Locations of amylose in normal corn starch revealed by phosphodiester cross-linking and phosphorus-31 NMR

by

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A Thesis submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Food Science and Human Nutrition
Major: Food Science and Technology

Signatures have been redacted for privacy

Iowa State University
Ames, Iowa

1991

TABLE OF CONTENTS

ABBREVIATIONS

g	acceleration due to gravity
BU	Brabender unit
°C	degree Celsius
$CaCl_2$	calcium chloride
CHO	total carbohydrate
D_2O	deuterium oxide
DP	degree of polymerization
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
g	gram
hr	hour
HCl	hydrochloric acid
H_2SO_4	sulfuric acid
H_3PO_4	phosphoric acid
id	inner diameter
KOH	potassium hydroxide
L	liter
µg	microgram
mg	milligram

ml	milliliter
mM	millimoles/liter
min	minute
M	moles/liter
nm	nanometer
N	normality
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance
ppm	parts per million
$POCl_3$	phosphorus oxychloride
rpm	revolutions per minute
sec	second
U	units
TMS	trimethyl silane
w/w	weight/weight
w/v	weight/volume

INTRODUCTION

Starch is the second most abundant biomass in the plant. Starch from most plant species is composed of two major types of polysaccharides: amylose and amylopectin. Normal corn starch contains about 28% amylose and 72% amylopectin. Amylopectin is a branched molecule which has α-1,4 linkage for the main chain and branched with α-1,6 linkage. The cluster model has been well accepted for the structure of amylopectin. The orientation of amylopectin in the starch granule has also been revealed. Amylose is an essentially linear macromolecule containing glucose units linked with α-1,4 linkage with few branches. The location of amylose is not well understood. Amylose can be located in bundles between the cluster of amylopectin, or it can be randomly interspersed over clusters of amylopectin and lined in both the amorphous and the crystalline region in the same way as amylopectin. Although chemical structural features of the starch components are now well established, studies on the organization of the starch granule yield only limited and often conflicting reports.

Objectives of this study were to investigate the locations of amylose in normal corn starch granules by cross-linking method. The locations of amylose in granules can help us understand the biosynthesis of normal starch granules,

and to explain the starch properties such as gelatinization, gelling, and retrogradation.

Jane et al. (1991b) studied the location of amylose by using cross-linking by epichlorohydrin. They found that with low degree of cross-linking, the amylose and amylopectin molecules were cross-linked and increased the blue value of the amylopectin peak in the gel permeation chromatography. They also did not find any amylose molecules cross-linked among themselves.

Starch in granules can be cross-linked by phosphorus oxychloride at alkaline pH. Phosphorus oxychloride provides phosphodiester cross-linking and phosphomonoester derivatives. The cross-linking can happen between amylopectin and amylose molecules, if amylose is interspersed among amylopectin molecules; and among amylose molecules, if amylose molecules are present in bundles. Molecular sizes of the soluble starches can be determined by gel permeation chromatography. Phosphomono- and diesters in the starch molecules can be revealed by phosphorus-31 NMR. The phosphorus-31 NMR can differentiate various phosphate ester structures by obtaining the signals at different chemical shifts.

LITERATURE REVIEW

Starch is a polysaccharide consisting of α-1,4 and α-1,6 linked anhydroglucose units. There are two structurally distinct components: amylose and amylopectin. A third component, often referred to as the intermediate fraction, has been reported in some starches (Lansky, 1949; Peat et al., 1952; Wolff et al., 1955). Starch molecules are organized into quasicrystalline macromolecular aggregates called granules (Hood, 1982). Corn starches prepared by different methods had different chemical compositions and properties (Takeda et al., 1988). Corn starch is isolated from the endosperm of corn kernels. The endosperm comprises 82-86% of the kernel (Earle et al., 1946) and consists of 86% starch (Watson, 1987). Starch from normal corn contains about 28% amylose and 72% amylopectin (Swinkels, 1985).

Amylose

Amylose has an essentially linear structure, consisting of glucose units linked by α-1,4 linkage (Greenwood, 1964; French, 1973). The hydrolysis of amylose with beta-amylase showed the incomplete maltose conversion (Bank, 1973; Greenwood, 1976). The beta-amylolysis of amylose ranges from 70-82% but with the addition of pullulanase, the hydrolysis is almost completed (Hizukori et al., 1981; Takeda and Hizukori, 1987). The main barrier to beta-

amylase hydrolysis are α-1,6 branches linkage (Peat et al., 1952; Hizukuri et al., 1981; Takeda and Hizukori, 1989). The degree of polymerization of corn amylose is in the range of 930-990 with an average chain length of 335 glucosyl units and 2.9 branch chains per molecule (Takeda and Hizukori, 1987; Takeda et al., 1988). Amylose in a neutral, aqueous salt solution forms a random coil, but with a complexing agent, it forms a helical structure with 6-8 glucose units per turn (Rundle and French, 1943; Banks et al., 1971; Bank and Muir, 1980; Davies and et al., 1980). The amylose form complex with complexing agents such as iodine, dimethyl sulfoxide (DMSO), and fatty acids and give V-patterns as studied by X-ray crystallography (Winter and Sarko, 1974; Davies et al., 1980). When amylose form a complex with iodine, it gives a dark blue color which is used for analysis of amylose (Banks et al., 1974).

Amylopectin

Amylopectin is the major polysaccharide in normal corn starch which has a multiple-branched structure. The main chain consists of α-1,4 linkages and branched by α-1,6 linkages (Greenwood, 1964; French, 1973). The X-ray diffraction pattern of normal corn starch is found in A type (Hizukuri et al., 1983). The average branched chain length is 20-25 glucose units, and the degree of polymerization (D.P.) of amylopectin is in the range of 10^4 - 10^5 glucose residue (Hizukuri et al., 1983; Zobel, 1984; Manners, 1985; Takeda et

al., 1988). Amylopectin structure contains three types of chains: A, B, and C chains. The A chain is the chain which is linked to B or C chains only by the reducing group with α-1,6 linkage and does not have any other chain attached. The B chain is the chain which is linked to another B or C chain with the only reducing group and also carries one or more other chains (A, B chain). The C chain is the only chain of the molecule which has a free reducing end and carries other chains. The structure of amylopectin was also studied with the ratio of A to B chain (Altwell et al., 1980). The ratio of A chains to B chains is referred to as the degree of multiple branching (Manners, 1985). The molecular structure of amylopectin has been studied widely and the cluster model is the most accepted (French, 1972). On the basis of the cluster structure, the properties of amylopectin can be explained, including viscosity and acid resistance (French, 1972). The crystalline region shown in amylopectin is composed of A-chain and the exterior part of B-chain (French, 1972). Amylopectin does not give iodine blue color as amylose-iodine does but gives purple to reddish-brown color depending upon its source (branch chain length).

Starch granular

Normal corn starch^X has a polygonal granular shape and a size of 3-26 micron (Swinkels, 1985). The starch granule consists of two parts: amorphous and crystalline. The starch granule is insoluble in cold water but soluble in

boiling water. Corn starch can also be dissolved completely in a 80-95% DMSO solution (Leach and Schoch, 1962; Wolf et al., 1970), concentrated alkaline solutions (1 M of NaOH or KOH), and some neutral salt solutions (2 M $CaCl_2$). Chemical compositions of starch granules varied by age and source. During the maturation of starch granules, starch contents and granular size increase, the ratio of amylose content also increases (Greenwood, 1979), the morphologies of the granules change, and the gelatinization temperature of the starch also change. The amylopectin forms the cluster and lines perpendicular to the growth rings and growing from the hilum to the surface of the granule in a radical arrangement (French, 1984). Amylopectin clusters present the amorphous and crystalline regions. The amylose is located in both crystalline and amorphous regions (Kainuma and French, 1971; French, 1972; Nikuni, 1978; Blanshard, 1986; Jane et al., 1991). The amylopectin has a highly crystalline structure which gives it compact and well organized-structure (Greenwood, 1979; French, 1984). Amylose has amorphous structure and much of the amylose formed complex with fat (Greenwood, 1979). Manners (1985) proposed that the crystalline regions were composed with much of amylose, but Kainuma and French (1971) reported that amylose was located primarily in the amorphous phase rather than crystalline regions. Blanshard et al. (1984) reported that amylose molecules were found in bundle in the amorphous region with the tangential direction to the growth ring. Kassenbeck

(1978) also proposed that amylose molecules were arranged in the radial amorphous arrangement and amylopectin was in crystallites in tangential lamellae. Blanshard (1986) proposed that amylose molecules were found complexing with amylopectin and also in the free form. Jane et al. (1991b) reported that amylose molecules were interspersed among amylopectin molecules. The starch granules are swollen in warm water to a limited extent because the swelling only happens in the amorphous regions; the crystalline regions hold the granule together to prevent the dispersion and solution of individual starch molecule (Leach et al., 1959; French, 1984). As the starch granules are exposed to water at the temperature below the gelatinization temperature, the granules are swollen reversibly until the temperature reaches the gelatinization temperature. Starch crystallites are melted at the gelatinization temperature, and it cannot be reversed to the native granule (French, 1984). After the starch granules gelatinize, the starch granules are ruptured, and the amylose and amylopectin are dispersed out. Viscosity of the solution increases with the changing of the granular structure. The crystalline organization of the starch granule can also be destroyed by a mechanical treatment such as ball milling (French, 1984). Physical properties of starch granules such as swelling and pasting can be detected by using a visco/amylo/graph. As the temperature increases, the starch granules are swollen and the viscosity increases until it reaches the highest viscosity (peak

viscosity). As the temperature further increases the granular structure cannot be held together, and the granules rupture and release the starch molecules; then the viscosity decreases. As the temperature decreases, the viscosity of the paste increases as the aggregation of amylose (Greenwood, 1979).

Nägeli dextrin

Nägeli dextrin is Lintnerized starch or amylodextrin are both acid resistant and remain insoluble after prolonged treatment of native starch with a strong aqueous acid at room temperature (Nägeli, 1874; French, 1972; Robin et al., 1974; Umeki and Kainuma, 1981). After three months of hydrolysis by acid, the insoluble residue of the starch or Nägeli dextrin still remains. The resistant starch can not be further hydrolyzed even kept for 6 years in the acid (French, 1972). The size of the Nägeli dextrin is dependent of the specificity of the hydrolytic agent. Resistant amylose can also be prepared by enzymatic hydrolysis of retrograded amylose (Jane and Robyt, 1984). After the starch granules are treated with acid, the amorphous regions are hydrolyzed leaving only the crystalline region intact (French, 1972; Kainuma and French, 1972; Yamaguchi et al., 1979; French, 1984). The starch granule still remains in the same shape and size, but the molecular weight of the resistant starch is reduced (Yamaguchi et al., 1979). The X-ray crystallography pattern was not changed but the spectrum became sharper than the parent starch (Kainuma

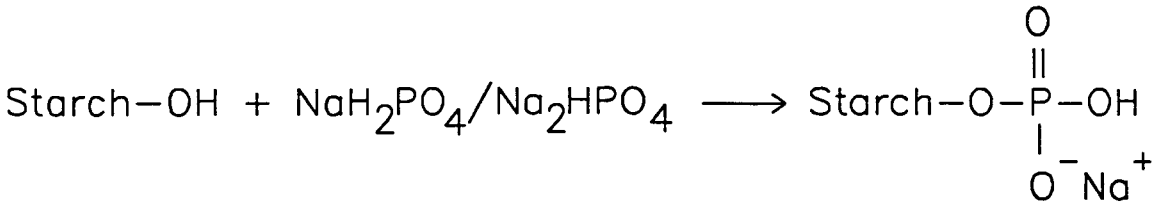
and French, 1971; Kainuma and French, 1972; Maningat et al., 1979; Jane et al., 1991). Kainuma and French (1971) proposed that the crystalline regions can protect the hydrolysis by two factors: The crystalline regions are packed so densely that acid cannot penetrate, but the amorphous regions are readily hydrolyzed by acids due to the loose structure. The hydrolysis can occur when the glucose molecule can change its conformation, but in the crystalline regions, changing conformations requires a high energy of activation which is not thermodynamically favorable (Kainuma and French, 1971). Robin et al. (1974) proposed that amylose content was decreased after three months of hydrolysis. The Nägeli dextrin is readily soluble in hot water without giving pasting or gelation (French, 1984). The degree of polymerization (D.P.) of Nägeli dextrins is in the range of 15 to 30 (Hall and Manners, 1980). The Nägeli dextrin can be fractionated into three peaks by Sephadex G-50 gel chromatography. The first peak contains the high molecular weight molecules with complex branching; the second peak contains the molecules with a single and branch and d.p. ~ 25, and the third peak is mostly linear molecule with d.p.~ 12 (Watanabe and French, 1980). Umeki and Kainuma (1981), Maningat et al. (1979), and Robin et al. (1975) proposed that Nägeli dextrin can be fractionated into two fractions: linear molecule with d.p. about 12-17 and multi-branch molecule with d.p. about 25-34.

Starch phosphate modification (Phosphorylation)

Phosphorylated starches are starch ester derivative of phosphoric acid (Solarek, 1986). The starch phosphates can be divided into two groups: phosphomonoester and phosphodiester. The starch phosphodiesters are cross-linked starches which have two hydroxy groups from starches involved in the starch-phosphate linkages. The starch phosphomonoester occurs in native starches such as potato starch and can also be synthesized by using chemical modification. The degree of modification can be controlled by the amount of the reagent, time, temperature, and the pH of the reaction (Harland, 1952).

Starch phosphomonoester

Starch phosphomonoester can be prepared by using orthophosphate (a mixture of monohydrogen and dihydrogen phosphate) with pH 5-6.5 and at a high temperature (Rutenberg and Solarek, 1984).

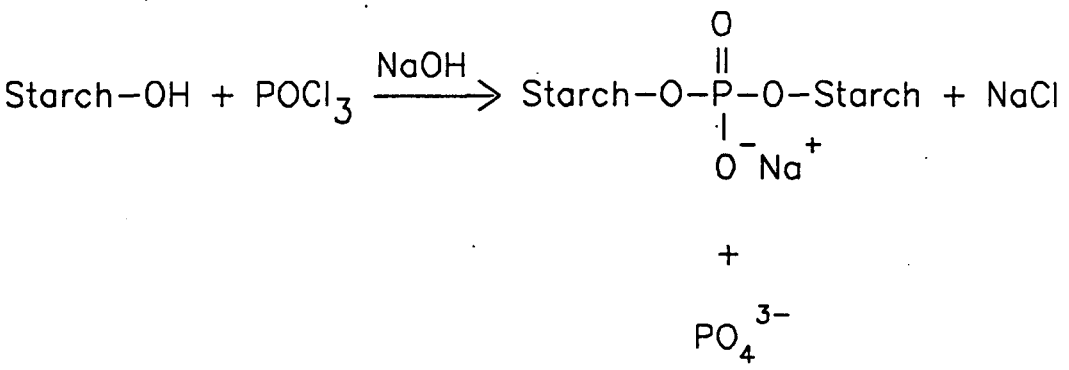


The dispersed starch phosphomonoester has high clarity, high viscosity, long cohesive texture, and stability to retrogradation (Hamilton and Paschall, 1967). The starch phosphomonoester, which has hydroxyl group substituted with bulky chemical groups carry negative charges, can inhibit the aggregation of the amylose and amylopectin (Hood et al., 1974). The starch phosphomonoester also reduces the enzyme hydrolysis (Lim, 1991).

Starch phosphodiester (Phosphate cross-linked starch)

Phosphate cross-linked starch is the starch which has two hydroxy groups forming ester bonds with one phosphoric molecule. The phosphate cross-linked starch can be prepared by many reagents such as phosphorus oxychloride and sodium trimetaphosphate (Wurzburg, 1986). The cross-linking can occur between phosphate and two hydroxy groups either on the same starch molecule or on different molecules (Wurzburg, 1986). Most of the cross-linking reagents also produce phosphomonoester derivatives as by products. The degree of cross-linking in the starch is very low and is difficult to determine directly (Hullinger, 1967; Rutenberg and Solarek, 1984; Wurzburg, 1986). The physical properties of the cross-linked starch such as viscosity (detected by visco/amylo/graph), and swelling power are generally used for determining the degree of cross-linking (Hullinger, 1967; Jarowenko, 1971; Rutenberg and Solarek, 1984). The ratio of the cross-linking and the monoester derivative can

be controlled by the pH of the reaction. At the high alkalinity (pH 8-12), the cross-linking reaction is predominant. At the low pH, the reaction shifts to produce more phosphomonoester starch (Felton and Schopmeyer, 1943; Wetzstein and Lyon, 1956; Patten et al., 1969; Lloyd, 1970; Rogols and Salter, 1979). The cross-linking by $POCl_3$ also requires high alkalinity (pH 8-12), and the reaction can be terminated by acidification to pH 5 (Wetzstein and Lyon, 1956; Felton and Schopmeyer, 1943; Wurzburg, 1986). Phosphorus oxychloride is a very reactive agent and the reaction rate is fast (Wurzburg, 1986; Wu and Seib, 1990). The cross-linking reaction is shown in the diagram below:



The starch modified with $POCl_3$ gives random cross-linking in starch molecules (Rutledge et al., 1974). Modified starches also give mixtures of phosphomonoesters and phosphodiesters (Smith, 1967; Abraham, 1979; Solarek, 1986). Koch et al. (1982) reported that the modified starch with 1.0% $POCl_3$ gave 3.4-4.0 phosphodiester groups per phosphomonoester group.

Since the starch granule was cross-linked, the granule was limited to swell and rupture (Felton and Schopmeyer, 1943; Rogols and Salter, 1979; Chilton and Collison, 1974; Ruterberg and Solarek, 1984). The water sorption isotherm for cross-linked starch is similar to the unmodified parent starch. One cross-linkage per hundred anhydrous glucose units retains the integrity of the swollen starch granule, eliminates the cohesiveness, and stabilizes the viscosity of the hot paste (Mellies, 1961; Hullinger, 1967; Hood et al., 1974). The cross-linked starch paste is resistant to hydrolysis by acid, enzyme, and mechanical shear (Suzuki et al., 1971; Chilton and Collison, 1974; French, 1984; Wurzberg, 1986; Wu and Seib, 1990). Since the cross-linkage suppresses the swelling and rupturing of starch granules, a highly cross-linked starch is unable to gelatinize and cannot give a high viscosity paste. A visco/amylo/graph of a low degree cross-linked starch shows a high peak viscosity, with a good stability while holding, but for a high degree of cross-linked starch, the peak viscosity cannot be detected; instead, the viscosity rises slowly: With an extremely high degree cross-linked starch the viscosity does not increase at all because of the highly restrained swelling of the starch (Hullinger, 1967; Chilton and Collison, 1974; Rutenberg and Solarek, 1984). The cross-linking also limits the leaching of amylose in the hot aqueous solutions (Wurzburg, 1986). The cross-linking lowers the solubility of the starch in aqueous and DMSO solutions. At a high degree of cross-linking the starch granules cannot be dissolved in aqueous or

DMSO solutions even after boiling at 95°C (Kerr and Cleveland, 1957; Leach, 1962; Wurzberg, 1986). Collison and Ogundiwin (1972) reported that the cross-linked starch did not affect the iodine binding capacity, but the rate of the reaction is much slower than the unmodified starch. The cross-linking of starch affected the digestibility of the starch. Ungelatinized cross-linked starch can be hydrolyzed better than ungelatinized native starch, but the gelatinized native starch can be hydrolyzed better than the gelatinized cross-linked starch (Hood and Arneson, 1976).

Phosphorus in native starch

Phosphorus found in native starch can be categorized into two groups: phosphomonoester derivatives and phospholipid. Most of the phosphorus in the starches is in the lipid form (Gracza, 1965; Hizukuri et al., 1983). The phosphomonoester derivative is found in many varieties of starch such as corn, potatoes, wheat, and rice (Tabata et al., 1975; Hizukuri et al., 1983). Tabata et al. (1975) reported that normal corn starch contains phosphorus (about 170 ppm), most of which is in the phospholipid form. Only 7.0% of the total phosphorus is in the phosphomonoester at the position 6 of the glucose. Corn, waxy corn, rice, and waxy rice starches contain significant amounts of phosphomonoester at the position 6 of glucose (Radomski and Smith, 1963, Tabata et al., 1975; Takeda and Hizukuri, 1982; Hizukuri et al., 1983). Hizukuri

et al. (1970) proposed that about 60-70% of the phosphate ester in potato starch is located in position 6 of glucose and the rest is located in the positions 2 and 3 of glucose. Radomski and Smith (1963) reported the distribution of phosphomonoester in the starch as follows: 64.45% located in amylopectin, 32.42% located in intermediate, and only 3.12% located in amylose. The incomplete β-amylase hydrolysis of starch is partially caused by phosphate esters because the phosphate esters are found mostly in β-limited dextrin, and phosphate esters are located closely to branching points (Radomski and Smith, 1963). Takeda and Hizukuri (1982) proposed that potato starch had phosphomonoester derivatives at position 3 and at position 6 of glucose and phosphate groups mainly attached to the center of unit chain in the amylopectin. About 1/3 of the phosphate groups are presented in the inner sections of the B-chain and 2/3 are in the A-chain and outer section of B-chain (Takeda and Hizukuri, 1982). Tabata and Hizukuri (1971) reported that the quantitative distribution of phosphomonoesters in potato starch was estimated to be 38% on the carbon-3 and 61% on the carbon-6 and about 1% possibly at carbon-2 of the glucose.

Nuclear magnetic resonance studies

Nuclear magnetic resonance (NMR) is a spectroscopic technique which detects the magnetic properties of atomic nuclei. Proton (^{1}H), ^{13}C, and ^{31}P

NMR are widely used to study the structure of carbohydrates (McIntyre et al., 1990). Quantitative analyses are also done by NMR. Degrees of branching, degrees of polymerization, and reducing residues can be analyzed by ^{13}C NMR (Gidley, 1985; McIntyre et al., 1990). Molecular conformations and branchings in starch chains can also be detected by ^{13}C NMR (Seymour et al., 1979; Jane and Robyt, 1985; McIntyre et al., 1990). Jane and Robyt (1985) reported conformational of amylose and Nägeli dextrin changed when complexed with various complexing agents by ^{13}C NMR. Carbon-13 NMR also can be used for studying the structure of each position of carbon in the sugar ring. The sensitivity of the NMR is dependent on the nuclei. The ^{1}H NMR is the most sensitive followed by ^{31}P, and ^{13}C NMR (McIntyre et al., 1990). ^{31}P NMR spectra can be obtained at a low concentration (1-10 mM) within a 2-4 hr (acquisition time) (McIntyre et al., 1990). ^{31}P NMR is used to detect the phosphate esters in the starch (Mcintyre, 1990; Lim, 1990). The pH and temperature effect chemical shifts of the ^{31}P signals because of the change of environment of nuclei (Bock and Sheard, 1975; Barany and Glonek, 1982; James, 1985; Tebby and Glonek, 1991). Resolution of the spectra can be improved by using chelating agent (EDTA) to reduce the broadening of the signals caused by cation interactions (Barany and Glonek, 1982). Some of the broadening of the ^{31}P NMR signals are caused by a number of molecules which give signals at similar chemical shifts, such as lecithin which has a mixture of

fatty acids. The signals are overlapped and cannot be resolved by the spectroscopy (Barany and Glonek, 1982). At pH 8-12, most of the sugar phosphomonoesters show the signals at the chemical shift δ 2 to 5 ppm; inorganic phosphate shows the signal at the chemical shift δ 2.6 ppm, and phosphodiester shows the signal at the chemical shift δ -1.0 to 1.0 ppm from an external standard, 85% phosphoric acid, (0.0 ppm) (Tebby and Glonek, 1991; Barany and Glonek, 1982; James, 1985). Barany and Glonek (1982) also reported the chemical shifts of the phospholipid at 0.0 ppm, phosphatidylcholine at -0.9 ppm, and phosphatidylethanolamine at -0.3 ppm. Tebby and Glonek (1991) proposed that cyclic phosphate such as glucose 1,2-cyclic-phosphate, myo-inositol 1,2-cyclic-phosphate, have the chemical shift at 15.5 ppm. Lim (1990) reported the spectra of the native potato starch which contains the phosphate monoester. The spectra of D-glucose 2-, 3-, 6-phosphate shows the signals at the chemical shift δ 1.38, 1.85, and 2.0 ppm from inorganic phosphate(Pi), respectively (Lim, 1990). The ^{31}P NMR spectrum of α-limit dextrins of phosphorylated potato amylose shows signals at chemical shift of δ 0.8, 1.03, 1.15, and 1.6 ppm downfield from Pi (0.0 ppm) (Lim, 1990). Lim (1990) also reported ^{31}P NMR spectrum of the α,γ-phosphodextrin (hydrolysis by α-amylase and glucoamylase) of phosphorylate potato amylose showed signals at δ 1.95, 1.62, 1.03, and 0.8 ppm from Pi. The signals at δ 1.95 and 1.62 ppm (from Pi, 0.0 ppm) indicated phosphomonoesters at carbon-6 and phosphomonoesters at carbon-3 on the non-reducing end, respectively. The

signals at δ 1.03 and 0.8 ppm (from Pi, 0.0 ppm) indicated phosphomonoesters at carbon-6 and phosphomonoesters at carbon-3 at the internal chain of repeating anhydroglucose unit in the α,γ-phosphodextrin. The phospholipids in wheat starch, lysophosphatidylcholine and lysophosphatidylethanolamine which have phosphate diester groups, show chemical shifts at about -2.73, -2.99 ppm from Pi (Lim, 1990). Lim (1990) also reported chemical shifts of ^{13}C NMR of the α-methyl glucoside, α-methyl group showed at 55.6 ppm, carbon-1 at about 99.9 ppm, carbon-2 at about 71.9 ppm, carbon-3 at about 73.8 ppm, carbon-4 at about 70.3 ppm, carbon-5 at about 72.2 ppm, and carbon-6 at about 61.3 ppm from standard TMS (0.0 ppm). The C-13 NMR chemical shift of a carbon, which is phosphorylated, shifts downfield for about 2 to 4 ppm from the original signals.

MATERIALS AND METHODS

Materials

Normal corn starch was purchased from Sigma Chemical Company (St. Louis, MO). Phosphorus oxychloride (99.999%) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). The agent was used without further treatment. Sepharose CL-2B and Sephacryl S-500 were products of Pharmacia Inc. (Piscataway, NJ). Bio-gel P-6 was a product of Bio-Rad Laboratories (Richmond, CA). A strong basic anion exchange resin (AG1-X8) was purchased from Bio-Rad Laboratory (Richmond, CA). Crystalline *Bacillus amyloliquefaciens* α-amylase was purchased from Sigma Chemical (St. Louis, MO). Activity of the α-amylase was 1400 unit/mg protein. One unit of α-amylase liberates 1 mg of maltose from starch in 3 min at pH 6.9 at 20°C. The reference compounds for NMR spectroscopy, 85% phosphoric acid (H_3PO_4), Trimethyl silane (TMS), glucose-1-phosphate, glucose-6-phosphate were products of Aldrich Chemical Co., Inc. Glucose-2-phosphate and a mixture of glucose-3 and 6-phosphate were received from Dr. Lim. All other chemicals were reagent grade and were used without further treatment unless otherwise specified.

Methods

Moisture determination

Moisture contents of the starches used in the experiments were determined by the procedure of Smith (1964a) with modification. The aluminum pan was dried and was accurately weighed (A). About 2 g of starch sample in the pan was accurately weighed (B). The pan with starch was placed in the oven at 120°C for 2 hr, then the pan was taken out of the oven and was cooled to room temperature in a desiccator. The pan with dried starch was accurately weighed (C). The percentage of moisture content was calculated by using the formula below.

$$\text{Percentage of moisture} = 100[B - C / B - A]$$

Defatting of starch

The starch was defatted by the method of Schoch (1942). Normal corn starch was defatted by extraction using 85% methanol for 24 hr in a Soxhlet extractor. After extraction, the starch was washed 2 times with 100% methanol and then dried in an air-draft oven (Isotemp model 655F, Fisher Scientific, Pittsburgh, PA) at 70°C.

Nägeli dextrin

Normal corn starch and waxy corn starch were hydrolyzed with acid. One hundred grams of starch was slurried in 1000 ml of 16% H_2SO_4 following the method of Kainuma and French (1973). The mixture had been kept in a 2 L flask for about 6 months at room temperature. During the period, flask was shaken once a week to mix the suspension. After the mixture was kept for about 6 months, the starch was neutralized with 1.0 M NaOH and was washed with about 10 volumes of distilled water. After that, the starch was dehydrated with 100% methanol and filtered with a Whatman No. 4 filter paper and was dried in an air-draft oven at 40°C.

Small particle starch

Small particle starch was prepared by the method of Jane et al. (1991a). Small particle starch was obtained by the acid hydrolysis of starch in an absolute ethyl alcohol. Normal corn starch (909 g, dsb) was suspended in 2 L. of absolute ethyl alcohol containing 1.8% w/w of HCl. The treatment was carried out in a three-neck, round-bottom flask equipped with a Liebig condenser and a heavy-duty propeller mixer. The suspension was heated with a heating mantle to its boiling temperature (80°C) and refluxed for 3 hr. Then the suspension was cooled to room temperature (25°C), and the starch was isolated by filtration with a Whatman No. 4 filter paper. The starch was

resuspended in 1 L of distilled water and neutralized with 1 N NaOH, then drained and washed two times with 1 L of distilled water each time. After washing, the starch was dehydrated with 1 L of 100% methyl alcohol and dried in the draft-air oven at 70°C for 4 hr. The hydrolyzed starch was milled at 70 rpm with a ball-mill (0.5 cm glass-beads) for 8 hr using 100% ethyl alcohol (1 L).

Phosphorus oxychloride modification

Normal corn starch, Nägeli dextrin, small particle starches, and defatted corn starch were modified by $POCl_3$. For low degree $POCl_3$ modifications, tetrahydrofuran (THF) was used for diluting the reagent.

Normal corn starch modification

Normal corn starch and defatted normal corn starch were modified with $POCl_3$. One hundred grams dry weight of starch was dissolved in 120 g of distilled water. The starch slurry was adjusted to the desired pH with 1.0 M NaOH or 1.0 M HCl, then $POCl_3$ was added to the slurry, stirred by a propeller. The pH of the slurry was controlled by a pH controller (Chemcadet, Cole parmer Instrument Co., Chicago, IL) at room temperature. After 30 min, the pH of the slurry was adjusted to 5.5 to stop the reaction. The slurry was washed 3 times with distilled water, and 2 times with 100% methyl alcohol. Then the

washed starch was filtered with a Whatman No.4 filter paper. The modified starch was dried in the draft-air oven at 40°C.

The starch was modified with different pH to observe the optimum pH for modification. pH 7, 8, 9, 10, and 11 were used in 0.005% w/w $POCl_3$ starch modification. pH 7, 8, 9, 10, 11, and 12 were also used to modify starch with 0.05% w/w $POCl_3$. The degree of the modification was determined by using a Brabender visco/amylo/graph (VA-VE MODEL, C.W. Brabender Instruments, Inc., S. Hackensack, NJ).

After the optimum pH was identified, the starch was modified with different concentrations of $POCl_3$ (0.005%, 0.05%, 0.06%, 0.07%, 0.08%, 0.1%, 0.12%, 0.14%, 0.18%, 0.2%, 0.5%, and 1.0%) at the optimum pH. The viscosities of the modified starches with various concentrations of $POCl_3$ were determined by using the Brabender visco/amylo/graph.

Nägeli dextrin and small particle starch modification

The procedure of Nägeli dextrin modification is the same as that of the starch modification except that the concentration of the Nägeli dextrin or small particle starch was 20% w/v instead of 83.33% (1:1.2 = starch : distilled water). After the modification, no washing step was performed because the Nägeli dextrin and small particle starch swelled in the solutions and did not retain in granular form. The modified Nägeli dextrin and small particle starch slurry were

diluted with 2 volumes of distilled water to dissolve the inorganic phosphate which left from the reaction. The solution was then precipitated with 6 volumes of 100% methyl alcohol, and the precipitant was dried in the draft-air oven at 70°C.

α-Methyl glucoside modification

The procedure of α-methyl glucoside modification is the same as that of the Nägeli dextrin and small particle starch modification except no dilution and precipitation steps is preformed, and it is dried by vacuum evaporator at 40°C.

Starch phosphomonoester

Orthophosphate (mixture of mono- and dihydrogen phosphate at pH 5-6.5) gives the maximum of starch phosphomonoester at 0.2 degree of substituent (DS) (Paschall, 1964). The starch phosphate was prepared by the method of Paschall (1964). One hundred grams dry weight of normal corn starch was mixed with 106 ml of distilled water, 57.7 g of sodium dihydrogen phosphate ($NaH_2PO_4 . H_2O$), and 83.7 gm of disodium hydrogen phosphate (Na_2HPO_4) at pH 6.1. The mixture was stirred with a stirring bar for 10 min, then filtered with a Whatman No. 4 filter paper and dried in a draft-air oven at 40-45°C for about 18 hr. The dried starch was ground with a coffee grinder, was kept in the draft-air oven at 65°C for 90 min, and then heated in the same oven at 155°C for 3 hr (modified from Paschall, 1964). After heating, the starch

diluted with 2 volumes of distilled water to dissolve the inorganic phosphate which left from the reaction. The solution was then precipitated with 6 volumes of 100% methyl alcohol, and the precipitant was dried in the draft-air oven at 70°C.

α-Methyl glucoside modification

The procedure of α-methyl glucoside modification is the same as that of the Nägeli dextrin and small particle starch modification except no dilution and precipitation steps is preformed, and it is dried by vacuum evaporator at 40°C.

Starch phosphomonoester

Orthophosphate (mixture of mono- and dihydrogen phosphate at pH 5-6.5) gives the maximum of starch phosphomonoester at 0.2 degree of substituent (DS) (Paschall, 1964). The starch phosphate was prepared by the method of Paschall (1964). One hundred grams dry weight of normal corn starch was mixed with 106 ml of distilled water, 57.7 g of sodium dihydrogen phosphate ($NaH_2PO_4 . H_2O$), and 83.7 gm of disodium hydrogen phosphate (Na_2HPO_4) at pH 6.1. The mixture was stirred with a stirring bar for 10 min, then filtered with a Whatman No. 4 filter paper and dried in a draft-air oven at 40-45°C for about 18 hr. The dried starch was ground with a coffee grinder, was kept in the draft-air oven at 65°C for 90 min, and then heated in the same oven at 155°C for 3 hr (modified from Paschall, 1964). After heating, the starch

was cooled to room temperature, suspended in 750 ml of 50% methyl alcohol, and stirred with a magnetic stirrer for 30 min. The starch was then washed with 250 ml of 100% methyl alcohol 3 times and dried in the draft-air oven at 70°C.

Viscosity

Viscosities of native and modified starches were obtained by using the Brabender visco/amylo/graph which determines the viscosity continuously during heating, cooking, and cooling of the starch paste. Thirty-two grams dry weights of starch were suspended in 368 g of distilled water and put in an amylograph cup. The suspension was heated at the rate of 1.5°C per min with a stirring rate at 78 rpm. The suspension was continuously heated to 95°C (45 min) and was held for 30 min, then it was cooled to 50°C at the same rate (30 min). The amylogram was reported in viscosity (Brabender unit, [BU]) vs time (min). The pasting temperature was determined when the viscosity started to increase.

Starch fractionation

Modified starches were fractionated into soluble and insoluble fractions. Thirty grams of the modified starch was dissolved in 1 L of 90% dimethylsulfoxide (DMSO). The slurry was boiled with stirring in a water bath (95°C) for 2 hr and continuously stirred at the room temperature for 24 hr, then

the slurry was centrifuged at 6400 g for 20 min. After centrifuging, the supernatant and the precipitate were collected separately. The precipitate was washed twice with methyl alcohol and dried in the draft-air oven at 70°C. The supernatant was precipitated with about 10 volumes of 100% methyl alcohol and was filtered by a Whatman No. 4 filter paper, then dried in the draft-air oven at 70°C.

Determination of total carbohydrate by phenol-sulfuric acid method

Total carbohydrate of the sample was determined by the method of Dubois et al. (1956). One milliliter of diluted sample solution was mixed with 1 ml of 5% phenol solution, and 5 ml of concentrated sulfuric acid was added quickly into the mixture. The solution was then mixed and was kept for 30 min at room temperature. The absorbance of the solution was measured at 470 nm with spectrophotometer (Beckman DU series 50, Beckman Instruments, Inc., Fullerton, CA). A standard curve was constructed with solutions containing 10-100 μg of glucose.

Gel permeation chromatography

Molecular weight distributions of native normal corn starch, the soluble fraction of the modified normal corn starch, small particle starch, modified small particle starch, Nägeli dextrin, and modified Nägeli dextrin were determined by

using gel permeation chromatography. One gram of sample was suspended in 100 ml of 90% DMSO. The suspension was boiled and stirred in the boiling water bath for 2 hr and continuously stirred at room temperature for 24 hr. Fifteen milliliter of the suspensions (about 150 mg of samples) was precipitated with about 100 ml of 100% methyl alcohol. The precipitate was separated by centrifugation at 5000 g for 20 min. The precipitate was used for the gel permeation chromatography as follows:

Gel permeation chromatography on Sepharose CL-2B

The precipitates (about 150 mg of starch samples) were dissolved in 50 ml, 75 ml, and 150 ml of boiling distilled water depending on peak height on the profile, and about 10 mg, 15 mg, and 30 mg of glucose was added respectively as a marker. Five milliliter solutions (containing 15 mg, 10 mg, or 5 mg) of normal corn starch and soluble fraction of the modified starch were injected into a 2.6 x 80 cm column (Pharmacia Inc., Piscataway, NJ) packed with Sepharose CL-2B gel. Distilled, deionized water containing 0.02% sodium sulfate (Na_2SO_4) was used to elute the samples in an ascending direction with the flow rate of 30 ml per hr. Fractions of 4.8 ml were collected and analyzed by Autoanalyzer II (Technicon Instruments Corp., Elmsford, NY). The total carbohydrate (anthrone-sulfuric acid reaction) and amylose-iodine blue value of the fractions were measured at 630 nm and 640 nm, respectively. Glucose solution (0.005%

w/v) was used as a standard marker for total carbohydrate determination. Molecular weight distributions were calculated on the basis of total carbohydrate. The blue value was used to identify locations of amylose and amylopectin in the chromatograms.

Gel permeation chromatography of Sephacryl S-500

About 150 mg of starch sample which was precipitated from 15 ml of 90% DMSO solutions (1 g of starch/100 ml 90% DMSO) was dissolved in 75, 150 ml of boiling distilled water. Five milliliter of the solutions was injected into a 2.6 x 80 cm column (Pharmacia Inc., Piscataway, NJ) packed with Sepharcyl S-500 gel. The samples were eluted, and the fractions were collected and analyzed by the same procedure of gel permeation chromatography of Sepharose CL-2B.

Gel permeation chromatography of Bio-gel P-6

Molecular weight distribution of the Nägeli dextrins and the small particle starch were determined by using gel permeation chromatography of Bio-gel P-6. One gram each of the Nägeli dextrins and the small particle starch was dissolved in 100 ml of 90% DMSO. The solution was heated and stirred in the boiling water bath for 2 hr. The solution was then cooled to room temperature and stirred for 24 hr. After stirring, 50 ml of the solution (about 500 mg of

sample) was precipitated from DMSO solution with 5 volumes of 100% methyl alcohol. The precipitates were redissolved in 50 ml of boiling distilled water, and 20 mg of glucose was added as a marker. One milliliter of sample solution was injected into a 1.5 x 80 cm Bio-Rad Econo-column (Bio-Rad laboratories, Richmond, CA) packed with Bio-gel P-6. Samples were eluted with distilled and deionized water in a descending direction. Fractions of 2.3 ml per cup were collected and were analyzed by the Autoanalyzer II for total carbohydrate content at 630 nm.

Enzymatic hydrolysis of starch, small particle starch, and Nägeli dextrin

The starch samples were hydrolyzed by *Bacillus amyloliquefaciens* α-amylase. One gram of starch or modified starch (4 gm of small particle starch, modified small particle starch, Nägeli dextrin, or modified Nägeli dextrin) samples were dissolved in 20 ml of 0.1 M acetate buffer (pH 6.9). The suspensions were heated and stirred in the boiling water bath for 20 min, then cooled down to room temperature. The cooled solutions were mixed with 1 ml of 0.1 M acetate buffer (pH 6.9) and 250 U of the crystalline Bacillus α-amylase (1000 U of α-amylase for small particle, modified small particle, Nägeli dextrin, and modified Nägeli dextrin). The mixtures were incubated in a water bath shaker (Versa-Bath S, model 236, Fisher Scientific) at 70°C, 100 strokes per min for 2 hr. After incubation, the starch samples were boiled and stirred in the

boiling water bath for 20 min to stop the enzyme activity. The hydrolyzed samples were subjected to anion exchange chromatography and NMR measurements.

Anion exchange chromatography

The hydrolyzed samples of $POCl_3$ modified Nägeli dextrin and small particle starch were subjected to anion exchange chromatography (AG1-X8). The anion exchange resin would absorb those oligosaccharides carrying phosphomono- and -diesters preferably, thus, separated the sugars with phosphate derivatives from those without phosphate.

Five grams of hydrolyzed sample was filtered by a Whatman No. 4 filter paper. The filtrate was adjusted to pH 7.5 and was injected into a 1.0 x 20 cm Flex-column (Kontes scientific glassware/instruments, Vineland, NJ) packed with a strong basic anion exchange resin (AG1-X8) in the bicarbonate form. The column was washed with 1 L of deionized water to remove all unmodified (phosphate ester) glucans. Ammonium bicarbonate (0.4 M) was used as an eluent. One hundred and twenty fractions of 19 ml per fraction were collected and were determined for total carbohydrate by phenol-sulfuric acid method. The fractions containing carbohydrate were combined, and the ammonium bicarbonate was removed by repeated vacuum evaporation. The evaporated sample was used for analysis by ^{31}P NMR measurement.

NMR measurement

The $POCl_3$ modified normal corn starch, soluble fractions, and insoluble fractions separated from the modified starch, Nägeli dextrins, modified Nägeli dextrins, small particle starch, and modified small particle starch were analyzed by NMR to determine structures and locations of the phosphate ester.

Phosphorus-31 NMR spectroscopy

Nuclear magnetic resonance spectra of the modified starches were analyzed by using a Bruker WM-200 NMR spectrometer (USA Bruker Instruments, Inc., Mountain View, CA), unless otherwise specified. ^{31}P NMR spectra were acquired with the following conditions: a spectrometer frequency of 81 MHz, using a 65° flip angle (15 μsec), 16 K data points, 20,000 Hz sweep width, and a recycle time of 1.4 sec. Typically, the spectrum was collected with about 30,000 scans. All samples were dissolved in aqueous solution containing 0.02 M EDTA to sharpen the signal and deuterium oxide (D_20) to lock the signal. The pH was adjusted to 8.0 ± 0.1 by 0.1 M NaOH prior to NMR measurement. All chemical shifts were reported in ppm from 85% H_3PO_4 as an external reference (0.0 ppm). The chemical shifts were recorded with ^1H-decoupled spectra. All chemical shifts were obtained by using NMR spectrometer at room temperature (25°C) unless otherwise specified. The solid NMR spectra were conducted with Bruker MSL-300 spectrometer (USA Bruker

Instruments, Inc., Mountain view, CA). The solid NMR spectra were acquired with 121.5 MHz, at 90° flip angle (6.5 μsec), 125,000 Hz sweep width, and 10 sec recycle time. The spectra were typically obtained in 20,000 scans.

Three milliliter of hydrolysed sample solution (5% concentration for modified starch and fractions of modified starch, 20% concentration for modified small particle starches and modified Nägeli dextrin) and 2 ml of D_2O containing 0.2 M EDTA was adjusted to pH 8.0 ± 0.1 and was injected into 10 mm (id) NMR sample tubes. The mixtures were used for liquid ^{31}P NMR measurement.

To identify chemical shifts of ^{31}P signals, the chemical shifts of reference compounds need to be revealed. For phosphate esters, glucose-1-phosphate, glucose-2-phosphate, mixture of glucose-3 phosphate and glucose-6-phosphate, glucose-6-phosphate, starch phosphomonoester, and lecithin were used as internal models. The chemical shifts were obtained by the same parameters as the samples except the references were acquired only about 16 acquisitions (except phosphate starch was obtained in about 1000 acquisitions). The glucose-1-phosphate, glucose-6-phosphate, starch phosphomonoester, and lecithin reference compounds were prepared in the same procedure as the samples without enzyme hydrolysis (except starch phosphomonoester was hydrolyzed by α-amylase). For phosphomonoester, the derivative of α-methyl glucoside, glucose-1-phosphate, glucose-6-phosphate, and starch phosphate monoester were used as model references. For phosphodiester (cross-linking),

lecithin was also used as a model reference. Sodium phosphate (pH 8) was used as an inorganic free phosphate internal reference. A spectrum of $POCl_3$ was obtained for identification of $POCl_3$ residue. Concentrated phosphoric acid (85% H_3PO_4) filled and sealed in capillary tube and was inserted into the middle of NMR tube which contained sample solutions. It was used as the external reference at δ 0.0 ppm.

The solution of 20% α-methyl glucoside (dsb) was modified with 5.0% $POCl_3$ in the same procedure as Nägeli dextrin except the solution was dried by vacuum evaporator at 45°C. The modified methyl glucoside was also measured by ^{31}P NMR for comparison.

Carbon-13 NMR spectroscopy

Normal corn starch, $POCl_3$ modified normal corn starch, starch phosphomonoester, waxy corn Nägeli dextrin, $POCl_3$ modified waxy corn Nägeli dextrin, and waxy corn Nägeli dextrin phosphomonoester were prepared for ^{13}C NMR measurement in the same procedure as ^{31}P NMR measurement except that EDTA was not added and Nägeli dextrin samples were not hydrolyzed by α-amylase. The ^{13}C NMR spectra were obtained by using Bruker WM-200 NMR spectrometer and recorded at 50.3 MHz with 60° pulse at 10 μsec, 16 K data points, 14,300 Hz sweep width, 1.6 recycle time in 40% D_2O solution. The chemical shifts were reported in ppm from trimethylsilane (TMS).

Trimethylsilane (δ 0.0 ppm) was used as an external reference for ^{13}C NMR measurement. The spectra were acquired within 4000 scans.

RESULTS AND DISCUSSION

Starch modification

Phosphorus oxychloride modification of starch at different pH

Normal corn starch was modified with $POCl_3$ at various pH. When $POCl_3$ was required to use at a low concentration, it was diluted with Tetrahydrofuran. Viscosities of the modified starches revealed types and degrees of the modification. The viscosity was determined by visco/amylo/graph from the method of Smith (1964b). A high degree cross-linking (phosphodiester linkage) in starch granules causes limited swelling which results in reducing the viscosity and increasing the pasting temperature. The phosphate derivative (phosphomonoester linkage) in starch will result in increasing the viscosity and lowering the pasting temperature because of charge repelling.

Normal corn starch was also modified with 0.05% $POCl_3$ at pH 7-12. With this concentration the result can explain the reaction rate of the modification by viscosity. The viscosity of the modified starches were detected by visco/amylo/graph. The amylogram indicates that modified starch at pH 7 to 11 do not show different viscosity. The amylogram also shows that the modified starch at pH 12 gives the highest viscosity because of the highest cross-linking rate. The cross-linked modified starch prepared at pH 12 showed

the highest pasting temperature at about 86 °C. The pasting temperature has shifted to the higher temperature because the cross-linking in the starch granule retarded swelling and pasting. The viscosity of this modified starch stays at the constant viscosity during the holding period because of the cross-linking which provides resistance to the shear thinning. The amylogram also suggests that the degree of modification increases as increasing the pH of reaction, and the highest degree of modification happens at pH 12. The viscosity profile is shown in Figure 1.

Modified starch with different concentration of $POCl_3$

Normal corn starch was modified with various concentrations of $POCl_3$ at pH 12. Phosphorus oxychloride can provide phosphodiester linkage for cross-linking and phosphomonoester derivative at the same time. Low concentration of $POCl_3$ can give a few phosphodiesters (cross-linking) which result in higher viscosity because of the cross-linking forming an apparent larger molecular weight. At a high concentration of $POCl_3$, the starch granules are highly cross-linked and the granules have limited swelling which results in the lower viscosity. Normal corn starch was modified at pH 12 with 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, and 0.2% of $POCl_3$. The modified starches with $POCl_3$ concentration from 0.005 to 0.1% gave viscosities higher than native normal corn starch. The viscosity of the

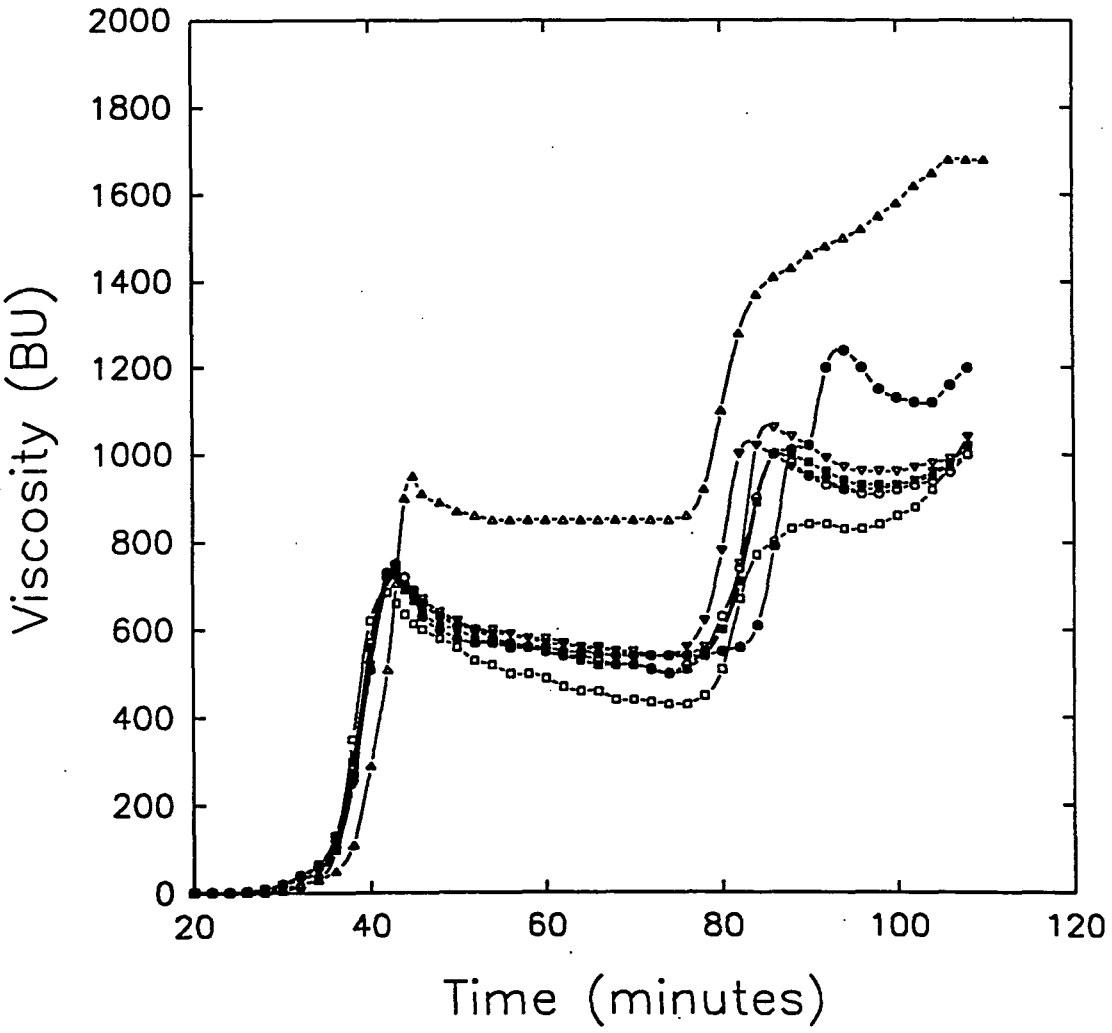


Fig. 1. Brabender amylogram of slurries (8% solid) of native and cross-linked normal corn starch in water. Native normal corn starch: □ - □ ; cross-linked corn starches prepared with 0.05% $POCl_3$ at pH 7: ● - ● , pH 8: ∇ - ∇ , pH 9: ▾ - ▾ , pH 10: o - o , pH 11: ■ - ■ , and pH 12: ▵ - ▵ .

modified starch increased when the concentration of $POCl_3$ increased from 0.005 to 0.04%, but if the concentration of $POCl_3$ was higher than 0.04% it caused a decrease in viscosity. The modified starch prepared with 0.2% $POCl_3$ showed no viscosity increase at all because the very high cross-linking of starch granules totally restrained from swelling. The modified starches made with a $POCl_3$ concentration higher than 0.1 had some insoluble parts and precipitated from the solution the samples. The viscosity profiles are shown in Figure 2-4.

Fractionation of starch

Phosphorus oxychloride modified starch was dispersed in 90% DMSO and was separated into soluble and insoluble fractions. Soluble fractions of the 0.04, 0.08, 0.1, 0.14, 0.18, and 0.2% $POCl_3$ modified starches were analyzed for the molecular weight distribution. All the chromatograms of the soluble fractions were compared to the chromatogram of normal corn starch. The soluble fractions showed only amylose (Figure 5-11). Modified starches that were made with concentrations of $POCl_3$ lower than 0.1% could not be separated into the insoluble and the soluble fractions. The cross-linking in starch samples formed network and caused the starch to become insoluble. The amylopectin in the modified starch was preferentially cross-linked and became insoluble. Amylose is a relatively linear and smaller molecule compared to amylopectin, so it is less susceptible to cross-linking. Amylopectin

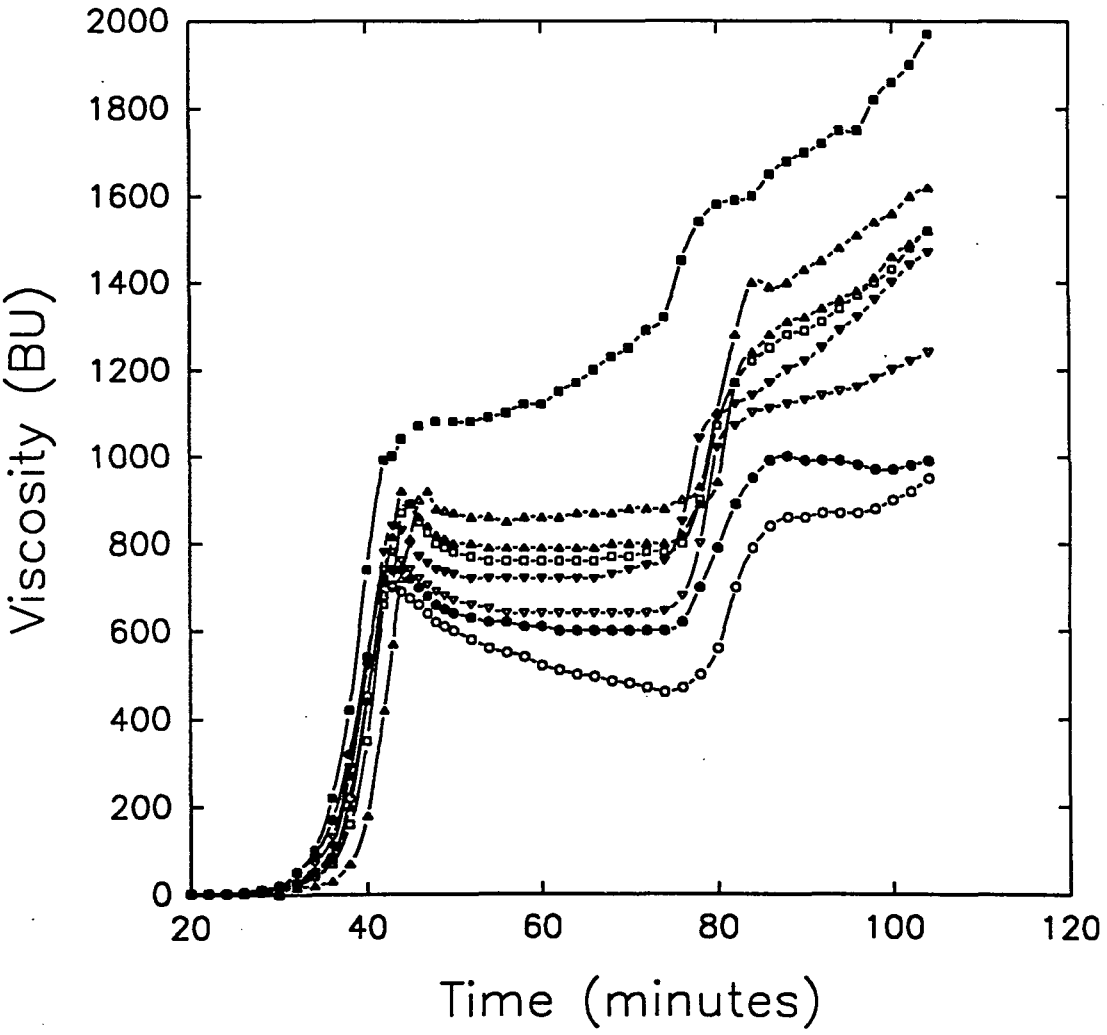


Fig. 2. Brabender amylogram of slurries (8% solid) of native and cross-linked normal corn starches in water. Native normal corn starch: o - o ; cross-linked corn starches prepared at pH 12 with 0.005% $POCl_3$: ● - ● , 0.01% $POCl_3$: ∇ - ∇ , 0.02% $POCl_3$: ▾ - ▾ ,0.03% $POCl_3$: □ - □ , 0.04% $POCl_3$: ■ - ■ , 0.05% $POCl_3$: ▵ - ▵ , and 0.06% $POCl_3$: ▲ - ▲ .

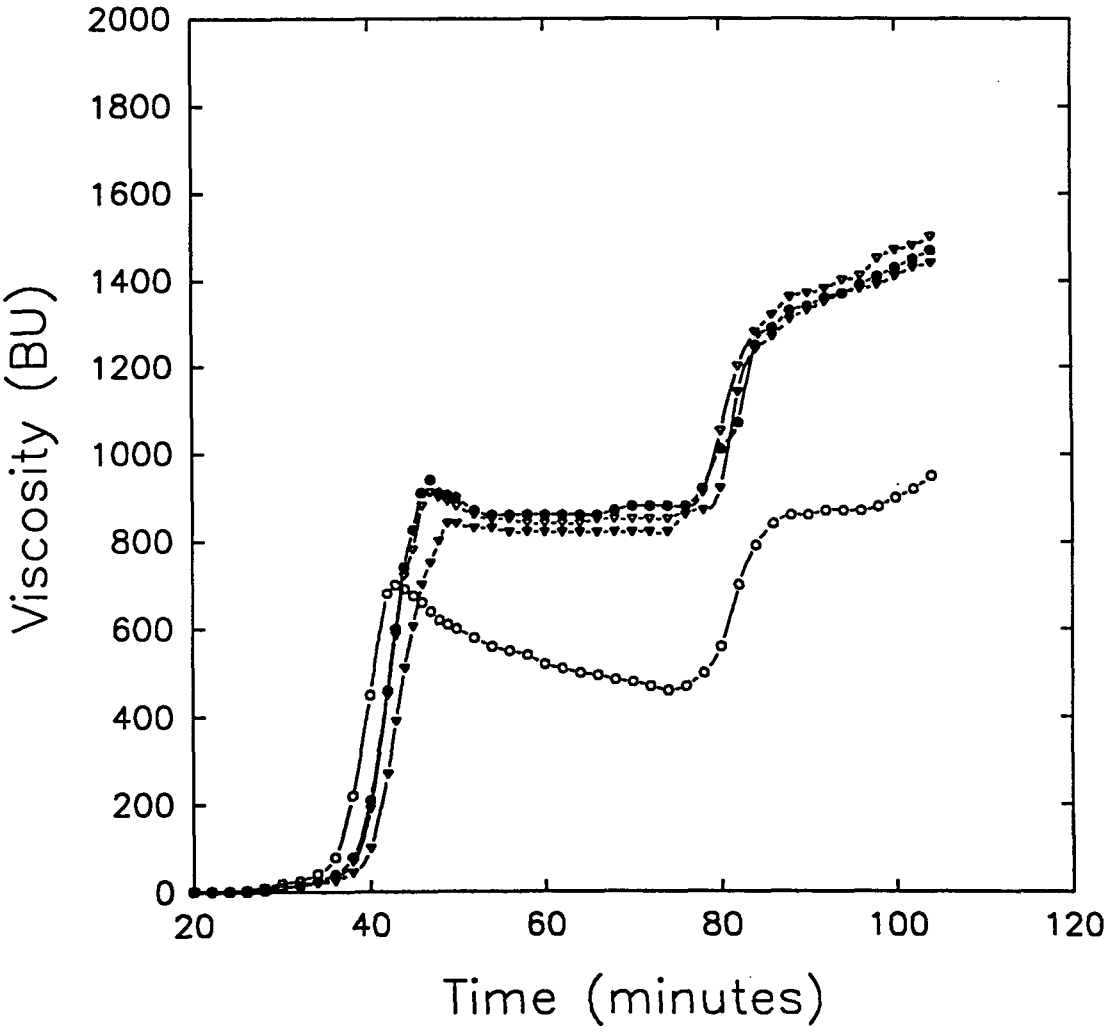


Fig. 3. Brabender amylogram of slurries (8% solid) of native and cross-linked normal corn starches in water. Native normal corn starch: o - o ; cross-linked corn starches prepared at pH 12 with 0.07% $POCl_3$: ● - ● , 0.08% $POCl_3$: ∇ - ∇ , 0.1% $POCl_3$: ▾ - ▾ .

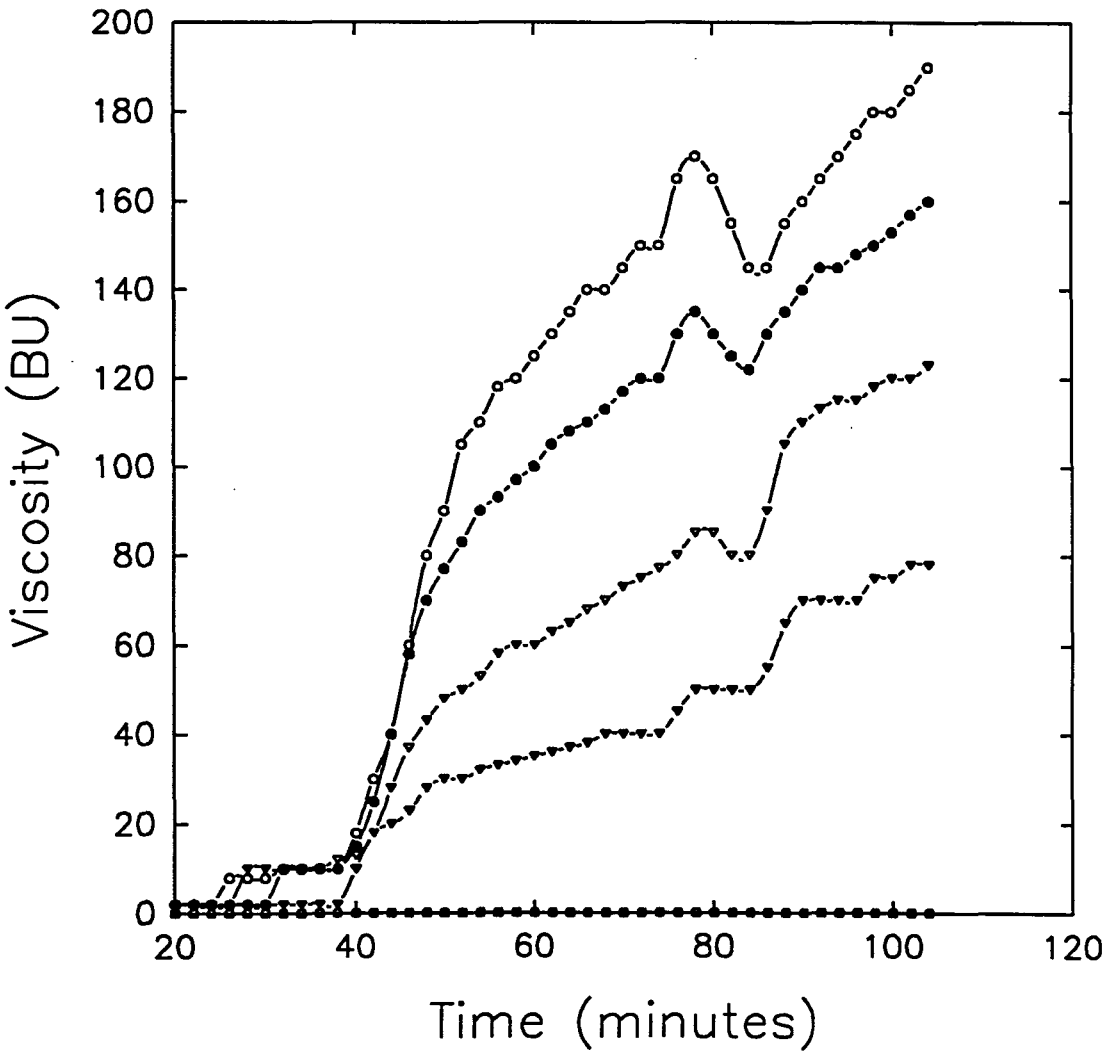


Fig. 4. Brabender amylogram of slurries (8% solid) of cross-linked normal corn starches in water. Cross-linked corn starches prepared at pH 12 with 0.12% $POCl_3$: o - o , 0.14% $POCl_3$: ● - ● , 0.16% $POCl_3$: ∇ - ∇ , 0.18% $POCl_3$: ▾ - ▾ , 0.2% $POCl_3$: □ - □ .

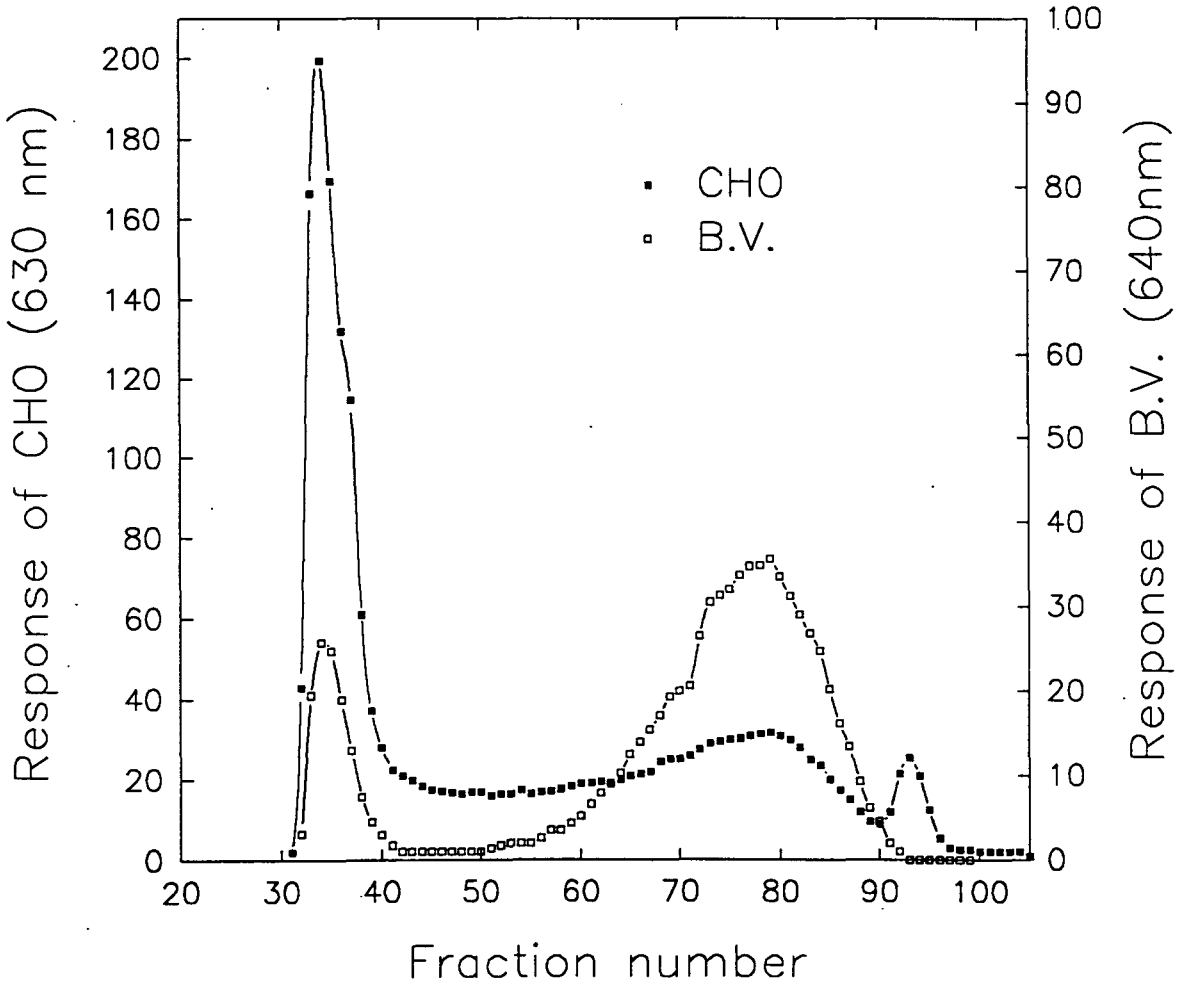


Fig. 5. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 10 mg of native normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

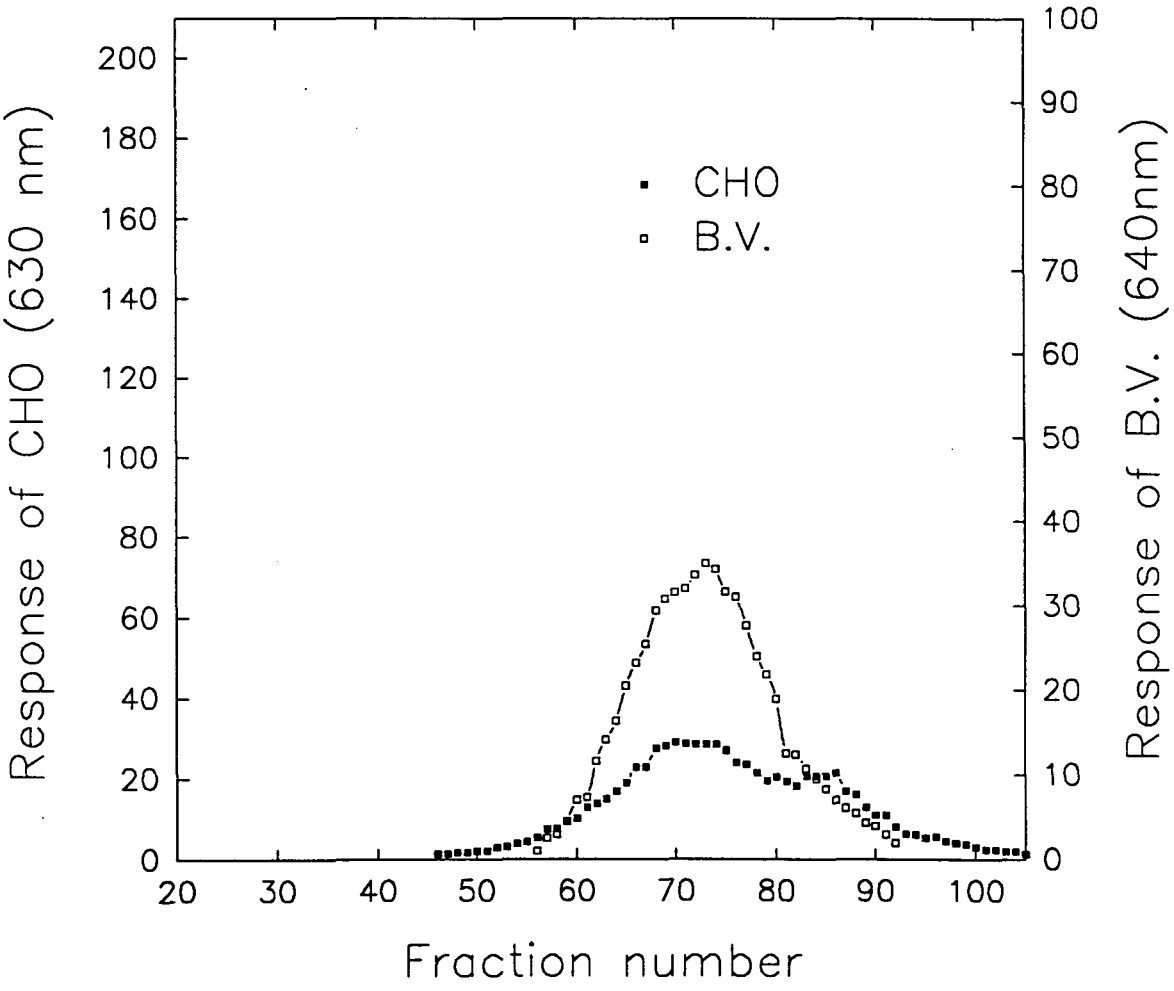


Fig. 6. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 10 mg of the soluble fraction which was fractionated from 0.04% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

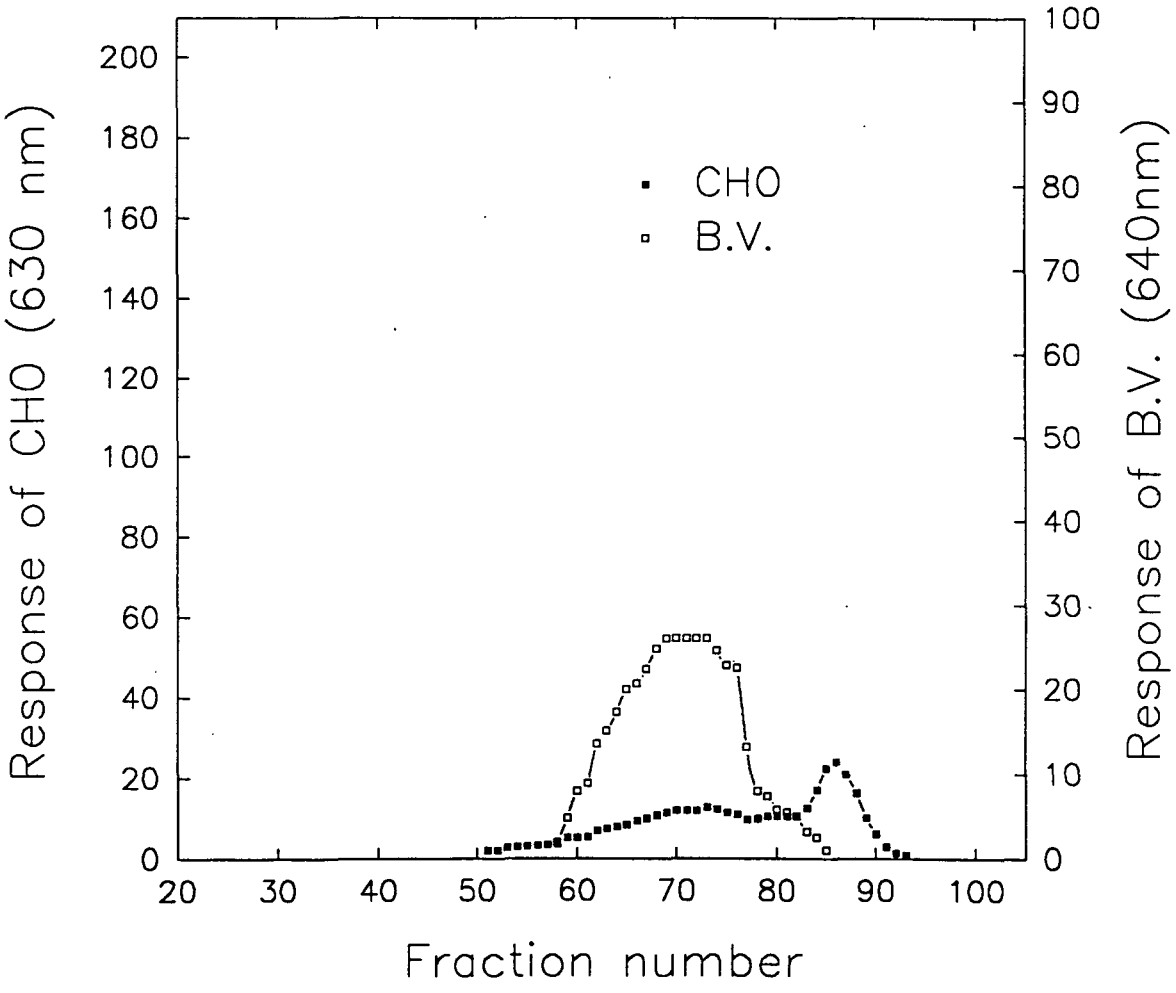


Fig. 7. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 10 mg of the soluble fraction which was fractionated from 0.08% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

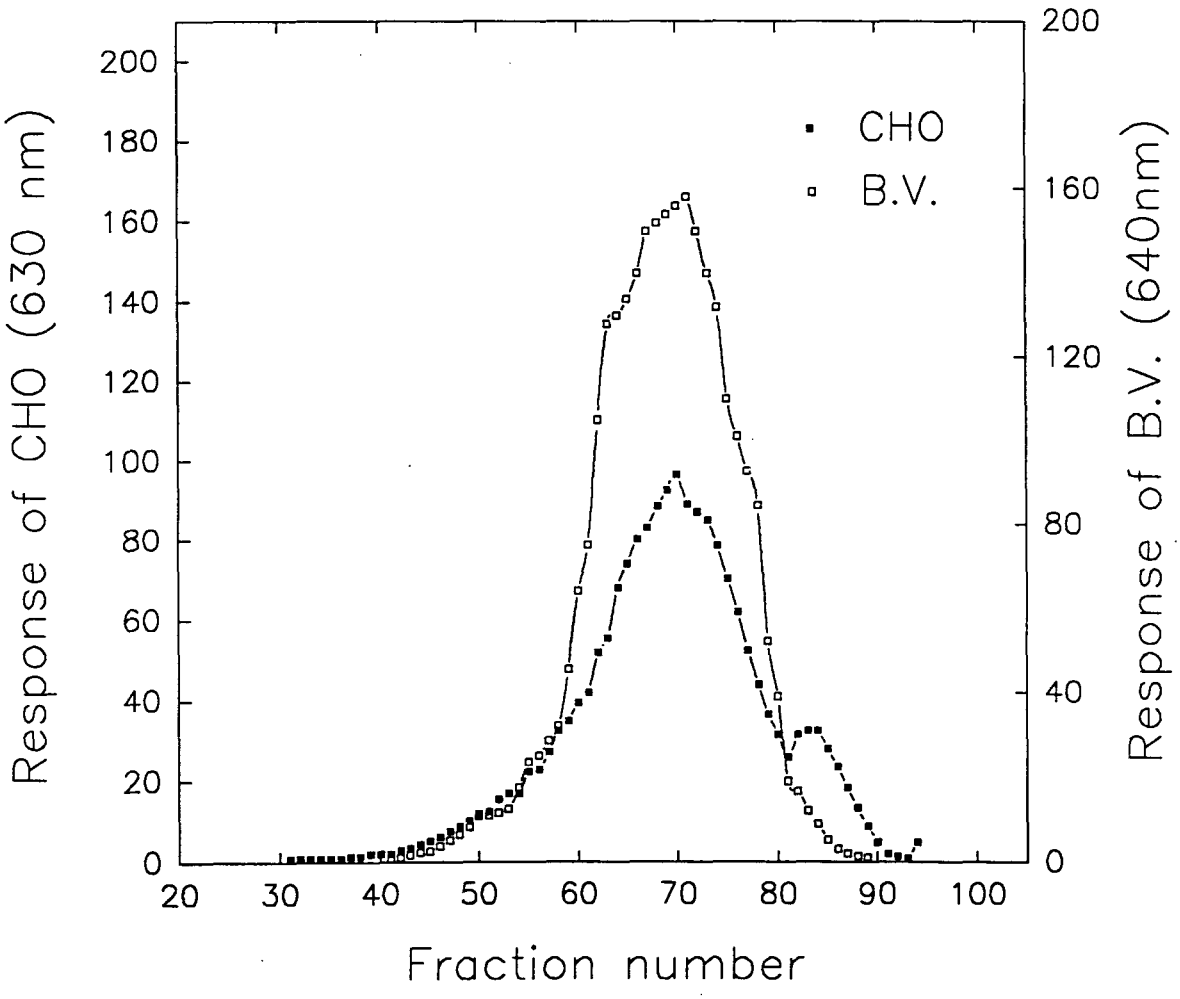


Fig. 8. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 5 mg of the soluble fraction which was fractionated from 0.1% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □. Glucose was used as a marker.

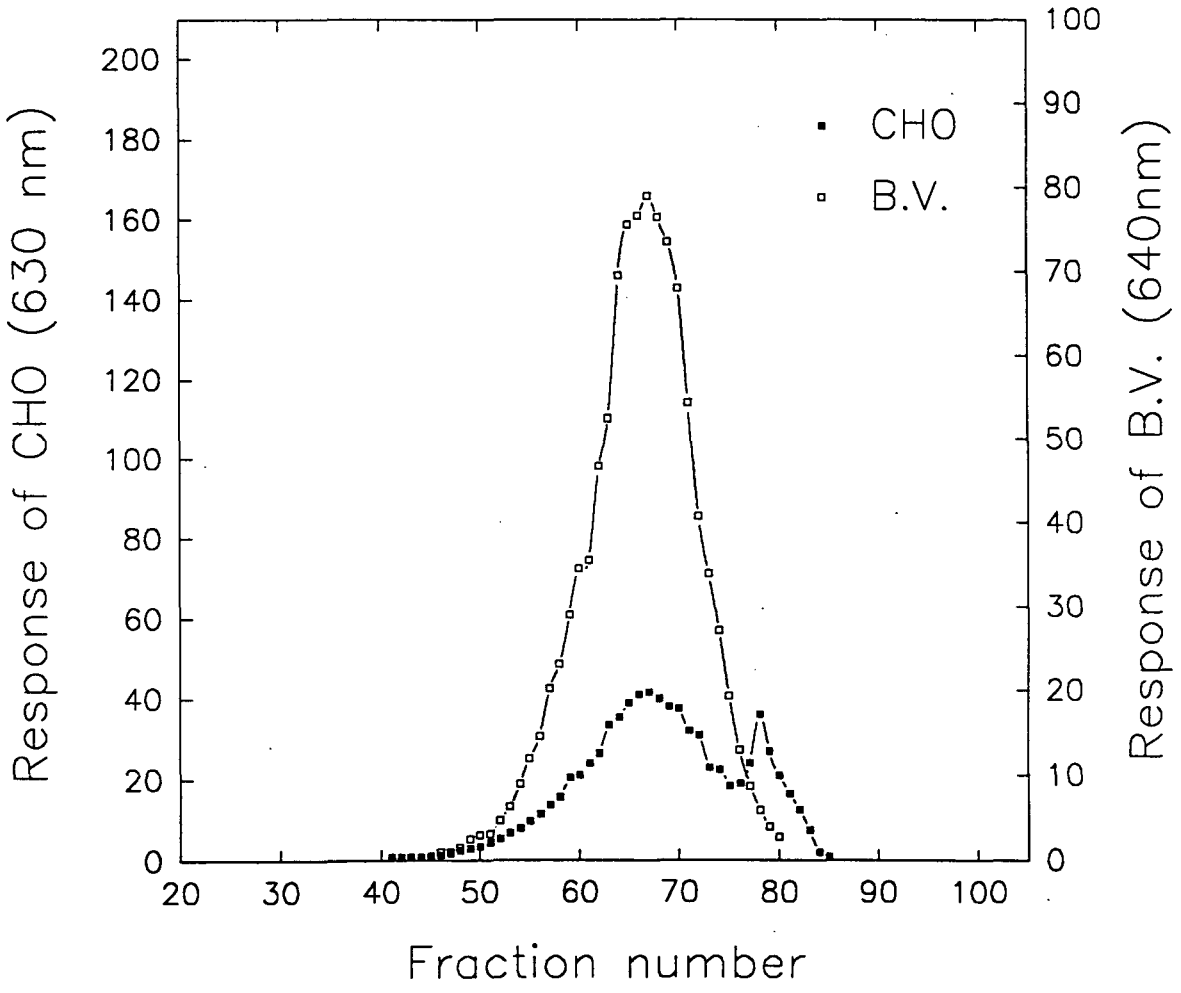


Fig. 9. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 5 mg of the soluble fraction which was fractionated from 0.14% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

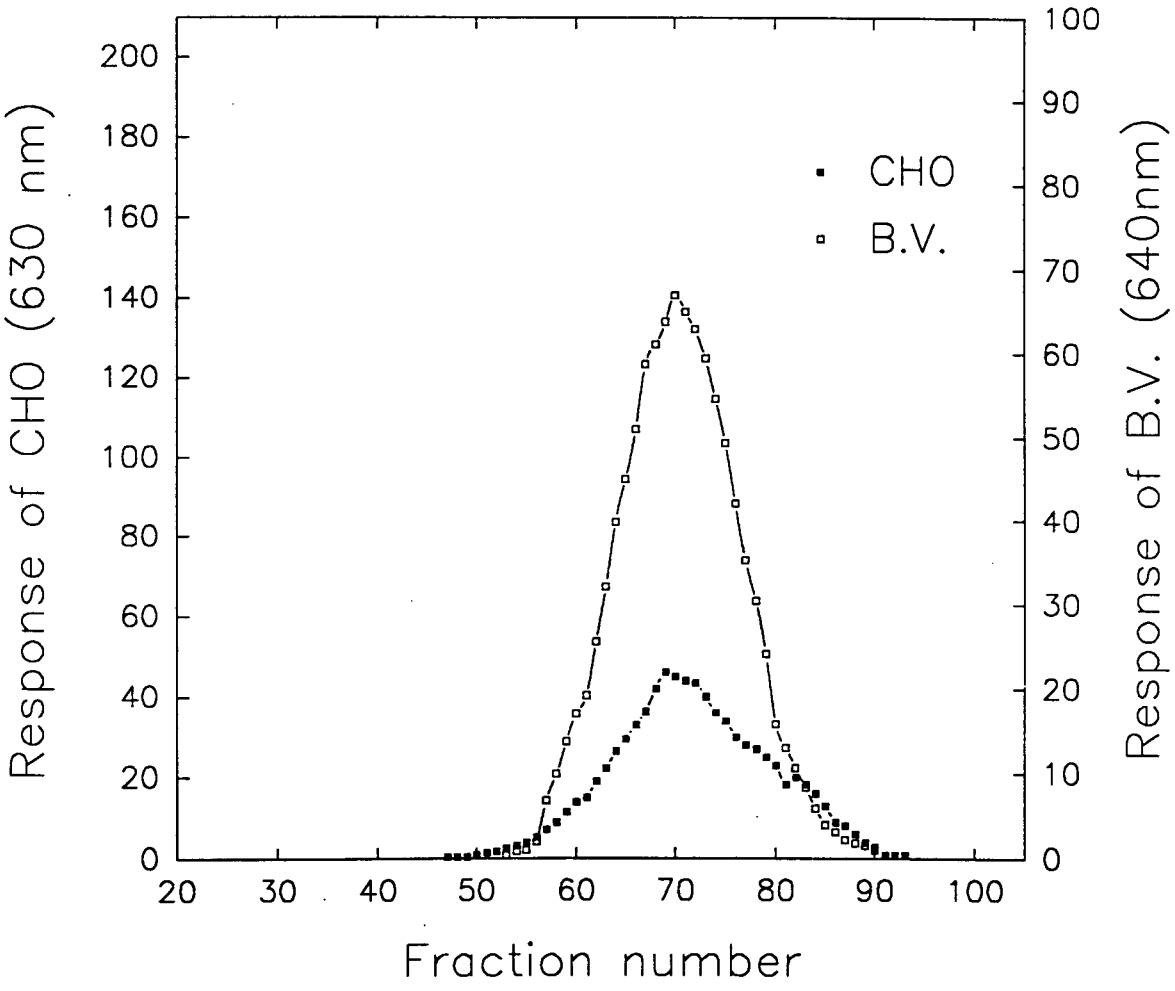


Fig. 10. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 5 mg of the soluble fraction which was fractionated from 0.18% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

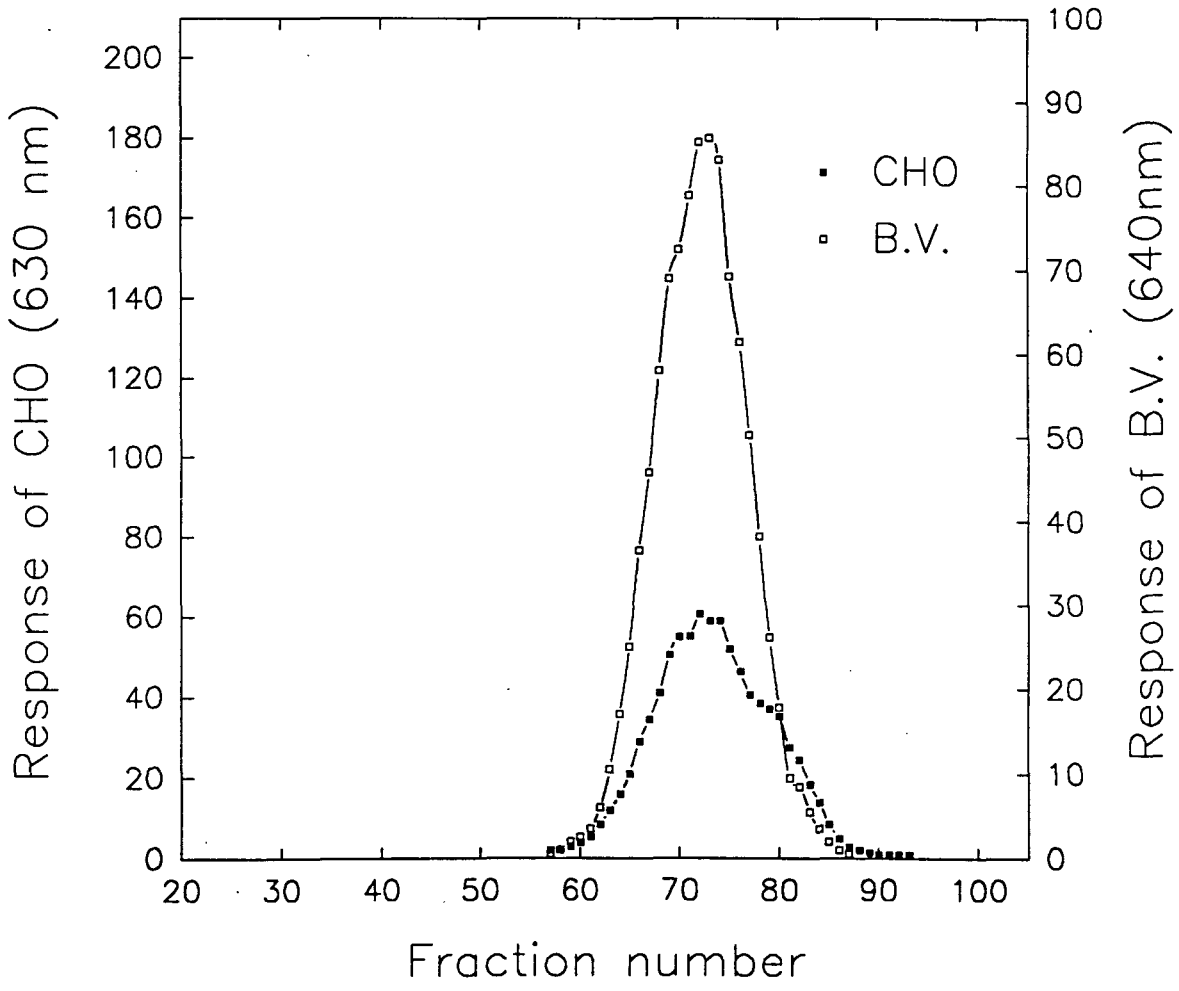


Fig. 11. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 10 mg of the soluble fraction which was fractionated from 0.2% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

was found cross-linked to modified starch samples, it appeared that amylopectin and large molecules of amylose were preferably cross-linked and became insoluble. Only small molecules of amylose were remained soluble and detected by gel permeation chromatography.

The insoluble sample was resuspended in 90% DMSO, stirred for 24 hr and then centrifuged to separate the second soluble portion which might have been entangled within the network. The molecular weight distribution of second soluble fraction was determined by the same procedure as the previous. The second soluble fraction showed the same small molecular weight of amylose. The chromatograms are shown in Figures 12 to 15.

The third soluble fraction was separated by the same procedure and was redissolved and determined for the molecular weight distribution. A small amount of small molecule of amylodextrin was detected. The chromatograms are shown in Figures 16 to 19. Only the modified starches made with 0.1-0.2 % of $POCl_3$ were used for the analysis because of the separatability of the insoluble and soluble portions. The modified starch with lower concentration of $POCl_3$ could not be separated into insoluble and soluble fractions because it formed a gel-like structure and entangled together. The modified starch with higher concentration of $POCl_3$ did not give a soluble portion because the starch was highly cross-linked.

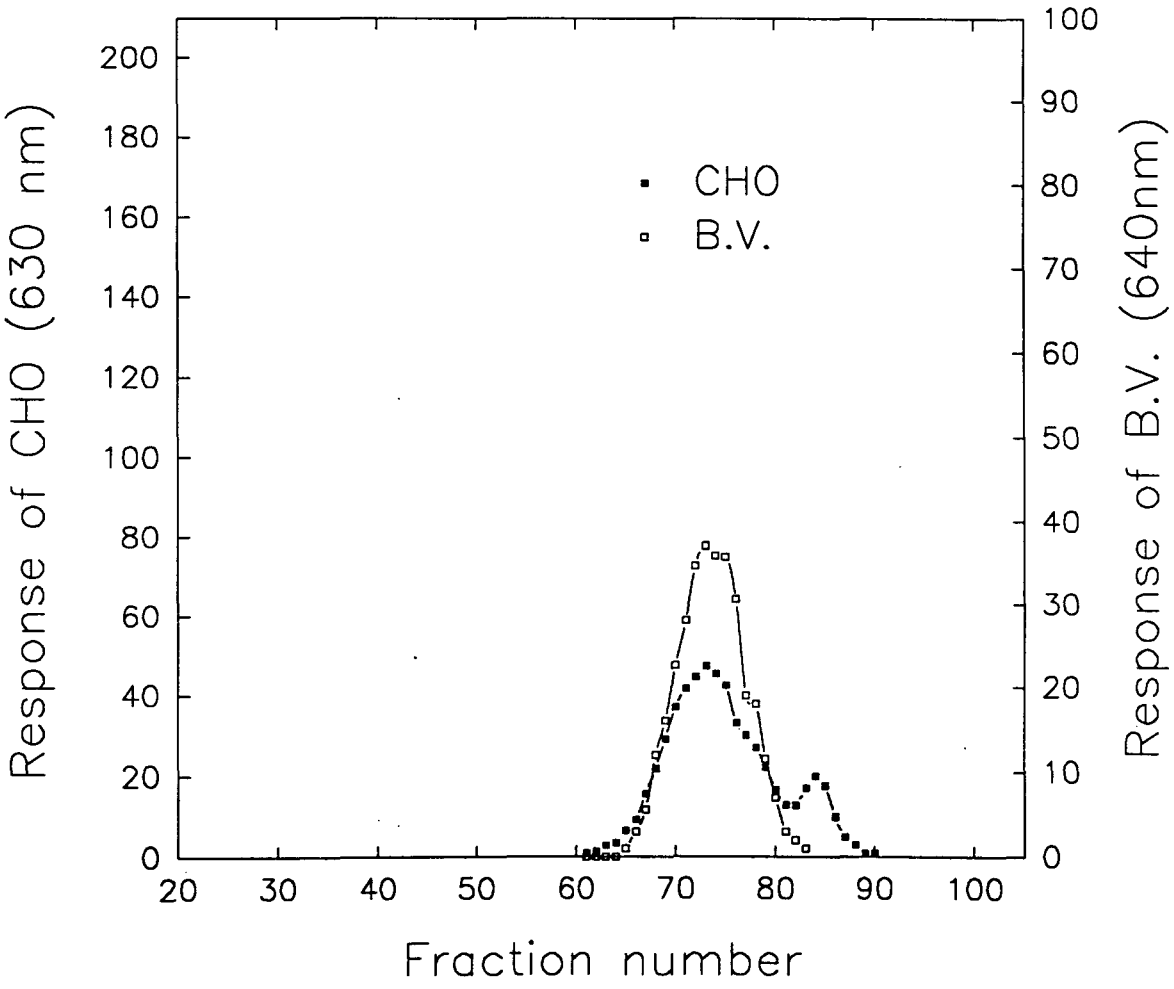


Fig. 12. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 5 mg of the second soluble fraction which was fractionated from 0.2% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

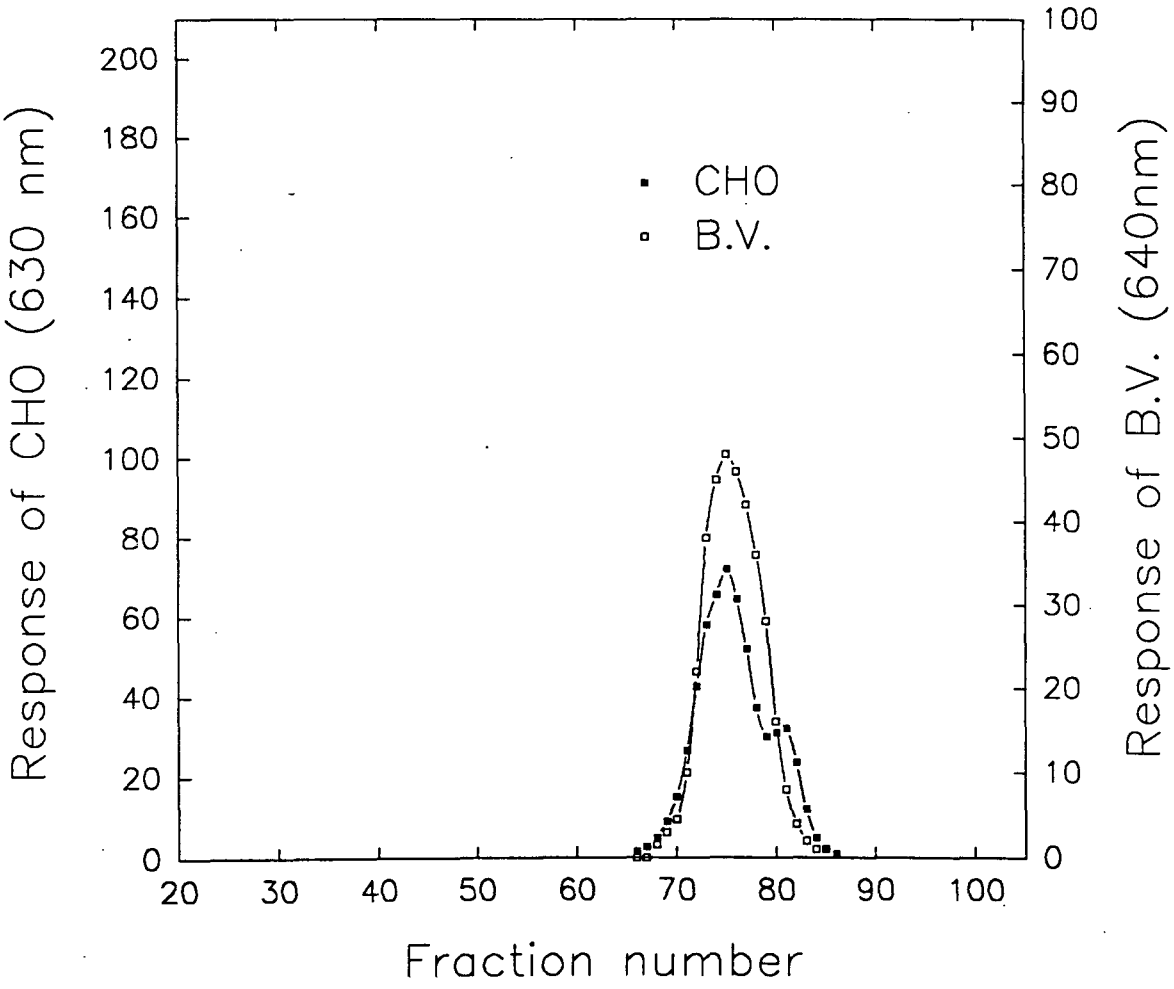


Fig. 13. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 5 mg of the second soluble fraction which was fractionated from 0.14% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

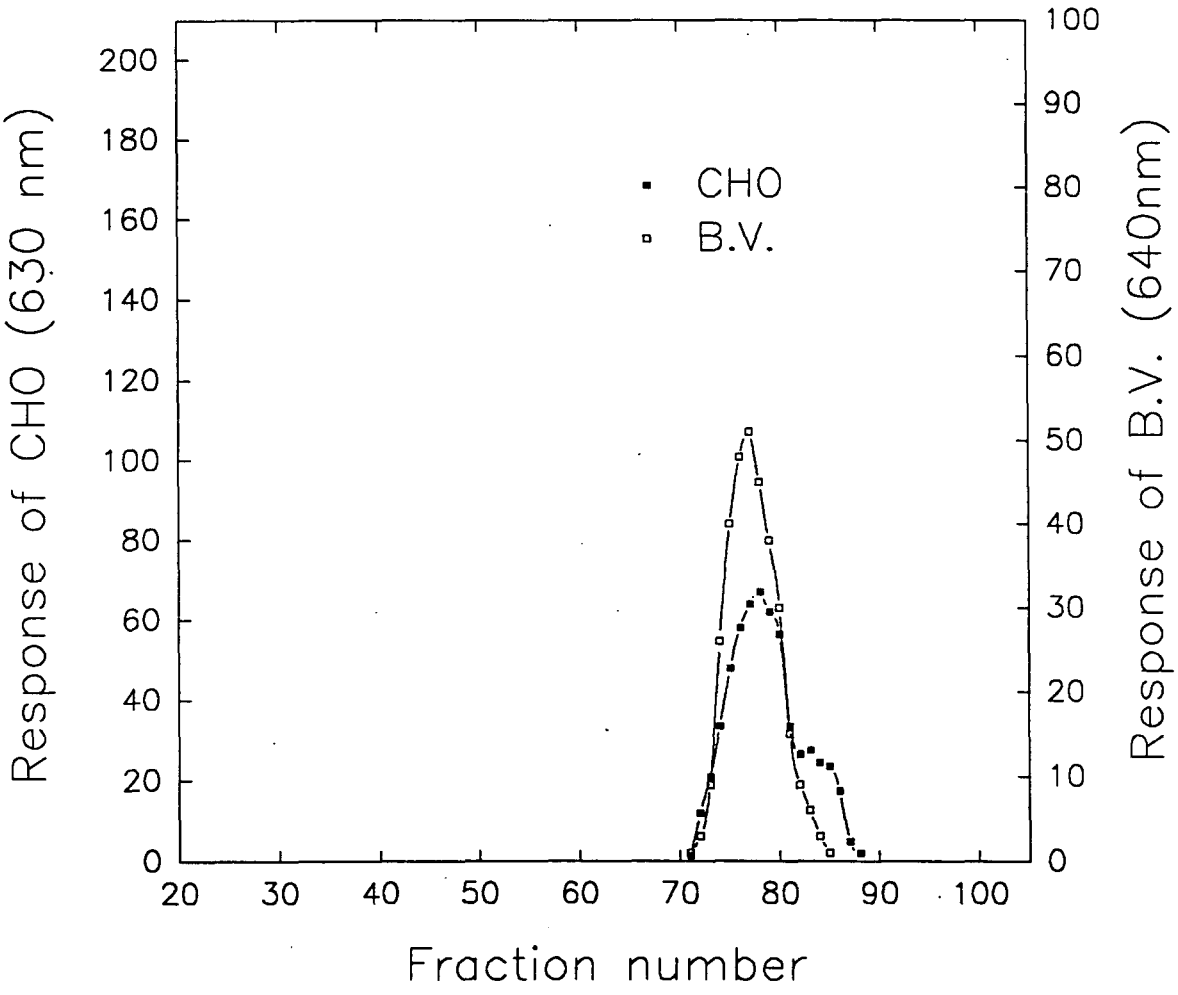


Fig. 14. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 5 mg of the second soluble fraction which was fractionated from 0.18% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

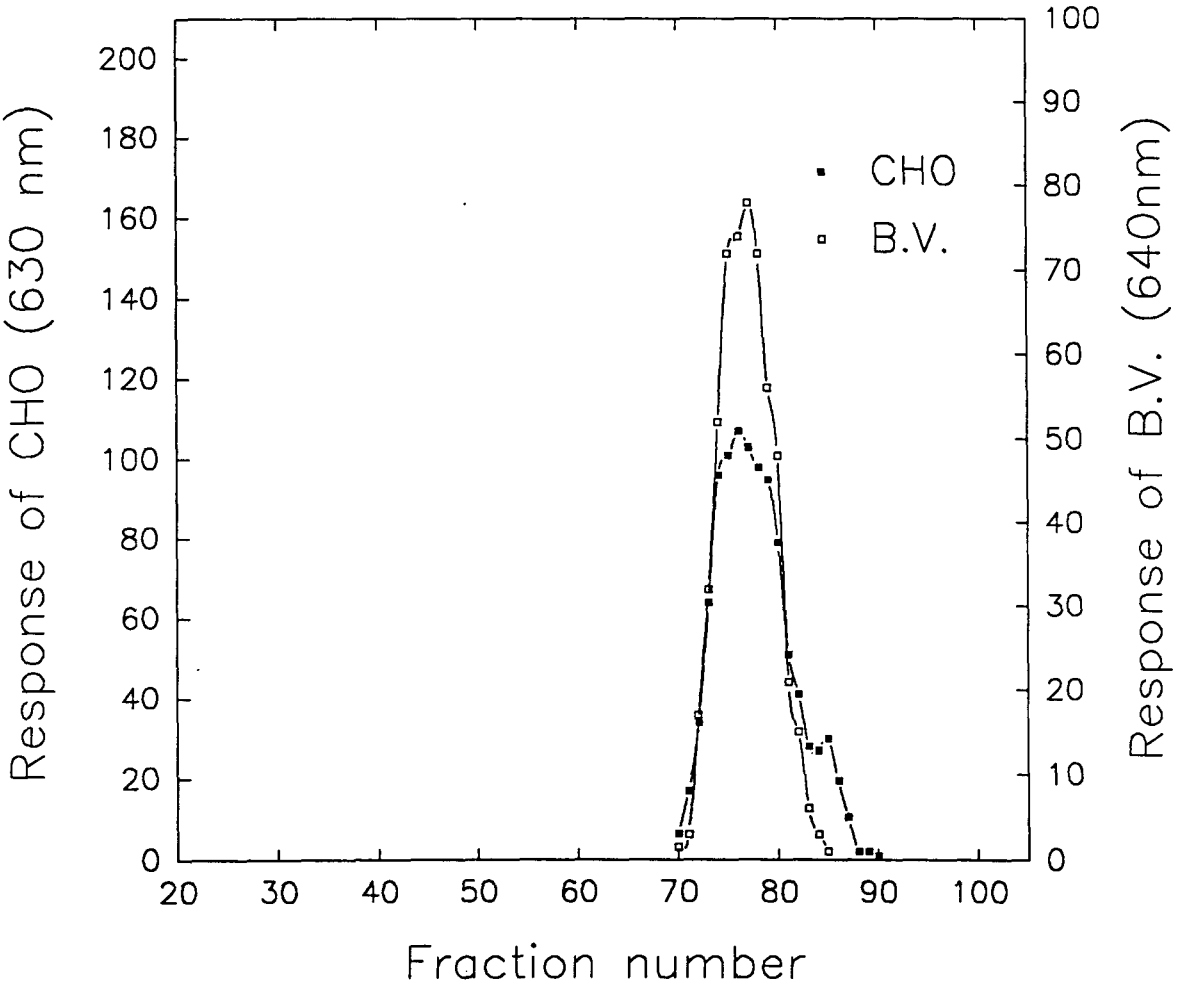


Fig. 15. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 5 mg of the second soluble fraction which was fractionated from 0.2% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

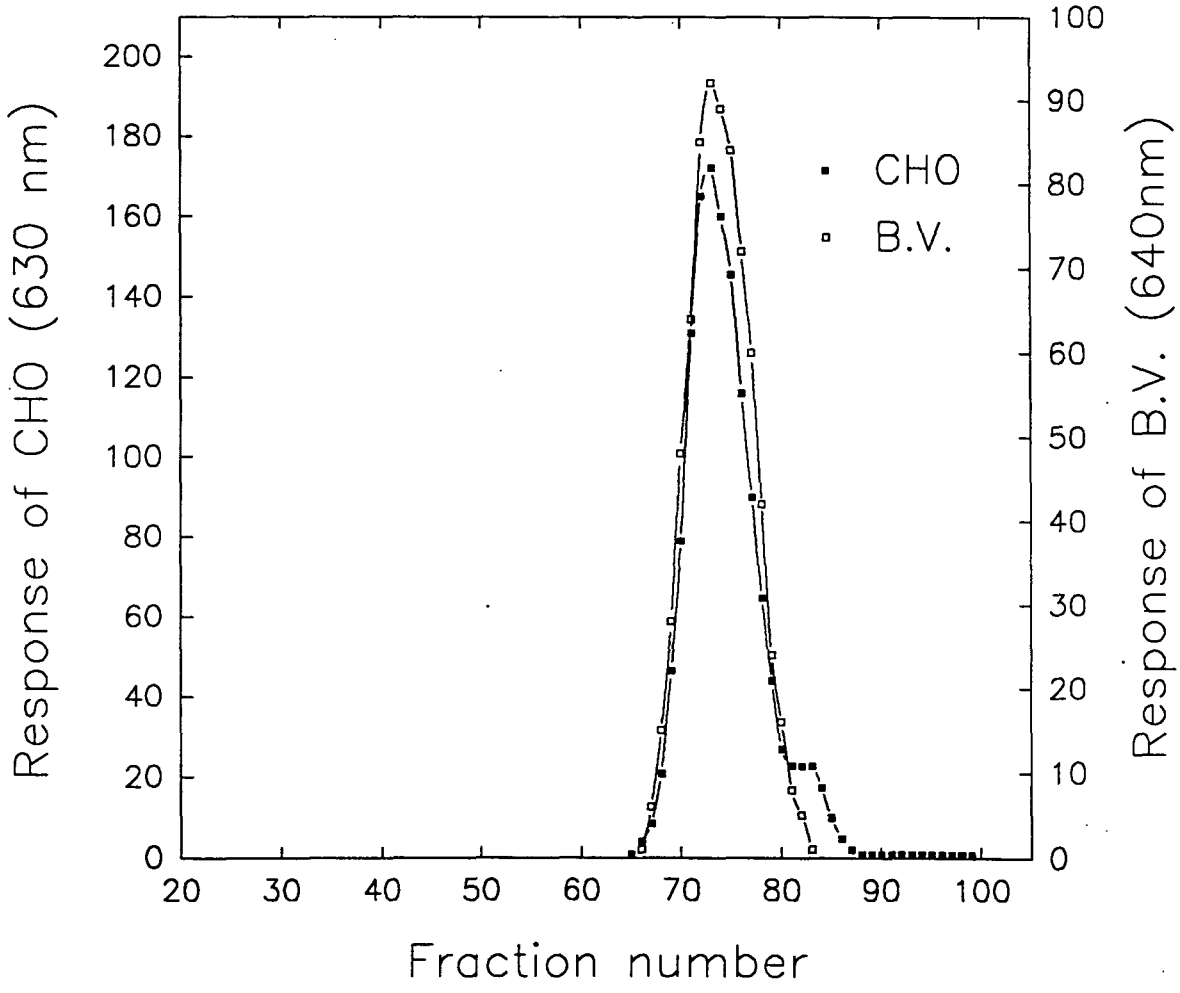


Fig. 16. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 10 mg of the second soluble fraction which was fractionated from 0.1% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

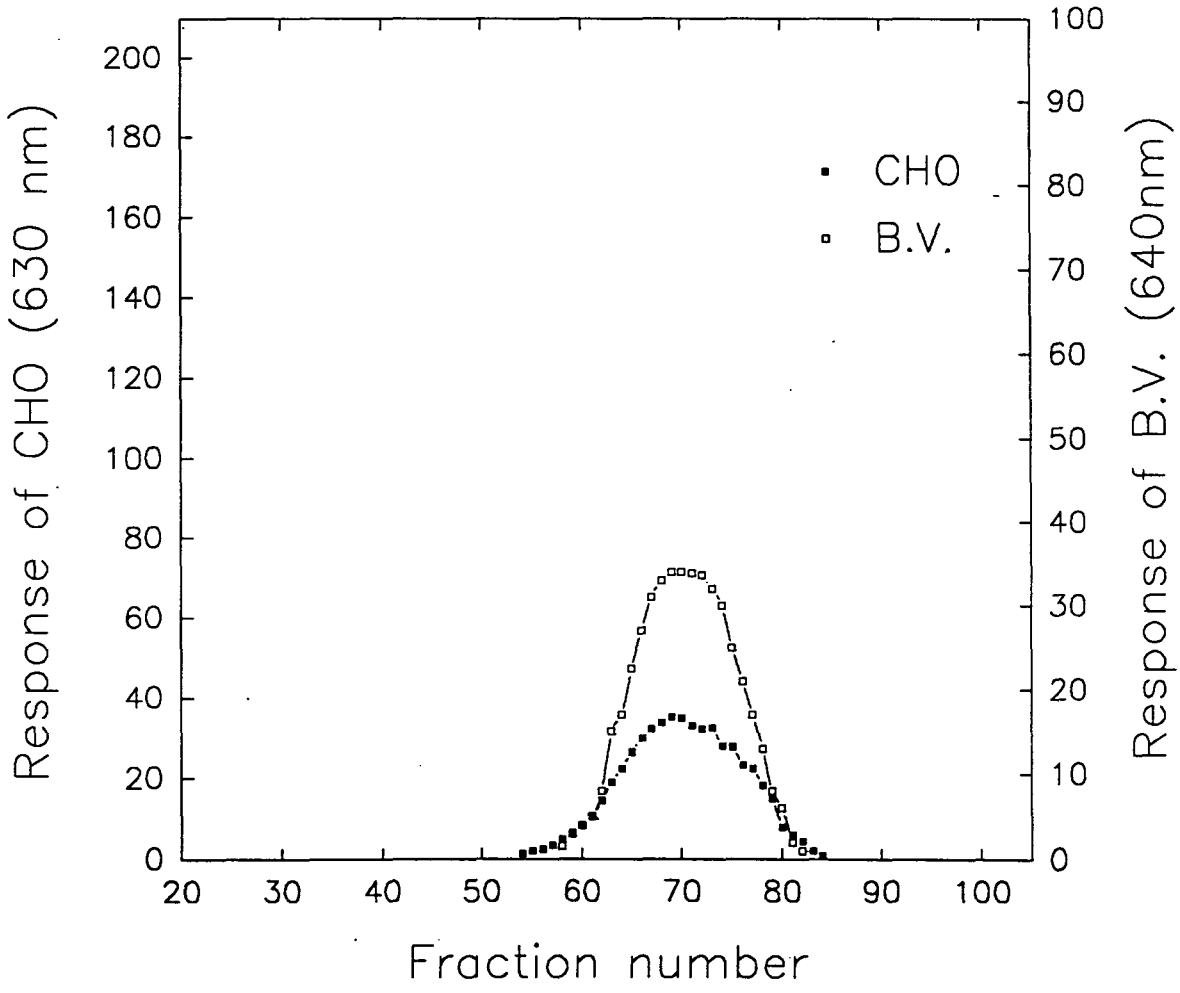


Fig. 17. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 5 mg of the third soluble fraction which was fractionated from 0.14% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

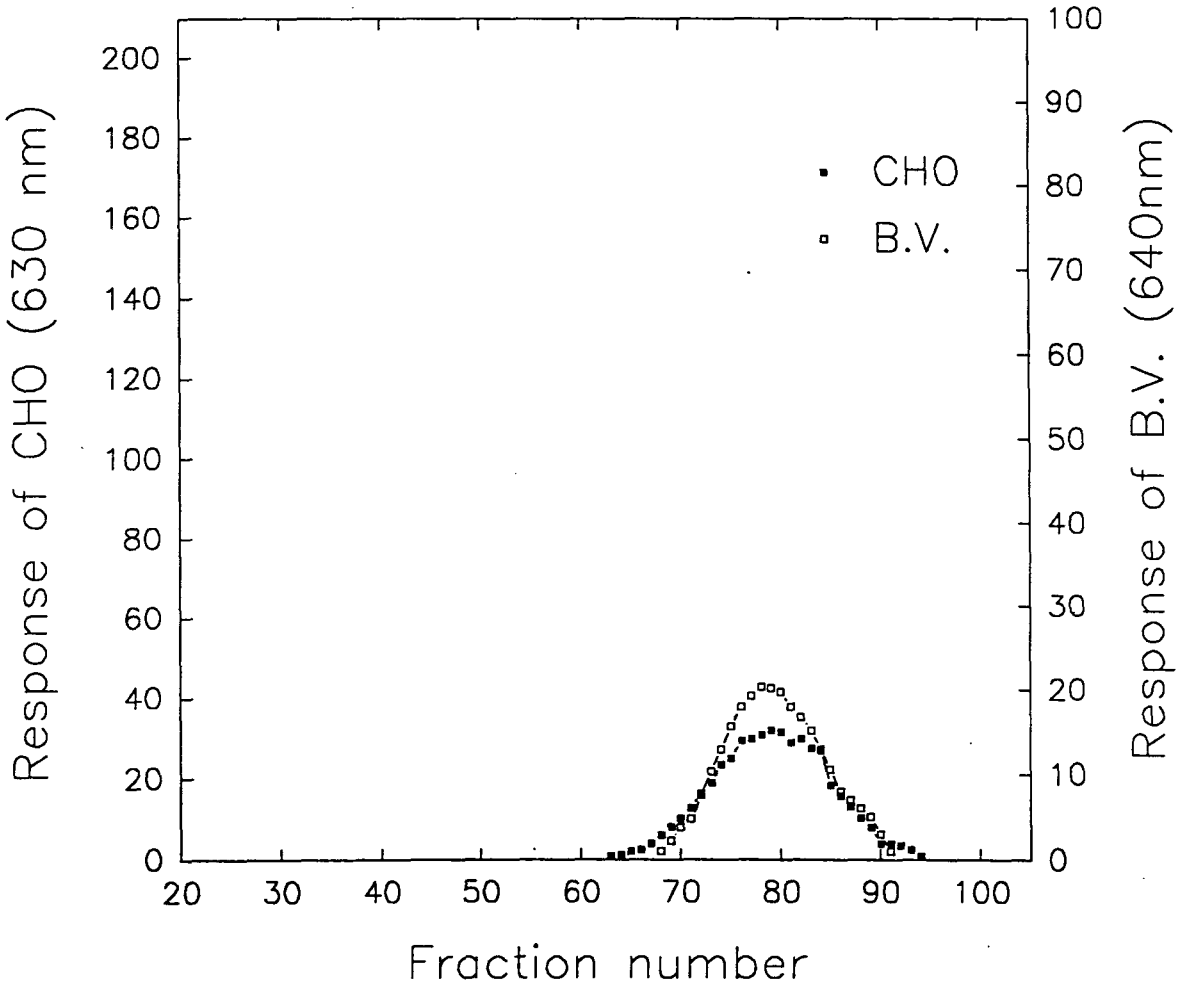


Fig. 18. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 5 mg of the third soluble fraction which was fractionated from 0.18% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

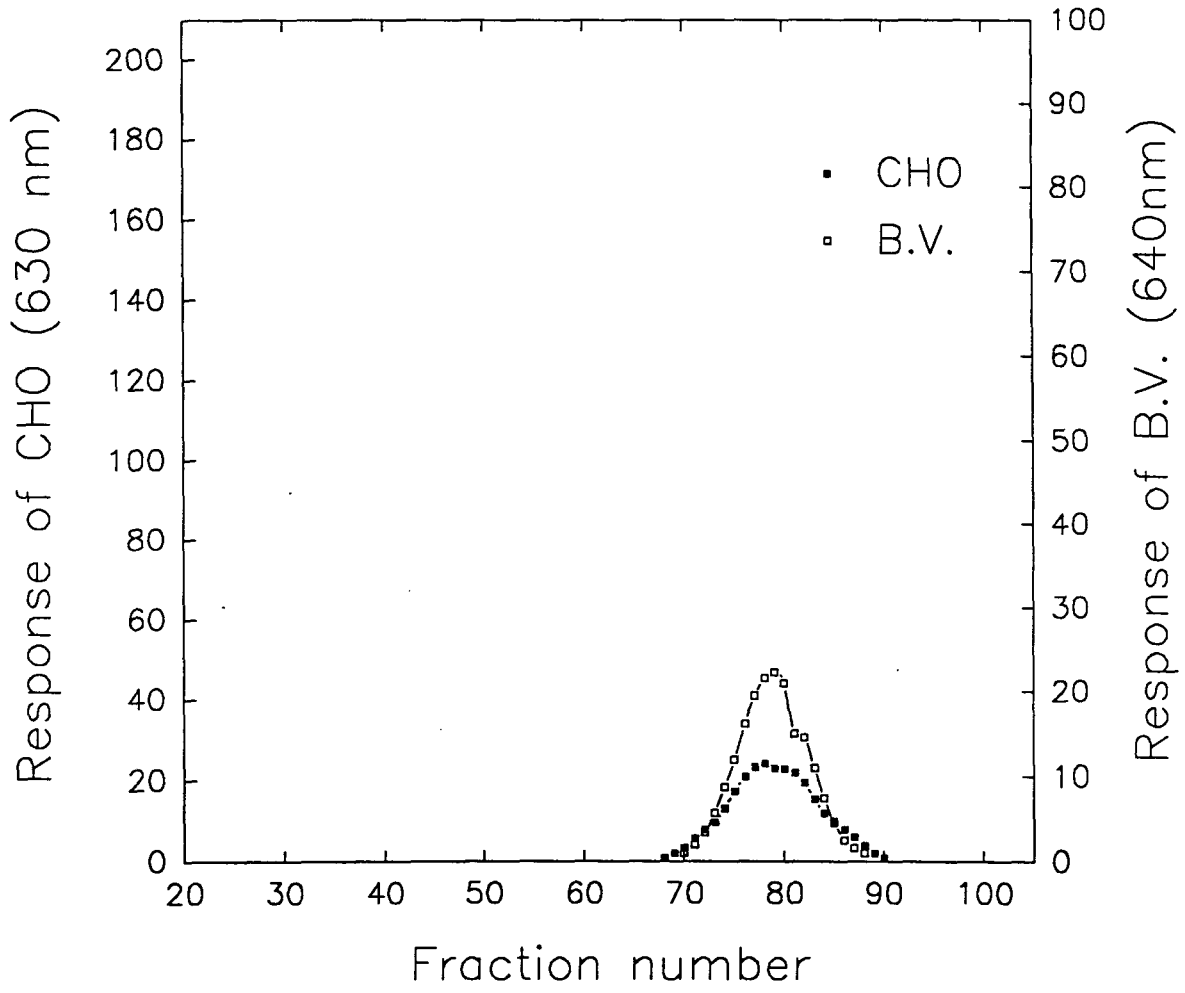


Fig. 19. Sepharose CL-2B column (2.6 I.D. x 90 cm) profile of 5 mg of the third soluble fraction which was fractionated from 0.2% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

Molecular weight distributions of normal corn starch and the soluble fractions of the 0.1, 0.14, 0.18, and 0.2% $POCl_3$ modified starch analyzed by Sephacryl S-500 gel are shown in Figures 21 to 25. The chromatogram of normal corn starch showed that the amylose and amylopectin peaks were well separated but not the intermediate fraction (Figure 20). Chromatograms of the soluble fractions also showed amylose only (Figures 21-24). Results of the molecular weight distributions form both gel permeation chromatography (Sepharose CL2B and Sephacryl S-500) were identical. The results from the molecular weight distribution indicated that the starch granules were cross-linked by $POCl_3$ even at the low concentration (0.05%), and the chromatogram showed only small amylose. Amylopectin and large amylose disappeared because of being the cross-linked and insolubilized. The soluble fractions dissolved in 90% DMSO derived from the amylose that leached out of the starch granules. At the high concentration of $POCl_3$, the starch granules were heavily and more evenly cross-linked so that more of the amylose was cross-linked with amylopectin and became insoluble. Total carbohydrates of the soluble fractions of the 0.1, 0.14, 0.18, and 0.2 % $POCl_3$ modified which were separated from 5% (w/v) modified starch suspensions in 90% DMSO were measured using the supernatant. The total carbohydrate of the soluble fraction of the starch which was cross-linked with higher concentration of $POCl_3$ was less than the one with lower concentration of cross-linking (Table 1).

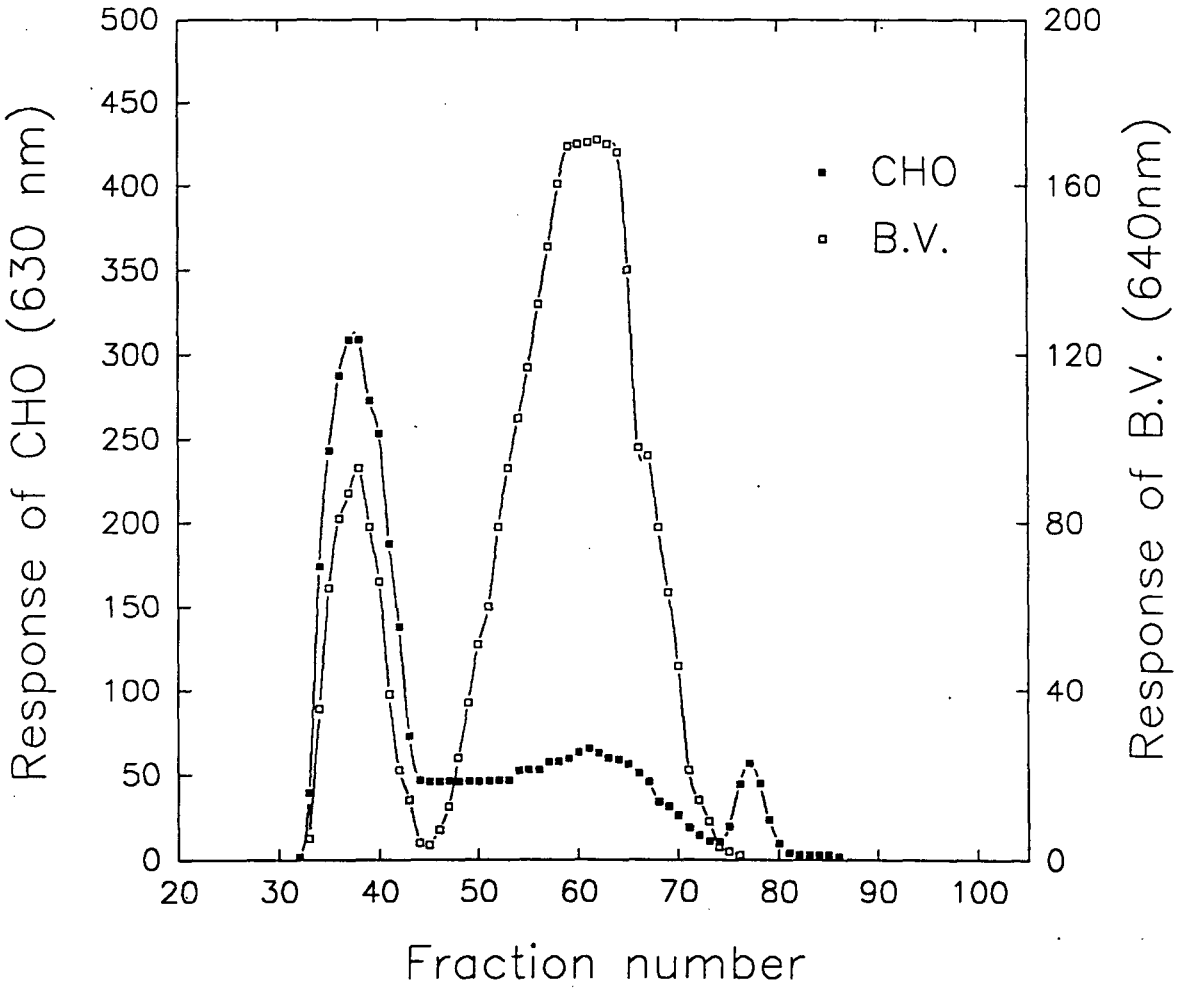


Fig. 20. Sephacryl S-500 column (2.6 I.D. x 90 cm) profile of 10 mg of native normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min.Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

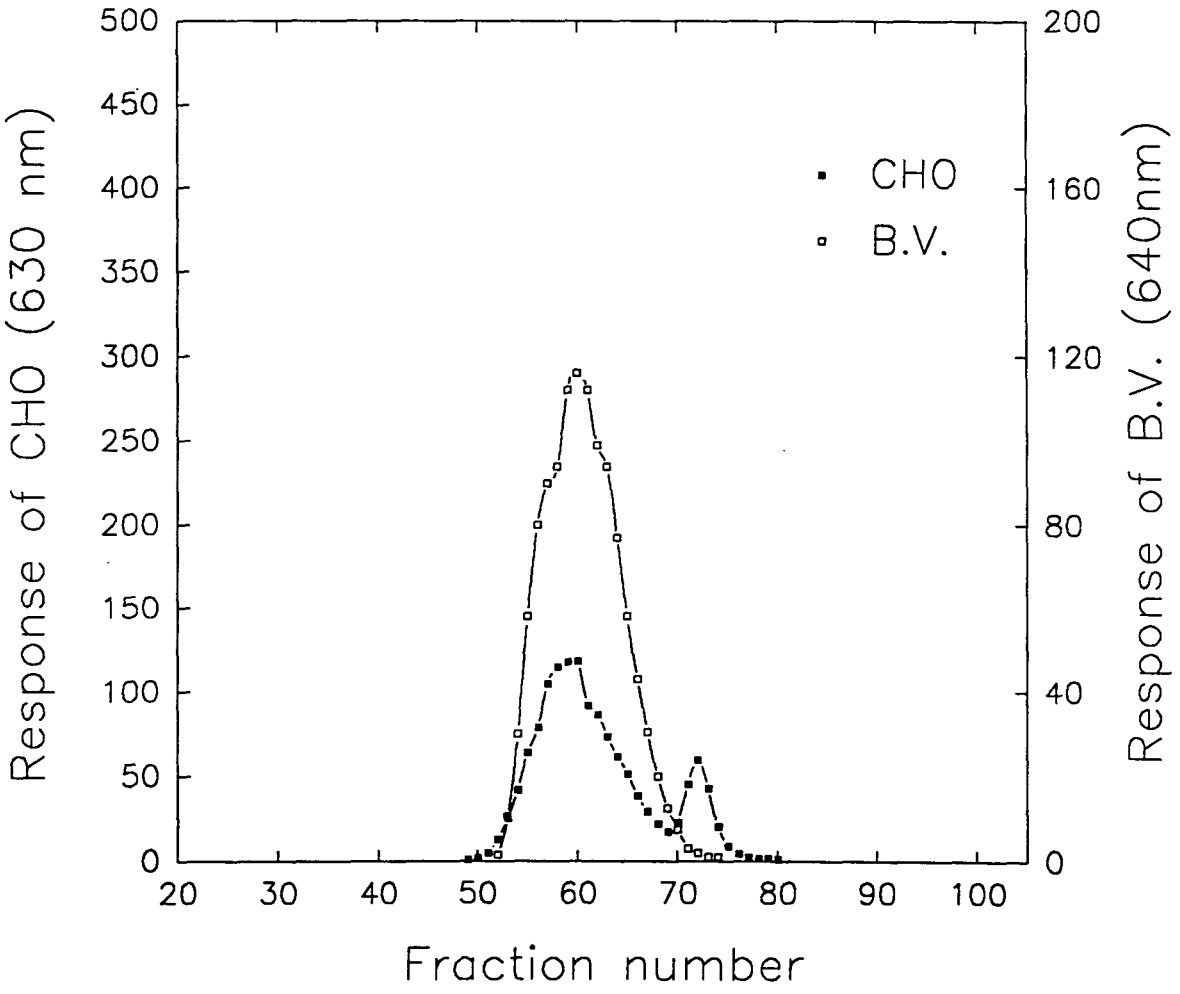


Fig. 21. Sephacryl S-500 column (2.6 I.D. x 90 cm) profile of 5 mg of the soluble fraction which was fractionated from 0.1% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □. Glucose was used as a marker.

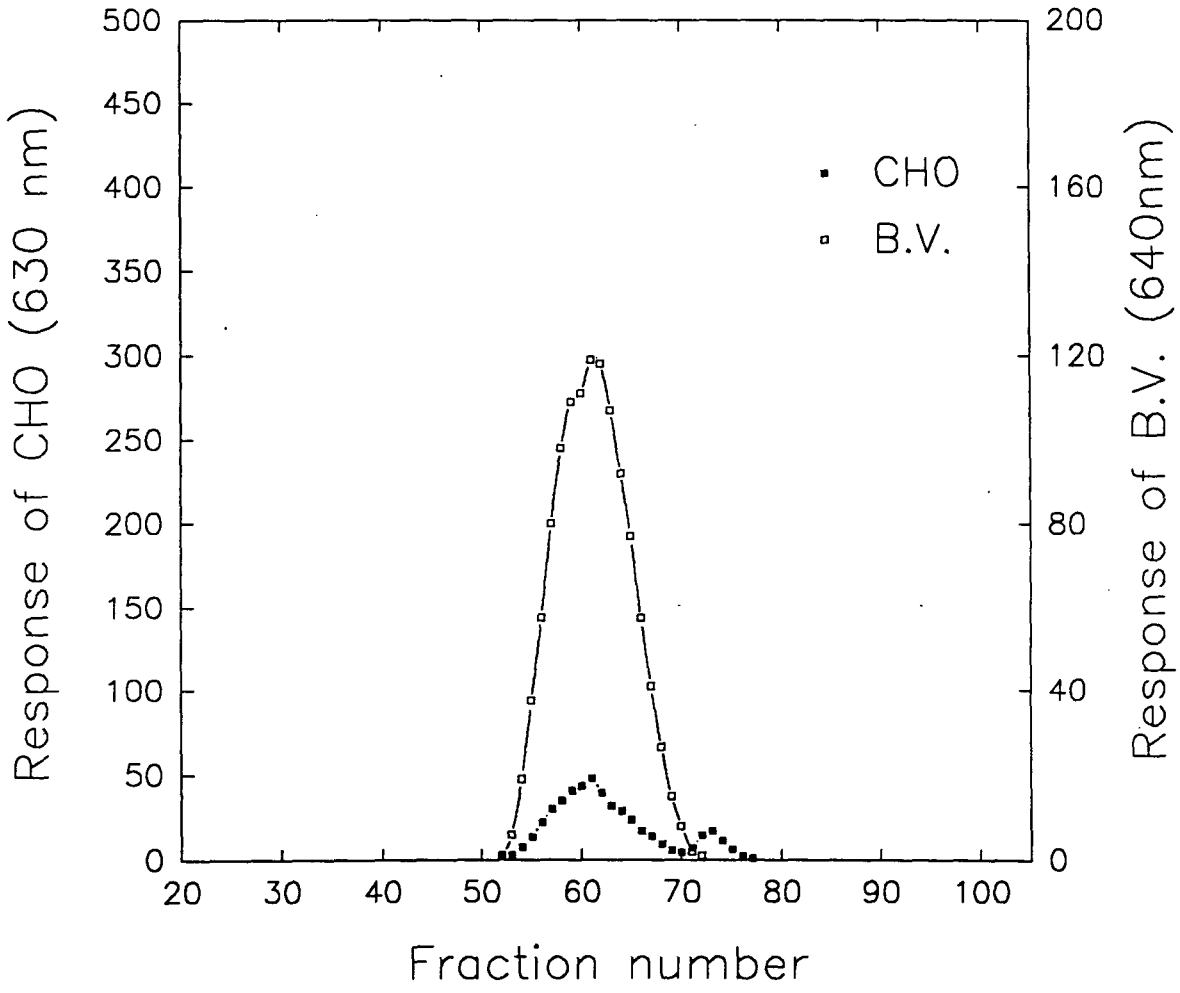


Fig. 22. Sephacryl S-500 column (2.6 I.D. x 90 cm) profile of 5 mg of the soluble fraction which was fractionated from 0.14% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

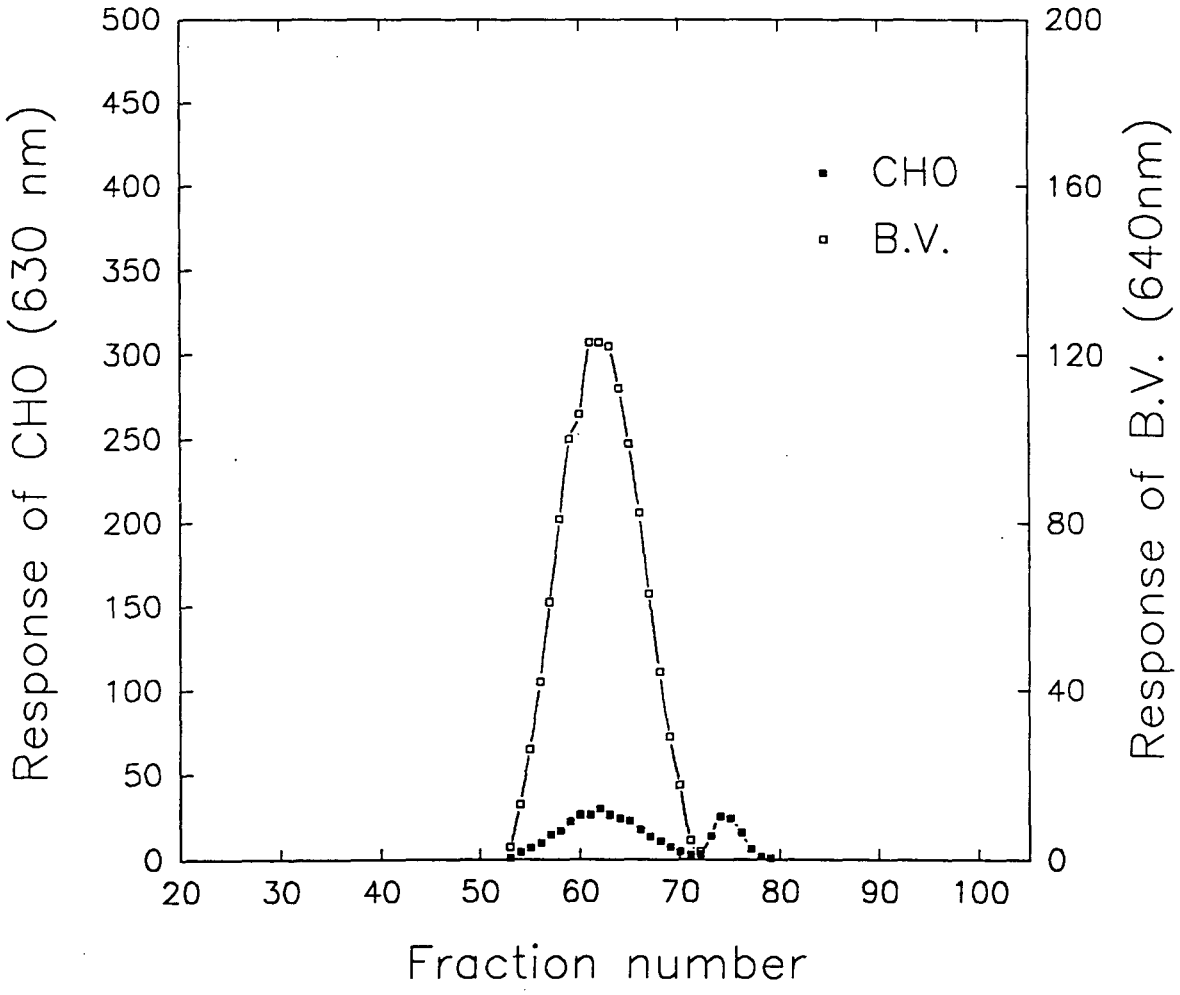


Fig. 23. Sephacryl S-500 column (2.6 I.D. x 90 cm) profile of 5 mg of the soluble fraction which was fractionated from 0.18% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □ . Glucose was used as a marker.

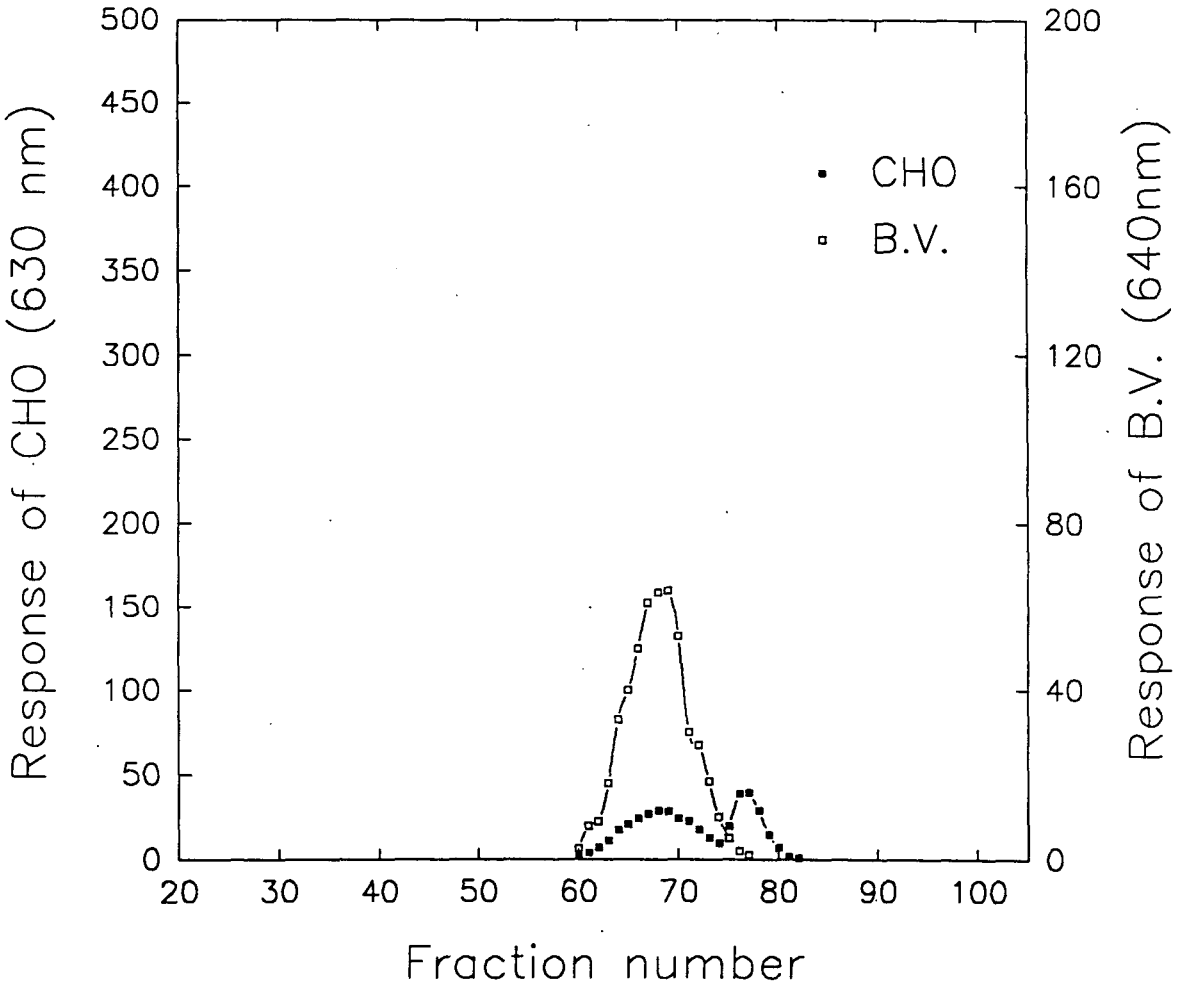


Fig. 24. Sephacryl S-500 column (2.6 I.D. x 90 cm) profile of 5 mg of the soluble fraction which was fractionated from 0.2% $POCl_3$ modified normal corn starch. The column was eluted with 0.02% sodium sulfate aqueous solution, and flow rate was 0.5 ml/min. Fractions (5 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): ■ - ■ , and blue value (amylose-iodine complex): □ - □. Glucose was used as a marker.

Table 1. Total carbohydrate of the 1st, 2nd, and 3rd soluble fractions which were separated from 5% (w/v) modified starch suspension in 90% DMSO.

Sample	Total carbohydrate (mg/ml)			
	0.1% $POCl_3$ modified starch	0.14% $POCl_3$ modified starch	0.18% $POCl_3$ modified starch	0.2% $POCl_3$ modified starch
1st soluble fraction	8.7	5.5	4.15	0.6
2nd soluble fraction	5.32	0.63	0.65	0.2
3rd soluble fraction	1.56	0.21	0.2	0.01

Molecular weight distributions of small particle starch and Nägeli dextrin were analyzed by Bio-gel P-6 gel permeation chromatography. The chromatogram (Figures 25-26) showed one peak at the low molecular weight, compared to glucose marker. They were also indicated that hydrolysis was complete.

NMR Spectroscopy

^{31}P NMR spectrum of the reference compounds

Spectra of reference compounds, including glucose-1-phosphate, glucose-2-phosphate, a mixture of glucose-3 and glucose-6-phosphate, glucose-6-phosphate, starch phosphomonoester, and lecithin are shown from Figures 27 to 36. The ^{31}P signal of phosphomonoester derivative at carbon 1 of glucose unit is shown at δ 4.8 ppm (Figure 27). The ^{31}P signal of phosphomonoester derivative at carbon 2 of glucose unit is shown at δ 4.6 ppm (Figure 28). A mixture of phosphomonoester at carbons 3 and 6 of methylglucoside gave signals at δ 4.9, 4.7, and 4.5 ppm (Figure 29). Phosphomonoester at carbon 6 of methylglucoside showed a ^{31}P signal at δ 4.8 ppm (Figure 30).

The spectrum of free phosphate (inorganic phosphate) was obtained from diluted phosphoric acid at pH 8. The phosphoric acid was dissociated and

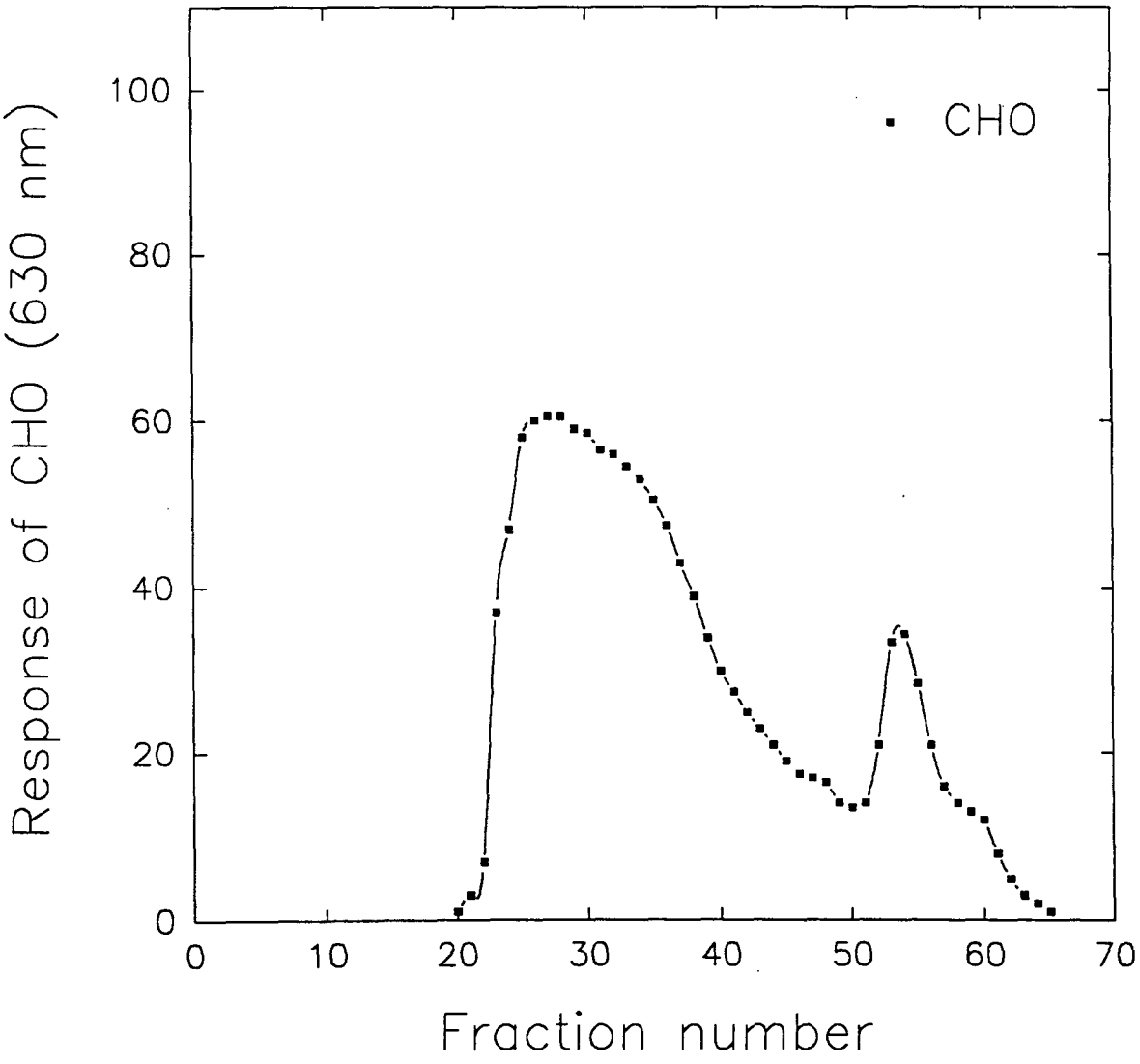


Fig. 25. Bio-gel P-6 column (1.5 I.D. x 80 cm) profile of 5 mg of the small particle starch. The column was eluted with deionized and distilled water. Fractions (2.3 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): - . Glucose was used as a marker.

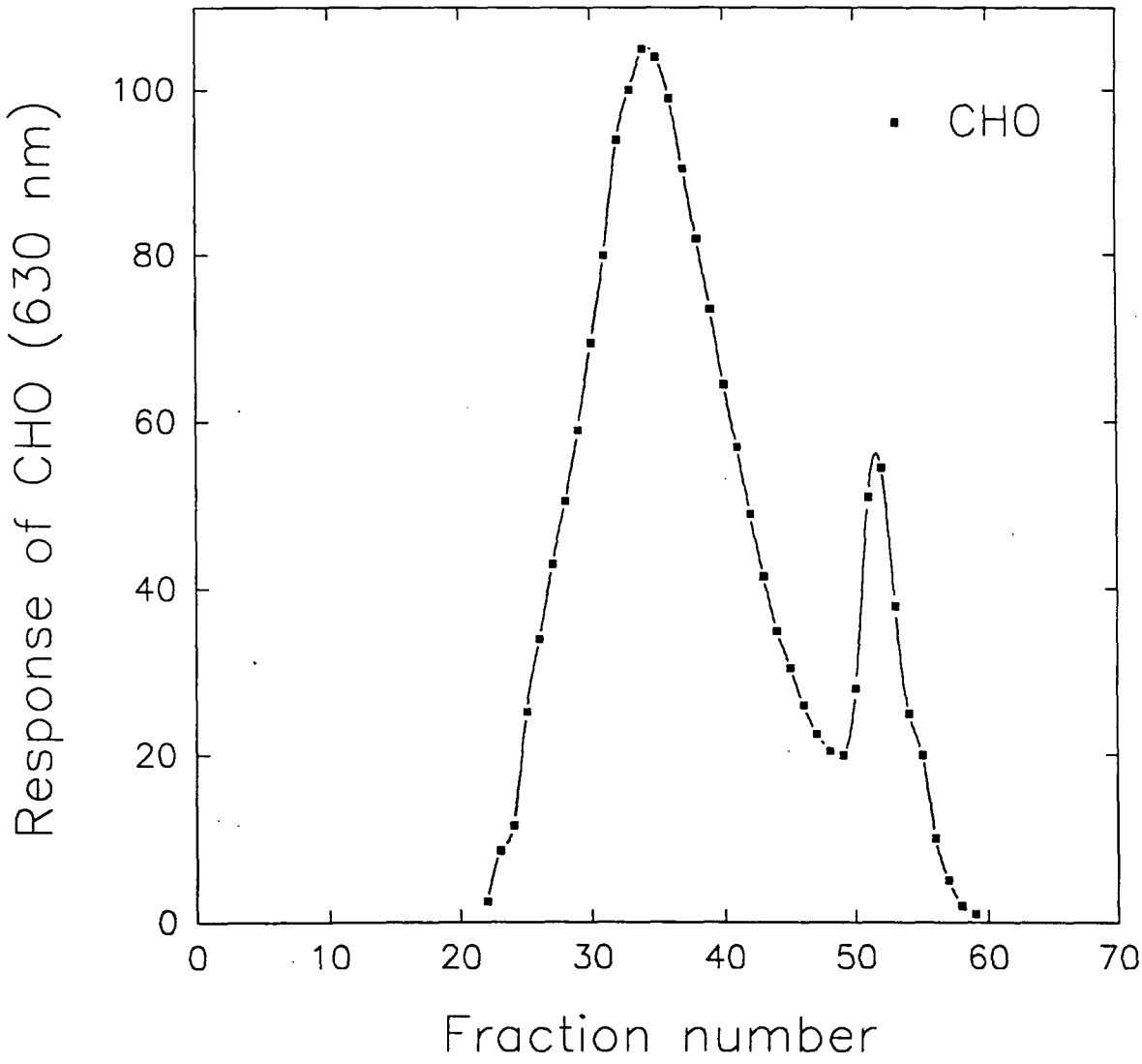


Fig. 26. Bio-gel P-6 column (1.5 I.D. x 80 cm) profile of 5 mg of the normal corn Nägeli dextrin. The column was eluted with deionized and distilled. Fractions (2.3 ml) were analyzed for total carbohydrate (anthrone-sulfuric acid procedure): - . Glucose was used as a marker.

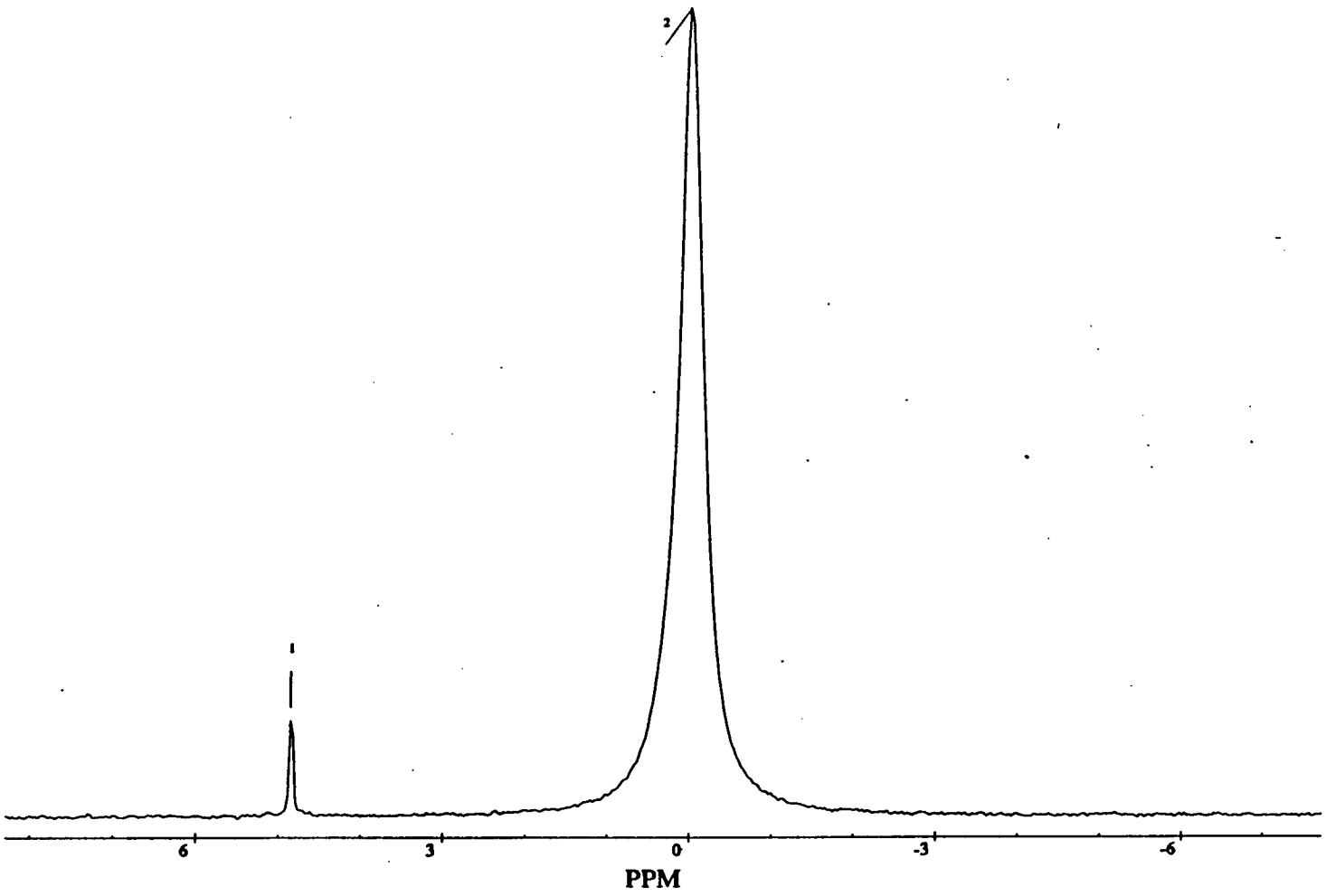


Fig. 27. ^{31}P NMR spectrum of glucose-1-phosphate, at pH 8.0±0.1 and 81 MHz with standard 85% H_3PO_4 as an external reference.
(signal 1: 4.8 ppm, signal 2: 0.0 ppm)

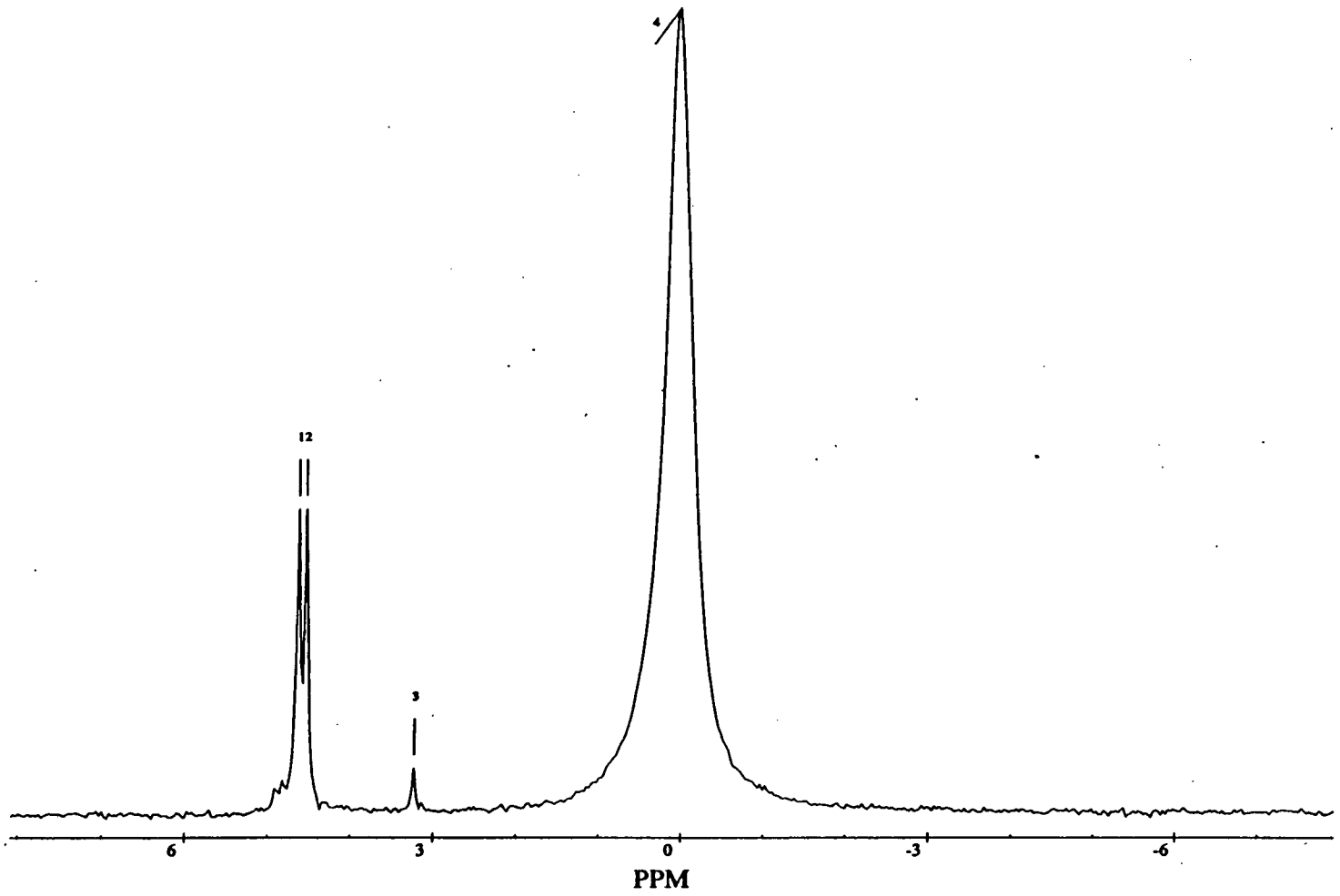


Fig. 28. ^{31}P NMR spectrum of glucose-2-phosphate, at pH 8.0±0.1 and 81 MHz with standard 85% H_3PO_4 as an external reference.
(signal 1: 4.65 ppm, signal 2: 4.6 ppm, signal 3: 3.2 ppm, signal 4: 0.0 ppm)

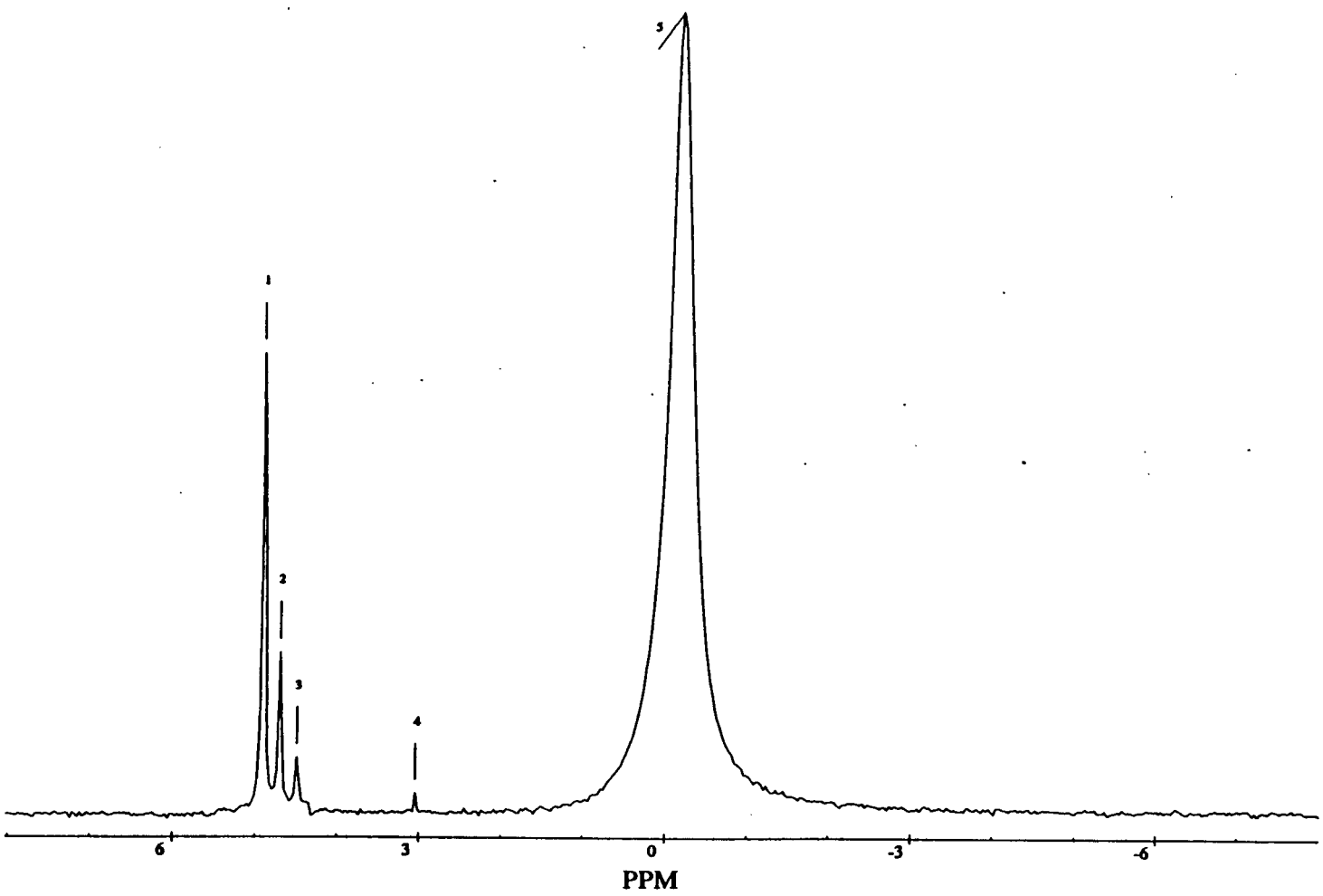


Fig. 29. ^{31}P NMR spectrum of glucose-3 and glucose-6-phosphate, at pH 8.0±0.1 and 81 MHz with standard 85% H_3PO_4 as an external reference.
(signal 1: 4.9 ppm, signal 2: 4.7 ppm, signal 3: 4.5 ppm, signal 4: 2.9 ppm, signal 5: 0.0 ppm)

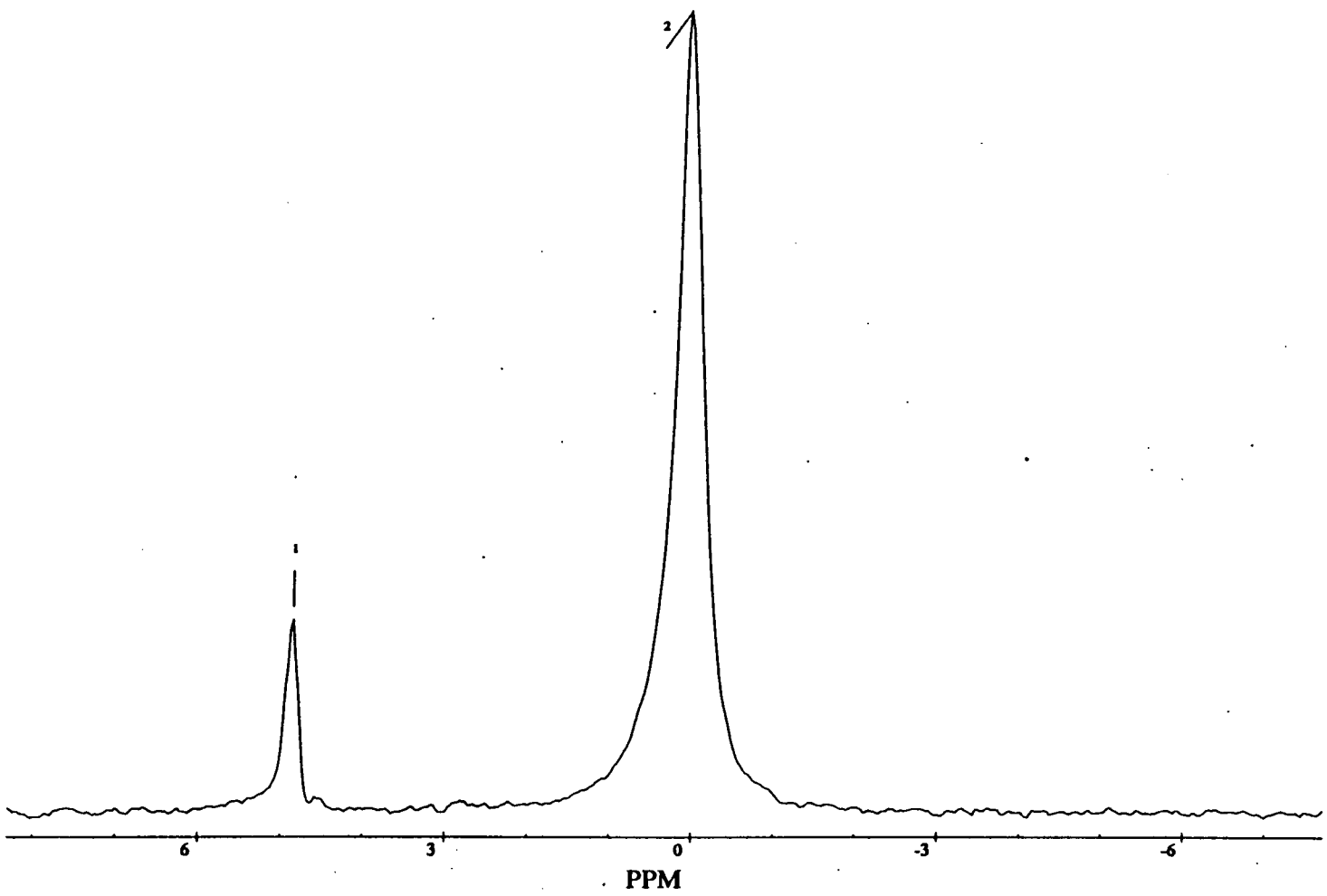


Fig. 30. ^{31}P NMR spectrum of glucose-6-phosphate, at pH 8.0±0.1 and 81 MHz with standard 85% H_3PO_4 as an external reference.
(signal 1: 4.8 ppm, signal 2: 0.0 ppm)

gave inorganic phosphate at alkaline pH and showed a signal at about δ 2.9 ppm. The spectrum is shown in Figure 31.

The spectrum of $POCl_3$ at pH 8 is shown in Figure 32. The inorganic pyrophosphate spectrum at pH 8 showed a chemical shift at δ -6.3 ppm (Figure 33).

Lim (1990) reported ^{31}P chemical shifts of phosphomonoesters in native potato starch, i.e., glucose 2-phosphate, glucose 3-phosphate, and glucose 6-phosphate gave signals at the chemical shift of δ 4.04, 4.51, 4.66 ppm from the H_3PO_4 (0.0 ppm), respectively.

Lim (1990) also reported ^{31}P chemical shift of phosphate groups at carbon-3 and carbon-6 of at non-reducing end of α,γ phosphodextrin gave signals at chemical shift of δ 4.5 and 4.8 ppm from H_3PO_4 (0.0 ppm), respectively. Phosphomonoesters at carbon-3 and carbon-6 on the internal chain of α,γ phosphodextrin showed signals at chemical shifts δ 3.6, 3.8 ppm from H_3PO_4 (0.0 ppm), respectively.

Starch phosphomonoester was prepared by the Paschall method (1964), which gave only phosphomonoester not -diester (cross-linked). A spectrum of the phosphomonoester starch showed ^{31}P signals at δ -6.6, 2.9, 3.7, 4.3, 4.6, and 4.9 ppm. The signals in the upper field (4.3, 4.6 and 4.9 ppm) indicated the phosphomonoesters at positions 2, 3, and 6 of the glucose at the non reducing terminal of α-limit dextrins. The signal at the chemical shift δ 3.7 ppm indicated phosphomonoester on the internal chain of α-limit dextrins. The

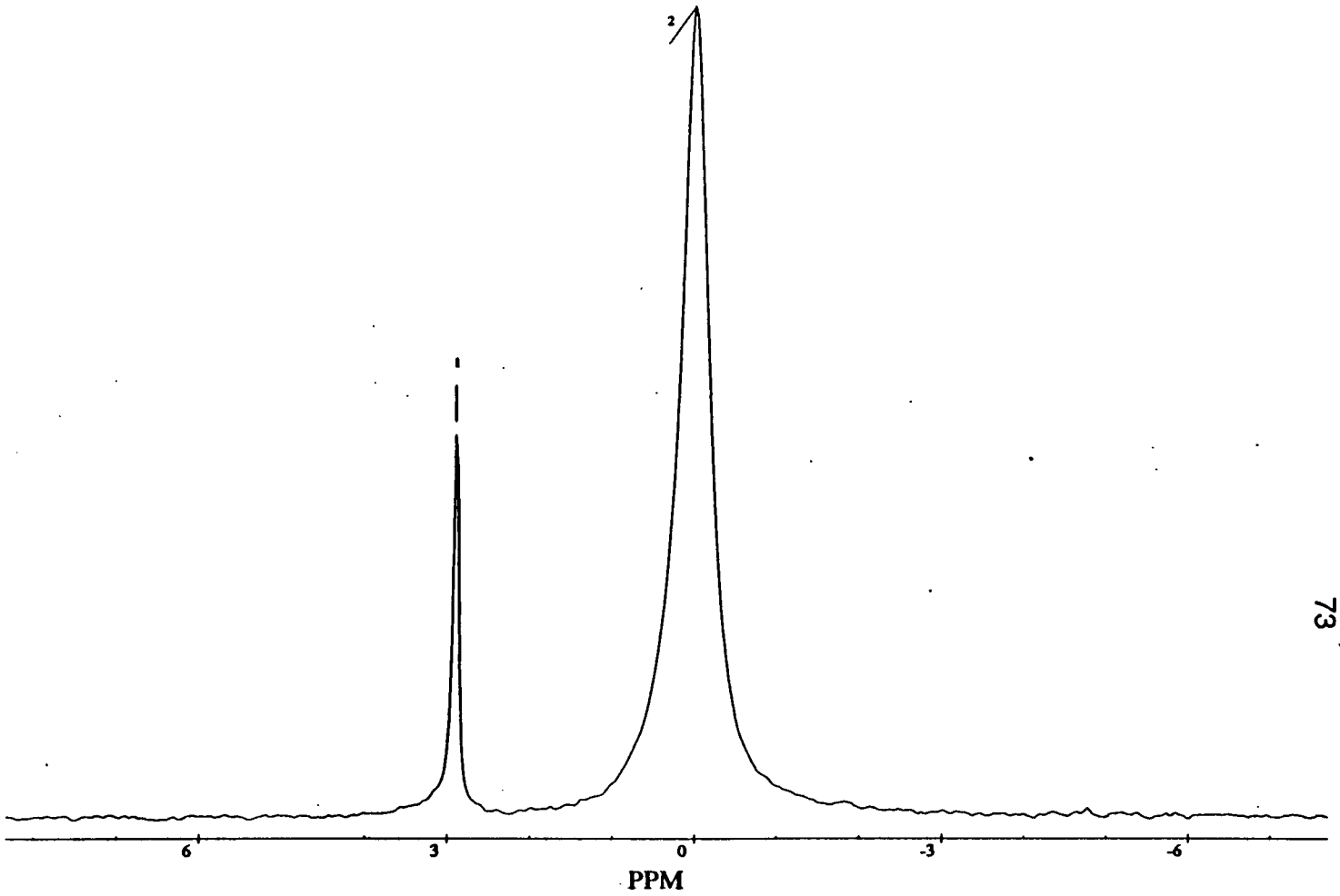


Fig. 31. ^{31}P NMR spectrum of inorganic phosphate, at pH 8.0±0.1 and 81 MHz with standard 85% H_3PO_4 as an external reference.
(signal 1: 2.9 ppm, signal 2: 0.0 ppm)

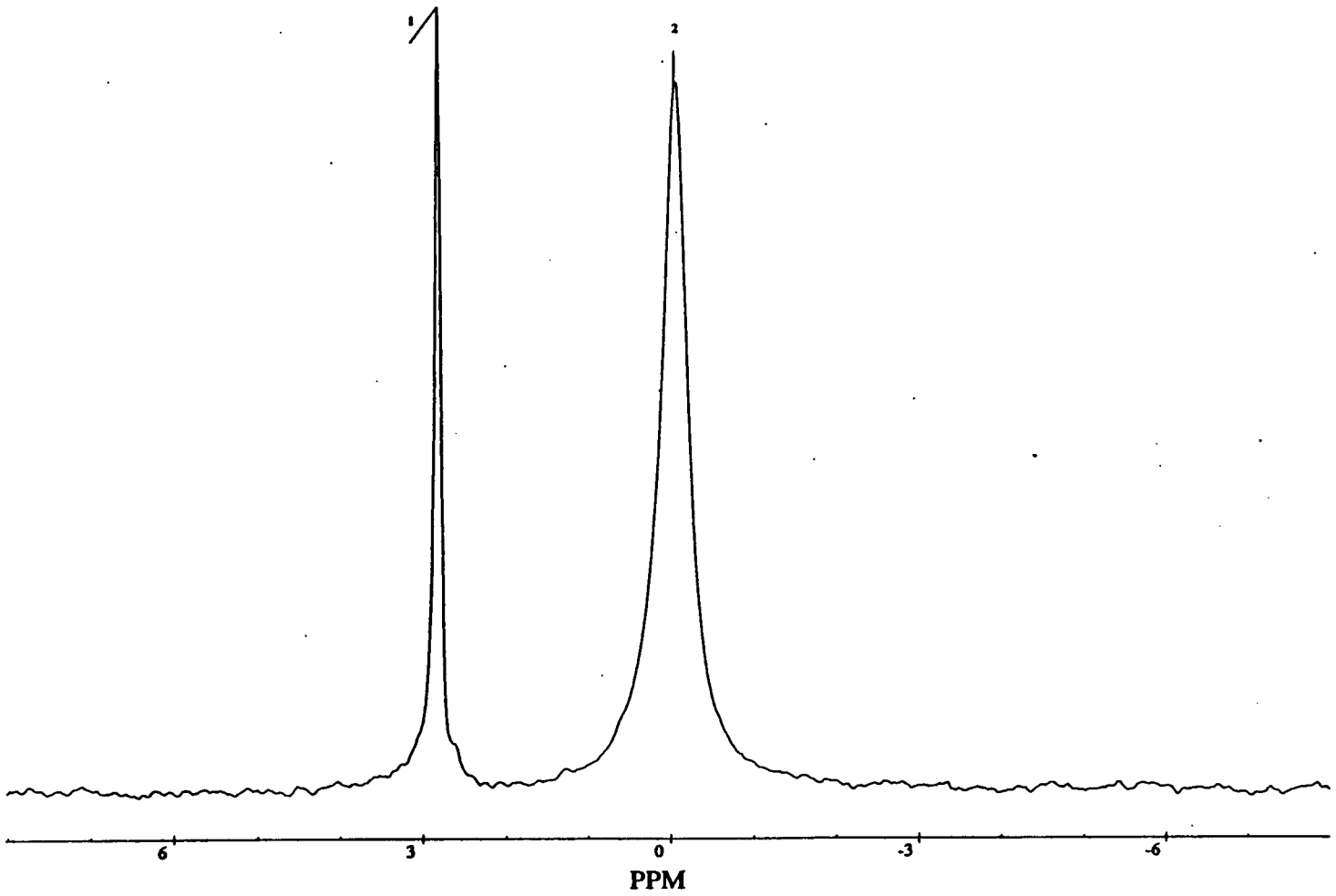


Fig. 32. ^{31}P NMR spectrum of phosphoric acid, at pH 8.0±0.1 and 81 MHz with standard 85% H_3PO_4 as an external reference.
(signal 1: 2.8 ppm, signal 2: 0.0 ppm)

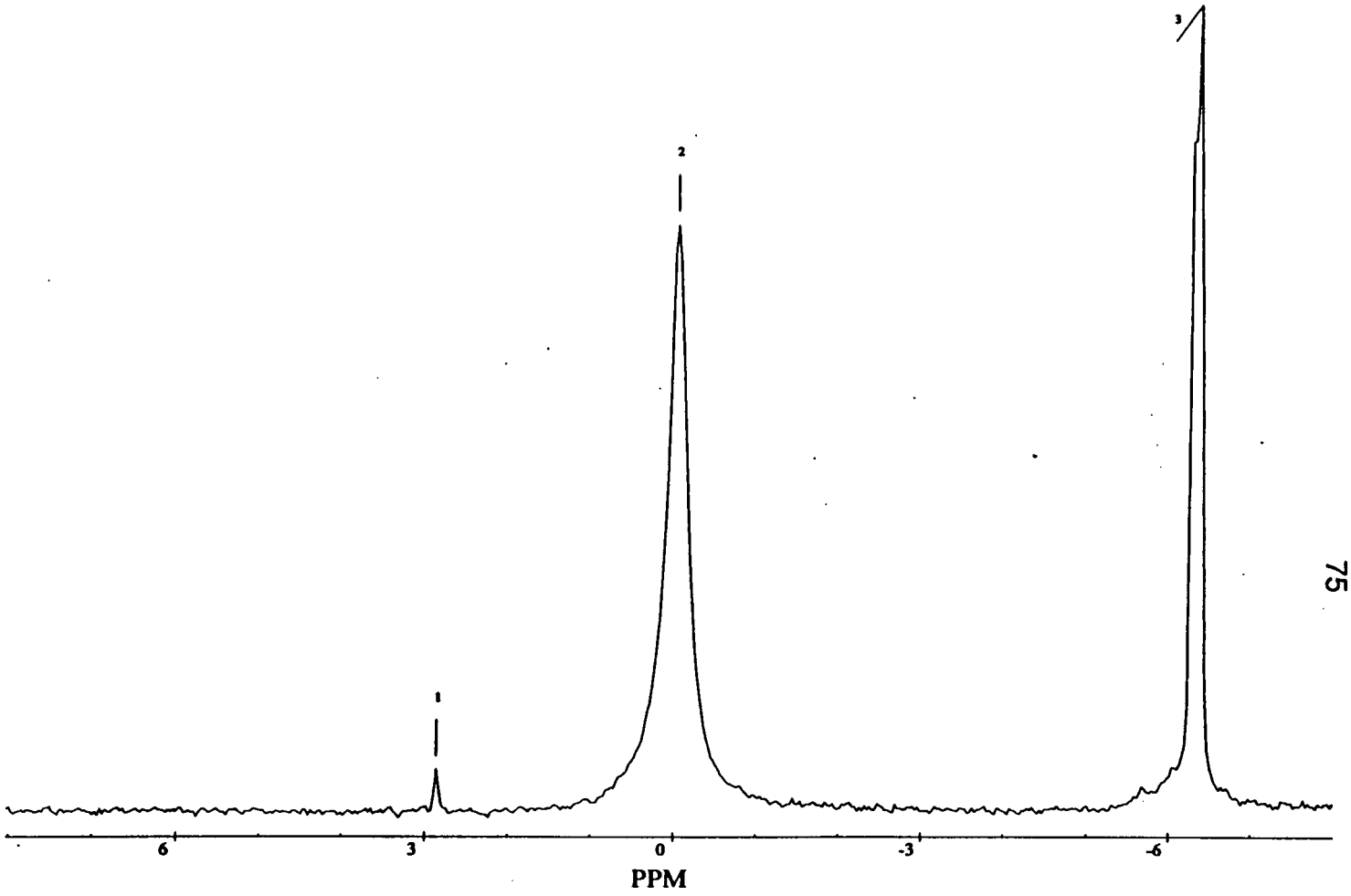


Fig. 33. ^{31}P NMR spectrum of inorganic pyrophosphate phosphoric acid, at pH 8 at pH 8.0±0.1 and 81 MHz with standard 85% H_3PO_4 as external reference.
(signal 1: 2.8 ppm, signal 2: 0.0 ppm, signal 3: -6.3 ppm)

signal at the chemical shift δ -6.6 ppm indicated the pyrophosphate salt residues (sodium pyrophosphate). The spectrum is shown in Figure 34.

Lecithin was used as a reference compound for phosphodiester linkage (cross-linking). Because it contains a mixture of diglycerides of stearic, palmitic, and oleic acids linked to the choline ester of phosphoric acid. Lecithin has a limited solubility in aqueous solution, and, thus, a high noise level is observed in the spectrum (Figure 35). The major signal at the chemical shift -1.0 to 1.0 ppm (phosphodiester) is broad because of the number of similar molecules (mixture of fatty acids) which gave signals in the close range and created overlapping (Barany and Glonek, 1982). Minor signals at 2.9, 4.2, and 4.6 ppm could be from contaminants such as free phosphate (δ 2.9 ppm).

Myo-inositol 1,2-cyclic phosphate which had two hydroxy groups from the same sugar ring formed cyclic phosphate diester gave a signal at the chemical shift about δ 15.5 ppm (Tebby and Glonek, 1991).

α-Methyl glucoside was also cross-linked with $POCl_3$ following the same method as that of normal corn starch. The spectrum (Figure 36) shows the signal at δ -0.7, 0.1, 0.3, 1.0, 2.7, 4.3, 4.5, 4.7 and 15.5 ppm. They are identified as phosphodiester (-1.0 to 1.0 ppm), inorganic phosphate (2.7 ppm), phosphomonoester of methyl glucoside at carbon 2, 3, and 6 of glucose (4.3, 4.5, 4.7 ppm), and cyclic phosphate (15.5 ppm)

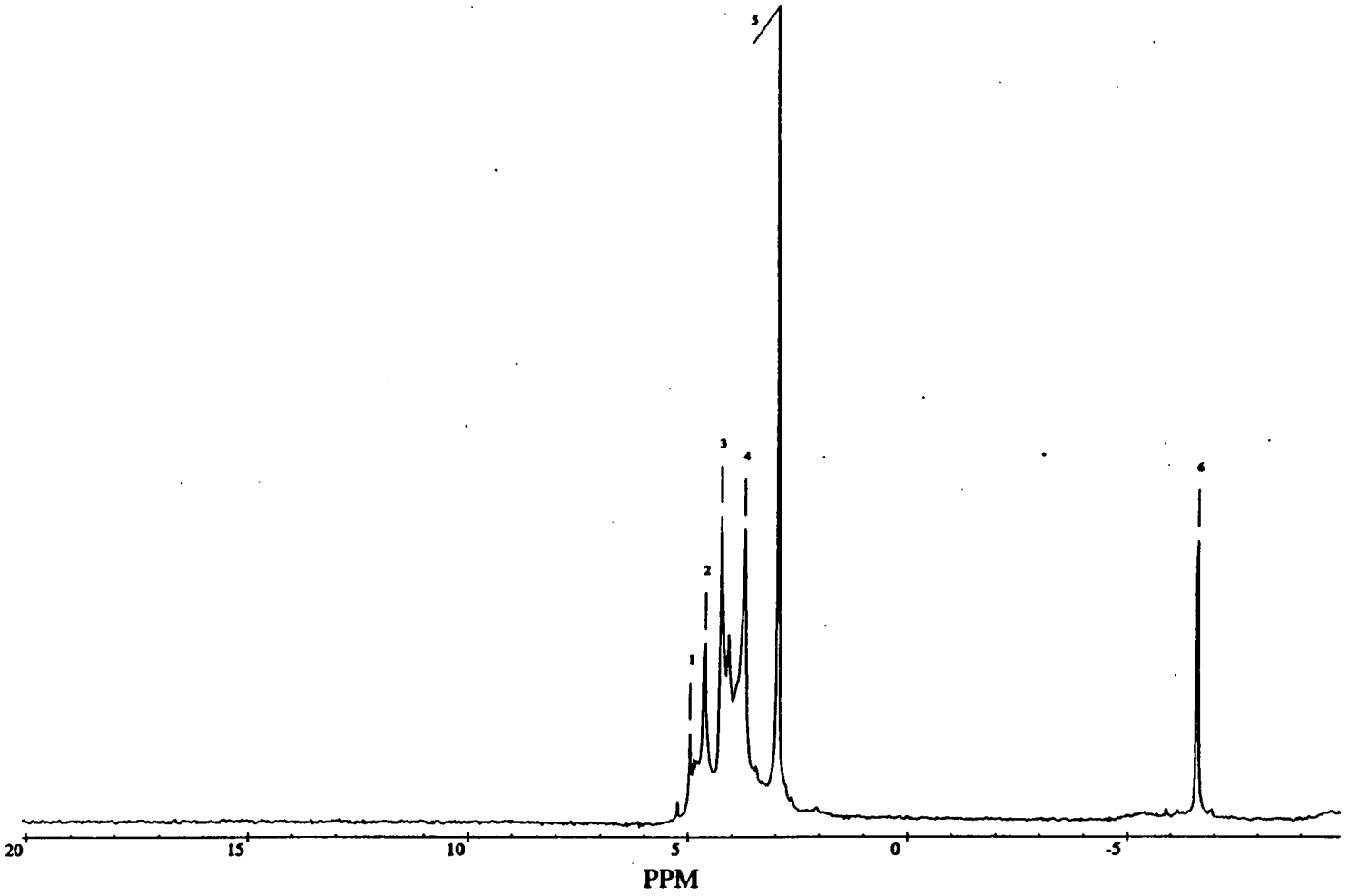


Fig. 34. ^{31}P NMR spectrum of α-limit dextrins from starch phosphomonoester, at pH 8.0±0.1 and 81 MHz with standard inorganic phosphate as an internal reference.
(signal 1: 4.9 ppm, signal 2: 4.6 ppm, signal 3: 4.3 ppm, signal 4: 3.7 ppm, signal 5: 2.9 ppm, signal 6: 6.6 ppm)

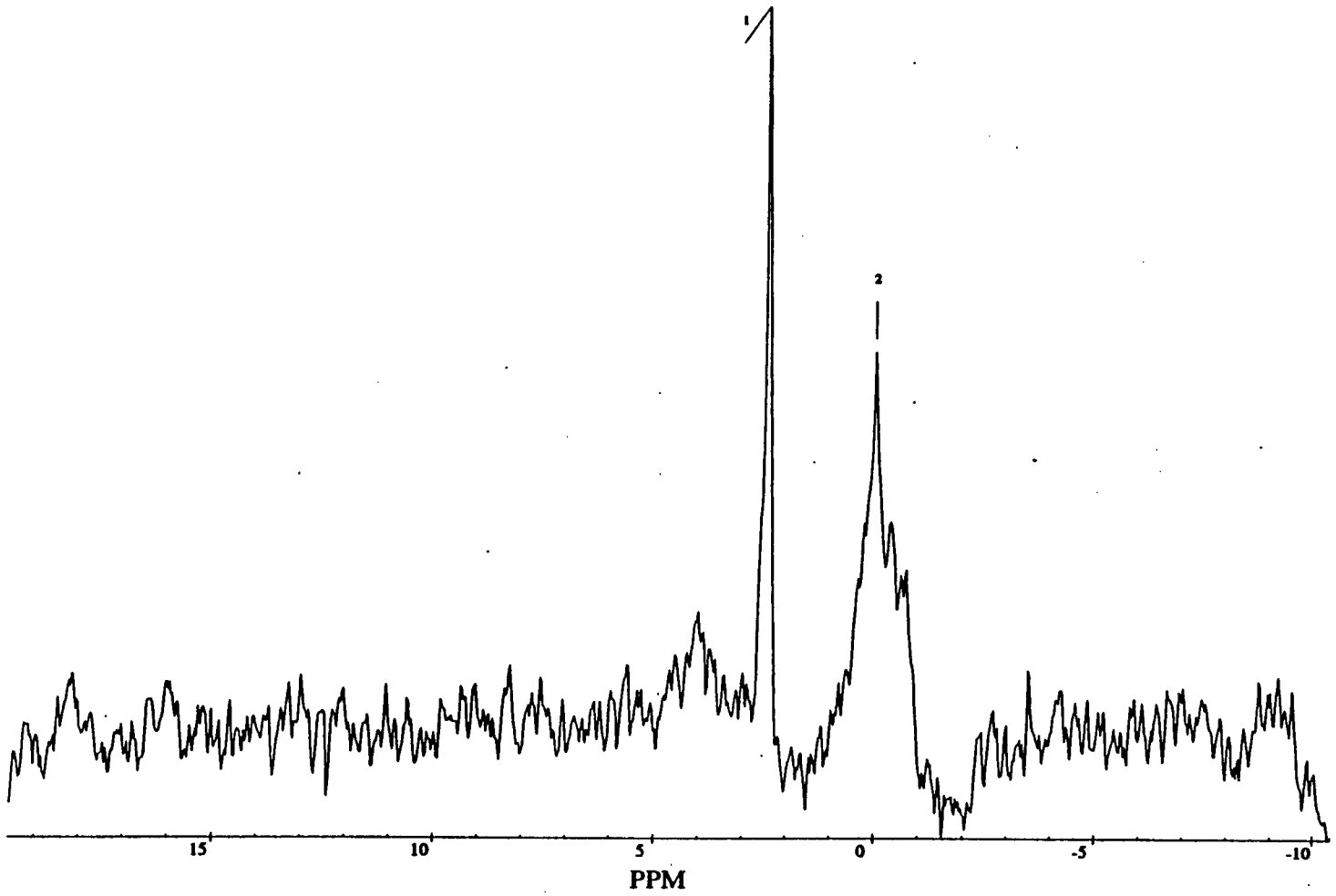


Fig. 35. ^{31}P NMR spectrum of lecithin, at pH 8.0±0.1 and 81 MHz with standard 85% H_3PO_4 as an external reference.
(signal 1: 2.4 ppm, signal 2: 0.0 ppm)

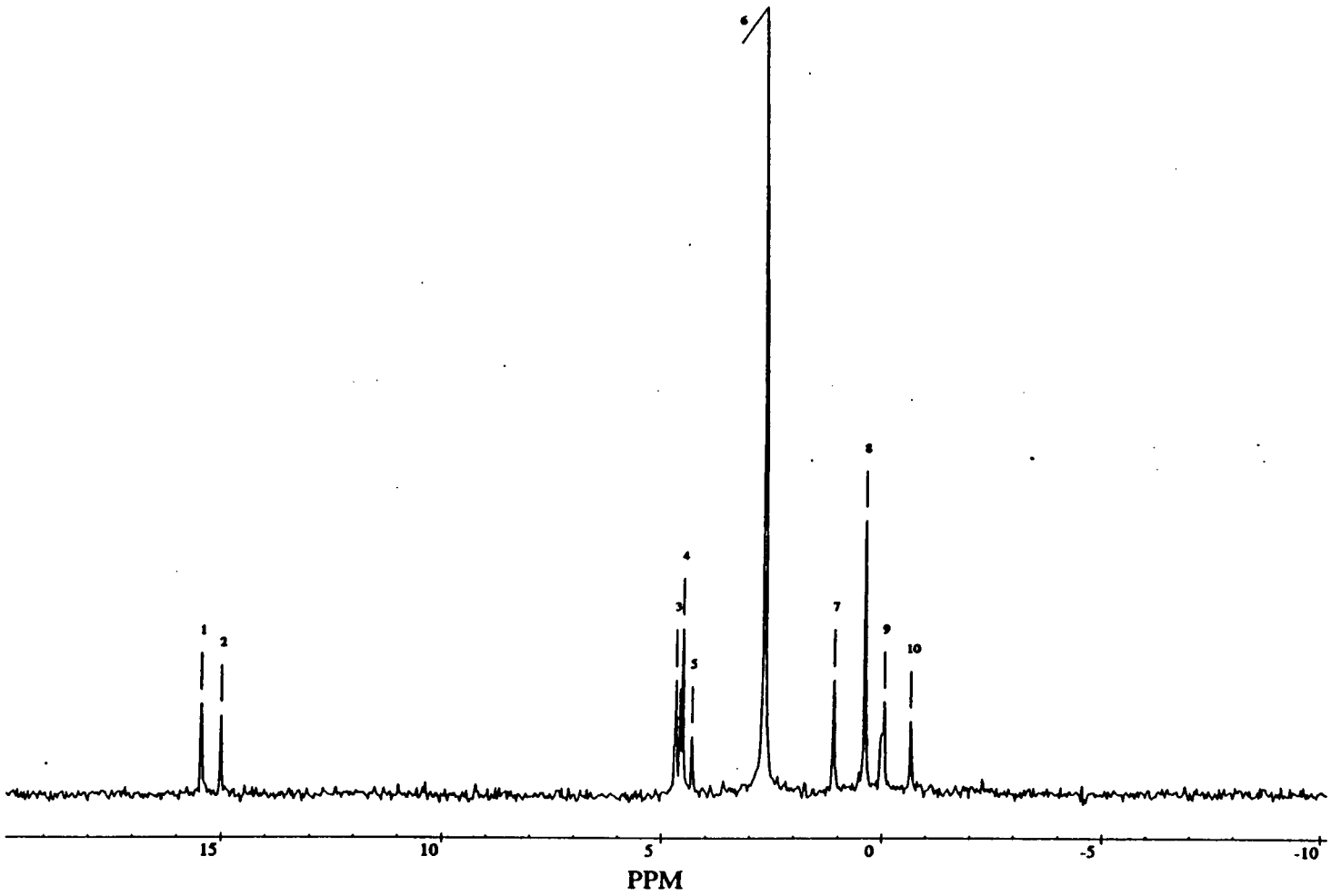


Fig. 36. ^{31}P NMR spectrum of cross-linked α-methyl glucoside, at pH 8.0±0.1 and 81 MHz with standard inorganic phosphate as an internal reference.
(signal 1: 15.5 ppm, signal 2: 15.0 ppm, signal 3: 4.7 ppm, signal 4: 4.5 ppm, signal 5: 4.3 ppm, signal 6: 2.7 ppm, signal 7: 1.0 ppm, signal 8: 0.3 ppm, signal 9: 0.1 ppm, signal 10: -0.7 ppm)

^{31}P NMR spectrum of normal corn starch.

Normal corn starch modified with 0.2% $POCl_3$ was most suitable for separation into soluble and insoluble parts. The higher and the lower concentrations of $POCl_3$ were also prepared and were investigated, but the spectra showed a high noise ratio and were difficult to identify the signal because of the low solubility or low concentration of the phosphorus in the sample. Spectra of these samples (Figures 37 and 38) had high noise ratios. Since $POCl_3$ modification of starch generated high concentration of cross-linking, starch granule could not swell and disperse, and caused less solubility in aqueous solution, thus, resulted in weaker detection by NMR.

The cross-linked starch samples were also attempted to dissolve in dimethylsulfoxide (DMSO), but the noise ratio of the NMR spectrum was not improved (data not shown). The same sample was subjected to NMR measurement at high temperature (70°C) for improving the solubility of the sample, but the spectrum was not much improved either (data not shown).

The spectrum of the 0.2% $POCl_3$ modified starch before separation showed signals at the chemical shift δ -0.1, 0.0, 4.6, and 15.5 ppm (Figure 39). The signal at δ 0.0 ppm (phosphodiester) had the highest intensity, whereas the signal at δ 4.6 ppm indicated the signal of the phosphomonoester at the non reducing terminal of α-limit dextrins. The signal at δ 15.5 ppm was identified as the cyclic phosphate group.

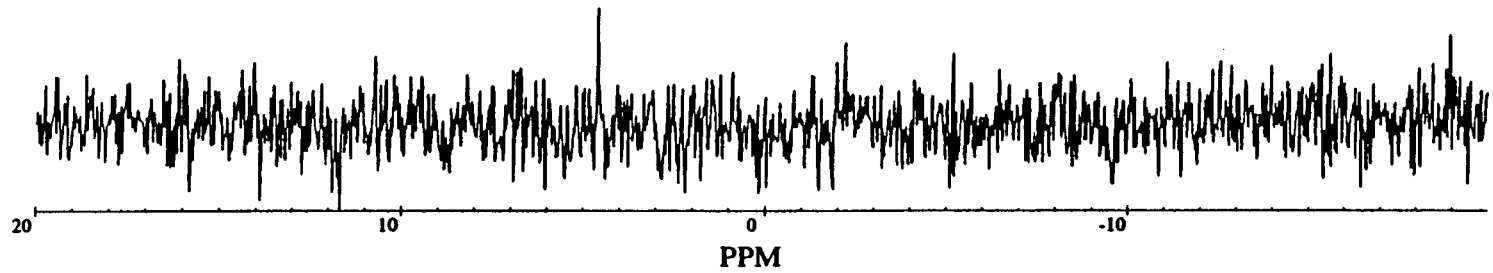


Fig. 37. ^{31}P NMR spectrum of α-limit dextrins from 5.0% $POCl_3$ modified normal corn starch, at pH 8.0±0.1 and 81 MHz.

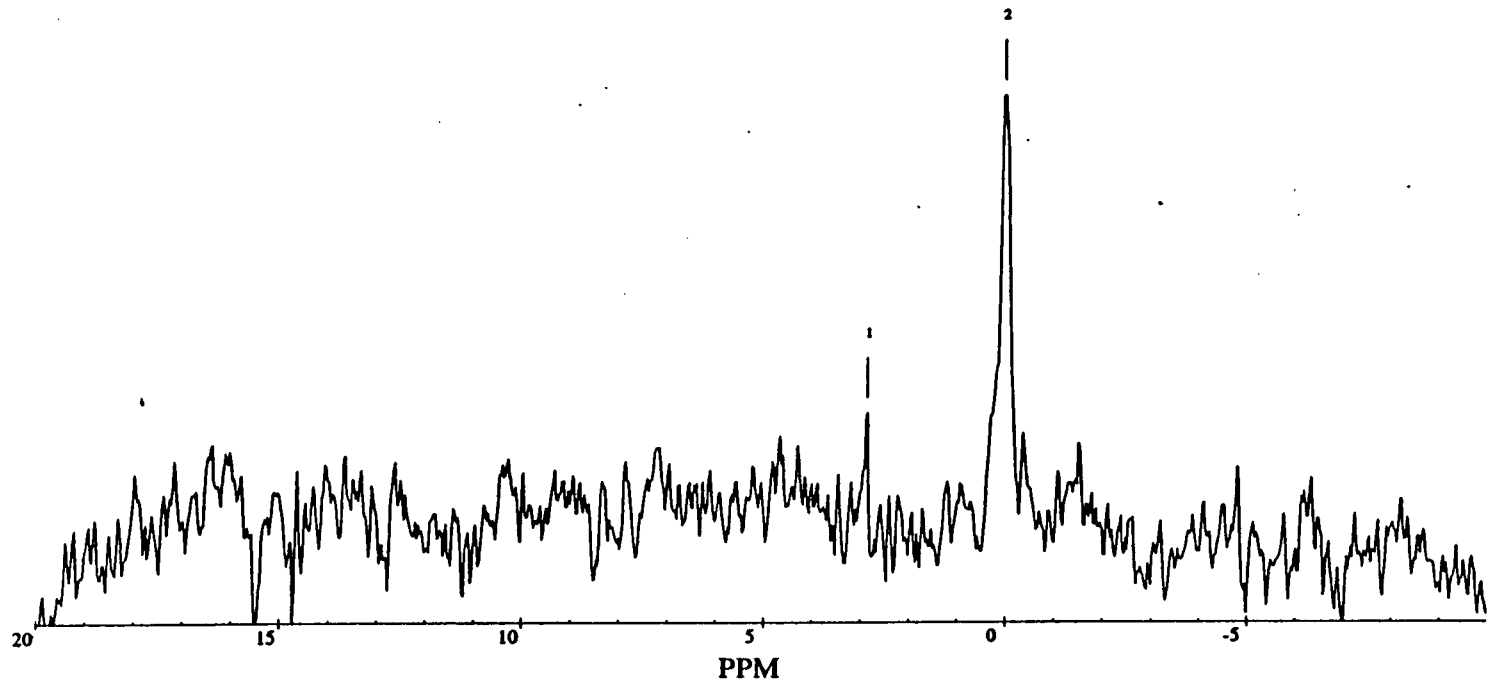


Fig. 38. ^{31}P NMR spectrum of α-limit dextrins from 1.0% $POCl_3$ modified normal corn starch, at pH 8.0±0.1 and 81 MHz.
(signal 1: 2.9 ppm, signal 2: 0.0 ppm)

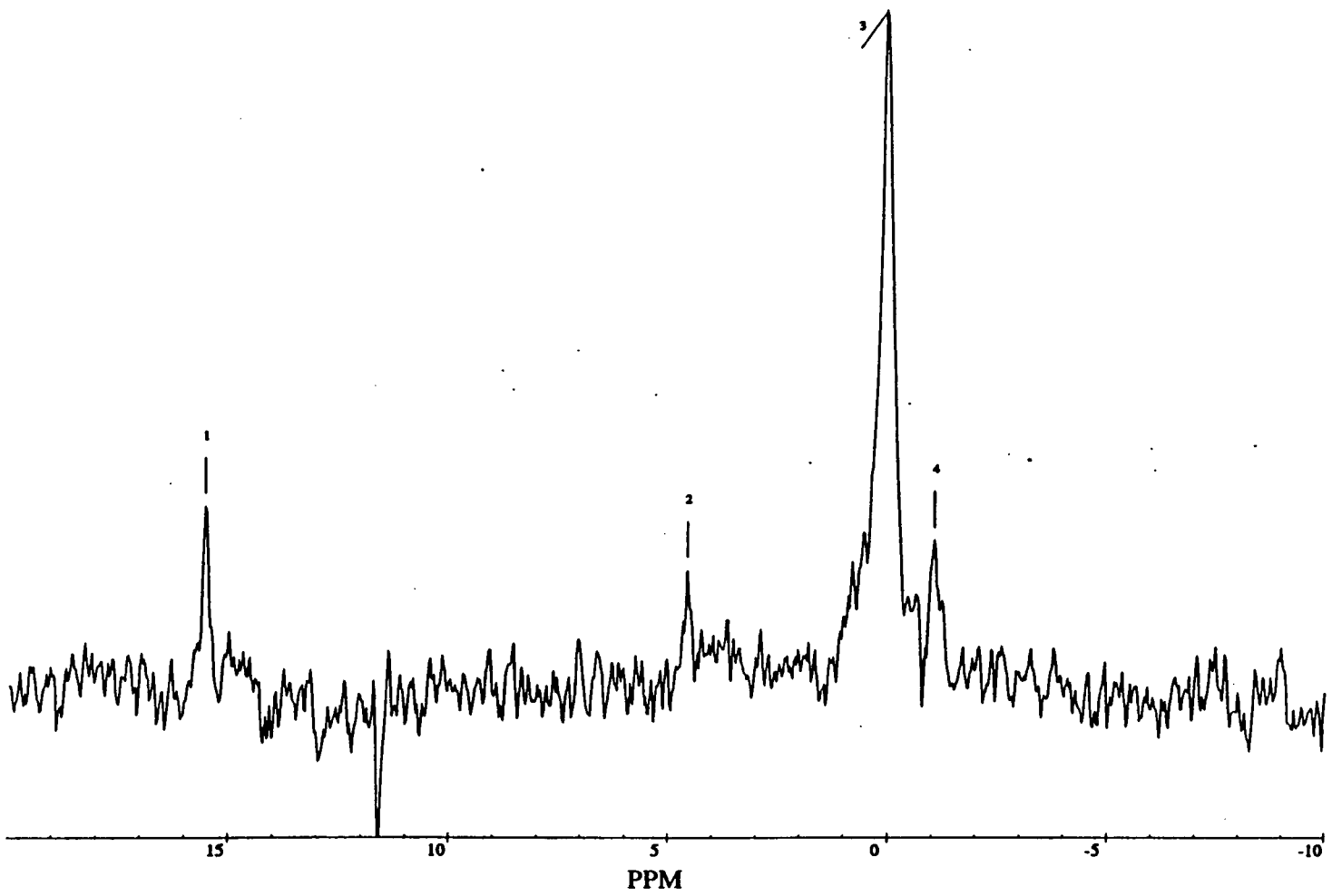


Fig. 39. ^{31}P NMR spectrum of α-limit dextrins from 0.2% $POCl_3$ modified normal corn starch without fractionation, at pH 8.0±0.1 and 81 MHz.
(signal 1: 15.5 ppm, signal 2: 4.6 ppm, signal 3: 0.0 ppm, signal: -1.0 ppm)

A defatted 0.2% $POCl_3$ modified starch was also used for NMR measurement to eliminate a possible interference by the presence of phospholipid. The spectrum was the same as that without defatting process (spectrum not shown).

The 0.2% $POCl_3$ modified starch was dissolved in DMSO and was separated into soluble and insoluble portions. The insoluble portion was subjected to enzyme hydrolysis following the same method as the sample without separation. The samples retained some insoluble portion after enzyme hydrolysis. The spectrum had a high noise ratio (Figure 40). The signals showed in the chemical shift at about δ 0.0, 3.6, 4.6, and 15.5 ppm. The signals showed at δ 0.0 ppm had the highest intensity and indicated the phosphodiester linkage. The signal δ 4.6 ppm indicated the phosphomonoester at the non-reducing terminal of the α-limit dextrins. The signals at the chemical shift at δ 3.6 indicated the phosphomonoester derivative in the internal chain of α-limit dextrins, and the signal at 15.5 ppm indicated as glucose cyclic phosphate.

Soluble starch fraction in DMSO solution was recovered by precipitating with large volume of alcohol. The spectrum showed three signals: the first at δ 2.6 ppm standing for free phosphate, the second at δ 4.5 ppm corresponding to phosphomonoester derivative at non reducing terminal of α-limit dextrins, and the third at 11.6 ppm was not identified (Figure 41). This sample was also

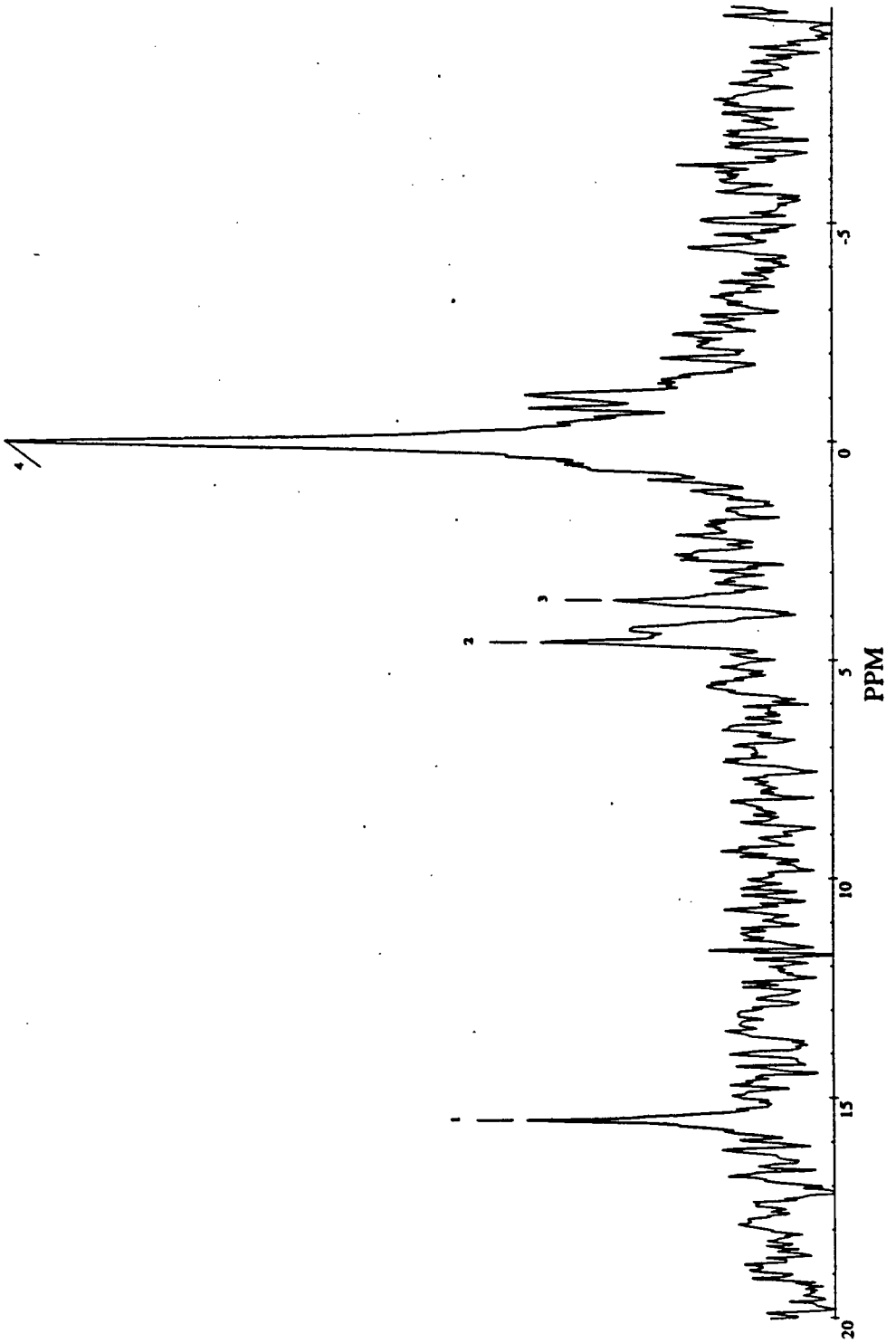


Fig. 40. ^{31}P NMR spectrum of α-limit dextrins from the insoluble fraction which was separated from 0.2% $POCl_3$ modified normal corn starch, at pH 8.0±0.1 and 81 MHz.
(signal 1: 15.5 ppm, signal 2: 4.6 ppm, signal 3: 3.6 ppm, signal: 0.0 ppm)

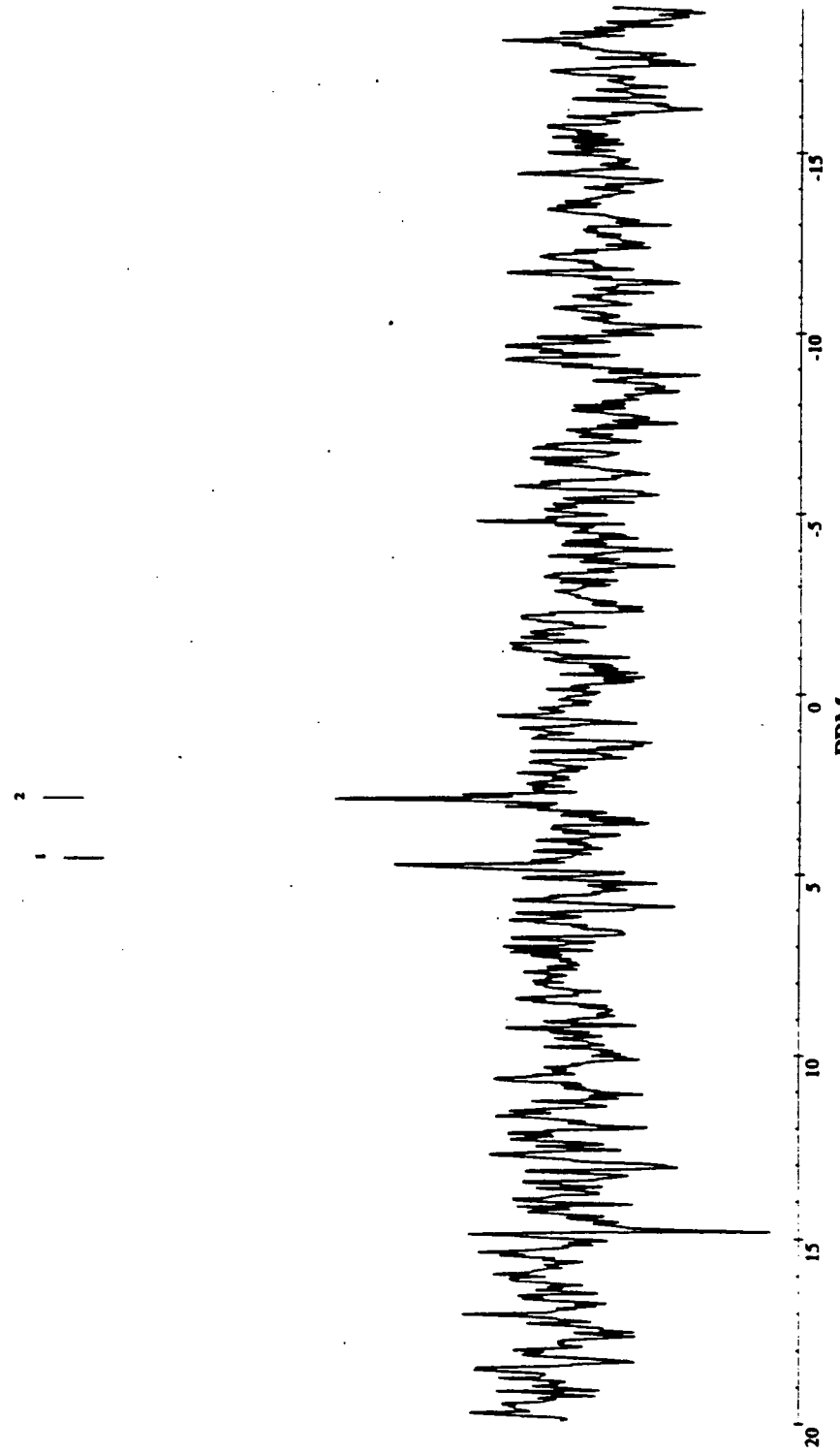


Fig. 41. ^{31}P NMR spectrum of α-limit dextrins from the soluble fraction which was separated from 0.2% $POCl_3$ modified normal corn starch at pH 8.0±0.1 and 81 MHz. (signal 1: 4.5 ppm, signal 2: 2.6 ppm)

measured by the Bruker MSL 300 NMR spectrometer, and the spectrum showed the same chemical shifts of signals. (data not shown).

The MSL 300 solid NMR was also used for measuring the modified starch samples (before separation, soluble fraction, and insoluble fraction). The spectra showed broad signals. The signals overlapped and definite chemical shifts could not be identified. The spectra are shown in Figures 42 to 44.

The normal corn starch was suspended in 90% DMSO, then boiled and stirred continuously for 24 hr. The starch was dispersed and lost the granular structure. The dispersed starch was washed with alcohol and was dried. Five percent of $POCl_3$ was used to modify it with 5% of the dispersed sample (dsb). The sample was measured with ^{31}P NMR spectroscopy. The spectrum had a low noise ratio and gave signals at the chemical shifts of δ 0.0, 2.9 3.7, 4.6, and 15.5 ppm (Figure 45). The signals at -1.0 to 0.0 ppm were identified as cross-linkages; the signal at δ 2.9 ppm was free phosphate; the signal at δ 4.6 ppm was identified as phosphomonoester at the non-reducing terminal of the α-limit dextrins. The signal at the chemical shift δ 3.6 was identified as the phosphomonoester derivative in the α-limit dextrins chain; and the signal at 15.5 ppm was identified as glucose cyclic phosphate.

^{31}P NMR spectrum of Nägeli dextrin

Waxy corn Nägeli dextrin was modified with 0.2% $POCl_3$, and was measured by ^{31}P NMR without enzyme hydrolysis. The spectrum showed

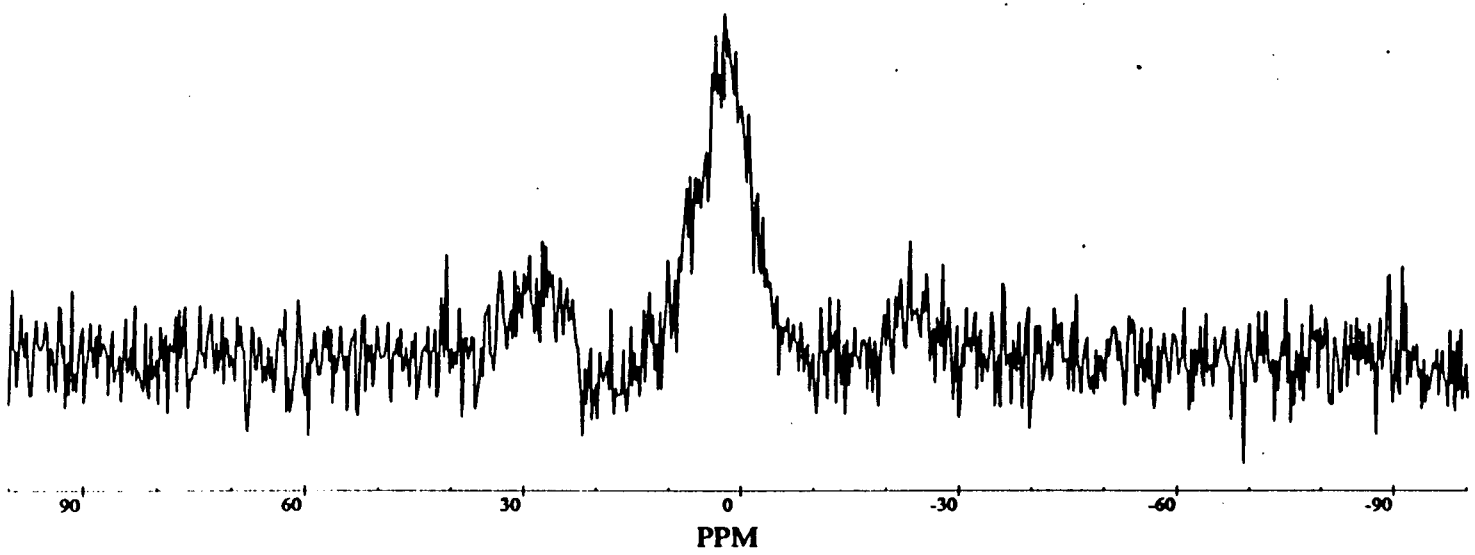


Fig. 42. ^{31}P solid state NMR spectrum from 0.2% $POCl_3$ modified normal corn starch without fractionation, at pH 8.0±0.1 and 81 MHz.

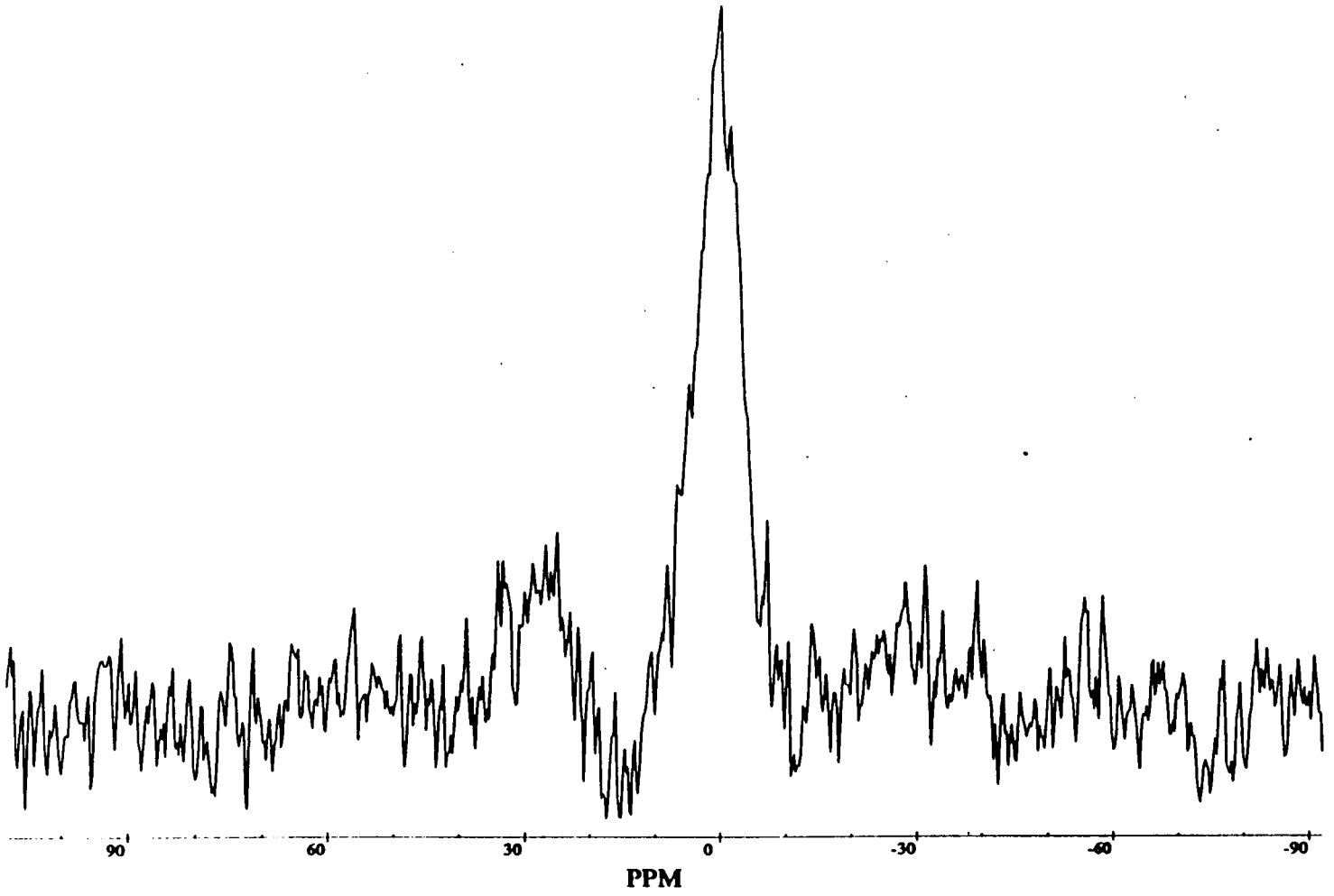


Fig. 43. ^{31}P solid state NMR spectrum from the insoluble fraction which was separated from 0.2% $POCl_3$ modified normal corn starch, at pH 8.0±0.1 and 81 MHz.

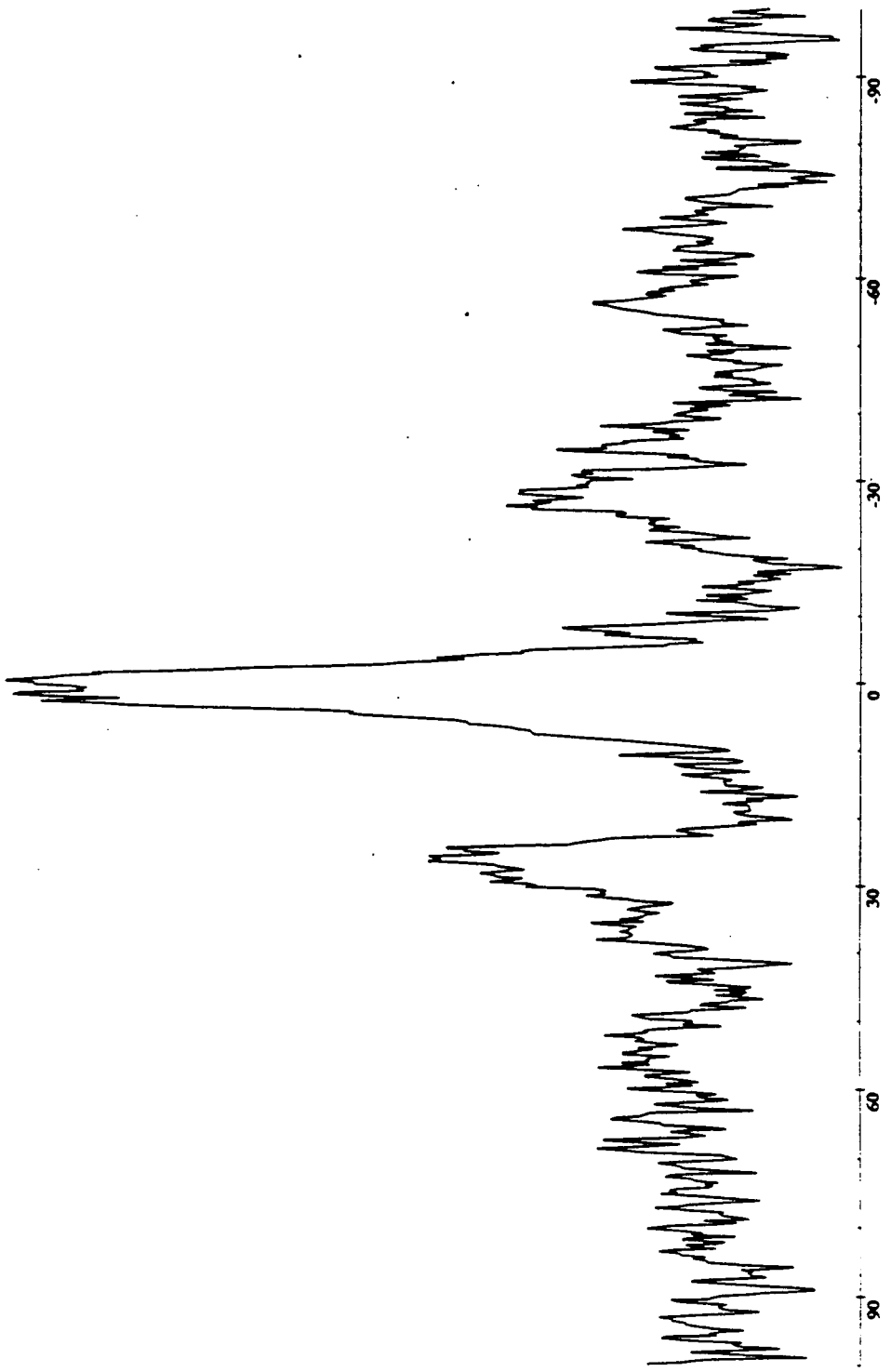


Fig. 44. ^{31}P solid state NMR spectrum from the soluble fraction which was separated from 0.2% $POCl_3$ modified normal corn starch, at 8.0±0.1 and 81 MHz.

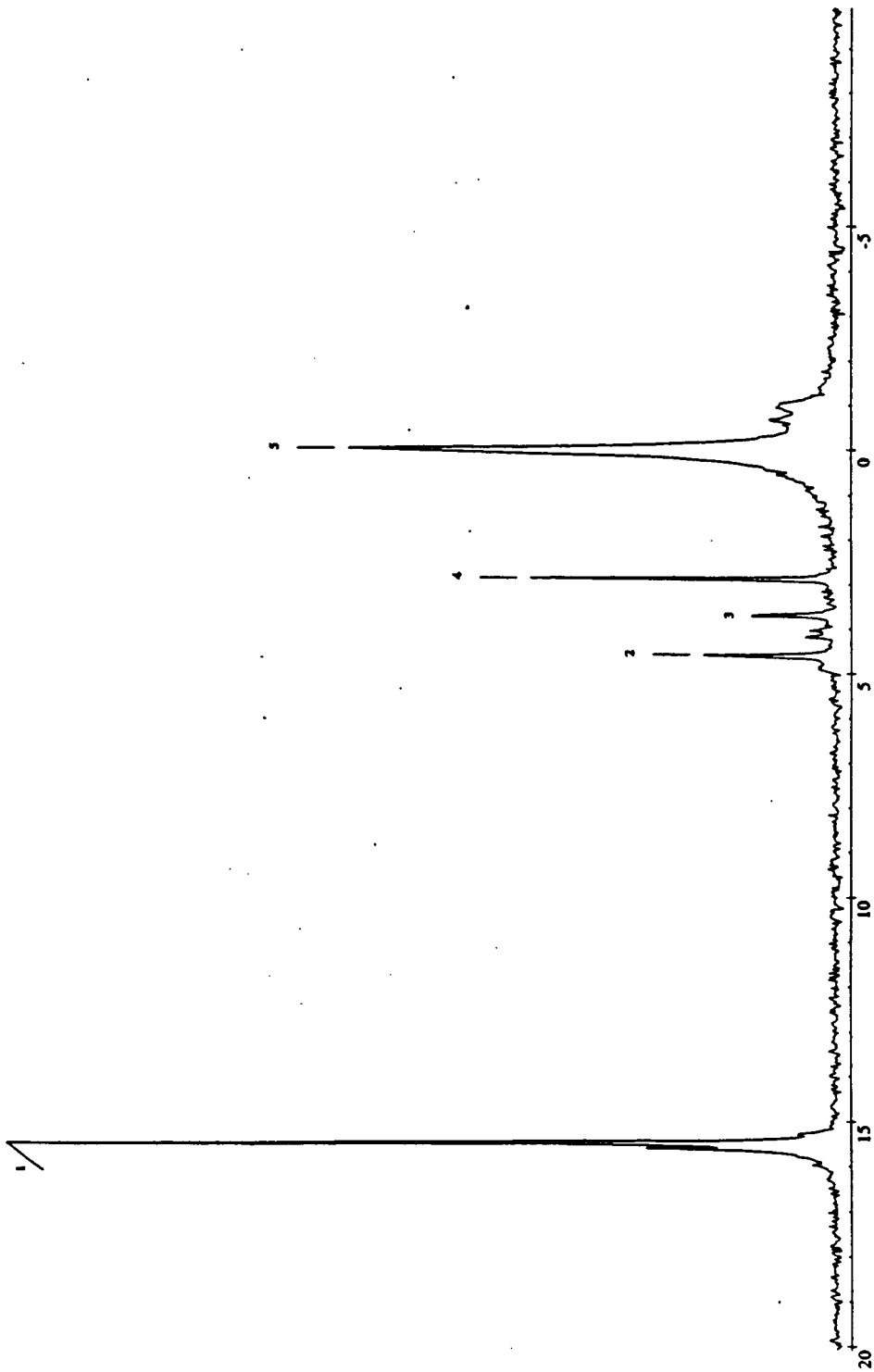


Fig. 45. ^{31}P NMR spectrum of α-limit dextrins from 5.0% $POCl_3$ modified dispersed normal corn starch, at pH 8.0±0.1 and 81 MHz.
(signal 1: 15.5 ppm, signal 2: 4.6 ppm, signal 3: 3.7 ppm, signal 4: 2.9, signal 5: 0.0 ppm)

signals at δ 0.0, 2.6, 3.6, 4.5, and 15.5 ppm (Figure 46). The signals of δ 0.0 and -1.0 indicated the phosphate cross-linkages of the Nägeli dextrin. The signal of δ 4.5 ppm indicated the phosphomonoester at the non-reducing terminal of Nägeli dextrins. The signal at δ 3.6 ppm indicated the phosphomonoester in the internal chain of Nägeli dextrin. The largest signal at δ 2.6 ppm indicated the large amount of free phosphate in the sample. The signal at the chemical shift 15.5 ppm indicated the glucose cyclic phosphate.

Waxy corn Nägeli dextrin modified with 1.0% $POCl_3$ was hydrolyzed with α-amylase and was measured by ^{31}P NMR. The spectrum showed signals at δ -1.1, 0.0, 2.7, 3.6, 4.0, 4.6, and 15.5 ppm (Figure 47). The highest intensity signal was at δ 0.0 and -1.1 ppm which signified the cross-linking in Nägeli dextrin. The signals also indicated inorganic phosphate, glucose cyclic phosphate, and phosphomonoester at the non-reducing terminal and on the internal chain of the α-limit dextrin. The cross-linked Nägeli dextrin was also predominant.

From the spectra of hydrolyzed and without hydrolyzed modified waxy corn Nägeli dextrin, it suggested that the enzyme hydrolysis improved the noise ratio of the spectra, and the signals could be identified clearly.

Normal corn Nägeli dextrin modified with 5% $POCl_3$ was hydrolyzed with α-amylase before being analyzed by ^{31}P NMR. The spectrum showed signals at the chemical shifts of δ 0.0, 2.9, 3.7, 4.6, and 15.5 ppm (Figure 48). The signals also indicated the same (phosphomonoester, phosphodiester, inorganic

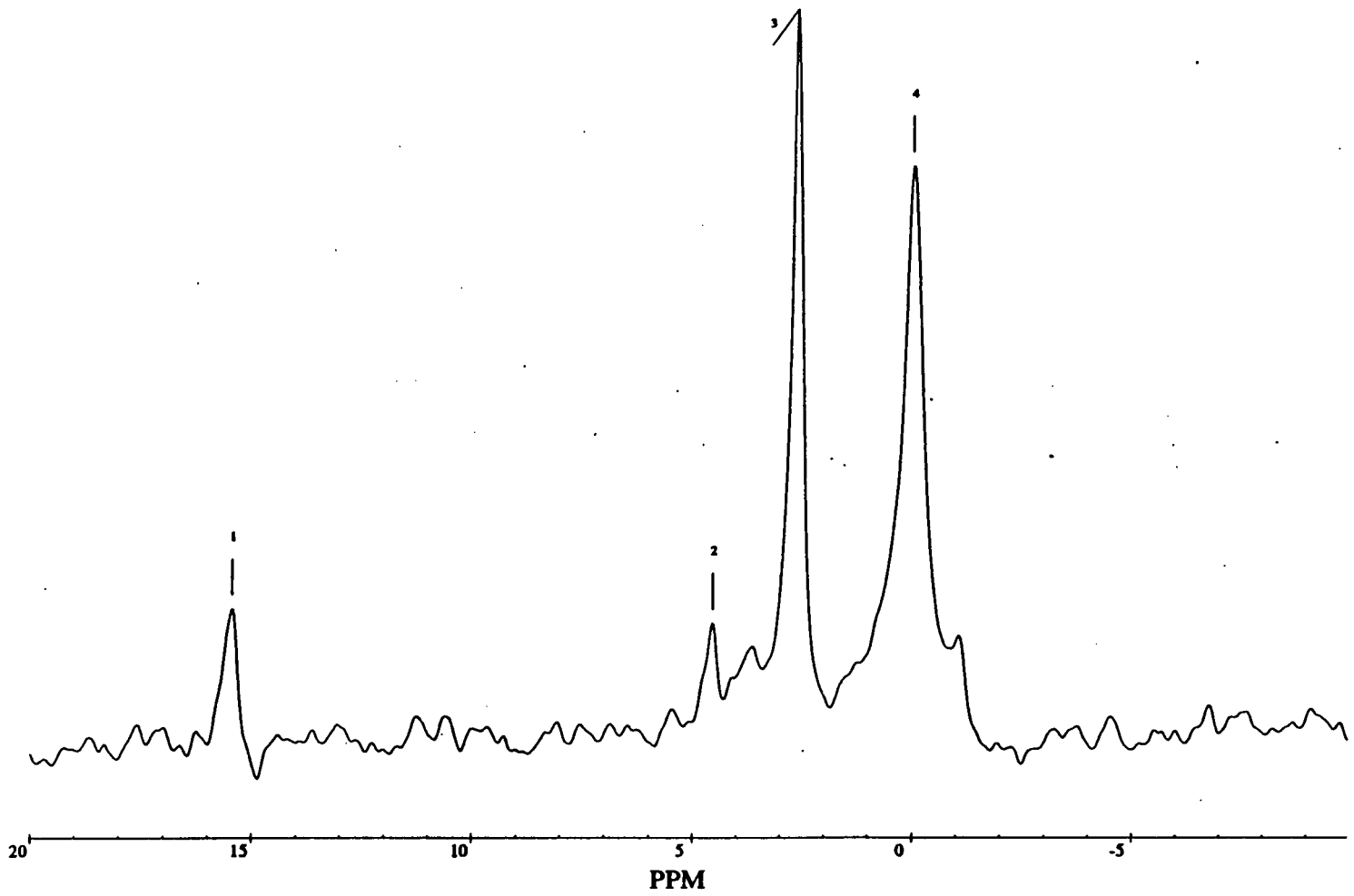


Fig. 46. ^{31}P NMR spectrum of 0.2% $POCl_3$ modified waxy corn Nägeli dextrin (without hydrolyzed), at pH 8.0±0.1 and 81 MHz.
(signal 1: 15.5 ppm, signal 2: 4.5 ppm, signal 3: 2.6 ppm, signal 4: 0.0 ppm)

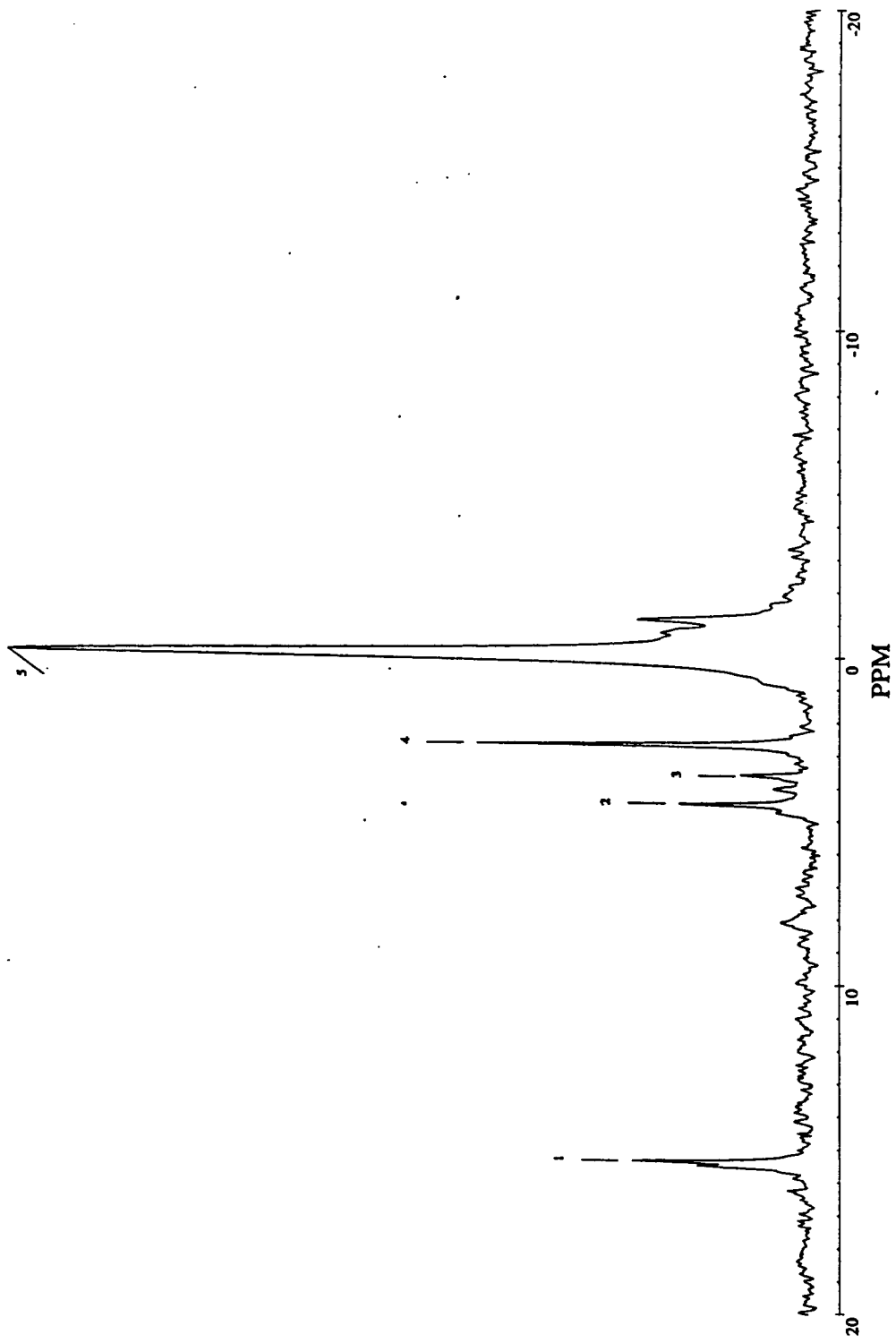


Fig. 47. ^{31}P NMR spectrum of α-limit dextrins from 1.0% $POCl_3$ modified waxy corn Nägeli dextrin, at pH 8.0±0.1 and 81 MHz.
(signal 1: 15.5 ppm, signal 2: 4.5 ppm, signal 3: 3.6 ppm, signal 4: 2.6 ppm, signal 5: 0.0 ppm)

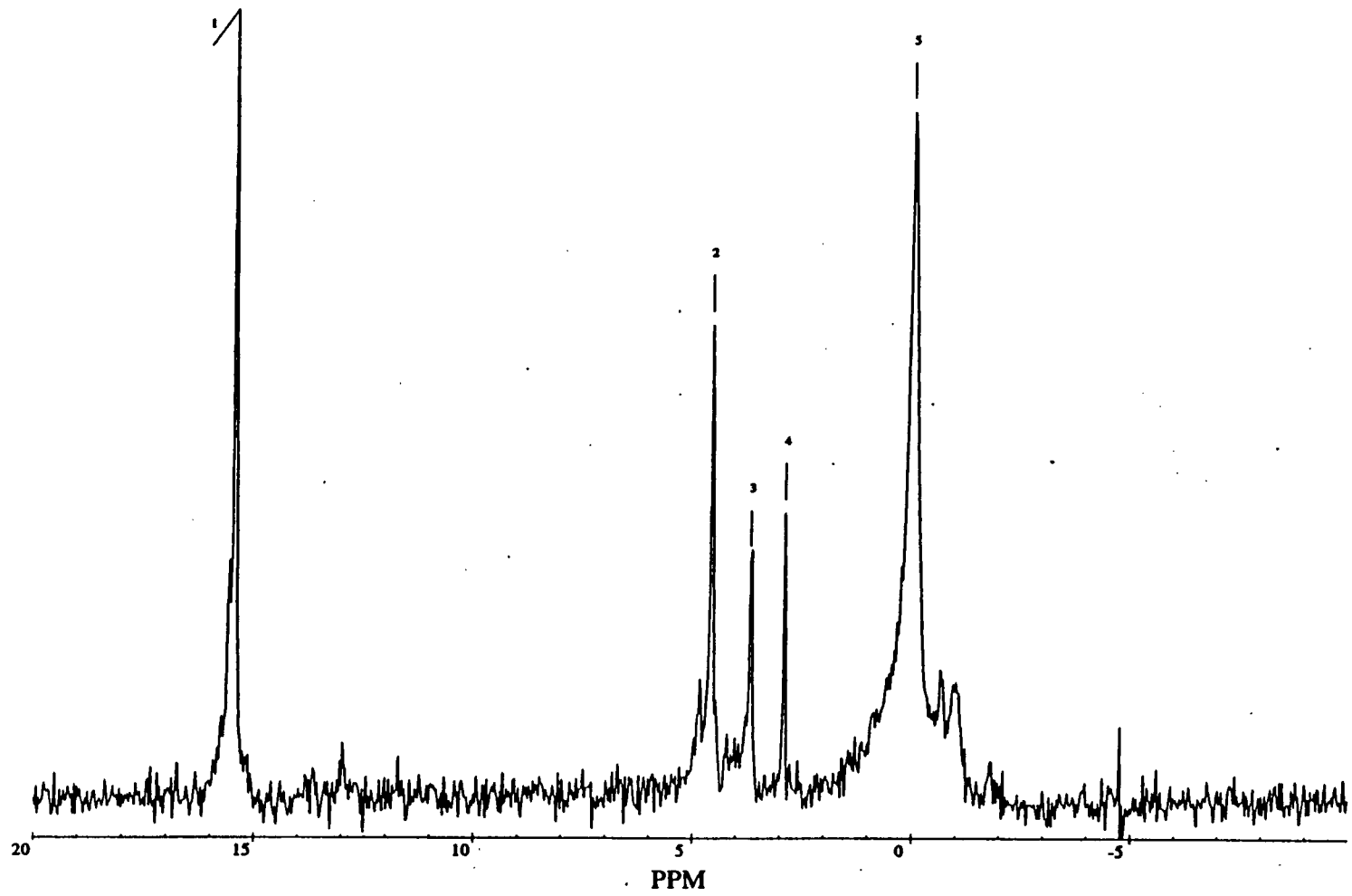


Fig. 48. ^{31}P NMR spectrum of α-limit dextrins from 5.0% $POCl_3$ modified normal corn Nägeli dextrin, at pH 8.0±0.1 and 81 MHz.
(signal 1: 15.5 ppm, signal 2: 4.5 ppm, signal 3: 3.6, signal 4: 2.6 ppm, signal 5: 0.0 ppm)

phosphate, and glucose cyclic phosphate) as the other modified starches, and modified waxy corn Nägeli dextrin. The ratio of phosphomonoester linkages to phosphodiester linkages was higher than other modified starches. The noise ratio of this sample was also low, and the signals were also easy to identify. From the intensity of the signal, it could confirm that the number of phosphodiester linkages (cross-linkage) was more than the phosphomonoester linkage.

^{31}P NMR spectrum of small particle starch

Small particle starches were prepared by the method previous described. The small particle starches were modified with various concentrations of $POCl_3$. A twenty percent concentration of small particle starch solutions was used to prepare for NMR measurement. All samples were prepared in the same method as the Nägeli dextrin.

Small particle starch with 1.0% $POCl_3$ modification was hydrolyzed by α-amylase and was measured by NMR. The spectrum showed clear and low noise ratio signal. The spectrum contained signals at δ 0.0, 2.5, 3.6, 4.6, and 15.5 ppm (Figure 49). The signals at δ -1.1 and 0.0 ppm indicated the cross-linking in small particle starch. The signal at δ 2.5 ppm indicated inorganic phosphate that retained in the sample. The signal at δ 4.6 ppm indicated phosphomonoester at the non-reducing terminal of α-limit dextrin. The signals at δ 3.5 indicated phosphomonoester in the internal chain of α-limit dextrins,

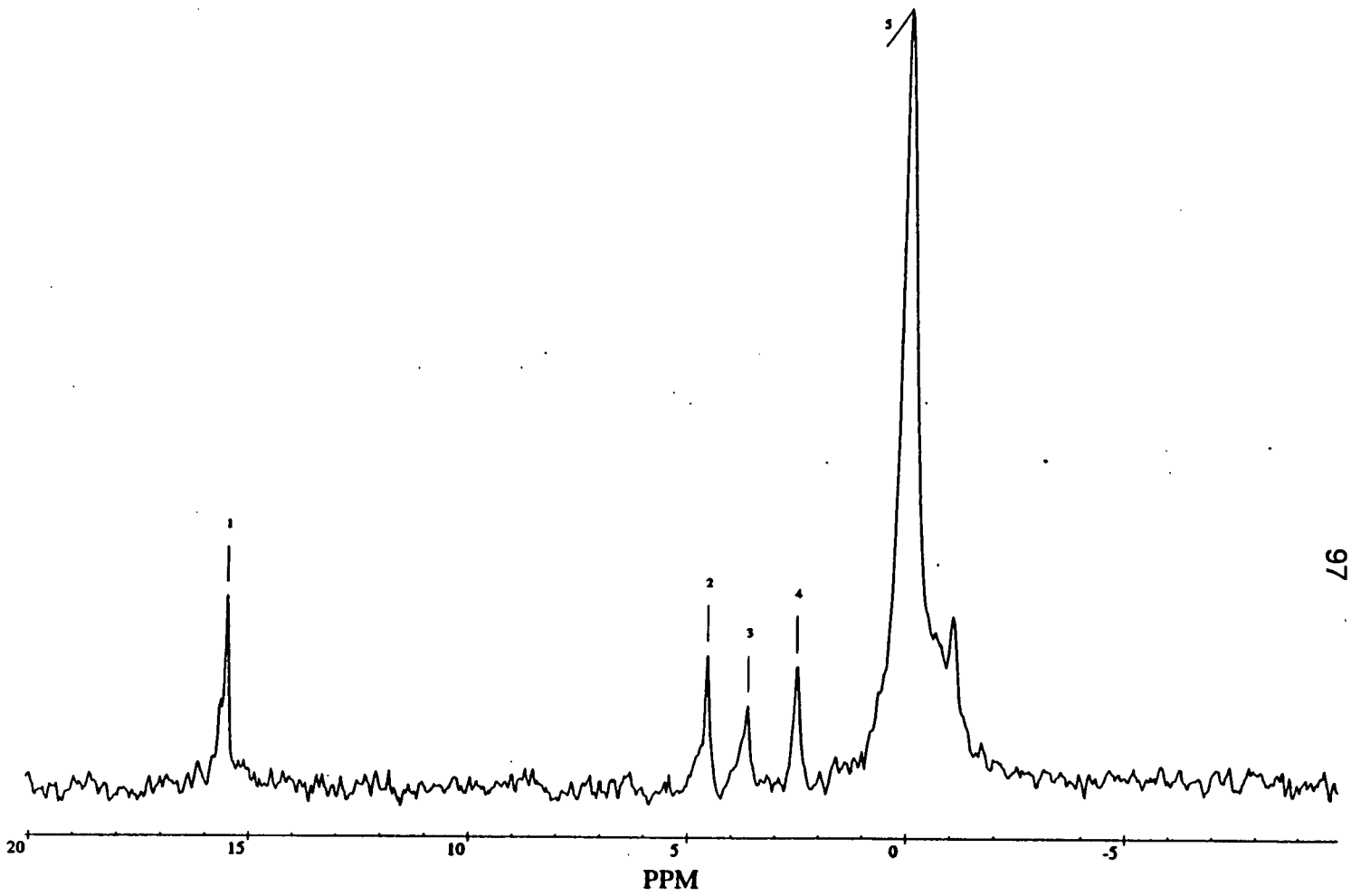


Fig. 49. ^{31}P NMR spectrum of α-limit dextrins from 1.0% $POCl_3$ modified small particle normal corn starch, at pH 8.0±0.1 and 81 MHz.
(signal 1: 15.5 ppm, signal 2: 4.6 ppm, signal 3: 3.6 ppm, signal 4: 2.5, signal 5: 0.0 ppm)

and the chemical shift of 15.5 ppm indicated glucose cyclic phosphodiester. The signal intensity of the cross-linking was also predominant.

Small particle starch modified with 5% $POCl_3$ was hydrolyzed by α-amylase and measured by ^{31}P NMR. The spectrum gave clear and low noise ratio. The spectrum showed signals at δ 0.0, 2.7, 3.6, 4.0, 4.6, and 15.5 ppm (Figure 50) which indicated the same structure as other modified starch. From the intensity of the signals and the ratio of noise, it indicated that the higher concentration of $POCl_3$ provided the higher cross-linking.

A twenty percent solution (dsb) of small particle starch was gelatinized and then was modified with 5% $POCl_3$. The modified sample was hydrolyzed by α-amylase and subjected to ^{31}P NMR. The sample was prepared in the same method as other small particle starch. The spectrum shows signals clearly with low noise ratio. The spectrum displays signals at δ 0.0, 2.9, 3.6, 4.6, and 15.5 ppm (Figure 51). The spectrum was measured by ^{31}P NMR in lower number of scan and the noise ratio was also low. The spectrum was identical to the $POCl_3$ modified dispersed starch (Figure 43). The $POCl_3$ modified Nägeli dextrin, small particle starch, and dispersed starch showed the clear spectra as indicated that modification with $POCl_3$ provided mostly cross-linking, small amount of phosphomonoester, and glucose cyclic phosphate.

The MSL 300 NMR with 20 mm tube probe, which has large capacity of sample holding, was also attempted to improve the noise ratio. The 5.0% $POCl_3$

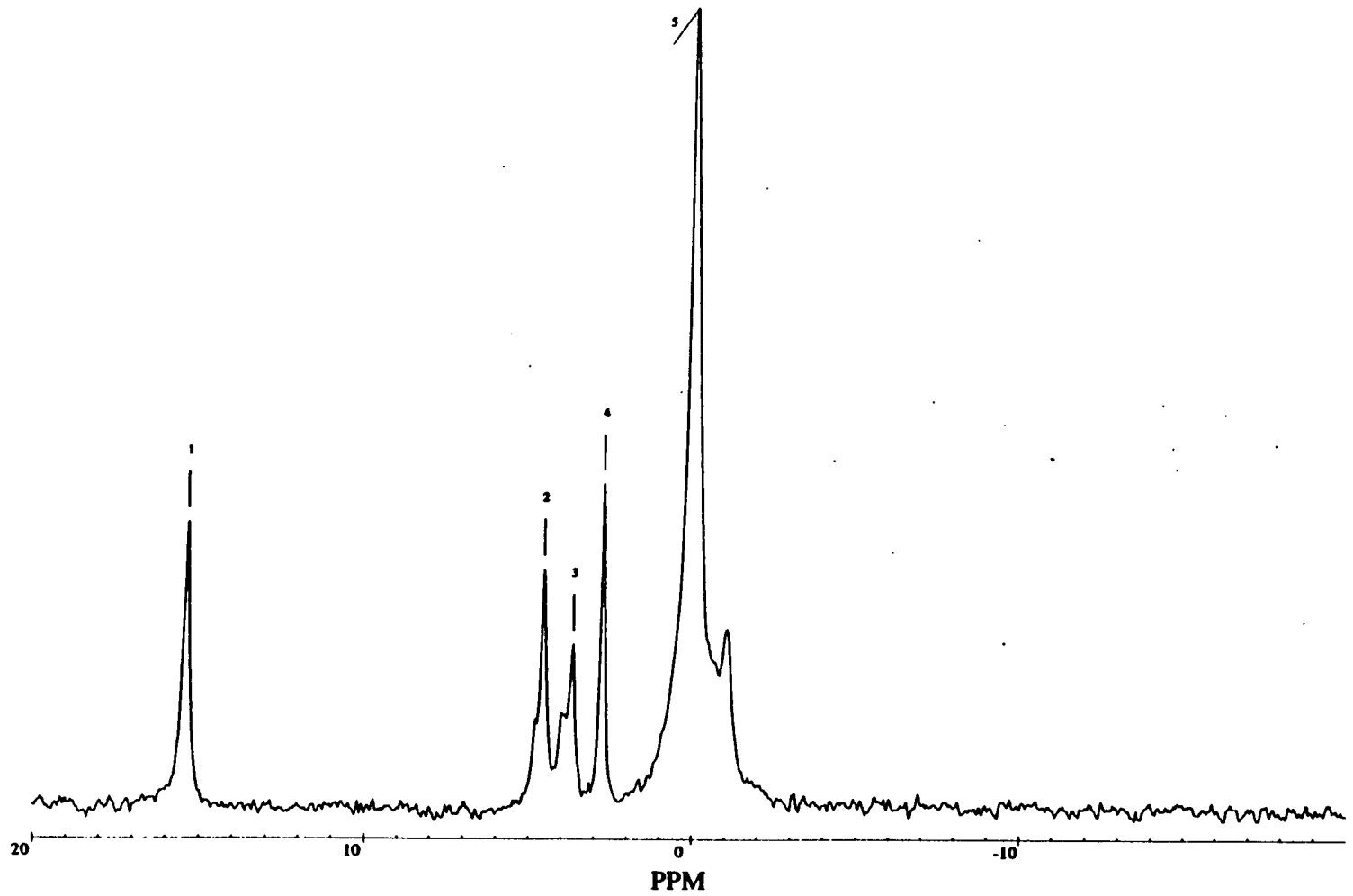


Fig. 50. ^{31}P NMR spectrum of α-limit dextrins from 5.0% $POCl_3$ modified small particle normal corn starch, at 8.0±0.1 and 81 MHz.
(signal 1: 15.5 ppm, signal 2: 4.8 ppm, signal 3: 3.6 ppm, signal 5: 0.0)

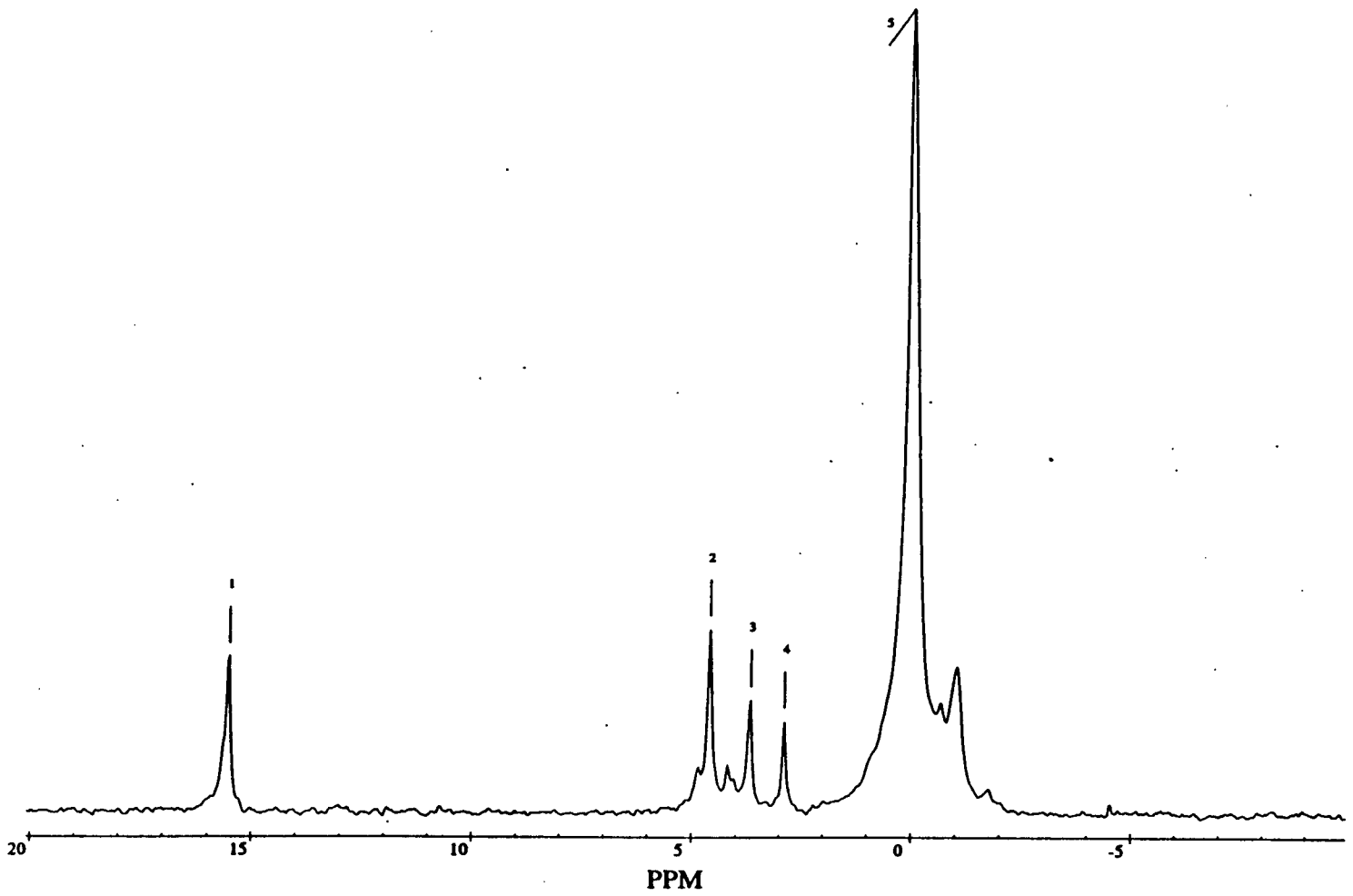


Fig. 51. ^{31}P NMR spectrum of α-limit dextrins from 5.0% $POCl_3$ modified small particle normal corn starch (which was gelatinized before modified), at pH 8.0±0.1 and 81 MHz.
(signal 1: 15.5 ppm, signal 2: 4.6 ppm, signal 3: 3.6 ppm, signal 4: 2.9, signal 5: 0.0 ppm)

modified normal corn Nägeli dextrin and the 1.0% $POCl_3$ modified small particle were measured with this tube probe, but the spectrums were not improved and showed the signals at the same chemical shift as the previous instrument (data not shown).

The strong basic anion exchange resin (AG1-X8) was attempted for purification of the phosphate ester in the sample. The spectrum of the sample purified by anion exchange chromatography did not improve the noise ratio of the spectrum because of the lost of some of the phosphate ester samples during recovery. The spectrum showed signals at the chemical shifts about δ 2.6 and 4.6 ppm which indicate inorganic phosphate and phosphomonoester (spectrums not shown).

The normal corn starch was equilibrated with $POCl_3$ by stirred the starch with $POCl_3$ for 2 hr at pH 6.5, and was adjusted to pH 10, then was stirred for 30 min. The reaction was stopped by adjusted to pH 5.5, then the starch was washed and dried as the previous modification. Modified starch with 0.2 and 2.0% $POCl_3$ (equilibrated) was fractionated by precipitated amylose with n-butanol. The amylose fraction was subjected to ^{31}P NMR. The spectrum did not show the signal. Instead, they suggested that $POCl_3$ was a very reactive reagent and could decompose very fast, and the cross-linking required high pH so at the time that pH was increased (pH 10), the $POCl_3$ was already composed (data not shown).

^{13}C NMR spectroscopy

The spectra of native normal corn starch, cross-linked starch, and starch phosphomonoester showed the same result. It showed the signal at δ 0.0, 54.9, 60.5, 69.5, 71.2, 71.5, 73, and 99 ppm, which can be identified as standard TMS, α-methyl group, carbon-6 of glucose, carbon-4 of glucose, carbon-2 of glucose, carbon-5 of glucose, carbon-3 of glucose, and carbon-1 of glucose respectively. The results from all of the samples did not provide any difference in chemical shifts because of the low amount of the esterified carbons. The spectra of α-methyl glucoside, cross-linked α-methyl glucoside, and α-methyl glucoside phosphomonoester (prepared the same method as starch phosphomonoester) also showed the same results (data not shown).

CONCLUSION

Native corn starch can be modified with $POCl_3$ at pH 12 and shows the highest degree of cross-linking. The modified starches made with concentrations of $POCl_3$ lower than 0.04% showed that the pasting viscosity increased proportional to the concentration of the reagent. At concentrations of $POCl_3$ higher than 0.04%, the viscosity decreased with increasing $POCl_3$ concentration. The starch modified with 0.04% $POCl_3$ showed the highest pasting viscosity, and when modified with 0.2% $POCl_3$ the starch pasting viscosity disappeared. The phosphorus oxychloride modification provides mostly cross-linkage and some phosphomonoester derivative. At the low concentration of the $POCl_3$ modification, the starch obtained a sufficient amount of cross-linking to increase the apparent molecular size which resulted in the high pasting viscosity. At a high degree of cross-linking, the starch molecules were highly cross-linked and could not swell which caused a low pasting viscosity. The molecular weight distribution of the modified starches which was dispersed in 90% DMSO showed only the fractions which were similar to the normal amylose fraction. It can be explained that amylopectin molecules were cross-linked among themselves and also cross-linked with some portions of amylose, and became insoluble. It cannot be detected by gel permeation chromatography; only small amylose which was not cross-linked remained soluble. The phosphorus-31 NMR of the insoluble fractions of the modified

starch showed phosphomonoester and phosphodiester (cross-linkage); it also showed cyclic phosphate within a glucose unit. The phosphorus-31 NMR of the soluble fraction indicated phosphomonoester and inorganic phosphate (no cross-linking was found). The results from the ^{31}P NMR indicated that the amylopectin and amylose were interspersed and, thus, were cross-linked. Only small amounts of amylose which had short chain were not cross-linked and could be separated as "soluble fraction." If the amylose molecules were located in bundle, the amylose molecules should be cross-linked, and the amylose peak should be shifted to higher molecular weight in the gel permeation chromatography. From the results of the gel permeation chromatography and ^{31}P NMR spectroscopy, it showed that amylose molecules were interspersed among amylopectin molecules.

Nikuni (1978) proposed the model (Figure 52a)in which amylose molecules located in bundle and separated from amylopectin. Our model showed that amylose molecules interspersed among amylopectin and located in both crystalline and amorphous regions (Figure 52b).

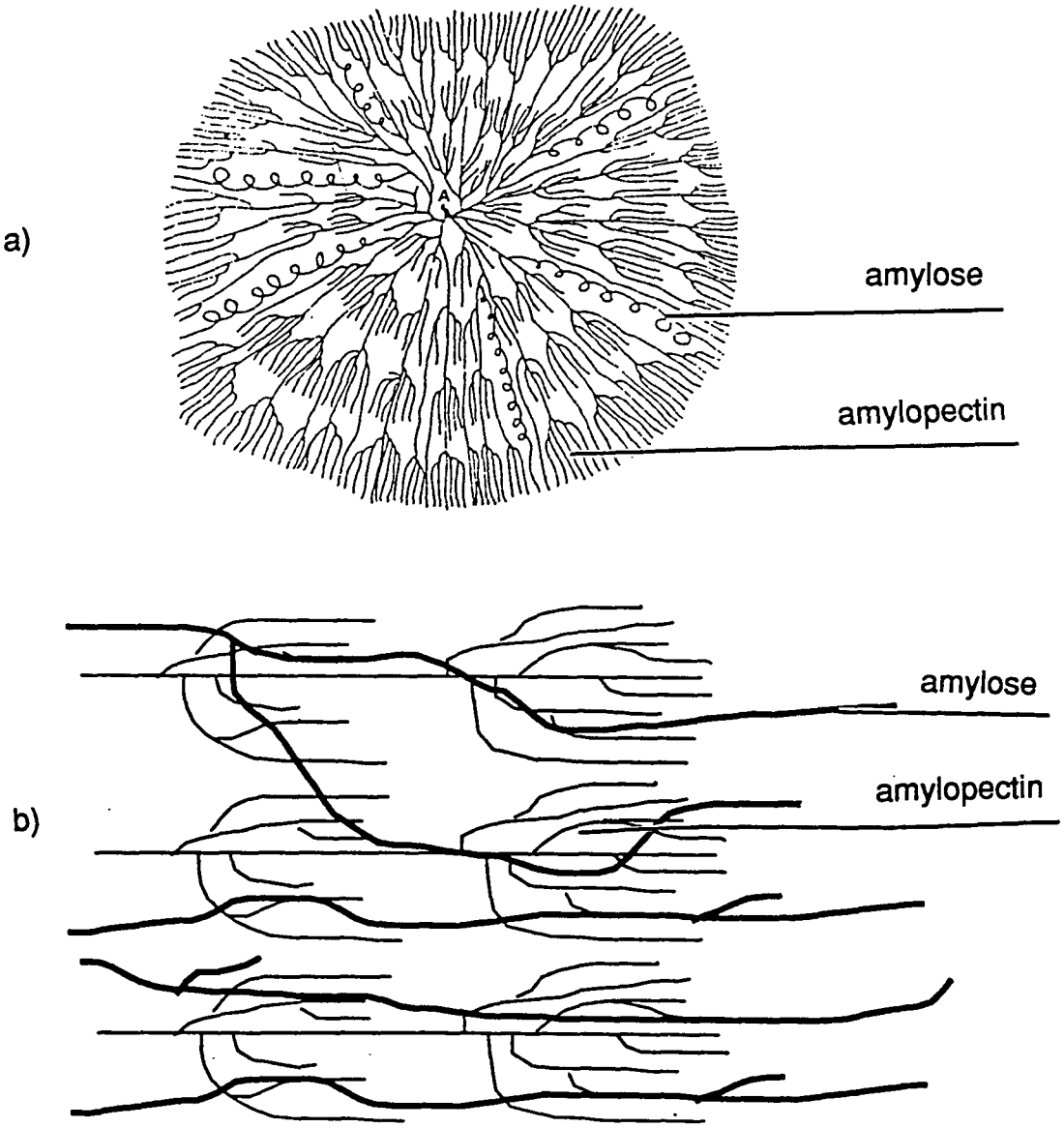


Fig. 52 Shematical models of starch granule organization a) proposed by Nikuni, b) proposed on the basis of present studies.

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ACKNOWLEDGMENTS

I want to express my sincere appreciation to the following for their valuable contributions:

To my major professor, Dr. Jay-lin Jane, for her invaluable suggestions of this research, and her superb guidance throughout the cause of this endeavor.

To my POS committee, Dr. John F. Robyt and Dr. Zivko L. Nikolov, for their interest and valuable help.

To Nan Morain, Shantilal Parmer, and Wei Sung, for being unselfish with their time, and invaluable assistants.

To Dr. Lim, Jerry, Lu, Aki, Inke, and Ting-Jang, for their suggestions, and great company.

To my Thai friends, Sang, Nitus, Chittinun, Ing, Noke, for their unsolicited help and great company.

To my grandmother and my parents, for their support, understanding and encouragement.

Finally, I want to thank everyone that helped, supported, and contributed to my research and **MADE THIS THING HAPPENED.**