Location of Phosphate Esters in a Wheat Starch Phosphate by ^{31}P-Nuclear Magnetic Resonance Spectroscopy[1]

S. LIM[2] and P. A. SEIB[3]

ABSTRACT

Phosphate esters of D-glucose, methyl α-D-glucopyranoside, and maltose, as well as the α,γ-limit phosphodextrins from native potato starch (degree of substitution [DS] 0.0033) and phosphorylated amylose (DS 0.016) served as model compounds in ^{31}P-nuclear magnetic resonance experiments to locate the phosphate groups on a phosphorylated wheat starch (DS 0.012). The α,γ-limit phosphodextrins were isolated by ion-exchange chromatography after exhaustive digestion of amylose or starch phosphates with *Bacillus amyloliquifaciens* α-amylase followed by *Aspergillus niger* glucoamylase. The endogenous orthophosphate groups

on potato starch were confirmed to be the 6- and 3-esters. The wheat starch phosphate, prepared by heating starch with sodium tripolyphosphate under semidry conditions at an initial pH of 6, contained mainly 6-monophosphate esters along with lower levels of 3- and probably 2-monophosphates. The wheat starch phosphate contained orthophosphate groups at the nonreducing ends of starch molecules, whereas endogenous phosphate esters in potato starch occur on inner anhydroglucose units.

The phosphate ester groups on potato starch contribute to its high clarity and viscosity when it is cooked to a paste. Phosphorylation of wheat and corn starches increased paste clarity and consistency, but potato starch remained superior in those properties in spite of the fourfold higher level of phosphate esters on the modified wheat and corn starches and their low level of cross-linking (Lim and Seib, 1993). The location of endogenous phosphate esters on potato starch apparently differs from that on chemically phosphorylated starches.

Gramera et al (1966) prepared corn starch phosphate with a degree of substitution (DS) of 0.016 (0.3% phosphorus [P]) by reacting corn starch at 150°C with sodium tripolyphosphate under unspecified conditions. The starch phosphate was subjected to Smith degradation followed by mild acid hydrolysis and anion-exchange chromatography to give three main fractions. The components in the main fractions were investigated using paper chromatography, periodate analysis, and optical rotation. The results indicated that 6-, 2-, and 3-phosphates made up 63, 28, and 9% of the fractions, respectively.

The location of phosphate esters on native potato starch has been examined extensively over the past 20 years by Hizukuri and his colleagues. Of the total P on potato starch (0.036–0.092%, or 1 atom of P per 209–532 anhydroglucose units [AGUs]), practically all was found on the amylopectin fraction (Abe et al 1982), with two thirds occurring as 6-phosphate, one third as 3-phosphate, and a trace as 2-phosphate (Hizukuri et al 1970, Tabata and Hizukuri 1971). The phosphate esters were not found on the nonreducing terminus of the α-1,4-linked unit chains in potato amylopectin (Tabata et al 1978), and 88% or more were on B-chains (Takeda and Hizukuri 1982). The phosphate esters were located a minimum of 9 AGUs inward from a branch point (Takeda and Hizukuri 1982).

Glucoamylase that was free of both α-amylase and phosphatase was unable to bypass phosphorylated AGUs in potato starch (Abe et al 1982). Exhaustive digestion of a potato starch, which contained 1 P per 209 AGU, with glucoamylase gave 17% γ-limit dextrin with 1 P per 36 AGU and an average unit-chain length of 14. The γ-limit dextrin was of high molecular weight, as indicated by its high viscosity in water. Since the P in potato starch was concentrated in the 17% γ-limit dextrin, Abe et al (1982) concluded that either a few amylopectin molecules are highly phosphorylated or that phosphate groups are concentrated locally as a structural feature of the potato starch granule.

Recently, Muhrbeck and Tellier (1991) measured the ^{31}P-nuclear magnetic resonance (NMR) spectra of eight samples of potato starch dissolved in methyl sulfoxide. As the total-P level increased from 0.0153 to 0.0221% of amylopectin, the level of 3-P remained relatively constant (from 0.0044 to 0.0063%), whereas the 6-P level increased (from 0.0104 to 0.0165%). Furthermore, the crystallinity and enthalpy of gelatinization decreased with the level of 6-phosphorylation, but not with the level of 3-phosphorylation (Muhrbeck et al 1991).

In this work, we used ^{31}P-NMR spectroscopy on model P compounds to explore the position of phosphorylation on a wheat starch phosphate.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade unless otherwise stated. Potato starch (0.063% P), potato amylopectin, methyl α-D-glucopyranoside (MG), D-glucose, and D-glucose 6-phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Potato amylose, diphenyl phosphorochloridate, and platinum oxide were from Aldrich Chemical Company, Inc. (Milwaukee, WI). Wheat starch was Midsol 50 provided by Midwest Grain Products Co. (Atchison, KS).

Crystalline *Bacillus amyloliquifaciens* α-amylase (Type IIA), a solution of *Aspergillus niger* glucoamylase with glucose and preservative, and bovine intestinal alkaline phosphatase (Type I-S) were from Sigma Chemical Co. The enzyme activity was 930 units [U]/mg for α-amylase, 6,100 U/ml for glucoamylase, and 6.8 U/mg for phosphatase. One unit of α-amylase liberated 1 mg of maltose from starch in 3 min (1.0 μmol/min) at pH 6.9 and 20°C; 1 U of glucoamylase released 1 mg of glucose from starch in 3 min (1.9 μmol/min) at pH 4.5 and 55°C; and 1 U of phosphatase hydrolyzed 1 μmol/min of p-nitrophenol phosphate at pH 10.4 and 37°C.

The α-amylase and glucoamylase were found to have no phosphatase activity according to the following methods. D-Glucose 6-phosphate (0.1 g) was mixed with 0.01M acetate buffer (pH 4.5, 6 ml) and glucoamylase (0.1 ml). After 1 hr at 40°C, the mixture was adjusted to pH 8, and its ^{31}P-NMR spectrum was examined. No increase was observed in the intensity of the signal for orthophosphate. It was estimated that 5% hydrolysis of D-glucose 6-phosphate would increase the orthophosphate concentration by 3 mM so that the orthophosphate would be readily detectable. α-Amylase (1 mg) was added to a solution of D-glucose 6-phosphate in water at pH 6-7. After digestion for 1 hr at 70°C, the mixture was adjusted to pH 8.0, and its ^{31}P-NMR spectrum was examined. The spectrum showed no increase in the intensity of the orthophosphate signal. The commercial glucoamylase solution was found to contain a trace of inorganic phosphate.

General Methods

Total carbohydrate was measured by the phenol-sulfuric acid method (Dubois et al 1956) and reducing end groups by the modified Park-Johnson method (Hizukuri et al 1981), using D-glucose as the reference standard. Phosphorus in starch and dextrins was measured by the method of Smith and Caruso (1964).

NMR spectra were recorded on a Bruker WM400 NMR spectrometer (Bruker Instruments, Inc., Billerica, MA). ^{31}P-NMR spectra were measured at 162 MHz on aqueous solutions at pH 8.0 ±0.1; the solutions contained $0.02M$ ethylenediaminetetraacetate to sharpen the signals. Chemical shifts are reported in parts per million from orthophosphate (sodium salt) as internal reference standard. ^{13}C and ^{1}H-NMR spectra were recorded at 100.6 and 400 MHz, respectively, in $CDCl_3$ or D_2O solution, and chemical shifts (δ) are reported (in ppm) from tetramethylsilane (δ 0.0 ppm). Methanol (49.6 ppm) was used as the internal reference for ^{13}C-NMR spectra.

Phosphorylation of Amylose and Wheat Starch

Wheat starch was phosphorylated at an initial pH of 6 using 5% sodium tripolyphosphate (STPP) in the presence of 5% sodium sulfate as described by Lim and Seib (1993). The P content of the modified wheat starch (0.28%) was corrected for endogenous P in its associated lipids (0.05%). The net 0.23% phosphorus was equivalent to DS 0.012.

Potato amylose was phosphorylated as follows. Amylose (5 g) was dissolved in $0.3M$ sodium hydroxide (200 ml), and STPP (0.5 g) was added, followed by $2M$ hydrochloric acid to bring the mixture to pH 7. Ethanol (1,200 ml) was added slowly with stirring, and the mixture was kept at 4°C overnight. The precipitate was collected by centrifugation and vacuum-dried at room temperature over calcium sulfate until its moisture level was below 10%. The amylose-STPP mixture was heated at 130°C for 3 hr; then the phosphorylated amylose was separated from contaminating salts by dialysis. The retained material was isolated by precipitation with ethanol and dried in a vacuum desiccator. The yield of amylose phosphate, which contained 0.30% P (DS 0.016), was 3.1 g.

D-Glucose 2- and 3-Phosphates and Methyl α-D-Glucopyranoside 2- and 6-Phosphates

D-Glucose 2- and 3-phosphates were prepared according to Farrar (1949) and Brown et al (1957), respectively. The esters were converted to their cyclohexylammonium salts using a strongly acidic cation-exchange resin in the cyclohexylammonium form. The cyclohexylammonium salt of α-D-glucopyranose 3-phosphate (mp 131°C) crystallized from ethanol, whereas the 2-phosphate remained a syrup.

Methyl 4,6-O-benzylidene-α-D-glucopyranoside (1 g, mp 168–170°C) (Fletcher 1963) was reacted at 0°C with diphenyl phosphorochloridate (1.2 equivalents) in dry pyridine (2 ml) (Ballou and MacDonald 1963). After 2 hr, water (0.05 ml) was added, and the mixture was evaporated to a syrup. The syrup was dissolved in chloroform (50 ml), and the organic layer was washed repeatedly with water. Vacuum evaporation of the chloroform solution gave syrupy diphenylphosphate ester, from which the last traces of pyridine were removed by high vacuum at 25°C. The phenyl and 4,6-acetal blocking groups were removed by hydrogenation over platinum oxide (0.05 g) at 1 atm in methanolic solution (50 ml). After removal of the catalyst by filtration, the solution was evaporated to a syrup that was dissolved in water (50 ml). The solution was adjusted to pH 8.0 with $0.1M$ NaOH, and an aliquot (10 ml) of the solution was applied to a column (10 × 200 mm) of strongly basic anion-exchange resin (AG1-X8, Bio-Rad Laboratories) in the bicarbonate form. The column was washed with water (500 ml) and developed with $0.4M$ ammonium bicarbonate. Fractions (5 ml each) were collected and assayed for carbohydrate, and the desired fractions were combined. Ammonium bicarbonate was removed by repeated addition of water and evaporation to a syrup. The residual MG 2-phosphate ammonium salt, which contained a trace of the 3-phosphate ester, was identified by [13]C- and [31]P-

NMR.

MG (1.0 g) was phosphorylated using the same procedure described above. Anion-exchange purification gave an amorphous solid containing predominately MG 6-phosphate mixed with low levels of the 3-phosphate ester and a trace of the 2-phosphate, as shown by ^{31}P- and ^{13}C-NMR.

Maltose 6-Phosphate

Phenyl 4-0-(4',6'-O-benzylidene-α-D-glucopyranosyl)-β-D-glucopyranoside (PBG) was synthesized according to the procedure of Takeo et al (1974). PBG was purified on a column of silica gel using chloroform as the developing solvent. The 4',6'-acetal was crystallized from acetone-petroleum ether and had a melting point of 139–143°C. PBG (0.5 g) was reacted at 25°C with diphenyl phosphorochloridate (1.5 equivalent) in pyridine (5 ml). After reaction for 8 hr, the product was isolated in the usual manner, with care taken to remove all traces of pyridine. The syrupy product was hydrogenated to give a syrup that was purified on a strongly basic anion-exchange column, as described for MG 2-phosphate. Two components were resolved on the column upon eluting with $0.4M$ ammonium bicarbonate. The first component (30 mg) was maltose 6-phosphate, as determined by ^{13}C- and ^{31}P-NMR spectroscopy. The second component (32 mg) was cyclohexyl β-maltopyranoside 6-phosphate. Its ^{31}P-NMR spectrum showed a singlet at 1.05 ppm, and its ^{13}C-NMR spectrum showed two anomeric signals at 99.2 and 100.6 ppm, two primary carbons at 61.6 and 64.2 ppm, and five cyclohexyl signals at 33.5, 32.0, 25.6, 24.5, and 24.3 ppm. No attempts were made to isolate the ammonium salts of the two derivatives of maltose. Their yields were determined by total carbohydrate assay.

α-Limit Dextrins

Ten grams of native potato starch or phosphorylated wheat starch (DS 0.012) was dispersed in distilled water (45 ml) containing 2 mM calcium chloride (2.5 ml), and the pH was adjusted to 6.5 with $0.1M$ hydrochloric acid or sodium hydroxide. For phosphorylated amylose (DS 0.016), the polymer (5 g) was dissolved in $0.4M$ sodium hydroxide (45 ml) containing 2 mM calcium chloride (2.5 ml), and the pH was adjusted to 6.5 with $3M$ hydrochloric acid.

One milliliter of 0.2% aqueous α-amylase (2 mg) was added to the aqueous dispersion of potato starch, wheat starch phosphate, or amylose phosphate. Each mixture was heated with stirring in a boiling-water bath for 10 min and then cooled to 25°C. The pH of a mixture was readjusted to 6.5; an additional 3 ml of α-amylase (3 mg) was added; and the digest was warmed to 70°C. The total α-amylase amounted to 465 U/g of starch and 930 U/g of amylose phosphate. After 1 hr at 70°C, the reducing power of the three hydrolysates remained constant, indicating formation of α-limit dextrins.

An aliquot (25 ml) was removed from each digest, and α-amylase was inactivated by heating in a boiling-water bath for 15 min. Each mixture was centrifuged at 3,000 × g for 10 min, and any precipitate was removed. The precipitate from the digest of potato starch or wheat starch phosphate contained 3–5% of the carbohydrate and 3–4% of the P present in the α-amylase digests. The precipitate in the digest of amylose phosphate amounted to 24% of original carbohydrate; its P content was not measured. The average degree of polymerization (DP) of the α-limit dextrins in the supernatant solutions was 4.2 for potato starch, 2.0 for phosphorylated amylose, and 4.0 for phosphorylated wheat starch. Each solution was evaporated under reduced pressure to approximately 10 ml, and a portion (5 ml) of the concentrate was mixed with deuterium oxide (2 ml), ethylenediaminetetraacetate dihydrate disodium salt (52 mg), 0.2% sodium azide (70 μl), and $1M$ sodium phosphate buffer (pH 8.0, 50 μl). Before measurement of the ^{31}P-NMR spectra, each mixture was adjusted to pH 8.0 ± 0.1 (measured with a pH meter) by addition of $0.5M$ sodium hydroxide.

α,γ-Limit Phosphodextrin

The remaining solution (~25 ml) from an α-limit hydrolysate,

which contained approximately 5 g of carbohydrate from potato starch or wheat starch phosphate and 2.5 g from amylose phosphate, was adjusted to pH 4.5 by addition of $1M$ acetic acid. Sodium acetate buffer ($0.01M$, pH 4.5, 25 ml) was added, followed by glucoamylase (1 ml, 6,100 U), and digestion was done at 40°C for 1 hr, after which the average DP remained constant at 1.5–2.0. Each hydrolysate was adjusted to pH 7 using $0.5M$ sodium hydroxide, and the mixture was heated in a boiling-water bath for 15 min. The precipitated solids were removed by centrifugation ($3,000 \times g$ for 10 min) and assayed for P. The precipitate from potato starch or wheat starch phosphate contained less than 5% of the initial carbohydrate and P, while that from amylose phosphate amounted to 27% of the starting carbohydrate. P content in the precipitate from amylose phosphate was not measured.

After the supernatant was adjusted to pH 7.5 with $0.5M$ sodium hydroxide, the mixture was added to a column (10×200 mm) of strongly basic anion exchange resin (BioRad AG1-X8) in the bicarbonate form. The column was washed with water (500 ml) to remove glucose, and the phosphorylated maltooligosaccharides (α,γ-phosphodextrins) were eluted using $0.4M$ ammonium bicarbonate. The fractions (5 ml each) testing positive for carbohydrate and P were combined, and ammonium bicarbonate was removed by four successive additions of water followed by evaporation at reduced pressure. The yields of α,γ-phosphodextrins were approximately 50, 210, and 65 mg from 5 g of potato starch, 5 g of phosphorylated wheat starch, and 1.8 g of phosphorylated amylose, respectively. Phosphorus recovered in the α,γ-phosphodextrins was approximately 90% of the initial P in potato starch or wheat starch phosphate. The average DPs of the α,γ-phosphodextrins were 4.4 from native potato starch, 4.5 from wheat starch phosphate, and 4.9 from amylose phosphate. ^{31}P-NMR measurements were made on the phosphodextrins according to the procedure described for the α-limit dextrins.

One half of the solution ($\sim$7 ml) that had been used to record the ^{31}P spectrum of the α,γ-phosphodextrin from potato starch was evaporated under vacuum to a small volume. The concentrate was dissolved in D_2O (5 ml), and the solvent exchange was repeated. The residue was dissolved in D_2O (2 ml), and its ^{1}H-NMR spectrum was recorded. The solution was then adjusted to pH 10.4 with $1M$ NaOD, and alkaline phosphatase (30 mg) was added. The digest was held overnight at 25°C; its pH was adjusted to 7 with DCl; and the ^{1}H-NMR spectrum was recorded.

RESULTS AND DISCUSSION

Phosphorylated Model Compounds

Phosphate esters of MG served as model compounds for phosphorylated nonreducing ends of α-1,4-linked AGUs in starch, whereas maltose 6-phosphate represented the reducing ends or inner AGUs. Phosphorylated limit dextrins from potato starch and potato amylopectin also served as model substances. The structures of two model substances are shown in Figure 1.

Synthesis of Model Compounds

Glucose 2- and 3-phosphates were prepared by well-established methods, whereas the 2-, 3-, and 6-phosphate esters of MG were prepared here for the first time. Methyl 4,6-O-benzylidene-α-D-glucopyranoside was reacted with diphenyl phosphorochloridate in pyridine, and the intermediate product was catalytically reduced
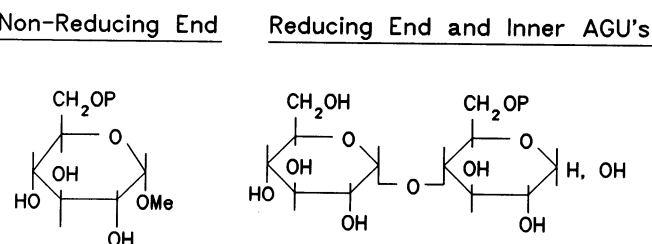
Fig. 1. Model compounds of phosphorylated starch.

Non-Reducing End   Reducing End and Inner AGU's

TABLE I
^{13}C-Chemical Shifts of Methyl α-D-Glucopyranoside (MG) and Its 2-, 3-, and 6-Phosphate Esters

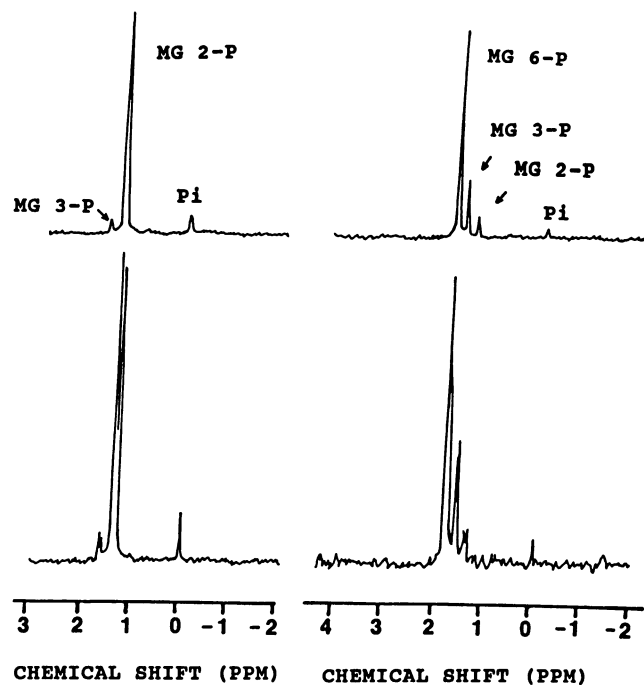| Compound	Chemical Shift (ppm)						
	CH_3	C-1	C-2	C-4	C-4	C-5	C-6
MG[a]	55.6	99.9	71.9	73.8	70.3	72.2	61.3
MG 6-P	55.7	100.0	71.8	73.2	69.5	72.0	63.0
MG 3-P	55.5	99.5	70.5	77.0	68.8	71.7	61.3
MG 2-P	55.7	98.9	74.0	73.1	70.2	71.7	60.9

[a] Assignments from Perlin et al (1970).

Fig. 2. ^{31}P spectra of methyl α-D-glucopyranoside (MG) 2-phosphate with a trace of 3-phosphate (left) and a mixture of MG 6-, 3-, and 2-phosphate (right). Top, proton-decoupled; bottom, proton-coupled. Spectra were recorded in water at pH 8.0 ± 1 with orthophosphate (Pi) an internal reference.

TABLE II
^{31}P-Nuclear Magnetic Resonance Chemical Shifts[a] (ppm) of Model Compounds Relative to Orthophosphate in Water at pH 8.0

Model[b,c]	Chemical Shift
α-Glu 2-P	1.85 (d, 6.7)
α-Glu 3-P	1.87 (ND)
α-Glu 6-P	2.03 (t)
α-Glu 1-P	1.93 (ND)
α-Mal 6-P	1.04 (ND)
β-Mal 6-P	1.10 (ND)
Cy α-Mal 6-P	1.05 (ND)
Potato amylopectin	1.32 (major)
	1.63 (minor)
β-Glu 2-P	1.54 (d, 7.8)
β-Glu 3-P	1.80 (d, 6.5)
β-Glu 6-P	2.03, (t, ND)
MG 2-P	1.38 (d, 8.3)
MG 3-P	1.68 (d, 5.8)
MG 6-P	1.87 (t, 5.4)

[a] The splitting pattern (d = doublet, t = triplet, ND = not determined) and the magnitude of a splitting (Hz) are given for the ^{1}H-coupled ^{31}P-NMR spectra.

[b] Anomeric assignments based on the β-pyranose form having the higher intensity at equilibrium.

[c] Cy = cyclohexyl, Glu = D-glucose, Mal = maltose, MG = methyl α-D-glucopyranoside.

to remove the blocking groups. Preparative ion-exchange chromatography gave MG 2-phosphate (MG 2-P, 1.38 ppm, $^3J_{POCH}$ 8.3 Hz) with traces of the 3-phosphate (MG 3-P, 1.68 ppm, $^3J_{POCH}$ 5.8 Hz) as shown by ^{31}P-NMR (Fig. 2, left) and ^{13}C-NMR spectroscopy (Table I).

Phosphorylation of unprotected MG under the same reaction and isolation conditions as used on the 4,6-acetal gave a mixture of three phosphorylated compounds. The predominant product was the 6-phosphate ester (MG 6-P), with a ^{31}P signal at 1.87 ppm (Fig. 2, right, top). In accord with that assignment, the ^{1}H-coupled ^{31}P spectrum showed a triplet with a small splitting ($^3J_{POCH}$ 5.4 Hz) of the 6-P signal (Fig. 2, right, bottom).

A trace of MG 2-P was also present in the product from direct phosphorylation of MG, as evidenced by the ^{31}P signal at 1.38 ppm (Fig. 2, right). The other phosphorus resonance (1.68 ppm) in the mixture was assigned to MG 3-P based on the ^{13}C-NMR spectrum of the mixture (Table I). All ^{31}P resonances measured in this investigation are compiled in Tables II and III. It is considered unlikely that any of the ^{31}P signals at 1–2 ppm in Figure 2 (right) were due to higher phosphate esters of MG, because 1.2 molar equivalents of phosphorylating agent were used.

The ^{13}C spectrum of the reaction mixture from phosphorylation of MG was consistent with the presence of mostly MG 6-P mixed with some MG 3-P, but the trace of MG 2-P was not detected. The spectrum showed two signals for primary carbons at 61.3 and 63.0 ppm (Table I), the latter of which was consistent with the presence of MG 6-P due to deshielding by 2–3 ppm. The other phosphorylated component in the mixture was the 3-phosphate according to the following arguments. The ^{13}C signal of C-3 of MG 6-P was observed at 73.2 ppm, which matched the C-3 (73.8 ppm) in MG. However, another signal was observed at 77.0 ppm, or 3.2 ppm downfield, and was consistent with MG 3-P. Furthermore, the signals of C-2 and C-4 in MG 3-P were seen 1.5 ppm upfield of those in MG.

Maltose 6-phosphate was prepared by reacting phenyl 4′,6′,O-benzylidene-β-D-maltopyranoside with 1.5 equivalents of diphenyl phosphorochloridate. Catalytic reduction of the intermediate product followed by anion-exchange chromatography separated the desired maltose 6-phosphate from a compound tentatively identified as cyclohexyl β-D-maltopyranoside 6-phosphate. Carbon-13 and ^{31}P-NMR were used to deduce those structures. The data in Table IV show that only one of the ^{13}C signals, a primary carbon, was shifted significantly (3.7 ppm downfield) in the phosphorylated maltose compared to its position in maltose. Because C-6′ was blocked during phosphorylation, the phosphorylated position was C-6 at the reducing end.

The ^{31}P spectrum of maltose 6-phosphate gave two signals (Fig. 3) because of the presence of α- and β-pyranose forms. The ^{31}P signal of α-maltose 6-phosphate was at 1.04 ppm. Thus, the chemical shift of the 6-phosphate residue on the reducing end of α-maltose was to a position approximately 0.8 ppm upfield from the signal of the 6-phosphate on MG 6-P (Fig. 1, Table II).

On the other hand, the ^{31}P signals of D-glucose 2-, 3-, and 6-phosphates were shifted only slightly downfield (~0.1 ppm) from the corresponding signals of MG 2-P, MG 3-P, and MG 6-P (Table II). In other words, α-glucosylation at the O-4 position of D-glucose 6-P caused a substantial upfield shift of its ^{31}P signal, whereas methylation at the α-anomeric hydroxyl caused only a slight upfield shift. These results suggest that α-maltose 6-phosphate serves as a model compound for all 6-phosphorylated α-1,4-linked AGUs in starch, except those on the nonreducing end.

^{31}P- and ^{1}H-NMR Spectra of Phosphodextrins from Potato Starch

The mixture of α-limit phosphodextrins and oligosaccharides from potato starch gave three ^{31}P signals (Fig. 4, top). The signal at 1.54 ppm was from a phosphate ester on a secondary hydroxyl because it gave a doublet in its ^{1}H-coupled spectrum (not shown), whereas the signals at 0.98 and 1.09 ppm were from phosphate esters on primary hydroxyls because they showed narrow triplets in the ^{1}H-coupled spectrum. Potato starch is known to be phosphorylated at the 3- and 6- positions of α-1,4-linked AGUs (Tabata and Hizukuri 1971). Moreover, those workers found no phosphate esters on either end of its α-limit phosphodextrins. For those reasons, we assigned the signal at 1.54 ppm to 3-P on inner AGUs and the two signals at 0.98 and 1.09 ppm to 6-P esters on inner AGUs. Those assignments were supported

TABLE III
^{31}P-Nuclear Magnetic Resonance Chemical Shifts[a] (ppm) of Limit Phosphodextrins Relative to Orthophosphate in Water at pH 8.0

Source	Position of Phosphorylation				
	Inner C-2	Inner C-6		Inner C-3	Nonreducing C-6
Potato starch					
α-Limit	...	0.98	1.09	1.54	...
α,γ-Limit	...	...	1.12 (t, ND)	1.70 (d, 6.8)	1.95 (t, ND)
Amylose phosphate					
α-Limit	0.81	1.03	1.15	1.62	...
α,γ-Limit	0.79 (d, 7.3)	...	1.12 (t, ND)	1.65 (d, 6.9)	1.95 (t, 5.3)
Wheat starch phosphate					
α-Limit	0.87	1.06	1.12	1.71	1.98
α,γ-Limit	0.72 (d, 6.2)	...	1.01 (t, ND)	1.64 (d, 5.8)	1.88 (t, 5.1)

[a] The splitting pattern (d = doublet, t = triplet, ND = not determined) and the magnitude of a splitting (Hz) are given for the ^{1}H-coupled ^{31}P-NMR spectra.

TABLE IV
^{13}C-Chemical Shifts (ppm) of Maltose and Maltose 6-Phosphate at pH 8.0

Compound	Anomer	Nonreducing End						Reducing End					
		C-1′	C-2′	C-3′	C-4′	C-5′	C-6′	C-1	C-2	C-3	C-4	C-5	C-6
Maltose, literature[a] values	α	101.1	73.2	74.3	70.8	74.0	62.0	93.2	72.7	74.5	78.9	71.4	62.0
	β	101.1	73.1	74.3	70.8	74.0	62.0	97.2	75.4	77.6	78.6	76.0	62.2
Maltose, this work	α	99.8	71.9	73.0	69.5	72.8	60.6	92.0	71.4	73.3	77.1	70.1	60.7
	β	99.8	71.8	73.0	69.5	72.8	60.6	95.9	74.1	76.3	76.9	74.7	60.8
Maltose 6-phosphate[b]	α	99.3	72.5	73.3	69.8	72.4	61.6	92.6	70.6	74.0	77.0	70.6	64.4
	β	99.4	72.0	73.5	69.8	72.4	61.6	96.5	74.3	75.9	77.0	74.8	64.4

[a] From Kahn (1981).

[b] Assignments were deduced from those of maltose. The ^{13}C-spectrum of maltose 6-phosphate gave a total of 18 resolved signals.

by the [1]H-decoupled ^{31}P spectrum of potato amylopectin (not given). That spectrum gave a minor signal at 1.63 ppm (width at half height = 13 Hz), which was assigned to 3-P on inner AGUs, and a major signal at 1.32 (width at half height = 13 Hz), which was due to the 6-P on inner AGUs.

Our assignments of the ^{31}P signals in the α-limit dextrin of potato starch were also supported by the decoupled ^{31}P spectrum of its α,γ-limit phosphodextrin, which contained 90% of the P in potato starch (Fig. 4, bottom). It is known that *A. niger* glucoamylase can trim glucosyl residues from the nonreducing end of linear phosphodextrins until a 6-P AGU is reached or until an AGU adjacent to a 3-P AGU is reached (Abe et al 1982, Takeda et al 1983). That action of glucoamylase explains the appearance of the ^{31}P signal at 1.95 ppm for 6-P on the nonreducing end of the α,γ-phosphodextrin (Fig. 4, bottom, and Fig. 5) with the simultaneous disappearance of the signal at 0.98 ppm. All the while, the signal at 1.70 ppm for the 3-P on the α,γ-phosphodextrin moved slightly from its position (1.54 ppm) in the α-limit phosphodextrin. Thus, the 3-P groups remained on inner AGUs in the α,γ-phosphodextrin. In addition, the ^{31}P chemical shifts of the phosphodextrins moved downfield by ~0.16 ppm upon removal of the free sugars from a digest.

Integration of the ^{31}P signals in the spectra shown in Figure 4 top and bottom gave approximately 80% 6-P and 20% 3-P on potato starch compared to the 66 and 33% previously found (Tabata and Hizukuri 1971). The level of 6-phosphorylation varies in potato starch (Muhrbeck and Tellier 1991).

The ^{1}H-NMR spectrum of the α,γ-phosphodextrins from potato starch was also measured; the anomeric region of the spectrum is shown in Figure 6. At least six anomeric protons were resolved. The signals at 4.70 and 5.27 ppm were due to the β- and α-anomeric pyranoses, respectively, at the reducing end (Gidley 1985). Only a trace of signal was seen at 5.0 ppm, indicating negligible levels of α-1,6 branch points in the phosphodextrins, which concurred with the findings of Tabata et al (1978). The two signals at high field (5.57 and 5.73 ppm) were due to anomeric protons on phosphorylated AGUs, since those signals disappeared upon treatment with alkaline phosphatase (spectrum not shown). Some of the signals between 5.33 and 5.47 ppm, which are due to H-1 signals on inner AGUs, may be due to phosphorylated AGUs, since that complex of signals, after treatment with phosphatase, narrowed (0.10 vs. 0.14 ppm) and lost intensity (25%) relative to the reducing-end signals.

Unfortunately, the resolution of our spectrum of the dephosphorylated sample was not sufficient to determine which peaks disappeared between 5.33 and 5.47 ppm in Figure 6 after treatment with phosphatase. We conclude that ^{1}H-NMR of the anomeric region of phosphodextrins provides an alternate approach to ^{31}P-NMR data for determining the position of phosphorylation, especially if a 600-MHz instrument is available.

^{31}P-NMR of Phosphodextrins from Amylose Phosphate

The α,γ-limit phosphodextrin of amylose phosphate was also prepared. Potato amylose, which is known to be void of naturally occurring phosphate esters (Abe et al 1982) was phosphorylated in the semidry state by reaction with sodium tripolyphosphate. The purified amylose phosphate with 1P/66 AGU was converted to its α,γ-phosphodextrin, which after purification from D-glucose accounted for 90% of the P in the amylose phosphate and had a DP of 4.9, with an average of 1.7 P groups per chain.
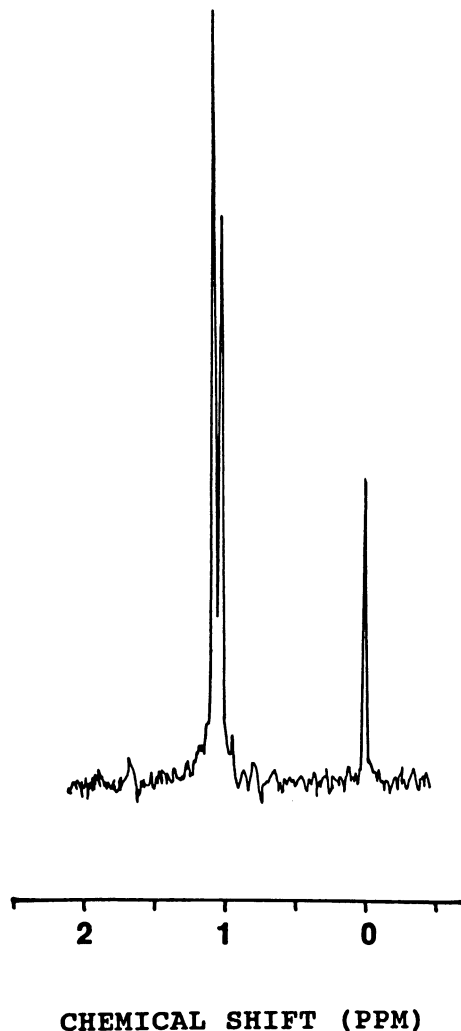

CHEMICAL SHIFT (PPM)

Fig. 3. Proton-decoupled ^{31}P-nuclear magnetic resonance spectrum of maltose 6-phosphate in water at pH 8.0.

α-limit dextrin

α,γ-limit phosphodextrin

CHEMICAL SHIFT (PPM)
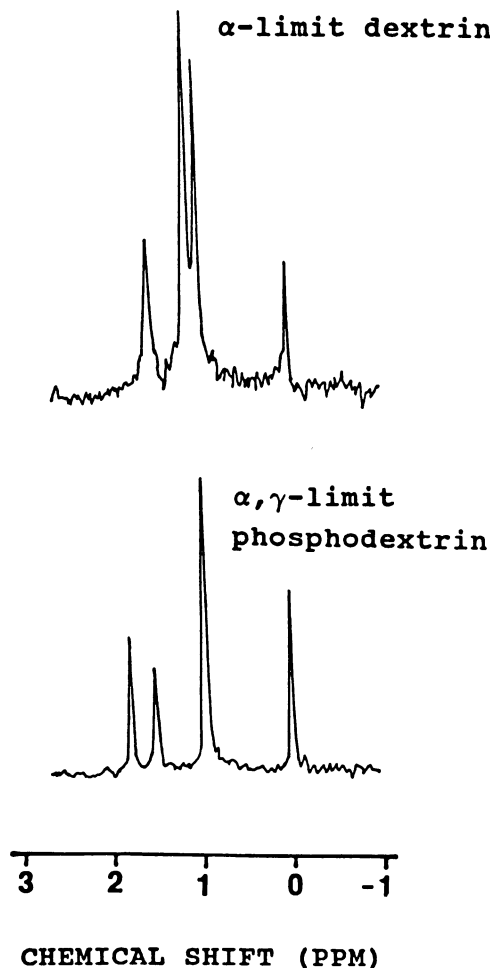
Fig. 4. Proton-decoupled ^{31}P-nuclear magnetic resonance spectra (in water, pH 8.0) of the α-limit dextrin and the α,γ-phosphodextrin from potato starch (1 P per 303 anhydroglucose units).

The ^{1}H-decoupled ^{31}P-NMR spectrum of the α,γ-phospho-dextrin from amylose phosphate is shown in Figure 7 (bottom), and the resonances are listed in Table III. The signal at 1.95 ppm was assigned to 6-P on the nonreducing end because of its similarity with the signal of MG 6-P (1.87 ppm) and the signal of 6-P (1.95 ppm) on the α,γ-phosphodextrin of potato starch. The signals at 1.65 and 1.12 ppm were assigned to 3-P and 6-P, respectively, on inner AGUs, based on the ^{31}P signals observed for the limit phosphodextrins of potato starch and for maltose

6-P. In agreement with those assignments, the ^{1}H-coupled ^{31}P-NMR spectrum of the α,γ-phosphodextrin of the amylose phosphate in Figure 8 showed a narrow triplet, a doublet, and a narrow triplet for the signals at 1.95, 1.65, and 1.12 ppm, respectively.

There remains one unidentified signal at 0.79 ppm in Figure 7 (bottom), which is a phosphate on a secondary hydroxyl (Fig. 8). That signal was not from a diphosphate or a triphosphate ester because no signal was observed at -9 or -24 ppm for β- or γ-phosphorus atoms, respectively, in a polyphosphate chain (Barany and Glonek 1982). We also discounted the possibility of a 1-P at the reducing end of the phosphodextrin. Formation of amylose 1-P is statistically improbable. Moreover, the signal centered at 0.79 ppm in the [1]H-coupled spectrum (Fig. 8) was a reasonably sharp doublet. Phosphorylation at the 1-OH of amylose would give α- and β- anomers, and the coupled ^{31}P signal of amylose 1-P would then be a multiplet.

The unknown signal may be from inner 3-P groups contiguous to other phosphorylated AGUs, or it may be from inner 2-P groups. We favor the latter. Electrophilic substitution on starch or amylose often gives reaction at the 2- and 6-OHs (Sugihara 1953). In addition, ^{31}P resonances of all the 2-phosphorylated
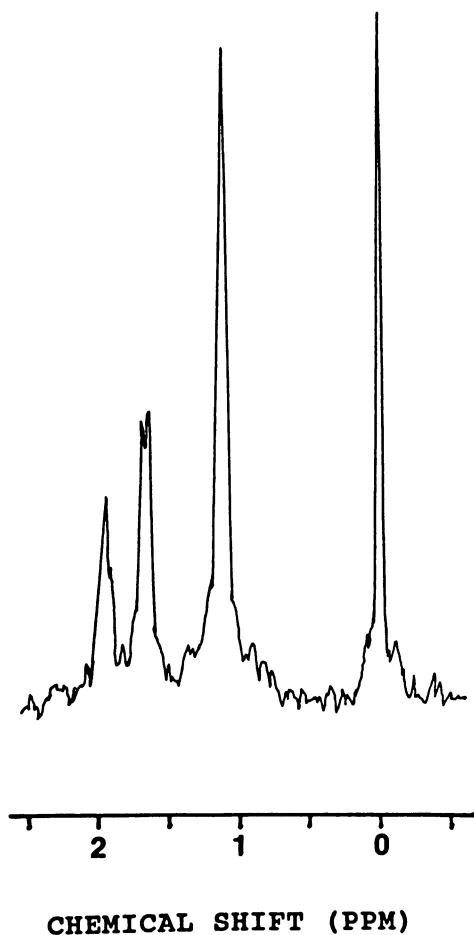

CHEMICAL SHIFT (PPM)

Fig. 5. Proton-coupled ^{31}P-nuclear magnetic resonance spectrum (in water, pH 8.0) of α,γ-phosphodextrin from potato starch.

CHEMICAL SHIFT (PPM)
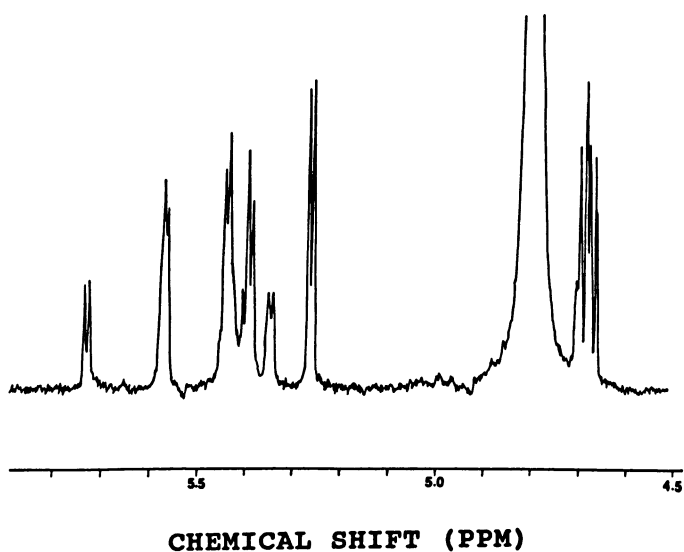
Fig. 6. Anomeric region of the [1]H-nuclear magnetic resonance spectrum (in D$_2$O, pH 7) of the α,γ-phosphodextrin from potato starch.
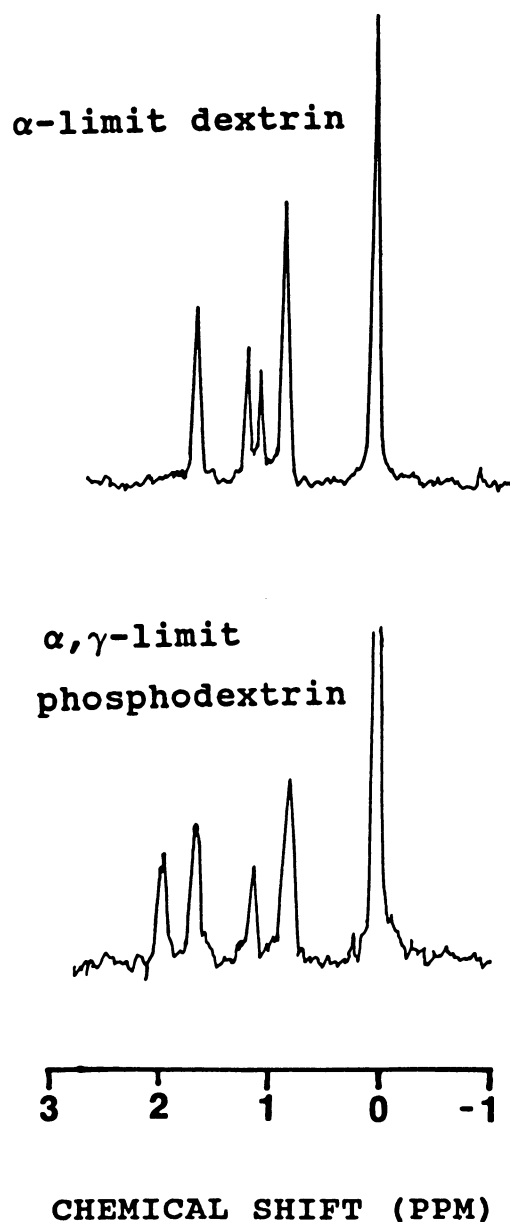

CHEMICAL SHIFT (PPM)

Fig. 7. Proton-decoupled spectra (in water, pH 8) of the α-limit dextrin and the α,γ-limit phosphodextrin from amylose phosphate (1 P per 66 anhydroglucose units).

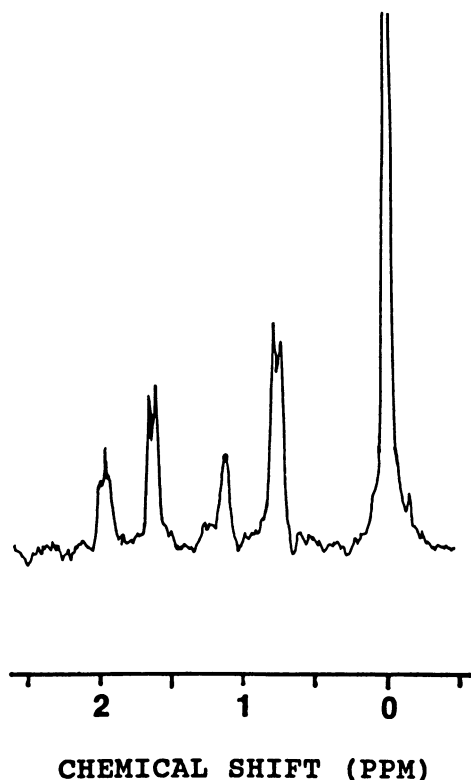CHEMICAL SHIFT (PPM)

CHEMICAL SHIFT (PPM)

Fig. 8. Proton-coupled spectrum (in water, pH 8.0) of the α,γ-phosphodextrin from amylose phosphate (1 P per 66 anhydroglucose units).
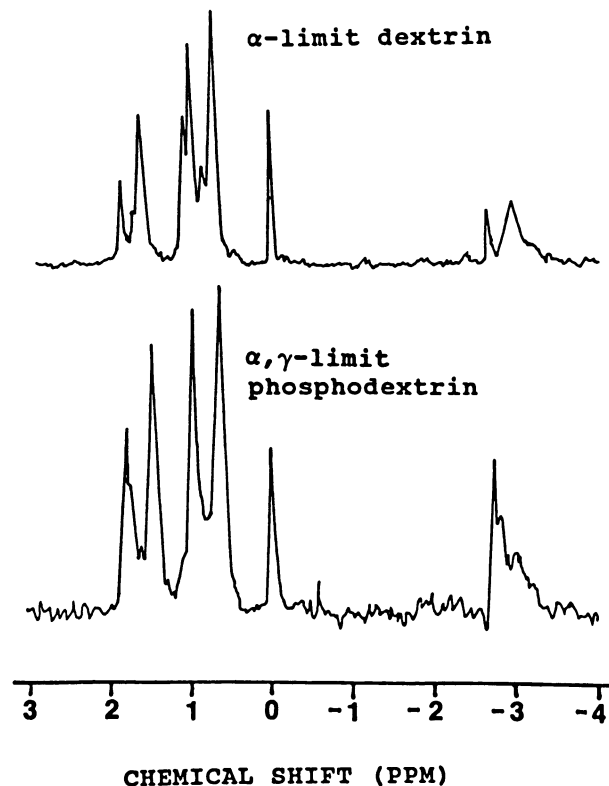
Fig. 9. Proton-decoupled spectra (in water, pH 8.0) of the α-limit dextrin and the α,γ-phosphodextrin from phosphorylated wheat starch (1 P per 77 anhydroglucose units).

model compounds occurred upfield from those of the corresponding 3-phosphorylated derivatives (Table III), which is consistent with the upfield position of the presumed 2-P signal (0.79 ppm) on inner AGUs vs. the 3-P signal (1.65 ppm). The difference between the ^{31}P signals of inner 2-P and those of inner 3-P may be magnified by H-bonding between a 3-OH and a 2-P on adjacent α-1,4-linked AGUs. St. Jacques et al (1976) found that the 3-OH in amylose, when dissolved in dimethyl sulfoxide, donates a proton to form an H-bond with the 2-OH on an adjacent AGU.

The α-limit dextrin of the amylose phosphate, which was a mixture of phosphodextrins and oligosaccharides, showed four ^{31}P signals (Table III) in its [1]H-decoupled spectrum (Fig. 7, top). The signal at 1.62 ppm was assigned to 3-P on inner AGUs and the signal at 0.81 ppm tentatively to 2-P on inner AGUs. At the same time, the signal at ~2.0 ppm for 6-P on the nonreducing end was absent, but two signals were found upfield at 1.03 and 1.15 ppm, both of which were primary phosphate esters on inner AGUs. Exhaustive glucoamylase digestion of the α-limit dextrin caused the signal at 1.03 ppm to move downfield to 1.95 ppm in the α,γ-phosphodextrin (compare top and bottom spectra in Fig. 7). Apparently the 2-P, like the 3-P, blocks the action of glucoamylase on an unmodified AGU at the nonreducing end if it is contiguous to a 2- or 3-phosphorylated AGU. Thus, the ^{31}P signal for the inner 2-P in the α-limit dextrin did not shift its position in the spectrum of the α,γ-limit dextrin (Fig. 7).

Wheat Starch Phosphate

Wheat starch has been shown to contain no covalently bonded phosphate groups (Tabata et al 1975), so the positions of phosphorylation were due to chemical synthesis. The ^{31}P-NMR spectrum of the α,γ-limit phosphodextrin from wheat starch phosphate (Fig. 9, bottom) showed four major peaks that could be assigned to nonreducing 6-P (1.88 ppm, triplet, 5.1 Hz), inner 3-P (1.64 ppm, doublet, 5.8 HZ), inner 6-P (1.01 ppm, triplet), and tentatively to inner 2-P (0.72 ppm, doublet, 6.2 Hz). Once again the splitting in the [1]H-coupled ^{31}P spectrum supported those assignments (spectrum not shown). The spectrum of the α-limit

dextrin (Fig. 9, top) gave a peak for 6-P on the nonreducing end, a structure that cannot be formed by α-amylolysis of an inner 6-P along a unit chain (Takeda et al 1983, Takeda and Hizukuri 1986, Robyt 1989). Instead, the 6-P arose from phosphorylation of nonreducing AGUs in wheat starch, of which 99% occur on the amylopectin molecules. The nonreducing ends would be accessible on one end of the clusters of unit chains in amylopectin. Phosphorylation near branch points in the amorphous region of the amylopectin fraction of wheat starch may account for the extra peaks in Figure 9 (top). The phosphodiester ^{31}P signal at −2.7 to −3.1 ppm is due to the presence of lysophospholipids in the enzymolysis reaction mixtures.

Our results indicate that phosphorylation occurred on all three hydroxyls of AGUs in wheat starch when it was heated with sodium tripolyphosphate. From the relative intensities of the ^{31}P signals measured on the α,γ-limit dextrin (Fig. 9), the 6-OH was the most reactive (sum of signals at 1.88 and 1.01 ppm), whereas the 2- and 3-OHs showed approximately equal reactivity (compare intensities at 0.72 and 1.64 ppm, respectively, in Fig. 9). Based on previous work (Sugihara 1953, Gramera et al 1966), we expected more phosphorylation at the 2-OH than at the 3-OH. However, phosphorylation of starch with STPP was done in the semidry state at high temperature, and the reaction mixture became acidic, which may have increased reactivity at the 3-OH.

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Assessment of Extrusion-Induced Starch Fragmentation by Gel-Permeation Chromatography and Methylation Analysis

PANAYOTIS RODIS,[1,2] LU-FANG WEN,[1,3] and BRUCE P. WASSERMAN[1,4]

ABSTRACT

Three commercial corn starches (high-amylose, amylopectin-enriched, and corn meal) were extruded under various conditions and analyzed for changes in solubility, molecular size distribution, and linkage content. Each of the extruded starches dissolved in dimethyl sulfoxide at a faster rate than unprocessed material. Gel-permeation chromatography confirmed the occurrence of starch fragmentation. Methylation analysis showed only small changes in linkage distributions, suggesting that under the conditions tested, the total number of fragmentation points is small relative to the the total number of glycosidic linkages in starch. Furthermore, the absence of glucose or maltodextrins indicates that fragmentation sites are largely internal, rather than adjacent to the reducing or nonreducing termini of the amylopectin molecule.

Many physical and chemical changes are known to take place during the extrusion of starch-based materials. One of the most significant and well-documented of these changes is the fragmentation of starch. Starch fragmentation has been observed indirectly, as evidenced by parameters such as decreases in its intrinsic viscosity (Colonna and Mercier 1983, Gomez and Aguilera

1983, Colonna et al 1984, Davidson et al 1984, Diosady et al 1985), and directly, by gel-permeation chromatography (Colonna and Mercier 1983, Colonna et al 1984, Davidson et al 1984, Chinnaswamy and Hanna 1990, Jackson et al 1990, Wen et al 1990, Wasserman and Timpa 1991). Gel-permeation studies uniformly show that extrusion induces the transformation of native (unextruded) starches into low molecular weight fragments. The extent of fragmentation is a function of extrusion conditions and, possibly, of the starch source. Although covalent reassociation of the fragments when starch was subjected to temperatures ranging from 125-213°C has been suggested (Greenwood 1967, Srivastava et al 1970), it has not been confirmed by recent studies (Wen et al 1990, Wasserman and Timpa 1991). Heating starch and other carbohydrates may result in the formation of anhydro linkages (Thompson and Wolfrom 1958, Greenwood 1967, Theander and Westerlund 1988).

[1]Department of Food Science, New Jersey Agricultural Experiment Station, Cook College, Rutgers University, New Brunswick.
[2]Present address: Agricultural University of Athens, Department of Food Science and Technology, Votanikos, Athens, 11855, Greece.
[3]Present address: Procter and Gamble, Sharon Woods Technical Center, Cincinnati, OH.
[4]Author to whom correspondence should be addressed.