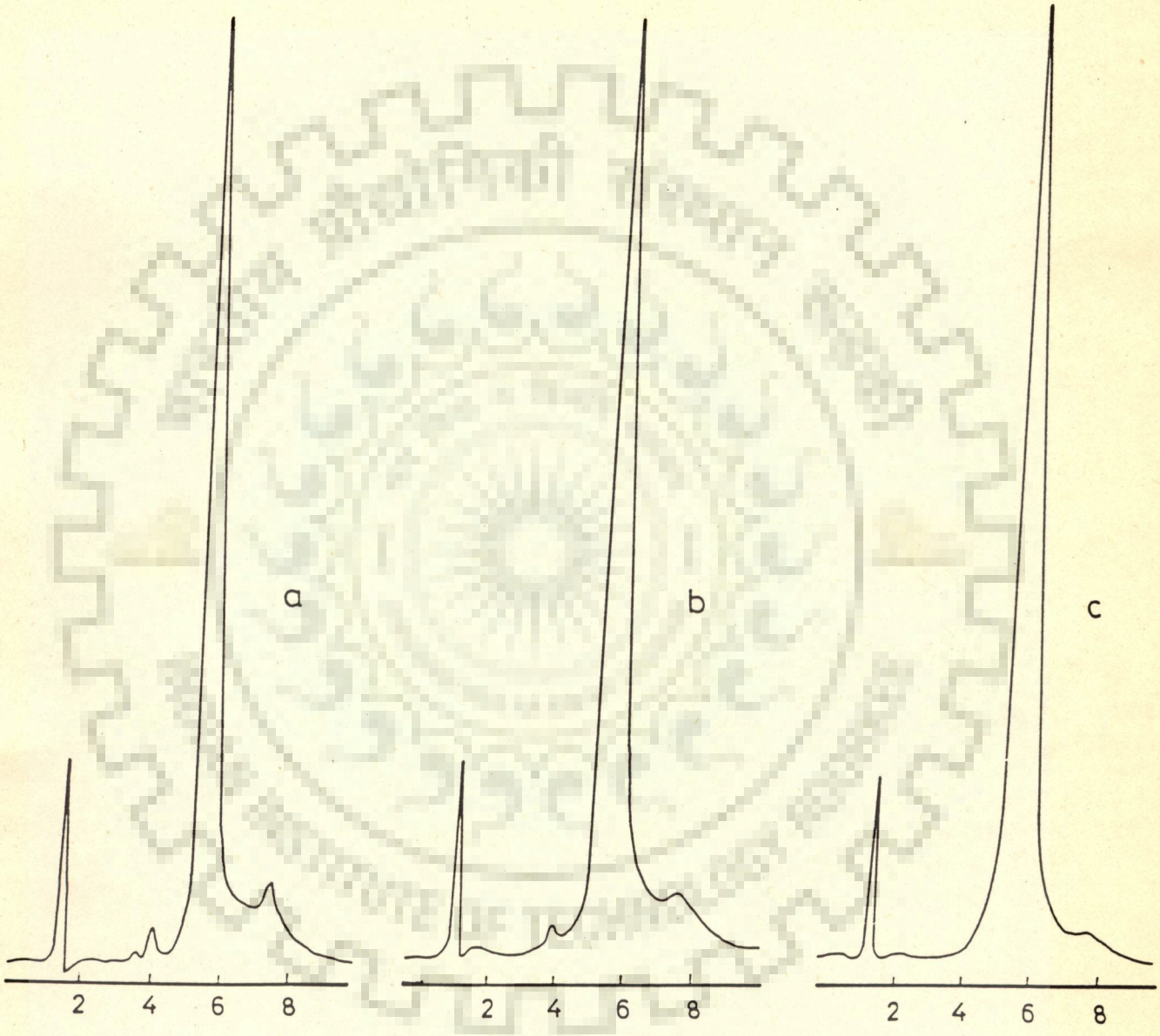
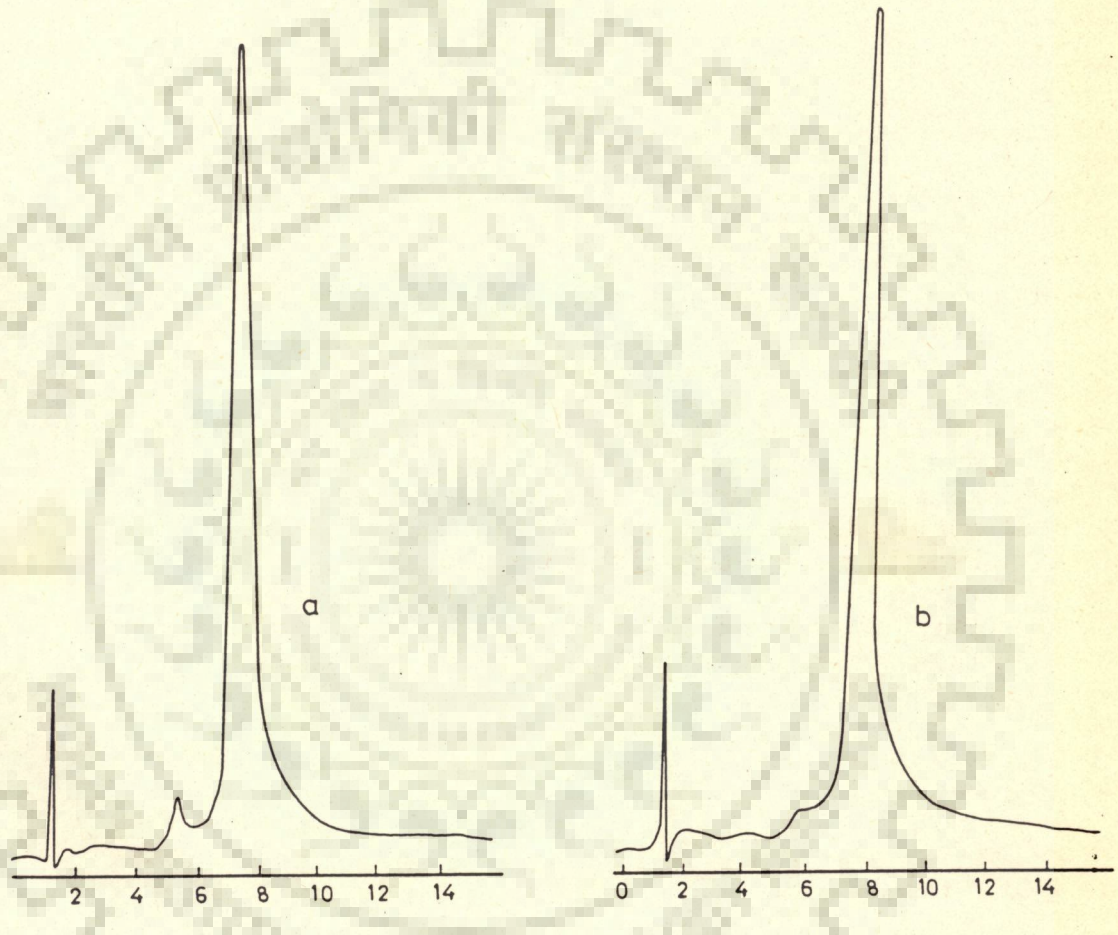


Fig. 9 - Reverse phase chromatograms of the compound P-V during various steps of purification. HPLC was carried out on a Zorbax C-18 column using water as the mobile phase at a flow rate of 1 ml/ min. The elution was monitored by UV absorption at 202 nm. Fractions were manually collected :

(a) after the first preparative run (88% pure) and
(b) after the second preparative run (97% pure).


a
b
2
4
6
8
10
12
14
0
2
4
6
8
10
12
14

Zorbax-NH_2 column. A mixture of acetonitrile : water (60:40) was used as the mobile phase at a flow rate of 1 ml/min. The normal phase elution profiles of the P-I, P-II, P-III, P-IV and P-V oligosaccharides, purified on C-18 column are shown in Figure 10(a-e). As is evident from these chromatograms, none of the compounds showed any significant contamination by any cross peak. Furthermore, in each case the purity was found to be 95% or above. The retention time and relative retention values (k') of the compounds on the normal phase column are listed in Table VIII. Since, the purity of the compounds was above 95%, these were good enough for characterization by ^1H Nuclear Magnetic spectroscopy (NMR).

4.1.2.3 Carbohydrate Composition of Oligosaccharides by HPLC

Table IX shows the chemical composition data of the various goat milk oligosaccharides obtained by HPLC of the benzoylated methyl glycosides using Jentoft's method. The results show that compound P-I is a pentasaccharide containing one Glc, one GlcNAc and three Gal residues whereas P-II is a tetrasaccharide containing one Glc, one GlcNAc and two Gal residue. Compounds P-III and P-IV are fucose-containing pentasaccharides having one Glc, one GlcNAc, two Gal and one Fuc residues each. Compound P-V consists of one residue each of Glc, GlcNAc and Gal.

4.2 Characterization of Oligosaccharides by ^1H NMR

4.2.1 Structure determination of oligosaccharide P-V by ^1H NMR

Figure 11 shows the 400 MHz ^1H NMR spectrum of the oligosaccharide P-V and the ^1H NMR chemical shifts for various monosaccharide residues of the P-V oligosaccharide are listed in Table X. Integration of the anomeric region, that is, from 4.0-5.5 ppm, shows that the compound is a trisaccharide. The presence of glucose (Glc) residue at the reducing

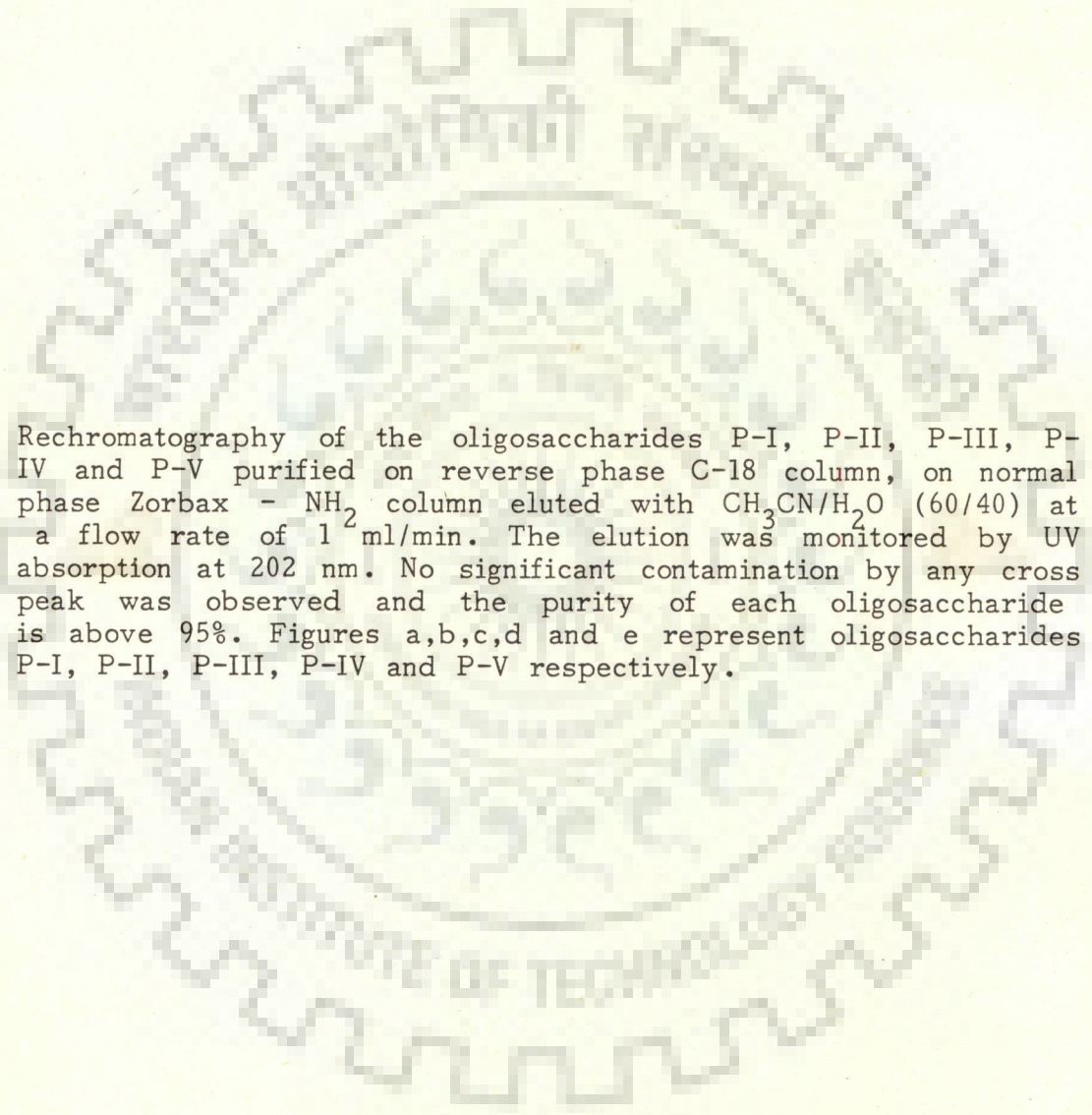
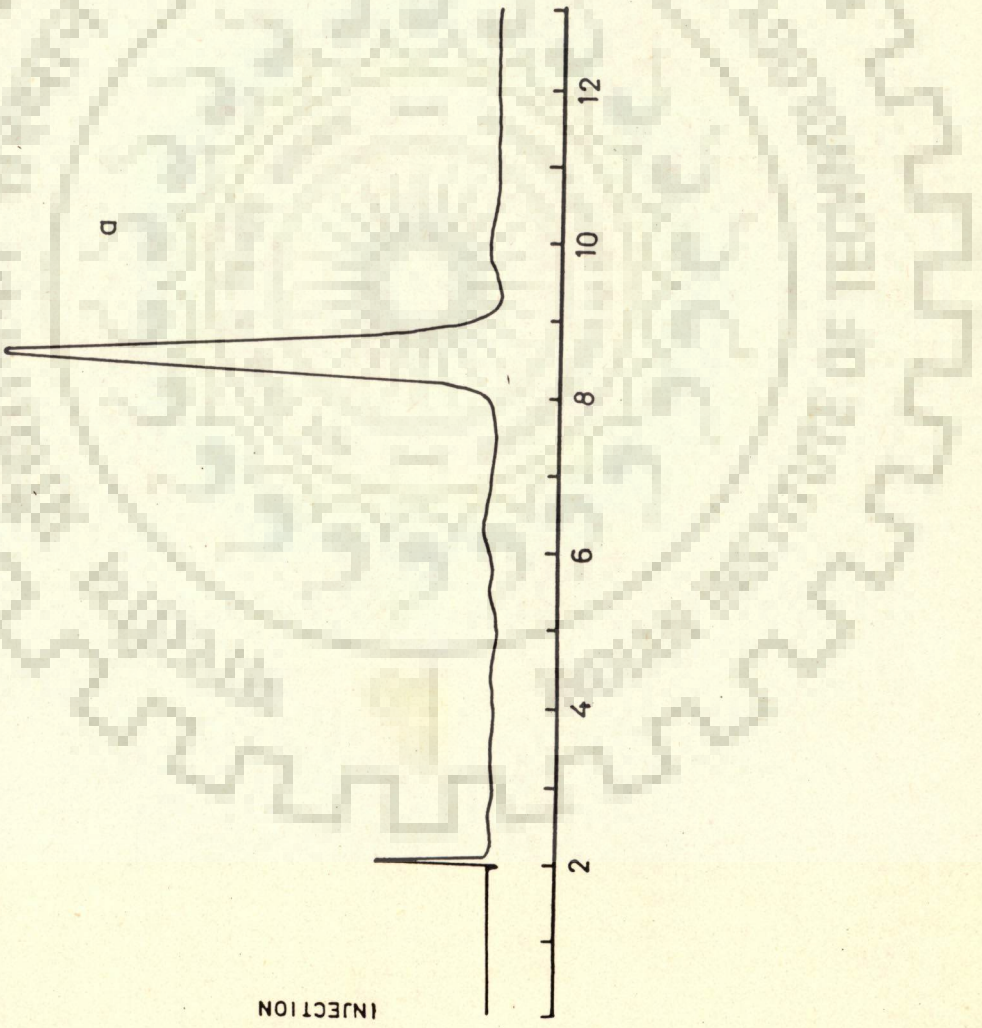
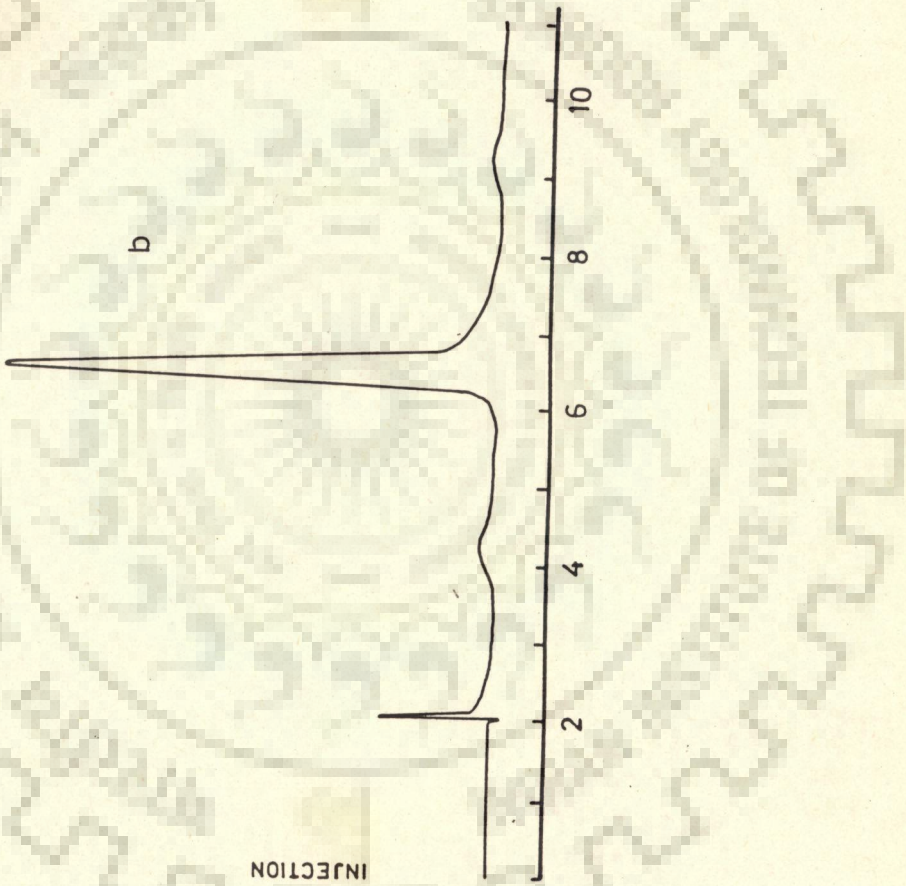
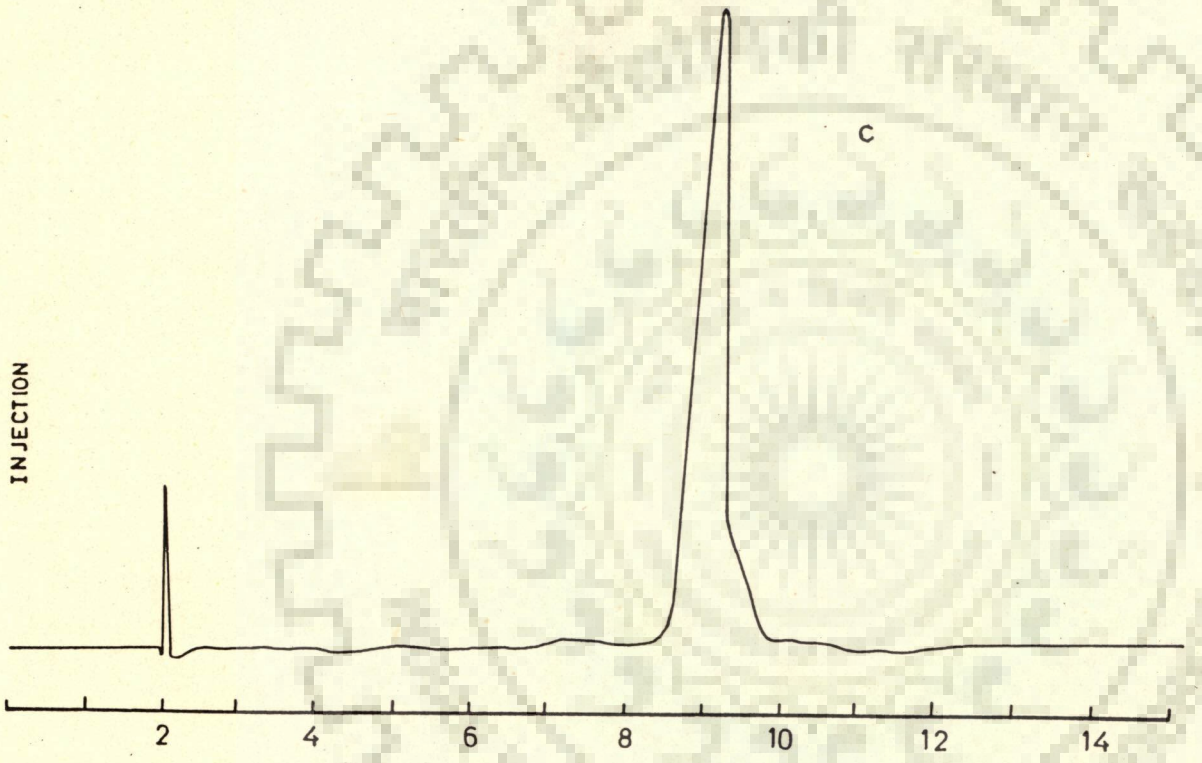
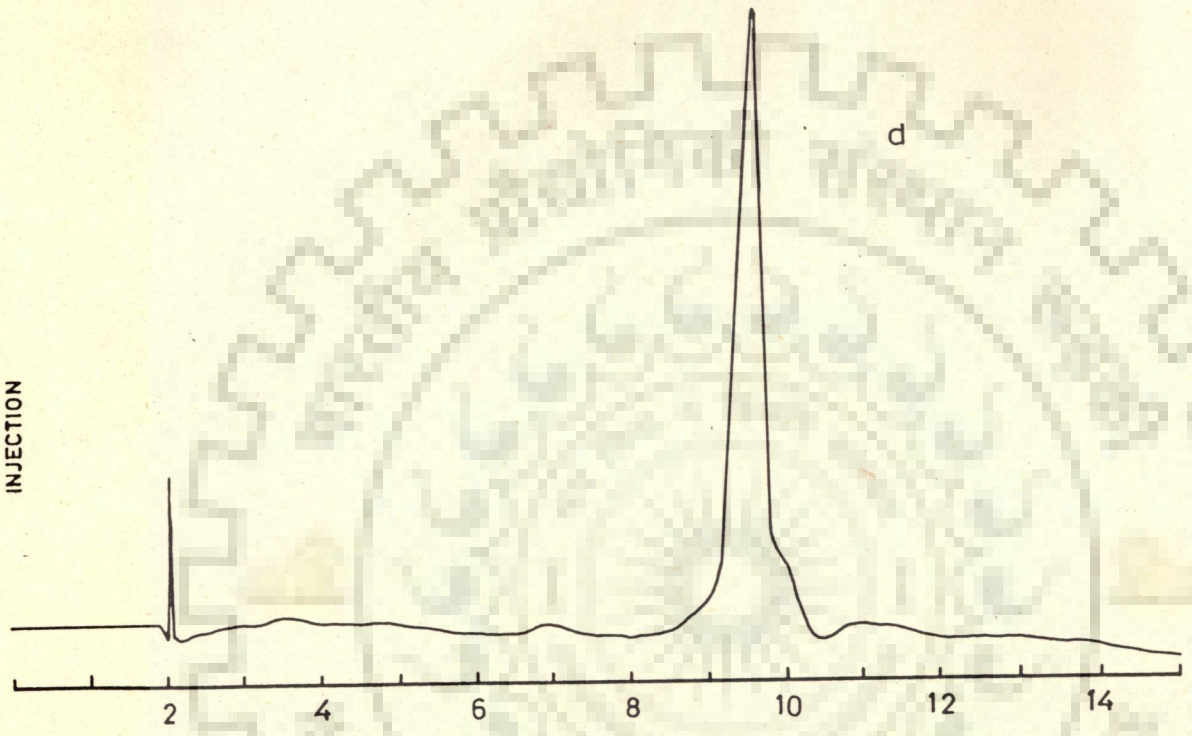


Fig. 10 - Rechromatography of the oligosaccharides P-I, P-II, P-III, P-IV and P-V purified on reverse phase C-18 column, on normal phase Zorbax - NH_2 column eluted with CH_3CN/H_2O (60/40) at a flow rate of 1 ml/min. The elution was monitored by UV absorption at 202 nm. No significant contamination by any cross peak was observed and the purity of each oligosaccharide is above 95%. Figures a,b,c,d and e represent oligosaccharides P-I, P-II, P-III, P-IV and P-V respectively.


INJECTION
2
4
6
8
10
12
a


b
INJECTION
2
4
6
8
10


C
INJECTION
2
4
6
8
10
12
14


d
INJECTION
2
4
6
8
10
12
14

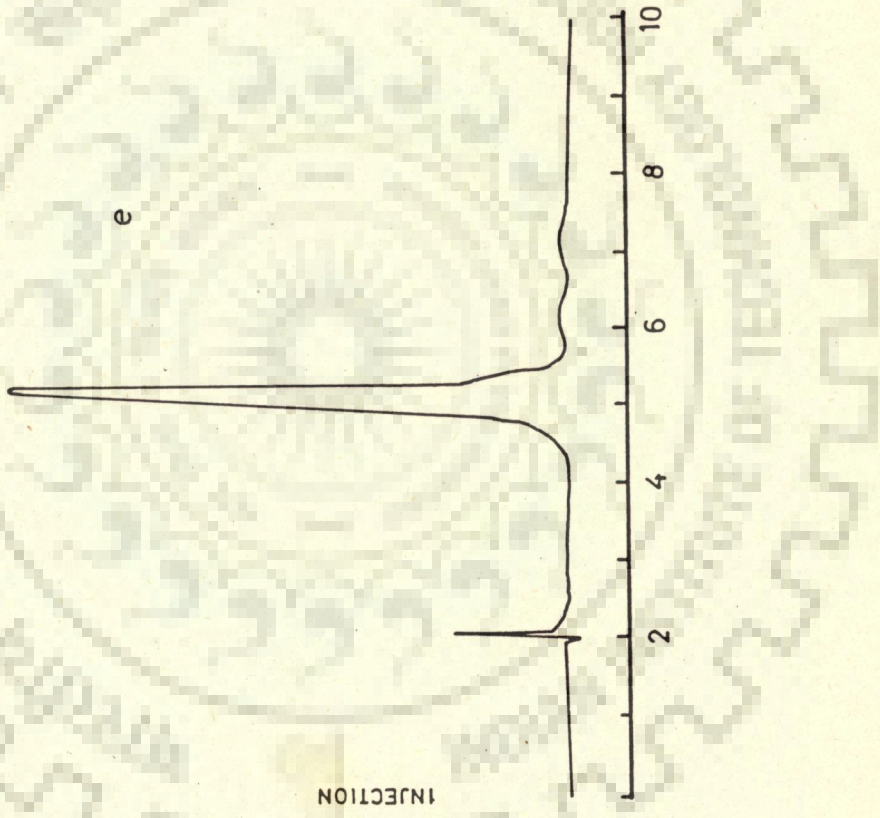

e
2
4
6
8
10
INJECTION

TABLE VIII - NORMAL PHASE HPLC VALUES OF GOAT MILK OLIGOSACCHARIDES

Oligosaccharide	Retention time (t_r)	Relative retention *(k')
P-I	8.2	4.1
P-II	6.4	3.2
P-III	9.0	4.5
P-IV	9.6	4.8
P-V	5.0	2.5

Chromatographic conditions

Column	Du-Pont Zorbax - NH_2
Solvent	Acetonitrile/water (60:40)
Flow	1 ml/min

* Values have been obtained by dividing the retention time (t_r) by the column dead time (t_o).

TABLE IX - CARBOHYDRATE ANALYSIS OF GOAT MILK OLIGOSACCHARIDE BY REVERSE PHASE HPLC

Oligosaccharide	Residues			
	Glc	GlcNAc	Gal	Fuc
P - I	1	0.91	3.2	-
P - II	1	0.95	2.1	-
P-III	1	1.04	1.9	0.9
P - IV	1	1.09	2.2	1.1
P - V	1	0.97	1.1	-

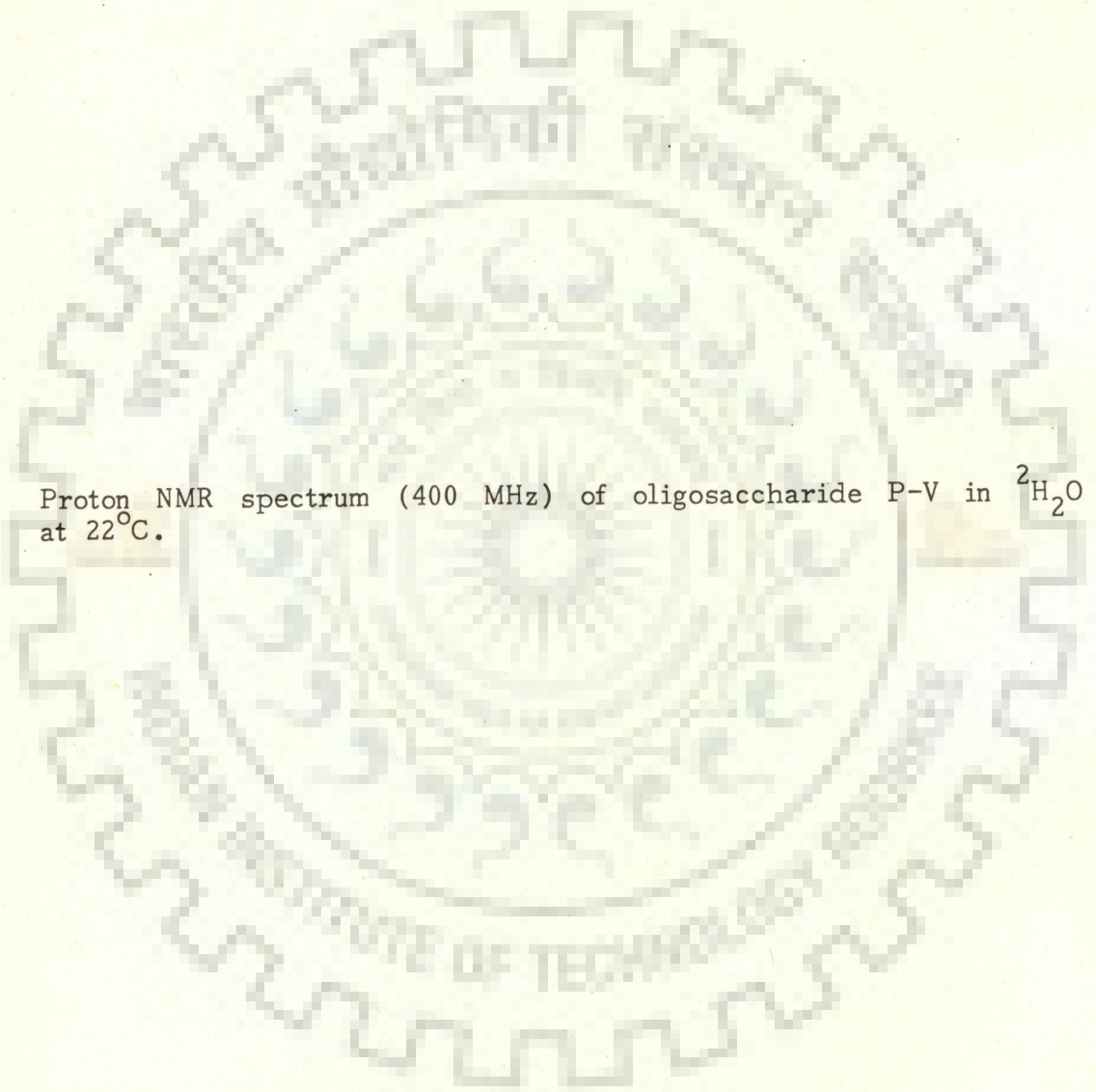


Fig. 11 - Proton NMR spectrum (400 MHz) of oligosaccharide P-V in $^{2}H_{2}O$ at 22°C.

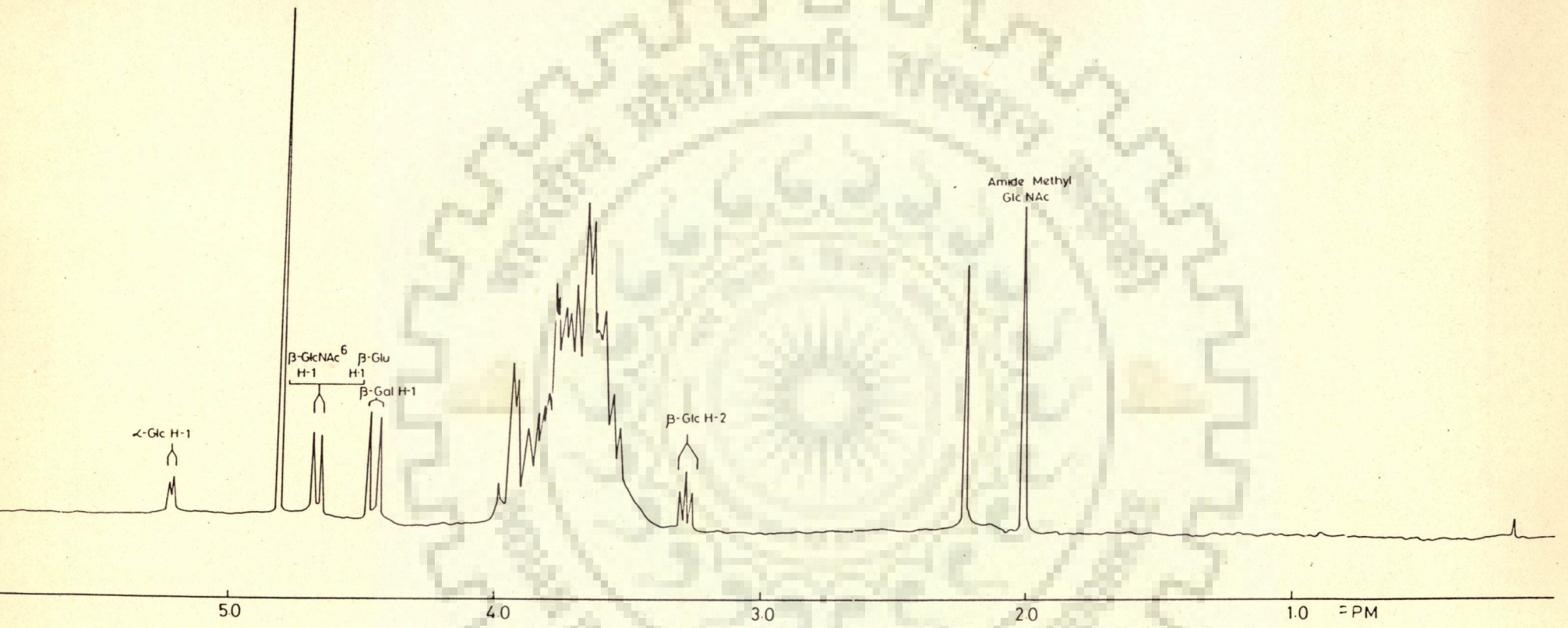

α-Glc H-1
β-GlcNAc 6
H-1
β-Glu
H-1
β-Gal H-1
β-Glc H-2
Amide Methyl
GlcNAc
5.0
4.0
3.0
2.0
1.0
PPM

TABLE X - ^{1}H NMR CHEMICAL SHIFTS OF GOAT MILK OLIGOSACCHARIDE P-V

The chemical shifts are reported relative to internal (DSS) using acetone as internal standard (δ = 2.225 pm downfield from DSS).

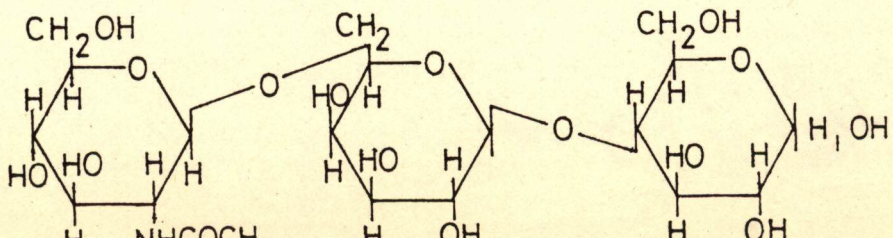
Glc = Glucose; Gal = Galactose; GlcNAc = N-acetylglucosamine

Residue	Proton	Chemical shift, δ, ppm
α-Glc	H_1	5.228
	H_2	3.585
β-Glc	H_1	4.680
	H_2	3.286
	H_3	3.639
β-Gal^4	H_1	4.436
	H_2	3.574
	H_3	3.738
	H_4	-
β-Gal^{4,6}	H_1	-
	H_2	-
	H_3	-
β-Gal^3	H_1	-
	H_2	-
	H_3	-
β-Gal^{4,3}	H_1	-
	H_2	-
β-GlcNAc^6	H_1	4.680
	H_2	3.745
	N-acetyl	2.059
β-GlcNAc^3	H_1	-
	H_2	-
	N-acetyl	-

terminal is indicated by the doublet at 5.228 ppm (J = 3.7 Hz) assigned to α- Glc H-1 and a triplet at 3.286 ppm assigned to β -Glc H-2 (41). The singlet at 2.059 ppm (3 proton by integration) is assigned to the amide methyl of one N-acetylglucosamine (GlcNAc) residue. This is further supported by the presence of a doublet at 4.680 ppm with a coupling constant, (J) of 7.8 Hz, due to β-GlcNAc H-1. The presence of one β-galactose (Gal) is shown by the doublet at 4.436 ppm. The β-Glc H-1 signal has overlapped with that of β-Glc NAc H-1 at 4.680 ppm, which is confirmed by decoupling of the H-2 of β-Glc at 3.286 ppm. The comparision of the spectrum with that of lactose (15), lacto-N-tetraose(LNT) and lacto-N-neotetraose (Ln neoT) structures reported by Dua and Bush (41), indicates the presence of a lactosyl, Gal (β-1,4) Glc, residue at the reducing terminal. The absence of a downfield shift of the H-4 resonance of galactose confirms that β-Gal is not substituted at C-3 position by β-Glc NAc (41). This implies that the GlcNAc may be 1 → 6 linked to Gal.

The β-GlcNAc H-1 chemical shift (4.680 ppm) of P-V (Table X) is in excellent agreement with the H-1 chemical shift of β-Glc NAc^6 (4.666 ppm) of the compounds lacto-N-hexaose (LNH) reported by Dua et al. (44). The resonance of β -GlcNAc H-2 (3.745 ppm) obtained by irradiation of β-GlcNAc H-1, also compares favourably with the H-2 resonance of β-Glc NAc^6 (3.741 ppm) of LNH, leading to the conclusion that β-GlcNAc is 1 → 6 linked to β -Gal. Based on the above interpretations, the compound P-V, was assigned the following structure :

Glc NAc (β -1,6) Gal(β -1,4) Glc



The remaining proton resonances were assigned by SDDS experiments- Decoupling at α-Glc H-1 (5.228 ppm) gave the position of α-Glc H-2 at 3.585 ppm (Fig. 12(a)). The resonance of β-Glc H-1 at 4.680 ppm was confirmed by irradiating the β-Glc H-2 resonance at 3.286 ppm, which also gave decoupling effect at 3.639 ppm assigned to β-Glc H-3 (Fig. 12b, Decoupling of the β-GlcNAc H-1 signal at 4.680 ppm yielded the position of its corresponding H-2 at 3.745 ppm (Fig. 13b). Irradiation of the β-Gal H-1 doublet at 4.436 ppm showed effect at 3.574 ppm due to β-Gal H-2 (Fig. 13a). Decoupling of β-Gal H-2 yielded the position of its H-3 at 3.738 ppm, thus completing all the assignments.

4.2.2 Structure determination of oligosaccharide P-II by ^{1}H NMR

The ^{1}H NMR spectrum of compound P-II is given in Fig. 14, and the ^{1}H NMR chemical shifts for various protons in P-II are listed in Table XI. The integration of the anomeric region reveals that the compound is a tetrasaccharide. The presence of Glc at the reducing terminal is indicated by the doublet at 5.225 ppm (J = 3.6 Hz) assigned to α-Glc H-1 and a triplet at 3.282 ppm due to β-Glc H-2 (41). The 3 proton singlet at 2.057 ppm is assigned to the amide methyl of one GlcNAc residue, which is further supported by the presence of a doublet at 4.680 ppm due to β-GlcNAc H-1. The presence of one β-Gal (by integration) is shown by the doublet at 4.438 ppm. The β-Glc H-1 signal has merged with that of β-GlcNAc H-1 at 4.680 ppm which is confirmed by decoupling at β-Glc H-2. Comparision of the spectrum of compound P-II to that of compound P-V (Fig. 11) shows the presence of an additional doublet at 4.543 ppm with a coupling constant of 7.7 Hz. This doublet is assigned to the H-1 resonance of a second β-Gal residue present at the non-reducing terminal. The downfield shift of the H-1 resonance of this β-Gal residue is derived from the

Fig. 12 - Spin Difference Decoupling Spectra (SDDS) of compound P-V. (a) Decoupling at α-Glc H-1 giving the position of α-Glc H-2; (b) Decoupling at β-Glc H-1 and β-GlcNAc H-1 signal giving the position of β-Glc H-2 and β-Glc NAC H-2 respectively.

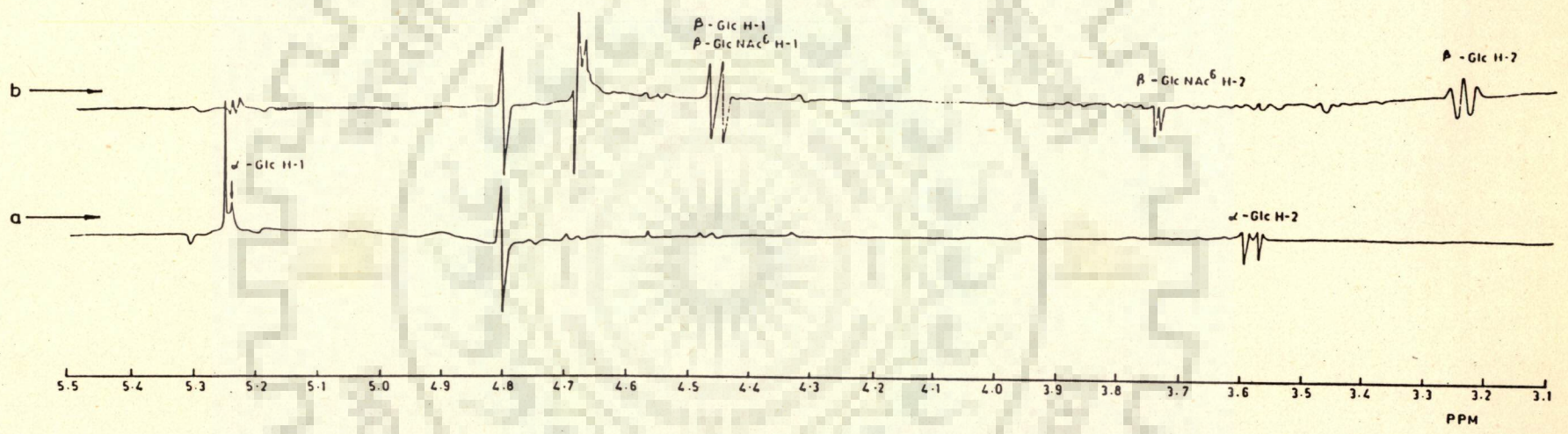

b
a
β - Glc H-1
β - Glc NAc^{6} H-1
β - Glc NAc^{6} H-2
β - Glc H-2
α - Glc H-1
α - Glc H-2
5·5 5·4 5·3 5·2 5·1 5·0 4·9 4·8 4·7 4·6 4·5 4·4 4·3 4·2 4·1 4·0 3·9 3·8 3·7 3·6 3·5 3·4 3·3 3·2 3·1
PPM

Fig. 13 - Spin Difference Decoupling Spectra of compound P-V. (a) Decoupling at β-Gal H-1 giving the corresponding H-2; (b) Decoupling at β-Glc H-2 identifying β-Glc H-3 as well as β-Glc H-1.

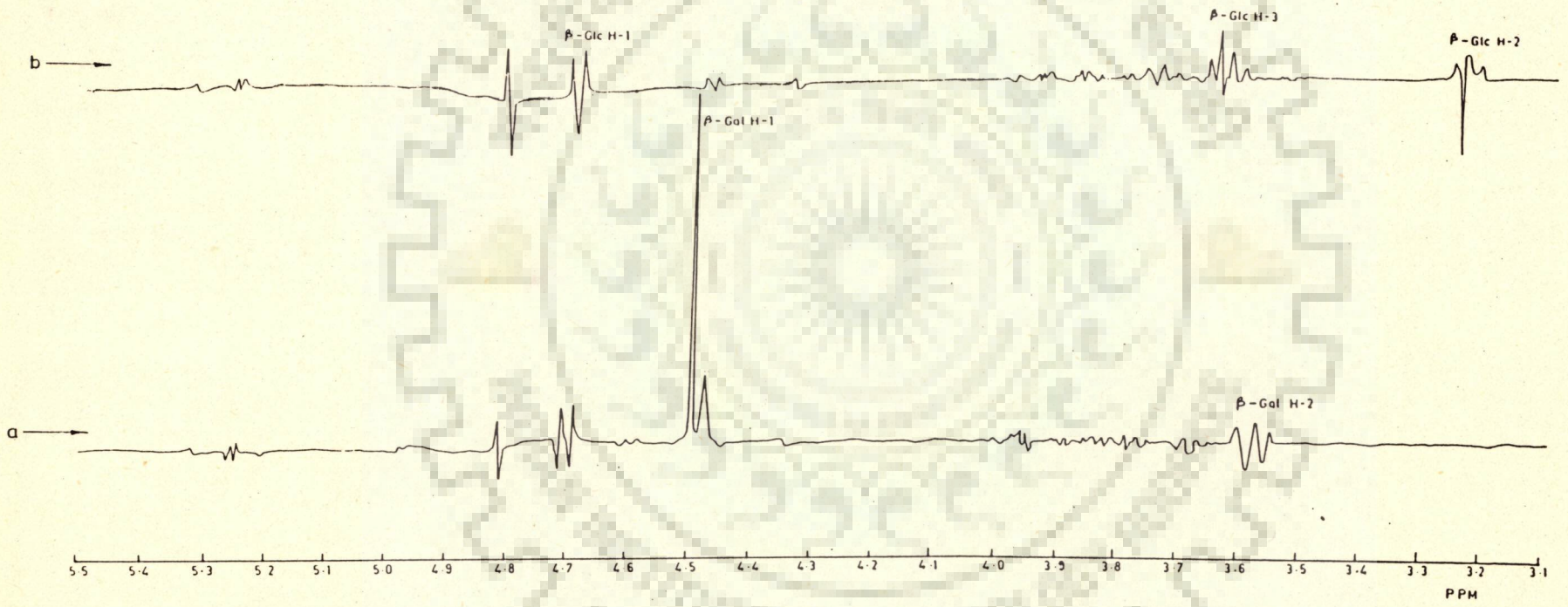

b
a
β-Glc H-1
β-Gal H-1
β-Glc H-3
β-Glc H-2
β-Gal H-2
5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1
PPM

Fig. 14 - Proton NMR spectrum (400 MHz) of oligosaccharide P-II in $^{2}H_{2}O$ at 22°C.

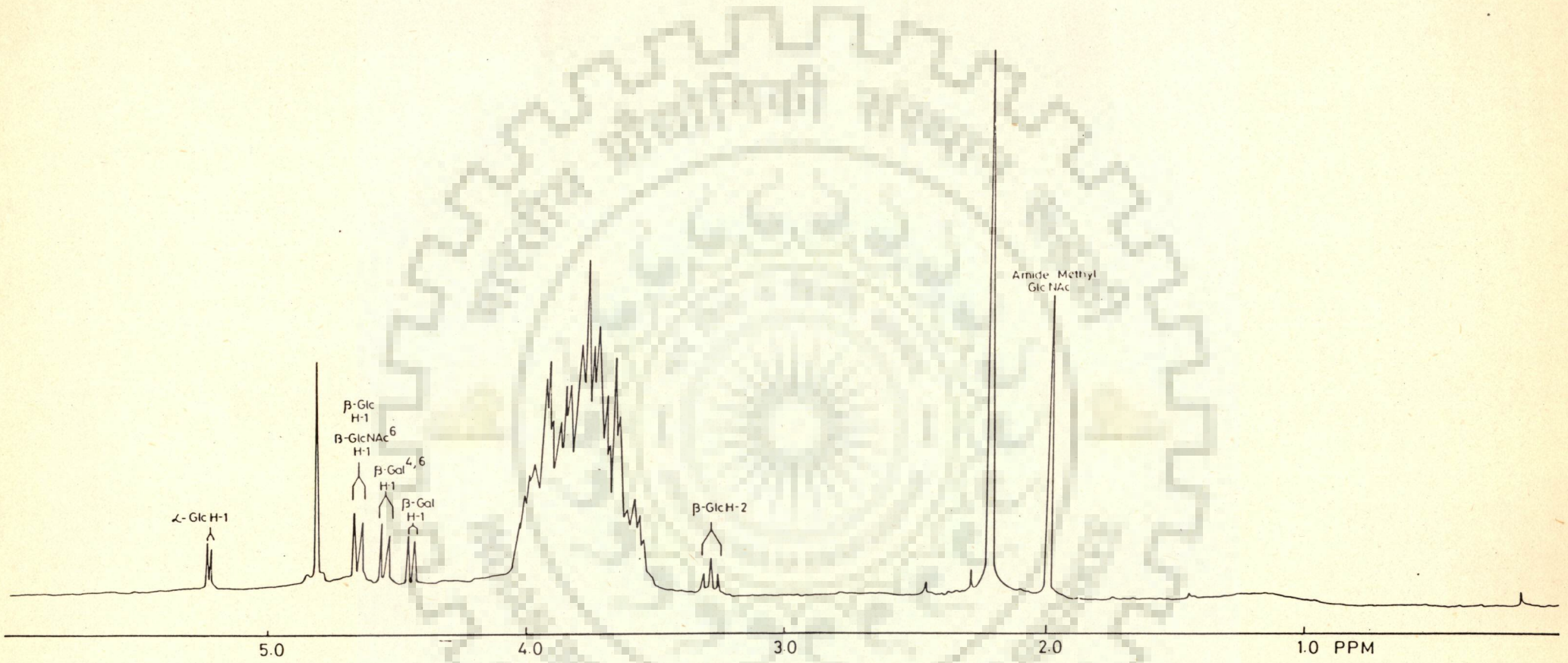

α-Glc H-1
β-Glc H-1
β-GlcNAc 6 H-1
β-Gal 4,6 H-1
β-Gal H-1
β-Glc H-2
Amide Methyl GlcNAc
5.0
4.0
3.0
2.0
1.0 PPM

TABLE XI - ^1H NMR CHEMICAL SHIFTS OF GOAT MILK OLIGOSACCHARIDE P-II

Chemical shifts are reported relative to internal(DSS) using acetone as internal standard (δ = 2.225 ppm downfield from DSS)

Glc = Glucose; Gal = Galactose; GlcNAc = N-acetylglucosamine

Residue	Proton	Chemical shift, δ ,ppm
α-Glc	H_1	5.225
	H_2	3.587
β-Glc	H_1	4.680
	H_2	3.282
	H_3	3.635
β-Gal^4	H_1	4.438
	H_2	4.580
	H_3	3.732
	H_4	-
β-Gal^{4,6}	H_1	4.543
	H_2	3.520
	H_3	-
β-Gal^3	H_1	-
	H_2	-
	H_3	-
β-Gal^{4,3}	H_1	-
	H_2	-
β-GlcNAc^6	H_1	4.680
	H_2	3.759
	N-acetyl	2.057
β-GlcNAc^3	H_1	-
	H_2	-
	N-acetyl	-

comparision with LNH structure and is assigned to the non-reducing terminal β-Gal which is 1 → 4 linked to a β-GlcNAc which in turn is 1 → 6 linked to the subsequent galactose. Similar downfield shift in the position of $Gal^{4,6}$ H-1 resonance has been reported by Dua et al. (44). Further comparision of the spectrum with those of LNT and LNneoT structures confirms the presence of lactosyl residue at the reducing terminal. Since there is no downfield shift of the β-Gal H-4 resonance, it can easily be concluded that none of the Gal residue is substituted at C-3 position (41). The SDDS and chemicals shift analogies of GlcNAc 1 → 6, as have been derived in the case of compound P-V, show that in this compound too, β-GlcNAc is 1 → 6 linked to β Gal. Based on the above interpretations, the structure of compound P-II was assigned as follows :

Gal(β -1,4) GlcNAc(β-1,6) Gal(β-1,4) Glc

It may be pointed out that the ^1H NMR spectrum of the compound shows it to be more than 95% pure.

The rest of the proton assignments were made by SDDS experiments. Irradiation of the H-1 resonances of α-Glc (5.225 ppm), β-GlcNAc (4.860 ppm) β-$Gal^{4,6}$ (4.543 ppm) and β-Gal^4 (4.438 ppm) gave the corresponding H-2 resonances at 3.587, 3.759, 3.520 (Fig. 15) and 3.580 ppm, respectively. Decoupling at β-Glc H-2 (3.282 ppm) and β-Gal H-2 (3.580 ppm) gave the positions of their H-3 resonances at 3.635 and 3.732 ppm respectively, thereby completing all the assignments.

4.2.3 Structure determination of the oligosaccharide P-I

Figure 16 shows the ^1H NMR spectrum of the compound P-I and the ^1H NMR chemical shifts for various protons in P-I are listed in Table XII.

Fig. 15 - Spin Difference Decoupling spectrum of compound P-II-Decoupling at β-Gal ^{4,6}H-1 identifying the corresponding H-2 resonance.

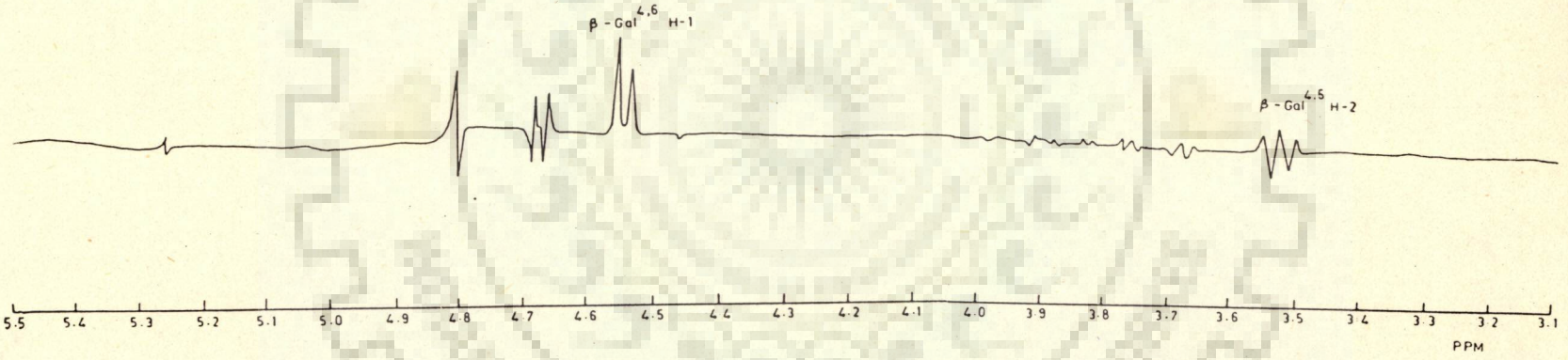

β - Gal 4,6 H-1
β - Gal 4,5 H-2
5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1
PPM

Fig. 16 - Proton NMR spectrum (400 MHz) of oligosaccharide P-I in $^{2}H_{2}O$ at 22°C.

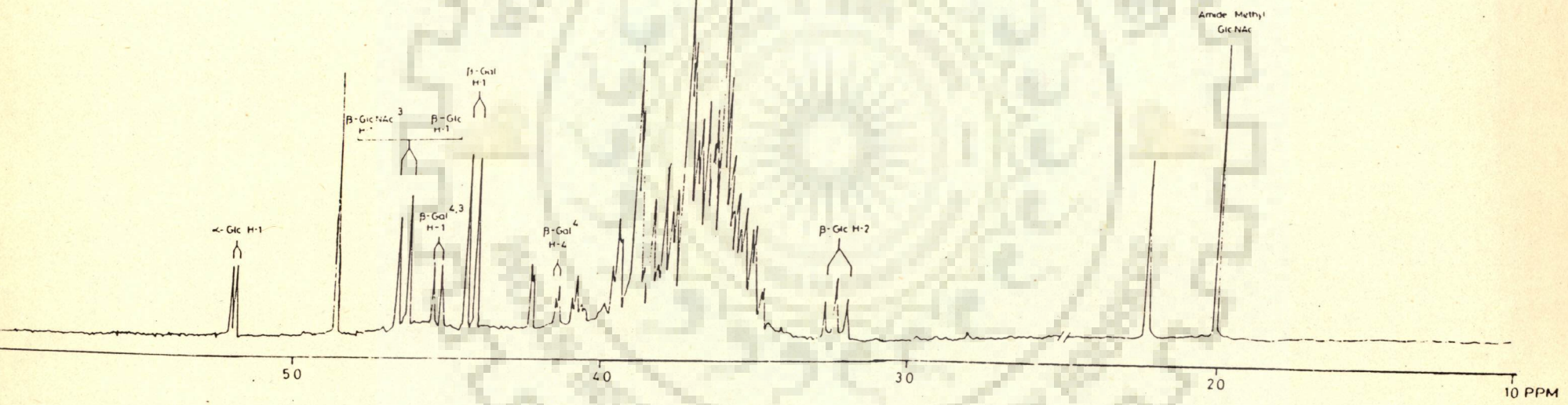

α-Glc H-1
β-GlcNAc 3 H-1
β-Glc H-1
β-Gal H-1
β-Gal 4,3 H-1
β-Gal 4 H-4
β-Glc H-2
Amide Methyl GlcNAc
50
40
30
20
10 PPM

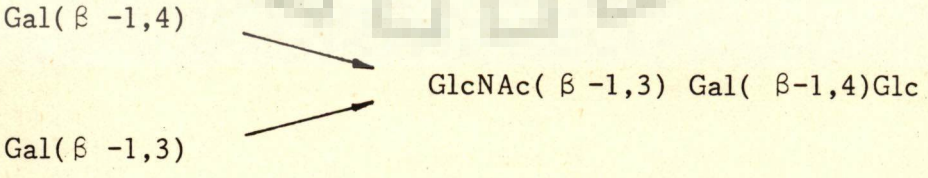
TABLE XII - ^{1}H NMR CHEMICAL SHIFTS OF GOAT MILK OLIGOSACCHARIDE P-I

The chemical shifts are reported relative to internal (DSS) using acetone as internal standard (δ = 2.225 ppm downfield from DSS).

Glc = Glucose; Gal = Galactose; GlcNAc = N-acetylglucosamine

Residue	Proton	Chemical shift, δ ,ppm
α -Glc	H_1	5.212
	H_2	3.587
β -Glc	H_1	4.652
	H_2	3.283
	H_3	3.630
β -Gal^{4}	H_1	4.411
	H_2	3.571
	H_3	3.728
	H_4	4.140
β -Gal^{4,6}	H_1	-
	H_2	-
	H_3	-
β -Gal^{3}	H_1	4.411
	H_2	3.517
	H_3	-
β -Gal^{4,3}	H_1	4.542
	H_2	3.493
β -GlcNAc^{6}	H_1	-
	H_2	-
	N-acetyl	-
β -GlcNAc^{3}	H_1	4.652
	H_2	3.904
	N-acetyl	2.050

The integration of the anomeric region shows it to be a pentasaccharide. The Gal H-4 resonance in this case is clearly shifted downfield to 4.140 ppm, which implies that the compound is not similar to P-II and P-V. But it may be of the type LNT or LNneoT structures. The presence of glucose at the reducing terminal is indicated by the doublet at 5.212 ppm assigned to α-Glc H-1 and the triplet at 3.283 ppm due to β-Glc H-2. Integration of the amide methyl signal at 2.050 ppm shows the presence of one N-acetyl glucosamine residue. The β-GlcNAc and β-Glc H-1 resonances have again overlapped at 4.652 ppm. The 2 proton signal at 4.411 ppm is assigned to H-1 of β-Galactose by analogy to LNT structure. All the above mentioned assignments are in excellent agreement with those of the LNT structure indicating that the compound has a tetrasaccharide core identical to that of LNT with an additional sugar residue shown by the presence of a doublet at 4.542 ppm in the anomeric region. This doublet is assigned to a third β-Gal residue linked through a 1 → 4 linkage to β-Glc NAc. The downfield shift of the H-1 resonance of this β-Gal residue may be attributed to crowding and steric hindrance, due to the presence of two β-Gal residue at the non-reducing end, linked to the same β-GlcNAc residue at C-3 and C-4 positions respectively. The compound can thus be assigned the following structure



SDDS experiments were carried out to obtain the assignments of the remaining ring protons. Decoupling at α-Glc H-1 (5.212 ppm) gave the corresponding H-2 resonance at 3.587 ppm. Irradiation of the doublet at 4.652 ppm identified the β-GlcNAc H-2 resonance at 3.904 ppm as well

as β-Glc H-2 resonance at 3.283 ppm (Fig. 17a). The β-Glc H-2 triplet at 3.283 ppm on irradiation yielded the corresponding H-3 resonance at 3.630 ppm and H-1 resonance at 4.652 ppm confirming the assignment of β-Glc H-1 resonance. Decoupling at B-Gal^{4,3} H-1 doublet identified the corresponding H-2 resonance at 3.493 ppm (Fig. 17b). Irradiation of the doublet at 4.411 ppm gave the effect at 3.571 ppm due to β-Gal^{4} H-2 (Fig. 17c) and at 3.517 ppm due to β-Gal^{3} H-2 (Fig. 17c). Decoupling at β-Gal^{4} H-2 resonance identified the corresponding H-3 resonance at 3.728 ppm. Decoupling of the β-Gal^{4} H-4 resonance at 4.140 ppm also yielded the corresponding H-3 at 3.728 ppm, thus confirming its assignment.

4.2.4 Structure determination of the oligosaccharide P-III

The ^{1}H NMR spectrum of the compound P-III is shown in Fig. 18 and ^{1}H NMR chemical shifts for various protons are listed in Table XIII. The integration of the anomeric region of the spectrum shows it to be a pentasaccharide. The presence of a doublet at 5.225 ppm assigned to α-Glc H-1 and a triplet at 3.295 ppm due to β-Glc H-2, indicate the presence of a Glc residue at the reducing terminal. The presence of one GlcNAc residue is inferred by the singlet (3 proton by integration) at 2.059 ppm, due to its amide methyl. The doublet at 4.663 ppm is assigned to β-GlcNAc H-1. The β-Glc H-1 resonance has again overlapped with that of β-GlcNAc H-1 at 4.663 ppm. Deriving analogies of Gal^{4,6} H-1 from mono- and di-fuco lacto-N-hexaoses (44) and the compound P-II, the doublet at 4.555 ppm is assigned to the H-1 of Gal^{4,6}. The one proton doublet at 4.451 ppm is due to H-1 of Gal^{4} (44). The presence of an α-fucose (Fuc) is indicated by its characteristic H-1, H-5 and H-6 resonances at 5.225 (J = 3.2Hz), 4.861 and 1.292 (J = 6.4 Hz) ppm, respectively (44). The assignment of α-Fuc H-5 at 4.861 ppm was accomplished by recording the spectrum at

Fig. 17 - Spin Difference Decoupling Spectra of compound P-I. (a) Decoupling at β-Gal^{3} H-1 giving the position of its H-2; (b) Decoupling of β-Gal^{4,3} H-1 resonance identifying the corresponding H-2 resonance; (c) Decoupling of β-Glc H-1 and β-GlcNAc^{3} H-1 resonance yielding the corresponding H-2 resonances.

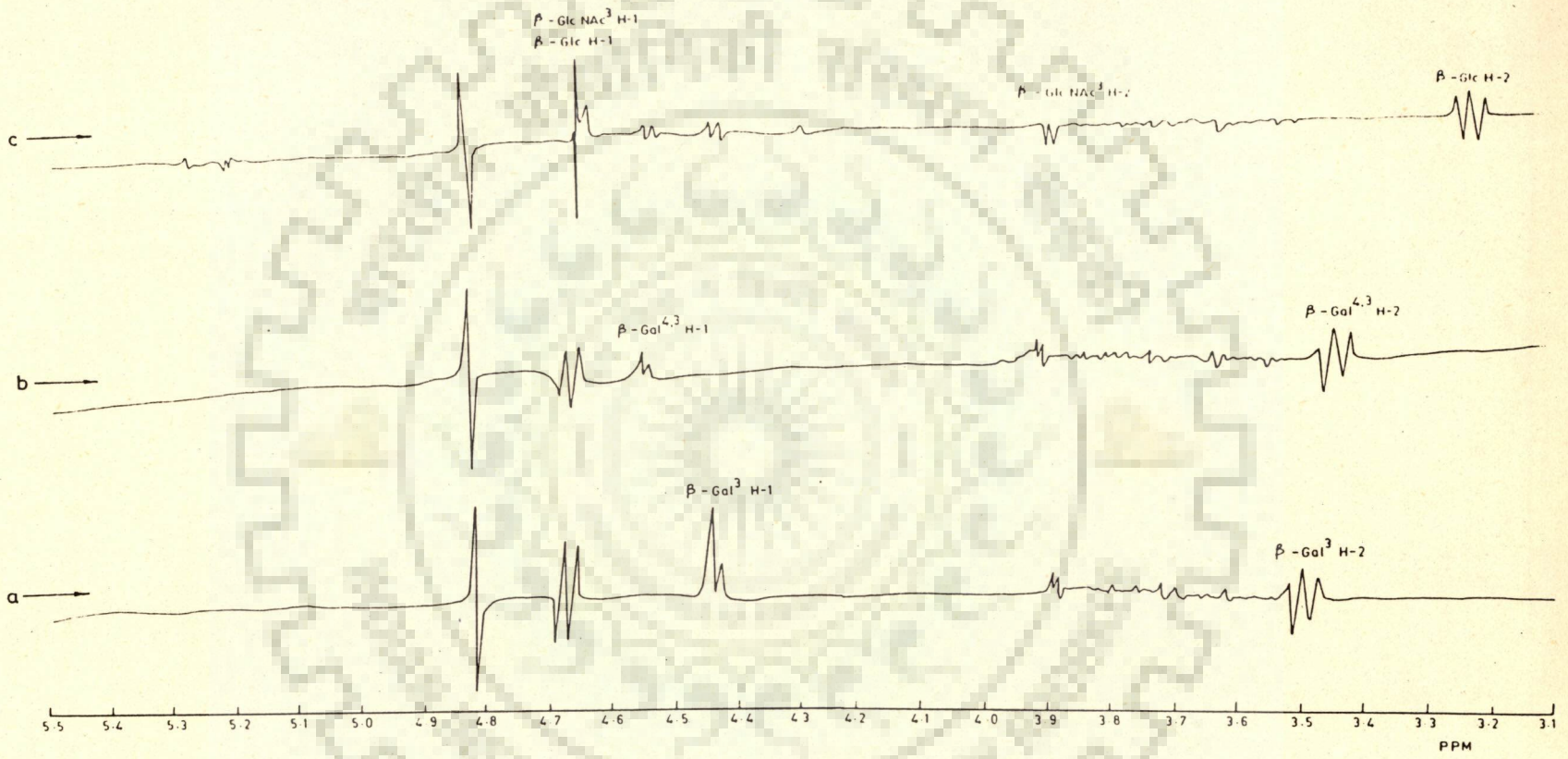

β - GlcNAc³ H-1
β - Glc H-1
β - GlcNAc³ H-2
β - Glc H-2
c
β - Gal⁴·³ H-1
β - Gal⁴·³ H-2
b
β - Gal³ H-1
β - Gal³ H-2
a
5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1
PPM

Fig. 18 - Proton NMR spectrum (400 MHz) of the oligosaccharide P-III in ^2H_2O at 22°C.

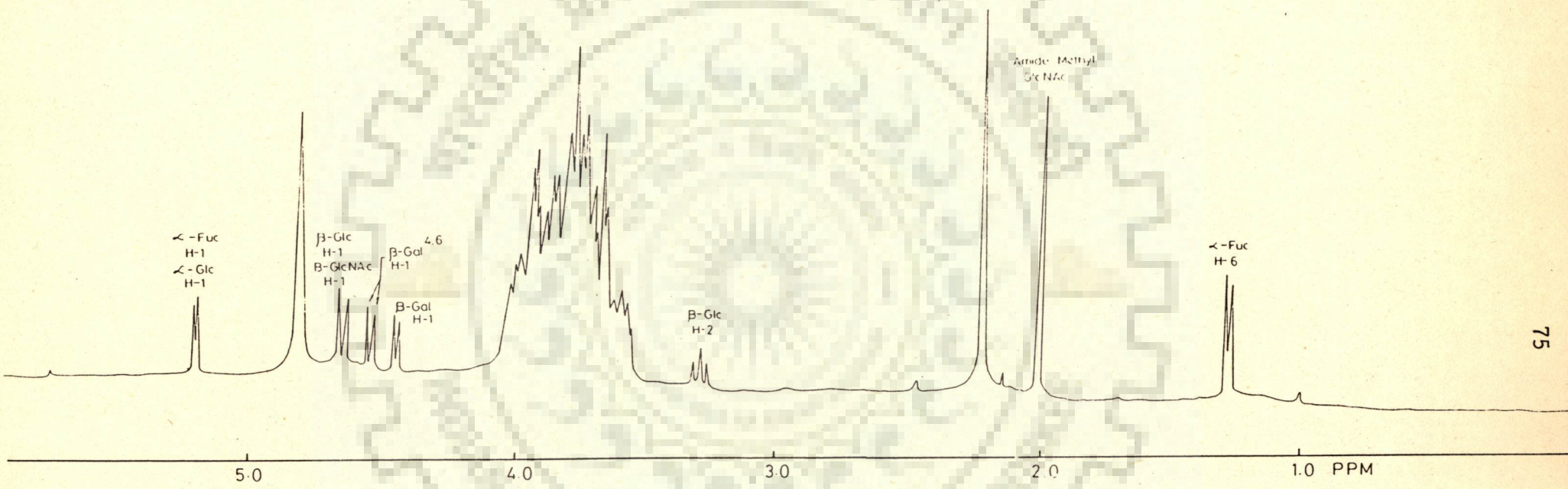

α-Fuc
H-1
α-Glc
H-1
β-Glc
H-1
β-GlcNAc
H-1
β-Gal
H-1
4,6
β-Gal
H-1
β-Glc
H-2
Amide Methyl
of NAc
α-Fuc
H-6
5.0
4.0
3.0
2.0
1.0 PPM

TABLE XIII - ^1H NMR CHEMICAL SHIFTS OF GOAT MILK OLIGOSACCHARIDES

The chemical shifts are reported relative to internal (DSS) using acetone as the internal standard (δ= 2.225 ppm downfield from DSS).

Glc = Glucose; Gal = Galactose; GlcNAc = N-acetylglucosamine; Fuc = Fucose

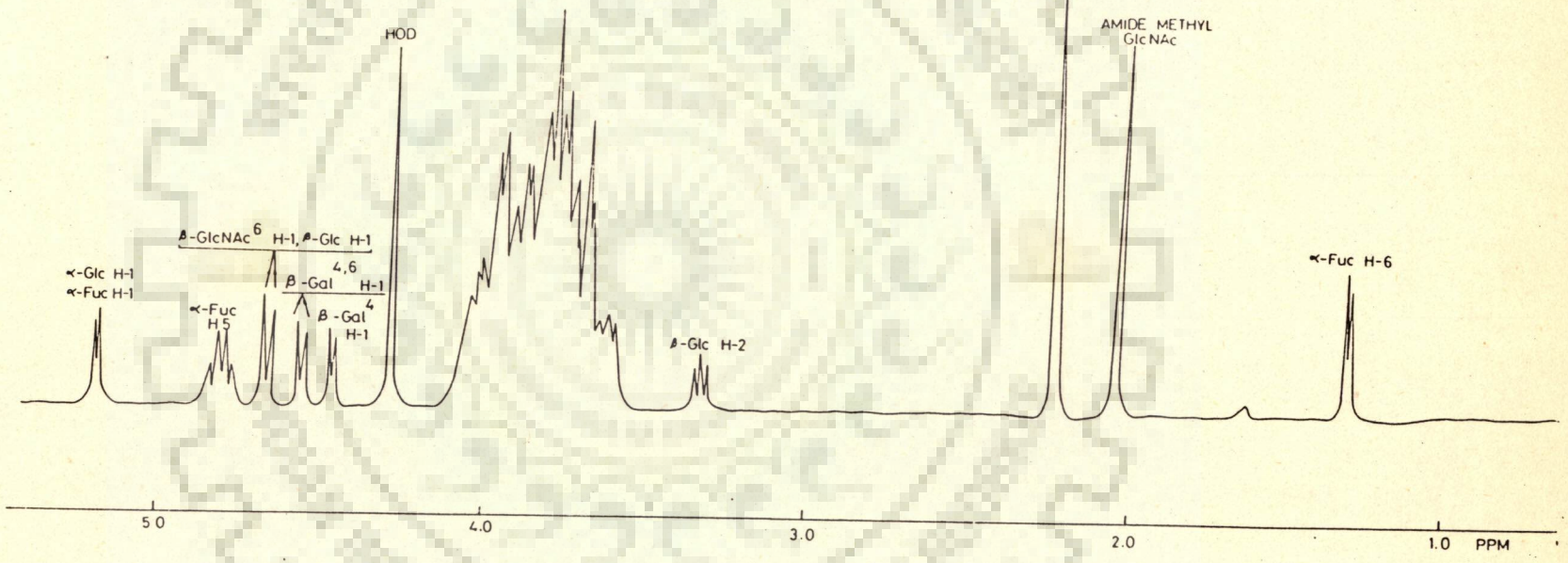
Residue	Proton	Oligosaccharide	
		P-III (δ, ppm)	P-IV (δ, ppm)
α-Glc	H_1	5.225	5.221
	H_2	3.583	3.582
β-Glc	H_1	4.663	4.658
	H_2	3.295	3.283
	H_3	3.632	3.709
$\beta\text{-Gal}^4$	H_1	4.451	4.453
	H_2	3.581	3.580
	H_3	3.714	3.691
	H_4	-	-
$\beta\text{-Gal}^{4,6}$	H_1	4.555	-
	H_2	3.524	-
$\beta\text{-Gal}^{3,6}$	H_1	-	4.453
	H_2	-	3.517
$\beta\text{-GlcNAc}^6$	H_1	4.663	4.658
	H_2	3.880	3.875
	N-acetyl	2.059	2.053
$\alpha\text{-Fuc}^3$	H_1	5.225	5.221
	H_2	3.682	3.688
	H_4	3.913	3.907
	H_5	4.861	4.865
	H_6	1.292	1.294

a high temperature (77°C) when the H-5 clearly shows its characteristic shape, as the HOD peak shifts upfield at this temperature (Fig. 19) (42). Comparision of its spectrum to that of the tetrasaccharide P-II (Fig. 14) shows that it has an identical tetrasaccharide core structure with an additional α-Fuc residue. The tetrasaccharide core structure can be assigned on the basis of the same interpretations as have been extended in the case of compound P-II. Comparision of the spectrum of P-III, to those of lactose (15), lacto-N-tetraose and lacto-N-neotetraose reveals the presence of a lactosyl residue (Gal(β-1,4)Glc) at the reducing end. Since, there is no downfield shift of the H-4 resonance of β-Gal, none of the β-Gal residues is substituted at C-3 position (41). This implies that the β-GlcNAc may be 1 → 6 linked to β-Gal, which is assigned by SDDS and chemical shift analogies of $GlcNAc^6$ as reported by Dua et al. (44) for lacto-N-hexaose (LNH), mono-fuco LNH and difuco LNH. The linkage of α-Fuc to β-GlcNAc was assigned in the following manner : The absence of α-Fuc H-5 resonance in the region 4.2-4.3 ppm suggests that α-Fuc is not linked via a 1 → 2 linkage (42). The chemical shift analogies of α-Fuc H-1 resonance (5.225 ppm) indicate that α-Fuc is linked through a 1 → 3 and not by a 1→4 linkage to β-GlcNAc (44). Since, in the tetrasaccharide core structure, the C-4 position of β-GlcNAc is occupied by a β-Gal residue, α-Fuc can only be 1 → 3 linked to β-GlcNAc which further supports the above interpretation. Based on the above data and interpretations, the compound P-III has been assigned the following structure :

Gal(β-1,4) GlcNAc(β-1,6) Gal(β-1,4) Glc
↑
Fuc(α-1,3)

Assignments of the remaining protons were made by SDDS experiments. Irradiation of the doublet at 5.225 ppm identified the α-Glc H-2 at 3.585

Fig. 19 - Proton NMR spectrum (400 MHz) of the oligosccharide P-III in ^2H_2O at 77°C.


HOD
AMIDE METHYL
GlcNAc
β-GlcNAc 6 H-1, β-Glc H-1
α-Glc H-1
α-Fuc H-1
α-Fuc
H 5
β-Gal 4,6
H-1
β-Gal 4
H-1
β-Glc H-2
α-Fuc H-6
5.0
4.0
3.0
2.0
1.0
PPM

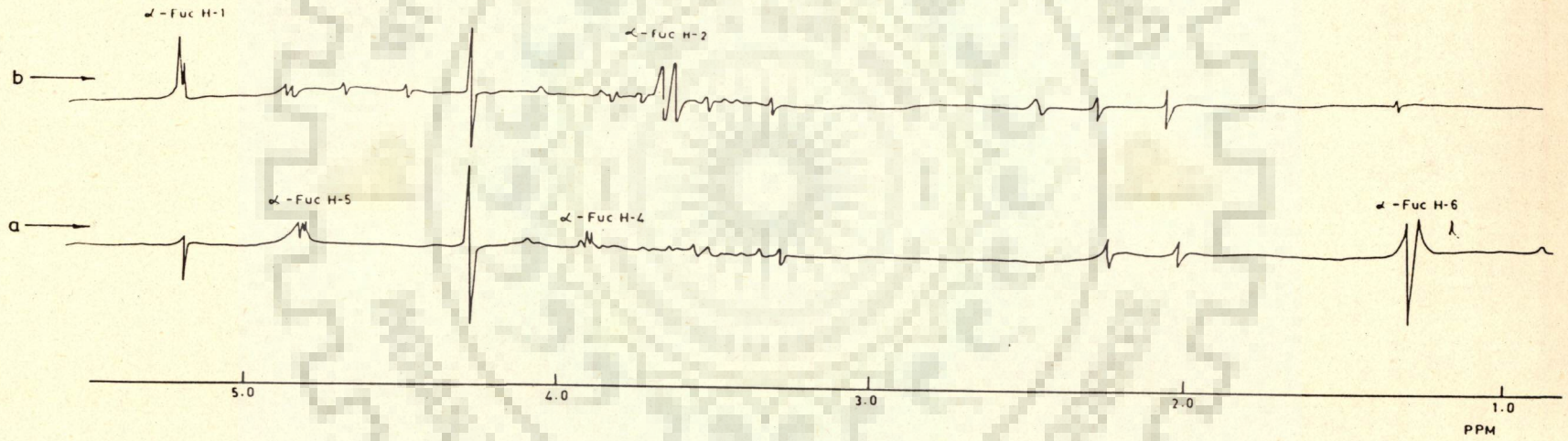
and the α-Fuc H-2 at 3.682 ppm (Fig. 20b), respectively. Decoupling of β-Glc H-2 resonance at 3.295 ppm yielded the β-Glc H-1 resonance at 4.663 ppm as well as β-Glc H-3 resonance at 3.632 ppm. The β-GlcNAc^{6} H-2 position was obtained by irradiating its corresponding H-1 resonance at 4.663 ppm. Decoupling at β-Gal^{4} H-1 (4.451 ppm) gave the corresponding H-2 resonance at 3.581 ppm, which, in turn, on irradiation yielded the H-3 resonance at 3.714 ppm. Irradiation of β-Gal^{4,6} H-1 at 4.555 ppm identified the corresponding H-2 at 3.524 ppm. The position of α-Fuc^{3} H-4 3.913 ppm was obtained by decoupling the H-5 at 4.861 ppm (Fig. 20a).

The structure of the compound P-III assigned by ^{1}H NMR studies was further confirmed by enzymatic digestion by α-1,3 fucosidase enzyme. Fig.21 shows the chromatogram of compound P-III obtained after 12 hours of digestion with α-1,3 fucosidase. The figure shows two peaks-one of the compound P-III with a retention time of 4.0 minutes and a second peak, which is the product of fucosidase digestion, having a retention time of 3.0 minutes. The retention time of the product is identical to that of the parent tetrasaccharide i.e. compound P-II, thus further confirming the assigned structure. Approximately 70% of P-IV was converted into P-II at this stage.

4.2.5 Structure determination of oligosaccharide P-IV

The ^{1}H NMR spectrum of the compound P-IV is shown in Figure 22 and the chemical shifts are listed in Table XIII. The integration of the anomeric region indicates that the compound is a pentasaccharide. The presence of Glc at the reducing terminal is shown by the presence of a doublet at 5.221 assigned to the H-1 of α-Glc and a triplet at 3.283 ppm due to β-Glc H-2. The presence of a 3 proton singlet at 2.053 ppm is assigned to the amide methyl of one GlcNAc residue which is further supported by the presence of a doublet at 4.658 ppm due to β-GlcNAc H-1. The

Fig. 20 - Spin Difference Decoupling spectra of compound P-III. (a) Decoupling at α-Fuc H-5 identifying the corresponding H-4 and H-6 resonances; (b) Decoupling of α-Fuc H-1 yielding the position of α-Fuc H-2.


α-Fuc H-1
α-Fuc H-2
b
α-Fuc H-5
α-Fuc H-4
α-Fuc H-6
a
5.0
4.0
3.0
2.0
1.0
PPM

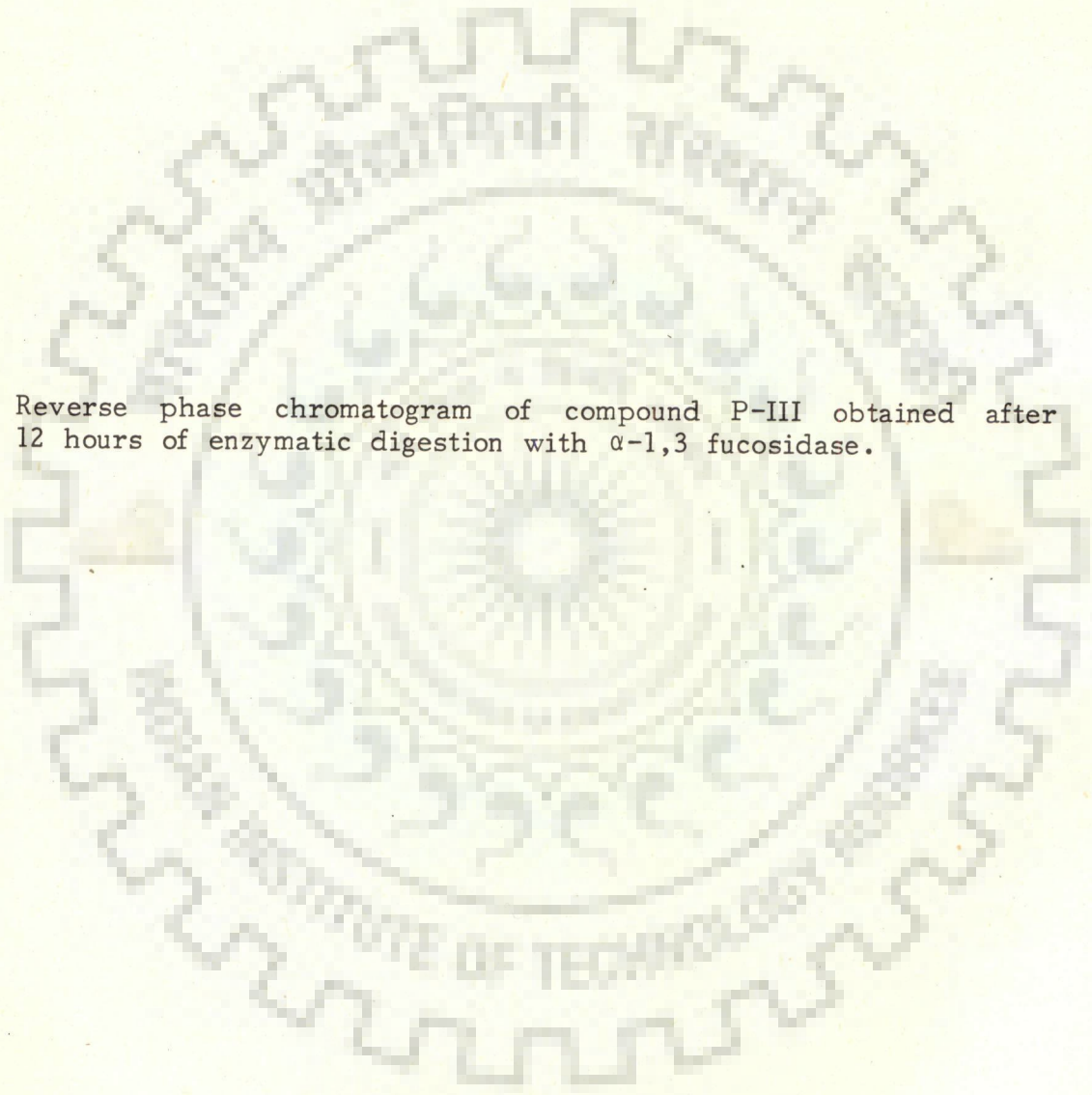
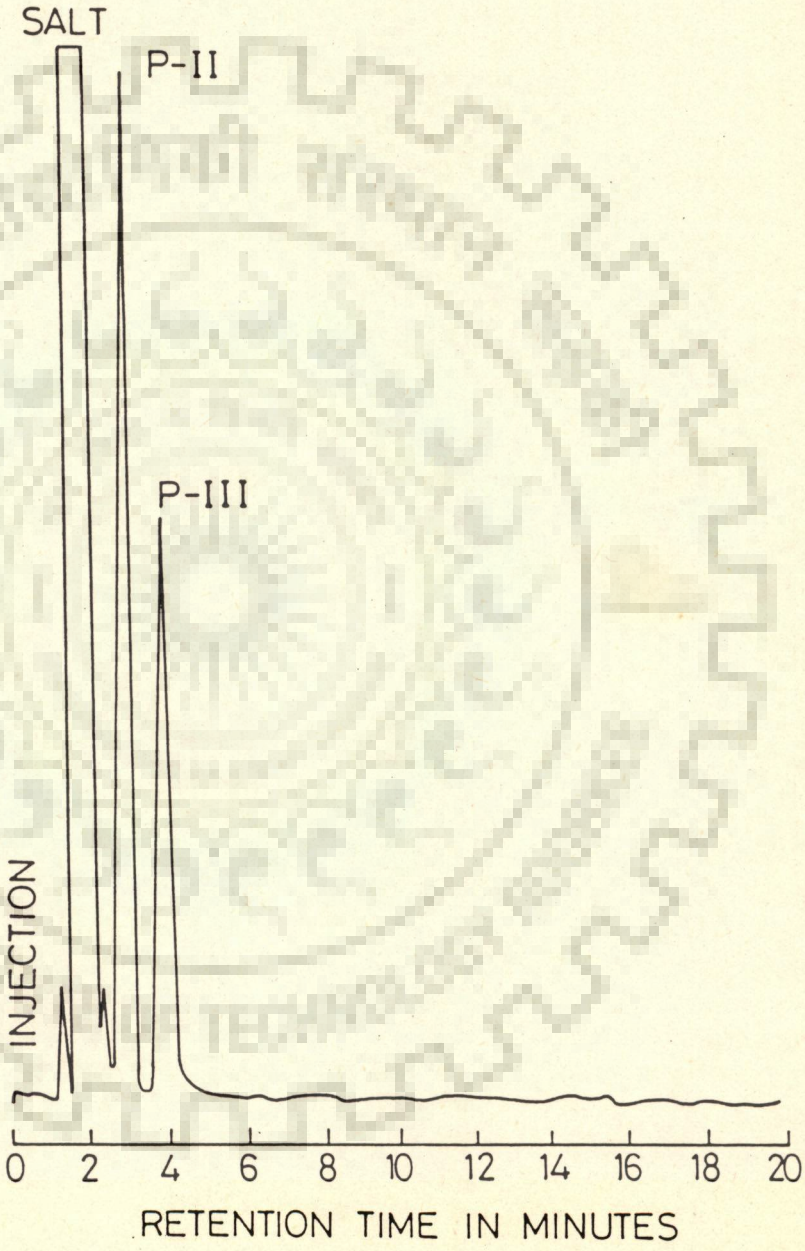
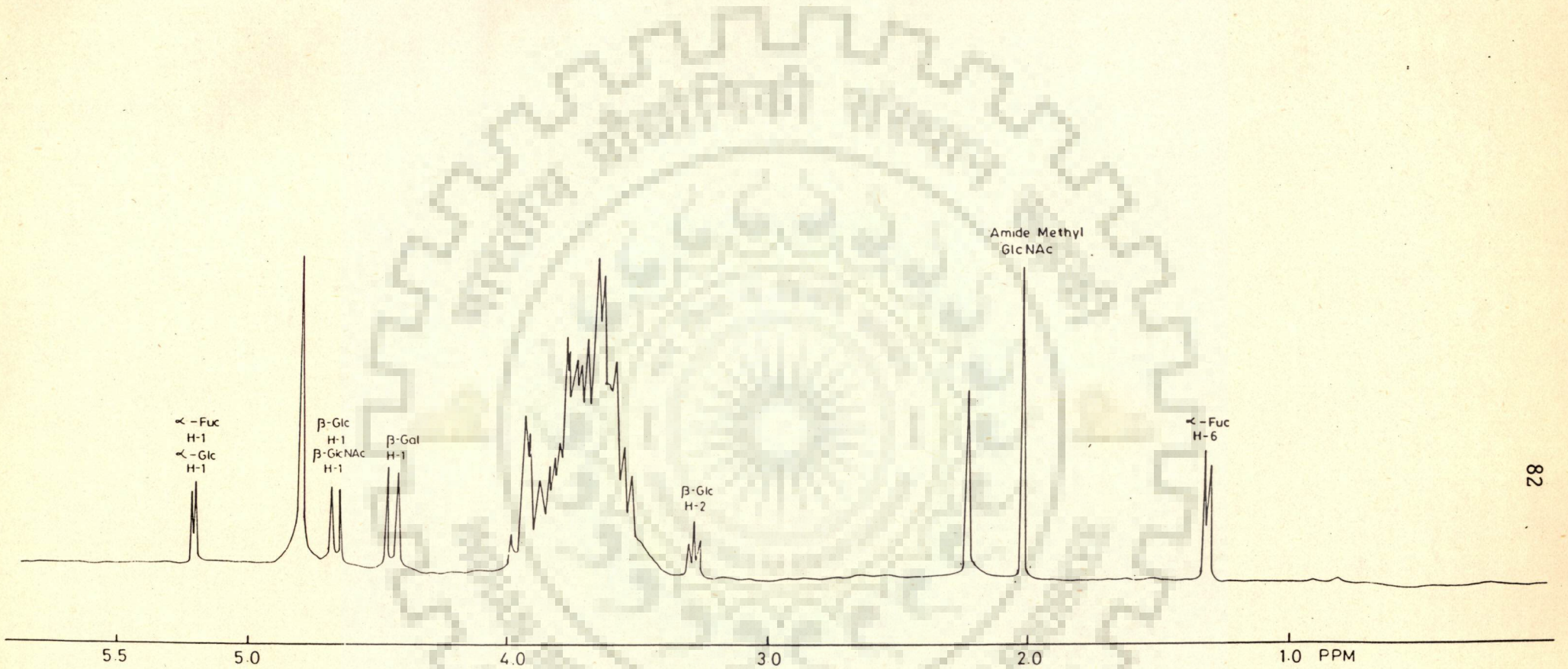


Fig. 21 - Reverse phase chromatogram of compound P-III obtained after 12 hours of enzymatic digestion with α-1,3 fucosidase.


SALT
P-II
P-III
INJECTION
0
2
4
6
8
10
12
14
16
18
20
RETENTION TIME IN MINUTES

β-Glc H-1 has overlapped with H-1 of β-GlcNAc at 4.658 ppm. The doublet at 4.453 is assigned to H-1 of β-Gal. Integration of this doublet indicates the presence of two β-Gal residues. The presence of an α-Fuc residue is inferred by the presence of its characteristic H-1, H-5 and H-6 resonances at 5.221 (J = 3.4 Hz), 4.865 and 1.294 (J = 6.1 Hz) ppm, respectively. The α-Fuc H-5 resonance was assigned by recording the spectra at 77°C, when the H-5 clearly shows its characteristic shape, as the HOD peak shifts upfield at this temperature, (Fig. 23) The α-Fuc H-5 position was further confirmed by decoupling α-Fuc H-6 resonance at 1.294 ppm. Comparision of the spectrum to those of lactose (15), LNT and LNneoT reveals the presence of a lactosyl residue at the reducing end. The absence of downfield shift of the β-Gal H-4 resonance indicates that none of the β-Gal residue is substituted at C-3 position. The absence of α-Fuc H-5 resonance in the 4.2 - 4.3 ppm (Fig. 23) suggests that α-Fuc is not 1 → 2 linked. The chemical shift analogies of α-Fuc H-1 (5.221 ppm) indicates that α-Fuc is linked via a 1 → 3 and not by a 1 → 4 linkage. However, in this compound the C-3 position of β-GlcNAc is occupied by β-Gal. Therefore, the α-Fuc residue can be linked either to β-Gal at the non-reducing end or to Glc at the reducing end. Decoupling of the β-Glc H-2 resonance identified its corresponding H-3 resonance at 3.709 ppm (Fig. 24). This downfield shift of β-Glc H-3 resonance as compared to the position of β-Glc H-3 resonance at 3.632 ppm of the compound P-III (Table XIII) suggests that Glc may be substituted at C-3 position. Hence, α-Fuc could be 1 → 3 linked to Glc at the reducing end. The α-Glc H-2 position (3.582 ppm) lies in the crowded region of the spectrum. Therefore, our assumptions are based on the position of β-Glc H-3 only. Based on the above interpretations, the compound P-IV was assigned the following structure :

Fig. 23 - Proton NMR spectrum (400 MHz) of the oligosaccharide P-IV in $^{2}H_{2}O$ at 77°C.


α-Fuc
H-1
α-Glc
H-1
β-Glc
H-1
β-GlcNAc
H-1
β-Gal
H-1
β-Glc
H-2
Amide Methyl
GlcNAc
α-Fuc
H-6
5.5
5.0
4.0
3.0
2.0
1.0 PPM

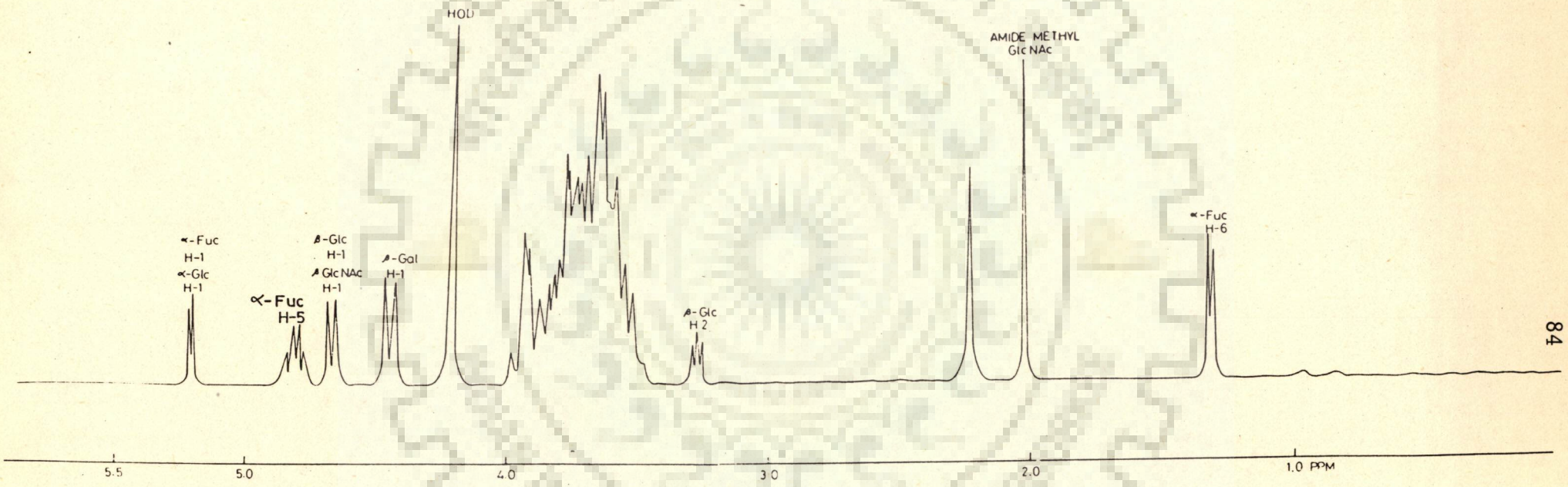
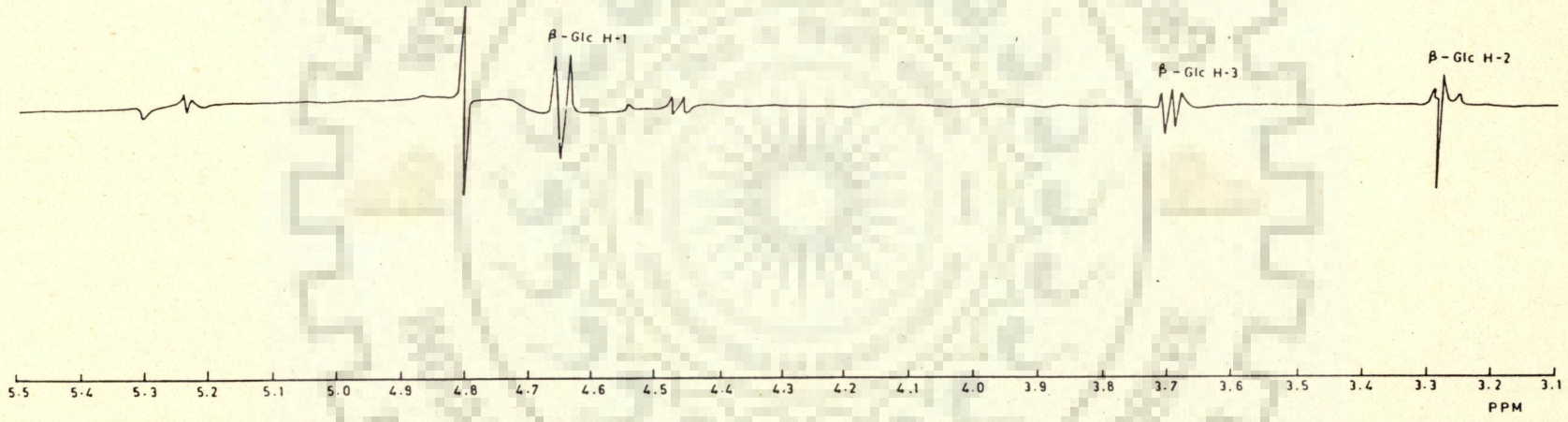

HOD
AMIDE METHYL
GlcNAc
α-Fuc
H-6
α-Fuc
H-1
α-Glc
H-1
α-Fuc
H-5
β-Glc
H-1
β GlcNAc
H-1
β-Gal
H-1
β-Glc
H 2
5.5
5.0
4.0
3.0
2.0
1.0 PPM

Fig. 24 - Spin Difference Decoupling Spectrum of Compound P-IV - Decoupling at β-Glc H-2 identifying the corresponding β-Glc H-3 as well as β-Glc H-1.


β - Glc H-1
β - Glc H-3
β - Glc H-2
5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1
PPM

Gal(β -1,3) GlcNAc(β-1,6) Gal(β -1,4) Glc
↑
Fuc (α -1,3)

This structure was further confirmed by SDDS experiments which gave the remaining resonances. Irradiation of the doublet at 5.221 ppm gave effect at 3.582 ppm due to α-Glc H-2 and at 3.688 ppm assigned to α -Fuc H-2. Decoupling of β -Gal H-1 resonance at 4.455 ppm identified the β -Gal^{4} H-2 at 3.580 ppm and the β -Gal^{3,6} H-2 at 3.517 ppm. The β -Gal^{4} H-3 at 3.691 ppm was obtained on irradiation of its H-2 resonance at 3.580 ppm. Decoupling of β-GlcNAc^{6} H-1 at 4.658 ppm identified the corresponding H-2 at 3.875 ppm. The α -Fuc^{3} H-4 was identified by decoupling of α -Fuc^{3} H-5 at 4.865 ppm, thus completing all the assignments.

The site of linkage of fucose was confirmed by N.O.E. experiment. The N.O.E.s obtained at 70°C on saturation of α -Fuc H-1 are shown in Fig. 25. Positive N.O.E.s were observed at α -Fuc H-2 and β -Glc H-3 confirming that α -Fuc was linked to Glc at the reducing terminal. Enzymatic digestion of compound P-IV with α -1,3 fucosidase yielded a compound with a retention time of 3.4 minutes which is different from that of tetrasaccharide P-II. This further confirmed the presence of a α-1,3 linked fucose residue in this pentasaccharide.

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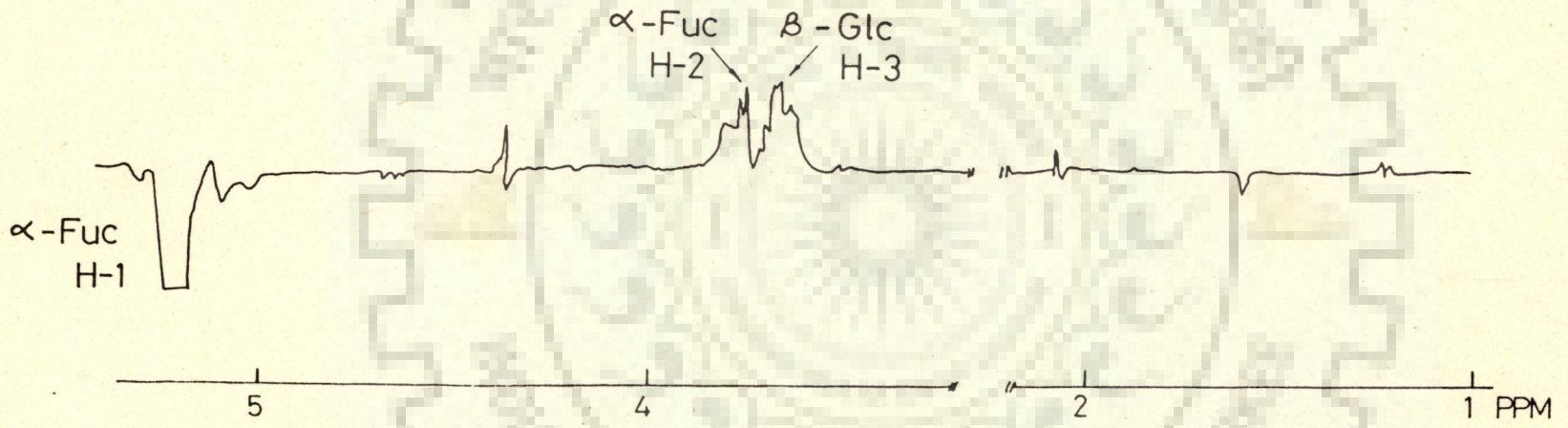
Fig. 25 - N.O.E's obtained at 70°C on saturation of α -Fuc H-1 in compound P-IV.


α-Fuc
H-2
β-Glc
H-3
α-Fuc
H-1
5
4
2
1 PPM

CHAPTER - V

5.0 DISCUSSION

The basic idea behind the present study was to compare the goat milk oligosaccharides with that of the human milk, recently reported by Dua and Bush (41, 4), with respect to the structure and the microheterogeneity that may be found in them. Our approach was to purify the oligosaccharides to over 96 percent purity and then determine the structure by the high field ^1H NMR. The preliminary purification of the milk oligosaccharides was done by Bio-Gel P-4 filtration which yielded oligosaccharide fractions of varying molecular sizes. In the present study we concentrated only on small molecular weight oligosaccharides mainly, containing 3 to 5 monosaccharides, since this fraction accounted for about 60% of the total carbohydrates in the milk. The reverse phase HPLC on C-18 column with water as the mobile phase (41) was successfully used for both analytical and preparative separations of the N-acetylglucosamine-containing oligosaccharides from the goat milk. The fractions, as tested on amino column (normal-phase chromatography) using CH_3CN and water as the mobile phase, were free from cross contamination and the purity of the oligosaccharides was greater than 96% making them suitable for ^1H NMR studies. The HPLC and ^1H NMR results are discussed in detail below.

The results of the reverse phase HPLC of the oligosaccharides reaffirm the earlier observations that the main mode of retention and fractionation of oligosaccharides containing N-acetylamino sugars is hydrophobic bonding and reverse phase chromotography. This means that the retention is controlled by the repulsion of sample molecules from the mobile phase, that is, water and not by the attraction between the solute and stationary phase. The primary solvent effect in reversed-phase liquid chromatography is that the mobile

phase polarity controls retention. Although the separation mechanism is unclear it certainly is structure dependent as shown by the different retention times of various oligosaccharides of the same molecular weight but having different linkage type. In general, the higher the molecular weight, the longer is the retention time of structurally similar oligosaccharides although exceptions to this generalization occur . The retention time remained practically unchanged even at sufficiently high column loading for semipreparative chromatography indicating that the retention does not critically depend on minor polar sites on the column (13).

Contrary to the general observation of the reverse phase separation of oligosaccharides, resulting in the resolution of the α- and β- anomeric forms, no resolution of the goat milk neutral oligoccharides into their α- and β- anomeric forms was observed. This is rather an unusual phenomenon and might be due to the unfavourable mutarotation time of the sugars, as suggested earlier by Blumburg et al. (13). They pointed out that the ability of the reverse-phase chromatographic system to resolve the anomeric pairs of the reducing terminal residues of oligosaccharides could complicate fractionation of complex oligosaccharide mixtures. The resolution of reducing terminal anomers doubles the number of peaks and may lead to broadening for cases in which the mutarotation time is comparable to the chromatographic time. This is precisely what seems to be happening in the case of the goat milk oligosaccharides. This observation is consistent with the finding that a partial resolution into anomeric pairs was possible when the chromatographic analysis was carried out at a lower temperature of about 7-8°C (Fig. 3 and Fig. 4), since at low temperatures, the mutarotation time is decreased, thereby leading to a partial separation into anomeric forms.

The compound P-V, a trisaccharide gave the highest relative retention value (Table VIII) which was quite unexpected. This might be explained

by the fact that amide substituents were the predominant sites of hydrophobic bonding in reverse phase fractionation of oligosaccharides containing N-acetyl amino sugars. Since, in the structure of this trisaccharide, the β - GlcNAc residue is present at the non-reducing terminal, the amide methyl group is fully exposed for interaction with the non-polar C-18 stationary phase. This will lead to a relatively higher retention time for compound P-V.

The tetrasaccharide P-II gives a k' value of approximately 2.2 min (Table VIII) which is less than that of the trisaccharide, P-V. This decrease might be attributed to the presence of the additional $Gal^{4,6}$ at the non-reducing end, thus making the amide group of GlcNAc less accessible for interaction with the C-18 stationary phase. Comparision of the structure of P-II to that of LNneoT of the human milk tetrasaccharide, shows only one difference, i.e., the linkage between GlcNAc and Gal of the lactosyl residue is 1 → 6 in the former whereas it is 1 → 3 in the latter. The LNneoT gives a k' value of 2.8 min (9) which is significantly more than that of compound P-II (2.2 min). This leads to the conclusion that the presence of a 1 → 6 linked GlcNAc results in lesser retention time on C-18 reverse phase column as compared to 1 → 3 linked GlcNAc This observation shows that the separation of oligosaccharides on C-18 reverse phase column is linkage dependent, which is in agreement with the findings of other authors (41).

The compound P-I, a pentasaccharide, has been assigned a novel structure in which two β - Gal residues are linked to the same GlcNAc residue via a 1 → 3 and 1 → 4 linkage, respectively, at the non-reducing terminal. The behaviour of this compound on reverse phase is also quite unique, in the sense that it has the shortest k' value of 1.3 min (Table VIII) and elutes very rapidly from the C-18 column, although as pointed out above

a 1 → 3 linked GlcNAc is expected to increase retention on reverse phase in this case. This anamolous behaviour is probably due to branching at the non-reducing end. This interpretation is based on the observations made by Cheetham and Dube in 1983 (28) in the course of their studies on milk oligosaccharides. They pointed out that the human milk LND-I and LND-II eluted earlier than LNT, LNneoT, LNF-I, LNF-II and LNF-III on the reverse phase C-18 column (28). They suggested that this was due to the branched structure of LND-I and LND-II. This finding led to the tentative conclusion that linear molecules interact most with the C-18 stationary phase. Another factor influencing the short retention time of P-I might be the crowding at β-Glc NAc, thereby not allowing the amide methyl group to interact fully with the non-polar stationary phase. Apparently, retention on the reverse phase column is partially governed by the accessibility of the functional groups of the oligosaccharide in some folded three-dimension conformation.

The fucose-containing pentasaccharide P-III gives a k' value of 2.9 min (Table VIII) which is significantly higher than the k' value of tetrasaccharide, P-II (2.2 min). This increase may be attributed partly to the increase in molecular weight of P-III and partly to the addition of an α-Fuc residue. Since the mechanism of retention of oligosaccharides containing N-acetyl amino sugars on reverse phase column is believed to be hydrophobic in nature, the methyl group of fucosylated oligosaccharides would be expected to lead to generally longer retention times than that of the parent tetrasaccharides (41). Comparision of the structure of P-III with that of LNF-III (Table II) shows only one difference, i.e., P-III has a 1 → 6 linked GlcNAc whereas in LNF-III Glc NAc is 1 → 3 linked. Although a 1 → 3 linked GlcNAc would be expected to be retained longer on C-18 stationary phase, the converse was the case for the oligosaccharide P-III. LNF-III gives a k' value of approximately 2.3 min, whereas under similar chromato graphic

(60:40) as the mobile phase. The relative retention values indicate that the separation was based on molecular weight or more precisely, on the number of hydroxyl functions, as has earlier been observed by Mellis and Baenziger (92). However, there are small differences in retention times of the isomeric pentasaccharides P-III and P-IV.

The purified oligosaccharides have been characterized here with the help of high field ^{1}H NMR spectroscopy only, which just goes to show its suitability for the characterization of underivatized oligosaccharides. The advantage of NMR method is that with no chemical work-up between sample and analysis, there is less chance for inadvertant cleavage of relevant chemical entities such as acyl groups from the original structure. It also immediately reveals any inadequacies of preparative chromatography because of its inherent stringent criteria of purity. Few other techniques offer so much structural information in a single experiment. The concept of 'structural reporter' groups developed by Vliegenthart and co-workers (124) was the basis for finding out the size, the different type of monosaccharide residues, their anomeric configuration as well as the molar ratios in which they are present in the chain. Moreover, the purity of the compounds could also be checked from the spectrum by observing the signal to noise ratio. Owing to the combination of the strong magnetic field of the 400 MHz spectrometer and the computer resolution enhancement routine, the chemical shifts of structural reporter groups could be determined more precisely as well as more details of the splitting patterns of their signals were discovered.The coupling constants and the line shape of the various signals are also quite characteristic in some cases and yield useful information about the anomeric configuration and identification of some particular resonances. Spin Difference Decoupling experiments proved to be a valuable adjunct for confirming the assigned structure, for example, in all the five compounds as will be discussed later,

decoupling of the doublet at approximately 4.65 ppm assigned to H-1 of β-Glc and β-Glc NAc identified both β-Glc and β-GlcNAc H-2 resonances, confirming the assignment. Similarly, in the pentasaccharide P-IV, the assignment of the α-Fuc position attached to Glc at the reducing end was made on the basis of the downfield shift of β-Glc H-3 resonance obtained by decoupling at β-Glc H-2.

The interpretation of the NMR spectra using Vliegenthart's concept of 'structural reporter' groups (124) was greatly helped by the reference spectra of pure and well-characterized similar compounds available in the literature, specially those for the human oligosaccharides (41, 44).

The compound P-V a trisaccharide gives a NMR spectrum which was very much similar to lactose barring the presence of the amide methyl resonance of GlcNAc at 2.059 ppm. The anomeric region of P-V shows the characteristic α-Glc H-1, β-Glc H-1 and β-Gal H-1 resonances as are present in the spectrum of lactose (Table IX). The difference lies in the intensity of the half proton β-Glc H-1 signal in lactose as compared to a one and a half proton signal in the case of P-V. This increase in intensity of the signal is due to the β-GlcNAc H-1 resonance at the same position. These chemical shift analogies clearly indicated that the compound P-V has a lactosyl structure (Gal β-1, 4 Glc) at the reducing end with an additional β-GlcNAc residue attached to the β-Gal residue. The linkage of β-GlcNAc to β-Gal was derived by chemical shift analogies of $GlcNAc^6$ of the human milk hexasaccharide lacto-N-hexaose (LNH) structure (44). Thus, it was possible to assign the structure of the trisaccharide with the help of chemical shift analogies of the reference compounds available in the library of pure and well-characterized oligosaccharides.

The examination of the ^{1}H NMR spectrum of compound P-II clearly indicated that the compound is a tetrasaccharide, with a trisaccharide core identical to that of P-V. So, the total assignment required the identification of the additional residue and its position and linkage in the chain. A closer look at the anomeric region showed an additional doublet, the chemical shift analogies of which were similar to those of β-Gal^{4,6} in human milk LNH. This observation led to the conclusion that the compound P-II was a tetrasacharide with a trisaccharide core structure identical to P-V and an additional β-Gal residue attached through a 1 $\rightarrow$ 4 linkage to β-GlcNAc. Thus, the availability of ^{1}H NMR data, even of the relatively simple, well characterized compounds, were extremely useful in the characterization of the higher complex ones. There was practically no change in the chemical shift values of any other anomeric proton in P-II as compared to P-V, suggesting that the substitution of β-GlcNAc at C-4 by β-Gal did not affect the chemical environment of β-GlcNAc or the neighbouring residue.

The interpretation of the ^{1}H NMR spectrum of the compound P-I was greatly facilitated by the reference spectra of LNT and LNneoT structures isolated from human milk (41). A comparision of the spectrum of P-V to those of LNT and LNneoT revealed that the 2 proton β-Gal doublet of P-V was identical to that of LNT indicating that the compound had the LNT type of structure. The additional doublet in the anomeric region was assigned to a β-Gal^{4} residue. The downfield shift of the H-1 of this Gal^{4} could only be accounted for if it is attached to the same β-GlcNAc at C-4 position. The steric hinderance caused due to crowding at the non-reducing end could then be responsible for the downfield shift. On the basis of the above interpretations, the compound P-I was assigned a novel structure in which two galactose residues are linked via β 1 $\rightarrow$ 3 and β 1 $\rightarrow$ 4 linkage, respectively, to the same GlcNAc residue at the non-reducing end. It was probably this

structure which was responsible for the extremely short retention time of this compound on the C-18 reverse phase column.

The compounds P-III and P-IV were found to be fucose-containing pentasaccharides. The ^{1}H NMR spectrum of P-III was similar to that of P-II, but for the presence of the α-Fuc H-6 resonance and the increase in the intensity of the doublet at 5.225 ppm. This increase was attributed to the α-Fuc H-1 resonance by chemical analogies of α-Fuc[3] of mono-and di-fuco LNH. The α-Fuc H-5 resonance with its characteristic shape was then located by recording the spectrum at a higher temperature. The rest of the chemical shifts in the anomeric region were almost identical to those of P-II suggesting that P-III had an identical tetrasaccharide core with an additional α-Fuc residue. The identification of the α-Fuc resonances were also helped by the characteristic coupling constant of α-Fuc H-1 (3-4 Hz) and the α-Fuc H-6 (6-7 Hz).

The interpretation of the NMR spectrum of P-IV was a bit problematic as the spectrum was almost similar to that of P-III, except for the absence of the downfield shifted H-1 resonance of Gal[4,6] which suggested that the Gal in this compound was 1 → 3 linked to GlcNAc. The problem lied in the assignment of the position of α-Fuc. It could not have been linked to Glc NAc as the C-3 position of GlcNAc was already occupied by Gal. In this case, the assignment was helped by the SDDS and NOE experiments. The decoupling of β-Glc identified the corresponding H-3 resonance which was slightly downfield shifted, indicating that α-Fuc was probably attached to Glc at the C-3 position at the reducing terminal. The NOE experiments gave further indication that α-Fuc was 1 → 3 linked to Glc at the reducing end. Irradiation of α-Fuc H-1 resonance at 70°C gave positive NOE's at α-Fuc H-2 and β-Glc H-3 supporting the assignment made by SDDS experiments.

Comparision of the α-Fuc^3 chemical shifts of P-III and LNF-III shows that both the H-1 and H-6 resonance are shifted downfield in the case of P-III. This suggests that the difference in the linkage of β-GlcNAc residue to which α-Fuc is attached, influences the chemical environment of Fuc H-1 and H-6 protons. Similarly, in the case of the compound P-IV, there is a slight downfield shift in the H-1 resonance of the β-Gal of the lactosyl residue at the reducing end. It was thought to be due to the presence of the α-Fuc 1 → 3 linked to Glc at that end.

The enzymatic studies also proved to be quite useful in confirming the structure assigned by NMR studies in the case of fucose-containing pentasaccharides, P-III and P-IV. Enzymatic digestion of P-III with α-1, 3 fucosidase yielded a product with a retention time identical to that of tetrasaccharide P-II, confirming that P-II did have a tetrasaccharide core similar to that of P-II, with an additional 1 → 3 linked α-Fuc residue. Since the fucosidase enzyme used was specific for 1 → 3 and 1 → 4 linkage, and the fact that the C-4 positions of both, GlcNAc as well as Glc residue were already occupied, the fucose can only be 1 → 3 linked to GlcNAc, thus confirming the structure assigned by ^{1}H NMR.

Similarly, in the case of compound P-IV, enzymatic digestion with α-1, 3 fucosidase gave a compound with a retention time that was different from that of tetrasaccharide P-II, which was expected also on the basis of the structure assigned to P-III and P-IV by ^{1}H NMR studies. Unfortunately, the structure of the compound obtained by enzymatic digestion of P-IV could not be confirmed by ^{1}H NMR experiments due to limited amount available. Nevertheless, enzymatic digestion does confirm the presence of a 1 → 3 linked α-Fuc residue .

Thus, I-d NMR spectroscopy used here, has been found quite adequate in carbohydrate resonance assignments which show characteristic coupling constants of axial and equatorial protons. Although 2-dimensional (2-d) NMR spectroscopy could be used for proton assignments, the limited digital resolution of 2-d methods generally obscures multiplet structures.

5.1 A COMPARISION WITH CORRESPONDING HUMAN MILK OLIGOSACCHARIDES

The tri- and the tetra-saccharides (P-V and P-II) reported here, differ from the LNT and LNneoT structures isolated from the human milk (41) in that the former have a 1 → 6 linkage between GlcNAc and the galactose of the lactosyl residue at the reducing end compared to a 1 → 3 linkage in the latter ones. Such a linkage has so far not been reported for corresponding human milk oligosaccharides.

The compound P-I, a pentasaccharide, represents an entirely novel structure having two galactose residue linked via β-1,3 and β-1,4 linkage, respectively, to the same GlcNAc residue at the non-reducing end. Such oligosaccharide structure from the goat milk, and perhaps, as far as we are aware of, from other biological fluids also, has not been reported before.

The goat milk fucose-containing pentasaccharide

P-III, Gal(β-1,4)GlcNAc(β-1,6)Gal(β-1,4)Glc,
↑
Fuc(α 1,3)

also differs markedly from the corresponding human milk pentasaccharide LNF-III (41)

Gal(β-1,4)GlcNAc(β-1,3)Gal(β-1,4)Glc
↑
Fuc(β-1,3)

and pentasaccharide LNF-II (41)

Gal(β -1,3)GlcNAc(β -1,3)Gal(β -1,4)Glc
↑
Fuc(α -1,4)

The P-III pentasaccharide differs from the human milk pentasaccharide LNF-III (41) in having 1 → 6 linkage between GlcNAc and galactose of the lactosyl residue at the reducing end. From LNF-II, the P-III differs in the following respects :

(i) it has a 1 → 6 linkage between β GlcNAc and β galactose as compared to a 1 → 3 linkage in LNF-II.

(ii) it has a 1 → 4 linked β galactose at the nonreducing end while LNF-II has a 1 → 3 linked β-Gal at the same position.

(iii) it has a 1 → 3 linkage between α -fucose and β -GlcNAc compared to a 1 → 4 linkage in LNF-II.

The goat milk pentasaccharide P-IV

Gal(β -1,3)GlcNAc(β -1,6)Gal(β -1,4)Glc
↑
Fuc(α -1,3)

differs from the corresponding human milk pentasaccharide LNF-I [Fuc (α -1,2)Gal(β -1,3)GlcNAc(β -1,3)Gal(β -1,4)Glc](6) in two respects - firstly P-IV has a 1 → 6 linkage between GlcNAc and the galactose of the lactosyl residue as compared to a 1 → 3 linkage in the case of LNF-I, and secondly, in having a α -Fucose which is 1 → 3 linked to glucose at the reducing end as compared to LNF-I, where fucose is 1 → 2 linked to β -galactose at the non-reducing end.

conditions, P-III oligosaccharide shows a k' value of 2.9 min. Apparently, in the above case the stereochemistry of the compound seems to be the major factor governing the retention of the oligosaccharide on C-18 column.

The other fucose-containing pentasaccharide P-IV (k' = 4.2 min) differs from P-III in the position of α-Fuc residue only, which is linked to Glc at the reducing terminal in the former and to GlcNAc in the latter. The wide difference in their k' values may be due to the more accessible amide methyl of GlcNAc, in the structure of P-IV, as the α-Fuc is at quite a distant from it. This concept is in line with the folded structure proposed for the human milk oligosaccharides by Ginsburg, et al. (134).

From the preceeding discussion, four generalizations regarding the retention of N-acetyl amino group-containing sugars on C-18 reverse phase, emerge clearly :

(i) Mechanism of retention of oligosaccharides containing acetamido sugars is dependent on stereochemical differences.

(ii) The presence of β-GlcNAc at the non-reducing terminal enhances retention greatly due to hydrophobic interaction with C-18 stationary phase.

(iii) Addition of fucose to the parent compound generally increases retention time due to the relatively higher hydrophobic nature of fucose.

(iv) Branching leads to early elution on reverse phase.

However, inspite of these generalization, it is difficult to predict retention times of oligosacharides on reverse phase due to the fact that more than one factors may influence the retention time on it.

The five oligosaccharides purified by reverse phase HPLC were tested for any cross-contamination on the normal phase-NH_2 column using CH_3CN : H_2O

Thus, structurally all the five neutral oligosaccharides from goat milk reported here differ markedly from that of the corresponding oligosaccharides isolated from human milk. The biological functions of these oligosaccharides are unknown, however, they can serve as substrates for various glycosyltransferases involved in the synthesis of complex carbohydrates of milk. They would also be useful in characterization of the related structure from glycolipids and glycoproteins as well as in studies of the conformation of the oligosaccharides. Furthermore, it is of interest to note that the goat milk tetrasaccharide, P-II, reported here has Gal(β -1,4)GlcNAc(β -1,6) type of structure at the non-reducing end. This is commonly known as the 'Type 2' Gal(β -1,4)GlcNAc(β -1,3 or β-1,6) chain sequence. The 'Type 1' chain sequence, Gal(β-1,3)GlcNAc(β -1,3) are the proponderant linear-backbone sequence in ovarian cyst and gastric glycoproteins of man, whereas 'Type 2' chains occur at branch points. In the gastric mucins of hog, horse and sheep, the preponderant sequence detected have been of 'Type 2' joined by (1 → 3) linkage in linear chains and 1 → 6 linkage at branch point. Branched and linear chain sequences of Type 2 are antigenic determinants of blood group I and i antigens, respectively (60). In the present work, purified oligosaccharides could not be tested for antigenic activities on account of the limited amounts available.

Anti-I antibody Ma specifically reacts with the sequence Gal(β -1,4) GlcNAc(β -1,6) and substitution of the terminal galactose with Fuc(α -1,2) blocks this reactivity whereas substitution with Gal(α -1,3) or sialic acid (α -2,3) does not (135). The pentasaccharide P-III has a α -Fuc 1 → 3 linked to β-GlcNAc of the tetrasaccharide core of P-II. Such structures are related to Le^a activity (107). It is difficult to predict about the Gal(β -1,3)GlcNAc (β -1,6) type of structure present in the pentasaccharide P-IV as no such blood group active sequence has been reported so far. Similarly, it is not

possible to predict about the blood group activity of pentasaccharide P-I, although it has a 'Type 1' sequence at the non reducing end, but with an additional β-Gal 1 → 4 linked to β-GlcNAc. It will be interesting to see what effect does this substitution has on its activity. Conversely, the sequence Gal(β-1,4)GlcNAc(β-1,3) Gal-, which is also present in this compound with an additional β-Gal 1 → 3 linked to GlcNAc, is involved in the antigenic determinants recognised by anti-I antibodies, other than those of 'Ma type' and the majority of anti-i antibodies (127).

It can be predicted that the higher oligosaccharides of goat milk may yield compounds which could be used as blood group I and i antigenic determinants.

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CHAPTER - VI

6.0 SUMMARY AND CONCLUSIONS

Five novel neutral oligosaccharides have been purified from the fresh goat milk using Bio-Gel P-4 and reverse phase C-18 high performance liquid chromatography (HPLC) and their structures determined by high field ^{1}H NMR. The Bio-Gel P-4 gave four distinct carbohydrate-containing peak (peak A, B, C and D) corresponding to higher oligosaccharides (k' value = 1.3-1.4) medium size oligosaccharides containing 7 to 12 monosaccharide residues (k' value = 1.9-2.1), small oligosaccharide containing 3 to 6 monosaccharide units (k' value = 2.2-2.5) and disaccharide (k' value = 2.5-2.7, mainly lactose), respectively. The small oligosaccharides (peak C) accounted for about 60% of the total oligosaccharides recovered from the Bio-Gel column.

The reverse phase C-18 HPLC with water as the mobile phase and uv absorbance at 202 nm as the detecting device has been found to be an excellent procedure for the separation of N-acetylglucosamine (GlcNAc)-containing oligosaccharides. Under these conditions, the Bio-Gel P-4 peak C (small oligosaccharides) was further resolved into five distinct peaks, designated as P-I, P-II, P-III, P-IV and P-V with k' values of 1.3, 2.2, 2.9, 4.2 & 5.5 respectively. The k' values of various oligosaccharide fraction on the C-18 did not bear relationship with the molecular weight, but instead the structure, polarity and conformation of the oligosaccharides seem to determine the k' values. For instance, presence of β-GlcNAc at the non-reducing end enhances retention greatly due to its hydrophobic interaction with the C-18 stationary phase and branching in the oligosaccharide chain enhances the elution rate.

The individual oligosaccharide fraction were repeatedly chromatographed on the reverse phase C-18 column until the desired purification level (96% or above) was obtained. Multiple runs were made in order to collect sufficient amount of the purified compounds.

The fractions prepared by preparative reverse phase C-18 column were found free of cross peak contamination by normal phase amino column HPLC and the purity of compounds was above 96%. The oligosaccharides prepared this way were found suitable for ^{1}H NMR.

On the basis of ^{1}H NMR data, the goat milk oligosaccharides, purified by reverse phase C-18 HPLC from the Bio-Gel P-4 fraction C, were assigned the following structures :

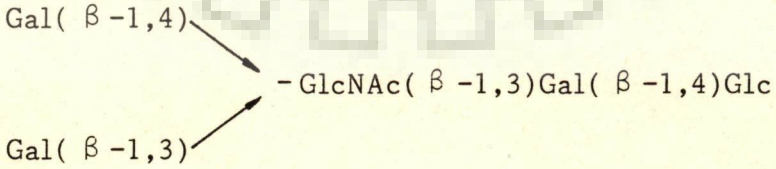
Trisaccharide, P-V

GlcNAc(β -1,6)Gal(β -1,4)Glc

Tetrasaccharide, P-II

Gal(β -1,4)GlcNAc(β -1,6)Gal(β -1,4)Glc

Pentasaccharide, P-I



Fucose - containing pentasaccharides

P-III : Gal(β -1,4)GlcNAc(β -1,6)Gal(β -1,4)Glc

↑

Fuc(α -1,3)

P-IV : Gal(β-1,3)GlcNAc(β-1,6)Gal(β-1,4)Glc
↑
Fuc(α -1,3)

Structurally, the goat milk oligosaccharides differ markedly from that of the corresponding oligosaccharides from human milk [Dua, V.K. and Bush, C.A. (1983) Anal. Biochem. 133, 1-8]. The pentasaccharide P-I, has a novel structure in the sense that it has two galactose residues linked via β-1,3 and β-1,4 linkage, respectively, to the same GlcNAc residue at the non-reducing end.

Although, we have not tested the antigenic activities of the oligosaccharides, it is highly likely that the tetrasaccharide P-II which has a 'Type 2', Gal (β-1,4)GlcNAc(β -1,3 or β-1,6), may exhibit antigenic activities since both linear and branched 'Type 2' chain sequence are antigenic determinants (60). Similarly, pentasaccharide, P-III, has an α-Fuc 1 → 3 linked to β-GlcNAc of the tetrasaccharide core of P-II. Such structures are related to Le^a activity (115).The work on the antigenic activities of these oligosaccharides is in progress.

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Goat milk oligosaccharides: purification and characterization by HPLC and high-field ^{1}H-NMR spectroscopy

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Three oligosaccharides were isolated from goat milk using Bio-Gel P-4 and reverse-phase C-18 HPLC and were characterized by high-field ^{1}H-NMR spectroscopy as a trisaccharide, GlcNAc(β1–6)Gal(β1–4)Glc, a tetrasaccharide, Gal(β1–4)GlcNAc(β1–6)Gal(β1–4)Glc, and a pentasaccharide,

Gal(β1–3)
\>GlcNAc(β1–3)Gal(β1–4)Glc
Gal(β1–4)/

Introduction

Oligosaccharides of milk are used in studies on the acceptor specificity of glycosyltransferases, the substrate specificity of glycosidases and the presence of antigenic determinants [1]. It is now well known that the oligosaccharides present in individual samples of milk can vary with ABO or Lewis blood type of the donor as the enzymes involved in their synthesis are also responsible for the formation of the structural determinants of these blood types [2,3]. The oligosaccharides have been isolated as reducing sugars from human milk by gel filtration and preparative paper chromatography [4,5]. Recently, the small neutral milk oligosaccharides have been fractionated using reverse-phase HPLC and reference ^{1}H- and natural abundance ^{13}H-NMR spectra for these compounds have been provided [6–8]. Egge and coworkers [9–11] have isolated some milk oligosaccharides as the reduced and per-*O*-acetylated derivatives using HPLC and characterized them by mass spectrometry and high-field ^{1}H-NMR spectroscopy. Whether these oligosaccharides are also present in milk from other mammals is not known. Since goat milk is commonly used by inhabitants of hilly areas in India and is also preferred for infants because of its easy digestibility and low fat content compared to cow or buffalo milk, it was considered worthwhile to analyse the goat milk neutral oligosaccharides and compare them with those found in human milk.

In this communication, we report the isolation of three neutral oligosaccharides from goat milk by reverse-phase HPLC. In addition, high-field ^{1}H-NMR spectra of the oligosaccharides are given which will serve as reference spectra for characterization of similar oligosaccharides from other biological sources.

Materials and Methods

Materials

Goat milk was procured from a local dairy. Bio-Gel P-4 was purchased from Bio-Rad. High-

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purity ^2H_2O was obtained from Merck, Sharp and Dohme Co., U.S.A.

Isolation of oligosaccharides

The goat milk was processed as described by Kobata and Ginsburg [12]. The milk was centrifuged at 4°C and the lipid layer removed by filtration through a loosely packed glass wool column in the cold. Lactose and proteins were precipitated by the addition of ethanol up to a final concentration of 68%, separated by centrifugation and the supernatant was lyophilized. The lyophilized material (250 mg) was then fractionated on a Bio-Gel P-4 column (1.5 × 60 cm) using glass-double-distilled water as eluent. Fractions (0.75 ml) were collected at a flow rate of 0.25 ml/min and aliquots (50 μl) from every fraction were analysed for sugars by the phenol-sulphuric acid method [13]. Carbohydrate-containing fractions under various peaks were pooled, lyophilized, dissolved in glass-double-distilled water (15 mg/ml) and analysed further by HPLC.

Purification of oligosaccharides by HPLC

The oligosaccharide fractions obtained from the Bio-Gel P-4 column were further purified by reverse-phase HPLC [6,14,15] using a Shimadju Model LC-4A Liquid Chromatograph fitted with a Du-Pont Zorbax C-18 column (4.6 × 250 mm), UV detector and a Shimadju Chromatopac Model C-R2AX data processing system. Oligosaccharides were eluted with HPLC-grade distilled water at a flow rate of 1 ml/min. The effluent was monitored by UV absorbance at 202 nm. In order to collect sufficient amounts of various peaks eluting at various time intervals, multiple runs were made on the same column and fractions were collected manually at the detector outlet. Each fraction was then repeatedly chromatographed on the reverse-phase column (C-18 Zorbax column) until a purification of over 95% was achieved. In addition, the purified fractions were checked for any cross-contamination by HPLC on the normal phase column (-NH_2 Zorbax column) using CH_3CN/H_2O (60 : 40) at a flow rate of 1 ml/min. The purified fractions were assayed for carbohydrate by the phenol-sulphuric acid method [13].

Structure determination by ^1H-NMR

^1H-NMR experiments were carried out on a 400 MHz Bruker spectrometer at the Regional Sophisticated Instrument Facility, Central Drug Research Institute, Lucknow and on a 270 MHz Bruker spectrometer at Sophisticated Instrument Facility, Indian Institute of Science, Bangalore, India. For ^1H-NMR spectroscopic analysis, samples were dissolved in ^2H_2O and then repeatedly exchanged with ^2H_2O followed by lyophilization. The samples were then dissolved in 0.3 ml high-purity ^2H_2O (Merck, Sharp & Dohme). The observed chemical shifts were reported relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) using acetone as an internal standard ($\delta = 2.225$ ppm) downfield from DSS. The spin difference decoupling experiments were carried out at 22°C.

Results

Fig. 1 shows the elution profile of the goat milk oligosaccharides from a Bio-Gel P-4 column. The

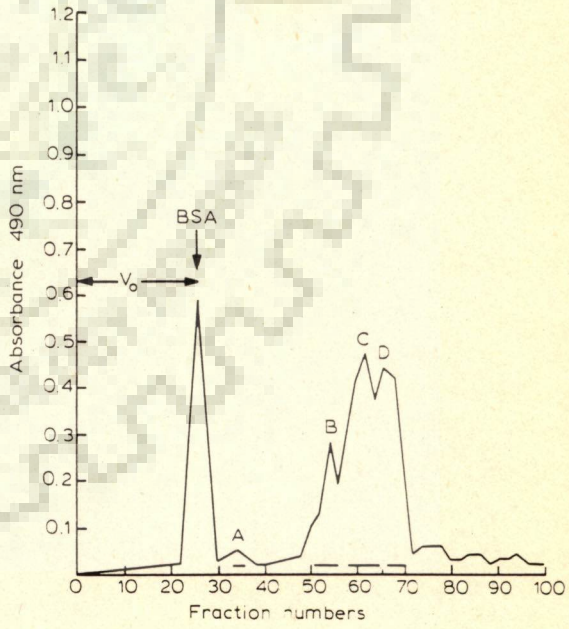

Fig. 1. Bio-Gel P-4 chromatography of goat milk oligosaccharides. A 1 ml sample (approximately equal to 250 mg glucose) was loaded on the Bio-Gel column (1.5 × 60 cm) and oligosaccharides were eluted with water at a flow rate of 0.25 ml/min as described in the text. Fractions indicated by a bar (—) in each peak were pooled. BSA, bovine serum albumin.

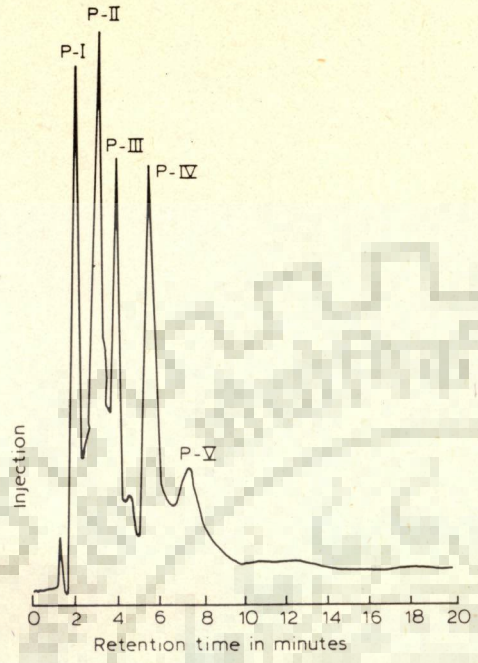

Fig. 2. Reverse-phase HPLC of the Bio-Gel P-4 fraction C. A 10 μl sample was injected on a Zorbax C-18 column and eluted with water at a flow rate of 1 ml/min. The elution was monitored by UV absorption at 202 nm. Each fraction was manually collected.

results show that a substantial amount of sugar was eluted in the void volume. The bovine serum albumin standard was also eluted at the same elution volume, indicating that this peak is a high molecular weight polymer. Four distinct carbohydrate-containing peaks, designated as A, B, C and D were obtained. Of these oligosaccharide peaks, D was mostly lactose whereas peaks C, B and A contained a mixture of oligosaccharides of varying sizes. Fractions under these peaks were pooled separately and lyophilized. Fraction C, which appeared to be the most dominant fraction, was used for further analysis by HPLC on the reverse-phase C-18 column using water as eluent. The results are shown in Fig. 2. As can be seen, fraction C was clearly resolved into five distinct oligosaccharide peaks designated as P-I, P-II, P-III, P-IV and P-V. It was, however, observed that the α and β anomeric forms of the reducing-terminal monosaccharide residue were not resolved as is generally observed for reducing sugars in reverse-phase chromatography [6]. This is probably due to unfavourable mutarotation time of the sugars [14].

The purified oligosaccharide fractions from the C-18 column were checked for contamination on a normal-phase Du Pont Zorbax-NH_2 column. No cross-contamination peaks were observed when the C-18 fractions were run on the -NH_2 column. The relative retention values (k') of the various peaks on both C-18 and -NH_2 columns are given in Table I.

Characterization of oligosaccharides by ^{1}H-NMR

The ^{1}H-NMR spectrum of the oligosaccharide P-V isolated by preparative HPLC is shown in Fig. 3. The integration of the anomeric region (4.0–5.5 ppm) shows that the compound is a trisaccharide. The presence of a glucose (Glc) residue at the reducing terminal is indicated by the doublet at 5.228 ppm ($J = 3.7$ Hz) assigned to α-Glc H-1 and a triplet at 3.286 ppm assigned to β-Glc H-2 [6]. The singlet at 2.059 ppm (3 protons by integration) is assigned to an amide methyl of one *N*-acetylglucosamine (GlcNAc) residue. This is further supported by the presence of a doublet at 4.680 ppm due to β-GlcNAc H-1 ($J = 7.8$ Hz). The presence of one β-galactose (Gal) (by integration) is shown by a doublet at 4.436 ppm. The β-Glc H-1 signal has overlapped with that of β-GlcNAc H-1 at 4.680 ppm which is confirmed by decoupling at β-Glc H-2. The comparison of the spectrum with that of lactose [16], lactose-*N*-

TABLE I

HPLC RELATIVE RETENTION VALUE (k') OF MILK OLIGOSACCHARIDES

Oligosaccharide	Relative retention value, k'	
	reverse-phase column	amino column [a]
P-I	1.3	4.1
P-II	2.2	3.2
P-III	2.9	4.5
P-IV	4.2	4.8
P-V	5.5	2.5
Chromatographic conditions		
column	Du-pont Zorbax C-18	Du-pont Zorbax-NH_2
solvent	water	acetonitrile/water (60:40)
flow	1 ml/min	1 ml/min

[a] Oligosaccharides purified on a reverse-phase C-18 column were rechromatographed on an amino column.

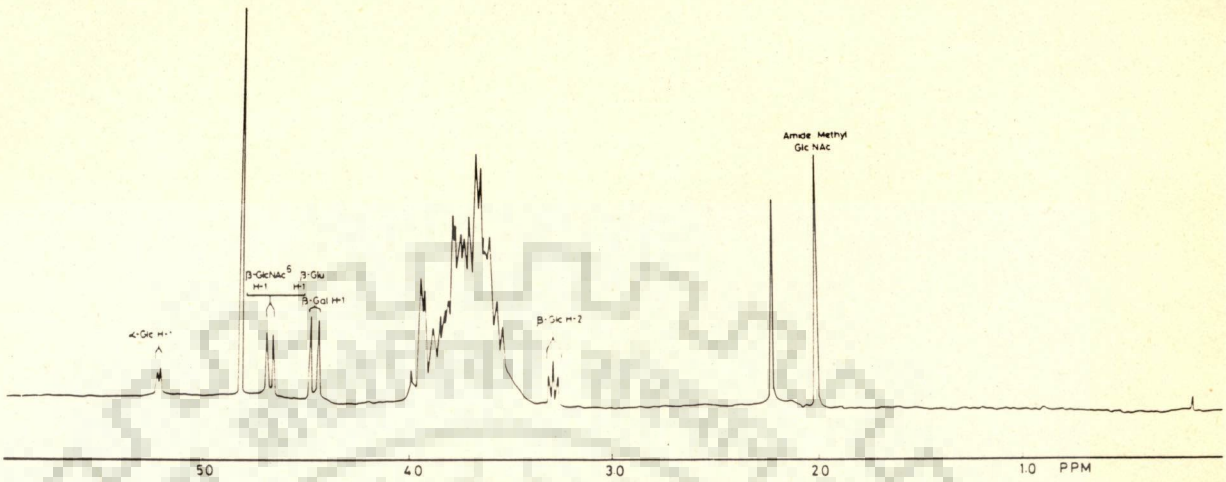

Fig. 3. ^{1}H-NMR spectrum (400 MHz) of oligosaccharide P-V in ^2H_2O at 22°C.

tetraose (LNT) and lacto-*N*-neotetraose (LN-neoT) structures reported by Dua and Bush [6] indicates the presence of a lactosyl, Gal(β1–4)Glc, residue at the reducing terminal. The absence of a downfield shift of the H-4 resonance of galactose confirms that β-Gal is not substituted at the C-3 position by β-GlcNAc. This implies that the GlcNAc may be 1 → 6 linked to Gal, which is assigned by spin difference decoupling and chemical shift analogies of GlcNAc 1 → 6 as reported by Dua et al. [17]. Assignments of α-Glc H-2, β-Glc H-1, β-GlcNAc H-2, β-Gal H-2 as well as remaining resonances (Table II) were determined by spin difference decoupling experiments. Based on these data, the structure of P-V trisacharide was assigned as follows:

GlcNAc(β1–6)Gal(β1–4)Glc

The ^{1}H-NMR spectrum of compound P-II is given in Fig. 4. The integration of the anomeric region shows that the compound is a tetrasaccharide. Comparison of its spectrum with that of P-V shows (Fig. 3) the presence of an additional β-Gal residue which is identified by the doublet at 4.543 ppm assigned to its H-1 resonance (by integration). This downfield shift of the β-Gal H-1 is derived from the comparison with LNT and LN-neoT and assigned to a non-reducing terminal β-Gal which is 1 → 4 linked to a GlcNAc which in turn is 1 → 6 linked to the subsequent galactose. A similar downfield shift in the position of Gal^{4,6}

TABLE II

^{1}H-NMR CHEMICAL SHIFTS OF SOME MILK OLIGOSACCHARIDES

Chemical shifts are given relative to internal DSS using acetone as the internal standard (δ = 2.225 ppm downfield from DSS)

Residue		Oligosaccharide		
		P-I	P-II	P-V
α-Glc	H-1	5.212	5.225	5.228
	H-2	3.587	3.587	3.585
β-Glc	H-1	4.652	4.680	4.680
	H-2	3.283	3.282	3.286
	H-3	3.630	3.635	3.639
β-Gal^4	H-1	4.411	4.438	4.436
	H-2	3.571	3.580	3.574
	H-3	3.728	3.732	3.738
	H-4	4.140	–	–
β-Gal^{4,6}	H-1	–	4.543	–
	H-2	–	3.520	–
	H-3	–	–	–
β-Gal^3	H-1	4.411	–	–
	H-2	3.517	–	–
	H-3	–	–	–
β-Gal^{4,3}	H-1	4.542	–	–
	H-2	3.493	–	–
β-GlcNAc^6	H-1	–	4.680	4.680
	H-2	–	3.759	3.745
	N-acetyl	–	2.057	2.059
β-GlcNAc^3	H-1	4.652	–	–
	H-2	3.904	–	–
	N-acetyl	2.050	–	–

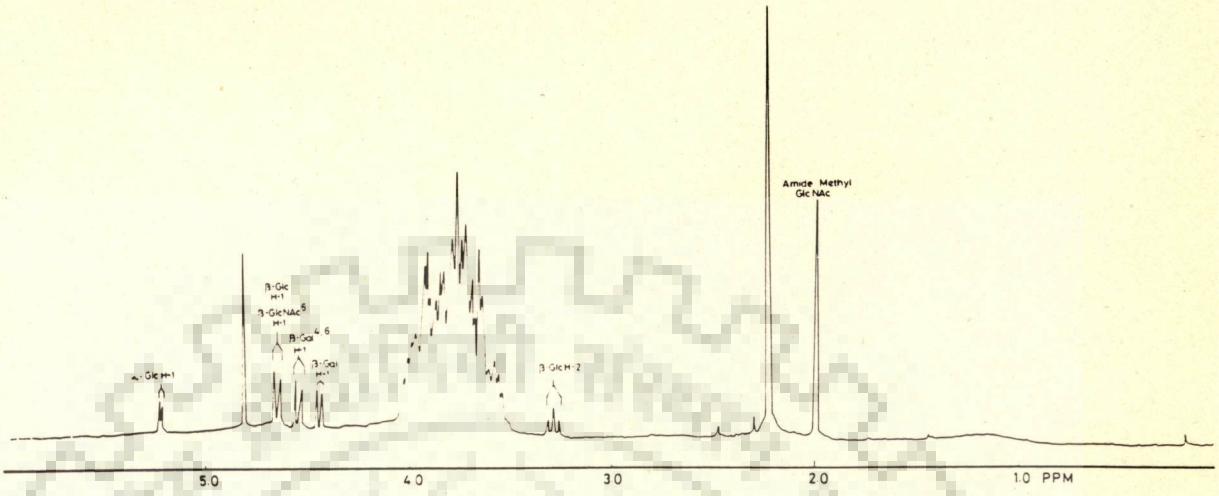

Fig. 4. ^{1}H-NMR spectrum (400 MHz) of oligosaccharide P-II in $^{2}H_2O$ at 22° C.

H-1 resonance was reported by Dua et al. [17]. The remaining resonances of the anomeric region are similar to those of P-V (Table II). A comparison of the spectrum with that of LNT and LN-neoT structures confirms the presence of a lactosyl residue at the reducing terminal. Again, the absence of the downfield shift of the Gal H-4 resonance confirms that none of the galactose residues is substituted at the C-3 position. This fact further supports our interpretation that the β-GlcNAc is $1 \rightarrow 6$ linked to galactose. On the basis of the above data, the compound P-II is assigned the following structure:

Gal(β1–4)GlcNAc(β1–6)Gal(β1–4)Glc

It may be pointed out that the ^{1}-NMR spectrum of the compound shows it to be more than 95% pure.

The ^{1}H-NMR spectra of compound P-I is shown in Fig. 5. The integration of the anomeric region shows it to be a pentasaccharide. The Gal H-4 resonance in this case is clearly shifted downfield to 4.140 ppm which implies that the compound is not similar to P-II and P-V, but it may be of the LNT or LN-neoT type. By comparison of the spectrum of compound P-I with that of LNT and LN-neoT structures and on the basis of chemical shift analogy, the compound appears to have the LNT structure with an additional galactose residue $1 \rightarrow 4$ linked to GlcNAc identified by its H-1 resonance at 4.542 ppm. This downfield shift of the Gal H-1 may be due to crowding and steric hindrance at the non-reducing end. The rest of the assignments were made by analogy to LNT structure. The presence of glucose at the reducing terminal is indicated by the doublet at 5.212 ppm

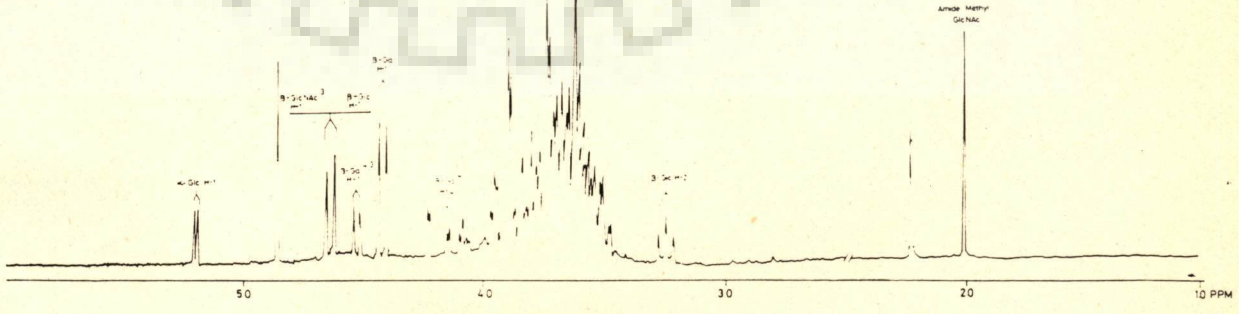

Fig. 5. ^{1}H-NMR spectrum (400 MHz) of oligosaccharide P-I in $^{2}H_2O$ at 22° C.

assigned to α-Glc H-1 and the triplet at 3.283 ppm due to β-Glc H-2. Integration of the amide methyl signal at 2.050 ppm shows the presence of one GlcNAc residue.The β-Glc H-2 and β-GlcNAc H-1 resonance have again overlapped at 4.652 ppm. The 2-proton signal at 4.411 ppm is assigned to H-1 of β-Gal by analogy to LNT structure whereas the 1-proton signal at 4.542 ppm is assigned to the third β-Gal H-1 which is 1 → 4 linked to β-GlcNAc. The compound can thus be assigned the following structure:

Gal(β1–4)
GlcNAc(β1–3)Gal(β1–4)Glc
Gal(β1–3)

Irradiation of the doublet at 4.652 ppm identifies the β-GlcNAc H-2 resonance at 3.904 ppm as well as β-Glc H-2 resonance at 3.283 ppm. Decoupling of the β-Glc H-2 resonance at 3.283 gives the corresponding H-3 resonance at 3.630 ppm, and H-1 at 4.652 ppm. The $Gal^{4,3}$ H-1 doublet at 4.542 ppm on irradiation identified the corresponding H-2 resonance at 3.493 ppm. The remaining assignments were made as in the case of P-II and P-V (Table II).

The P-III and P-IV peaks appear to be fucose-containing oligosaccharides. Their structures are under investigation and will be reported separately.

Discussion

The tri- and tetrasaccharides (P-V and P-II) reported here differ from the LNT and LN-neoT structures isolated from human milk in that the former have a 1 → 6 linkage between GlcNAc and the galactose of the lactosyl residue at the reducing end compared to a 1 → 3 linkage in the latter ones. Such a linkage has so far not been reported for corresponding human milk oligosaccharides. The compound P-I, a pentasaccharide, has been assigned a novel structure having two galactose residues linked via 1 → 3 and 1 → 4 linkage, respectively, to the same GlcNAc residue at the non-reducing end. The structures of these oligosaccharides would further add to the growing library of oligosaccharides of known structure. The ^{1}H-NMR assignments would be useful in the characterization of related structures isolated from glycolipids or glycoproteins as well as in studies of the conformation of the oligosaccharides.

As mentioned in the above text, the oligosaccharides are not clearly resolved into their α- and β-anomeric pairs on the reverse-phase C-18 column. This might be a consequence of poor resolution of anomers and line-broadening which occurs for cases in which the mutarotation time is comparable to the chromatographic time [14]. This is further supported by the finding that the partial resolution into the anomeric pairs was possible when the chromatographic analysis was carried out at lower temperature (7–8 °C). The relatively late elution of the compound P-V, a trisaccharide (Table I), from the reverse-phase column might be due to the presence of the β-GlcNAc residue at the non-reducing terminal, thereby exposing the amide methyl group for interaction with the non-polar C-18 stationary phase. The unexpected shorter retention time of the compound P-I, a pentasaccharide, than that of tetrasaccharide may be attributed to branching at the non-reducing end [7].

One-dimensional spectroscopy used here has been found to be adequate for carbohydrate resonance assignments which show characteristic coupling constants of axial and equatorial protons. Although two-dimensional NMR spectroscopy could be used for proton assignments, the limited digital resolution of such methods generally obscures multiplet structures.

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