SOIL POLYSACCHARIDES SYNTHESIZED
BY MICROORGANISMS IN SOIL

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The undersigned, appointed by the Dean of the Graduate Faculty, have
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SOIL POLYSACCHARIDES SYNTHESIZED
BY MICROORGANISMS IN SOIL

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a candidate for the degree of Master of Science

and hereby certify that in their opinion it is worthy of acceptance.

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CHAPTER 1

INTRODUCTION

The carbohydrate fraction of soil organic matter consists mainly of a mixture of heteropolysaccharides of high molecular weight. The major monosaccharide components are several sugars, uronic acids, amino sugars, and amino acids (10, 42, 50, 71, 72, 74).

Since mannose, rhamnose, and hexosamine are not normally found in higher plants, their presence indicates that soil polysaccharides are largely microbially derived (28, 42, 48, 61, 77). The persistence of polysaccharide in the soil is due either to its resistant nature or to some combination with other soil constituents rendering it resistant to decomposition.

It is well known that microbially produced soil polysaccharides are highly effective in stabilizing soil aggregates (14, 15, 24, 30, 35, 45, 49, 64, 70). Microbial cells and metabolic products affect soil structure by binding inorganic soil particles into water-stable aggregates. Chester et al. (14) and Rennie et al. (64) found a significant correlation between the proportion of water-stable aggregates and the polysaccharide content. Mehta et al. (51) concluded from their results that polysaccharide is not the only agent responsible for natural aggregation; other active agents such as humic acids or proteins may

contribute a considerable effect.

A comparison of soil polysaccharide fractions isolated from an old pasture soil with those from a cropped soil was made by Swincer et al. (72). The most significant result was that a considerably larger amount of high molecular weight material was found in the pasture soil. Furthermore, a high proportion of deoxyhexoses and a low proportion of galactose and ribose, in the high molecular weight material suggested that it was predominantly an exocellular microbial polysaccharide.

In order to gain information about the breakdown and synthesis of soil polysaccharides by microorganisms, an incubation of soil with ^{14}C dextran and ^{14}C glucose was conducted in the laboratory. The study involved the application of procedures for the extraction, purification, fractionation and characterization of sugar components of the soil polysaccharides after incubation of the soil with ^{14}C labeled substrates. By means of gel filtration soil polysaccharides with different molecular weights were obtained. By this means it was proposed to examine the distribution of the ^{14}C among different molecular weight fractions of polysaccharides at different times of incubation up to 28 days.

CHAPTER 2

LITERATURE REVIEW

A. THE OCCURENCE OF CARBOHYDRATE IN SOIL ORGANIC MATTER

Soil humus is an accumulation of the more resistant products of decomposition of original plant, animal and microbial tissue. Carbohydrates may be of considerable significance as constituents of humus. Total carbohydrate has been estimated to constitute between 5 and 20% of soil organic matter (33, 50, 61). Soil carbohydrates are derived chiefly from plant residues and from the remains of microorganisms and animals. The organic fraction of the soil is a complex of substances whose composition is determined in part by the plant and animal residues added to the soil, and to a greater extent, by the transformation of these substrates through biological, physical, and chemical means.

Plant residues contribute carbohydrates in the form of monosaccharides, oligosaccharides, and polysaccharides. All plant carbohydrates are more or less rapidly decomposed in the soil, the stability of some of them in increasing order follows: monosaccharides, oligosaccharides, starch, pectin, xylan, and cellulose. Microorganisms contribute indirectly to soil carbohydrates. Bacteria, actinomycetes, and fungi are chiefly responsible for the decomposition of

plant and animal remains. They in turn synthesize polysaccharides and other carbohydrates in their cells, frequently as major metabolic products. The microbial polysaccharides are not resistant to decomposition, but the rate of decomposition is lower than that of some plant polysaccharides (48, 77).

The main portion of soil carbohydrates is thought to occur as polysaccharide (32, 50). Only trace amounts of monosaccharides have been detected in soils. Glucose, galactose, xylose, and rhamnose have been identified in dilute alkaline extracts of various Scottish soils (25), among which glucose was the main constituent. Gupta and Sowden (33) extracted free sugars from a Northern Canadian Podzol with cold water, and glucose, galactose, mannose, arabinose, xylose, and fucose were identified. However, glucose constituted about 41% of the total free sugars.

It has been found that the polysaccharides from different sources will give the same sugars on hydrolysis (4, 26, 77). The sugars identified were glucose, galactose, mannose, arabinose, xylose, amino sugars, and uronic acids. Ribose was also invariably present in a small amount. The presence of ribose indicates polysaccharides are synthesized by soil microorganisms and strengthens the hypothesis that the polysaccharide complex is a synthetic microbial product (23, 25, 26). Duff (20) isolated from the soil a water-soluble polysaccharide complex which gave, on

hydrolysis, the same sugars identified previously in the material obtained by Forsyth (26) and, in addition, rhamnose and at least two methylated sugars. Demethylation and examination of the products on the paper chromatograms indicated that the products were hexoses, probably galactose and glucose. Duff (21) has shown that one of the methylated sugars from soil extract was identical with an O-methylated sugar which was found to be a constituent of a polysaccharide produced by Bacillus megaterin (27). 4-O-methyl-D-galactose and 2-O-methyl-L-rhamnose have been found in the hydrolyzate of the soluble polysaccharide fraction from a well-decomposed peat (22). Four to seven different high R_f sugars have been detected from some samples studied by Gupta et al. (34). They were considered to be microbial in origin and could be similar to the methylaldoses which Duff (21) identified.

B. METHODS FOR ANALYZING SOIL POLYSACCHARIDES

In order to understand the nature, origin, and function of the polysaccharides in soil, it is very important to find suitable methods to extract, purify, and identify them.

1. Extraction methods:

There are several kinds of extractants that have been used to extract polysaccharides from soil: such as hot water (10, 20, 21), aqueous buffers (4, 26), complexing reagents (2, 3, 4, 45), dilute mineral acids (2, 3, 11, 71),

alkalis (1, 24, 26, 31, 58, 71, 72), and organic reagents (2, 3, 61). No method of extraction appears to represent the whole carbohydrate fraction of the soil organic matter. So far, extractants have been evaluated with respect to yield and polymer degradation. High yield and an undegraded polysaccharide material free from inorganic and organic impurities are most important in studying isolation of soil polysaccharides.

Some polysaccharide can be extracted from soil with warm water, but the yield is low. Polysaccharides were extracted from a Scottish soil with hot water in yield of 0.3% of total soil (20). Whistler and Kirby (77) found that 1.45% of soil organic matter as polysaccharides was extracted from Indiana soil with warm water and this amount was equivalent in yield to that for 2% NaOH. Although the water extract contained only a small amount of humic substances, heating during extraction may cause extensive degradation of polysaccharides (71). Bernier (4) extracted a series of forest soils with a phosphate buffer solution at pH 7 and, after purification, he obtained a yield of polysaccharide material less than 1%. Complexing agents, such as sodium pyrophosphate and disodium EDTA have been used. Barker et al. (2, 3) studied several extractants to isolate soil polysaccharides, among which they found that 7% EDTA gave the highest yield, but the extracted polysaccharides were inseparable by gel filtration from colored

components in the mixture. Consequently, complexing reagents are not suitable for studying the whole soil polysaccharide fraction.

It has been shown that inorganic acids are more effective for soil polysaccharide extraction than other reagents. Barker (2) estimated that the organic matter in 0.6N H_2SO_4 extract gave the highest yield of polysaccharide (29% of the total organic matter extracted) which was separated from the colored materials by gel filtration through Sephadex G-100. From the results obtained by Swincer et al. (71), hydrochloric acid was considered to be the most effective extractant in soils containing calcium carbonate and free aluminum and iron oxide. Extraction of soil polysaccharide with 90-100% formic acid containing 0.2N LiBr has been attempted from a variety of soils (61). The materials extracted with formic acid contained a relatively high proportion of carbohydrates, such as reducing sugar, uronic anhydride, and hexoamines. Not all the polysaccharides were extracted. Dimethyl formamide containing 0.4M boric acid, 0.4M oxalic acid, and 0.2M lithium chloride also has been used as extracting reagents (71, 78). These methods would not successfully separate polysaccharides from non-polysaccharide components. Degradation also may occur during the extracting process.

Several workers have reported the use of dilute NaOH to extract soil polysaccharides (1, 14, 24, 26, 31,

71, 72). Forsyth (26) made the first successful attempt to isolate polysaccharide material from the "fulvic acid" fraction of an alkali extract. Acton et al. (1) showed that peptizing soil with dilute alkali prior to acid hydrolysis substantially increased the carbohydrates measured with anthrone. Swincer et al. (71) also concluded from his results that maximum extraction of polysaccharide had been obtained by shaking soil with 0.5N NaOH for 16 hr at 20C. Although hot 0.5N NaOH gives a higher yield, considerable degradation of polysaccharides occurs. It has been shown that alkali always extracts a large amount of humic materials which may influence the subsequent determination of polysaccharides (72, 73). In 1968, Swincer et al. (71) studied a sequential extraction treatment and found that about 80% of the carbohydrate was isolated from soils by using 1N HCl followed by 0.5N NaOH, and then acetic anhydride containing 2.5% concentrated H_2SO_4 at 60C for 2 hr.

2. Purification of extracted polysaccharides:

Duff (20), Whistler and Kirby (77) and Forsyth (26) have suggested the presence of non-carbohydrate substances in soil polysaccharide extracts. The non-carbohydrate materials included humic materials and protein (50). Neutralization of acid extracts has been used by some workers as an initial purification treatment to precipitate humic materials (2, 3, 6, 61, 71). Non-carbohydrate materials in

acid extracts could be precipitated with 40% NaOH (6) and NaHCO_3 (2, 3). The precipitated polysaccharides could be recovered by adding the respective acids before the application of further purification treatments. Acidification has been used as a method to precipitate humic acid in alkaline extracts of polysaccharide (1, 44, 71). Acton et al. (1) indicated that about 12% of the total carbohydrate was coprecipitated with humic acid by direct addition of acid to the NaOH extract. Later Swincer et al. (71) reported that only 1% of the polysaccharide was precipitated with the humic acid fraction by passing through the hydrogen form of Dowex 50.

Dialysis, decolorization, deproteinization and precipitation of the polysaccharides with ethanol or acetone are general procedures applied to soil polysaccharide extracts after the primary purification treatment. Dialysis against distilled water has been used by many workers (2, 3, 4, 20, 74, 77) as a method to remove salts, solvents, and some other low molecular weight materials. This method can be applied in all kinds of extractions. However, losses of carbohydrates during dialysis against distilled water have been shown to be considerable (71, 77). Swincer et al. (71) showed that about 40% of the carbohydrate in the fulvic acid fraction was lost during dialysis.

Animal charcoal was used by Forsyth (24) to adsorb all the color and most of the organic matter in fulvic

acid solutions. It has been shown that recoveries of polysaccharides from charcoal are poor (71, 77). Cross-linked polyvinylpyrrolidone was very useful for separating colored materials in fulvic acid fraction from the polysaccharide at low pH (71), and the recovery of carbohydrate was greater than 95%. Barker et al. (2, 3) introduced the use of gel filtration to separate the colored components from the high molecular weight polysaccharide materials on a Sephadex G-100 column. But only acidic extracts yield good separation. Swincer et al. (71) used Sephadex G-25 to separate low molecular weight solutes from the high molecular weight polysaccharide. And considerable amounts of the colored materials were found to be retarded by the Sephadex at low pH and the separation of these materials from polysaccharides was achieved.

Precipitation of polysaccharide with excess ethanol or acetone is another method to recover soil polysaccharides from aqueous solution (1, 4, 20, 24, 26). Protein materials remaining with soil polysaccharide fractions can be removed either by the Sevag method (4, 74) or by cadmium sulfate precipitation (4). However, complete removal of proteins from polysaccharide fractions is not possible.

3. Fractionation of soil polysaccharides:

Soil polysaccharides have been found to be heterogeneous in nature (4, 77). Many attempts have been made to fractionate the purified polysaccharides. The most com-

monly used methods are electrophoresis with phosphate or borate buffers (4, 52, 74, 77); fractional precipitation with ethanol and quaternary ammonium compounds (4, 61, 77); anion exchange chromatography on a cellulose column (10, 74) or DEAE Sephadex column (2, 3, 10); and gel filtration (3, 71). It has been shown that there is no significant qualitative differences in the sugar components between polysaccharide fractions (77).

Gel filtration is a new technique with great potential and great advantage due to a shorter time requirement for the fractionation of soil organic matter. Separation of the sulfuric acid extracted polysaccharides on a Sephadex G-100 column has been reported by Barker et al. (3). Three fractions were obtained. No difference in sugar composition in these fractions was indicated.

It has been shown that there is a wide range in molecular weight distribution of soil polysaccharides (58, 71, 72). This has been studied by using various grades of Sephadex and Bio-gel and several polysaccharide fractions are obtained, each with a definite molecular weight range. The composition of the extracted polysaccharides depends on the molecular size. Oades and Swincer (58) concluded that most of the uronic acids were in polymers of molecular weight 4000-100,000, neutral sugars and amino acids were largely in polymers of molecular weight 4000. The significant amounts of rhamnose and fucose in the high molecular

weight fraction suggest that it is largely an exocellular microbial polysaccharide (72).

Pospisil (63) used different grades of Sephadex to separate humus substances from chernozem and podzol soils. Three and four fractions differing in color quotient were obtained on Sephadex G-25 from chernozem soil and podzol soil, respectively. He attempted to determine humic substances with different molecular weights. However, the adsorption of humic substances on the matrix of the gel was observed and influenced the fractionation process.

4. Determining the monosaccharides present in soil polysaccharides:

The most important initial step in any structural determination of a polysaccharide is the identification of the monosaccharide components produced by acid hydrolysis. So far, no perfect conditions for the hydrolysis have been developed. In most cases, sulfuric acid (1N or 3N) has been used to hydrolyze the purified polysaccharide (1, 3, 4, 10, 20). A 2 hr pretreatment of a polysaccharide preparation using 72% H_2SO_4 at 20C followed by a 16 hr reflux in 1N H_2SO_4 has been found to release large amounts of monosaccharides (37, 56, 71) especially neutral sugars. Oades et al. (57) showed that total sugar contents obtained by reflux in 5N H_2SO_4 for 20 minutes were similar to those obtained by a 26N H_2SO_4 soak followed by a 1N H_2SO_4 reflux if the plant materials had been removed.

Colormetric methods have been employed for determining uronic acids, hexoses, and pentoses in the hydrolyzates of purified soil polysaccharides. MacLean and Delong (46) first applied the anthrone reagent to the measurement of carbohydrate material in a soil system. Brink et al. (7) concluded that a 0.2% solution of anthrone in 95% H_2SO_4 gave a relative measure of the total polysaccharide contents present in acid hydrolyzates of soil. The anthrone method is satisfactory for hexoses, methyl hexoses, and 6-deoxyhexoses; it is of limited use for pentoses and uronic acids. Orcinol in H_2SO_4 has been shown to give a matching color reaction with hexoses, methyl pentoses, and pentoses (11). However, both anthrone and orcinol reagents react with acid degradation products of sugars.

Phenol in the presence of H_2SO_4 has been used for the quantitative determination of the composition of polysaccharides and their methyl derivatives (19). Ivarson and Sowden (37) used this method to measure purified hydrolyzate of soil polysaccharide. Acton et al. (1) reported that the percentage of the total soil polysaccharides measured by the phenol- H_2SO_4 method resulted in slightly higher values than the anthrone method, because the phenol- H_2SO_4 reagent also measures pentoses, methyl pentoses and β -keto acids. Cheshire and Mundie (11) concluded that the alkaline ferricyanide method of Hoffman (36) was more suitable

for measuring reducing sugars in soil hydrolyzates than either anthrone or the orcinol- H_2SO_4 method.

Paper chromatography has been used widely as a method to separate and identify the monosaccharides in the hydrolyzate of soil polysaccharide (1, 4, 20, 26, 77). Chromatographic separation and identification of the soil polysaccharide constituents by Bernier (4) and Whistler and Kirby (77) have indicated that these substances contain primarily glucose, galactose, mannose, arabinose, rhamnose, and ribose, as well as amino sugars and methylated sugars. Jermyn and Isherwood (41) suggested that two three-component mixtures, ethyl acetate-acetic acid-water and ethyl acetate-pyridine-water, are both satisfactory for the qualitative and quantitative determination of sugars in mixtures of simple hexoses and pentoses. However, this method has not proved completely adequate for precise quantitative work. Furthermore, paper chromatography is not a suitable method for the separation of some ^{14}C labeled sugars in soil hydrolyzates (59).

During recent years gas-liquid chromatography has been developed for analyzing sugars in soil hydrolyzates (56, 57, 59, 71, 72). The gas-liquid chromatography procedure depends on the quantitative preparation of a volatile derivative of monosaccharide and complete separation of the derivatives of a monosaccharide mixture must be achieved. Reduction of monosaccharides to their alditols and then

separation of the alditol acetate derivatives have been found to offer possibilities for their quantification and resolution (65). Oades et al. (57) developed a quantitative procedure for the determination of sugars in soil hydrolyzates by gas-liquid chromatography of the derived alditol acetates using either myoinositol or quebrachitol as an internal standard.

C. USE OF ^{14}C IN SOIL ORGANIC MATTER STUDIES

The radioactive isotope, ^{14}C , has been used chiefly as a method for studying turnover of soil organic matter. Generally, there are two kinds of experiments: 1) by adding ^{14}C labeled plant materials into the soil, it could be determined whether or not there is any change in the decomposition of the native soil organic matter (5, 8, 9, 42, 66, 68); 2) carbon transformation can be identified by the addition of a suitable carbohydrate substrate to the soil (10, 12, 13, 38, 42, 47, 53, 59, 69, 76).

Microorganisms are responsible for the decomposition of plant and animal residues and are involved in the formation of soil organic matter. A very rapid initial development of microbial activity usually occurs with the incorporation of the carbohydrate substrate into soil organic matter. If the added substrates are labeled, the metabolic products will be labeled, too. The degradation of carbohydrates leads, directly and indirectly, to various

products, including CO_2 , organic acids, microbial polysaccharides, and humic substances. Carbohydrate such as glucose (10, 13, 42, 47, 59, 62, 76), starch (13), hemicellulose, cellulose, and plant material (42, 66, 67) have been used in these studies.

It has been shown that soil polysaccharide is largely of microbial origin (13, 26, 27, 28, 50). To study the biosynthesis of soil polysaccharides, labeled glucose is a most suitable energy source (10, 13, 60). This can account for its complete and rapid metabolism after incorporating it into soil. Other carbohydrates are metabolized in the same way as glucose, but more slowly.

Keeffer and Mortensen (42) added ^{14}C glucose and ^{14}C alfalfa to the soil and the decomposition rates of the materials were determined. Glucose was found to be metabolized more rapidly than alfalfa tissue. Furthermore, they suggested that the remaining ^{14}C labeled materials were probably converted into metabolic products which may have been constituents of polysaccharides. It is apparent that soil polysaccharides can be synthesized by soil microorganisms from simple carbohydrates and that the resulting polysaccharides undergo continual degradation and resynthesis.

Chahal (10) incubated ^{14}C labeled glucose with alkaline, neutral, and acid soil. The polysaccharides biosynthesized in these soils were characterized. Glucose

was metabolized more slowly in alkaline soil than in acid and neutral soil. At the end of the incubation period (45 days), more glucose carbon remained in neutral and alkaline soil samples than in acid soil sample. This may be due to different soil microorganisms responsible for decomposition of the added glucose. He also indicated that some of the remaining glucose carbon had been utilized by soil microorganisms for the biosynthesis of polysaccharides. The data showed that there were differences in the amounts of radioactivity among different "fraction" of each polysaccharide and among polysaccharide fractions from different soil samples. These differences may be attributed to the variation in microbial population. The radioactivities in the monomer sugars in different fractions from different soil samples were also given. In general, galactose, glucose, and mannose from all fractions had high amounts of radioactivity.

In 1969, Cheshire et al. (13) studied the transformation of ^{14}C glucose and ^{14}C starch in soil for different periods of time. Soil polysaccharides were extracted, hydrolyzed, and purified. They concluded that the specific activity of glucose changed ~~vgreatly~~ during incubation of both treatments. Glucose remained as the most highly labeled sugar even after 12 weeks. Other hexoses also received significant amounts of radioactivity whereas the specific activity of pentoses, arabinose and

xylose, tended to remain at their initial levels. The transformations observed in this experiment represented a synthesis of exocellular polysaccharide.

In addition, Oades and Wagner (59) incubated ^{14}C glucose in soil samples collected from a corn plot and a timothy plot on Sanborn Field. The transformation of labeled sugars was studied. They also concluded that the sugar composition of the soil was changed little by the incubation except for glucose. Uronic acids and hexoses accounted for more than 80% of the activity. But Oades and Wagner showed very little incorporation of ^{14}C into xylose and arabinose. A rapid synthesis of glucose and galactose was indicated. However, this kind of sugar transformation could be explained from the pathways of saccharide synthesis (43). Furthermore, incorporation of ^{14}C into sugars had occurred by one month and changes in the radioactivities of sugars were not significant after this period. This indicated that glucose carbon was rapidly utilized by soil microorganisms.

A general conclusion of the studies reported above is that the decomposition rate of soil polysaccharides was rather steady and slow.

CHAPTER 3

METHODS AND MATERIALS

A. DESCRIPTION OF THE EXPERIMENT

1. SOIL. The soil used in this experiment was a Mexico silt loam from the upper 6 inches of the profile from plot 6 of Sanborn Field. Corn had been grown continuously on the soil with complete fertilization since 1950. The soil had a total organic carbon content of 1.25%, and had a pH in water of 6.0. A composite sample was collected in November of 1965, air-dried, and passed through a 2 mm sieve.

2. ^{14}C GLUCOSE TREATMENT. Uniformally labeled ^{14}C glucose was obtained from Mallinkrodt Nuclear, St. Louis, Missouri. Glucose examination by GLC of its derived alditol acetate (57) showed no impurities. The labeled glucose was added to the soil along with water to maintain the soil at 80% of field capacity. For the incubation, 225 mg glucose containing 3.40 μCi ^{14}C was added to 100 g-units of soil (60).

3. ^{14}C DEXTRAN TREATMENT. Dextran is a mucilaginous polymer of glucose. It may be produced by the fermentation of sucrose with Leuconostoc mesenteroides. The ^{14}C dextran used here was obtained from the culture

of Leuconostoc mesenteroides, ATCC 10830a. The organism was cultured by Oades and Wagner (60) at 25C for 5 days in a medium containing uniformly labeled ^{14}C sucrose (Isotopes Inc., Westwood, N.J.), tryptone, phosphate, and supplemented with yeast extract and malt extract (40). At the end of the incubation, the dextran was isolated from the culture by precipitating with 95% ethanol through a continuous supercentrifuge to remove bacteria cells, and insoluble material. Then ^{14}C dextran was added to soil (i.e. 200 mg containing 4.51 μCi to 100 g-units of soil) with sufficient moisture to provide the soil with 80% of field capacity.

4. INCUBATION EXPERIMENT. Five replicates of 100 g-units of treated soil were incubated by Oades and Wagner (60) in conical flasks at 25C. During the incubation period, moist, CO_2 -free air was passed through each flask continuously at the rate of about 0.5 ml per second. Soil samples from each flask were removed individually at 0, 2, 5, 12, and 28 days of incubation. They were freeze-dried on a Virtis vacuum freeze dryer and stored in air-tight glass containers for sugar analysis.

B. DEVELOPMENT OF THE EXPERIMENTS

1. TOTAL ORGANIC CARBON.

(1) PROCEDURE. Total organic carbon of each

soil sample was determined by combusting a sample in an electric furnace with a constant flow of dry oxygen during the combustion. The carbon dioxide evolved from the soil was trapped in a scrubbing tower (69) containing 25 ml of 1N NaOH. The carbon dioxide absorbed in NaOH was then precipitated with 40% BaCl₂ as BaCO₃. The excess base was titrated with standard HCl to determine quantitatively the total CO₂ evolved from the soil.

The precipitated BaCO₃ was collected on a sintered glass funnel, washed free of salts with hot distilled water and oven dried at 105°C. Weighed amounts (15-20 mg) of BaCO₃ from each sample were placed into scintillation vials, and then suspended in 10 ml scintillation solution (2 g 2,5-diphenyl oxazole + 3 mg 1,4-bis-2-(5-phenyl-oxazolyl)-benzene + 1000 ml toluene) by using CAB-O-Sil gel (0.4 g) as a suspending material (55). Radioactivity was measured using a Packard Tri-Carb liquid scintillation counter. Standard BaCO₃ and blank samples were counted to determine efficiency and background, respectively.

(11) CALCULATION.

$$(a) \% \text{ Carbon} = \frac{(N_{\text{NaOH}} \times \text{ml}_{\text{NaOH}}) - (N_{\text{HCl}} \times \text{ml}_{\text{HCl}})}{\text{mg soil sample}} \times 6 \times 100$$

$$(b) A (\mu\text{Ci}/100 \text{ g soil}) = \frac{(N_G - N_B)}{K \times \text{mg BaCO}_3} \times \frac{M_B}{M_C} \times \frac{\%C \times 10^3}{2.2 \times 10^6}$$

Where: N_G = Gross counts per minute of BaCO_3 and background

N_B = Number of background counts per minute

K = Counting efficiency (expressed as decimal)

M_B = Molecular weight of BaCO_3

M_C = Atomic weight of carbon

2. ANALYSIS OF FREE SUGARS IN SOILS.

Free sugars are present in soils in such small quantities that it is difficult to achieve satisfactory separation.

(1) PRELIMINARY EXPERIMENT. In order to develop a suitable method to extract simple sugars (especially glucose) in soil, a preliminary experiment was conducted.

Soil preparation. A 2 g/100 ml glucose solution was prepared with distilled water. Then 5 ml (0.1 g) of this glucose solution was added to a 50 g soil sample along with 4 ml of distilled water.

Experiments. The following five extraction methods were compared by measuring the recovery of added glucose.

1. 5.9 g soil + 25 ml cold water, shaken 2 hr at 20C.

2. 5.9 g soil + 25 ml 70% ETOH, shaken 2 hr at 20C.
3. 5.9 g soil + 25 ml 70% ETOH, shaken 15 hr at 20C.
4. 5.9 g soil + 25 ml 70% ETOH, shaken 15 hr at 25C.
5. 5.9 g soil + 25 ml 70% ETOH, shaken 4 hr at 45C in a water bath.

Extracted sugars were determined quantitatively by the Anthrone method (using a glucose standard) (7). It was shown that treatment 5 gave the best recovery (97%). Therefore, this method was used in subsequent extractions.

(11) ANALYSIS OF FREE CARBOHYDRATES FROM SOILS INCUBATED WITH C-14 GLUCOSE AND C-14 DEXTRAN.

Extraction of simple sugars. Duplication of all analytical procedures was followed. Five gram samples of the incubated lyophilized soils were extracted by addition of 25 ml 70% ethanol to form slurries which were shaken for 4 hr in a 45C water bath. The soil suspensions were filtered under suction through medium porosity sintered funnels and the soil residues were washed three times with 70% ethanol. The filtrates were diluted to 50 ml in volumetric flasks with 70% ethanol. The sugars present in the filtrates were determined quantitatively by the Anthrone method.

Anthrone test (7). (a) Anthrone reagent: Two hundred mg of anthrone (Fisher Scientific Company) was dissolved in 100 ml of 95% sulfuric acid to make a 0.2%

solution. Since this reagent deteriorates with time, freshly prepared solutions were used each day. (b)

Preparation of standard solution: A 25 $\mu\text{g}/\text{cc}$ glucose solution was prepared with distilled water. Solutions containing glucose at 5, 10, 15, 20, and 25 $\mu\text{g}/\text{cc}$ were made from it. Five ml of each standard glucose solution was pipetted into a 150 mm by 24 mm test tube. Then 10 ml of anthrone reagent was rapidly poured into the glucose solutions using a graduate cylinder and the tubes were shaken immediately for a moment to mix the contents. After 15 minutes the contents were transferred to Beckman absorption tubes and the intensity of green color developed was measured on a Bausch and Lomb Spectronic 20 adjusted for maximum transmittance at 625 $\text{m}\mu$ wave length, against a water-anthrone blank. A standard plot of percent transmittance vs. concentration was prepared. Standard solutions were prepared daily. (c) Preparation of soil samples for Anthrone test: One to five ml of the soil filtrates from the 70% ethanol extractions were pipetted into 150 mm by 24 mm test tubes. To prevent any interference of ethanol in the color development using anthrone, the ethanol was removed by freeze-drying on a Virtis vacuum freeze dryer. Next, 5 ml of distilled water was added to each tube to dissolve sugars. Ten ml of anthrone reagent was added to each sugar solution and the color developed was read on the spectrophotometer as described

above. The amounts of soil sugars in ethanol extract were obtained from the standard curve.

Measurement of radioactivity. One ml of each ethanol extract was pipetted into a scintillation vial with 20 ml of scintillation solution (200 ml dioxane + 200 ml toluene + 120 ml methanol + 41.6 g naphthalene + 2.6 g 2,5-diphenyl oxazole + 0.052 g 1,4-bis-2(5-phenyloxazolyl)-benzene). The radioactivity was measured using a Packard Tri-Carb liquid scintillation counter. A counting efficiency was obtained from a glucose standard of known activity previously standardized. A background count was obtained with 70% ethanol employed as the sample.

3. ANALYSIS OF SOIL POLYSACCHARIDES.

(1) SOIL SAMPLES. The soil residues remaining after the ethanol extractions were transferred using acetone from sintered funnels to Erlenmyer flasks. Acetone was removed by freeze-drying.

(11) EXTRACTION PROCEDURE. Soil polysaccharides in the residual samples of soil were extracted with 25 ml of 1N H_2SO_4 at 20C under continuous shaking for 15 hr. After filtering through sintered funnels the filtrates were diluted to a volume of 100 ml with distilled water. The residues were again washed from the funnels with acetone, freeze-dried, and stored.

(111) PURIFICATION AND FRACTIONATION OF
POLYSACCHARIDES.

The sulfuric acid extracted polysaccharides were purified and fractionated using gel filtration with Sephadex G-25 and Bio-gel P-100. The mechanism of separation by gel filtration can be visualized as follows: Molecules larger than the pore size of the gel are sterically prevented from entering the beads of gel. Smaller molecules, however, can diffuse into the gel. During chromatography, movement of the smaller molecules is retarded upon entering the gel, and are therefore eluted after the large molecules.

Description of the techniques. (a) Preparing the column: Gel chromatography is conducted exclusively in a chromatographic column. A glass column 47 cm long x 4 cm in diameter was used. To avoid the possibility of zone remixing the dead space at the outlet of the column was reduced by filling it with glass beads. The column was also coated with a solution of a 1% dichlorodimethylsilane in benzene to avoid wall effects. This procedure was repeated, after which the column could be used for several months without further treatment.

(b) Packing the column: Using a rubber stopper, attach a wide funnel to the top of the chromatographic column. Mount the column to a support and check that it is vertical. Fill the column completely with distilled

water. Mix the swollen gel (Sphadex G-25 and Bio-gel P-100, respectively) with distilled water to form a slurry and add to the funnel (stirring constantly to prevent blockage of the funnel) to form a gel bed. Before the actual experiment is run the column must be eluted with several volumes of distilled water to stabilize the bed. The surface of the gel bed in the column was stabilized by covering it with a piece of filter paper equal to the inner diameter of the column. The column was filled with distilled water and connected to a distilled water reservoir.

(c) Column calibration: The substance used for calibration purpose should be closely related to the substance to be studied. In this case, the columns were calibrated using Blue Dextran 2000 (Pharmacia, Sweden) to determine the void volume and the volumes required for elution of high and low molecular weight materials.

Chromatography. A 50 ml aliquot of each soil polysaccharide solution extracted by 1N H_2SO_4 was placed on the top of the column of Sephadex G-25 (Pharmacia, Sweden) and eluted with distilled water. This enabled colorless polysaccharide materials of molecular weight > 4000 to be separated from low molecular weight polysaccharides, salts, and colored components. The collected fractions of colorless polysaccharide materials (molecular weight > 4000) from each sample were concentrated in vacuo at 45-50°C to

a volume less than 50 ml and then made to a volume of 50 ml with distilled water. Gel filtration of aqueous solutions of these concentrates on Bio-gel P-100 (Bio-Rad Lab.) was employed to separate polymers of molecular weight greater and less than 100,000. Thus, three molecular weight fractions were obtained by gel-filtration and will be referred to as < 4000 , $4000-100,000$, and $> 100,000$ molecular weight fractions.

(1v) QUANTITATIVE AND QUALITATIVE DETERMINATION OF MONOSACCHARIDES BY GAS-LIQUID CHROMATOGRAPHY (57).

Hydrolysis of polysaccharides. (a) The three fractions from each soil sample obtained by gel-filtration were transferred into 1000 ml round bottomed flasks and were evaporated in vacuo at 45-50C to remove water.

(b) The residues were transferred using 10 ml of 5N H_2SO_4 to 50 or 250 ml round bottomed flasks and refluxed for 20 minutes.

(c) Water was used to wash down the condenser to cool and dilute the hydrolyzates at the conclusion of the reflux period.

Purification of soil hydrolysates. (a) One ml of standard quebrachitol (i.e. 100 μg) was added to each warm hydrolyzate as an internal standard. The liquid hydrolyzates were filtered through Whatman No. 541 papers onto approximately 6 g of powdered $SrCO_3$ in 500 ml Erlenmeyer

flasks. These suspensions were warmed and the pH of the filtrates were adjusted near 7 by further addition of SrCO_3 . The neutralized hydrolyzates were allowed to stand overnight at room temperature and this insured complete precipitation of yellow-brown iron-organic complexes (57).

(b) The neutralized suspensions were filtered through Whatman No. 41 papers and the filtrates were evaporated to dryness on a rotary evaporator at 45-50C in one liter round bottomed flasks.

(c) Sugars and internal standard were extracted with 3 to 5 lots of 2 to 4 ml each of methanol and filtered through Whatman No. 41 papers to 125 by 16 mm. Kimax screw topped test tubes. The volume of methanol was reduced to about a half by evaporating under a small stream of air.

Preparation of alditol acetates. (a) About 1 to 2 mg of solid sodium borohydride was added to the mixtures of sugars in methanol. The reduction was allowed to proceed overnight.

(b) The methanol was removed by blowing a stream of air into the tubes while they were heated in a water bath at 50-60C.

(c) One to 2 ml of methanol containing 10% (v/v) glacial acetic acid was added to each tube in the water bath to remove borate. The resulting solutions were blown to dryness by an air jet. This treatment was performed a

total of five times.

(d) About 2 ml of acetic anhydride was added to each tube, the tubes were sealed tightly with screw caps lined with teflon inserts, and heated at 130C for 2 hr.

(e) The liquid acetylating agent was removed using an air stream blown into the tubes which were heated in 50-60C water bath.

(f) Alditol acetates along with brown materials were extracted by chloroform and filtered through a column (5 by 40 mm) of silica gel G to a small vial (10 by 45 mm) using chloroform as eluent.

(g) Chloroform extracts containing the purified alditol acetates were evaporated to dryness by a small stream of air then they were ready for gas-liquid chromatography analysis.

Gas-liquid chromatography of alditol acetates. (a)

Apparatus: The gas-liquid chromatograph used in this work is a dual column Micro Tek MT 220 instrument equipped with a differential flame ionization detector, linear temperature programmer and precise flow control for the carrier gas. The Micro Tek was used in conjunction with a Honeywell Electronic 15 single-pen recorder. A Model 201-B Disc Chart Integrater was used for determining peak areas. The injection port of the instrument was modified by using a rubber septum to reduce the vaporizing volume of the injected sample.

Chromatographic glass "U" columns (183.6 cm x 3.5 mm ID) were used and they were packed with 4% (w/w) ECNSS-M on 100-120 mesh Gas Chrom Q (Applied Sci. Lab.). The columns were employed at a nitrogen flow rate of 50 to 70 ml/min at 60 psi. The column was operated by programming from 140 to 190C at 2C/min. with an injection port temperature of 200C and a detector temperature between 250 and 280C.

(b) Monosaccharide analysis: After removing chloroform from the mixture of alditol acetates, the resulting acetate residues were dissolved in 20 to 30 μ l of methylene chloride and 1 to 2 μ l of this solution was injected into gas chromatography for monosaccharide analysis.

In order to determine the sugars in a soil sample qualitatively and quantitatively, the alditol acetates of standard monosaccharides (D-Glucose, D-galactose, D-mannose, D-xylose, D-arabinose, D-ribose, D-rhamnose, D-fucose) were prepared as previously described for GLC analysis. The basis of qualitative analysis in GLC is a comparison of the retention times of standard monosaccharides with those of the soil samples. Calibration with an internal standard is performed by the addition of an accurately measured weight proportion of the internal standard (quebrachitol) to a specified volume of several synthetic mixtures containing varying known amounts of the

standard monosaccharides. Therefore, the values of Equal Weight Response were established in calibration with reference to the peak area response of a unit weight of internal standard. The absolute amount of a sugar in a soil sample can be calculated from its value of Equal Weight Response.

(c) Calibration: The following equation is used to determine the quantity of the individual sugars in soil.

$$\text{mg sugar per 100 g soil} = \frac{A \times W_{\text{queb.}} \times 100 \text{ ml} \times 100}{A_Q \times K \times V_a \times W_s \times 100}$$

Where: A = Chromatographic area of soil sugar component peak

A_Q = Chromatographic area of quebrachitol peak

$W_{\text{queb.}}$ = Weight of quebrachitol added to each soil sample (100 μg)

K = Calibration factor for the individual component; 1.14 for glucose and galactose, 1.17 for mannose, 1.19 for xylose, 1.13 for arabinose, 1.03 for rhamnose and fucose

V_a = Volume of sulfuric acid extract for polysaccharides analysis

W_s = Weight (g) of soil sample

4. MEASUREMENT OF RADIOACTIVITY IN SULFURIC ACID EXTRACTS AND SAMPLES PREPARED FOR GAS CHROMATOGRAPHY.

(1) DETERMINATION OF RADIOACTIVITIES IN SULFURIC ACID EXTRACTS.

One-half millimeter of a sulfuric acid extract
(Section 3, subsection 11, of this chapter) from each soil

sample was transferred to a scintillation vial. Twenty ml of scintillation solution (200 ml dioxane + 200 ml toluene + 120 ml methanol + 41.6 g naphthalene + 2.6 g 2,5-diphenyl oxazole + 0.052 g 1,4-bis-2-(5-phenyloxazolyl)-benzene) was added to each scintillation vial and the radioactivity was measured using a Packard Tri-Carb liquid scintillation counter. A blank for background measurement was prepared using the same amount of sulfuric acid extract from the control soil. A counting efficiency was obtained using a glucose standard with a known radioactivity.

The following equation was used to calculate the amount of radioactivity in each sample:

$$A = \frac{(N_G - N_B) \times 2}{K \times 2.2 \times 10^6} \times \frac{100}{W_S} \times 100$$

Where: A = Specific activity ($\mu\text{Ci}/100 \text{ g soil}$)

N_G = Gross counts per minute per $\frac{1}{2}$ ml of sample and background

N_B = Number of background counts per minute per $\frac{1}{2}$ ml

K = Counting efficiency (%)

W_S = Weight of soil sample

(11) DETERMINATION OF RADIOACTIVITIES OF ALDITOL ACETATES PREPARED FOR GLC.

To each small vial containing alditol acetates was added 5 ml of chloroform and shaken to dissolve all alditol acetates. Then 1 ml of each solution was pipetted into a

scintillation vial. The radioactivity of each sample was counted after adding 10 ml of toluene scintillation solution (2g 2,5-diphenyloxazol + 3 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene + 1000 ml toluene) using a Packard Tri-Carb liquid scintillation counter.

A number of the samples contained yellow material which produce a quenching effect in the scintillation solution. Corrections for quench were made after counting each sample with a known external standard. All radioactivity measurements were also corrected for efficiency and background.

The amount of radioactivity was calculated by the following equation:

$$A = \frac{(N_G - N_B) \times 5 \text{ ml}}{K \times 2.2 \times 10^6} \times \frac{100 \text{ ml}}{\text{g soil}} \times \frac{1}{50 \text{ ml}} \times 100$$

Where: A = Specific activity ($\mu\text{Ci}/100 \text{ g soil}$)

N_G = Gross counts per minute per 1 ml of sample and background

N_B = Number of background counts per minute per ml

K = Counting efficiency

TABLE 1

INFLUENCE OF INCUBATION TIME ON TOTAL C
IN SOIL AMENDED WITH C-14 LABELED DEXTRAN AND GLUCOSE

Days of Incubation	Added ^{14}C Source	
	Dextran	Glucose
	% total C	
0	1.338	1.347
2	1.320	1.316
5	1.291	1.294
12	1.258	1.317
28	1.225	1.261

CHAPTER 4

RESULTS AND DISCUSSION

1. DECOMPOSITION OF ^{14}C LABELED GLUCOSE AND DEXTRAN

The percentage of total organic carbon and the radioactivity of the soils incubated with ^{14}C substrates at the various times of sampling are shown in Tables 1 and 2, respectively. When ^{14}C labeled glucose and ^{14}C labeled dextran were incorporated into the soil, there was an immediate development of microbial activity which resulted in the decomposition of the carbonaceous substrates. The activity of total carbon decreased as the incubation was extended because $^{14}\text{CO}_2$ was liberated from the added glucose and dextran. The results show that most of the labeled glucose had been decomposed by 5 days. As can be calculated from the activity prior to incubation and the activity at the end of the incubation (Table 2), about 26% of the added activity remained in the soil at 28 days. In other words, 74% of the ^{14}C labeled glucose was liberated as carbon dioxide during the incubation of 28 days which was in agreement with the report by Oades and Wagner (60). An initial lag in CO_2 evolution from ^{14}C labeled dextran appeared (60). A lag in the decline of total activity for the dextran soil was in agreement with this. It was concluded that the decomposition of dextran was slower than glucose. This is probably due to a period

TABLE 2

INFLUENCE OF INCUBATION TIME ON TOTAL ACTIVITY
IN SOIL AMENDED WITH C-14 LABELED DEXTRAN AND GLUCOSE

Days of Incubation	Added ^{14}C Source	
	Dextran	Glucose
	$\mu\text{Ci}/100 \text{ g soil}$	
0	4.44	3.43
2	3.59	1.64
5	2.60	1.21
12	1.89	1.07
28	1.60	0.89

of adaptation and increase of native soil microorganisms participating in the decomposition process for dextran. A nearly constant level of activity for the dextran treated soil was reached by 12 days, and only about 36% of the added dextran remained in the soil at 28 days. This indicates that about 64% of added dextran had been released as CO_2 at the end of 28 days.

Since most of the ^{14}C glucose was metabolized by 5 days, there is little doubt that the glucose carbon remaining in the soil after this period had been transformed into microbial tissue. After 12 days of incubation, the dextran carbon presumably had been transformed into microbial tissue. Therefore, the evolution of $^{14}\text{CO}_2$ after 12 days from both incubating soils probably resulted from the decomposition of recently synthesized microbial tissue. It is apparent that the process of glucose decomposition was divided into two parts: primary and secondary oxidation. Primary oxidation was the period (about 3 days) that glucose was predominantly oxidized by enzymatic systems originally present in the soil sample in microorganisms. Furthermore, a portion of the glucose was converted into the cellular materials of the microorganisms by forming reserve compounds and new enzymatic systems. Secondary oxidation (after 5 days) probably resulted from the decomposition of more difficultly decomposable metabolites (polysaccharides) and dead microbial cells. The process

of dextran decomposition was similar to glucose, but was less rapid. Table 1 shows that the total organic carbon content of the incubated soil gradually approached that of the original soil (1.25%) as the incubation was extended to 28 days.

2. TOTAL FREE SUGARS OF THE INCUBATED SOILS

Soluble sugars in the soil were extracted with 70% ethanol, and it may be assumed that sugars other than the added glucose were probably of minor significance (33, 54). The amount of free sugars extracted from the soil at various period of incubation are given in Table 3. After 2 days the amounts of free sugars in the soil incubated with ^{14}C glucose decreased to an approximately constant level and were the same as or slightly less than that in the original soil. In other words, the return of the free sugar content to its previous value after glucose addition was reached by 2 days. In the incubation of dextran with soil, the amount of free sugars shows a large difference from the control soil (11.13 against 6.97 mg/100 g soil) in the zero-time sample. This high amount of free sugars of dextran added soil prior to incubation may be either due to the presence of some sugars produced by freeze-drying the soil sample (32) or a small amount of ethanol extractable carbohydrate in the dextran preparation. The amounts of free sugars in the soil incubated with dextran reached

TABLE 3

INFLUENCE OF INCUBATION TIME ON ETHANOL EXTRACTABLE
CARBOHYDRATES* IN SOIL INCUBATED WITH C-14 LABELED
DEXTRAN AND GLUCOSE

Days of Incubation	Added ^{14}C Source		
	Control	Dextran	Glucose
	mg/100 g soil		
0	6.97	11.13	165.0
2		18.61	6.91
5		16.32	7.04
12		9.79	7.05
28		8.05	5.41

*Calculated with reference to a glucose standard.

an almost constant level after about 12 days and were higher than that of the original soil (6.97 mg/100 g soil). The higher equilibrium level of free sugars after 12 days incubation with dextran was probably accounted for by glucose released from the hydrolysis of dextran.

Table 4 shows the activities of the 70% ethanol extractions. The soil incubated with labeled glucose appears to have a slightly lower level of radioactivity in the zero-time sample as compared to the total activity (3.40 μ Ci/100 g soil) added to the soil. As can be calculated from the total activity added to the soil (Table 2) and the activity extracted prior to incubation (Table 4), about 79% of the added 14 C labeled glucose was recovered by this procedure. This is probably due to the adsorption of 14 C glucose on soil colloid surfaces during the mixing process, and that the adsorbed 14 C labeled glucose was not extractable with 70% ethanol. The activity of the free sugars in the soil incubated with labeled glucose decreased rapidly to an almost constant level and by 5 days was 2.6% of the total activity at 0 days. This is in agreement with the result of the total activity remaining in the soil (Table 2) and thus it can be assumed that most of the added glucose was metabolized by 5 days. Radioactivity in the free sugars at 5 days was 5.7% of the total activity remaining in the soil (Table 2). It is apparent that the assimilation of the glucose carbon by

TABLE 4

INFLUENCE OF INCUBATION TIME ON TOTAL ACTIVITY
EXTRACTED WITH ETHANOL FROM SOIL INCUBATED WITH
C-14 DEXTRAN AND GLUCOSE

Days of Incubation	Added ^{14}C Source	
	Dextran	Glucose
$\mu\text{Ci}/100 \text{ g soil}$		
0	0.0072	2.66
2	0.43	0.11
5	0.34	0.069
12	0.17	0.068
28	0.12	0.048

microorganisms was almost completed at this period, and the remaining ^{14}C present was largely in microbial tissue.

Since dextran is a highly polymerized molecule, it must be transformed into simpler molecules to allow the microorganisms to derive energy from its oxidation. An initial hydrolysis is necessary in order to convert dextran into the glucose which could be metabolized. Therefore, as shown in Table 4, the activity of the free sugars in the soil incubated with labeled dextran was higher than that soil incubated with labeled glucose except in the zero-time sample. A constant level of activity was reached by 12 days, and this amounted to about 9% of the total activity in the soil at this time. This result indicates that glucose was released by the hydrolysis of dextran throughout the incubation and that a small amount of unaltered dextran may still remain in the soil at the end of 28 days.

3. RADIOACTIVITY OF POLYSACCHARIDES EXTRACTED WITH N H_2SO_4 IN THE INCUBATED SOILS

Table 5 shows the activities of the sulfuric acid extracts at the different days of incubation. The results indicate that only about 55% of the added dextran (4.51 $\mu\text{Ci}/100\text{ g soil}$) could be extracted prior to incubation. The reason for this extraction difficulty is probably that the added dextran was adsorbed by clays and humic substances in the soil. It was also observed that about 17%

TABLE 5

INFLUENCE OF INCUBATION TIME ON TOTAL ACTIVITY
EXTRACTED WITH SULFURIC ACID FROM SOIL INCUBATED
WITH C-14 DEXTRAN AND GLUCOSE

Days of Incubation	Added ^{14}C Source	
	Dextran	Glucose
$\mu\text{Ci}/100 \text{ g soil}$		
0	2.48	0.58
2	1.39	0.27
5	0.675	0.17
12	0.295	0.14
28	0.19	0.11

of the added glucose carbon ($3.40 \mu\text{Ci}/100 \text{ g soil}$) was extracted with sulfuric acid prior to incubation. This together with the 79% extracted with ethanol yields a total recovery of 96%. Apparently, the acid extracted glucose had been adsorbed on soil colloid surfaces and was inaccessible to extraction by 70% ethanol.

As can be calculated from Tables 2, 4, 5, about 20% and 18%, respectively, of the ^{14}C dextran and ^{14}C glucose remaining in the soils at the end of the incubation occurred as saccharides. Presumably, most of the saccharides were present as constituents of microbial tissue or extracellular polysaccharides in the soil incubated with ^{14}C labeled glucoses. However, as already mentioned, the activity remaining in the free sugars was a little high in the soil incubated with ^{14}C labeled dextran. Probably a portion of the saccharides in this case were not utilized by microorganisms as energy sources for growth and protoplasmic synthesis during the time of incubation.

4. PURIFICATION AND FRACTIONATION OF POLYSACCHARIDES

Sulfuric acid hydrolyzate would be expected to contain large amounts of non-carbohydrate material which may influence the actual characterization of polysaccharides (20, 77). Gel filtration of polysaccharide extracts on Sephadex G-25 was employed to separate the yellowish, acid soluble components of soil organic matter

and low molecular weight polysaccharides (molecular weight < 4000) from the polysaccharides of high molecular weight (molecular weight > 4000). The polysaccharide fractions with high molecular weight was subsequently further separated on Bio-gel P-100 to obtain two fractions of molecular weight greater and less than 100,000.

Fig. 1 shows that the presence of soil sugars on Sephadex G-25 column from 320 ml to 750 ml and that of NaCl from 335 ml to 800 ml. Therefore, we determined that the fractionation of aqueous solutions of polysaccharide on Sephadex G-25 resolved colorless polysaccharide materials in the 250-500 ml eluate. The colored low molecular weight fraction was obtained in the 500-750 ml eluate. Fig. 2 shows the elution diagram of high molecular weight polysaccharides on Bio-gel P-100. Two fractions were obtained from the 100-215 ml eluate (molecular weight $> 100,000$) and the 215-415 ml eluate (molecular weight 4000-100,000).

5. DISTRIBUTION OF RADIOACTIVITY IN NEUTRAL SUGARS AMONG DIFFERENT MOLECULAR SIZE FRACTIONS

After fractionation of the soil polysaccharide extracts, all the fractions were hydrolyzed and the neutral sugars determined by gas-chromatography. The results in Table 6 represent the activity of the neutral sugars in the incubated soils among different molecular size fractions.

Figure 1. Elution diagram of a polysaccharide extract and 0.1 N NaCl on Sephadex G-25; gel bed 4 x 47 cm (813 ml); sample volume 50 ml; eluent distilled water.

Figure 2. Elution diagram of Blue Dextran and 0.1 N NaCl on Bio-gel P-100; gel bed 4 x 47 cm; sample volume 50 ml; eluent distilled water.

TABLE 6

INFLUENCE OF TIME AND MOLECULAR WEIGHT ON TOTAL
ACTIVITY IN POLYSACCHARIDES FROM SOIL INCUBATED
WITH C-14 DEXTRAN AND C-14 GLUCOSE FOLLOWING EXTRACTION
WITH SULFURIC ACID, FRACTIONATION BY GEL FILTRATION AND
PURIFICATION BY HYDROLYSIS, DERIVATIZATION,
AND METHYLENE CHLORIDE EXTRACTION

Days of Incubation	Molecular Weight Fraction	Added ^{14}C Source	
		Dextran	Glucose
$\mu\text{Ci}/100 \text{ g soil}$			
0	<4000	0.014	
	4000-100,000	0.16	
	>100,000	0.55	
2	<4000	0.035	0.0076
	4000-100,000	0.084	0.0014
	>100,000	0.18	0.0011
5	<4000	0.028	0.0033
	4000-100,000	0.025	0.0020
	>100,000	0.031	0.0020
12	<4000	0.021	0.0022
	4000-100,000	0.004	0.0017
	>100,000	0.0075	0.0011
28	<4000	0.020	0.0023
	4000-100,000	0.0018	0.000065
	>100,000	0.0015	0.0047

It has been reported in the literature (71, 72, 77) that soil hydrolyzate of polysaccharides contain amino sugars, uronic acids, and amino acids other than neutral sugars. Swincer et al. (72) suggested that the polysaccharides extracted with dilute acid were mostly those newly synthesized by microorganisms. Furthermore, Wagner and Mutatkar (75) have shown that significant amounts of ^{14}C were present as carbon of amino compounds, which are important constituents of some microbial cell walls (16), after the incubation of labeled glucose in soils. Thus a portion of ^{14}C in the polysaccharide extracts must be retained in these organic compounds which were not determined in this experiment. After the incubation of the soils for 28 days, the amount of the sulfuric acid extracted ^{14}C accounted for in neutral sugars was 12% and 2.6% respectively, for the soil incubated with ^{14}C dextran and ^{14}C glucose. However, as can be seen from the results in Tables 5 and 6, only about 30% of the ^{14}C in the sulfuric acid extracts for the zero-time sample of the soil incubated with ^{14}C labeled dextran was recovered in the fractions separated by gel filtration and prepared for GLC of the individual sugars. This low recovery indicates that losses of polysaccharides during gel filtration or during the hydrolysis and preparation of the derivatives for GLC were considerable. Therefore, the data in Table 6 does not represent quantitatively the activity of poly-

saccharides distributed in different molecular weight fractions but indicate the relative distribution. Because all soil samples were handled under the same condition, the figures are useful for comparative evaluations.

Little change in activity in the fractions of polysaccharide occurred after 12 days and 5 days, respectively, for the soil incubated with ^{14}C labeled dextran and ^{14}C labeled glucose (Table 6). Since it had previously been concluded that the sugars resulted from the microbial tissues after these periods, these results indicate that the rate of decomposition of microbial tissues were slower than that for the original substrates.

Studies (3, 72) have suggested that the easily extracted polysaccharides, which are predominantly of microbial origin, are high molecular weight materials. According to this conclusion, most of the ^{14}C material should be present in the high molecular weight fraction. Instead of this we found from Table 6 that, after 5 days, low molecular weight fractions contained relatively high amount of radioactivity in both incubation series. This anomaly probably can be explained in two ways: 1) Soil organisms are able to synthesize polysaccharides containing glucose, galactose, mannose, xylose, arabinose, rhamnose, fucose, amino sugars, and uronic acids from added glucose or dextran (59, 75). But amino compounds and uronic acids were not determined in this experiment,

and these compounds may add a considerable amount of radioactivity to the high molecular weight fractions. 2) As mentioned above, adsorption of polysaccharides on the gel may occur during gel filtration. It has been reported (17) that substances of high molecular weight are adsorbed more strongly than lower molecular weight substances of similar structure. Thus a portion of high molecular weight polysaccharides must be retarded on the gel in the presence of an adsorption effect.

The activity of the neutral sugars changed very little in the low molecular weight fraction after 5 days in the soil incubated with labeled dextran. This may indicate that most of the radioactivity had resulted from the undecomposed but partially hydrolyzed added dextran remaining in the soil. In addition, changes in activity of the neutral sugars with time in the high molecular weight fractions indicate that they are of microbial origin and under continual degradation.

After 5 days of incubation with labeled glucose the activity of the neutral sugars in the low molecular weight fraction remained nearly constant. This result shows that these labeled substances are stable and considerably resistant to decomposition by microorganisms. However, it has been shown (29) that radioactivity was found to be present in the newly synthesized humic substances after incubating ^{14}C glucose in the soils. Jansson and Persson

(39) concluded from their results that a small amount of stable ^{14}C labeled substances would rapidly build up during the first week after the addition of labeled glucose. Obviously, the biochemistry of the formation of humic substances is based on the intensive transformations which occur in the added glucose utilized by microorganisms in their life processes. The addition of labeled glucose to the soil resulted in a rapid increase of microbial activity. When the added glucose was exhausted the autochthonous microorganisms were dominant and responsible for the slow metabolism of the dead microorganisms. According to this theory, the humic substances are developed in the tissue of dead microorganisms. In this experiment, colored humic substances, including those newly synthesized, should be present in the low molecular weight fractions (73). Colored acid-soluble humic substances clearly contain some carbohydrates (1, 18) which are more readily hydrolyzed with dilute acid and upon hydrolysis will give glucose, galactose, and mannose. Therefore, it seems reasonable to expect that these stable humic substances synthesized by microorganisms made up the contribution to the activity of the neutral sugars in the low molecular weight fraction. After the decomposition of labeled glucose (about 5 days) continued changes in activity of the neutral sugars in the high molecular weight fraction is observed. In fact, ^{14}C was liberated as

$^{14}\text{CO}_2$ which came from the decomposition of secondary microbial substances radioactively marked in the course of the initial assimilation of glucose carbon.

6. NEUTRAL SUGAR COMPOSITIONS OF POLYSACCHARIDE FRACTIONS SEPARATED BY GEL FILTRATION IN INCUBATED SOILS

Tables 7 and 8 show the neutral sugar compositions of three polysaccharide fractions, which were obtained by the sequential gel filtration procedure, of the incubated soils treated with dextran and glucose respectively. Neutral sugars were determined by gas-liquid chromatography using quebrachitol as internal standard. Typical chromatograms of the neutral sugars of the incubated soils resulting from the polysaccharides of high and medium molecular weight fractions are shown in Figs. 3 and 4, respectively. Both figures show some unidentified components.

a. Sugar transformations during incubation of soil with added C-14 dextran

The results in Table 7 show rapid disappearance of glucose from the high and medium molecular weight fractions of polysaccharides in the soil incubated with C-14 dextran. This indicates that dextran was metabolized during the incubation. After 12 days these two fractions contained amounts of glucose similar to the original soil. However, the amount of glucose in the low molecular weight fraction of polysaccharides showed very little change during the incubation and was significantly larger than

TABLE 7

NEUTRAL SUGAR COMPOSITIONS OF POLYSACCHARIDE FRACTIONS SEPARATED
BY GEL-FILTRATION OF SULFURIC ACID EXTRACTS OF SOIL INCUBATED WITH C-14 DEXTRAN
(sugars in mg/100 g soil)

Days of Incubation	Molecular Weight Fraction	Monosaccharide							
		GLU	GAL	MAN	XYL	ARA	RIB	FUC	RHA
0	<4000	2.57	1.25	1.20	0.66	1.60	0.24	0.50	0.30
	4000-100,000	11.72	0.89	0.48	0.77	0.53	0.00	1.09	0.50
	>100,000	55.20	0.20	0.61	0.44	0.44	0.00	0.35	0.27
2	<4000	3.75	1.45	1.60	0.91	1.86	0.36	0.66	0.40
	4000-100,000	5.85	0.38	0.89	0.48	0.44	0.00	0.06	0.34
	>100,000	18.96	0.35	1.27	0.38	0.35	0.00	0.31	0.38
5	<4000	5.29	1.76	1.27	0.68	2.07	0.31	0.67	0.36
	4000-100,000	3.51	0.58	7.79	0.48	0.24	0.00	0.18	0.41
	>100,000	2.29	0.55	12.73	0.24	0.07	0.00	0.07	0.06
12	<4000	3.64	1.27	1.26	0.10	1.61	0.22	0.42	0.33
	4000-100,000	0.87	0.53	0.53	0.43	0.31	0.00	0.25	0.25
	>100,000	0.96	0.51	3.28	0.48	0.35	0.00	0.17	0.15
28	<4000	3.57	2.09	1.75	1.05	2.06	0.23	0.43	0.32
	4000-100,000	0.83	0.60	0.50	0.38	0.35	0.00	0.19	0.20
	>100,000	0.91	0.50	2.02	0.48	0.41	0.00	0.33	0.24
Control soil	<4000	4.60	2.29	2.04	1.18	2.95	0.20	0.85	0.33
	4000-100,000	0.93	0.50	0.52	0.28	0.27		0.18	0.29
	>100,000	0.91	0.54	2.26	0.32	0.28		0.20	0.36

TABLE 8

NEUTRAL SUGAR COMPOSITIONS OF POLYSACCHARIDE FRACTIONS SEPARATED
BY GEL-FILTRATION OF SULFURIC ACID EXTRACTS OF SOIL INCUBATED WITH C-14 GLUCOSE
(sugars in mg/100 g soil)*

Days of Incubation	Molecular Weight Fraction	Monosaccharide							
		GLU	GAL	MAN	XYL	ARA	RIB	FUC	RHA
2	<4000	1.92	1.16	0.83	0.58	1.67	0.20	0.48	0.36
	4000-100,000	0.49	0.37	1.31	0.57	0.30	0.00	0.42	0.50
	>100,000	0.80	0.24	0.41	0.35	0.31	0.00	0.33	0.32
5	<4000	2.11	1.60	1.27	0.58	1.50	0.22	0.64	0.36
	4000-100,000	0.75	0.56	16.12	0.50	0.41	0.00	0.28	0.33
	>100,000	0.41	1.42	9.44	0.45	0.17	0.00	0.13	0.20
12	<4000	2.19	1.10	1.02	0.77	1.80	0.12	0.51	0.29
	4000-100,000	0.65	0.41	0.55	0.44	0.40	0.00	0.32	0.26
	>100,000	0.73	0.41	6.85	0.58	0.37	0.00	0.31	0.15
28	<4000	1.87	0.80	0.81	0.39	0.54	0.10	0.30	0.13
	4000-100,000	0.85	0.18	0.33	0.50	0.26	0.00	0.17	0.10
	>100,000	0.32	0.23	0.54	0.77	0.40	0.00	0.13	0.12

* Values for the control soil are given in Table 7.

Figure 3. Gas-liquid chromatogram of alditol acetates derived from the neutral sugars in a hydrolyzate of polysaccharides with molecular weight greater than 100,000.

Figure 4. Gas-liquid chromatogram of alditol acetates derived from the neutral sugars in a hydrolyzate of polysaccharides with molecular weights from 4000 to 100,000.

that in the medium and high molecular weight fractions after 12 days. This result suggests that a portion of added dextran was not accessible to the soil microorganisms during the incubation and is in agreement with the amount of activity of neutral sugars in low molecular weight fractions (Table 6). It is interesting to note that the amount of mannose in the high molecular weight fraction increased significantly at 5 days, but it was short lived and decreased rapidly after 12 days. This may indicate that microorganisms synthesized mannose from dextran to a greater extent. Since mannose is thought to be the most common sugar in fungi cells (16), it is probable that the increase in metabolic activity after adding dextran was largely due to the response of fungi. The composition of pentoses and deoxyhexoses changed little during the incubation and this is in agreement with previous studies (13, 59, 60). Even though the activity of each sugar was not determined, it could be expected from the present results that of the neutral sugars in the high molecular weight fraction only hexoses will be strongly labeled but not pentoses or deoxyhexoses. This conclusion is biochemically justified (43). It is also observed that only the low molecular weight material contains ribose which is not a common sugar occurring in microbial polysaccharides (72). The results generally support the conclusion that high molecular weight

material predominantly originates from microbial polysaccharides, especially those newly synthesized microbial tissue.

b. Sugar transformations during incubation of soil with added C- 14 glucose

In general, the changes of the individual sugars except glucose in different molecular weight fractions of the polysaccharides were similar for the glucose treated soil as they were for the dextran treated soil. After 5 days, soil incubated with ^{14}C glucose contained a smaller amount of glucose in all the molecular weight fractions than that soil incubated with ^{14}C dextran. This result confirms the more rapid decomposition of glucose than dextran after incorporating them into the soil. Furthermore, the concentration of mannose in high molecular weight polysaccharides increased by 5 days indicating synthesis by soil microorganisms from glucose of soil polysaccharides containing mannose. Consequently, after 5 days the activity of sugars in the high molecular weight fraction (Table 6) must be largely associated with mannose. It is definitely shown from this result that the added glucose was metabolized by 5 days and that ^{14}C in high molecular weight (probably a part of medium molecular weight) polysaccharide at this stage was newly synthesized microbial tissue.

CHAPTER 5

SUMMARY AND CONCLUSION

Glucose and dextran labeled with ^{14}C were incubated in a fertile silt loam soil for 28 days at 25C to study the effect of incubation time on sugars. Sample periods were at 0, 2, 5, 12, and 28 days of incubation.

Total organic carbon and its radioactivity were measured for each sample. Sugars were extracted from the soil with 70% ethanol solution. Soluble sugars were determined quantitatively by the Anthrone method. The activities of the 70% ethanol extracts were measured. The soil residues from 70% ethanol extraction were then extracted with sulfuric acid to isolate polysaccharides. The acid extractable soil polysaccharides were purified and fractionated using gel filtration with Sephadex G-25 and Bio-gel P-100. Molecular weight fractions obtained were < 4000 , $4000-100,000$, and $> 100,000$. The individual monosaccharides in each molecular weight fraction of polysaccharides from each sample were determined quantitatively and qualitatively by gas-liquid chromatography. Total activity in the polysaccharides fractions was measured after preparation of the sample for GLC analysis which eliminated most non-carbohydrate material.

Because of the metabolic activity of soil microorganisms, considerable ^{14}C from the added glucose and

dextran was liberated as $^{14}\text{CO}_2$ during the incubation. The decomposition of glucose was more rapid than dextran, and most of the labeled glucose had been decomposed by 5 days. Little change in total activity was observed after 12 days for either treatment. Presumably, the ^{14}C remaining after this period had been transformed into microbial tissue and was more resistant to decomposition than the added substrates. After 28 days, 26% and 36% of the added glucose and dextran, respectively, remained in the soil. Measurements of free sugars in the soils at different intervals of incubation suggested that glucose was metabolized more rapidly by soil microorganisms than was dextran. A portion of the dextran probably was not accessible to the microorganisms and remained unaltered at the end of 28 days. About 20% and 18% of the ^{14}C dextran and ^{14}C glucose, respectively, remaining in the soils after 28 days occurred as saccharides. It appeared that most of these remaining saccharides were present as constituents of microbial polysaccharides except for the undecomposed dextran.

The changes in activity of neutral sugars after 12 days and 5 days for the soil incubated with ^{14}C dextran and ^{14}C glucose, respectively, confirm the previous conclusion that the decomposition of microbial polysaccharides was rather slow. Glucose, galactose, mannose, xylose, arabinose, fucose, and rhamnose were chromatographically separated from the hydrolyzate of polysaccharides of each

molecular weight fraction. Only the low molecular weight material contained ribose. In addition, it was observed that the concentration of mannose in the high molecular weight fraction increased significantly at 5 days for soil amended with either substrates, whereas the amounts of pentoses and deoxyhexoses changed little during the incubation. These changes in activity of neutral sugars and in the amount of mannose during the incubation suggest that the high molecular weight material consists predominantly of microbial polysaccharides. A rapid synthesis of hexoses, but a much slower synthesis of pentoses and deoxyhexoses, from the added ^{14}C substrates was indicated during the incubation.

A more complete picture of the synthesis and transformation of soil polysaccharides from ^{14}C substrates by soil microorganisms would require an identification of other components of polysaccharides such as uronic acids and amino compounds in each molecular weight fraction. These additional components should be included in further studies of this nature.

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by FOX RIVER

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75% COTTON

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