THE ISOLATION AND CHARACTERIZATION OF TISSUE FRACTIONS FROM THE MIDDLE LAMELLA AND SECONDARY WALL OF BLACK SPRUCE TRACHEIDS

by

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FOREWORD

This thesis is an investigation of the differences between the components of the middle lamella and secondary wall of black spruce tracheids. After more than a hundred years of investigations, very little is known about these components as they actually exist in the wood. Such knowledge is of fundamental importance to researchers working in all areas of wood science.

The present work involves the development of a method for isolating tissue from the middle lamella and secondary wall of black spruce tracheids, and subsequent studies of the components contained therein. The research was carried out under the supervision of Dr. D.A.I. Goring as part of the postgraduate research program conducted in the Department of Chemistry in cooperation with the Pulp and Paper Research Institute of Canada at McGill University.

The thesis starts with a General Introduction in which wood morphology, wood chemistry, and the chemistry of lignin are reviewed. The main body of the thesis is presented in nine chapters which follow the Introduction. These chapters have been written in the form of scientific papers, each with its own Abstract, Introduction, Experimental, etc. Described in Chapter II is the development of a method for the isolation of tissue from the middle lamella and secondary wall of black spruce tracheids. Chapter III deals with the distribution of the polysaccharides in these tissue fractions. In Chapter IV, an account is given of the development of a new technique, based on turbid spectrophotometry, for the measurement of the phenolic hydroxyl content of wood tissue

while in Chapter V, a pyrolytic gas chromatographic technique is used for the same purpose. In Chapter VI, the contents of methoxyl and carbonyl groups in the lignin of the two tissue fractions are discussed along with the carboxyl and elemental analyses of the fractions themselves. Chapter VII concerns work done on the relative reactivities of middle lamella and secondary wall lignin to chlorine, chlorine dioxide, and sodium bisulphite. It is shown in Chapter VIII that there is a topochemical preference towards the dissolution of secondary wall lignin in kraft, acid-sulphite, and acid-chlorite pulping, and in Chapter IX, the kinetics of the kraft delignification of middle lamella lignin is compared with that of the secondary wall lignin. Finally, the morphological origin of milled wood lignin is considered in Chapter X.

Four Appendices are presented which give additional details and data. In Appendix I, the gas chromatographic carbohydrate analyses of Chapter III are confirmed by using high pressure liquid chromatography. Appendix II deals with bromination experiments performed on the tissue fractions. Appendix III is concerned with leaching of lignin during the washing of kraft pulps made from middle lamella and secondary wall tissue. The technique for obtaining weight average molecular masses by sedimentation equilibrium is described in Appendix IV.

The thesis concludes with two sections entitled "Original Contributions to Knowledge" and "Suggestions for Further Research".

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ABSTRACT

A method was developed for the isolation of tissue fractions from the middle lamella and secondary wall of black spruce (Picea mariana) tracheids. The concentrations of cellulose and glucomannan were found to be lower in the middle lamella tissue than in the secondary wall tissue, while the reverse was found for galactan and arabinan. The content of glucuronoarabinoxylan was essentially the same in both morphological areas. The concentration of phenolic hydroxyl groups in the lignin in the two tissue fractions was determined by turbidimetric spectrophotometry and by pyrolytic gas chromatography. Secondary wall lignin was found to contain more than twice as many phenolic hydroxyl groups per phenylpropane unit as middle lamella lignin. The concentration of methoxyl groups in secondary wall lignin was 1.6 times the concentration in middle lamella lignin, indicating that about 40% of the lignin polymer in the middle lamella consisted of para-hydroxyphenyl residues. Most of the carbonyl groups in the lignin in wood were found in the middle lamella lignin. The carboxyl content of the middle lamella was about three times that of the carboxyl content of the secondary wall. Secondary wall lignin was more reactive towards chlorine, chlorine dioxide and sodium bisulphite. Secondary wall lignin was also more quickly dissolved than middle lamella lignin in kraft, acid-sulphite, and acid-chlorite pulping. The activation energies, towards kraft pulping, of middle lamella and secondary wall "bulk" lignin were found to be essentially the same. However, it was discovered that while most of the secondary wall lignin followed first order reaction kinetics, 70% of

middle lamella lignin did not. It was also discovered that the majority of milled wood lignin originated in the secondary wall of the tracheid.

RESUME

Une méthode permettant d'isoler des fractions de tissu de la lamelle mitoyenne et de la paroi secondaire des trachéides d'épinette noire (Picea mariana) a été développée. Les concentrations de cellulose et de glucomannane étaient plus basses dans le tissu de la lamelle mitoyenne que dans le tissu de la paroi secondaire, alors que le contraire a été observé dans le cas du galactane et de l'arabinane. Le contenu en glucuronoarabinoxylane était essentiellement le même dans les deux régions morphologiques. La concentration de groupes hydroxyle phénolique dans la lignine des deux fractions de tissu fut déterminée par spectrophotométrie turbidimétrique et par chromatographie pyrolytique en phase gazeuse. La lignine de la paroi secondaire contenait plus de deux fois plus de groupes hydroxyle phénolique par groupement phénylpropane que la lignine de la lamelle mitoyenne. La concentration de groupes méthoxyle dans la lignine était 1.6 fois plus élevée dans la paroi secondaire que dans la lamelle mitoyenne. Ceci indique qu'à peu près 40% de la macromolécule de lignine dans la lamelle mitoyenne consistait de résidus para-hydroxyphényle. La plupart des groupes carbonyle de la lignine du bois se trouvaient dans la lignine de la lamelle mitoyenne. Le contenu en groupés carboxyle était à peu près trois fois plus élevé dans la lamelle mitoyenne que dans la paroi secondaire. La réactivité de la lignine de la paroi secondaire envers le chlore, le bioxyde de chlore et le bisulfite de sodium, était plus élevée que celle de la lamelle mitoyenne. La paroi secondaire était aussi dissoute plus rapidement par les liqueurs chimiques réduisant le bois en pâte (kraft, sulfite-acide, chlorite-acide). Les énergies d'activation pour le procédé kraft

étaient essentiellement les mêmes pour la paroi secondaire et la lamelle mitoyenne. Par contre, bien que la majorité de la lignine de la paroi secondaire suivait une loi cinétique du premier ordre pour le procédé kraft, 70% de la lignine de la lamelle mitoyenne ne suivait pas une telle loi. La majorité de la lignine extraite du bois moulu ("milled wood lignin") provenait de la paroi secondaire de la trachéide.

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GLOSSARY OF PRINCIPAL SYMBOLS

A	= absorbance
A_{DL}	= absorbance of dissolved lignin
A_G^{OH}	= area of guaiacol chromatographic peak arising from free phenolic units
A_G^{OR}	= area of guaiacol chromatographic peak arising from units with aryl ether linkages
A_o	= measured absorbance of suspended sample
A_p	= pre-exponential factor
A_P^{OH}	= area of phenol chromatographic peak arising from free phenolic units
A_P^{OR}	= area of phenol chromatographic peak arising from units with aryl ether linkages
A_S	= apparent absorbance due to scattering
A_S'	= apparent absorbance due to scattering measured at 600 nm
A_{SL}	= absorbance arising from soluble lignin
$(A_S)_{300}$	= apparent absorbance due to scattering at 300 nm
$(A_S)_{600}$	= apparent absorbance due to scattering at 600 nm
A_1	= length of optical arm
A_{260}	= absorbance at 260 nm
A_{280}	= absorbance at 280 nm
c	= concentration
C_9	= phenylpropane unit of lignin
dn/dc	= refractive index increment

dn/dx	= refractive index gradient
D	= depth of ultracentrifuge cell
DHP	= dehydrogenase polymerized lignin
DHP-I	= dehydrogenase polymerized lignin, insoluble fraction
DHP-S	= dehydrogenase polymerized lignin, soluble fraction
E_a	= Arrhenius activation energy
EDXA	= energy dispersive X-ray analysis
E_{ML}	= percent lignin extracted from the compound middle lamella
E_S	= percent lignin extracted from the secondary wall
E_W	= percent lignin extracted from the whole wood
F_I	= fraction of structure I groups
F_{II}	= fraction of structure II groups
F_{III}	= fraction of structure III groups
F_{IV}	= fraction of structure IV groups
G.C.	= gas chromatography
G/C_9	= fraction of lignin built from guaiacyl groups
I	= percent transmittance at peak
I.D.	= internal diameter
I_o	= percent transmittance at baseline
I.R.	= infrared
k	= rate constant
k_1, k_2	= pyrolysis constants

K	= proportionality constant
K_ε	= correction factor for measured absorptivity
K_S	= correction factor for scattering measurements
ℓ	= path length
ℓn	= natural logarithm
L	= percent delignification
L.C.	= liquid chromatography
L_D	= percent of lignin that is difficult to pulp (residual lignin)
m	= cylindrical magnification
ML	= middle lamella tissue
ML-1, ML-2, ML-3	= middle lamella tissue samples of varying lignin contents
$\overline{M}_w$	= weight average molecular mass
MWL	= milled wood lignin
OCH_3	= methoxyl groups
OCH_3/C_9	= methoxyl groups per phenylpropane unit
PhOH	= phenolic hydroxyl groups
$PhOH/C_9$	= phenolic hydroxyl groups per phenylpropane unit
R	= ideal gas constant
SW	= secondary wall tissue
S_1, S_2, S_3	= layers of the secondary wall
t	= time
T	= absolute temperature
UV	= ultraviolet

$\bar{v}$	= partial specific volume
W_{ML}	= weight fraction of total lignin in middle lamella
W_S	= weight fraction of total lignin in secondary wall
WW	= whole wood tissue
X_m	= distance from centre of rotor to ordinate medium between meniscus and base of cell
Y_e	= height of centre ordinate

GREEK SYMBOLS

ε	= absorptivity
ε_{DL}	= absorptivity of dissolved lignin
ε_{SL}	= absorptivity of suspended lignin
ε_{280}	= absorptivity at 280 nm
ε_{350}	= absorptivity at 350 nm
$[\varepsilon]_6$	= absorptivity at pH 6
$[\varepsilon]_{12}$	= absorptivity at pH 12
$\Delta\varepsilon$	= change in absorptivity
$\Delta\varepsilon_{DL}$	= change in absorptivity of dissolved lignin
$\Delta\varepsilon_{SL}$	= change in absorptivity of suspended lignin
θ	= Schlieren angle
ρ	= density
ρ_s	= density of solution
ω	= angular velocity

CHAPTER I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

PHYSICAL AND CHEMICAL STRUCTURE OF WOOD

A. Classification of Woody Plants

Woody plants belong to phylum spermaphyta (seed plants)[1]. The plants can be divided into two sub-phyla, the gymnospermae (naked seeds) and the angiospermae (seeds enclosed within the ovary of the flower). Within the subphylum gymnospermae are found the coniferous or softwood trees (evergreens). Within the subphylum angiospermae are found the deciduous or hardwood trees. The softwood group contains such well known trees as pine, fir, hemlock and spruce while the hardwood group contains trees such as birch, maple, oak, ash, and aspen.

Softwoods are traditionally used more often in the manufacture of pulp and paper but recently hardwoods are being utilized to a larger extent.

Since the work in this thesis involves the study of black spruce (Picea mariana), a softwood, the introduction will focus on topics concerning the structure and chemistry of softwoods.

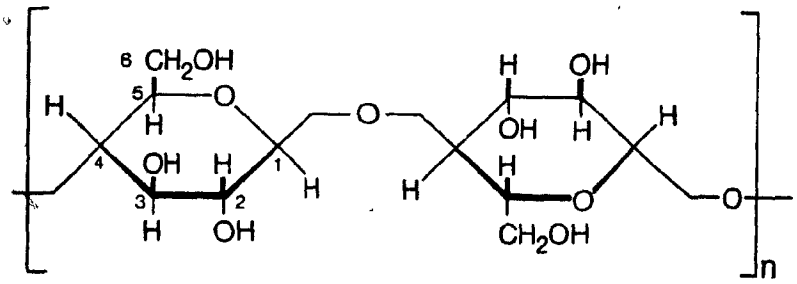
B. Wood Chemistry

Wood is composed of four classes of chemical compounds, cellulose, the hemicelluloses, lignin, and the extractives[2]. Cellulose and the hemicelluloses are polysaccharides and the term "holocellulose" is commonly used to describe the substance obtained from wood when the lignin and extractives are removed.

Definitions of the above four classes are given below.

Cellulose: Cellulose is a linear, partially crystalline polysaccharide which has a polydisperse molecular mass[3,4]. It is built up from anhydroglucose units connected to each other by β-(1→4')-glucosidic linkages.

CELLULOSE; β-D-ANHYDROGLUCOSE UNITS



The weight-average degree of polymerization of cellulose is between 10 000 and 17 000[5,6]. The basic repeat unit is the cellobiose unit. In wood, cellulose is present in the form of microfibrils varying in diameter from 10 to 40 nm[7]. The microfibrils are believed to be composed of protofibrils, considered to be the basic unit of cellulose, with a diameter of 3.5 nm[8-12]. The ultrastructure of the protofibrils is not well understood and is the subject of considerable debate.

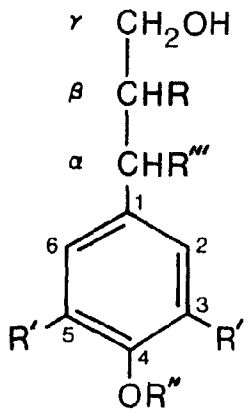
Cellulose is not soluble in water, dilute acids or dilute alkali at room temperature. It can, however, be dissolved using cuprammonium hydroxide, some salt solutions, and some strong acids[13]. However, during solubilization the cellulose is probably degraded to some extent so that molecular mass measurements are likely to be lower than that for the naturally occurring polymer.

Hemicellulose: Hemicelluloses are polysaccharides of considerably lower molecular mass than cellulose. They are found in wood cell walls together with cellulose and lignin. When isolated from wood they can be distinguished from cellulose by their solubility in dilute alkali. The hemicellulose polysaccharide chains are branched, unlike cellulose, and are believed to be amorphous in wood[14]. They have a degree of polymerization of 100 - 200[15].

There are three main groups of hemicelluloses, the xylans, the mannans, and the galactans. Only small amounts of the hemicelluloses can be removed from softwoods without chemical modification[16]. However, large quantities can be easily extracted from hardwoods by cold alkali[17]. The chemistry of the hemicelluloses has been reviewed by Timell[18].

Lignin: Lignin is found in all woody plants. Its structure is aromatic in nature[19] and contains phenylpropane units.

LIGNIN ; PHENYLPROPANE SKELETON



R = PHENYLPROPANE UNIT

R' = H, OCH_3, OR

R'' = H, R

R''' = OH, R

In softwoods, the lignin has a methoxyl group substituted at the number 3 position (guaiacyl unit). In hardwoods there is a mixture of guaiacyl units and units with methoxyl groups in both the 3 and 5 positions (syringyl unit). The relative proportions of syringyl to guaiacyl units in hardwoods varies considerably from species to species[20,21]. Interunit bonding in the polymer can occur through the α, β, γ, 2, 3, 4, 5 and 6 positions of the phenylpropane units giving rise to a highly cross-linked polymer with carbon-carbon and carbon-oxygen linkages[22].

A derived structure for softwood lignin is shown in Figure 1[23]. The structure shows all the types of interunit bonding proposed for lignin.

Lignin is almost completely insoluble in common organic solvents. A small amount may be dissolved in 95% aqueous ethanol (Brauns native lignin)[24] but this is usually less than 5% of the lignin in the wood. Lignin cannot be isolated from wood in an unchanged form[25]. This, plus the bonding complexity and large natural variation from species to species, has not allowed the determination of the exact chemical structure of lignin.

Extractives: The extractives are lower molecular mass compounds of various types, extractable from the wood with water or organic solvents. These compounds include polyphenols (other than lignin), fatty acids and resins (non-volatile ether soluble compounds), terpenes, and tannins. Wood extractives have been thoroughly reviewed by Hillis[26].

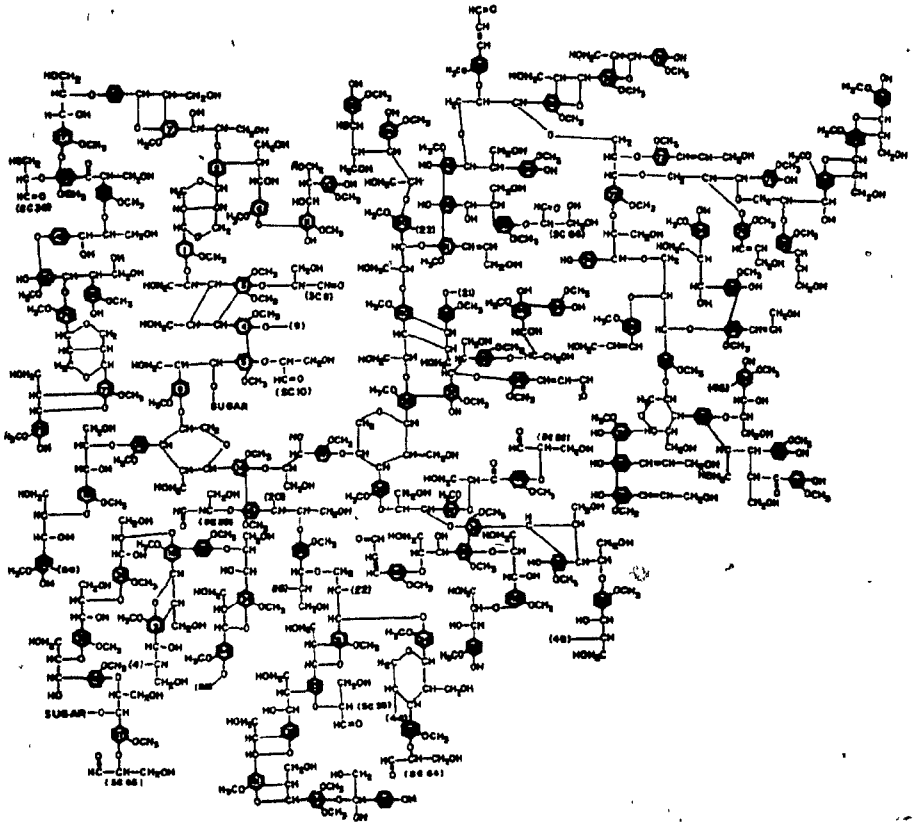


Figure 1. Glasser and Glasser's structure of softwood lignin (1981)[23].

C. Softwood Cells

Softwoods are of simpler structure than hardwoods. The greater part of softwood consists of tracheids, which are both water-conducting and supporting elements[1]. The axial (vertical) tracheids of softwoods are between 1 and 11 mm long, and are normally about 100 times longer than they are broad. Their diameters vary from 0.02 to 0.2 mm^2. Tracheids make up about 95% of the xylem of most softwoods[2,27].

The majority of the other 5% of the xylem of softwoods is made up by parenchyma cells (ray cells)[2,27]. These cells run radially in the tree and serve for transportation and storage of reserve materials. Figure 2 shows a cube of softwood with the cells labelled.

The conduction of liquids from one cell to another is achieved by means of pits with a thin membrane separating adjacent cells[1]. The transport of materials in solution takes place from one cell cavity or lumen to the other through these membranes.

D. Tracheid Structure

A structural model of a softwood tracheid is shown in Figure 3. The cell wall layers in sequence from the cell cavity or lumen are the secondary wall, primary wall, and middle lamella. The secondary wall is the dominant layer and can be subdivided into S_1, S_2, and S_3 layers, depending on the orientation of the cellulose microfibrils. The S_3 layer is adjacent to the fibre lumen and is sometimes referred to as the tertiary wall. The S_1 layer is adjacent to the primary wall and the S_2 layer is between the S_1 and S_3 layers.

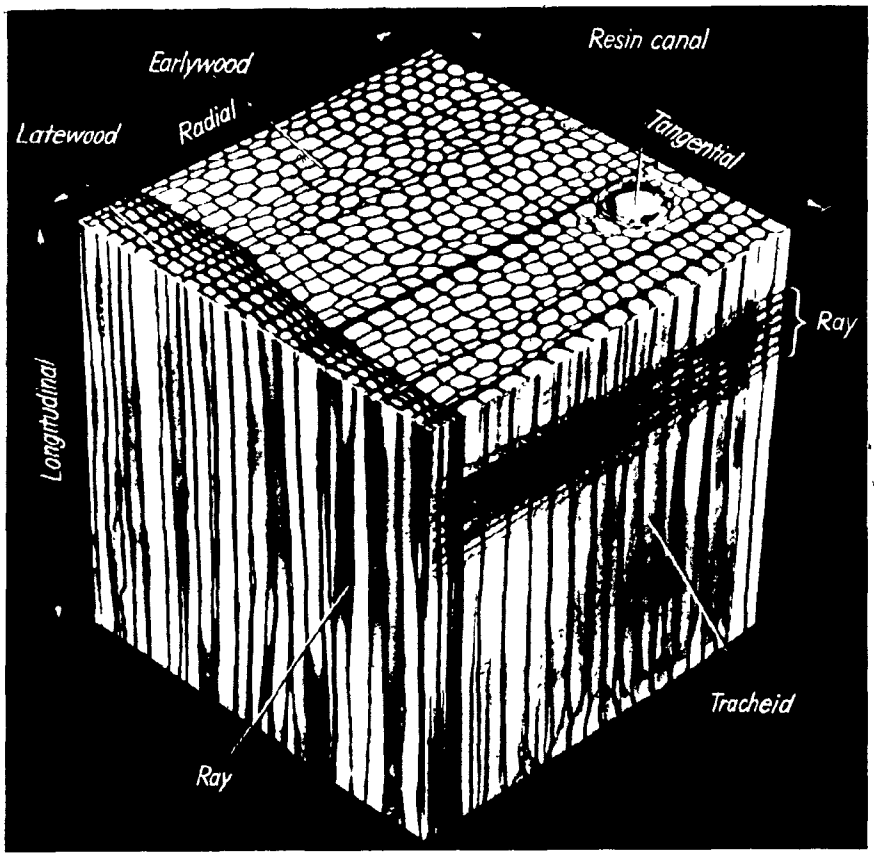


Figure 2. Softwood cube

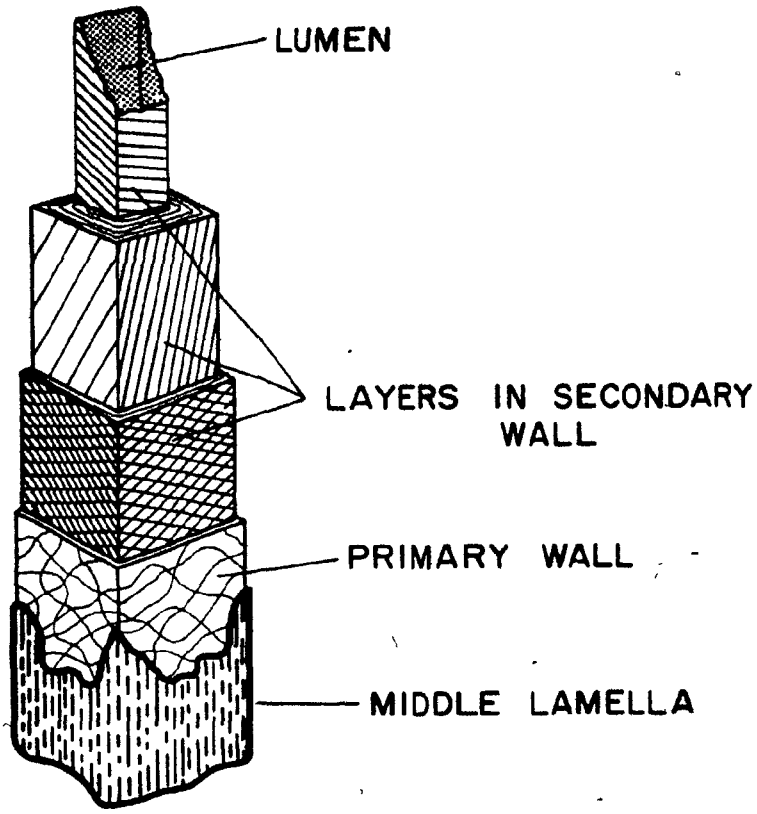


Figure 3. Diagram of a tracheid.

The primary wall lies outside the S_1 layer and consists of randomly oriented cellulose microfibrils embedded in an amorphous matrix of lignin and hemicellulose. The middle lamella region is amorphous and separates one tracheid from another. The structure of the cell wall has been described in greater detail by Wardrop and Dadswell[28].

The relative thicknesses of these layers have been measured for softwood tracheids and were found to be: primary wall, 7 - 14%, S_1, 5 - 11%; S_2, 74 - 84%; and S_3, 3 - 4%[29]. The middle lamella is about the same thickness as the primary wall, being about 0.1 μm[30,31]. The middle lamella, plus the two primary walls on either side, are often taken together and called the compound middle lamella.

E. Distribution of Tracheid Components

The distribution of lignin in wood cells was first studied by ultraviolet microscopy[32,33]. More recently, the techniques of ultraviolet microscopy have been made quantitative by Goring and co-workers[34-37]. Table 1 shows the distribution of lignin in the earlywood of black spruce as determined by this method[35].

The results show that the concentration of lignin in the middle lamella is considerably higher than in the secondary wall. However, because the secondary wall makes up a much larger proportion of the total cell volume, the majority of the lignin is found in the secondary wall.

Little work has been done on the distribution of the polysaccharides in the cell wall[38]. The most often quoted work on the subject

Table 1

The distribution of lignin in black spruce earlywood tracheids[15].

Tissue Type	Tissue Volume (%)	Lignin (% total)	Lignin Concentration ($g \cdot g^{-1}$)
Secondary Wall	87.4	72.1	0.225
Middle Lamella	8.7	15.8	0.497
Middle Lamella (cell corner)	3.9	12.1	0.848

was done by Meier[39]. Meier noted a decrease in relative cellulose and glucomannan contents on going from the secondary wall to the compound middle lamella. He also noted that the relative amounts of galactan and arabinan were highest in the middle lamella while the relative glucuronoarabinoxylan content was largest in the S_1 layer. More recently, Hardell and Westermark[40] studied the carbohydrate distribution in spruce tracheids. Their results showed smaller differences in the relative amounts of the polysaccharides in the middle lamella and secondary wall than were found by Meier[39].

LIGNIN CHEMISTRY

In earlier years chemists believed that wood was a chemical entity, that is, a pure compound[25]. It was not until 1838 that Anselme Payen[41-46] first performed a crude separation of wood into different components. He succeeded in isolating a uniform compound later called "cellulose" and a by-product, different from cellulose, which he termed "matière incrustante" (incrusting material). This material had a higher carbon and hydrogen content than did the cellulose. Payen tried to isolate this material, but failed.

Much more work was done on wood[25], mostly in an attempt to isolate pure cellulose, but little more was learned about lignin for many years. It was not until well after the first pulp mills using wood as a raw material were built, that any large advances came in the study of lignin. In 1893 Klason[47] produced the first of his classic publications on lignin. He suggested that a phenylpropane derivative of

the coniferyl type might be the basic parent substance of lignin. He also proposed a method for determining lignin content. The sample was treated with 72% sulfuric acid to dissolve the carbohydrates, leaving the lignin as a precipitate which could be weighed. The Klason analysis is still the standard method for lignin determination.

For all the work that had occurred up to then, no one had ever succeeded in isolating lignin from wood in a pure unaltered state. Much structural information on lignin was obtained from Klason lignin, dissolved lignins from pulping, etc., but methods were needed in which lignin could be prepared in a state near to that of the lignin in wood.

Other methods were then developed for the isolation of lignin from wood. They included dissolution of the carbohydrate with hydrochloric acid (Willstätter lignin); dissolution of the carbohydrate with cuprammonium hydroxide (Freudenberg lignin); dissolution of the lignin with boiling dioxane in the presence of hydrochloric acid (dioxane lignin); dissolution of the carbohydrate with periodic acid (periodate lignin); dissolution of the lignin with dimethyl sulphoxide and sulphur dioxide (dimethyl sulphoxide lignin); and others[48]. However, all of these methods have the fault that the isolated lignins are irreversibly altered from the natural state[49].

However, two methods have been developed which have proven very useful to lignin chemists over the years. In 1939 Brauns[50] extracted black spruce wood with ethanol at room temperature. The purified product was found to possess all the chemical properties hitherto associated with lignin although only a small percentage of the total lignin

in the wood could be obtained in this manner. Brauns' native lignin was used by many workers for many years in studies of lignin structure[49,50]. However, Brauns' lignin represents only the very low molecular mass portion of the lignin which did not completely polymerize during the formation of the cell. It is extremely doubtful if the results obtained from Brauns' lignin can be extrapolated to determine the structure of the bulk lignin in wood[49].

The most widely used lignin preparation in recent years has been milled wood lignin, made by the method of Björkman[51]. The wood is ground in toluene in a vibrating ball mill for 48 h. The sample is dried and extracted with dioxane and water. This method usually dissolves about 50% of the lignin. The soluble lignin has the advantage to lignin chemists that degradation of the lignin has been minimized. However, in a cross-linked polymer such as lignin, small chemical changes can cause vast changes in the properties of the compound. The problem is best stated by Lai and Sarkanen in "Lignins"[49].

"Remarkably, some of the exaggerated generalizations made in the interpretation of data on Brauns' lignins are now being repeated, in a somewhat modified form, in studies on the milled wood lignin (MWL) preparations. It is true that the milling process that precedes the isolation of these preparations does not cause profound chemical modification, but it is equally true that some modification does occur. It has again been tacitly (although not explicitly) assumed that the 30 - 50% of lignin isolated in MWL preparations represents a structural average of the total lignin."

Other structural information on lignin has been obtained from work on synthetic lignins. These lignins are made by the action of a dehydrogenase enzyme on 1-(4-hydroxy-3-methoxyphenyl)-1-propene-3-ol (coniferyl alcohol)[48].

Although much information on bonding between structural units and on the biosynthesis of lignin is available, the information may not be directly applicable to the structure of lignin in wood.

Since it is not possible to isolate pure unchanged lignin from wood, some researchers have attempted to study the lignin while it is still in the woody matrix[32-37]. As noted earlier, Goring and co-workers[34-37] have measured the distribution of lignin in wood cells by ultraviolet microscopy[34-37]. Later the ultraviolet microscopic technique was used to measure the free phenolic hydroxyl content of the lignin in wood cells. Yang and Goring[52] found that there are approximately twice as many free phenolic hydroxyl groups in secondary wall lignin as in middle lamella lignin. This difference was confirmed by Hardell et al[53]. In a later paper, Yang and Goring[54] showed that the moles of phenolic hydroxyl groups per phenylpropane unit (C_9) for secondary wall lignin was 0.12 while the value for middle lamella lignin was 0.06. These phenolic hydroxyl contents are considerably lower than the value for milled wood lignin (0.20)[54].

The discovery of a difference in phenolic hydroxyl content between middle lamella and secondary wall lignin was an important step in lignin science. For the first time a quantitative difference in lignin structure was found in the two morphological regions of the cell.

Earlier a difference had been noted, again by ultraviolet microscopy, in the rates at which the lignin from the secondary wall and middle lamella were extracted during pulping[55-58]. This effect was termed the "topochemical effect". In 1967, Procter et al[55] showed

that, for both kraft and acid-sulphite pulping, the lignin in the secondary wall dissolved faster than the lignin in the middle lamella. Results of further work[56-58] showed that this phenomenon occurred in other pulping media as well. Only acid-chlorite pulping showed no topochemical preference for lignin dissolution from the secondary wall. The topochemical effect may be related to the difference in phenolic hydroxyl content[52-54], a difference in the degree of crosslinking[59], other chemical differences not as yet found, or to physical effects[56]. Unfortunately, due to the nature of pulping experiments it is difficult to distinguish between the effect of chemical differences in the lignin in the two regions and the influence of physical factors such as the rates at which the degraded lignins diffuse from the fibre wall into the pulping liquor. Few experiments, designed to resolve this problem have, as yet, been performed. It is, therefore, not known if there are chemical differences other than phenolic hydroxyl content between the lignin in the middle lamella and secondary wall (e.g., methoxyl or carbonyl contents). Likewise the reactivities of middle lamella and secondary wall lignin to specific reagents, such as chlorine, bromine or bisulphite, have not been studied.

PURPOSE OF WORK

Although a large amount of work has been done over the years on lignin chemistry and on the differences between the components of the various morphological regions of the wood cell, there remain numerous questions to which answers have not been possible. They include:

A. How do the amounts of the various polysaccharides vary in the middle lamella and secondary wall regions?

B. Are the results obtained by ultraviolet microscopy, with respect to the phenolic hydroxyl contents of middle lamella and secondary wall lignin, correct?

C. Are there any other chemical differences between the structures of middle lamella and secondary wall lignin?

D. Is there a difference in the reactivities of middle lamella and secondary wall lignin?

E. Are the results obtained by ultraviolet microscopy, with respect to the topochemical effect during pulping, correct?

F. How similar are isolated lignins to the lignin in wood?

G. Is milled wood lignin representative of the whole lignin in wood?

In general, answers to the above questions have tended to be educated guesses based on data from experiments not designed specifically to answer the questions. The purpose of the work described in this thesis is to develop a method for the isolation of enough tissue from the middle lamella and secondary wall to enable experimental answers to the fundamental questions posed above.

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CHAPTER II

FRACTIONAL SEPARATION OF MIDDLE LAMELLA AND SECONDARY WALL TISSUE FROM BLACK SPRUCE WOOD

ABSTRACT

Differential sedimentation of finely ground wood has been used to isolate fractions of black spruce (*Picea mariana*) in which the lignin content varied from 20 to 65 percent. The fractions of low lignin content consisted mainly of secondary wall tissue while those of high lignin content came predominantly from the compound middle lamella.

INTRODUCTION

It is becoming increasingly apparent that the chemistry of wood polymers is not uniform but varies in different morphological regions of wood. Most of the evidence for this has been microscopic[1-6]. Few attempts have been made to isolate wood components from well-defined morphological regions. In 1936, Bailey[7] isolated 0.5 mg of middle lamella lignin by a method so laborious that no other researcher has attempted to repeat the work. In 1961 Meier[8] studied the distribution of polysaccharides in birch fibres by removing layers of cells near the cambium at different stages of maturation. By a chemical peeling technique, Luce[9] determined the radial variation in content of hemicellulose and cellulose in softwood fibres.

By differential extraction of lignin and chemical characterization of the fractions, Kolar et al[10] have studied the distribution of syringyl and guaiacyl residues in birch lignin. Lindstrom and Nordmark[11] noted that the fines from unbleached kraft pulps contain up to 50% lignin and that these fines contain fragments of cell corner which are responsible for the high lignin content. However this cell corner lignin is probably altered during the kraft cook.

Recently Hardell et al[12] have developed a method of separating wood particles into fractions of fibres, ray cells and fines which contain secondary wall, ray cell and middle lamella lignins, respectively. They applied the method to both spruce[12] and birch wood[13] and detected certain chemical differences in the lignins from the different morphological regions which confirm earlier results obtained by microscopic

methods[3,5,6]. The method developed by Hardell et al[12] involved collecting fractions of thermomechanical pulp of various particle sizes and manually separating the particles by rotation in a liquid on a watch glass. The main disadvantage of this method is that the yield is low.

The purpose of the work described in the present chapter is to develop a simple and reproducible method of preparing decigram scale fractions of wood tissue from the middle lamella and secondary wall of black spruce. The method is based on the difference in densities between lignin and carbohydrate material. The wood is ground to a fine powder, suspended in a separation medium and the suspension is centrifuged. The lignin is of lower density ($\rho \approx 1.4$ g $\cdot$ mL^{-1}) than the carbohydrates ($\rho \approx 1.5$ g $\cdot$ mL^{-1})[14]. If the proper density of the separation medium is chosen, particles from the middle lamella which will be high in lignin content will float and the other particles will sink. Conversely, the density of the medium may be set so that the particles from the secondary wall, which will be lowest in lignin content, will sink while all others will float.

One of the weaknesses of the previous work by Hardell et al[12,13] was the ambiguity in their results for lignin concentration. In the present work, the concentration of lignin is determined by four different methods. In addition, the fractions obtained are examined microscopically and characterized by ultraviolet and infrared spectroscopy.

EXPERIMENTAL

Preparation of Wood Flour

Freshly cut logs of black spruce (Picea mariana) were debarked by hand. The green wood was chipped in two stages. In the first a No. 24 Appleton Chipper was used which gives wood chips approximately 5 cm square by 0.5 cm thick. These chips were then rechipped in a Mead Chipper, Size 2, giving chips about 1 cm long, 0.3 cm wide, and 0.1 cm thick. These chips were then air dried for five days and ground to fine flour-like material in a Bauer No. 730 Hurricane Pulverizer (Figure 1). The Hurricane Pulverizer has refiner-type grinding action and incorporates a recycling system for particles which are too large. Material to be processed is fed into the mill near the bottom of the grinding chamber by a screw feeder driven by an automatically controlled variable speed drive. A controlled flow of air is passed through the grinding zone fluidizing the product, lifting the fines and near-fines up into the classifier zone where the oversize material is rejected and/or returned to the grinding chamber for further reduction. The fines passed by the classifier move into the integral fan chamber and are pneumatically conveyed to the dust collecting system, usually a cyclone followed by a constant back pressure dust filter.

The resulting wood flour was about the same color as the original spruce wood and contained many small fibres and fines with no large pieces of wood (Figure 2).

The wood flour was then extracted with acetone for 16 h. The lignin content of the dry extractive free wood flour was found by the Klason technique to be $26.8 \pm 0.2\%$[15].

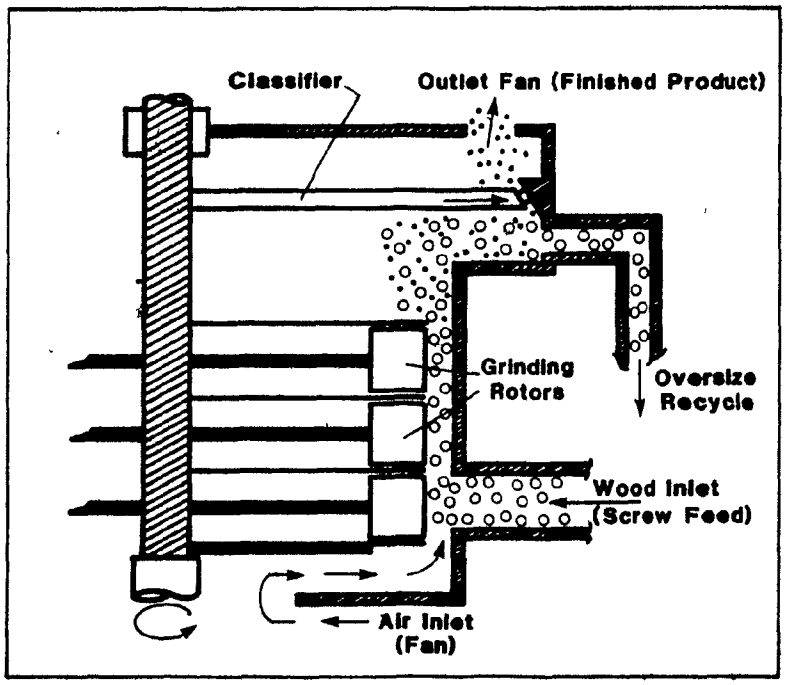

Figure 1. Hurricane Pulverizer (cross section).

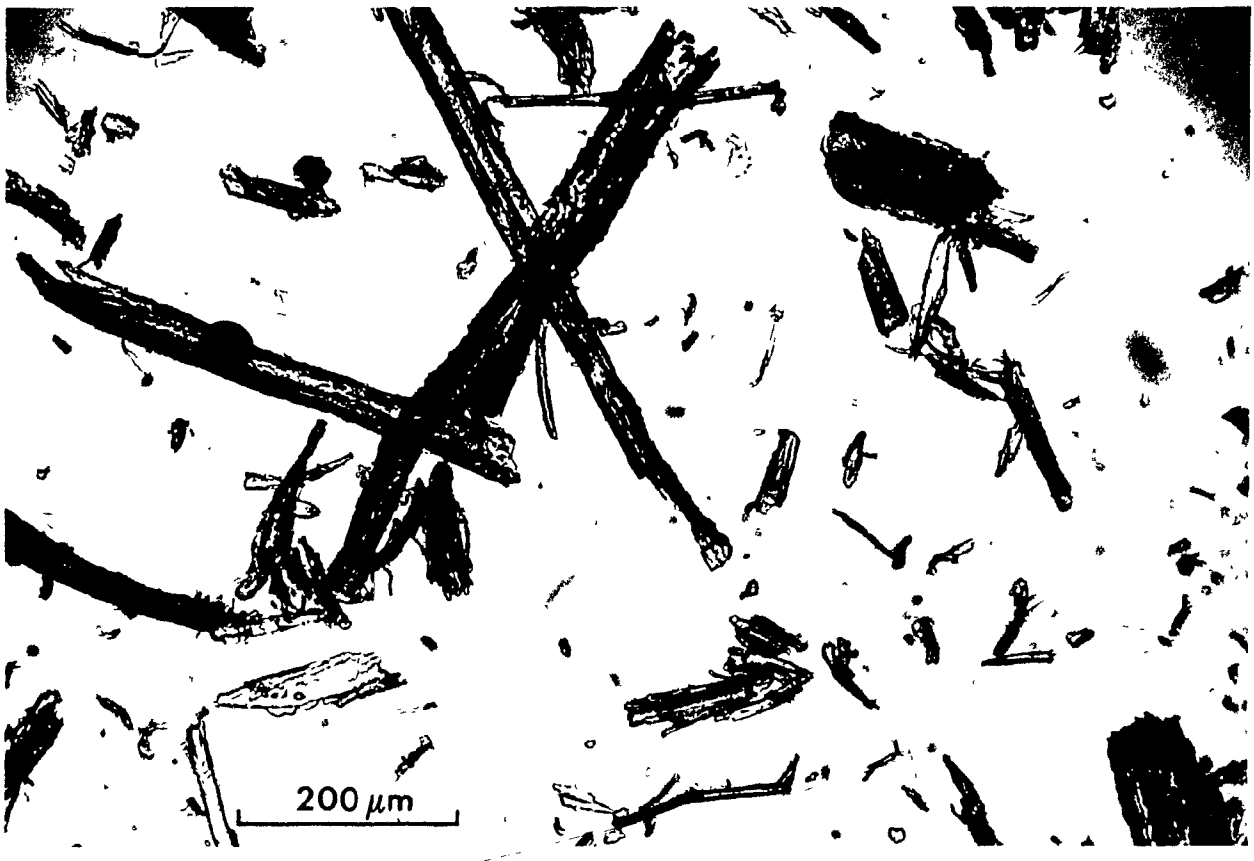

Figure 2. Photomicrograph of extractive free wood flour.

Approximately 30 g of dry wood flour was then placed in a 21 cm diameter sieve containing a 500-mesh screen. The hole size of the screen was 25 μm. The sieve was covered and placed on an Eberbach horizontal shaker for 15 min at full speed. The powder that passed the screen, approximately 30% of the original wood flour, was collected and saved as the working material.

Fractionation

The 500-mesh wood flour was a pale cream color and contained no large fibrous pieces. Approximately 2 g of the wood flour were placed in a 250 mL jar and 100 mL of separation liquid was added. The separation liquid was a mixture of carbon tetrachloride (tetrachloromethane) and 1,4-dioxane adjusted to give the desired density. The density was measured gravimetrically in a calibrated volumetric flask.

The resulting mixture was then placed in an MSE Ultrasonic Power Unit for five minutes. The unit is tuneable from 18 000 to 20 000 s^{-1} and has an output of 60 W. The mouth of the vessel was sealed with Parafilm to prevent the escape of vapors from the vessel as the solution warmed up on ultrasonication. The vessel was removed, covered, and cooled to room temperature. The wood flour was well dispersed. However the particles flocculated after standing for periods over 1 h.

The mixture was then transferred by pipette to centrifuge tubes (Figure 3) specially designed to allow the collection of a small amount of floating material without stirring up material at the bottom of the tube.

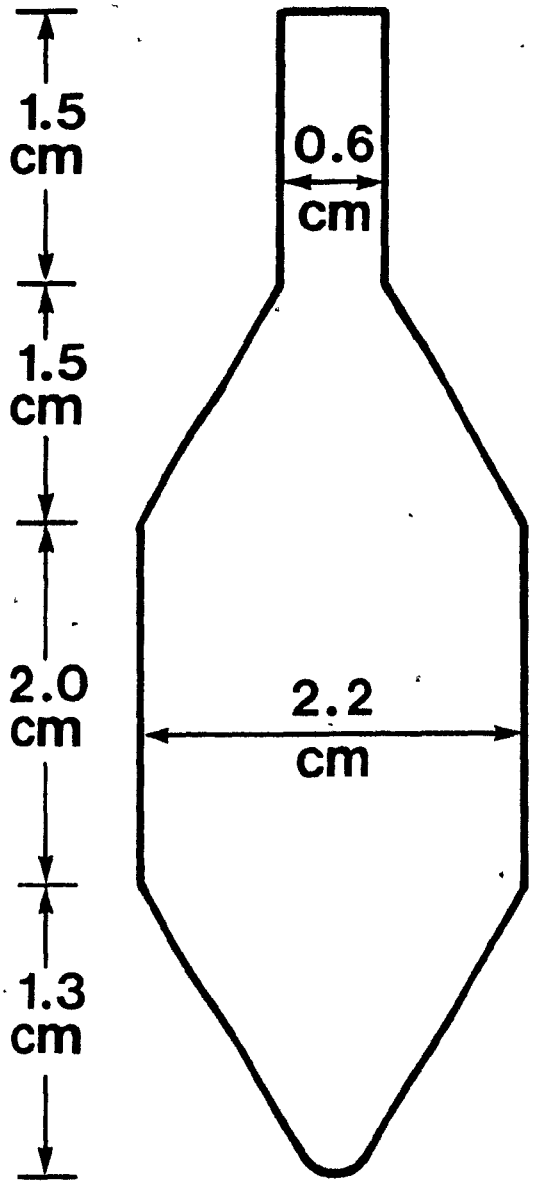

Figure 3. Cell used to collect fractions of wood material.

The tubes were covered, placed on a suitable swing bucket rotor (SW-25.1), and spun in a Beckman Model L Ultracentrifuge for fifteen minutes at 10,000 rpm at room temperature and under vacuum.

After centrifugation the tubes were carefully removed and the floating material collected by pipette. The sample was placed in a sample vial and the solvent evaporated in a vacuum oven at 60° C. In the isolation of samples of secondary wall lignin, the same procedure was followed except that the fraction which sank during centrifugation was collected.

The most important factor in obtaining reproducible samples was found to be the maintenance of constant density in the separation medium. The density of the carbon tetrachloride:dioxane mixture varied considerably with temperature; it was essential that the experiments be carried out in a room with a temperature variation of no more than plus or minus 2° C, with less variation preferable.

Measurement of Lignin Content

The measurement of the lignin content of the isolated samples was carried out by four methods: a micro-Klason technique, infrared absorption, solid state ultraviolet absorption, and ultraviolet absorption of samples dissolved in acetyl bromide (ethanoyl bromide) and acetic acid (ethanoic acid).

The micro-Klason technique was basically the same as the usual Klason analysis[15] except that samples as small as 1 mg were analyzed. From one to ten milligrams of extractive free wood tissue were weighed

accurately in a 10 mL boiling flask. For each milligram of wood, 0.015 mL of 72% sulfuric acid was added to the wood using a microburet. The wood was thoroughly mixed with the acid using a glass stirring rod and the mixture allowed to stand at 20° C for 2 h. Enough water was added to make a 3% sulfuric acid solution and the mixture was refluxed for 4 h. It was important that the condenser used for the refluxing be the same scale as the boiling flask as a significant amount of water in the form of droplets was found to stick to the inside of a large condenser raising the acid concentration above 3%. After refluxing, the mixture was cooled and filtered with care taken to ensure that all the material was transferred to the filter. Selas silver filters (0.8 μm) were used for the filtration. It was important that the water and sulfuric acid be filtered at least 3 times to ensure that small insoluble particles did not interfere with the results. After the lignin was filtered it was washed with 1 L of distilled water and dried in a vacuum oven at 60° C for 24 h. The lignin was then weighed. As a control in the micro-Klason technique a sample of black spruce with a known Klason lignin content was analyzed. The results agreed with the accepted value to within $\pm$ 2%.

For the infrared analysis the absorbance at 1510 cm^{-1} due to the stretching of aromatic carbon-carbon bonds was used. One to two milligrams of sample was pressed in a pellet with KCl and the peak height at 1510 cm^{-1} was measured in the same manner as the method of Kolboe and Ellefsen[16]. The absorbance (A) was calculated from the transmittance readings where $A = \log I_o/I$ (Figure 4). Samples of black spruce wood

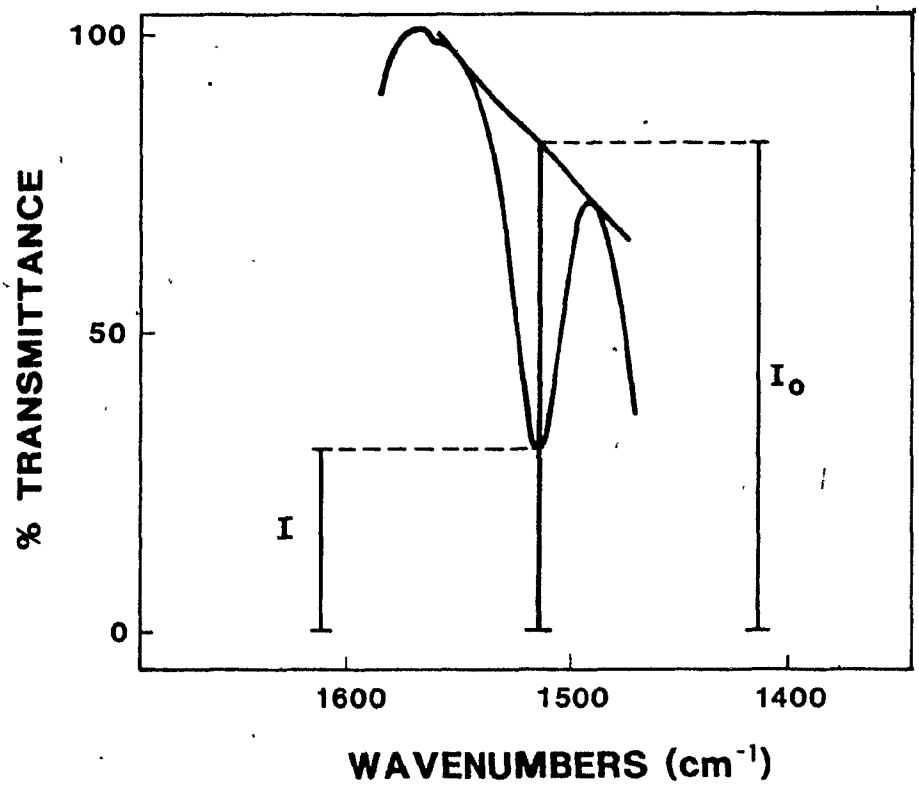

Figure 4. Baseline method of calculating absorbances from infrared peaks.

of varying mass were used to calibrate the technique. The method was found to be reproducible to within $\pm$ 1.5%.

For ultraviolet measurements in the solid state the method of Bolker et al[17] was adopted. The finely divided sample was pressed in a KCl pellet, similar to the type used for infrared spectroscopy. Pellets containing from 0.1 to 2.0 mg of tissue were prepared. The absorbance at 280 nm was measured for each sample and Beers law was found to be obeyed in this range. Black spruce of known Klason lignin content was used as a control. This technique provided a fast, convenient method for measuring lignin contents, on very small samples, with a reproducibility of $\pm$ 2%.

The lignin contents of the tissue fractions were also measured by the method of Johnson et al[18] in which the wood sample is dissolved in acetyl bromide and acetic acid. One to three milligrams of sample were placed in a reaction vessel with 2 mL of 25% acetyl bromide in acetic acid. The vessel was placed in an oil bath at 70° C. The method calls for a reaction time of 30 min but it was found that for samples high in middle lamella lignin, not all the lignin dissolved after this period. For this reason a reaction time of 2 h was used. With black spruce wood as a control it was possible to measure the lignin content of the various tissue fractions with a reproducibility of $\pm$ 1%. It is interesting to note that the long reaction time produced a higher absorptivity for black spruce lignin (ε = 27.3 cm^{-1} g^{-1} L) than found after 0.5 h reaction time (ε = 23.5 cm^{-1} g^{-1} L).

As discussed in the next section of the chapter, the agreement between the four methods was good. The three spectroscopic methods were

much more convenient to use than the micro-Klason technique. Therefore on the tissue samples of the secondary wall, only the spectroscopic methods were used.

Microscopy

Wood fractions were photographed microscopically on a Reichert Nr 235 980 light microscope using a Nikon F 35 mm camera with Kodak PX 135-36 black and white film.

RESULTS AND DISCUSSION

The fractionation of black spruce wood was carried out at various densities and the lignin contents of the fractions obtained are given in Table 1. As shown in Table 1, there is good agreement between the lignin contents measured by the different methods, the maximum deviation being $\pm$ 3%.

In Figure 5, the average values of lignin content and yield are plotted against the density of the separation medium. There was a gradual increase in lignin content of the floating fraction as the density was decreased from 1.460 to 1.420 g $\cdot$ mL^{-1}. This was as expected. However, it is interesting to note that the lignin content decreases below a separation density of 1.420 g $\cdot$ mL^{-1}. If lignin was the only low density component, the lignin content of the samples should increase until a density was reached at which nothing floated. The maximum in Figure 5 suggests that there is another component (or components) of wood which

Table 1

Lignin Analyses and Yields for Various Fractions of Black Spruce

Density of Separation ($g \cdot mL^{-1}$)	Sample Floats or Sinks	Klason Lignin (%)	Infrared Lignin (%)	UV Pellet Lignin (%)	Acetyl Bromide Lignin (%)	Average Lignin (%)	Yield (%)
1.459	Floats	31.8	31.2	29.7	30.1	30.7 ± 1.0	61.5
1.448	Floats	37.1	36.8	34.4	33.2	35.4 ± 1.9	22.0
1.439	Floats	41.3	43.2	37.0	40.5	40.5 ± 2.6	8.3
1.435	Floats	44.3	42.1	43.6	42.1	43.0 ± 1.1	4.6
1.430	Floats	40.8	42.2	39.2	37.9	40.0 ± 1.9	1.9
1.425	Floats	45.6	45.0	38.9	42.6	43.0 ± 3.0	0.5
1.423	Floats	50.3	48.3	49.5	48.3	49.1 ± 1.0	0.5
1.420	Floats	52.4	52.1	50.7	51.1	51.6 ± 0.8	0.4
1.415	Floats	-	44.6	46.9	42.3	44.6 ± 2.3	0.3
1.410	Floats	-	36.4	35.7	31.4	34.5 ± 2.7	0.2
1.459	Sinks	-	20.8	20.8	21.7	21.1 ± 0.5	2.3
1.455	Sinks	-	22.4	22.3	22.8	22.5 ± 0.3	26.9
1.450	Sinks	-	26.0	24.9	25.0	25.3 ± 0.6	52.1
1.440	Sinks	-	27.7	25.7	25.3	26.2 ± 1.3	78.0

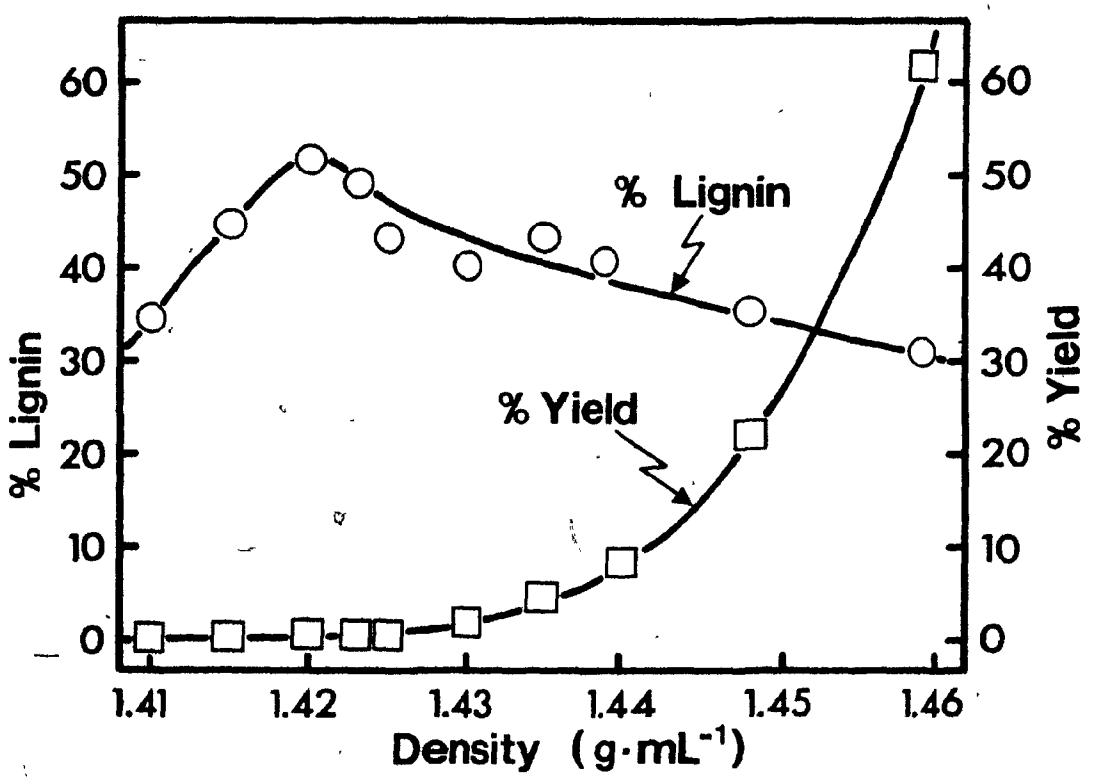

Figure 5. Plot to show lignin concentration and yield of floating tissue fractions collected at various densities of the separation medium.

is of density low enough to interfere with the isolation of the middle lamella lignin. There seems to be only a very small amount of this material and so it interferes significantly only at low densities where the quantity of tissue in the fraction is small. The nature and origin of this light, non-lignin substance is unknown but it could have been derived from some relatively minor wood component such as cutin[19]. It should be noted that in Table 1, Klason lignin contents are not shown for samples isolated at densities lower than 1.420 g · mL^{-1}. This is because the non-lignin material interfered with the Klason test and gave a result which was too high.

The presence of the light non-lignin substance made necessary an extra step in the isolation procedure for the middle lamella tissue. A sample with a lignin content of 52% which had been separated at a density of 1.420 g · mL^{-1} was reseparated at densities from 1.410 to 1.418 g · mL^{-1}. Fractions containing 55 - 65% lignin were obtained in the sediment. The floating fraction had a lignin content of about 35 to 45%. This result indicated that the light non-lignin material was not bound to the middle lamella fragments and could be separated by flotation.

It is interesting to note that Fergus *et al*[3] found the lignin content of the compound middle lamella to be 50% and that of the true middle lamella to be 85%. The tissue fraction with a lignin content of 65% must have contained a considerable proportion of the true middle lamella.

Optical microscopy confirmed that the particles of the high lignin content fraction indeed came from the middle lamella. Figure 6 shows

the middle lamella tissue under bright field and crossed polarized light. It can be seen from the photomicrograph in bright light that the particles are thin and flat, which is the shape of the middle lamella in wood. In the crossed polars, the image of the particles is virtually invisible indicating the amorphous nature of the material. It should be noted that some of the middle lamella particles have small holes in them, due to the bordered pits structure of the fibres. Bordered pits in wood fibres, seen through crossed polars (Figure 6 (inset)), give the characteristic maltese cross image due to the circumferential alignment of the cellulose microfibrils around the pits. Such maltese crosses were completely absent when holes in the middle lamella tissue were viewed through crossed polars because the oriented cellulosic material was not present.

In Figure 7 the lignin content and the yield of the sinking fractions are plotted against the density of the separation medium. It can be seen that at a density of 1.461 $g \cdot mL^{-1}$ a sample containing only 21% lignin was isolated. Fergus et al found the lignin concentration in the secondary wall to be 22%[3]; this fraction must therefore contain virtually all pieces of secondary wall.

In the microscope, the tissue fractions which sank were found to contain few or no fragments of middle lamella. Figure 8 shows a sample containing 21% lignin. In contrast to the material shown in Figure 6, it resembles the fragments of a fibre wall. Under crossed polars most of the particles give a bright image indicating the presence of cellulose. Note the presence of maltese crosses around the bordered pits.

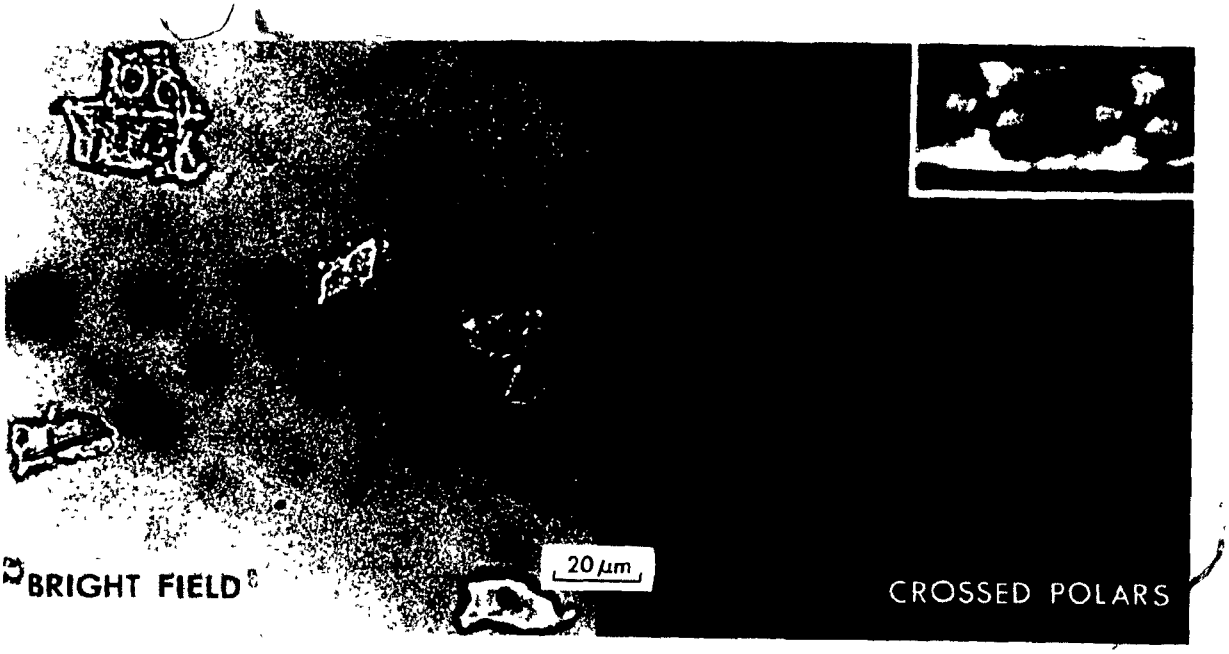

Figure 6. Middle lamella particles in bright field and cross polarized light. The inset in the upper right hand corner shows bordered pits in a fibre fragment under cross polarized light.

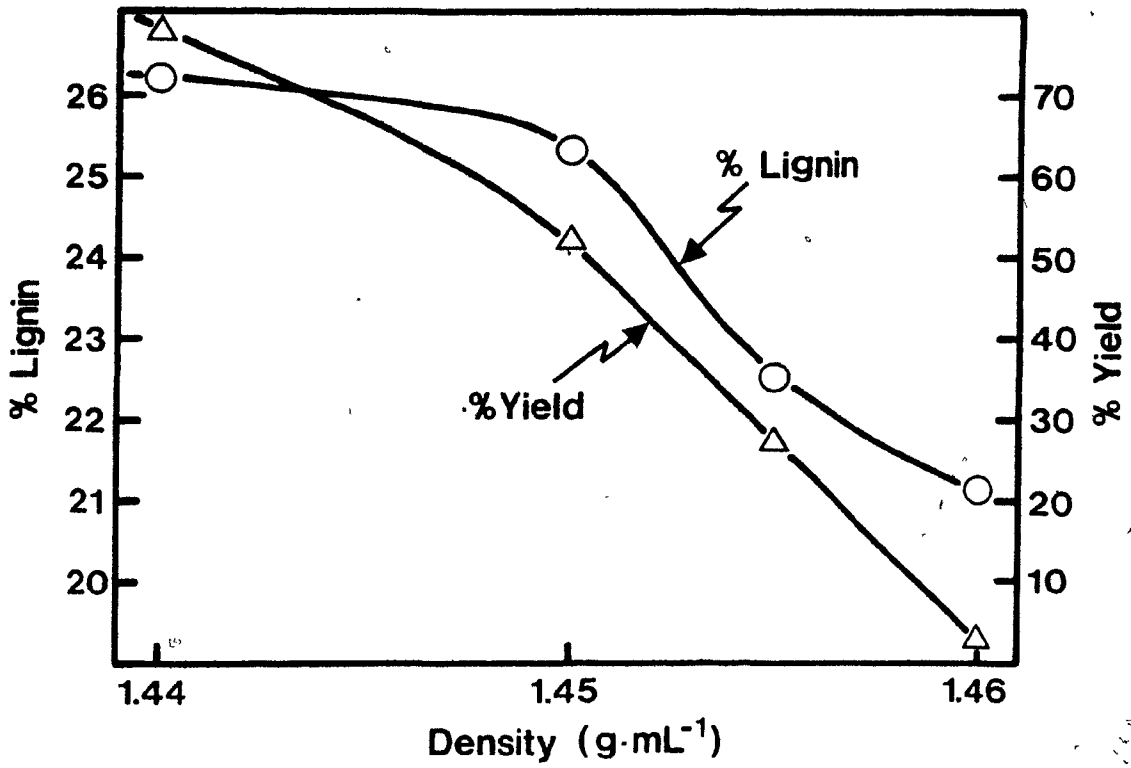

Figure 7. Plot of lignin content and yield of sinking tissue fractions at various densities of the separation medium.

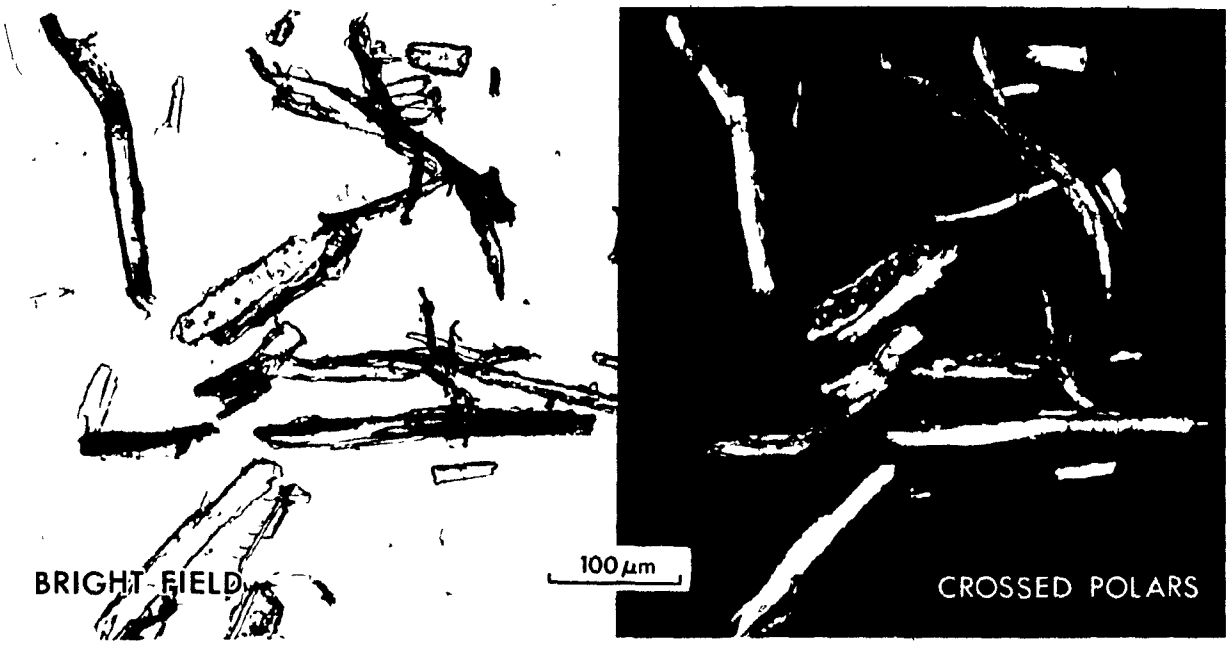

Figure 8. Secondary wall particles in bright field and cross polarized light.

The floating tissue fractions which contained a high percentage of lignin were further characterized by infrared and ultraviolet spectroscopy. The infrared spectra (Figure 9) showed a prominent peak at 1510 cm^{-1} (the aromatic carbon-carbon stretching frequency) which is characteristic of all lignins. The basic overall spectrum was very similar to that of other lignin preparations, especially subtraction spectra of lignin in wood[16]. The ultraviolet spectrum of the middle lamella sample (Figure 10) showed absorption bands at 205 and 280 nm, characteristic of lignin.

In conclusion, it has been shown that the separation of wood into fractions of high and low lignin content is possible by taking advantage of the difference in density between lignin and carbohydrate material. It has also been shown that the fractions high in lignin content originate in the middle lamella, and those low in lignin content originate in the secondary wall. These preparations should prove to be very useful to the lignin chemist studying lignin as it exists in the wood, and to lignin chemists studying the differences between middle lamella and secondary wall lignin. Further work is necessary to more fully characterize the lignin and polysaccharide fractions of the tissues. The polysaccharide contents of the tissue fractions will be studied in the next chapter.

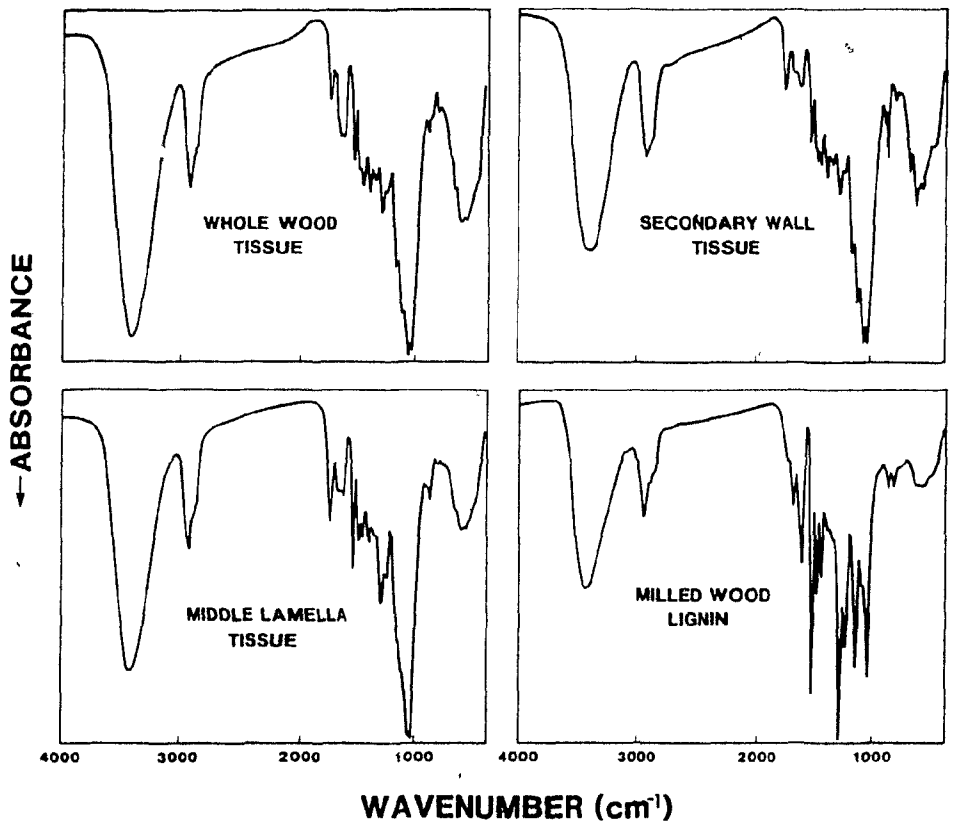

Figure 9. Infrared spectra of secondary wall tissue, whole wood tissue, middle lamella tissue, and milled wood lignin.

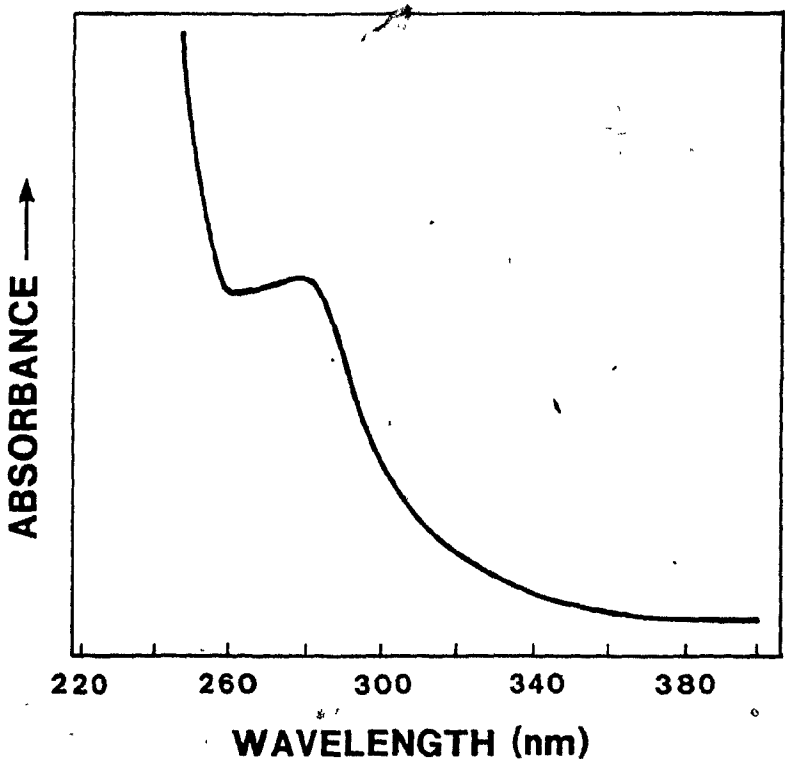

Figure 10. Solid state ultraviolet spectrum of middle lamella tissue (KBr pellet).

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CHAPTER III

THE POLYSACCHARIDE CONTENT OF THE MIDDLE LAMELLA AND SECONDARY WALL OF TRACHEIDS FROM BLACK SPRUCE WOOD

ABSTRACT

Carbohydrate analyses were performed on fractions of tissue from the middle lamella and secondary wall of black spruce tracheids. The concentrations of cellulose and glucomannan were found to be smaller in the middle lamella than in the secondary wall while the concentrations of galactan and arabinan were slightly larger in the middle lamella than in the secondary wall. The concentration of glucuronoarabinoxylan was essentially the same in the middle lamella as in the secondary wall.

INTRODUCTION

Little research has been published on the distribution of polysaccharides in the various morphological regions of wood cells[1]. Such work is difficult, and tends to give inconclusive results.

In 1961, Meier[2] measured the polysaccharide distribution in the cell wall of conifer tracheids by analysis of fractions obtained through microdissection. Fibres of different degrees of maturation were microscopically distinguished and isolated. For an individual polysaccharide in a particular tissue fraction, Meier expressed his results not as the weight concentration but rather as the percentage relative to the overall content of polysaccharide. He found that the relative amounts of cellulose and glucomannan were lower in the compound middle lamella than in the secondary wall. Galactan and arabinan showed the reverse trend, while the glucuronoarabinoxylan was at its highest concentration relative to the overall polysaccharide content in the S_1 layer of the secondary wall. Meier also noted that there was a higher relative percentage of pectic acid in the compound middle lamella than in the secondary wall.

By a chemical peeling technique, Luce[3] studied the radial distribution of the hemicelluloses in softwood fibres. He noted a larger hemicellulose content in the middle lamella of the fibres than in the secondary wall.

More recently, Hardell and Westermark[4] have developed a method for peeling layers of the cell wall from single fibres. Samples as small as 20 ng were analyzed for their carbohydrate contents. Only small differences in the relative amounts of carbohydrates were found

in the various cell layers of spruce tracheids. Most notably, the authors did not find a large reduction in the relative amounts of glucose and mannose in the compound middle lamella, as was noted by Meier[2].

Chapter II describes a new procedure for the separation of centigram quantities of tissue from the middle lamella and secondary wall of black spruce tracheids. In the present chapter, the analyses of the carbohydrate portions of these tissue fractions are described. Results are compared with those of previous workers.

EXPERIMENTAL

Five samples of black spruce (Picea mariana) tissue of varying lignin contents were isolated according to the method in Chapter II. The secondary wall tissue sample contained 22% lignin and the whole wood sample contained 27% lignin. Three samples of middle lamella tissue were prepared, ML-1, ML-2, and ML-3, with lignin contents of 39%, 50%, and 60%, respectively.

The polysaccharides in the tissue samples were hydrolyzed by means of a modified version of the method of Fengel and Wegener[5]. Five milligrams of dry tissue were weighed accurately into a 12 x 100 mm test tube and 1.6 mL of trifluoroacetic acid was added to each sample. The tube was covered with Parafilm and allowed to stand for 48 h at room temperature. The Parafilm was removed and 0.4 mL of H_2O was added. The contents were then frozen using dry ice and acetone, and the tube was sealed under vacuum. The sealed tubes were brought to

room temperature and then heated in a 100° C oven for 2 h. After cooling, the tubes were opened and dried over NaOH in a vacuum dessicator at room temperature. After drying, 1 mL of water was added and the evaporation and drying were repeated. A final cycle of water addition, evaporation, and drying was then performed on the sample. A known amount of myo-inositol was added as an internal standard.

The dried samples were dissolved in 0.8 mL of silylation-grade dimethylsulfoxide and derivatized to trimethylsilyl esters with 0.8 mL "Tri-Sil Concentrate" (Pierce Chemical Co.). The derivatized monosaccharides were separated by gas chromatography using a J & W 30 m fused silica capillary column (0.253 mm I.D.) coated with SE-30). The gas chromatograph was temperature-programmed as follows: 160° C to 220° C at 1.5° C per minute; 220° C for 2 min.

The quantity of a particular monosaccharide in each tissue fraction was obtained by comparing the total area of the appropriate peaks in the chromatogram with the peak area of the internal standard. An example of a gas chromatogram of hydrolyzed and derivatized wood flour is shown in Figure 1.

Each tissue fraction was analyzed at least three times, and the results expressed as grams of monosaccharide per gram of tissue. The reproducibility in measurement was about $\pm$ 4%. The monosaccharide composition of the tissue fractions was also measured by high pressure liquid chromatography. The HPLC data and a brief description of the method are given in Appendix I.

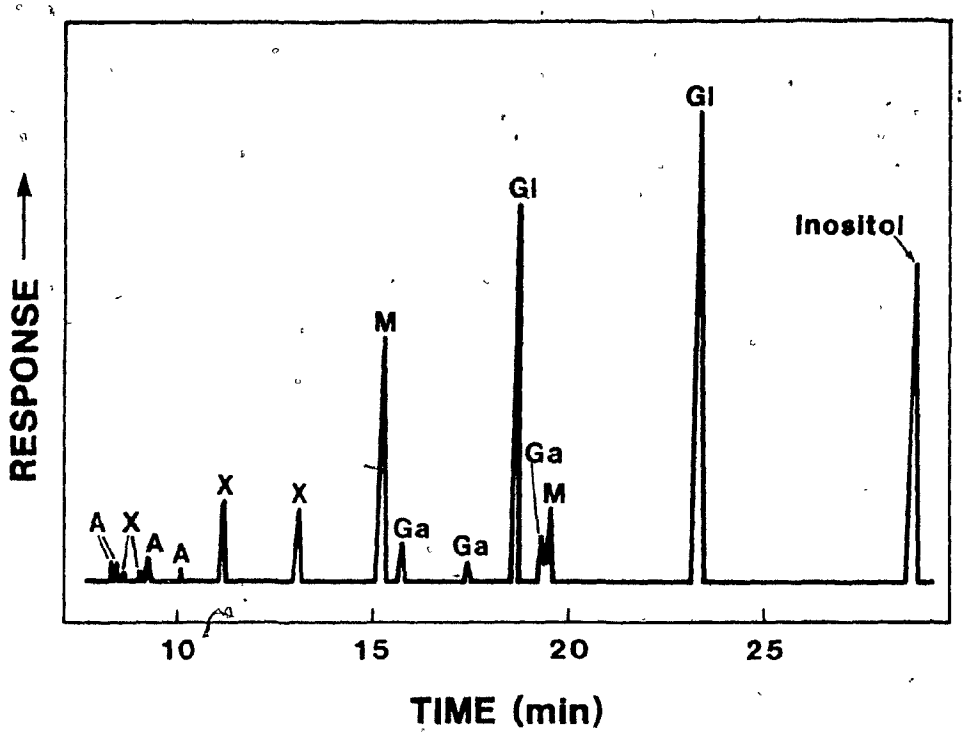

Figure 1. Gas chromatogram of hydrolyzed and derivatized black spruce wood flour. Code: A = Arabinose, Ga = Galactose, Gl = Glucose, M = Mannose, X = Xylose.

RESULTS AND DISCUSSION

The monosaccharide content of each of the tissue fractions is shown in Table 1. The monosaccharide contents were converted to polysaccharide contents by the method of Meier and Wilkie[6]. This method involves a step by step conversion of monosaccharide to polysaccharide contents as follows:

i) Convert all monosaccharide contents to anhydro-sugar contents (multiply monosaccharide contents by 162/180);

ii) assume all galactose is galactan;

iii) all the xylose is present as glucuronoarabinoxylan in the ratio 1.9:1:9 glucose to arabinose to xylose units;

iv) all the mannose is present as glucomannan in the ratio 1:3.5 glucuronic acid to mannose units;

v) the residual arabinose, after subtraction for arabinose present in glucuronoarabinoxylan, is present as arabinan;

vi) the residual glucose, after subtraction for glucose present in glucuronoarabinoxylan and in glucomannan, is present as cellulose.

The total polysaccharide content was then subtracted from the non-lignin content to give a portion called the residue.

The mass concentrations of the polysaccharides and the residue are plotted against the lignin content in Figure 2. Note that the trends obtained do not represent the distribution of components across the cell wall. They arise because each tissue fraction is a mixture of secondary wall, primary wall, and middle lamella in varying proportions. The straight lines allow extrapolation to a composition at which the

Table 1

Lignin and Monosaccharide Contents of Tissue

Fractions Expressed as Grams Component per Gram of Tissue

Component	SW[1]	WW[2]	ML[3]-1	ML-2	ML-3
Lignin	0.22	0.27	0.39	0.50	0.60
Glucose	0.551	0.470	0.361	0.227	0.137
Mannose	0.152	0.156	0.105	0.092	0.054
Xylose	0.066	0.072	0.072	0.076	0.072
Galactose	0.033	0.039	0.044	0.057	0.069
Arabinose	0.020	0.024	0.039	0.046	0.052

[1] SW: Secondary wall tissue

[2] WW: Whole wood flour

[3] ML: Middle lamella tissue.

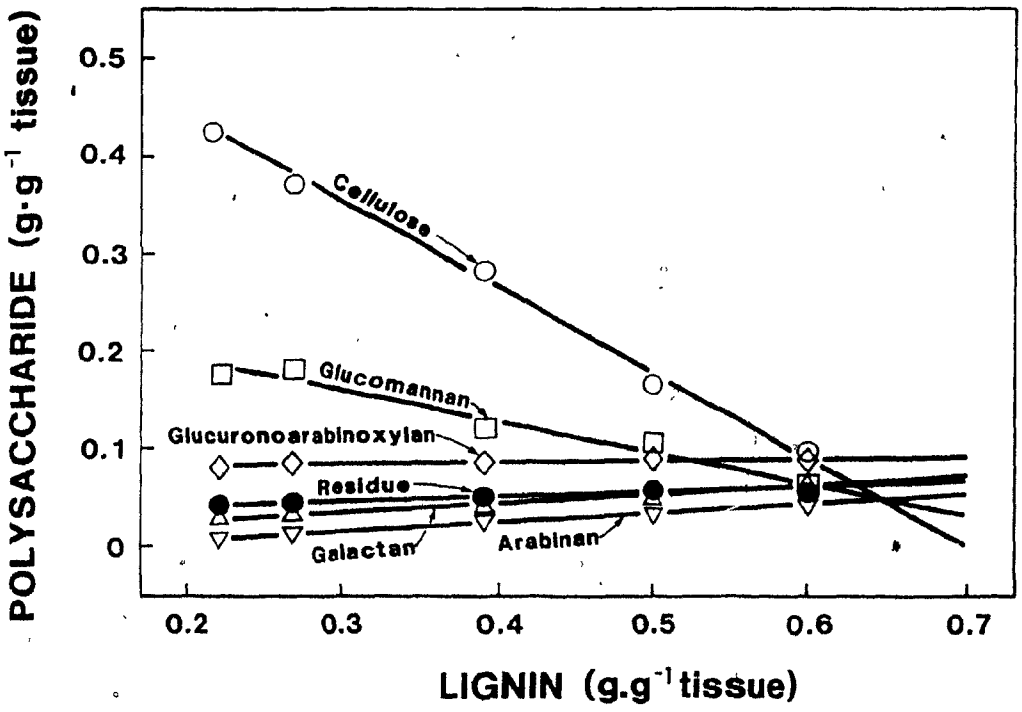

Figure 2. Plot of the polysaccharide contents of the tissue fractions against the lignin contents of the tissue fractions.

cellulose content becomes zero. At a lignin content of 70%, the extrapolated polysaccharide contents must be a fairly good representation of the polysaccharide contents of the true middle lamella.

Figure 2 shows that the contents of cellulose and glucomannan are much lower in the middle lamella than in the secondary wall. The content of glucuronoarabinoxylan is essentially the same in the middle lamella and secondary wall, while the contents of galactan and arabinan are higher in the middle lamella than in the secondary wall.

The residue content in the middle lamella is slightly higher than in the secondary wall. It should be noted, however, that the residue makes up 20% of the non-lignin fraction when the lignin content is extrapolated to 70%, while it makes up only 5% of the non-lignin fraction of the secondary wall tissue. Meier[2] noted that there was a larger proportion of pectic substance in the polysaccharide moiety of the middle lamella than in that of the secondary wall. Perhaps the unidentified residue noted in the present work contains a large proportion of pectic material.

In Table 2, the proportions of the individual polysaccharides, as percentages of total polysaccharide content, are given for the lignin-rich fractions. Included for comparison are the previous results of Meier[2] for the compound middle lamella. Also included are data for the compound middle lamella calculated from the monosaccharide composition given by Hardell and Westermark[4]. Meier's data seem to fit the present results for glucuronoarabinoxylan at 39% lignin content, cellulose and galactan at 50-60% lignin, and glucomannan and arabinan at > 70% lignin.

Table 2

Relative Polysaccharide Percentages of the Lignin-rich Tissue Fractions

Polysaccharide	Meier[1] (2)	Hardell, Westermark[1] (4)	Present Work			
			ML-1 (39% Lignin)	ML-2 (50% Lignin)	ML-3 (60% Lignin)	70% Lignin[2]
Cellulose	33.4	50.3	50.8	37.1	27.5	0.0
Glucomannan	7.9	22.6	21.6	23.7	18.0	12.5
Glucurono-arabinoxylan	13.0	13.3	15.4	20.2	24.9	37.5
Galactan	16.4	7.6	7.2	11.4	18.0	29.2
Arabinan	29.3	6.2	5.0	7.6	11.6	20.8

[1] For the compound middle lamella

[2] Extrapolated data from Figure 1.

These anomalies cannot be explained. In contrast, the results of Hardell and Westermark correspond well with the present data at 39% lignin content. The quantity of material obtained by these authors[4] for their compound middle lamella fraction was too small for a lignin determination[8]. However, their disintegrated wood fines, with a lignin content of 41%[7], had about the same polysaccharide composition as their middle lamella fraction[8], in agreement with the data from the present work.

The results from the carbohydrate analyses of the secondary wall are given in Table 3. Also included in Table 3 are data derived from the literature. Meier[2] did not report an analysis for the entire secondary wall. However, he did give data for the whole cell and for the compound middle lamella. This allowed a calculation of the polysaccharide content of the secondary wall using a proportion of 90% secondary wall and 10% middle lamella for the whole wood[9].

The data in Table 3 are in reasonable accord for the three investigations. The agreement between the results of Hardell and Westermark and those of the present work is particularly good. This is not unexpected since the secondary wall of the fibre is probably the tissue region most readily prepared in a pure state and most easily analyzed.

It should be noted that both Meier, and Hardell and Westermark worked with white spruce (Picea glauca) whereas black spruce (Picea mariana) was used in the present investigation. It is not expected that this species difference will invalidate the comparisons made above.

Table 3

Relative Polysaccharide Percentages of the Secondary Wall Tissue

Polysaccharide	Meier[1] (2)	Hardell and Westermark (4)	Present Work
Cellulose	63.0	58.1	60.0
Glucomannan	23.1	20.8	23.7
Glucurono-arabinoxylan	12.8	14.3	10.7
Galactan	1.1	4.8	4.1
Arabinan	0.0	2.0	1.5

[1] Calculated from data in reference 2.

CONCLUDING REMARKS

The results show clearly that the amounts of cellulose and glucomannan in the middle lamella of black spruce are small. These two polysaccharides account for nearly all of the difference between the carbohydrate contents of the middle lamella and secondary wall. The contents of other polysaccharides change little on going from the secondary wall to the middle lamella. These trends suggest that during the formation of the wood cell, the glucuronoarabinoxylan, galactan, and arabinan are produced at a fairly constant rate. In contrast, the rate of formation of cellulose, glucomannan, and lignin seem to vary considerably during the various stages of cell growth.

Significant differences between the polysaccharide contents of the middle lamella and secondary wall were noted in this chapter. The next three chapters will deal with other differences in the chemical compositions of the two morphological regions. Particular emphasis will be placed on differences between the lignin in the two regions.

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CHAPTER IV

A SPECTROPHOTOMETRIC METHOD FOR THE ANALYSIS OF PHENOLIC HYDROXYL GROUPS IN THE LIGNIN IN WOOD

ABSTRACT

A new method, based on the $\Delta\varepsilon$ technique, was developed for the analysis of phenolic hydroxyl groups in wood tissue. The method involves turbidimetric absorbance measurements of finely ground samples suspended in buffers. For lignin from the secondary wall, middle lamella and whole wood of black spruce, the $PHOH/C_9$ ratios were, respectively, 0.12, 0.05 and 0.08, in good agreement with the results previously obtained by ultraviolet microscopy.

INTRODUCTION

The measurement of free phenolic hydroxyl groups is important to the characterization of lignin preparations. Phenolic hydroxyl groups cause chemical activity at the ortho and para positions of the aromatic ring and therefore a knowledge of their abundance may lead to the understanding of the differences in chemical reactivity between various types of lignin.

Over the years, numerous chemical and physical techniques for the analysis of the phenolic hydroxyl content of lignins have been developed[1]. However, all these methods suffer from an inability to determine the phenolic hydroxyl content of insoluble samples such as the lignin in wood. In 1978, a new approach to the problem was developed by Yang and Goring[2]. These authors used ultraviolet microscopy to determine the ratio of free phenolic hydroxyl groups in the lignin of the secondary wall and middle lamella by measuring the pH-induced change in absorptivity of the lignin at 300 nm according to the method of Aulin-Erdtman[3]. This work was carried further by the use of milled wood lignin as a standard to calculate the absolute values of the phenolic hydroxyl content of the lignin in the secondary wall and middle lamella of black spruce wood[4]. However, there were some problems with the procedure, the most pronounced being in the lack of precision of the measurement of $\Delta\varepsilon_{300}$ with the ultraviolet microscope, as discussed by Yang and Goring[4].

The purpose of the present work was to develop a method for the measurement of the phenolic hydroxyl content of the lignin in finely

ground samples of wood tissue. An adaptation of the $\Delta\varepsilon_{300}$ method was applied to the wood flour, suspended in aqueous buffers and the results obtained compared with those found previously by UV microscopy.

THEORY

When the absorbance (A_0) of a sample of lignin suspended in an aqueous buffer is measured there are three contributions to the observed value. They are: 1) the actual absorbance of the suspended lignin (A_{SL}), 2) the apparent absorbance due to scattering (A_S), and 3) the absorbance due to a small amount of dissolved lignin (A_{DL}). Thus

$$A_0 = A_S + A_{SL} + A_{DL} \qquad (1)$$

A_{DL} and A_0 are easily measured. A_S can be obtained by measuring the apparent absorbance due to scattering (A_S') at a wavelength where the lignin does not absorb. This value can then be related to the scattering at the experimental wavelengths by

$$K_S(A_S') = A_S \qquad (2)$$

in which K_S is a correction factor obtained from a suspension of non-absorbing scattering material such as powdered cellulose. Thus A_{SL} can be determined from equation 1.

The absorptivity (ε_{SL}) is given by

$$\varepsilon_{SL} = \frac{A_{SL}}{C\ell} \qquad (3)$$

in which C is the lignin concentration and ℓ the path length of the cell.

NEW METHODS FOR THE CHEMICAL SYNTHESIS OF RIBONUCLEOTIDES AND THEIR ANALOGUES

A Thesis

by

© *Mona J. Nemer*

Submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

McGill University

Department of Chemistry

Montreal, Quebec, Canada

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October 1981

SYNTHESIS OF RIBONUCLEOTIDES AND THEIR ANALOGUES

.... life is indeed darkness save when there is urge,
And all urge is blind save when there is knowledge,
And all knowledge is vain save when there is work,
And all work is empty save when there is love;

Kahlil Gibran
"The Prophet"

To my parents, John and Wadad Nemer

whose support has been constant,
whose understanding has been great,
and whose love has been infinite.

Chemistry Ph.D.

NEW METHODS FOR THE CHEMICAL SYNTHESIS OF RIBONUCLEOTIDES AND THEIR ANALOGUES

by

Mona J. Nemer

ABSTRACT

The synthesis of 5'-monomethoxytrityl-2'-tert-butyldimethylsilyl-3'-levulinylribonucleosides of the four common bases as well as their 2',3' isomeric derivatives was accomplished. These units are suitable for block condensations via the phosphite procedure as demonstrated in the preparation, in good yield, of a hexadecauridylic acid.

A novel solid phase synthesis of ribonucleotides featuring the use of the phosphite procedure, along with a derivatized silica gel polymer, was fully investigated. The efficiency of the method is exemplified in the stepwise assembly of a nonadecamer corresponding to the dihydrouridine loop of $t\text{-}RNA_f^{Met}$. Several mild reagents capable of hydrolyzing nucleotides from the polymer were introduced. In addition, a new non-aqueous oxidation of phosphite triesters using meta-chloroperbenzoic acid was examined.

The isolation and properties of oligonucleoside phosphites is reported and their use in nucleotide synthesis described. Versatile routes to several nucleotide analogues modified at the phosphate linkage are suggested. The interactions of novel analogues with various enzymes are analyzed.

Using the phosphite procedure, symmetrically linked (5'-5', 3'-3', 2'-2') diribonucleoside monophosphates of uridine were easily prepared. The stability of these dimers to acid, base and enzyme hydrolysis is discussed.

Finally, the use of the levulinyl moiety as a new N-acyl protecting group for nucleosides is proposed.

Chimie Ph.D.

NOUVELLES METHODES POUR LA SYNTHESE CHIMIQUE DE RIBONUCLEOTIDES ET DE LEURS ANALOGUES

par

Mona J. Nemer

RESUME

On décrit la synthèse des monométhoxytrityl-5'-tert-butyldiméthylsilyl-2' lévulinyl-3' ribonucléosides des quatres bases communes ainsi que les dérivés isomères 2',3'. Ces unités permettent la synthèse d'oligonucléotides par blocs suivant le procédé de couplage au chlorophosphite. La méthode est illustrée par la préparation, en bon rendement, de l'acide hexadécauridylique.

Une nouvelle synthèse de ribonucléotides en phase solide fut étudiée. Le support utilisé est un gel de silice et le procédé de phosphorylation est celui du chlorophosphite. On met en évidence l'efficacité de la méthode en préparant, par étape, le nonadécamère correspondant à la boucle dihydrouridine du t-RNA$_f^{Met}$. On introduit aussi plusieurs réactifs capables d'hydrolyser les nucléotides du polymère. De plus, on examine une nouvelle oxidation nonaqueuse des phosphites de trialkyles à l'aide de l'acide métachloroperbenzoique.

On rapporte également l'isolement et les propriétés d'oligonucléoside phosphites ainsi que leur utilité pour la synthèse d'oligoribonucléotides. On suggère aussi des routes versatiles qui permettent l'obtention de nombreux analogues de nucléotides modifiés au lien phosphate et on analyse l'interaction de ces molécules avec plusieurs enzymes.

Les diribonucléosides monophosphates de l'uridine ayant un lien symmétrique (5'-5', 3'-3', 2',2') furent facilement préparés grâce à la méthode de couplage au chlorophosphite. La stabilité de ces dimères quant à l'hydrolyse en milieu acide, basique et enzymatique fut aussi étudiée.

Enfin, on propose l'utilisation du groupement lévulinyle pour la protection de la fonction amine des nucléosides.

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TABLE OF CONTENTS

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Tout ce que réalise l'homme tourne autour d'un seul axe: l'homme lui même.

Mikhaël Nuaymah
"Le Crible"

INTRODUCTION

In 1961, Nirenberg and Matthaei discovered that polyuridylic acid (poly U) stimulates the synthesis of poly L-phenylalanine in a cell free E.coli system[1]. This was the first in a series of dramatic experiments with synthetic polyribonucleotides which led in 1966 to the complete unravelling of the genetic code. The synthesis of all 64 possible triribonucleotides by Khorana and his co-workers[2] contributed significantly to this important development[3].

Since then, researchers have found several uses for synthetic polyribonucleotides in the area of genetic engineering. Tennant and his co-workers[4] reported that poly U and poly A inhibit leukemia viruses in cell culture and Torrence and DeClerq[5] found that synthetic double stranded RNA's are among the most potent interferon inducers. Synthetic ribonucleotides have also made possible studies on the structure-function relationship of t-RNA and have potential use in the enzymatic assembly of proteins[6]. Many other biological studies await the development of efficient procedures for the chemical synthesis of polyribonucleotides of defined sequences.

The importance of ribonucleotides arises from the fact that ribonucleic acids (RNA) are essential cellular components[7]. RNA synthesis accounts for one-third of all macromolecular synthesis in the cell and most of the RNA is involved in protein

synthesis. In addition, some ribonucleotides function as metabolic regulators or as energy carriers in biological systems (e.g. ATP); others are substrates for various enzymes or components of numerous coenzymes (e.g. NADH, FADH).

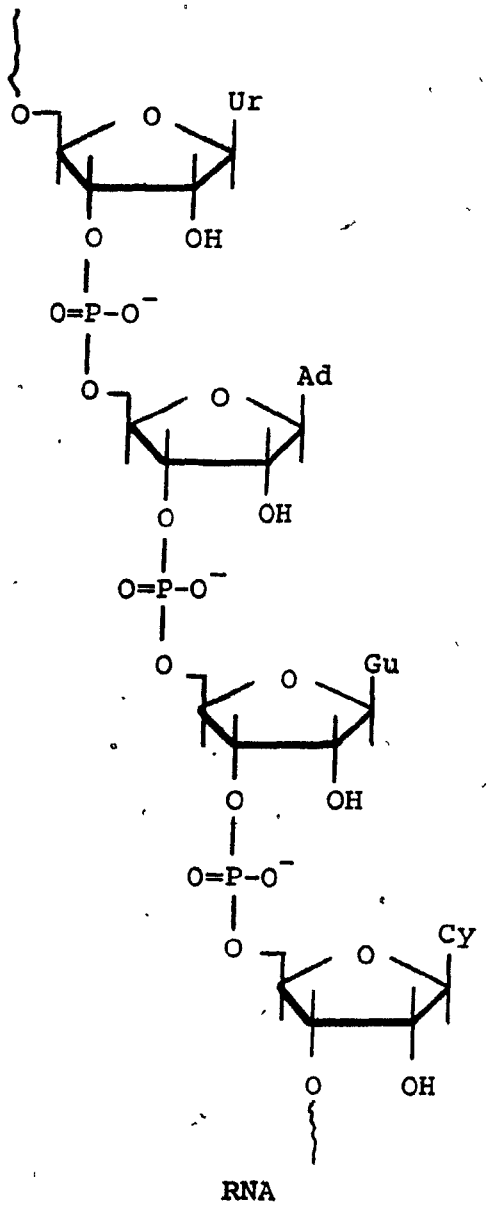
In view of the key role played by RNA in these biological processes, the chemical preparation of ribonucleotides and their analogues has generated a lot of interest in recent years.

CHEMICAL SYNTHESIS OF POLYRIBONUCLEOTIDES

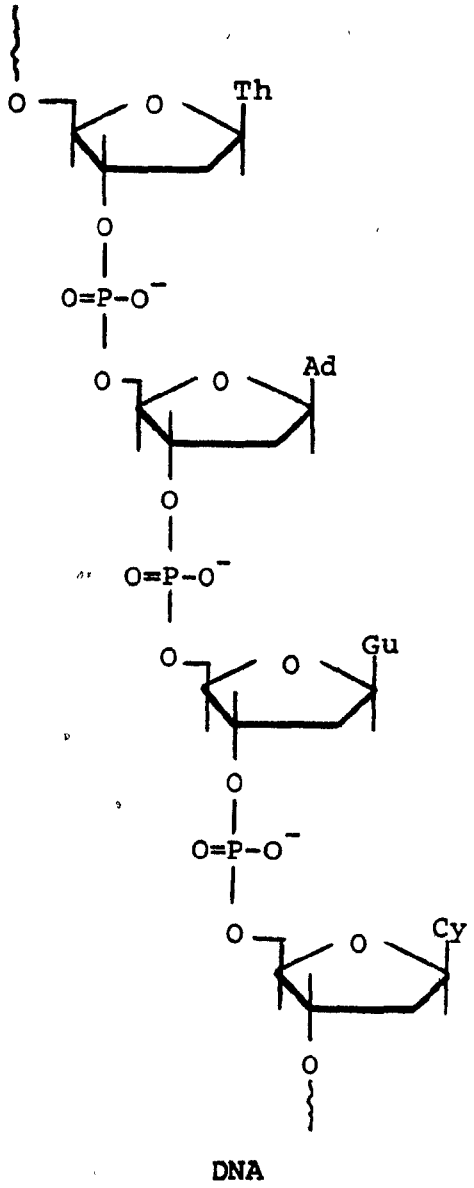
Although significant progress has been made in the synthesis of deoxynucleotides, allowing the assembly of biologically important DNA fragments[8-10], no development of equal magnitude has been reported in the ribonucleotide area. The presence of the 2'-hydroxyl in ribonucleosides profoundly influences the chemistry of RNA and complicates the synthesis of 3'-5' linked polyribonucleotides (Fig. 1).

Solution Phase Synthesis

The first successful chemical synthesis of a 3'-5' linked oligoribonucleotide was achieved only 20 years ago by Khorana and his co-workers[11]. They prepared uridylyl-(3'-5')-uridine (UpU) and uridylyl-(3'-5')adenosine (UpA) using a phosphodiester approach and dicyclohexylcarbodiimide (DCC) as



RNA

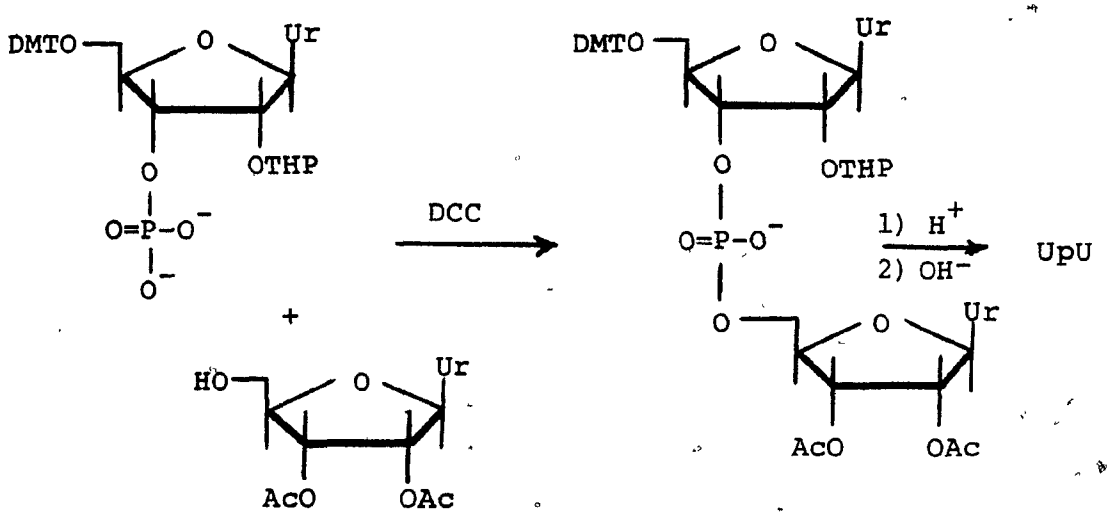


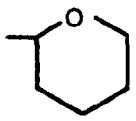
DNA

Figure 1

the condensing agent (Scheme 1). The Khorana group later applied the same procedure to the chemical preparation of the 64 possible triribonucleotides[2].

SCHEME 1



THP = 

Since then, other groups have used the phosphodiester method to prepare oligoribonucleotides either stepwise[12,13] or following a block condensation approach[14,15].

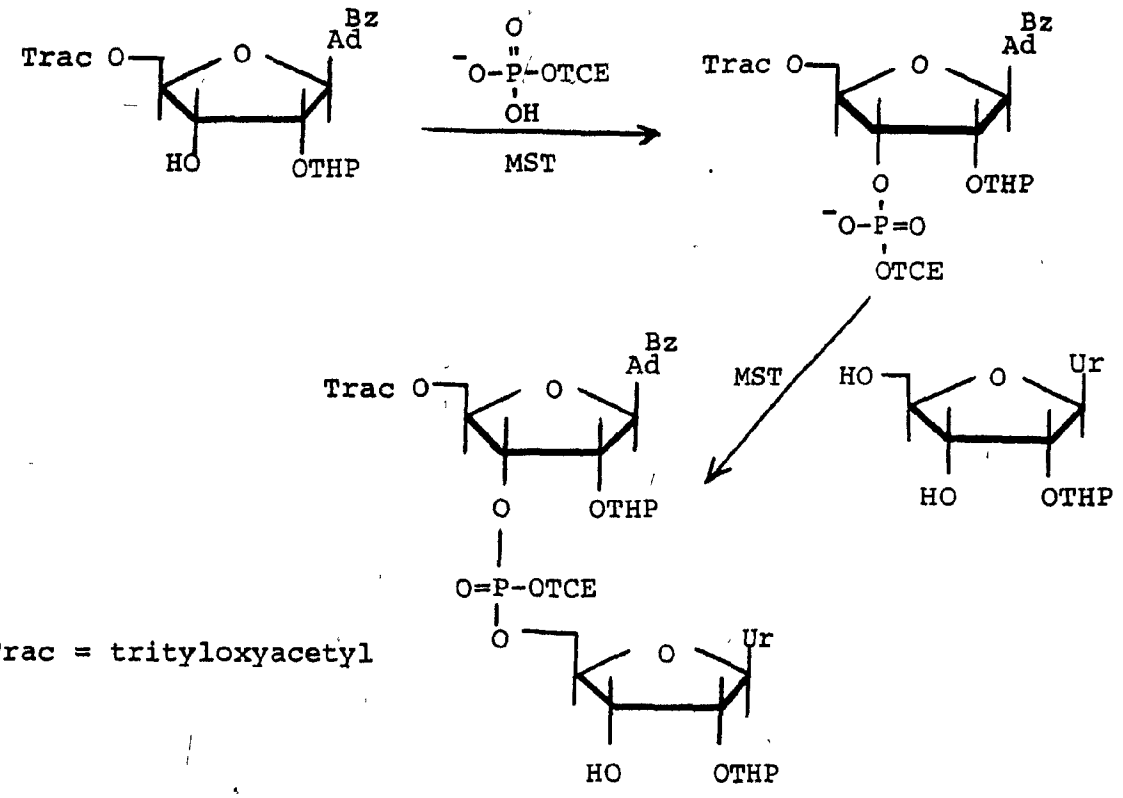
But it was soon realized that the diester method suffers from inherent disadvantages that are due to the nature of the internucleotide phosphodiester linkage. For example, the nucleophilic

phosphate moiety was found to participate in side reactions during phosphorylation resulting in the formation of pyrophosphates. As the chain length increased, the side reactions became more probable and higher oligomers were always obtained in low yield. The longest ribonucleotide prepared by this method was a nonamer isolated in 8% yield from the coupling of a trimer to a hexamer [15]. In addition to taking part in side reactions, the phosphodiester bridge decreases the solubility of the molecules in organic solvents; this in turn creates significant complications, during the isolation of the product.

In an attempt to eliminate these problems, Letsinger and Ogilvie[16], and Eckstein and Rizk[17], reintroduced the phosphotriester method initially used by Michelson and Todd in 1955[18]. Neilson[19] was the first to adapt this method to the synthesis of 3'-5' linked ribonucleotides (Scheme 2). Subsequently, van Boom and his co-workers[21] and Smrt[22] investigated stepwise triester synthesis involving extension of the nucleotidic chain from either the 3' or 5' end.

Using the procedure shown in Scheme 2, Werstiuk and Neilson tried the block condensation approach. By coupling a tetramer to a pentamer they obtained a nonamer in 48% yield[20]. The condensing agent was mesitylenesulfonyl 1,2,4-triazolide (MST) and the total reaction time was 18 days. A further aspect of this synthesis was the fact that the 3'-OH had to be left unprotected to allow extension of the chain in the

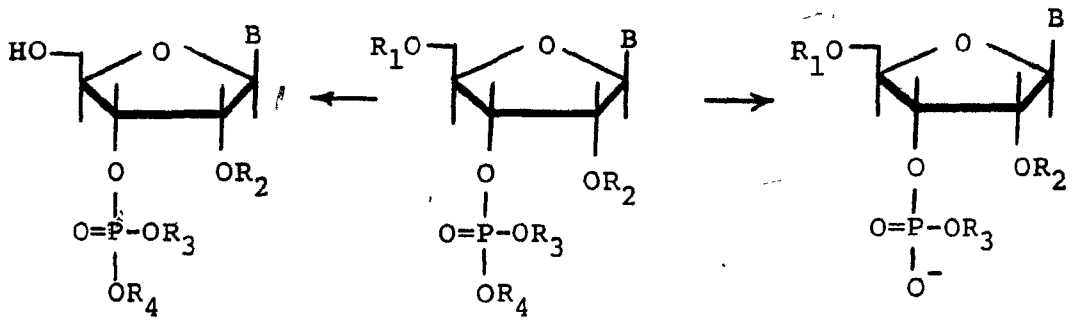
SCHEME 2



3' direction.

The next development in the synthesis of oligonucleotides came in 1973 with the introduction, by Catlin and Cramer[23] and Narang and co-workers[24], of the "modified triester approach". A few years later, van Boom and co-workers[25] reported the first synthesis of an oligoribonucleotide using this improved triester technique (Scheme 3).

The key intermediate in this approach is the fully protected mononucleotide 1 with the terminal phosphotriester moiety. Compound 1 can be functionalized at the 5' position by



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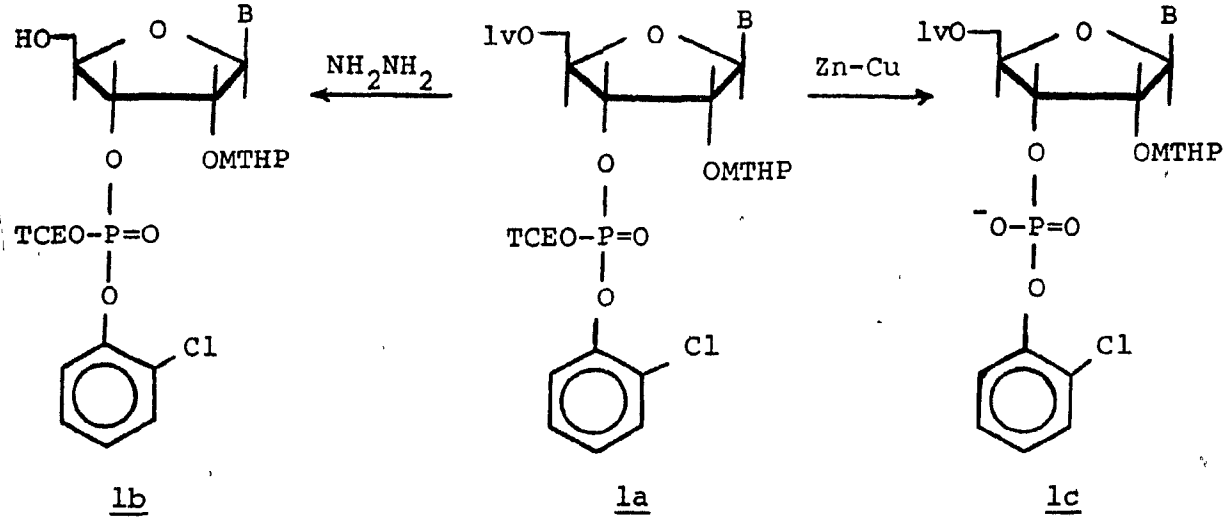
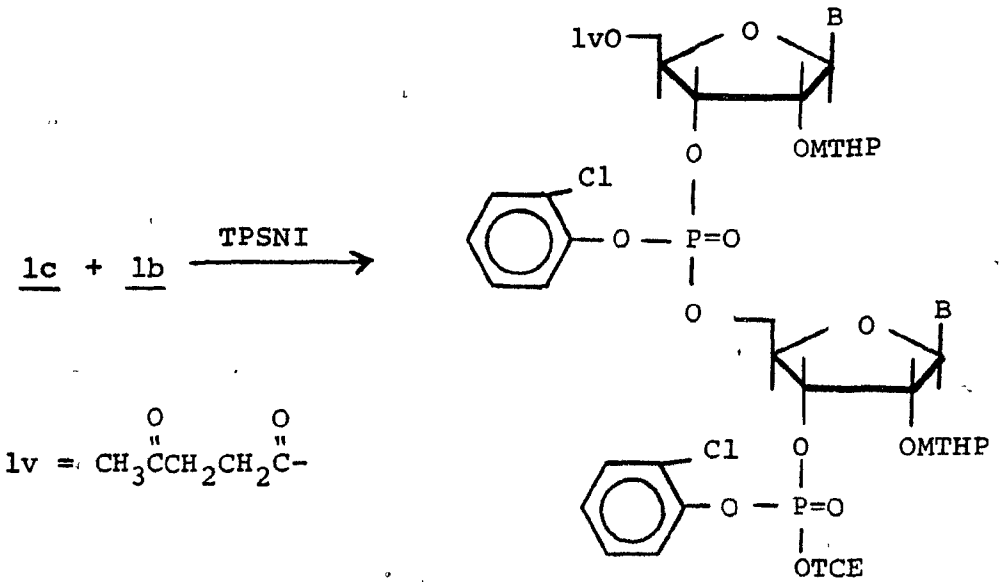
SCHEME 3



1b 1a 1c



1v = $CH_3\overset{O}{\overset{\|}{C}}CH_2CH_2\overset{O}{\overset{\|}{C}}-$

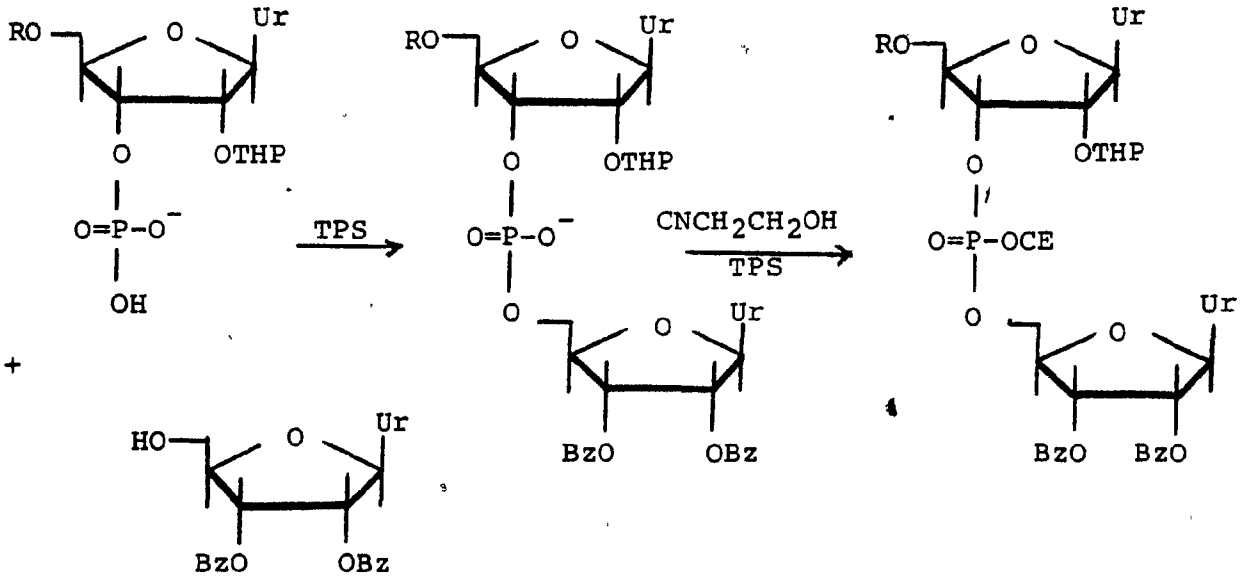
removal of the masking group and at the 3' end by selectively hydrolyzing one of the phosphate esters.

Following the procedure outlined in Scheme 3, van Boom and co-workers added a dimer to a tetramer using 2,4,6-triisopropylbenzenesulphonyl 4-nitroimidazolide (TPSNI) as a coupling agent. The reaction time was 2 days and the yield was 70%[26]. Subsequently, they prepared a tetradecamer by coupling a tetramer to a decamer (4:1 ratio) using the same procedure; the yield was 58% after a 3.5 day reaction[27].

Similarly, Wiwiorowski and co-workers[28] combined a trimer and a tetramer using triisopropylbenzenesulphonyl tetrazolide (TPST). The resulting heptamer was obtained after 8 h in 58% yield. Ikehara et al. have also used the modified triester approach to couple a trimer to a tetramer with TPS to yield a heptamer (30% yield) after a 72 h reaction[29]. The same group recently reported the block synthesis of a decamer starting with a trimer and a heptamer. The condensing agent used was mesitylenesulfonyltriazolide (MST), the reaction time was 4 days and the yield was 27%[30]. Reese et al.[31] very recently prepared a decamer by adding a trimer to a heptamer; they used mesitylene sulfonyl nitrotriazolide and relatively short reaction times. However, due to formation of side products, the isolation of pure fully protected decamer was not possible and the yield could only be estimated at 55%.

Smrt tried to combine both diester and triester approaches in the stepwise synthesis of a tetrauridylic acid[32] (Schême 4). His reasons were based on the observation that

SCHEME 4



activation of phosphomonoesters requires less time than activation of phosphodiesters. Once the internucleoside bond is formed, it can always be converted to the triester to avoid side reactions at the phosphate bridge. This method seemed interesting for the preparation of the diribonucleoside monophosphate; but as the chain length increased, yields dropped drastically from 90% for the dimer, 65% for the trimer to 47% for the tetramer.

All the aforementioned syntheses have one common feature: the use of a condensing agent to mediate the formation of the internucleotide bond. In addition to those already mentioned, a wide variety of aryl sulfonylimidazolides, arylsulfonyltriazolides and arylsulfonyltetrazolides are available. Recently, Ogilvie and Pon[33] have evaluated nine of the most commonly used condensing agents.

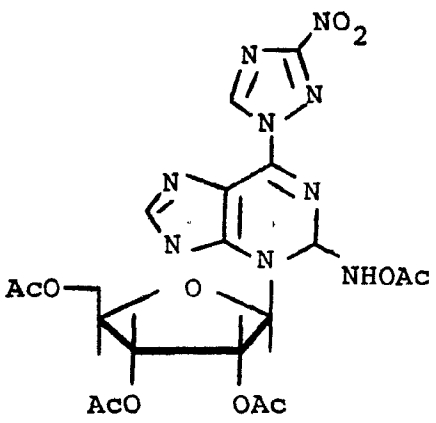
Generally, there are two major problems associated with the use of these condensing agents. The first is their short shelf life and the second is the formation of undesired side products during phosphorylation reactions. The most significant side reaction is, by far, sulfonation of the 5'-OH of the incoming block. Other than lowering the yield of the desired product, sulfonation of the free 5'-OH generates extra purification problems since the sulfonated derivatives often do not separate from the desired condensation product.

A study of the problem of sulfonation during the chemical synthesis of deoxynucleotides has been recently carried out by Seth and Jay[34]. They observed significant sulfonation with all the condensing agents investigated. Furthermore, as the chain length increased, the rate of phosphorylation appeared to drop, whereas the rate of sulfonation remained unaltered. As a result, sulfonation seems to become a serious problem with higher oligomers.

Other workers have also shown that greater sulfonation

takes place when the phosphate protecting group is alkyl rather than aryl[35]. Presumably, the less electron withdrawing alkyl group reduces the rate of phosphorylation while the rate of sulfonation is not affected.

In addition to sulfonation, other serious side reactions have been observed with some coupling agents. Of special significance is the report of Reese and Ubasawa[36] that MSNT and TPSNT react readily with the guanine and uracil residues of ribonucleosides to yield the modified nucleosides 2 and 3 respectively.



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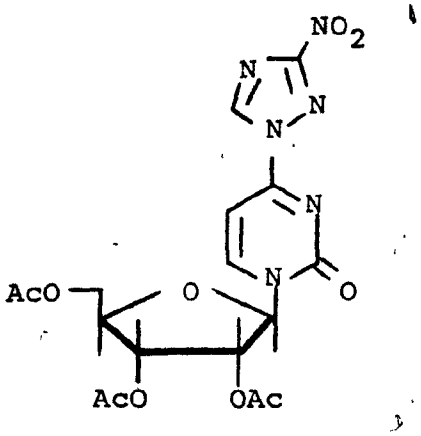
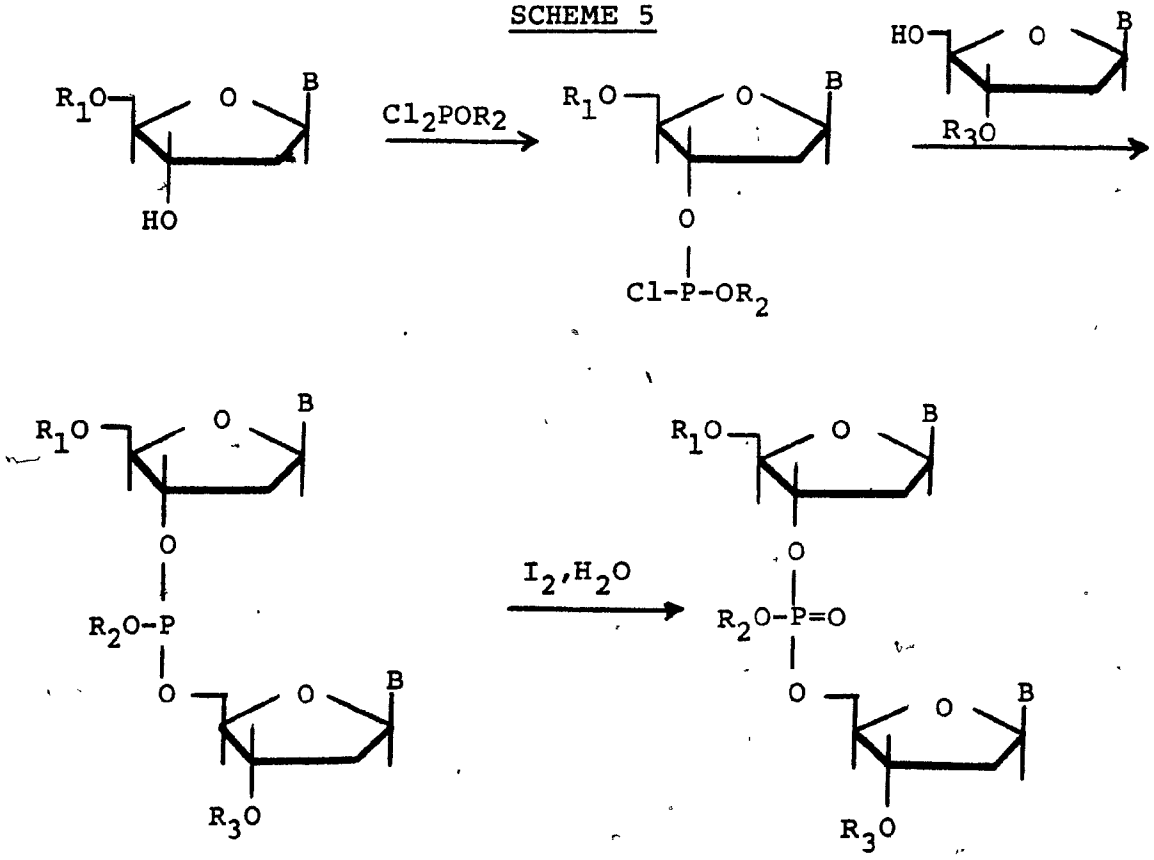


3

In an attempt to avoid the use of condensing agents, Letsinger and co-workers introduced the highly efficient "phosphite procedure" for the synthesis of deoxynucleotides[37,38]. This novel approach (Scheme 5) was based on the high reactivity of chlorophosphites with hydroxyl groups and the rapid oxidation

of nucleoside phosphites to the corresponding phosphates.

SCHEME 5



Soon after Letsinger's report[37], the phosphite procedure was adapted to ribonucleotide synthesis by Daub and van Tamelen[39] and Ogilvie and co-workers[40].

The Ogilvie group combined the phosphite procedure with the use of the tertiarylbutyldimethylsilyl group (TBDMS) as a masking group for the 2'-OH of nucleosides to prepare

several short oligomers in very high yield[41,42]. Later on, they demonstrated the efficiency of the method in the stepwise synthesis of a heptamer[43].

Recently, the same group carried out extensive studies on this new procedure and came up with interesting results[44]. For example, they found that coupling reactions can be carried out in a range of solvents, including pyridine, DMF and THF, at temperatures varying from -78°C to 20°C. They also observed that reaction times can be as short as 20 min. In addition, they explored the use of a series of phosphorodichloridites which incorporate the most commonly used phosphate protecting groups and settled on the 2,2,2-trichloroethyl (TCE) moiety.

In a comparative study of the phosphite procedure and the modified triester approach[33], the same researchers clearly showed the superiority of the former method for the synthesis of oligoribonucleotides. Nevertheless, a further development, namely block condensations, is necessary before their procedure could be useful for the synthesis of long polyribonucleotides like t-RNA's.

The total synthesis of a t-RNA molecule is an important challenge by itself. Furthermore, the chemical synthesis of t-RNA's will open the way to the preparation and study of unnatural or modified t-RNA's which in turn may help our understanding of protein synthesis.

Several groups have been interested in preparing

oligoribonucleotides having sequences of t-RNA[20,31,43]. Ikehara and co-workers have been attempting for many years to synthesize a t-RNA and have claimed many times the total synthesis of $t\text{-RNA}_f^{Met}$ although no such report has appeared in the literature yet. In fact, the group is still reporting the preparation of short fragments corresponding to various regions of t-RNA[29,30,45,46]; the longest ribooligomer they ever prepared chemically is a decamer (27% yield)[30]. However, they have prepared longer sequences by enzymatic joining of shorter chemically prepared oligomers[47].

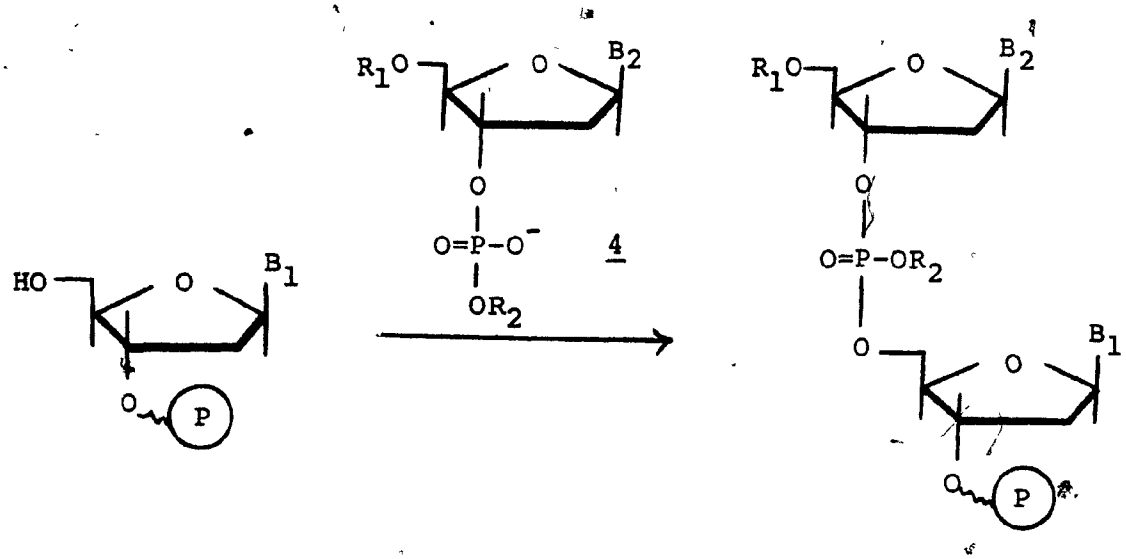
Solid Phase Synthesis

In spite of the numerous advancements previously discussed, the synthesis of oligoribonucleotides remains a time consuming operation. Much of this can be blamed on the lengthy purification steps required for the isolation of intermediate and final products. The only way to eliminate the need for these laborious separation techniques would be solid phase synthesis.

The general procedure is illustrated in Scheme 6. A 3' phosphorylated nucleoside (4) is added to a nucleoside linked through its 3' end to an insoluble support. When the reaction is over, the only materials chemically bound to the polymer are the dinucleotide product and any unreacted mononо-

nucleoside. Any excess of 4 is simply washed off. The chain can be further extended from the 5' end by simple removal of the protecting group R_1. This approach also offers the most

SCHEME 6



obvious route to automation of a reptetitive step synthesis like the assembly of polynucleotides.

The concept of polymer support synthesis was first developed by Merrifield in the peptide field[48] and introduced to the nucleotide area by Letsinger and Mahadevan[49]. Since the first solid phase synthesis of a dideoxynucleotide in 1965[49], there have been numerous attempts to develop an efficient polymer support procedure[50]. However, until recent months, the extensive investigations in this field made little practical

impact on the synthesis of nucleotides.

Classically, there have been two major problems associated with solid phase synthesis. One is the choice of the support, and the other is the phosphorylation method.

Most of the supports studied were polystyrene-type polymers which were inadequate for several reasons including excessive swelling, slow diffusion rate into the support and irreversible adsorption of reagents onto the polymer[50]. Only a few polar carriers were tested; among the more interesting ones are cellulose[51] and silica gel[52]. More recently, polymers consisting of polydimethylacrylamide[53] and polyacrylmorpholide[54] have been suggested and appear promising.

Until the mid-70's, the diester method was used almost exclusively for solid phase synthesis of nucleotides[50]. The presence of ionic phosphodiester residues along with low yields of coupling steps gave rise to many difficulties and prevented the application of the procedure to the preparation of polynucleotides.

In the past two years, several groups have reported successful assembly of polydeoxynucleotides using a triester approach. Thus, Gait and co-workers prepared two dodecamers in an overall yield of 1.2-2.5% on a polyamide support[55]. They later improved on their method and were able to obtain a heptadecamer in 8% yield[56]. Markham et al.[57] prepared several large deoxynucleotides on a polyamide support; overall yields ranged

between 7.8% for the nonamer to 0.4% for the heneicosamer (21 unit long). Crea and Horn[58] used a cellulose polymer to prepare a tridecamer in 7.5% yield. Itakura and his co-workers assembled many polydeoxynucleotides on a polyacrylylmorpholidate carrier[54,59,60]. In their latest communication[61], they described the synthesis of a hentriacontamer (31 unit) in 0.25% overall yield.

As can be seen, the yield of desired product in all these syntheses was quite low because individual conversions varied between 45 and 80%. Ideally, individual coupling steps should be as close to 100% as possible to avoid the difficult task of separating the final molecule from all the shorter unreacted sequences.

Another common feature to the aforementioned preparations is the use of the triester method. A serious drawback of this procedure is the fact that one of the major by-products, sulfonation of the 5'-OH cannot be eliminated during the synthesis since the growing chain is bound to the polymer.

In the past few months, a novel solid phase approach was proposed by Matteuci and Caruthers[62,63]. The method features the use of a derivatized silica gel polymer along with the phosphite procedure. Reaction times were very short and individual coupling steps exceeded 95%.

While intensive studies were pursued in the deoxy area, attempts to synthesize ribonucleotides using a solid phase

approach have been scarce. Today, there are only 4 published reports[64-67] - two of them in the last few months[66,67] - on polymer supported synthesis of ribonucleotides.

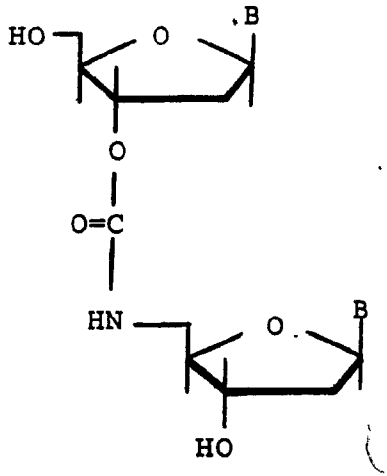
The first preparation of an oligoribonucleotide on an insoluble support was carried out in 1971 by Yip and Tsou[64]. Using a succinylated polystyrene polymer and a diester approach, they prepared a triuridylic acid in 25% yield. Unfortunately, only 95% of the isolated trimer had the desired 3'-5' linkage. A year later, Ikehara et al.[65] prepared several diribonucleotides and one trimer on a polystyrene polymer. However, their method had no practical utility because of low coupling yields and an efficient polymer support synthesis of oligoribonucleotides had yet to be achieved.

NUCLEOTIDE ANALOGUES

Synthetic ribonucleotides have been used in various biochemical studies and many of them have shown in vitro inhibitory effects[4-6]. Unfortunately, their activity in vivo is seriously hampered by their inability to cross the membranes of living cells and their susceptibility to enzymatic degradation. Both problems may be overcome by using nucleotide analogues that are resistant to enzyme hydrolysis and are less polar in nature. Since the phosphate residue is the site of many enzymatic reactions and because the polar nature of this bridge

presumably prevents transport into the cell, the preparation and study of oligonucleotide analogues having modified internucleoside linkages is of particular interest.

Various oligonucleotide analogues stable to enzyme hydrolysis, have been prepared and several have shown interesting biochemical activity. They include molecules containing carbamate (5)[68], carbonate (6)[69] and carboxymethyl (7) linkages[70].



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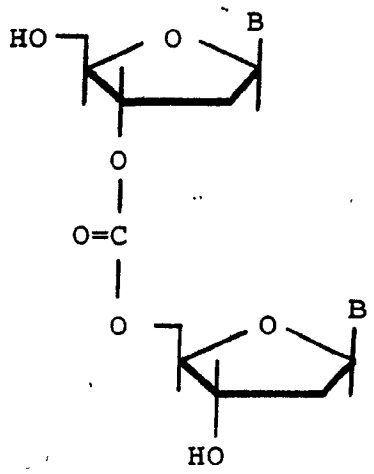

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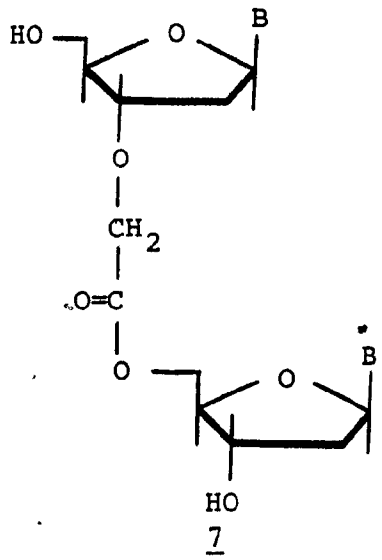
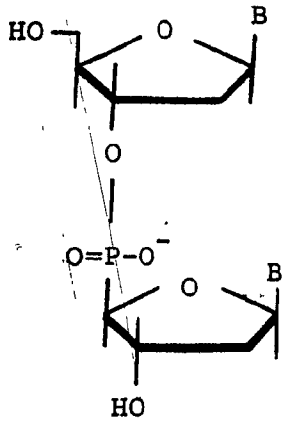


7

Of special interest are analogues having modified phosphate linkages like phosphonates (8)[71] and phosphoramidates (9)[72]. Ts'o and co-workers have also carried out extensive studies on oligodeoxynucleoside methyl phosphonates (10)[73-76]; they recently showed that these molecules have a great potential as selective regulators of bacterial or mammalian cellular

function[75,76].



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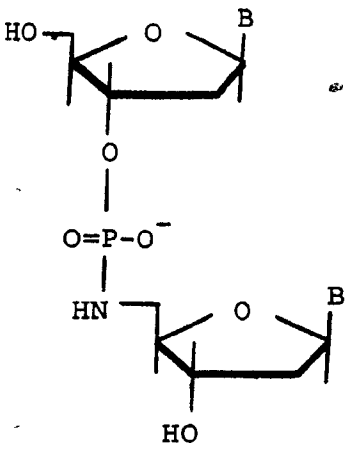

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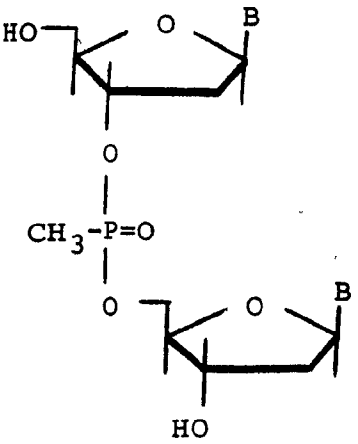

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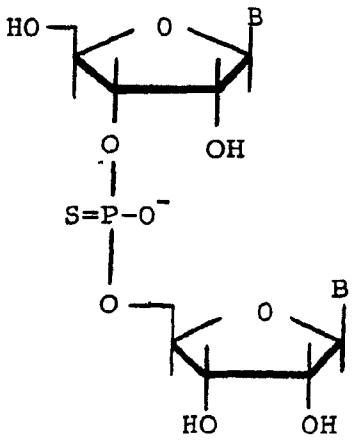
Oligonucleotide analogues that are substrate for various enzymes, especially those with a chiral phosphorus center can be very useful in investigating enzyme mechanisms. By making possible studies on the stereochemical requirements of the enzyme, they can also provide valuable information on its active site. One example of such compounds are the thiophosphate derivatives (11) prepared and widely used by Eckstein and his co-workers[77-82]. These analogues have helped immensely in establishing the stereochemical course of action of Rnase A[80] and polynucleotide phosphorylase[81].

In addition to helping improve "our still poor understanding of enzymatic reactions"[82], oligonucleotide analogues can be useful in investigating a variety of other cellular



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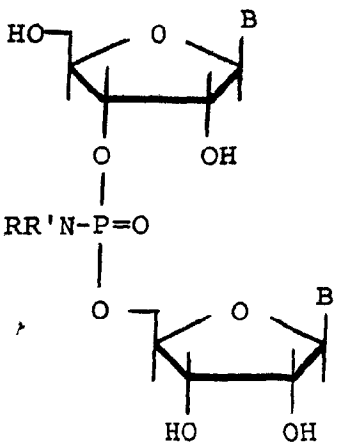


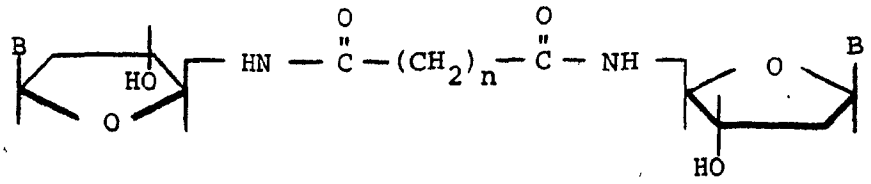
12

processes. The most striking example of such molecules may be phosphoramidate derivatives of type 12. Several nucleotide analogues of this type containing a nucleotide (P-N) aminoacid linkage (12, R = aminoacid) have been isolated from various biological sources. These compounds, generally referred to as nucleotide-peptides, are believed to be intermediates formed during the biosynthesis of proteins[83]. Unfortunately, "due to the very scanty knowledge of such compounds"[83], the biological role of nucleotide-peptides is not yet understood. It has been suggested that synthetic nucleotide-peptides may be useful as models of the enzymatic synthesis of internucleotide linkages and may greatly help elucidate the action of enzymes involved in nucleotide exchange[83].

In 1964, an interesting enzyme, ribonucleoside 5' phosphoamidase was discovered[84]. The enzyme seems to be

specific for nucleotide phosphoramidates but little else is known about its structural requirements. Its cellular role and mode of action are not clear yet mainly because synthetic oligonucleotide phosphoramidates have not been available.

Recently, a novel class of analogues, dinucleoside analogues designed on the basis of their possible resemblance to transition state substrates, has interested various researchers. Lin et al.[85] prepared several N,N'-bis-(5'-deoxynucleoside)-azeloamide (13) and Bornemann and Schlimme[86] synthesized 5',5" phosphate linked dimers (14).



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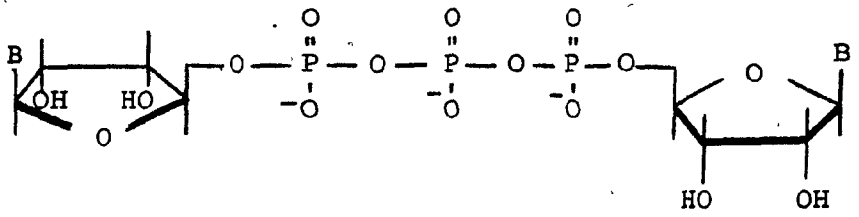


14

Wolfenden[87] had already pointed out that such models may help understand the structural geometry of the transition state complex for many enzymes. These analogues have also the possibility of being irreversible and highly specific

metabolic inhibitors[87]. Indeed, P^1, P^5 di(adenosine-5')penta-phosphate was found to be a potent inhibitor with respect to AMP and ATP[88].

The study of nucleotide analogues has made important contributions in many other areas of biophysical, biochemical and even clinical research[89]. The preparation and study of new analogues would undoubtedly shed light on the puzzling structure-function relationship of nucleotides.

In the present work the synthesis of ribonucleotides and their analogues is dealt with. The solution phase synthesis of polyribonucleotides using the phosphite procedure is extended to the block condensation approach. A novel solid phase procedure, suitable for the synthesis of long RNA chains and amendable to automation is fully investigated. The preparation of several new nucleotide analogues as well as their interaction with various enzymes is examined. Finally, the synthesis of symmetrically linked diribonucleotides is carried out; the stability of these molecules to acid, base and enzyme hydrolysis is studied in an effort to determine the influence of the phosphate position on the behavior of nucleotides.

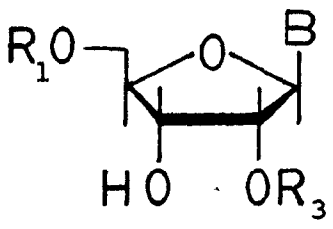
RESULTS AND DISCUSSION

CHAPTER 1

SYNTHESIS OF PROTECTED NUCLEOSIDES SUITABLE FOR BLOCK CONDENSATIONS

1.1 INTRODUCTION

The synthesis of adequately protected nucleosides is an essential prerequisite for nucleotide synthesis. In the phosphite procedure, compounds 15 and 16 represent the smallest building blocks. Compound 15 is used to extend the nucleotidic chain in the 5' direction. Compound 16 is useful for elongation of the chain at the 3' end, provided R_2 can be selectively removed in the presence of R_1 and R_3 and without causing any migration of R_3 from the 2' to the 3' position.



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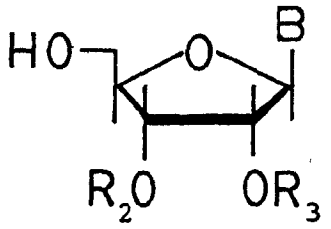
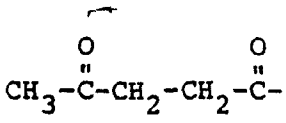


16

Compound 15, where R_1 = MMT, R_3 = TBDMS has already been shown to be compatible with the phosphite coupling procedure[41,42]. Compound 16, where $R_2 = R_3$ = TBDMS has been used in the stepwise synthesis of oligoribonucleotides[43].

However, for block condensations using the phosphite procedure, the choice of R_2 becomes critical. Since the monomethoxytrityl group is labile to acid, and since the isomerization of TBDMS from the 2' to the 3' position occurs in basic media[90], R_2 can be labile only to neutral conditions.

The levulinyl group (lv, 4-oxopentanoyl), which was



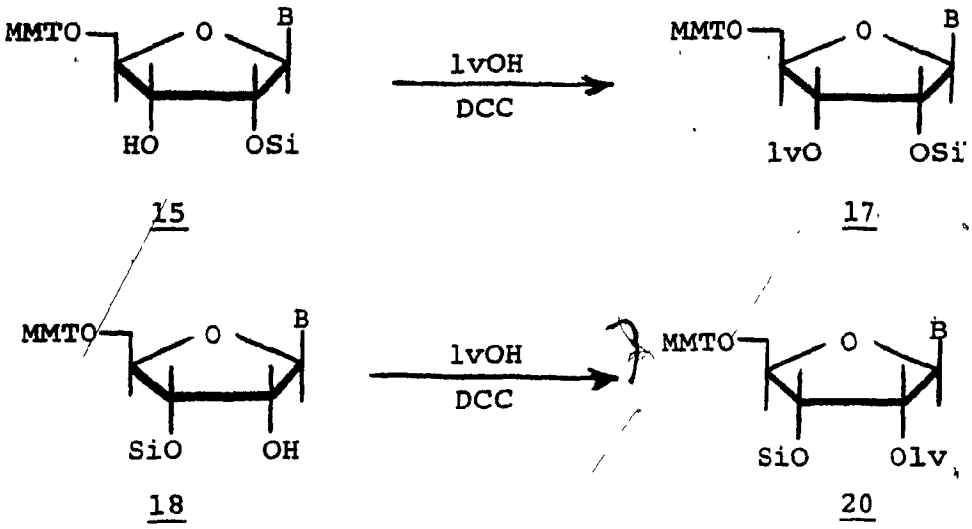
introduced to carbohydrates by Guthrie and Lucas[91], looked very promising. The levulinyl group can be rapidly cleaved under essentially neutral conditions[92]. It has been shown to be useful for the protection of the 5'-OH of nucleosides by two independent groups[93,94].

In this chapter, the synthesis and properties of the key intermediates 17 (a-f) having a 5'-MMT, 2'-TBDMS and 3'-lv and their 2',3' isomeric derivatives 20 (a-f) will be described.

1.2 SYNTHESIS OF THE FULLY PROTECTED NUCLEOSIDES 17 AND 20

The fully protected nucleoside derivative 17, was obtained in virtually quantitative yield within 6 h, by treating compound 15 with levulinic acid (lv OH) and dicyclohexylcarbodiimide (DCC) in dioxane (Scheme 7)[94].

SCHEME 7



15 17 18 20

a, B = Ur

b, = Ad

c, = Cy^{Bz}

d, B = Gu^{Bz}

e, = Cy

f, = Gu

The reaction was catalyzed by 4-dimethylaminopyridine (DAP) and was complete within 6 h.

A reasonable drop in R_f was observed upon the introduction of the levulinyl group which facilitated monitoring of the reaction by tlc.

Migration of the silyl group from the 2' to the adjacent 3' position during the reaction was not observed. This was verified by the preparation of the isomeric compound 20 from the 3',5'-diprotected nucleoside 18. The isomeric

pairs 17 and 20 had different chromatographic properties and were easily distinguished by tlc.

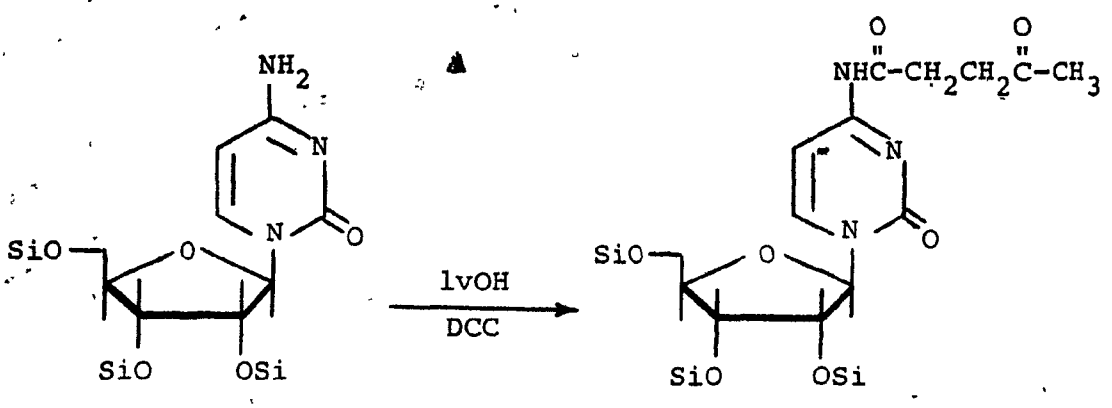
Adenosine derivatives lacking protection at the amino group have been used in conjunction with the phosphite procedure[41,43], because the glycoside bond in N-protected adenosine is more labile to acid than in adenosine. The elimination of the need to protect the amino function of nucleosides is one of the useful features of the phosphite procedure[37]. The synthesis of the adenosine derivative 17b was therefore important.

The position of the levulinyl group, (O-lv, or N-lv) can be easily determined by u.v. spectroscopy. Acylation of the exocyclic amino group of the base results in a noticeable change in the u.v. spectrum, whereas acylation of the sugar hydroxyl has no effect on the λ_{max}.

With the adenosine derivative 15b, no levulinylation occurred at the base; instead, levulinylation was exclusively at the 3'-OH. Selective O-levulinylation was later found to occur with the cytidine and guanosine derivatives 15e and 15f as well. Furthermore, attempts to prepare N-levulinylated adenosine and guanosine derivatives by reacting 2',3',5' tri-TBDMS adenosine[41] or 2',3',5' tri-TBDMS guanosine[42], with a large excess of levulinic acid and DCC for 24 h, were unsuccessful. However, when 2',3',5' tri-TBDMS cytidine 21[42]

was treated with an excess of levulinic acid and DCC, the corresponding N-levulinylated derivative 22 was isolated in 95% yield after 16 h (Scheme 8).

SCHEME 8



21 λ_{max} = 270 nm

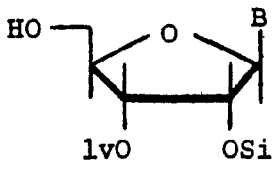
22 λ_{max} = 302, 219 nm

Compound 22, as expected, had a different u.v. spectrum than compounds 21 and 17e. It also had different chromatographic properties. The introduction of the levulinyl group on the NH_2 reduced the polarity of the molecule, thus 22 migrated faster on tlc than 21. On the other hand, derivatives such as 17e where the levulinyl is on the hydroxyl, migrated slower on tlc than the corresponding starting material 15e. The use of the levulinyl group as a protecting group for the exocyclic amino function of cytidine was investigated later on, and will be fully described in Chapter 6.

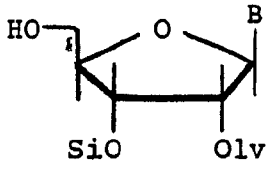
1.3 SELECTIVE REMOVAL OF PROTECTING GROUPS FROM 17 AND 20

1.3.1 Removal of MMT, Preparation of Compounds 16 and 19

Compounds 16 and 19 were obtained from 17 and 20 respectively following treatment with 80% acetic acid, to remove the 5'-MMT group.



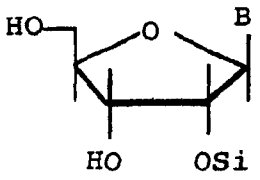
16



19

The fully protected nucleosides were generally treated with the acid at 90°C for 15 min. In the case of the N-benzoyl cytidine derivatives 17c and 20c, treatment with acid was done at room temperature for 15 h to minimize deamination of the starting materials and their conversion to the corresponding uridine derivatives[95].

In preparative conversions of 15 to 16, there was no need to isolate compound 17. However, it was important to make sure that 15 had been quantitatively converted to 17 (tlc) before the detritylation step. Presence of 15 in the mixture would give, upon detritylation, the corresponding derivative 23 whose chromatographic properties closely resemble those of



23

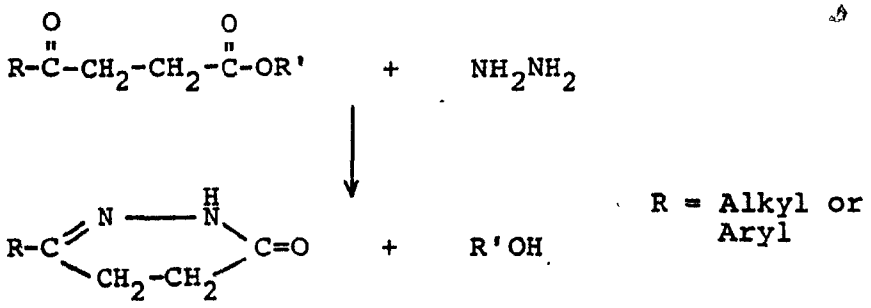
the corresponding levulinylated compound 16. This would create unnecessary complications during the final purification step.

1.3.2 Removal of the Levulinyl. Regeneration of Compounds 15 and 18

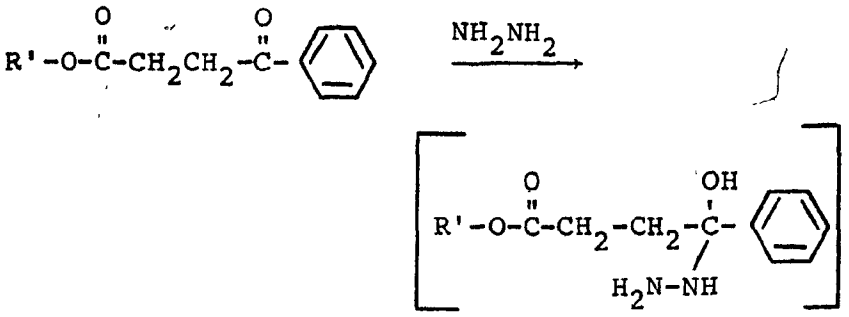
The levulinyl group was rapidly removed from fully protected nucleosides using mild conditions: 0.5 M hydrazine hydrate in pyridine-acetic acid. This solution was introduced by Letsinger et al.[92], for the removal of the β-benzoylpropionyl group. It was recently used by Van Boom and co-workers[94] to cleave the levulinyl group from the 5' position of nucleosides.

While the time required to remove the β-benzoyl propionyl was 3 h, the levulinyl group was completely cleaved within 5 min, under identical conditions (Scheme 9).

SCHEME 9



This increase in reactivity is due to the nature of the group next to the oxo function. Indeed, it has been argued[96], that when the substituent is aryl rather than alkyl, there is a loss of conjugation between the aryl and the carbonyl functions in the transition state for hydrazine addition, which in turn results in decreased reactivity.



It had been previously reported that N-benzoyl cytidine derivatives lose their benzoyl group in the presence of hydrazine[91]. The conditions used to removed the levulinyl did not affect N-protecting groups as monitored by both paper chromatography and tlc.

The hydrazine hydrate in pyridine-acetic acid was essentially a neutral medium. It was not sufficiently acidic to cause cleavage of the monomethoxytrityl group nor sufficiently basic to cause isomerization of the TBDMS during removal of the levulinyl. In fact, when pure 15a was treated with 10 or 50 equivalents of the hydrazine solution, isomerization to 18a was detected only after two hours.

1.4 CHARACTERIZATION AND PROPERTIES OF LEVULINIC NUCLEOSIDES

All levulinylated nucleoside derivatives were easily purified by silica gel chromatography using the appropriate solvent systems (Table 15, experimental section). Fully protected nucleosides 17 and 20 were converted to the previously characterized derivatives 15 and 18 upon removal of the levulinyl group. The elemental analysis of selected nucleoside derivatives agreed with the assigned structure (see experimental, p. 180). The position of levulinylation (sugar or base) was determined using u.v. spectroscopy as discussed earlier. The presence of the levulinyl group was easily confirmed through 60 MHz PMR, by the appearance of a distinct singlet (CH_3) around 2.2 ppm and a multiplet (CH_2) around 2.9 ppm. The methoxy group of the trityl appeared, as usual, around 3.9 ppm. The t-butyl and methyl protons on the silicon atom were below 1 ppm and the anomeric proton was visible between 5.5 and 6.1 ppm depending on the nucleoside.

The PMR spectra of all the prepared levulinylated derivatives were examined. The chemical shifts of interest are collected in Table 1 and the PMR spectra of 2 isomeric pairs are displayed in Fig. 2.

Several years ago, Reese and co-workers[97] had examined the PMR spectra of several acylated nucleoside derivatives. Two generalizations - or rules - were stated:

1. For a pair of 2' and 3' isomers, the H(1')

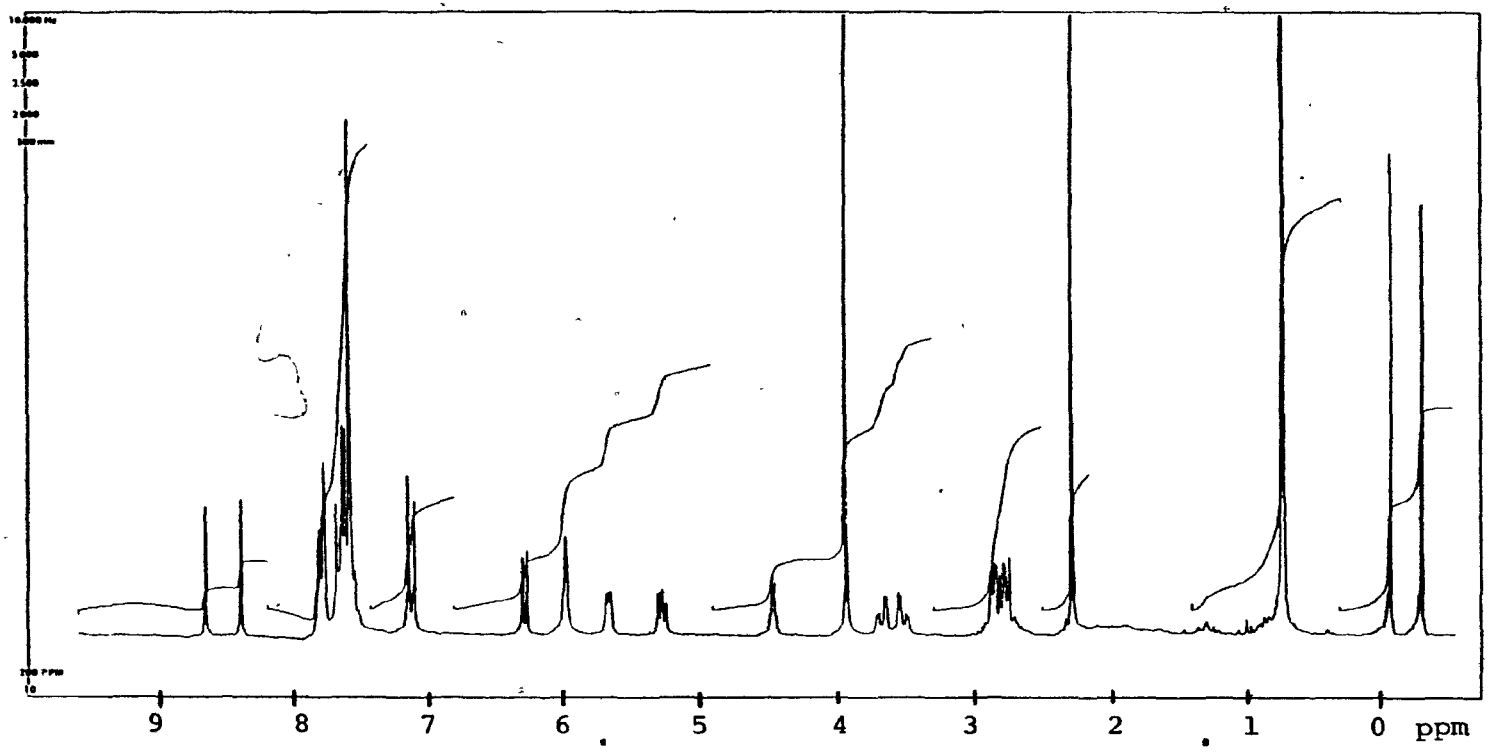


Fig. 2a: 200 MHz PMR of MMT A_{lv}^{si} in $CDCl_3$.

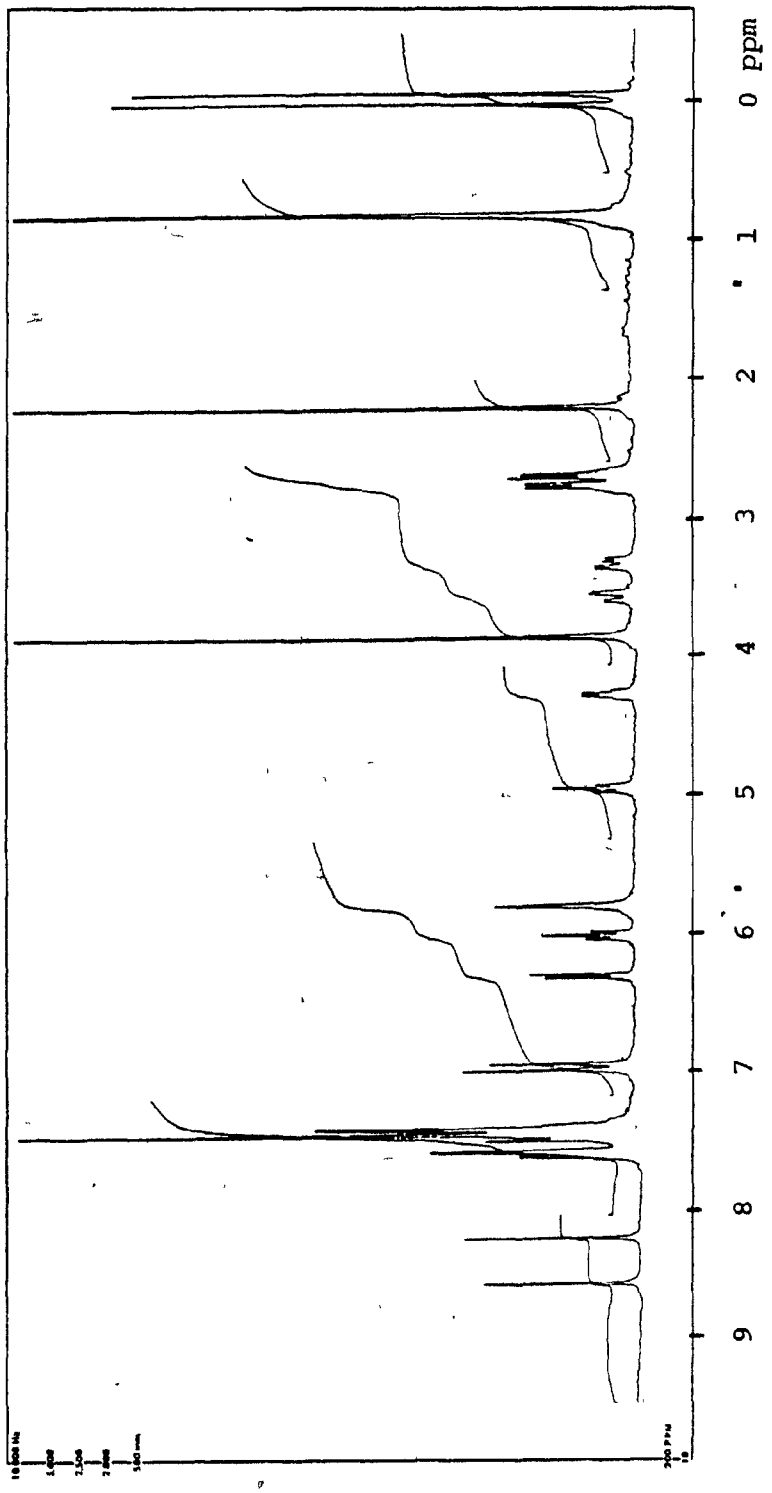


Fig. 2b: 200 MHz PMR of MMT A_{si}^{lv} in $\overset{\circ}{C}Cl_3$.

resonance is at lower field for the 2' than for the 3' isomer.

2. The coupling constant $J_{1',2'}$, is greater for the 3' than for the 2' isomer.

As shown in Table 1 , the chemical shift of the H-1' signal is greatest, in all cases, for the isomer having the levulinyl group in the 2' position. This is in agreement with the first "rule". For the levulinylated nucleosides listed in Table 1 , $J_{1',2'}$ is greater when the levulinyl group is on the 3' position of adenosine and guanosine derivatives or the 2' position of cytidine and uridine derivatives. It is also interesting to note that the magnitude of the coupling constants is greater for the purine derivatives than for the pyrimidine nucleosides. This trend was previously observed for the silylated nucleosides 15 and 18[41,42].

Nucleosides having a 5'-MMT and a 2' or 3'-TBDMS group (15 and 18) show interesting chemical shifts for the methyl and t-butyl protons of the TBDMS group[41,42]. For pyrimidines (C and U), the 3'-TBDMS protons are significantly shielded relative to the 2'-TBDMS isomer. For purines (A and G) it is just the reverse. This same trend is observed for compounds 17 and 20 which also bear a levulinyl group. Moreover, the presence of the neighbouring acyl group appears to have a deshielding effect on the methyl and t-butyl protons of the TBDMS group.

Table 1. Chemical Shifts of Levulinated Nucleosides.

Compound	$\delta^{\dagger}CH_3$-Si	$\delta(CH_3)_3C$-	$\delta CH_3\overset{O}{\overset{\|}{C}}$	δH-1' (d, $J_{1',2'}(H_2)$)
MMT U^{Si}_{lv} 17a	0.21	1.0	2.33	6.06 (4)
MMT U^{lv}_{si} 20a	-0.01, 0.05	0.86	2.20	6.13 (5)
MMT A^{Si}_{lv} 17b	-0.43, -0.2	0.53	1.98	5.76 (6)
MMT A^{lv}_{si} 20b	-0.21, 0.03	0.83	1.96	6.10 (6)
MMT $C^{Bz}_{lv}{}^{Si}$ 17c	-0.01, 0.08	0.60	2.05	5.85 (3.1)
MMT $C^{Bz}_{si}{}^{lv}$ 20c	-0.16, -0.06	0.37	2.06	6.06 (5)
MMT $G^{Bz}_{lv}{}^{Si}$ 17d	-0.46, 0.08	0.70	2.08	5.65 (7)
MMT $G^{Bz}_{si}{}^{lv}$ 20d	-0.38, -0.06	0.70	1.98	5.83 (5.5)
MMT C^{Si}_{lv} 17e	0.00, 0.08	0.8	2.10	5.95 (3)
MMT C^{lv}_{si} 20e	0.36, 0.48	1.18	2.25	6.16 (2.5)
MMT G^{Si}_{lv} 17f	0.17, 0.19	1.01	2.14	5.87 (7.2)
MMT G^{lv}_{si} 20f	0.08, 0.15	0.95	2.24	6.09 (6)
U^{Si}_{lv} 16a	0.13	0.86	2.23	5.9 (5)
U^{lv}_{si} 19a	0.15, 0.25	0.91	2.16	6.05 (6)
A^{Si}_{lv} 16b	-0.13, -0.43	0.70	2.20	5.70 (8)
A^{lv}_{si} 19b	0.05	0.90	2.10	5.95 (6)
$C^{Bz}_{lv}{}^{Si}$ 16c	0.06	0.86	2.08	5.50 (3.1)
$C^{Bz}_{si}{}^{lv}$ 19c	0.12	0.93	2.10	5.80 (4)
$G^{Bz}_{lv}{}^{Si}$ 16d	-0.22, -0.45	0.59	2.12	5.60 (8)
$G^{Bz}_{si}{}^{lv}$ 19d	-0.02	0.71	2.01	5.80 (6)

†Chemical shifts in ppm relative to TMS.

For the nontritylated compounds 16 and 19, the levulinyl group had little effect on the chemical shift of the TBDMS protons of the corresponding derivative of type 23. For compounds 16 and 19, the 3'-TBDMS protons all have greater chemical shifts than those of the 2'-TBDMS isomer.

1.5 CONCLUSION

The levulinyl group, along with the MMT and TBDMS groups, constitute an ideal protection system for ribonucleosides. These units have been used to build ribonucleotides as described in the next chapter. In addition, the synthesis of compounds 19 and 20 was useful in confirming the structure of 16 and 17, and is of obvious utility for the synthesis of any desired sequence involving 2'-5' phosphate linkages. With the observation of the production of 2'-5' linked oligoadenylates during the interferon process[98], it has become essential to be able to synthesize such linkages.

The desired protection system being achieved, the following chapter will deal with the incorporation of the nucleoside derivatives 16 into nucleotidic chains.

CHAPTER 2

SOLUTION PHASE SYNTHESIS OF RIBONUCLEOTIDES: BLOCK CONDENSATIONS IN THE PHOSPHITE PROCEDURE

2.1 INTRODUCTION

The utility of the combination of protecting groups described in the previous chapter was evaluated through the synthesis of oligouridylates.

The nucleotides were prepared by coupling smaller nucleotide blocks together rather than stepwise addition of a nucleoside to a growing chain in an effort to minimize the amounts of protected nucleosides needed and the time required in the preparation of long nucleotidic chains.

Two routes were investigated:

1. Phosphorylation of the 3' end, followed by coupling to the 5' hydroxyl of the second block.

2. Phosphorylation at the 5' terminal, followed by addition to the 3' hydroxyl of the second block.

The effect of temperature on the yields of coupling reactions was examined. The synthesis of nucleotide blocks possessing a 3'-3' linkage was undertaken. Finally, the overall efficiency of the procedure was evaluated in the synthesis of a hexadecauridylic acid.

2.2 PRELIMINARY STUDIES

2.2.1 Temperature and Direction of Phosphorylation

In the stepwise synthesis of ribonucleotides[43], the 3'-OH of a nucleoside was phosphorylated then added to the 5'-OH of a growing chain. This was the logical direction of phosphorylation, since the "cheapest" of the two components, the nucleoside, was added in excess.

In a block condensation approach, the above rationalization does not apply, since the two building units are valuable oligomers. In evaluating the best conditions for block condensations, it was therefore important to first determine the optimum direction of phosphorylation.

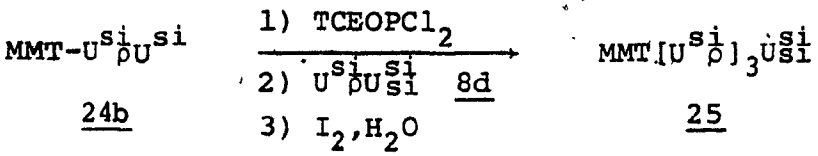
Coupling reactions in the phosphite procedure were generally carried out at -78°C both in the deoxy[37,38] and ribo area[40,43], although Ogilvie and co-workers[44] recently used higher temperatures. At the time of this work, it was felt that the coupling of two blocks may need to be carried out at a higher temperature, for steric considerations.

In order to determine the most suitable temperature and direction of coupling, the tetramer 25 was prepared by two different routes, at -78°C and 0°C (Scheme 10).

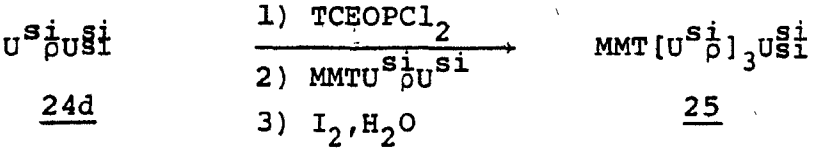
All reactions were carried out on the same scale. In all cases, the ratio of the first component to the second was

SCHEME 10

Route A



Route B



ρ = p-OTCE

1 to 0.8. The reaction time was held constant at 90 min. Increasing the time to 150 min did not increase the yield of couplings. The results of the study are summarized in Table 2.

Table 2 . Preparation of Tetramer 25 at Different Temperatures by Routes A and B.

Route	Temp. (°C)	% 25	% unreacted 24b
A	-78	51	19
A	0	76	5
B	-78	38	40
B	0	54	30

In route B, a large amount of 24b was recovered despite the fact that 24d reacted readily with the dichloridite. A significant amount (15%) of symmetrically linked 5'-5' tetramer was also isolated in this instance. The decreased reactivity of 24b can be attributed to steric hindrance at the 3'-OH of the nucleotide for the incoming phosphorylated block. An increase in temperature to 0°C, as expected, resulted in an improved yield of product.

Route A appeared to be the method of choice. Here again, best results were obtained at 0°C where 25 was isolated in 76% yield, and only 5% of 24b remained unreacted. Furthermore, a negligible amount (less than 5%) of 5'-5' linked tetramer was detected.

2.2.2 Formation of 3'-3' Linkages

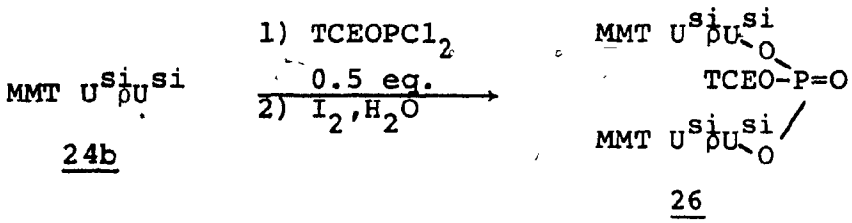
One of the side reactions in the synthesis of short nucleotides is the formation of 3'-3' linked dimers*. While this seldom represents a separation problem, it was essential to determine the probability of 3'-3' formation in block condensations, as well as the chromatographic properties of these side products.

Consequently, an attempt was made to determine the maximum amounts of 3'-3' linked tetramer 26 that could be

*For a detailed discussion of the synthesis and properties of symmetrically linked dimers, see chapter 5.

formed from compound 24b (Scheme 11).

SCHEME 11



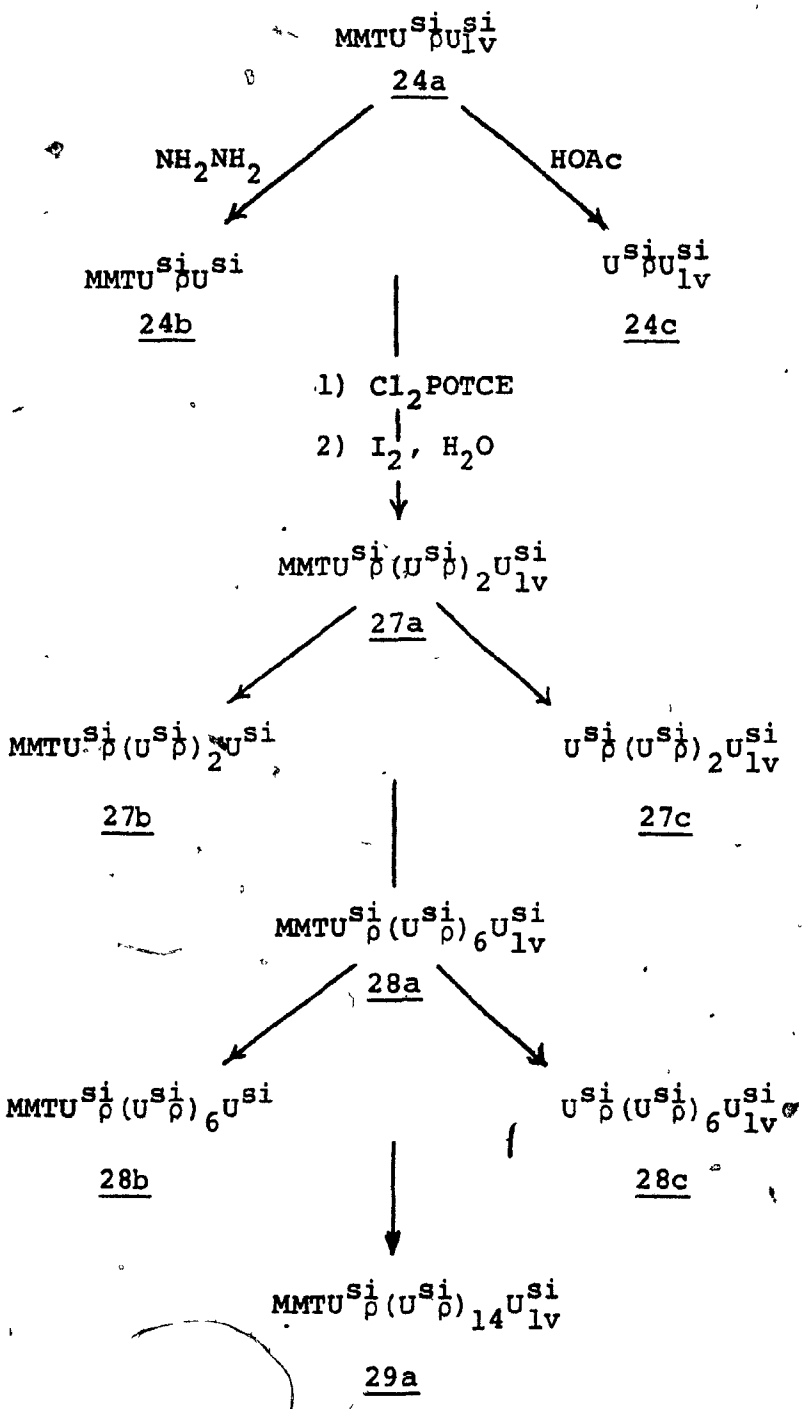
Compound 24b was treated with 0.5 eq. of phosphodichloridite at 0°C for 1 h. After oxidation and work-up, the starting material 24b was recovered to the extent of 46%. Compound 26 was obtained in only 10% yield and a large amount of nucleotidic material (presumably, phosphorylated 24b) remained at the baseline on silica gel plates. As expected, compound 26 had the same u.v. and PMR spectrum as the starting material 24b. However, it was clearly distinguishable from both 24b and the 3'-5' linked tetramer 25 on tlc (see experimental).

2.3 PREPARATION OF THE HEXADECAURIDYLIC ACID

To evaluate the efficiency of the block condensation approach in the phosphite procedure, and its utility for the chemical preparation of long ribonucleotides, the synthesis of a hexadecauridylic acid was undertaken.

The overall synthesis is outlined in Scheme 12 and

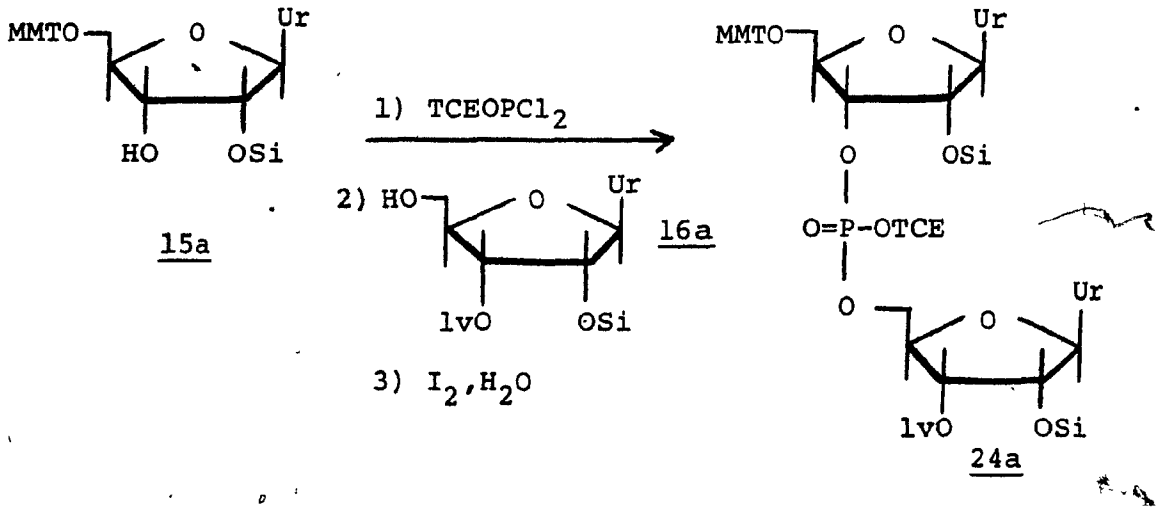
SCHEME 12


MMTU$^{si}_{p}U^{si}_{lv}$
24a
NH_2NH_2
HOAc
MMTU$^{si}_{p}U^{si}$
24b
$U^{si}_{p}U^{si}_{lv}$
24c
1) Cl_2POTCE
2) I_2, H_2O
MMTU$^{si}_{p}(U^{si}_{p})_2U^{si}_{lv}$
27a
MMTU$^{si}_{p}(U^{si}_{p})_2U^{si}$
27b
$U^{si}_{p}(U^{si}_{p})_2U^{si}_{lv}$
27c
MMTU$^{si}_{p}(U^{si}_{p})_6U^{si}_{lv}$
28a
MMTU$^{si}_{p}(U^{si}_{p})_6U^{si}$
28b
$U^{si}_{p}(U^{si}_{p})_6U^{si}_{lv}$
28c
MMTU$^{si}_{p}(U^{si}_{p})_{14}U^{si}_{lv}$
29a

the properties of all protected nucleotides are listed in Table 18 (experimental).

The smallest unit used in block condensations is the dinucleotide 24a. The dimer was prepared several times according to the procedure previously described for the stepwise synthesis of short nucleotides[40] (Scheme 13). The average yield from all preparations was 80%.

SCHEME 13



Treatment of 24a with 0.5 M hydrazine hydrate in pyridine acetic acid for 10 min gave 24b in over 90% yield after silica gel chromatography. The chromatographic properties of 24a and 24b were very similar. However, the purity of 24b was checked by PMR spectroscopy. The two peaks corresponding to the CH_3 (2.2 ppm) and $(CH_2)_2$- (2.9 ppm) of the levulinyl group were totally absent from the spectrum (Fig. 3).

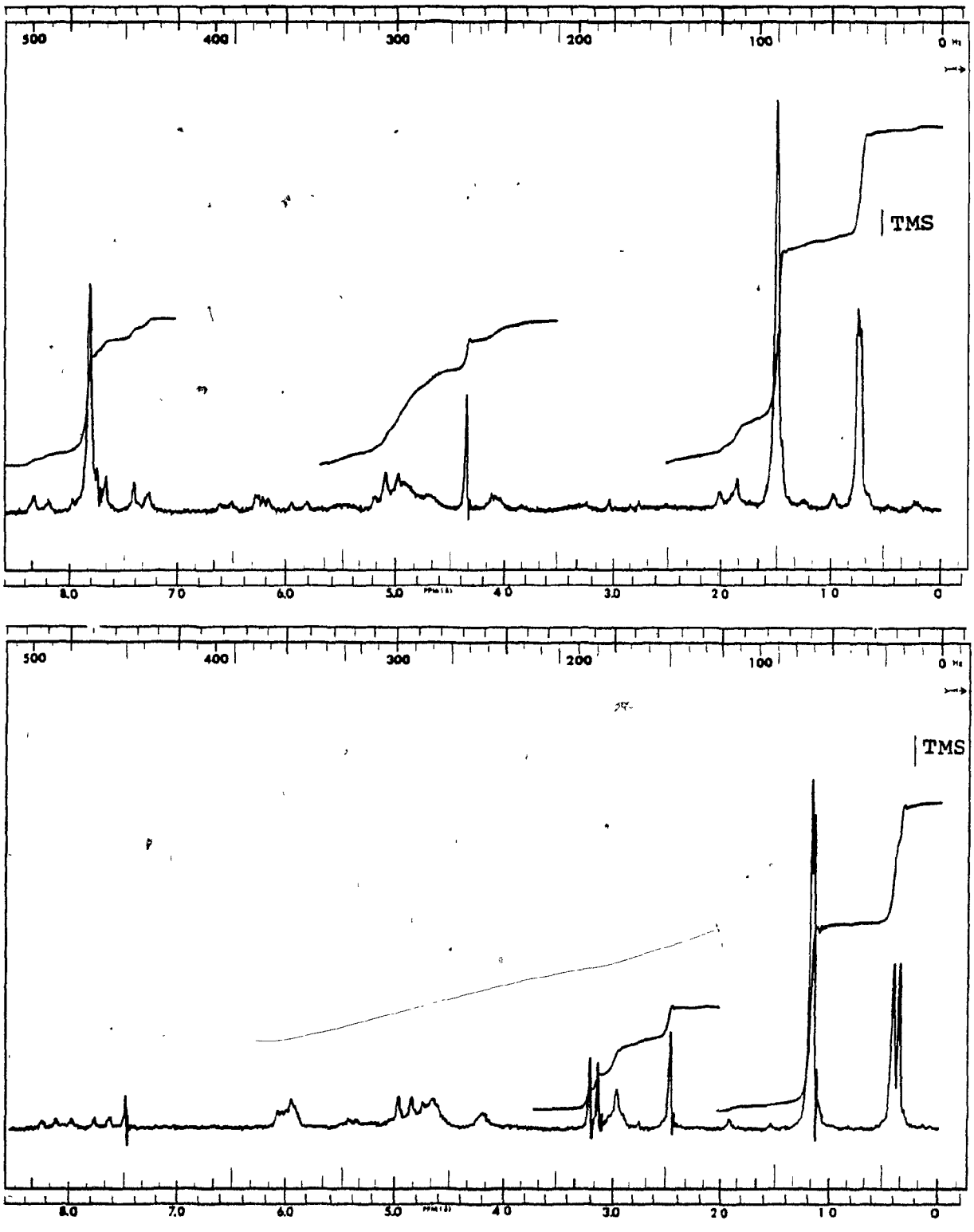
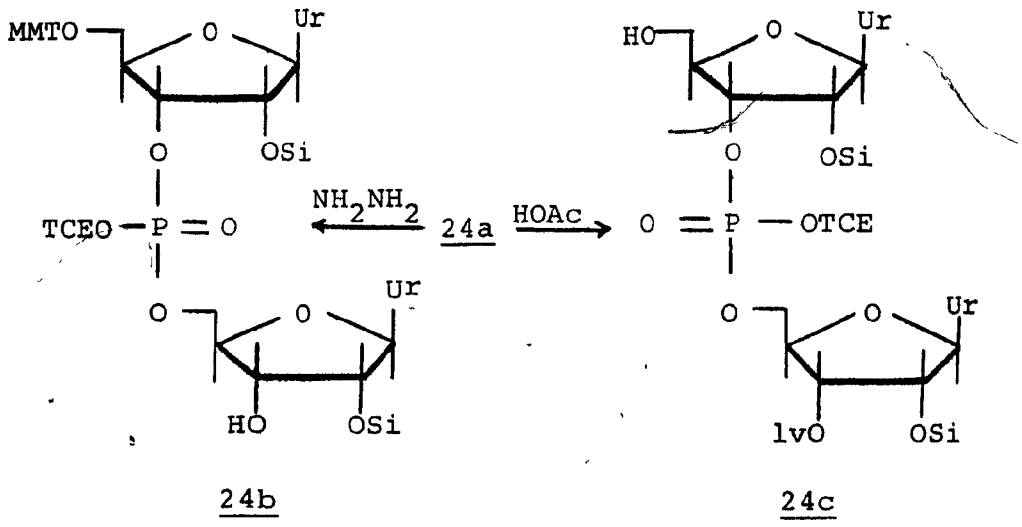


Fig. 3: 60 MHz PMR of MMT $U^{si}\rho U^{si}$ (Top) and $U^{si}\rho U^{si}_{lv}$ (bottom) in $CDCl_3$.

On the other hand, treatment of 24a with 80% acetic acid at 90°C for 15 min gave 24c in greater than 85% yield (Scheme 14).

SCHEME 14



Detritylation reactions were generally easily followed by tlc since the product of the reaction migrates much slower than the starting material on tlc. Compound 24c was purified on silica gel plates or columns. Its u.v. spectrum lacked the absorbance at 235 nm typical of the trityl group, and its PMR spectrum (Fig. 3) showed the disappearance of the methoxy protons (s, 3.9 ppm) but indicated the presence of the levulinyl protons (m, 2.9; s, 2.2). The utility of PMR spectroscopy in connection with this procedure, will be further

examined later on, in this chapter.

The two dimers 24b and 24c were coupled together following Route A at 0°C. The coupling procedure was very similar to the one outlined in Scheme 13. The tetramer 27a was purified on silica gel chromatography. Virtually no 24b remained unreacted, however, 10% of 24c was recovered. The tetramer was generally isolated in 70-75% yield.

Compound 27a was converted into the two tetramers 27b and 27c in the same manner as 24a. The two blocks were then coupled together at 0°C to yield 76% of the octamer 28a. Repeating the hydrazine treatment, and acetic acid deprotections on 28a gave the units 28b and 28c which were condensed at 0°C to produce the fully protected hexadecamer 29a in 51% yield.

In this synthesis, as in all coupling reactions involving the phosphite procedure, extra care had to be taken to eliminate moisture from the reactants, solvents, and equipment used in condensations. In small scale reactions, the elimination of the last trace of moisture was practically impossible. Since a tiny trace of water is enough to destroy a substantial portion of the phosphodichloridite, yields in small scale condensations were generally lower than yields obtained with large scale reactions. This is illustrated in Table 3 , in the preparation of the tetramer 27a and the octamer 28a at various scales.

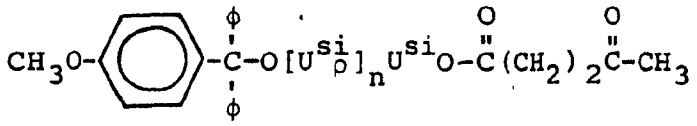
Table 3. Variation of Condensation Yields with Reaction Scale

Product	% Yield	1st Component mmole (mg)	2nd component mmole (mg)	Cl_2POTCE µl
27a	36	$6.3x10^{-2}$ (75)	$5.03x10^{-2}$ (51)	10.2
	52	$16.9x10^{-2}$ (200)	$13.5x10^{-2}$ (136)	27.3
	76	$32.7x10^{-2}$ (385)	$29.5x10^{-2}$ (300)	52.6
	75	2.5 (3000)	2.35 (2400)	450
28a	27	$2.6x10^{-2}$ (60)	$1.6x10^{-2}$ (35)	5
	50	$4.4x10^{-2}$ (100)	$4.1x10^{-2}$ (85)	8
	69	$8.3x10^{-2}$ (190)	$7.2x10^{-2}$ (150)	15
	76	$18x10^{-2}$ (420)	$14.8x10^{-2}$ (310)	33

An important feature of this procedure, is the fact that yields did not decrease significantly as building blocks became larger. Yields ranged from 80% for the dimer 24a, 76% for the tetramer 27a, 76% for the octamer 28a to 51% for the hexadecamer 29a. Furthermore, no condensation required more than 90 min to join two blocks together and the ratio of reactants was maintained at 1:0.8 even at the hexadecamer stage. At the same time, partial deprotection reactions proceeded smoothly and efficiently whether at the dimer, tetramer or octamer stage. Again, reaction times were held constant at 10 min for the removal of the levulinyl and 15-20 min for the removal of the monomethoxytrityl, regardless of

the chain length.

There is one additional feature of this procedure that is very useful. As mentioned earlier, the methoxytrityl and the levulinyl groups contain an isolated methyl group. The methyl resonances occur at different chemical shifts in the nmr. Typically, CH_3O has δ = 3.85 ppm while $CH_3\overset{O}{\overset{\|}{C}}$ has δ = 2.26 ppm. Since the two groups appear at opposite ends of the growing chain, the presence of the two methyl singlets confirms a successful coupling of two units in the correct fashion.



Indeed, only the formation of a 3'-5' linkage between two units can result in a molecule possessing both signals. The methyl peaks, therefore, serve as internal labels and can be observed even with small quantities of large molecules (Fig. 4).

By the same reasoning, the disappearance of one of these signals serves to establish the removal of one of the protecting groups during a partial deprotection step (Fig. 3).

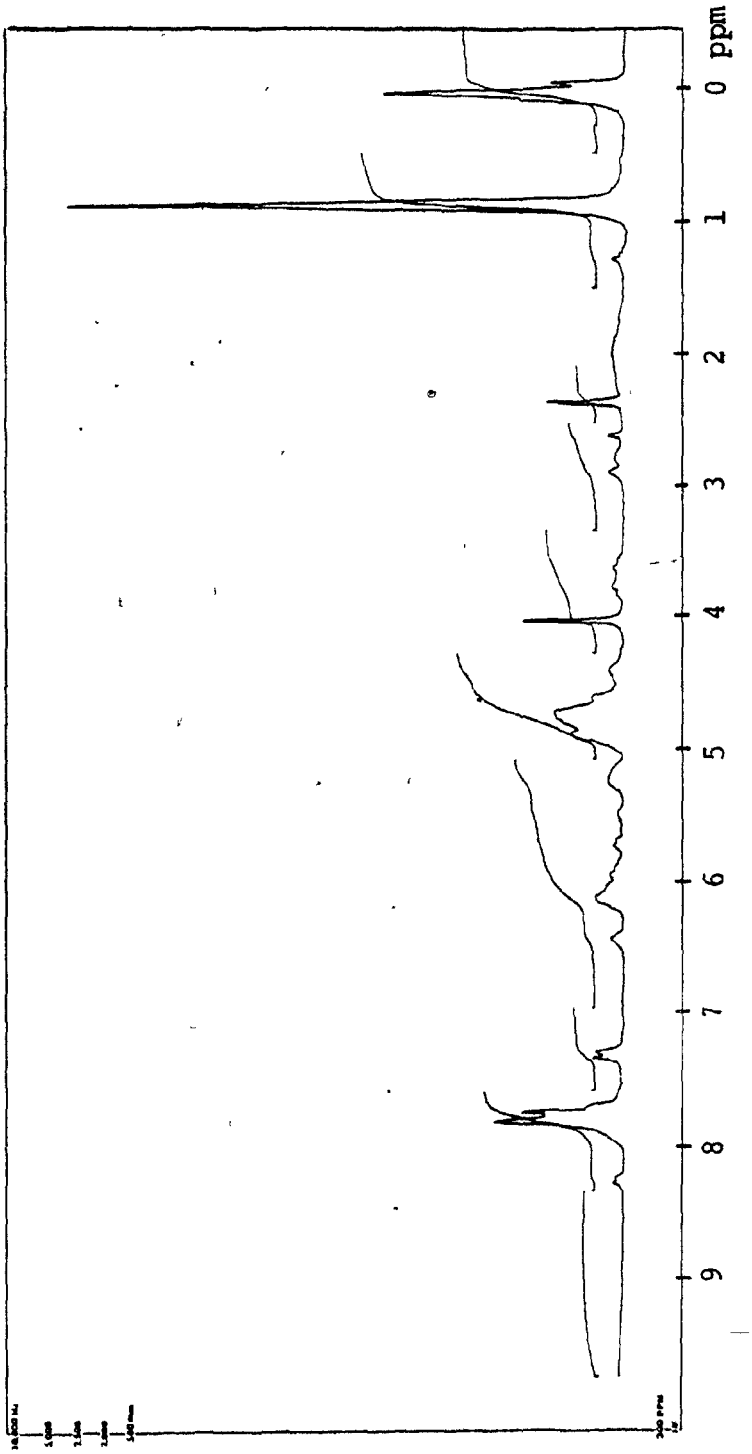


Fig. 4: 200 MHz PMR of MMT $U^{Si}_{1}U^{Si}_{1}U^{Si}_{1v}$ in $CDCl_3$.

2.4 DEPROTECTION AND CHARACTERIZATION OF RIBONUCLEOTIDES

The oligomers 24a, 27a, 28a and 29a were deprotected to the free nucleotides by first removing the levulinyl group using hydrazine hydrate for 10 min. The methoxytrityl group was then removed using HOAc for 15-20 min at 90°C, followed by the removal of the trichloroethyl group using freshly prepared Zn/Cu couple in DMF at 55°C for 6-13 h. The silyl protecting groups were finally removed with TBAF as has been previously reported[41,42]. It is worthwhile mentioning that as the chain length increased, it became more difficult to separate the nucleotides from the Zn-Cu by the usual means of paper chromatography. However, the problem was nicely resolved by applying the reaction mixture after the Zn-Cu treatment to pre-adsorbant Analtech silica-gel plates. The plates were developed in chloroform-methanol mixtures. Under these conditions, oligonucleotides separated cleanly as a tight band while the salts remained at the origin. Nucleotides were recovered by eluting the silica gel with methanol. All deprotected nucleotides were treated with Pancreatic Ribonuclease (Rnase A) to confirm the fidelity of the 3'-5' phosphate linkage. Rnase A is specific for 3'-5' linked ribonucleotides which it degrades to the nucleoside and nucleotide 3' phosphate. Oligomers 24e, 27e, 28e and 29e were completely digested by Rnase A to give the expected ratios

of uridine and uridine 3' phosphate.

The properties of deprotected nucleotides along with their enzyme degradation ratios are summarized in Table 19 (experimental).

In addition, the fully protected nucleotides 24a, 27a and 28a were characterized by ^{252}CF plasma desorption mass spectroscopy [99] (PDMS). As an example, the negative ion spectrum of 27a is illustrated in Fig. 5 and most relevant peaks are summarized in Table 4.

Table 4. Negative Ion Peaks of $MMTU^{si}_{p}U^{si}_{p}U^{si}_{p}U^{si}_{lv}$

M_{exp}	M_{calc}	Assigned Structure
2419.56	2419.85	$(M + Cl)^-$
2383.59	2383.39	$(M - H)^-$
2347.77	2348.94	$(M - Cl)^-$
2252.58	2254.01	$(M - TCE)^-$
1769.69	1770.58	$[pU^{si}_{p}U^{si}_{p}U^{si}_{lv}]^-$
1733.68	1733.46	$[MMTU^{si}_{p}U^{si}_{p}U^{si}_{p}]$
1392.95	1393.01	$[MMTU^{si}_{p}U^{si}_{p}]^-$
1218.65	1218.76	$[pU^{si}_{p}U^{si}_{lv}]^-$
841.64	841.18	$[MMTU^{si}_{p}]^-$
667.39	666.93	$[pU^{si}_{lv}]^-$

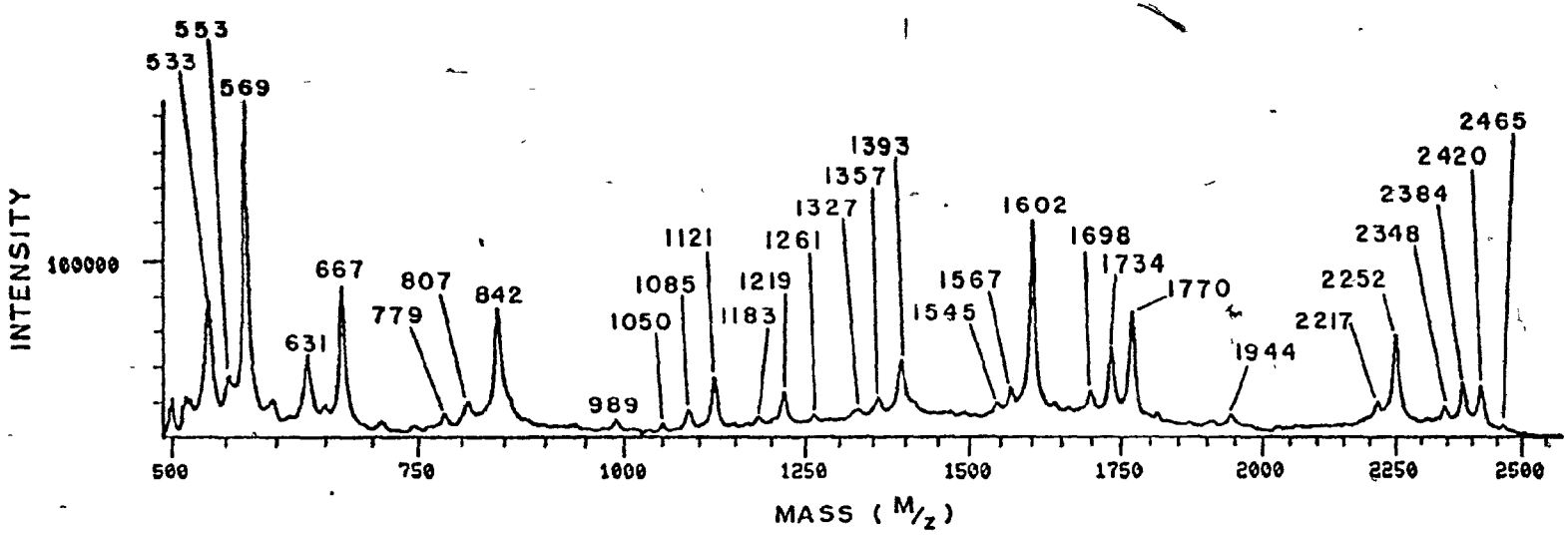


Fig. 5. Negative ion spectrum of MMT $U^{Si}\rho U^{Si}\rho U^{Si}\rho U^{Si}\rho Ulv$.

2.5 CONCLUSION

The formation of the internucleotide bond is one of the most important steps in nucleotide synthesis. The best coupling is one that gives the highest yields in the shortest time with very few side products. Side products and unreacted starting material lead to a lengthy purification process.

The procedure outlined in this chapter clearly demonstrates that the levulinyl group combined with the t-butyl dimethyl silyl group provides an ideal system for protecting nucleosides which in turn are useful in block condensations. Block coupling in the phosphite procedure led to rapid preparation of oligoribonucleotides on relatively large scale. The efficiency of the method was evaluated in the successful synthesis of a hexadecauridylic acid. The hexadecamer is the longest ribonucleotide reported in the literature, which was prepared by purely chemical means.

CHAPTER 3

SOLID PHASE SYNTHESIS OF RIBONUCLEOTIDES

3.1 INTRODUCTION

The procedure outlined in the previous chapter led to an efficient chemical preparation of oligoribonucleotides, however, it still involved time consuming purification steps. Solid phase synthesis of nucleotides offers the advantage that simple washing of the insoluble support after each step replaces the laborious purifications by silica gel chromatography.

There are three major considerations in any procedure involving solid phase synthesis of nucleotides. They include:

1. Choice of the support.
2. Strategy of phosphorylation and chain extention.
3. Removal of nucleotides from the support.

All three points were investigated and will be fully discussed in this chapter. In addition, the stepwise synthesis of a nonadecamer corresponding to bases 9-27 of t-RNA_f^{Met} from E.Coli will be described to illustrate the efficiency of the procedure.

3.2 CHOICE OF THE SUPPORT AND ITS DERIVATIZATION

Classically, a major problem with solid phase synthesis has been the choice of the support. In the past, various polymers have been tested, but most of them have been shown to be inadequate. Problems encountered vary from slow diffusion rates into the support, excessive swelling of various macroporous resins to irreversible adsorption of reagents onto the polymer[50].

Since the most attractive feature of solid phase synthesis is the prospect of automation, rigid, nonswellable polymers appear to be the most suitable for nucleotide synthesis. These polymers, eventually, can be packed into a column and the reactants merely pumped through the column.

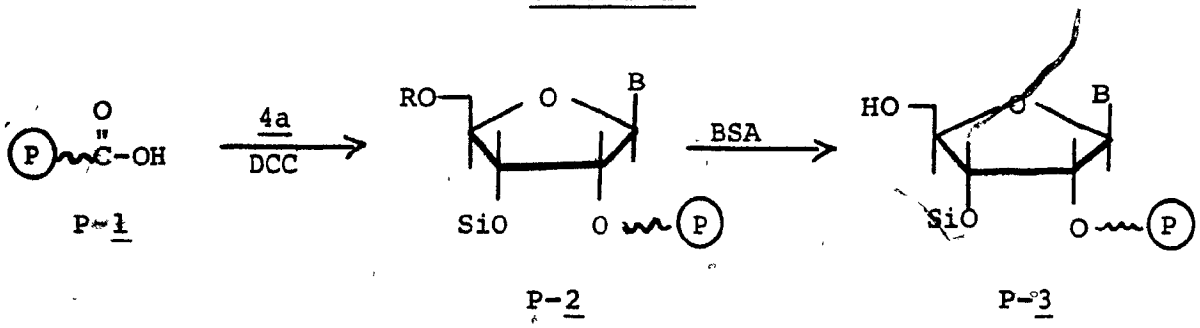
An early report by Köster[51] on the use of silica gel as a support for nucleotide synthesis along with the recent development of surface activated silica gels in general[100,101] prompted a full reinvestigation of the utility of silica gel as a solid support for nucleotide synthesis. The polymer P-1 used in this study was a generous gift from BioLogicals and was identical to the one that was being used at BioLogicals for deoxynucleotide synthesis[102]. The silica gel used was hplc grade because it was anticipated that reactants and reagents could be rapidly removed from this polymer. The derivatization of the silica gel to contain a carboxylic acid function is

outlined in Scheme 15.

The initial step involved activating the silica gel surface by refluxing with conc. HCl. The silica gel was then functionalized by first refluxing with 3-aminopropyltriethoxysilane in dry toluene. The resulting amino polymer was reacted with succinic anhydride to generate P-1. Finally, excess silanol groups were eliminated by treatment with trimethylchlorosilane. The amount of carboxylic acid groups was estimated to be 297 μmole/g of silica.

Nucleosides were linked to the polymer through the 2' or 3' hydroxyl group (Scheme 16).

SCHEME 16



P-1 P-2 P-3

R = MMT

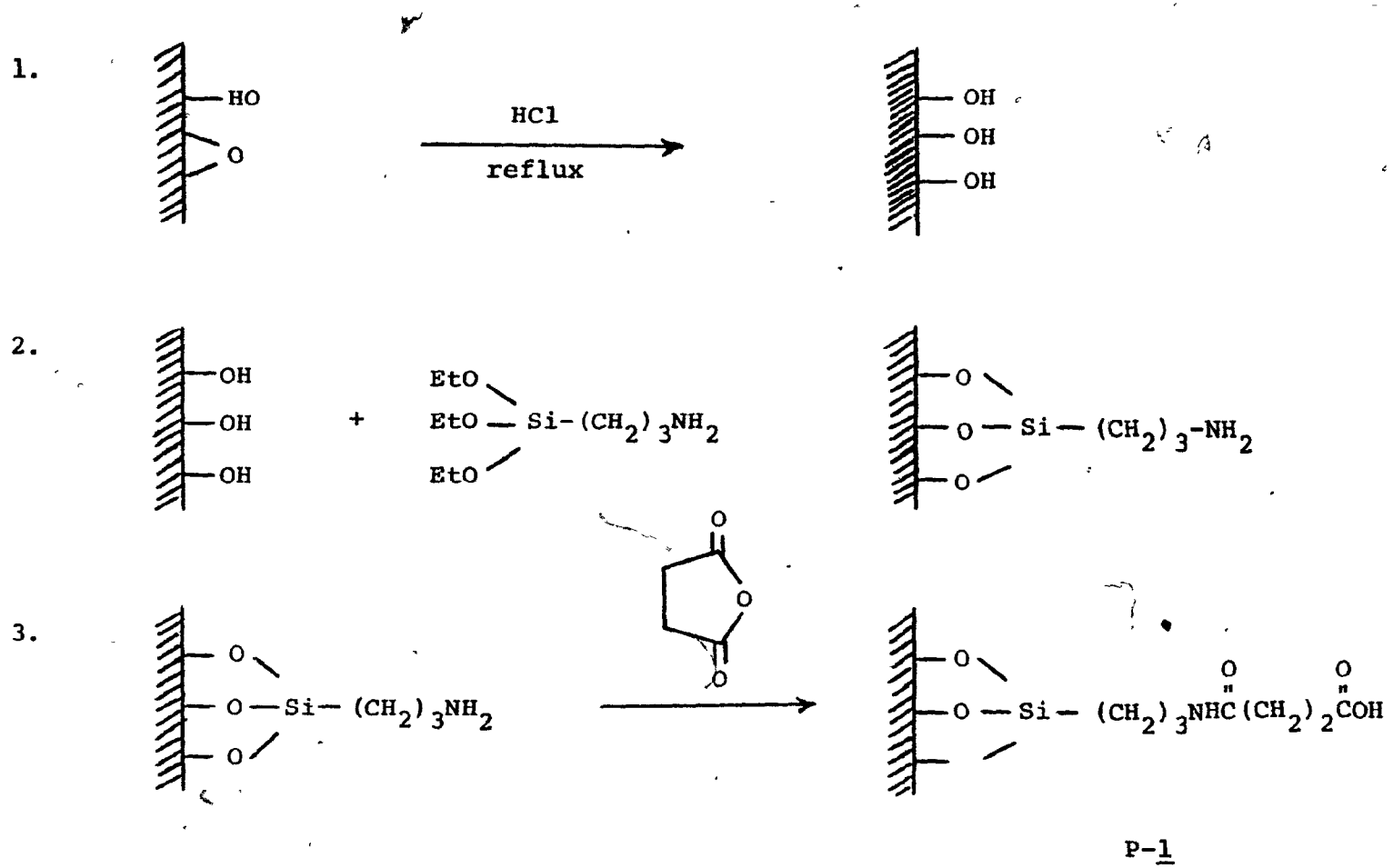
a, B = Ur

b, B = Ad

c, B = Cy^{Bz}

d, B = Gu^{Bz}

SCHEME 15: Derivatization of Silica Gel Polymer.

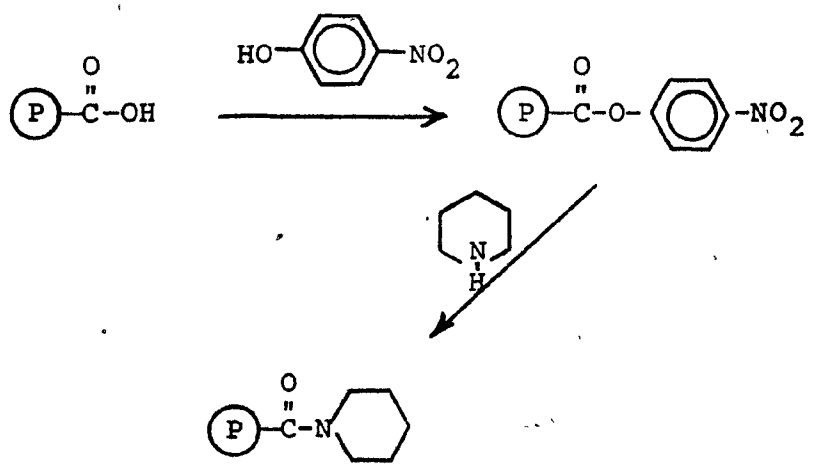


P-1

Linkage via the 2' hydroxyl was the preferred route since it gave about 30% higher yields of functionalized polymer P-2. This is presumably due to greater steric hindrance at the 3'-OH.

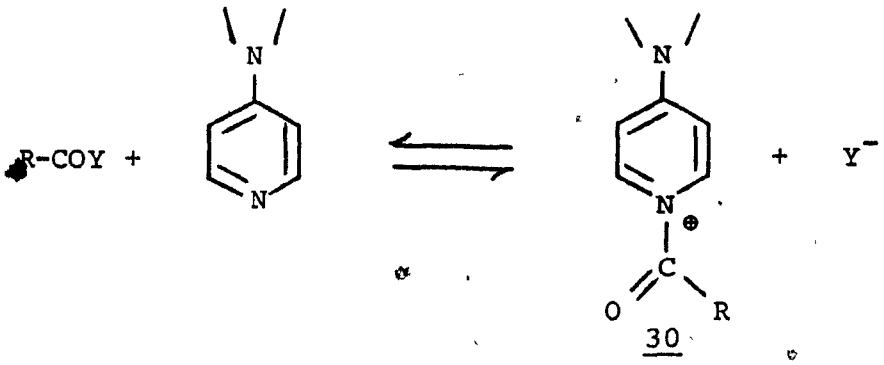
The coupling reaction outlined in Scheme 16 involved treatment of P-1 with a 5'-MMT-3'-TBDMS nucleoside (18, 2 eq.), DCC (10 eq.) and 4-dimethylaminopyridine (DAP, 25 mg/g) in pyridine. After 48 h, residual acid groups were converted to an inert amide by addition first of p-nitrophenol and then piperidine (Scheme 17).

SCHEME 17



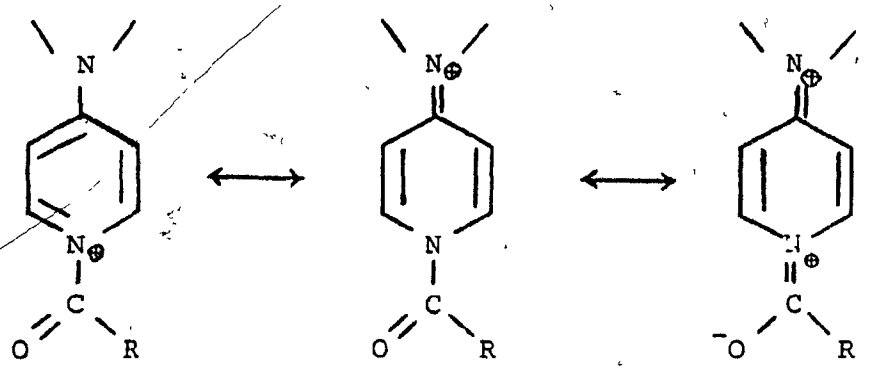
It is interesting to note that yields of P-2 were reduced five times when the acylation shown in Scheme 16 was carried

out in the absence of 4-dimethylaminopyridine. The catalytic role of DAP in numerous reactions has been recently[103] investigated and its effect on acylations is well documented. It is believed that the enormous catalytic activity of DAP is largely due to the formation of the N-acylpyridinium salts (30) which, because of their charge, are more capable of transferring an acyl residue to a nucleophile than the anhydride or even the acyl chloride.



30

In the case of DAP, the above equilibrium is more shifted to the right (compared with pyridine) due to mesomeric stabilization of the N-acyl-4-dialkylaminopyridinium ion 30 as shown below.



Polymer P-3, was obtained from P-2 after treatment at room temperature for 20 min with a 0.05 N solution of benzenesulfonic acid in acetonitrile. These conditions usually led to more than 50% depurination of deoxyadenosine. Adenosine, however, is more stable to acid because of the inductive effect of the 2'-OH on the glycoside bond[104]. In test experiments (monitored by tlc), depurination of MMT A was detected only after two hours of stirring with 0.05 N BSA. Derivatives of N-benzoylcytidine are also susceptible to acid. They decompose under certain acid conditions - like 80% acetic acid - to the corresponding cytidine and uridine derivatives[105]. However, no conversion of N-benzoylcytidine to either cytidine or uridine has been observed upon removal of the methoxytrityl group with 0.05 N BSA in 20 min.

Others have used different conditions to remove dimethoxytrityl groups from deoxynucleosides. Matteuci and Caruthers[106] and Köster and co-workers[107] have reported the use of zinc bromide in nitromethane, Gait et al.[108] have used a 10% solution of trichloroacetic acid in $CHCl_3$ and Cramer and Catlin[109] have shown that trifluoroacetic acid can be substituted for trichloroacetic acid.

Although these conditions look very interesting, the use of benzenesulfonic acid proved to be superior in the ribose area, mostly because of shorter reaction times.

The amount of nucleoside covalently bound to the polymer was

determined spectrophotometrically. An accurately weighed amount of P-3 (usually 50 or 100 mg) was stirred at room temperature with concentrated ammonium hydroxide and ethanol. The nucleoside was released from the support, within 2 h. However, in the case of P-3c and P-3d, treatment with ammonium hydroxide was extended to 16 h to remove the benzoyl group from the base. Washing the polymer after this deblocking step must be carried out with extra care. Deprotected nucleosides (only the TBDMS group is left) tend to "stick" to the silica gel surface due to their polar nature. This is even more so with deprotected nucleotides. It is important to stir the polymer with ethanol for a few minutes before filtering, and to repeat this procedure at least three times. To illustrate the significance of proper washing of the gel, 100 mg of P-3a were treated with conc. ammonium hydroxide-ethanol (3 ml, 2:1) for 2 h. The suspension was filtered, and 2 x 3 ml of ethanol were passed through the silica gel in a scintered glass funnel. The amount of nucleoside liberated was estimated to be 26 μmole. The gel was then stirred with 5 ml of ethanol and filtered; this operation was repeated three times. The amount of nucleoside that had not been washed off the support the first time was 49 μmole.

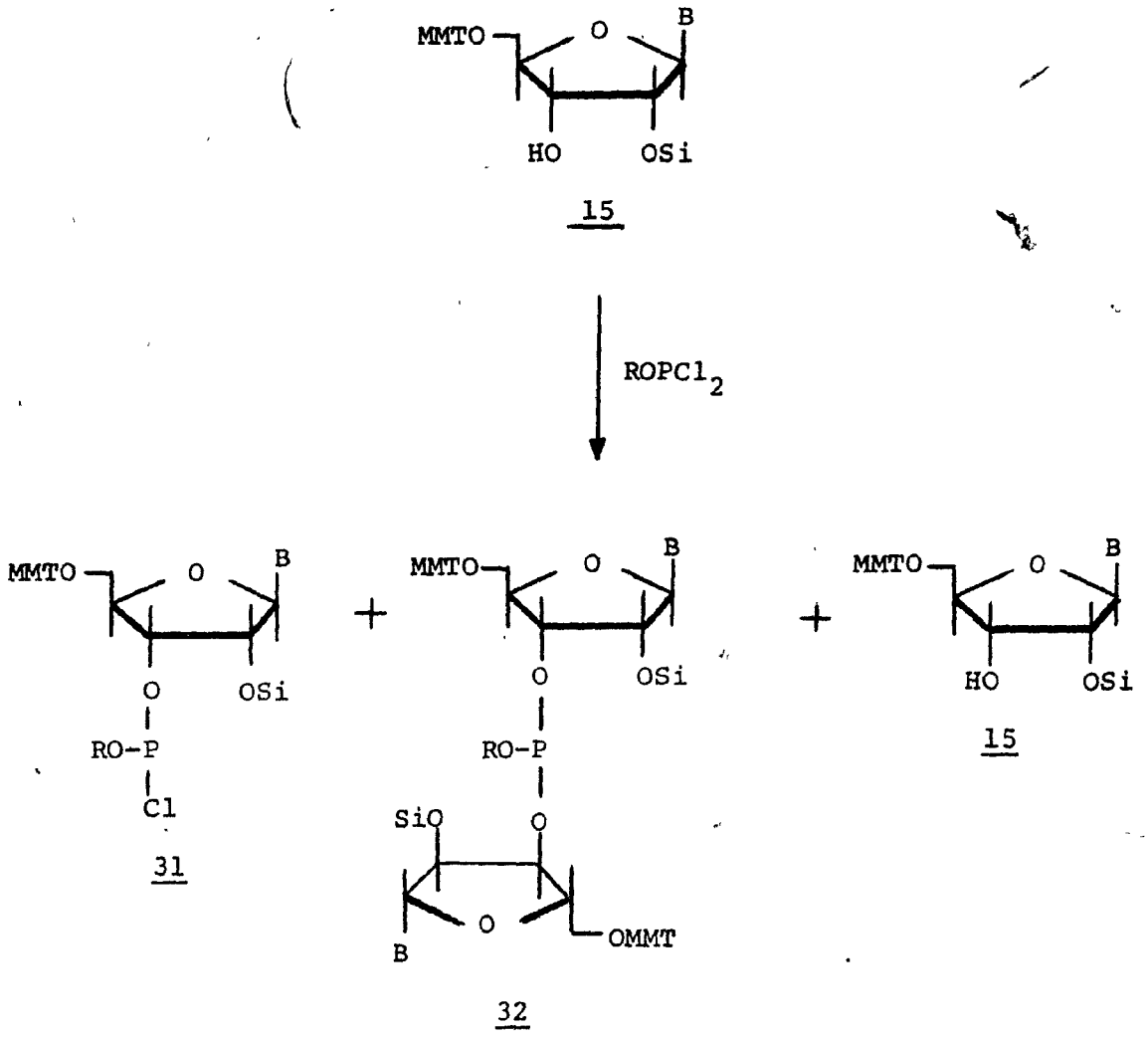
The amount of nucleoside bound to the polymer varied from 48 μmole of A/g of gel to 91 μmole of C. The results for all four common ribonucleotides are listed in Table 20 (p. 196).

3.3 PRELIMINARY INVESTIGATIONS ON PHOSPHORYLATION AND CHAIN EXTENTION

The phosphite procedure[38,40], was the method of choice for polymer supported ribonucleotide synthesis. Reactions, in the solution phase, have been shown to proceed rapidly and in high yields. They can be carried out at temperatures ranging from -78° to 22°C and in a number of solvents including THF, DMF, and pyridine[44]. Moreover, the only by-products formed during these reactions, the 3'-3' symmetrically linked dimers, would not be a problem in the solid phase synthesis.

Indeed, the first step in the phosphite procedure involves phosphorylation of the 3'-OH of a nucleoside - or nucleotide - with a highly reactive dichlorophosphite. This step, usually, generates three products (Scheme 18). The major product 31 is the only species that will add to the free 5'-OH of a growing chain. Both the starting material 15 and the by product 32 are inert toward further reaction. When the growing chain is attached to a solid support, only 31 will chemically react with its 5' end. Compounds 15 and 32 are simply washed off along with any excess 31. Since in a multistep solid phase synthesis, yields should be as close to 100% as possible, an excess of 31 can be used to drive the addition reaction to completion.

SCHEME 18: First Step in the Phosphite Procedure

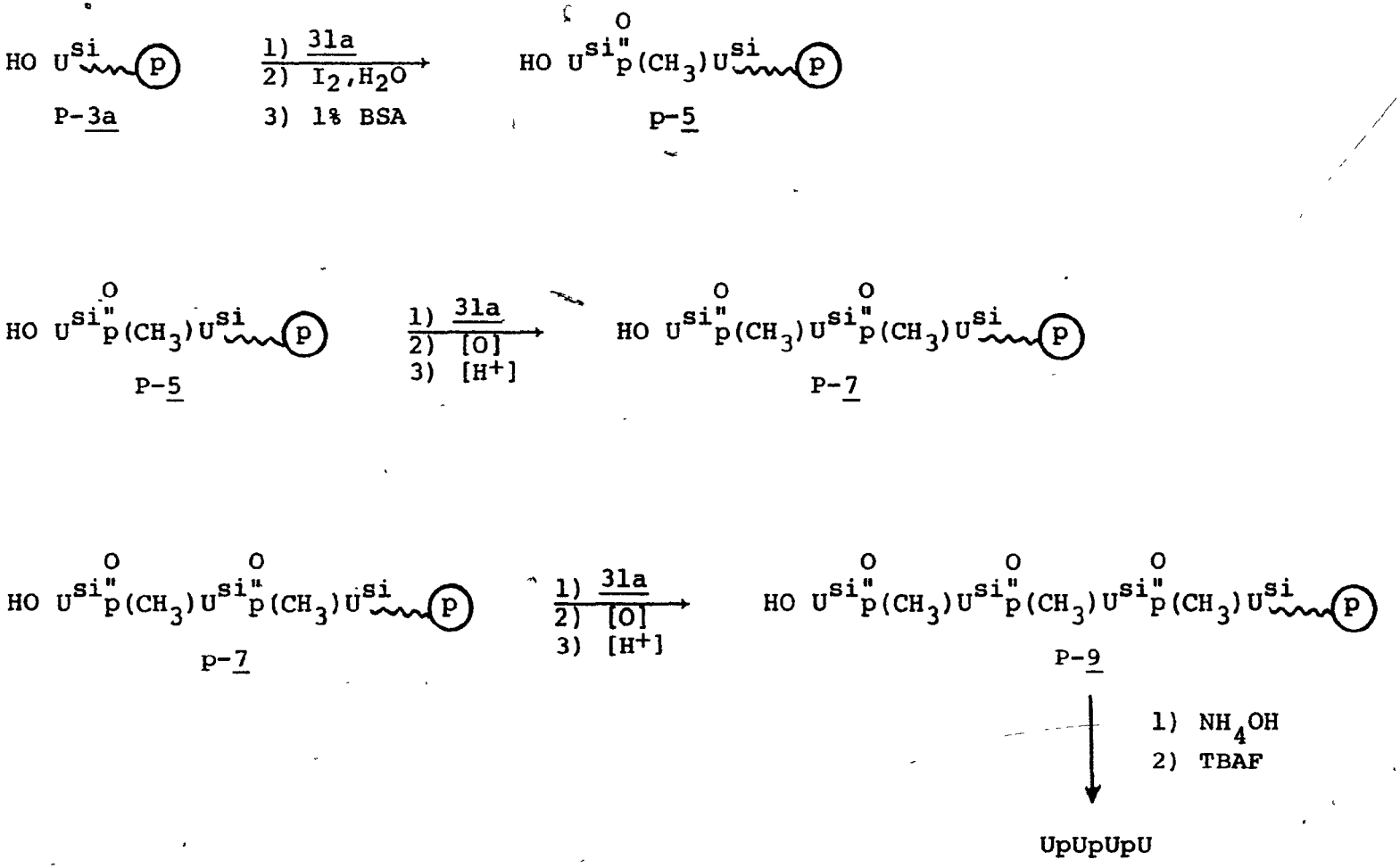


The methyl moiety was favored as a protecting group for the internucleotide phosphate linkages, mainly because its removal is easy and does not generate problems[44]. There are at least three known methods for deblocking methyl triesters without causing degradation of the nucleotidic chain: exposure to thiophenoxide for 5 min[39], or treatment with t-butylamine for 18 h[110]. In addition, the methyl group can be removed with conc. NH_4OH within one hour[111]. No chain degradation was observed under these last conditions[66] since the elimination of the methyl proceeds via an SN2 displacement at carbon. This observation was very interesting since treatment with conc. NH_4OH would not only release nucleotides from the support but also remove both methyl and benzoyl protecting groups.

3.3.1 Stepwise Synthesis of a Tetrauridylic Acid

The adaptation of the phosphite procedure to a polymer supported synthesis of ribonucleotides was first investigated in the stepwise synthesis of the tetramer UpUpUpU (Scheme 19). The first step in the general procedure was the preparation of the activated nucleotide 31a. Compound 31a was prepared by adding a THF solution of 15a to a solution containing CH_3OPCl_2 (0.9 eq.) and collidine (4 eq.) in THF. In contrast to solution phase synthesis, CH_3OPCl_2 was not used in excess.

SCHEME 19: STEPWISE PREPARATION OF A TETRAMER ON A POLYMER SUPPORT



In solid phase synthesis, excess CH_3OPCl_2 would produce side reactions - like phosphorylation of the 5'-OH - that are carried through to the next steps. On the other hand, any unreacted 15a is eliminated by washing the gel and does not cause any further problem. Phosphorylation of 15a was carried out at -78°C to minimize the formation of the 3'-3' dimer 32a. The reaction was generally over in 10 min. The activated nucleotide 31a was used without any purification for step 2. In the second step, the freshly prepared mixture of 31a was added to a suspension of P-3a in THF, at -78°C.

After 30 min, the reaction was allowed to warm to room temperature for 15 min. The mixture was oxidized with a solution of iodine in THF-H_2O for 5 min. The gel was then filtered and washed several times with CH_2Cl_2 and finally with ether. At this point, 100 mg of the polymer were treated with a 0.05 N solution of BSA in acetonitrile. A strong yellow color appeared corresponding to $[(CH_3O-C_6H_4)(C_6H_5)_2C]^+$, the cation of the methoxytrityl group. The yellow color is a qualitative indication of a successful coupling. When the detritylation was over (20 min), the gel was washed and then treated with conc. NH_4OH in ethanol for 2 h. The liberated nucleotide and nucleoside were completely deprotected by treatment with TBAF to remove the silyl groups. The mixture was purified by paper chromatography and the yields were

determined spectrophotometrically as % conversions. The yield of UpU was 92.5%. The remainder of the polymer was dried by stirring for 2 h with a 1% solution of phenylisocyanate in pyridine[53]. Phenylisocyanate would also block any unreacted 5'-OH. The gel was then detritylated and dried under high vacuum for at least 1 h before further reaction. The general procedure was repeated for the trimer UpUpU (94% yield) and finally the tetramer UpUpUpU which was isolated in an overall yield of 76.8% from the first nucleoside. The deprotected dimer, trimer and tetramer were identical to previously prepared samples[112]. They were also completely digested by pancreatic ribonuclease to U and Up in the expected ratios.

In the preparation of the dimer, a five-fold excess of the activated nucleotide 31a was used. This was decreased to a two-fold excess in the synthesis of the trimer and the tetramer. The large excess used in the first condensation was apparently not necessary as determined from the yields of both trimer and tetramer. A summary of all condensation steps is given in Table 5.

3.3.2 Synthesis of a Tetrauridylic Acid by Block Condensation

After the initial success with the stepwise synthesis, the preparation of the tetramer using a block condensation

Table 5 . Summary of the Stepwise Preparation of UpUpUpU on the Polymer.

Polymer (g)	Nucleoside	CH_3OPCl_2	Collidine	Product	(% Yield)
HO U^{si}-(P) (P- 3a, 2g)	MMT U^{si} (15a, 630 mg, 1 mmole)	86 µl	0.6 ml	MMT $U^{si}pU^{si}$-(P)	92.5
HO $U^{si}pU^{si}$-(P) (P- 5 , 2 g)	15a (315 mg, 0.5 mmole)	43 µl	0.3 ml	MMT$[U^{si}p]_2U^{si}$-(P)	94.5
HO $U^{si}pU^{si}pU^{si}$-(P) (1.9 g)	15a (315 mg, 0.5 mmole)	43 µl	0.3 ml	MMT$[U^{si}p]_3U^{si}$-(P)	90.0

p = $\overset{O}{\overset{\|}{p}}OCH_3$

approach was attempted.

Block condensations offer the advantage of speed, both in the assembly of long chains and the purification of the final product. The idea has been recently exploited by several workers in the solid phase synthesis of deoxy nucleotides[54,55,62]. Dinucleotides are usually used as building units, since it is relatively easy to prepare large quantities of the 16 possible dimers.

Block condensations using the phosphite procedure have resulted in good yields of ribonucleotides in the solution phase (see previous chapter). In an effort to adapt this approach to polymer supported ribonucleotide synthesis, the preparation of the tetramer UpUpUpU was undertaken. The efficiency of the method was evaluated by comparing it with the stepwise synthesis of the same molecule.

The strategy of the synthesis is outlined in Scheme 20. It involves adding an activated dinucleotide to a dimer, already linked to the polymer.

The general procedure was very similar to the one described for the stepwise addition, except that the reaction was carried out at 0°C, as is usual for block condensations. The time of the reaction was identical to the reaction time used in section 1. A summary of the condensation reactions used in the preparation of the tetramer is given in Table 6. The dinucleotide 24b was phosphorylated with CH_3OPCl_2 at 0°C

SCHEME 20: Block Condensation on a Polymer Support

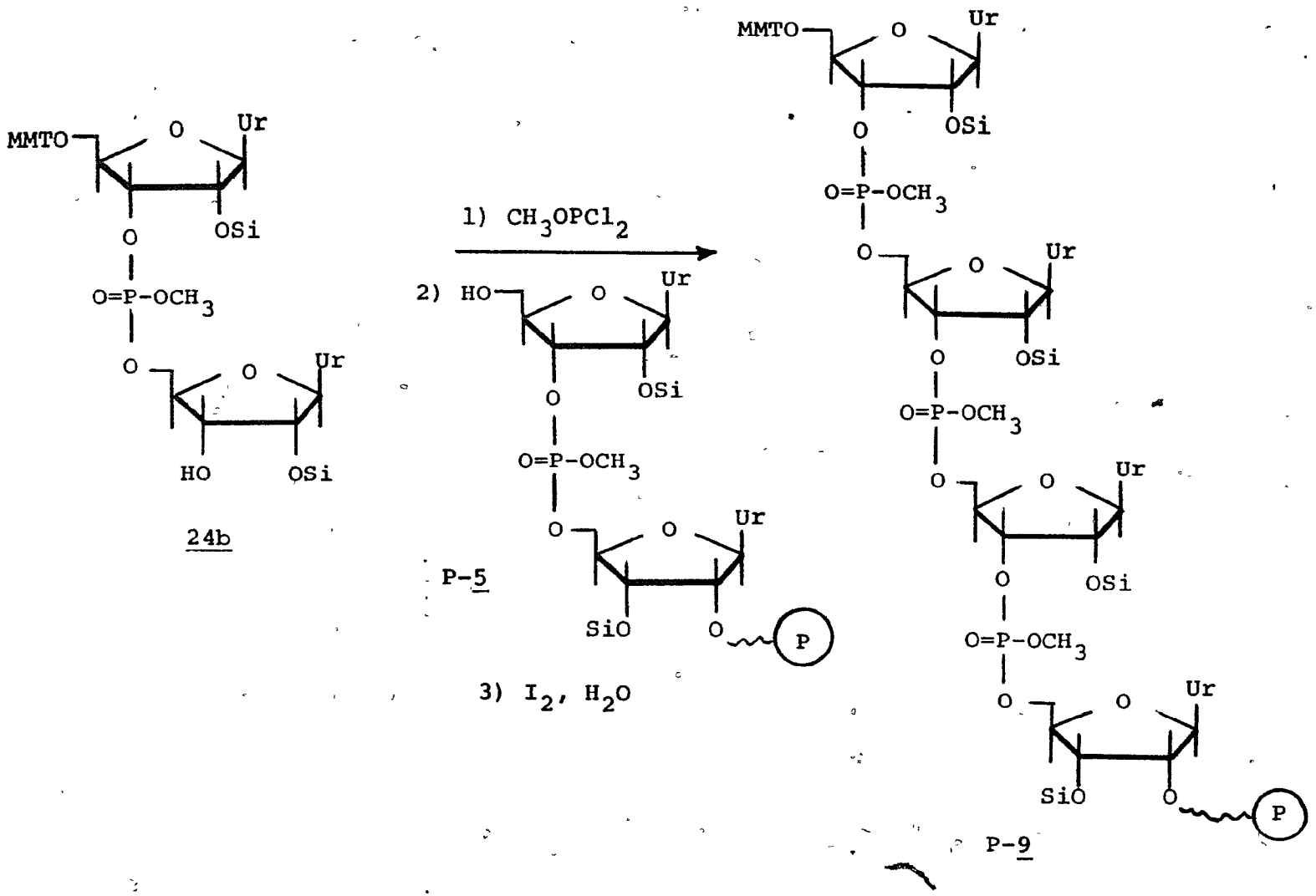


Table 6 . Summary of Block Condensation on the Polymer.

Polymer (g)	Nucleoside (or nucleotide)	CH_3OPCl_2	Collidine	Product	(% Yield)
HO U^{si}–(P) (P-3a, 2g)	MMT U^{si} (15a, 315 mg, 0.5 mmole)	43 µl	0.3 ml	MMT $U^{si}\rho U^{si}$–(P)	92.5
HO $U^{si}\rho U^{si}$–(P) (P-5, 1g)	MMT $U^{si}\rho U^{si}$ (24a, 160 mg, 0.12 mmole)	12 µl	0.1 ml	MMT $[U^{si}\rho]_3U^{si}$–(P)	71.0

ρ = $\overset{O}{\overset{\|}{P}}$–$OCH_3$

for 15 min. The mixture of the activated dinucleotide was added to the growing dimer on the polymer (P-5). This coupling was stopped after 45 min, and the subsequent steps were identical to the ones described in the synthesis of UpU. In this case, however, only 1.5 eq. of 24b were used to avoid the loss of large amounts of this valuable starting material. It was also rationalized that a smaller excess would be required since the yield of activated dinucleotide would not be reduced by the formation of a 3'-3' linked by-product (see discussion of chapter 2).

The yield of the coupling step was 71% and the overall yield of the tetramer from P-3a was 65.5%. Although this is a respectable yield, it is slightly lower than the yield obtained in the stepwise synthesis. However, it should be remembered that the reaction scale was smaller than in the previous preparation of the same tetramer. Only 12 µl of phosphite were used compared to 43 µl in the stepwise coupling. The yield can be probably optimized by using a larger excess of 24b - perhaps a one-fold excess - and carrying out the reaction on a larger scale.

3.3.3 Synthesis of the Hexamer AGCUCG

The procedure described in section 1 was extended to the synthesis of the ribonucleotide hexamer corresponding to

units 22-27 of t-RNA$_f^{Met}$ of E.Coli (Fig. 6, p. 77). The hexamer AGCUCG contains at least one unit corresponding to the four common ribonucleosides. The synthesis of the hexamer was initiated by coupling the activated cytidine nucleotide 31c with the polymer P-3d functionalized with the guanosine derivative. The chain was subsequently elongated in the 5' direction upon removal of the terminal monomethoxytrityl group and addition of the following nucleoside. A two-fold excess of nucleoside was used in each coupling step. Reactions were carried out at -78°C and the total time per addition was maintained at 45 min from the dimer to the hexamer stage. The progress of the synthesis was monitored by deblocking 100 mg of gel after each addition and analyzing the products obtained. All intermediate nucleotides and the final product were purified by paper chromatography. Yields ranged from 85 to 98%. Table 22 (experimental) lists the properties and yield of all five ribonucleotides isolated during the synthesis.

From Table 22, it can be seen that yields, within experimental error, did not drop as the chain length increased and all four ribonucleotides reacted equally well.

An important feature of the synthesis - as already pointed out - was the thorough drying of all reagents and reactants. Silica gel retains water easily, and careful drying of the polymer was an essential step that preceeded

all condensations.

Thorough washing of the polymer was also very important, particularly after the deblocking of nucleotides from the support. Deprotected nucleotides have a high affinity for silica gel and the longer the chain gets, the harder it becomes to wash it from the support. Thus, improper washing of the hexamer yields mostly the unreacted nucleoside, the smaller oligomers and only a fraction of the final product. This in turn leads to inaccurate assessment of the reaction progress.

The deprotected nucleotides were further characterized by their degradation with various enzymes. The dimer and tetramer were completely digested by Rnase A whereas the hexamer was degraded by Rnase T_2 (Table 23, experimental).

3.4 SYNTHESIS OF THE NONADECAMER GGAGCAGCCUGGUAGCUCG

The utility of the polymer supported synthesis of ribonucleotides was demonstrated in the rapid synthesis of the hexamer AGCUCG. However, the true value of a new synthetic approach must always be tested with the synthesis of polynucleotides of significant length and defined sequence. In order to prove that the procedure described in this chapter is, so far, the most efficient route to synthetic polyribonucleotides, the preparation of a nonadecaribonucleotide was undertaken. The nonadecamer corresponds to units 9-27 of

t-RNA$_f^{Met}$ of E.Coli, except that uridine was used instead of dihydrouridine at position 21 (Fig. 6).

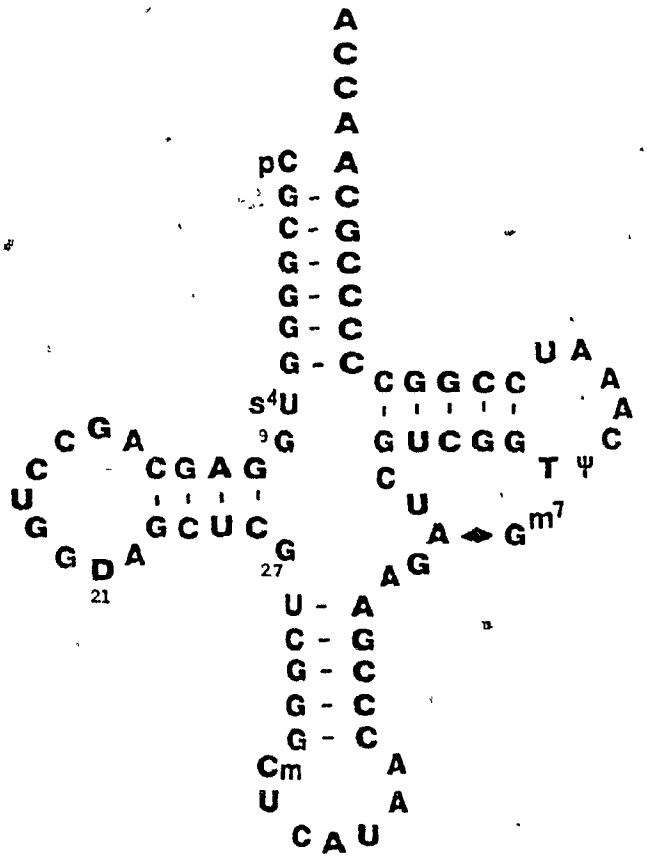


Fig. 6

t-RNA$_f^{Met}$ from E.Coli

The chemical principles involved in this synthesis were identical to the ones previously outlined (section 1 and 3). The essential features were:

1. The use of a derivatized silica gel as the solid support.
2. The use of the methyl group as a phosphate masking group.
3. Stepwise addition of 3'-phosphorylated nucleosides to the 5' end of the growing chain using the phosphite procedure.

4. Treatment of the gel with phenylisocyanate after the coupling reaction and before detritylation.

The general procedure was basically as previously detailed except that shorter reaction times were used; 1) for the coupling of the activated nucleoside to the growing chain and 2) for the treatment of the gel with phenylisocyanate. Khorana and Agarwal[194] had previously reported that the exocyclic amino group of deoxyadenosine reacts quantitatively with a 10% solution of phenylisocyanate in dry pyridine within 1 h. The resulting N^6-phenylureidodeoxyadenosine derivative has á u.v. absorption maxima at 278 nm which is very different form that of the starting material at 258 nm. However, when 5',3',2'-tri-O-TBDMS adenosine (50 mg) was reacted with a 1% (v/v) solution of phenylisocyanate in pyridine (5 ml), the starting material remained unchanged after 1 h. Furthermore, polymer bound adenosine (P-3b) was stirred with the 1% solution for 1 h, filtered, washed and treated with concentrated NH_4OH to cleave the nucleoside from the polymer. The released adenosine derivative had the same u.v. spectrum as adenosine indicating that no reaction had occurred between the base amino group and phenylisocyanate.

The addition cycle was the same throughout the sequence, and required a total of 90 min. The steps involved in each cycle are listed in Table 7. The progress of the synthesis was followed qualitatively by the appearance of a strong yellow color during the detritylation step of each cycle. The amount of methoxytrityl released could be quantitated by measuring the optical density at 470 nm in 1%

Table 7 . Addition Cycle in Solid Phase Synthesis.

STEP	REAGENT OR SOLVENT	AMOUNT (ml/lg of gel)	TIME (min)
1	Phosphorylated nucleoside (31)	3 eq.	30
2	4% I_2 / THF:H_2O	2	2
3	(i) CH_2Cl_2	5	1
	(ii) Ether	3	1
4	1% (v:v) Phenylisocyanate in pyridine	5	30-60
5	(i) CH_2Cl_2	5	1
	(ii) Ether	3	1
6	1% BSA in CH_3CN	5	20
7	(i) CH_2Cl_2	5	1
	(ii) Ether		

BSA in acetonitrile[113].

This method has been used in the deoxy area, to semi-quantitatively estimate the coupling yields. However, yields estimated from the analysis of the trityl absorbance have been reported to be 26 to 50% higher than the yields actually obtained after releasing the nucleotide from the support[57,59,60]. Based on these observations, visible light spectroscopic analysis was omitted as it was judged not accurate enough to be useful.

The extension of the nucleotidic chain was analyzed at the dimer, hexamer and decamer stages. A small amount of gel (100-200 mg) was deblocked; the nucleotidic material was fully deprotected and purified by paper chromatography. The yields and properties of these intermediate oligomers are described in the experimental section.

The dimer and the hexamer were identical to the ones previously prepared and characterized[66]. The yield of the hexamer was much better than in the first preparation. When the crude mixture of hexamer was purified on paper, only two bands were detectable. The minor one, was unreacted G, and the other band turned out to be pure hexamer (Fig. 7). The overall yield of the hexamer (75%) suggests that all couplings after the dimer stage proceeded with more than 95% yield. However, the first coupling - i.e. formation of the dimer - rarely gave more than 90% yield. Several attempts were made to increase this yield by either using a five-fold excess of phosphorylated nucleoside or repeating the condensation several times. But no improvement was detectable. Itakura and co-workers[114] also observed lower yields of the first nucleotide addition to the polystyrene resin. The lower yields of the first coupling might be due to steric hindrance of the polymer backbone. As the chain length increases, the terminal 5'-OH is further away from the polymer surface, and the steric effect of the support becomes virtually nonexistent.

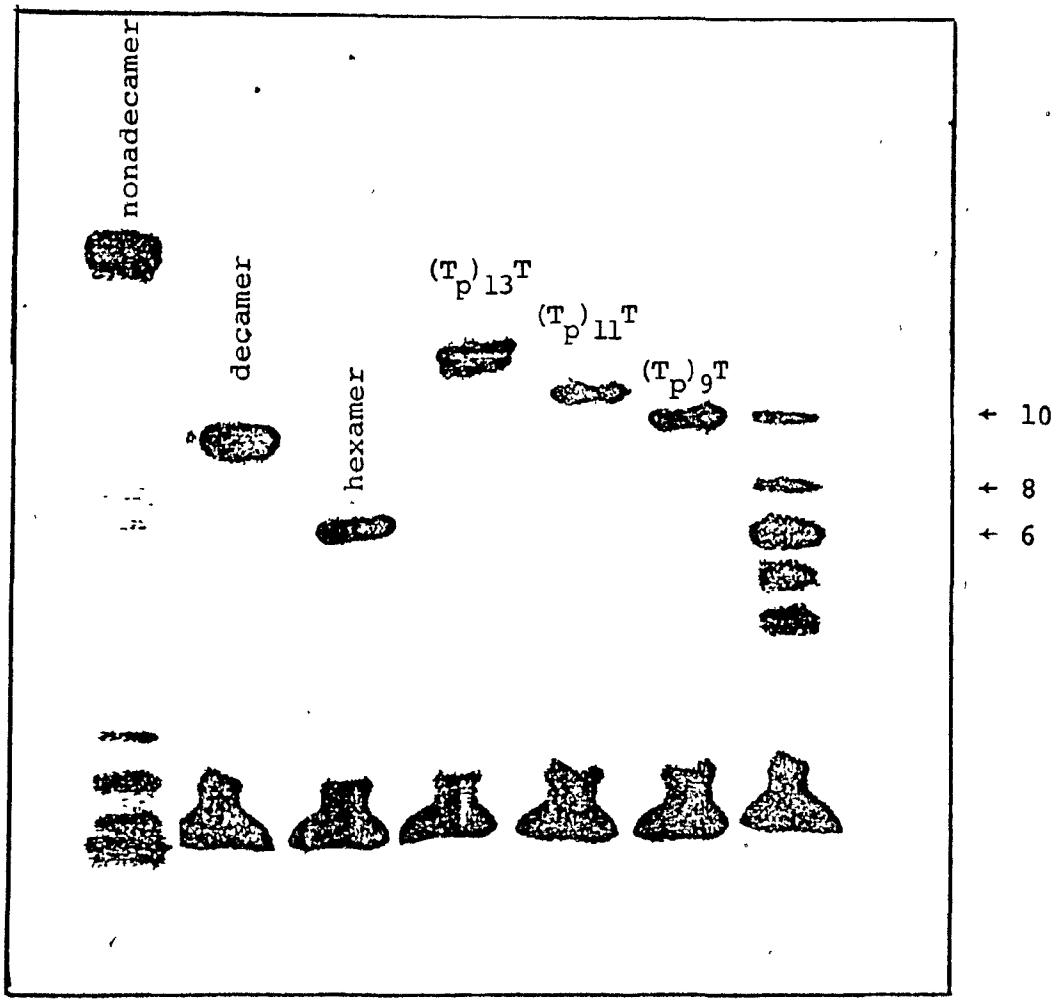


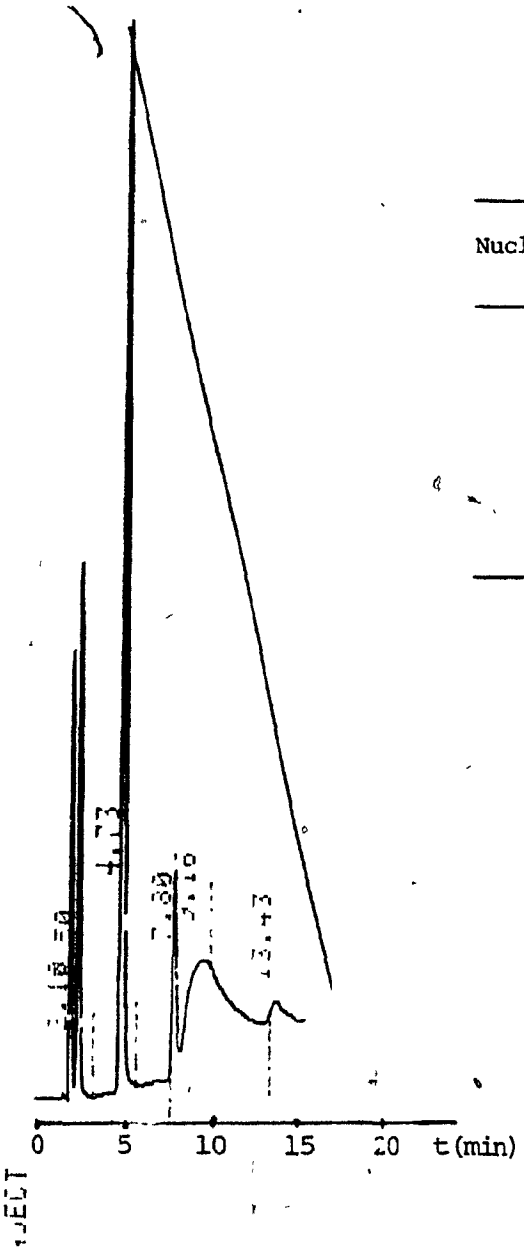
Fig. 7: Sizing gel of the oligoribonucleotides: from left to right, the nonadecamer, the decamer UGGUAGCUCG, the hexamer AGCUCG and $(T_p)_nT$ standards.

In the purification of UGGUAGCUCG, the decamer was again the only detectable nucleotidic material on paper chromatography, other than the unreacted first nucleoside. The purity of the paper purified decamer was checked by anion exchange hplc and further confirmed by gel electrophoresis. The sequence was verified by determining the relative ratios of the four constituent nucleosides. The ratios were obtained after simultaneous treatment of the oligomer with snake venom and phosphatase enzymes (Fig. 8a).

3.5 ISOLATION OF THE NONADECAMER

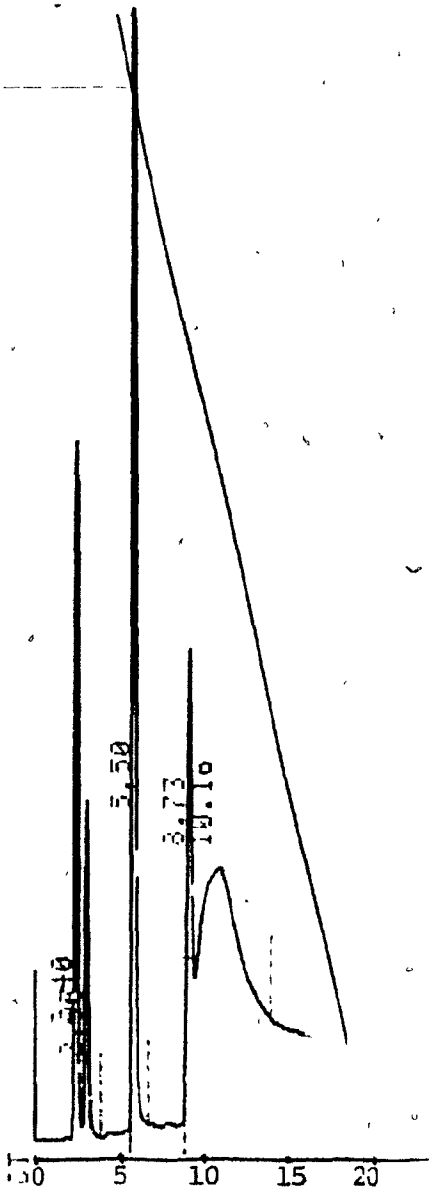
The target molecule was obtained in an overall yield of 35%. However, its isolation was rather complicated.

The nonadecamer could not be purified by paper chromatography alone, since it remained at the baseline even when the paper was developed for 48 h. Ion exchange high performance liquid chromatography alone was also inadequate since the products' peaks became very broad, almost undetectable from the base line as nucleotides increased in length. Others have reported the same observation and difficulties in separating deoxynucleotides larger than a decamer by ion exchange hplc [57]. Reverse phase hplc was not particularly useful either. When applied to the isolation of long deoxynucleotides, it has resulted in considerable losses



Nucleoside	Retention time	Absorbance corrected	Ratio
C	2.10	3255	2.0
U	2.50	5182	3.2
G	4.73	5983	3.7
A	7.80	1635	1.0

Fig. 8a: Base count of the decamer UGGUAGCUCG.



Nucleoside	Retention time	Absorbance corrected	Ratio
C	2.40	5983	4.9
U	2.93	3608	3.0
G	5.50	8987	7.5
A	8.73	3699	3.1

Fig. 8b: Base count of the nonadecamer GGAGCAGCCUGGUAGCUCG.

yield is high. The results of the present investigation suggest that this will not be an easy task. The concentrations of methoxyl and phenolic hydroxyl groups, both of which cause chemical activation, are lower in middle lamella lignin than in secondary wall lignin (Chapters IV, V, and VI). The middle lamella contains a much larger portion of residual lignin, which is difficult to pulp, than does the secondary wall. Moreover, it is unlikely that middle lamella lignin contains bonds which are not present in secondary wall lignin, and which might be preferentially broken by some new pulping procedure. Therefore, it seems that the best that can be done is to find methods in which the difference between the ratios of dissolution of the middle lamella and secondary wall lignin are minimized, as is the case with acid-chlorite pulping (Chapter VIII).

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CHAPTER X

THE MORPHOLOGICAL ORIGIN OF MILLED WOOD LIGNIN

ABSTRACT

Fractions of wood from the middle lamella and from the secondary wall of black spruce were subjected to ball milling and extraction with dioxane:water. The proportion of the lignin removed from the secondary wall fraction was approximately four times as great as that from the middle lamella fraction. These results indicate that the morphological origin of milled wood lignin in softwoods is the secondary wall of the tracheid.

INTRODUCTION

Over the years, many methods for isolating lignin from wood have been developed. Most of the methods involved chemical degradation and dissolution of the carbohydrate to yield the lignin as an insoluble residue[1]. However, the lignins obtained by these methods are known to be altered chemically[2]. In 1939, Brauns[3] discovered that a small portion of black spruce lignin is soluble in 95% ethanol in water. However, since the bulk of the lignin is insoluble in ethanol, Brauns native lignin probably represented only the very low molecular mass fractions of the lignin.

In 1956, Björkman[4] developed a method for the isolation of a soluble lignin in which the wood is vigorously ball milled in a non lignin-swelling solvent and the milled wood is extracted with dioxane and water. This method usually dissolves about 50% of the lignin in the wood. Björkman described the lignin as "a very useful material for lignin chemists" and, indeed, many researchers have made use of it in studies of lignin chemistry. However, there is probably some degradation of the lignin from the wood during the ball milling and subsequent dissolution[2]. Even more important to lignin chemists is the morphological origin of the milled wood lignin. Björkman himself[4] suggested that the bulk of the milled wood lignin comes from the middle lamella region of the wood and this view has been supported by others[5,6]. However, there is no direct experimental evidence indicating in which part of the wood tissue the milled wood lignin originates.

The purpose of the present work is to elucidate the morphological origin of milled wood lignin. Samples of black spruce (Picea mariana)

tissue enriched in middle lamella lignin and in secondary wall lignin have been prepared by the method described in Chapter II. Ball milling experiments have been carried out on these samples and the quantity of lignin extracted with dioxane:water has been measured.

EXPERIMENTAL

Fractions containing either the middle lamella or the secondary wall were prepared by the method described in Chapter II. The concentration of lignin in the middle lamella and secondary wall tissue fractions was 55 and 22%, respectively. Black spruce wood flour, with a lignin content of 27%, was also used..

Ball milling was carried out with an Apex No. 8000 Mixer/Mill. This mill was used because samples of small size could be treated in it. The wood sample, approximately 30 mg, was accurately weighed into the 50 mL stainless steel container which held seventy five stainless steel balls, 6 mm in diameter.

Twenty five millilitres of spectro grade toluene were then added to the container and the lid tightly closed. The container was cooled to 4° C and the sample was milled for 1.5 h in a room held at 4° C. It was found by trial and error that for this mill and sample size, 1.5 h ball milling gave results most similar to those obtained for black spruce by the method of Björkman[4]. Since the Apex ball mill was more violent than the vibratory ball mill used by Björkman, there was a noticeable temperature increase on ball milling in the Apex mill. When

the milling was done at a room temperature of 4° C, it was found that the temperature in the container rose only to 15 - 20° C after ball milling for 1.5 h.

After milling, the sample was washed from the ball mill with 200 mL fresh toluene, in 25 mL increments, into a 250 mL Nalgene bottle. The bottle was then centrifuged at 2000 rpm for 0.5 h. After centrifugation, the toluene was decanted from the solids and the sample was evaporated to dryness at 60° C in a vacuum oven.

After drying, 100 mL of dioxane:water (100:4) was added to the sample and the lid tightly closed. The sample was placed on a wrist shaker and shaken for the desired amount of time, usually 48 h. During the extraction, the shaker was stopped at various times and 5 mL samples were removed. The samples were centrifuged for 5 min at 2000 rpm and 1 mL of supernatant liquid was pipetted from the vessel. The sample was shaken and poured back in the bottle on the shaker and the 1 mL sample was diluted to the desired volume. The absorbance was then measured at 280 nm. The amount of lignin in solution was calculated from the absorbance using an absorptivity of 19.5 $L \cdot g \cdot cm^{-1}$[7].

The procedure described above was also carried out on a blank containing no wood. The absorbance of the blank was subtracted from the sample absorbance.

Ball milling and extraction were carried out twice on the middle lamella fraction, twice on the secondary wall fraction, and seven times on the whole wood. Values obtained were reproducible to $\pm$ 2% from sample to sample.

RESULTS AND DISCUSSION

The amount of lignin extracted into dioxane:water after ball milling, calculated as percent of the lignin originally present in the sample, is plotted against time in Figure 1. After 24 h, the percent lignin extracted from the secondary wall is 3.8 times as great as that from the middle lamella. Extractions were carried out for as long as two weeks and the ratio of percent lignin extracted from the secondary wall to percent lignin extracted from the middle lamella remained constant at about 3.8.

Assuming whole wood contains only compound middle lamella and secondary wall lignin, the amount of milled wood lignin extractable from the whole wood can be calculated from the amounts extracted from the middle lamella and secondary wall fractions. The amount of lignin that should be extracted from whole wood at a given time is given by:

$$E_w = W_{mL} \times E_{mL} + W_s \times E_s \qquad (1)$$

in which:

E_w = Percent lignin extracted from whole wood at that time

W_{mL} = Weight fraction of total lignin in the compound middle lamella

E_{mL} = Percent lignin extracted from the compound middle lamella at that time

W_s = Weight fraction of total lignin in the secondary wall

E_s = Percent lignin extracted from the secondary wall at that time.

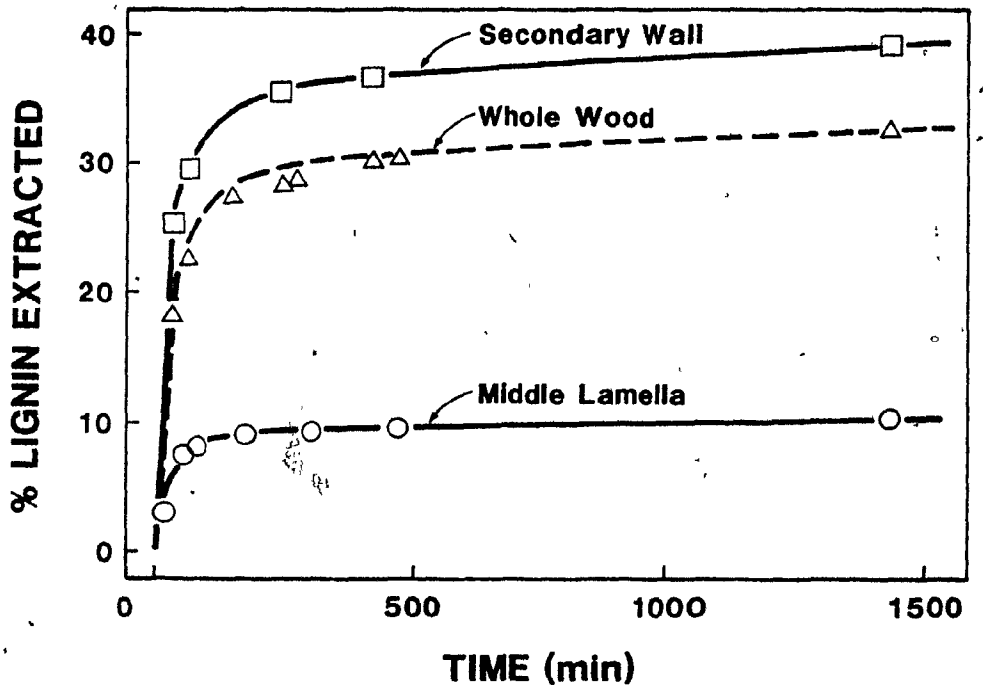

Figure 1. Plot of percent lignin extracted after ball milling against time (calculated as percent of lignin originally present in sample). Dashed line represents calculated curve for whole wood.

Fergus et al[8] showed that 77% of the total lignin in black spruce is present in the secondary wall, while 23% is present in the compound middle lamella. Using equation 1, an extraction curve can be calculated for the whole wood. The dashed line in Figure 1 represents the calculated curve. The fit of the calculated curve to the experimental points is good.

The most important conclusion which can be drawn from this work is that milled wood lignin is not representative of the whole lignin in wood, but originates primarily in the secondary wall of the cell. The difference in rate of extraction after ball milling is probably due to an inherent difference in the chemistry of the two lignin types. There is evidence to support this. Procter et al[9] showed that in kraft and acid sulphite pulping, there was a topochemical preference for removal of lignin from the secondary wall. This effect was confirmed[10] even when the middle lamella was fully exposed to the pulping lignin. Yang and Goring[11] demonstrated that the concentration of phenolic hydroxyl groups in middle lamella lignin is approximately half that in secondary wall lignin. This difference was confirmed by the work of Hardell et al[12] on fractions of spruce wood and by the present work in Chapters IV and V. A higher concentration of methoxyl groups has been found in secondary wall lignin than in middle lamella lignin (Chapter VI). Secondary wall lignin has been shown to react more quickly, and to a larger extent, with agents such as chlorine, chlorine dioxide, sodium bisulphite, (Chapter VII), and bromine (Appendix II). Taken together with the results of the ball milling experiments, this

evidence supports the hypothesis by Bolker and Brenner[13] that the lignin of the middle lamella is more highly crosslinked than the lignin of the secondary wall.

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APPENDIX I

CARBOHYDRATE ANALYSES OF TISSUE FRACTIONS BY LIQUID CHROMATOGRAPHY

The gas chromatographic results obtained for the carbohydrate compositions of the tissue fractions (Chapter III) were confirmed by liquid chromatography by M. Paice, Pulp and Paper Research Institute of Canada, Pointe Claire, Quebec. Samples of middle lamella and whole wood tissue were hydrolyzed with trifluoroacetic acid, as described in Chapter III. The hydrolyzed sugars were dissolved in water and separated by high pressure liquid chromatography using a 25 cm "BIO-RAD" HPX-65 column. The solvent flow rate was set at 0.5 mL $\cdot$ min^{-1} and a refractive index detector was used. An example of a liquid chromatogram of hydrolyzed wood flour is shown in Figure 1. The results of the liquid chromatographic analyses are compared with the results of the gas chromatographic analyses in Table 1. As can be seen from Table 1, good agreement between the two methods was obtained.

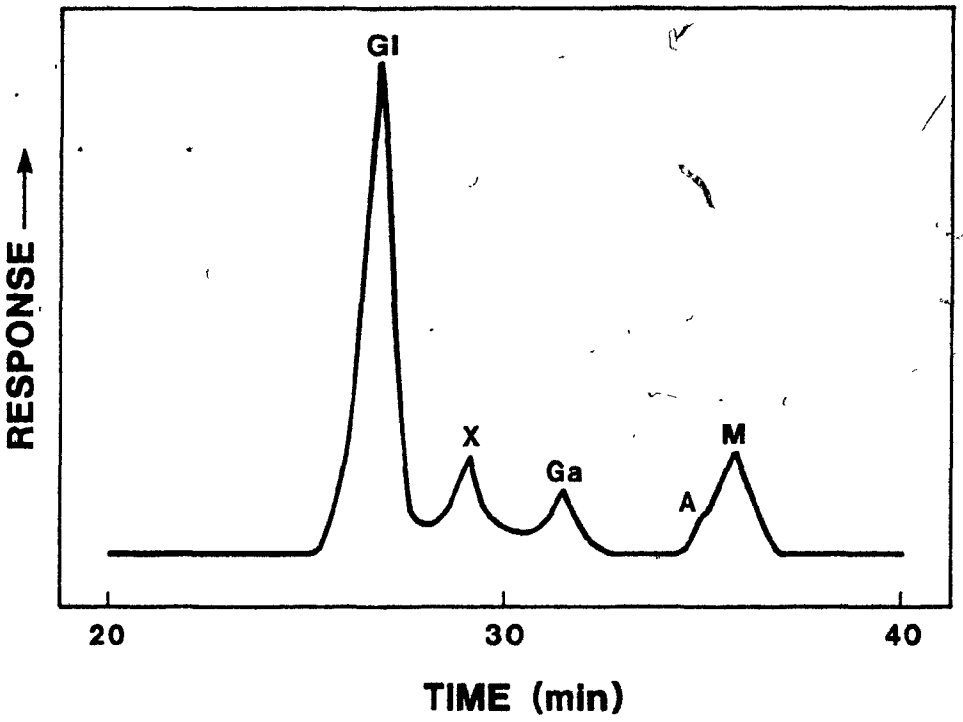

Figure 1. Liquid chromatogram of hydrolyzed black spruce wood flour. Code: A = Arabinose, Ga = Galactose, Gl = Glucose, M = Mannose, X = Xylose.

Table 1

Monosaccharide Contents of Tissue Fractions Expressed as Percent of Total Carbohydrate Contents

Component	WW[1] (G.C.)[3]	WW (L.C.)[4]	ML[2] (G.C.)	ML (L.C.)
Percent Lignin	22		50	
Glucose	67.0	67.1	45.6	47.6
Mannose	18.5	17.2	18.5	19.0
Xylose	8.0	9.0	15.3	12.6
Galactose	4.0	6.7	11.4	12.6
Arabinose	2.5	-	9.2	8.2

[1] WW: Whole wood tissue

[2] ML: Middle lamella tissue

[3] G.C.: Results obtained by gas chromatographic analysis

[4] L.C.: Results obtained by liquid chromatographic analysis.

APPENDIX II

BROMINATION OF MIDDLE LAMELLA AND SECONDARY WALL TISSUE FROM BLACK SPRUCE WOOD

In his Ph.D. thesis, Saka[1] measured the distribution of lignin in wood cells by a bromination technique. The wood was brominated and, since the bromine attached itself only to the lignin, its distribution was studied by electron microscopy using the EDXA technique (energy dispersive X-ray analysis). Saka found the concentration of bromine to be about twice as high in the middle lamella as in the secondary wall indicating that there is about twice as much lignin in the middle lamella as in the secondary wall. However, Fergus et al[2] found, by ultraviolet microscopy, that the ratio of lignin concentration in the middle lamella to that in the secondary wall was about 3.9, a value significantly different from that of Saka[1].

In his work, Saka[1] had to assume that the lignin in the middle lamella and the lignin in the secondary wall reacted to the same extent with the bromine. However, recent work (Chapter II) has allowed the isolation of tissue from the middle lamella and secondary wall of black spruce wood. This appendix, therefore, describes work, done in collaboration with Saka, on the relative reactivity of middle lamella and secondary wall lignin towards bromination.

Fractions of tissue from the middle lamella and secondary wall of black spruce (Picea mariana) were isolated as described in Chapter II. These tissues, along with black spruce wood flour, were used in all the experiments. The lignin contents of the middle lamella, secondary wall, and whole wood tissues were 60, 22, and 27%, respectively.

Brominations were carried out by the method described in Saka's thesis. To obtain samples of various extents of bromination, Saka's procedure was modified in several ways.

Procedure 1: About 5 mg of tissue were brominated. For each milligram of tissue, 0.3 µL of bromine in 0.9 mL of chloroform was added. The sample was shaken at room temperature for 3 h and then refluxed for 3 h. The brominated sample was then washed with chloroform for 10 - 12 days to ensure complete removal of any unreacted bromine. This procedure is virtually identical to that used by Saka.

Procedure 2: This procedure was the same as Procedure 1 except that pyridine was used as the solvent for the bromine and the 3 h reflux was replaced by a 3 h treatment at 60° C. Pyridine, and then methanol were used to wash the samples.

Procedure 3: This procedure was the same as Procedure 2 except that ten times the amount of bromine was used.

Procedure 4: This procedure was the same as Procedure 3 except that instead of a 3 h reflux, a 24 h treatment at 60° C was used.

The brominated samples were sent to Guelph Chemical Laboratories, Guelph, Ontario, for microanalysis. This company claims a reproducibility of $\pm$ 3% for samples of this size.

The results of the four bromination experiments are shown in Table 1. It can be seen that secondary wall lignin brominates to a larger

Table 1

Percent Bromine on the Lignin in

Tissue Fractions Brominated by Four Methods

Tissue Fraction	% Lignin	% Br Procedure 1	% Br Procedure 2	% Br Procedure 3	% Br Procedure 4
Secondary Wall	22	29.24	12.20	54.9	86.0
Whole Wood	27	25.19 (26.10)[1]	11.31 (11.01)	49.1 (49.4)	75.9 (78.7)
Middle Lamella	60	15.58	7.03	31.2	54.2
Ratio SW[2]/ML[3]	-	1.88	1.74	1.76	1.59

[1] Values in brackets calculated from middle lamella and secondary wall values based on 77% secondary wall lignin and 23% middle lamella lignin in whole wood[2]

[2] SW: Secondary wall lignin bromine content.

[3] ML: Middle lamella lignin bromine content.

extent than does middle lamella lignin, under all the reaction conditions used. Even for widely differing overall degrees of bromination, the ratio of the bromine content of secondary wall lignin to the bromine content of middle lamella lignin remains quite constant at an average value of 1.74 ± 0.12.

If the bromination is conducted by Procedure 1, the method used by Saka[1], the ratio of bromine content in secondary wall lignin to bromine content in middle lamella lignin is 1.88. Using this factor, together with the value of 2 reported by Saka for the ratio of bromine in the middle lamella region to that in the secondary wall region, the lignin content of the middle lamella is found to be 3.8 times that of the secondary wall. This is in good agreement with the value of 3.9 found by ultraviolet microscopy[2].

It is clear from the present results that secondary wall lignin brominates to a greater extent than does middle lamella lignin in accordance with the chlorination and sulphonation results of Chapter VII.

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APPENDIX III

THE LEACHING OF LIGNIN FROM THE MIDDLE LAMELLA AND SECONDARY WALL OF BLACK SPRUCE TISSUE COOKED BY THE KRAFT PROCESS

When unbleached kraft pulp fibres are stirred gently in water, some of the residual lignin in the pulps is slowly dissolved. This phenomenon, known as leaching, has been studied in depth by Favis[1]. In his work, he based his calculations and conclusions as to the mechanism of leaching on the assumption that all leachable lignin comes from the secondary wall of the fibre, not from the middle lamella. This view was supported by the ultraviolet microscopic work of Choi[2] in which no leaching of lignin from the cell corner was observed.

In order to determine if the above conclusions are correct, fractions of tissue from the middle lamella and secondary wall of black spruce were prepared by the method described in Chapter II. The lignin contents of the fractions were 60% and 22%, respectively. The fractions were cooked using a kraft liquor consisting of 18.7 g $\cdot$ L^{-1} NaOH and 6.7 g $\cdot$ L^{-1} Na_2S in water. The reaction temperature was 170° C and the reaction time was ten minutes. Lignin contents in the residues were determined by measuring the delignification by ultraviolet spectroscopy of the liquor. This measurement was used in cooperation with yield measurements to determine the lignin content of the residues.

The cooked samples were washed with distilled water by placing 20 mg samples in 50 mL Erlnemeyer flasks along with 30 mL H_2O and shaking the samples on a wrist shaker. The leaching was followed by measuring the absorbance of the wash water at 280 nm at various times.

The results of the leaching experiments are shown in Figure 1. The lignin leached is expressed as a percentage of the lignin available

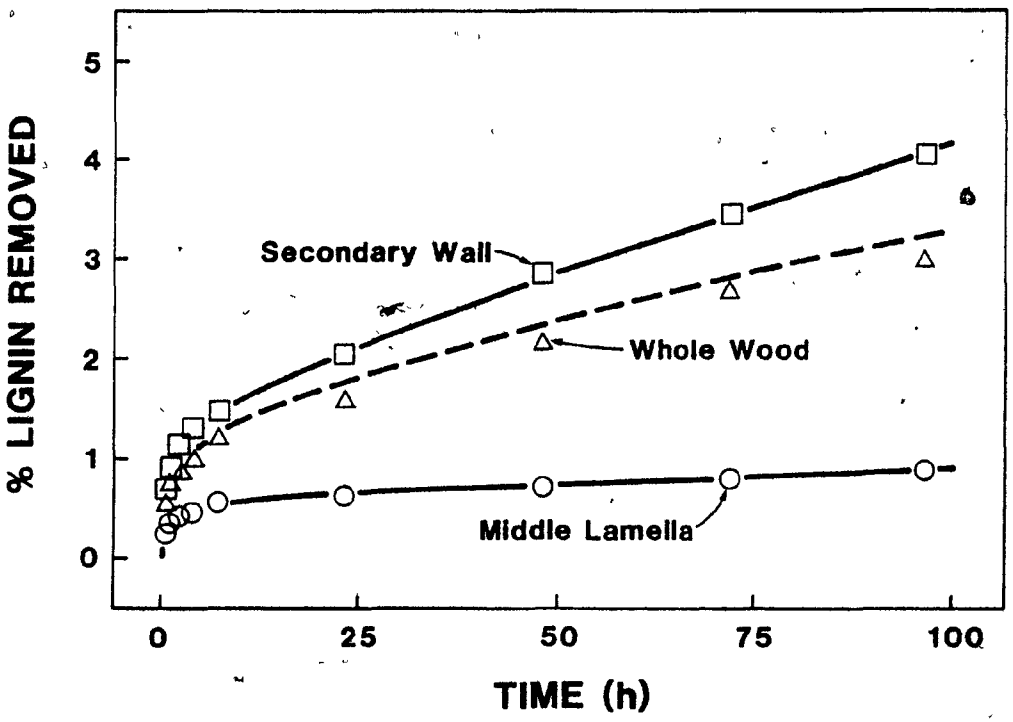

Figure 1. Plot of percent lignin leached during washing, against time. Dashed line represents calculated whole wood leaching curve.

in the residue after cooking. The dashed line represents the calculated extraction curve for whole wood lignin based on a proportion of 77% secondary wall lignin and 23% middle lamella lignin in the whole wood[3].

The results clearly show that only a small amount of lignin is leached from the middle lamella as compared to the secondary wall. The middle lamella extraction curve shows a fast initial extraction and then a very slow extraction after about 10 min. The initial fast extraction could be due to a small amount of residual secondary wall material in the middle lamella tissue sample. It is, therefore, expected that the leaching of lignin from middle lamella tissue rendered in pulps would be almost zero. This observation is in agreement with that of Choi[2], and supports the assumption of Favis[1] in his work.

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APPENDIX IV

DETERMINATION OF WEIGHT AVERAGE MOLECULAR MASS BY SEDIMENTATION EQUILIBRIUM

The sedimentation equilibrium technique is based on achieving a balance between opposing forces, the applied centrifugal field and diffusion. The centrifugal force causes the molecule to sediment while diffusion, resulting from a concentration gradient produced by the sedimentation, acts in the opposite direction. The distance that a molecule sediments before equilibrium is achieved is dependent, among other factors, on its molecular size.

The long time required to establish equilibrium is proportional to the square of the column length. Van Holde and Baldwin[1] developed a short column technique which significantly reduced centrifugation time. This technique was adapted to molecular mass studies on lignin by Yean and Göring[2] and has been developed into a reliable technique for measuring the weight average molecular mass of soluble lignins.

Samples of tissue from the secondary wall, whole wood, and middle lamella of black spruce were pulped in kraft liquors as described in Chapter X. Solutions of about 1% lignin were prepared from each tissue fraction. These solutions were then diluted with water by factors of 1.3, 2.0, and 4.0. These samples were then run at 25° C in a Spinco Model E Analytical Ultracentrifuge by Mr. W.Q. Yean. A 4 h centrifugation time was used to ensure that equilibrium had been reached. After 4 h, the Schlieren sedimentation equilibrium pattern was photographed. A schematic representation of a Schlieren pattern is shown in Figure 1. The procedure was repeated at two other speeds.

Each Schlieren photograph was enlarged by a factor of 8, the pattern was traced, and two distances were carefully measured: the distance

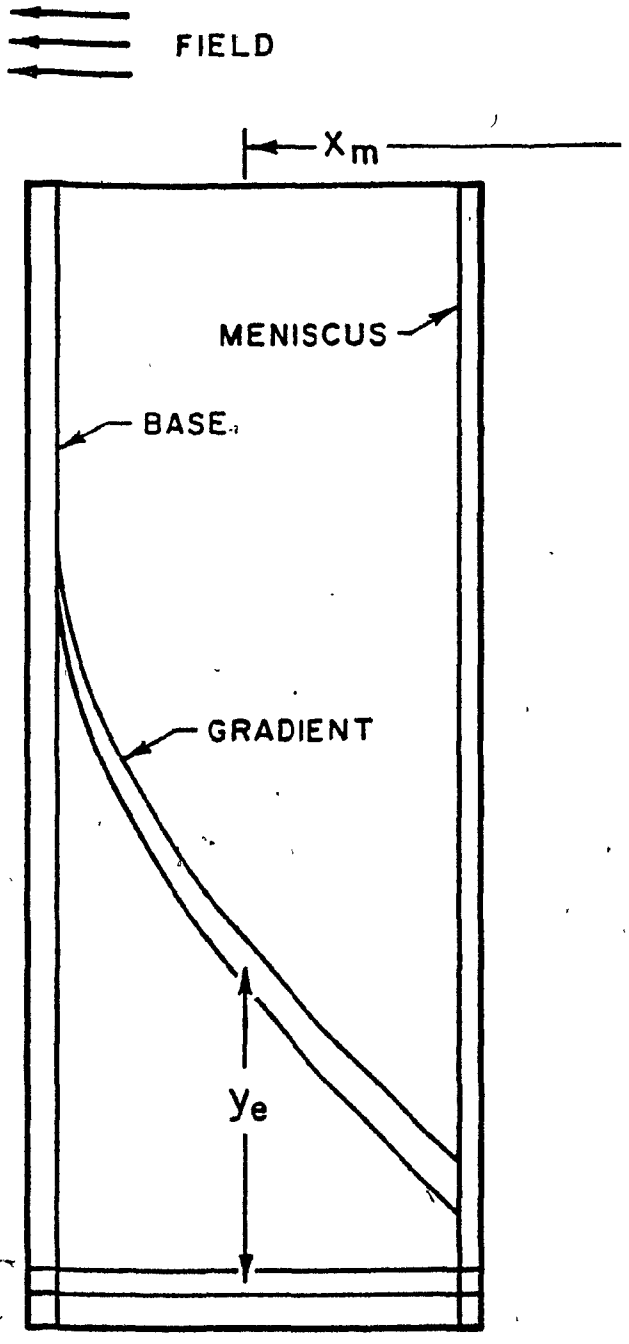

Figure 1. Schematic of a Schlieren pattern.

from the centre of the rotor to the ordinate midway between the meniscus and the base of the cell (X_m), and the height of that centre ordinate (Y_e) (Figure 1).

By using previously determined apparatus constants, the Y_e value was converted to a refractive index gradient (dn/dx) by the following equation

$$\frac{dn}{dx} = \frac{Y_e \tan \theta}{m D A_1} \qquad (1)$$

where θ = Schlieren angle = 65°

m = cylindrical magnification = 2.144

D = depth of ultracentrifuge cell = 1.2 cm

A_1 = length of the optical arm = 58.58 cm.

The weight average molecular mass can then be calculated by the following equation

$$\bar{M}_w = \frac{1}{X_m c} \frac{dn/dx}{dn/dc} \frac{RT}{\omega^2(1-\bar{v}\rho_s)} \qquad (2)$$

where $\bar{M}_w$ = weight average molecular mass

c = concentration of lignin in solution ($g \cdot mL^{-1}$)

dn/dc = refractive index increment = 0.663 $mL \cdot g^{-1}$ (Ref. 3)

R = ideal gas constant

T = absolute temperature

ω = angular velocity of the rotor = $\pi(rpm)/30$

$\bar{v}$ = partial specific volume of the lignin = 0.201 $mL \cdot g^{-1}$ (Ref. 3)

ρ_s = solution density ($g \cdot mL^{-1}$).

In order to calculate the molecular mass at zero concentration for each rotor speed, the reciprocal of the molecular mass was plotted

against concentration (Figure 2).

The measured molecular mass is also speed dependent. The molecular mass at zero speed was determined by plotting $1/\overline{M}_w$ from the zero concentration plots versus ω^2 and extrapolating to zero speed. Figure 3 shows such a plot for the experimental data from which the weight average molecular masses were obtained for the three tissue fractions. Table 1 shows the molecular mass data for each sample at each rotor speed and concentration.

The weight average molecular masses for the lignin dissolved from the secondary wall, whole wood, and middle lamella tissue fractions were found to be 25 000, 29 000, and 40 000 $g \cdot mol^{-1}$, respectively. The uncertainty in measurements of this type is about $\pm$ 20%.

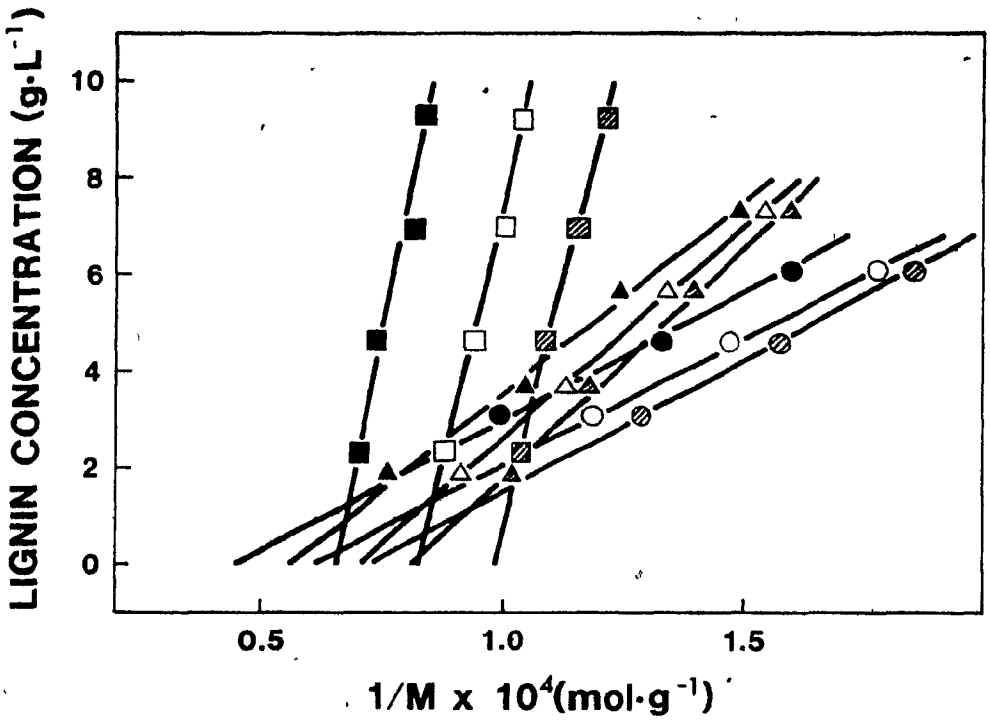

Figure 2. Plot of lignin concentration versus 1/M at various rotor speeds.

Legend:
- ■ - secondary wall lignin at 16 000 rpm
- □ - secondary wall lignin at 20 000 rpm
- ▨ - secondary wall lignin at 24 000 rpm
- ▲ - whole wood lignin at 16 000 rpm
- △ - whole wood lignin at 20 000 rpm
- ▲ - whole wood lignin at 24 000 rpm
- ● - middle lamella lignin at 16 000 rpm
- ○ - middle lamella lignin at 20 000 rpm
- ⊘ - middle lamella lignin at 24 000 rpm

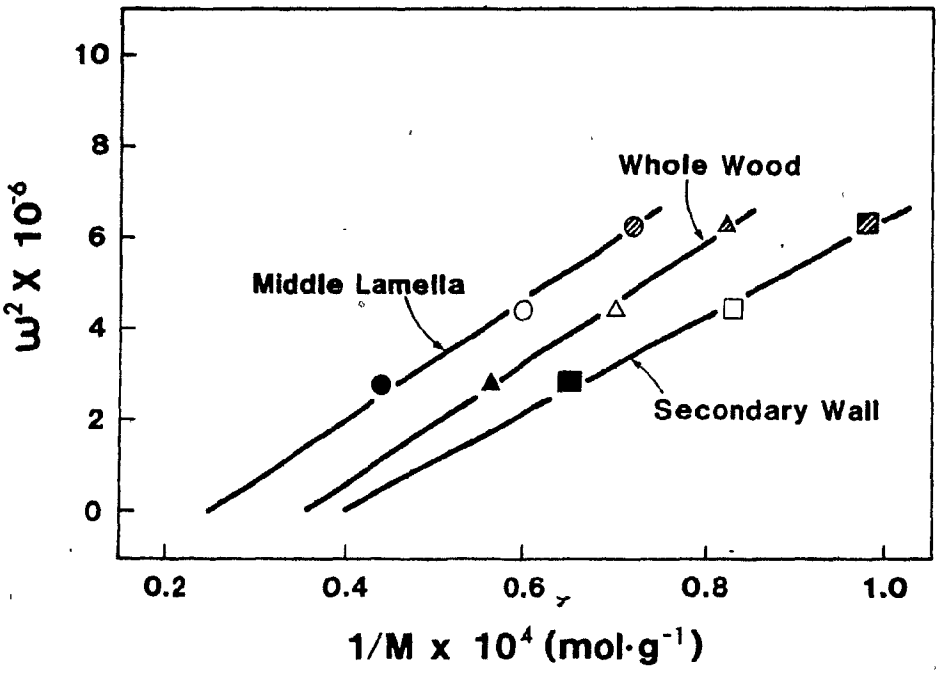

Figure 3. Plot of ω^2 versus zero concentration values of 1/M

Legend:
- ■ - secondary wall lignin at 16 000 rpm
- □ - secondary wall lignin at 20 000 rpm
- ▨ - secondary wall lignin at 24 000 rpm
- ▲ - whole wood lignin at 16 000 rpm
- △ - whole wood lignin at 20 000 rpm
- ▲ - whole wood lignin at 24 000 rpm
- ● - middle lamella lignin at 16 000 rpm
- ○ - middle lamella lignin at 20 000 rpm
- ⊘ - middle lamella lignin at 24 000 rpm

Table 1

Molecular mass values of soluble material from tissue fractions.

Values were measured at various rotor speeds and concentrations

ROTOR SPEED	SECONDARY WALL ($\bar{M}_w \times 10^{-3}$ ($g \cdot mol^{-1}$))				WHOLE WOOD $\bar{M}_w \times 10^{-3}$ ($g \cdot mol^{-1}$)				MIDDLE LAMELLA $\bar{M}_w \times 10^{-3}$ ($g \cdot mol^{-1}$)		
Concentration ($g \cdot L^{-1}$)	2.3	4.6	6.9	9.3	1.9	3.7	5.7	7.3	3.1	4.6	6.1
16 000 rpm	14.3	13.5	12.2	11.9	13.1	9.52	8.03	6.71	10.1	7.52	6.29
20 000 rpm	11.3	10.6	9.95	9.57	10.9	8.85	7.43	6.49	8.47	6.80	5.62
24 000 rpm	9.57	9.17	8.70	8.26	9.80	8.51	7.19	6.29	7.75	6.27	5.40

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3. J.G. McNaughton, W.Q. Yean, and D.A.I. Goring, Tappi 50 (1967) 548.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

The following original discoveries, which arose out of research designed to answer the questions listed in the section "Purpose of Work", are claimed.

1. A method was developed, based on differential sedimentation, by means of which centigram scale quantities of tissue from the middle lamella and secondary wall of black spruce tracheids could be isolated.

2. Two new methods, based on turbid spectroscopy and pyrolytic gas chromatography, were developed for the measurement of the phenolic hydroxyl content of insoluble lignins. The phenolic hydroxyl contents of cuoxam, periodate, and DHP-I lignins, previously unknown, were also measured.

3. The methoxyl content of secondary wall lignin in black spruce was found to be 1.6 times that of middle lamella lignin, indicating that about 40% of middle lamella lignin is based on para-hydroxyphenyl building units.

4. The carbonyl content of middle lamella lignin was found to be more than three times that of secondary wall lignin.

5. Absolute, as opposed to relative, amounts of polysaccharides were, for the first time, measured in the middle lamella and secondary wall of black spruce tracheids.

6. Secondary wall lignin was shown to be more reactive towards chlorine, chlorine dioxide, and sodium bisulphite than was middle lamella lignin. Reactivity ratios were calculated for each of these reactions.

7. In acid-chlorite pulping, the topochemical preference for removal of the lignin in the secondary wall was seen to be small below 50% delignification, but large above 50% delignification.

8. Arrhenius activation energies, in kraft pulping, were found to be the same for the so-called "bulk" lignin in the middle lamella and secondary wall. However, the secondary wall was found to contain mostly "bulk" lignin while middle lamella had a high proportion of "residual" lignin. The molecular weight of the dissolved "bulk" lignin was less than that of dissolved "residual" lignin on kraft pulping.

9. Milled wood lignin was shown to originate predominantly in the secondary wall of the tracheid.

SUGGESTIONS FOR FURTHER RESEARCH

In the light of the results described in this thesis, the following suggestions for further research can be made.

1. The isolation procedure, developed in Chapter II, should be adapted for use on other wood species. In particular, work on hardwoods would be very interesting, since the wood structure is different from that of softwoods. Tissue from the various morphological regions of a hardwood could be studied in similar ways as was done for black spruce in this thesis.

2. The isolation of ray cells in softwoods was not attempted in this work, although some experimental evidence pointed to a possibility to do so. It would be interesting to compare the components of these cells to those of the tracheids already studied.

3. The carbohydrate analyses in Chapter III indicated that the cellulose content of the true middle lamella is virtually zero. Attempts could be made to isolate tissue fractions of even higher lignin contents so that this hypothesis could be tested.

4. Recent developments in solid state "magic angle" NMR spectroscopy have allowed the recording of informative spectra of wood samples. This technique could be used for the measurement of the phenolic hydroxyl and methoxyl contents of lignin and tissue preparations.

5. It would be worthwhile to investigate more fully the distribution of the pectic substances across the cell wall in wood.

6. The results given in Chapter VI suggested that the lignin in wood has a lower carbon content than previously suspected. An in depth study should be undertaken to see if this is, indeed, the case.

7. Further research, with model compounds, could elucidate more fully the influence of methoxyl and phenolic hydroxyl groups on the reactivity of lignin in wood.

8. DHP lignin should be prepared using 40% para-hydroxyphenyl and 60% guaiacyl building units, so that a synthetic middle lamella lignin could be studied.

9. The relative reactivities of middle lamella and secondary wall lignin to other chemicals should be studied. In particular bleaching reactions of the lignin, left after pulping, could be studied.

10. Middle lamella and secondary wall tissue would be useful in research designed to find a new pulping method in which middle lamella lignin is preferentially dissolved.

11. More thorough molecular mass studies of the lignin dissolved from the different tissue regions should be undertaken. The lignin

component of the pulping liquor could be separated, by electrophoresis convection, from the carbohydrate component so that the measured molecular mass would more accurately represent the molecular mass of the dissolved lignin. The molecular masses of dissolved "bulk" and "residual" lignin could then be compared more precisely.

12. The pulping kinetics of the "residual" lignin should be investigated. In work on whole wood, this is difficult because of the small amount of residual lignin (about 10% of the total lignin). However, in the middle lamella tissue, the "residual" lignin makes up about 70% of the total lignin and could be more easily studied.

13. Biochemical studies on the mechanism by means of which a softwood tree produces both "bulk" and "residual" lignin should be undertaken.

14. The tissue fractions could be used to determine the morphological origins of lignin preparations other than milled wood lignin.

15. The fractionation obtained by differential sedimentation is rather crude. Electron microscopy, used in conjunction with EDXA (energy dispersive X-ray analysis) could be used to study differences in the lignin in the S_1, S_2, S_3, primary wall, and true middle lamella. Such fine separation of tissue would be very difficult or impossible by the physical techniques employed in the present thesis.