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Decomposition of Lignin by Soil Bacteria and Complex Formation between Autoxidized Lignin and Organic Nitrogen Compounds

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SUMMARY

Several bacteria able to decompose native lignin prepared according to Brauns (1939) were isolated from soil; they were aerobic, Gram-negative, motile or non-motile, non-endospore forming rods belonging to the genera *Pseudomonas* and *Flavobacterium*. The decomposition was studied in a neutral medium containing the lignin as a suspension of fine particles and solidified with silica gel. 20–30 % of the lignin added to the medium was decomposed by the bacteria. Independently of the biological decomposition, a non-biological transformation of lignin occurred during the later stage of the incubation when the medium dried out. The products of the non-biological transformation were dark brown, soluble in water but insoluble in ethanol. They reacted readily with nitrogen compounds in peptone, forming complexes containing about 2·5 % nitrogen, half of which was α-amino nitrogen. The relation of these complexes to soil humic acid is discussed.

INTRODUCTION

Lignin, cellulose and xylan are the major constituents of the plant residues being decomposed in the soil. Cellulose and xylan are known to be decomposed by a large number of aerobic and anaerobic micro-organisms by the action of hydrolytic enzymes. Lignin disappears from decomposing plant material, but slowly as compared with polysaccharides, and little is known about the organisms responsible for this decomposition. Certain fungi mostly belonging to the Basidiomycetes (*Polyporus* spp., *Marasmius* spp.) are known to be able to utilize lignin from straw and wood; these organisms, however, utilize the cellulose and xylan constituents as well. A few species appear to prefer the lignin to the polysaccharides (Lindeberg, 1946).

The sparse information available about this important process is partly due to the difficulties involved in preparing pure unaltered lignin. The preparations mostly used have been isolated by means which make it doubtful whether the material obtained represents unaltered lignin. The method introduced by Brauns (1939) which uses only neutral solvents was a great improvement in this respect. Lignin prepared according to this method was first used by Day, Pelczar & Gottlieb (1949) for investigating the microbial decomposition. Reviews of the literature dealing with the methods of preparation and biological decomposition of lignin have been presented by Norman (1937), Gottlieb & Pelczar (1951) and Lawson & Still (1957).

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Recent literature on lignin and formation of soil organic matter was reviewed by Bremner (1954). The present paper describes the isolation of lignin-decomposing bacteria from soil, the quantitative determination of the decomposition and the chemical analyses of the lignin and its decomposition products.

METHODS

Media

Culture media. The basal mineral solution had the following composition (%, w/v, dissolved in tap water): K_2HPO_4, 0·05; $MgSO_47H_2O$, 0·02; NaCl, 0·02; $FeSO_4$, 0·01. Medium GN contained in addition to the basal medium 0·1 % (w/v) glucose and 0·05 % (w/v) NH_4NO_3; medium GP 0·5 % (w/v) glucose, 0·1 % (w/v) Bacto peptone (Difco), and 0·05 % yeast extract. Agar to 1·8 % (w/v) was added when the media had to be solidified. Soil extract (25 %, v/v) was added to the media in some cases; this extract was prepared by autoclaving 1 kg. soil suspended in 1 l. water for 30 min. at 110°, followed by filtration.

The lignin preparation. Wood flour (a mixture of pine and spruce of commercial Norwegian origin) was extracted with water at room temperature until the extract was colourless. The wood flour was dried and then extracted with 96 % (v/v) ethanol in water for about a week, i.e. as long as lignin was extracted. The ethanol extract was evaporated to dryness under reduced pressure and the residue treated with ether and water until it became powdery, then purified by reprecipitations until the methoxyl ($—OCH_3$) content was constant as described by Brauns (1939). About 3 g. purified material was obtained from 1 kg. wood flour. The preparation was a light cream-coloured powder soluble in N-sodium hydroxide, dioxane, ethanol, methanol, pyridine and insoluble in water, ether, benzene, light petroleum. It gave the typical purple colour reaction with phloroglucinol + hydrochloric acid. Chemical analysis: 61·24 % C, 6·38 % H (determined by the Chemical Department, Danish Atomic Energy Commission Research Establishment Risö), 0·2 % ash, 86·0 % Klason lignin, 12·9 % $—OCH_3$, 0·17 % N, 0·03 % α-amino acid nitrogen. Paper chromatograms revealed the presence of amino acids and sugars in hydrolysates (see later). Brauns (1939) gave the following analytical data for native lignin from spruce wood: 63·89 % C, 6·07 % H, 14·9 % $—OCH_3$.

Preparation of sterile suspension of lignin in water. A homogenizer made from two close fitting Pyrex test tubes (see Umbreit, Burris & Stauffer, 1949, p. 136) was sterilized in the oven. A portion of lignin was brought into the tube of the homogenizer and absolute ether added until the lignin was covered by a layer 10 cm. deep; the tube was then closed with a sterilized cork stopper and left for 2–3 days. Most of the ether was removed by decantation and the cork stopper replaced by a sterilized cotton plug. When all the ether had evaporated through the cotton plug sterile water was added and a homogeneous suspension prepared by means of the pestle of the homogenizer, moved by the hand. The suspension was brought aseptically into a sterile flask, adding sterile water to make a 2 % (w/v) suspension. The exact concentration was determined by a dry wt. determination on a sample. The sterility of the suspension was tested by inoculation of nutrient agar and nutrient broth with a drop of the suspension. Microbial growth was never observed from a lignin suspension prepared in this way.

Silica gel + lignin medium. An aqueous solution which contained 1·8–2·0 % (w/v) sodium silicate was prepared from sodium water-glass. The solution was run through a column of Amberlite IR120 (H) (Taylor, 1950; Smith, 1951) and autoclaved at 110° for 20 min. The solution after autoclaving was at pH 3·0. Two mineral solutions were prepared: solution (A) contained (%, w/v): NaCl, 1·0; NH_4NO_3 or KNO_3, 0·6; K_2HPO_4, 0·3, $MgSO_47H_2O$, 0·1; $FeSO_4$, 0·05; solution (B) contained the same constituents but in double strength. The salts were dissolved in tap water and sterilized by autoclaving. A few ml. of sterile N-sodium hydroxide was added to the mineral solutions to adjust the final medium to about pH 7·0. Four ml. of solution A and 20 ml. of the silicic acid solution were mixed in Petri dishes (10 cm. diameter). The dishes were left overnight to allow the mixture to solidify. Four ml. silicic acid solution, 0·3 ml. solution B and 0·7 ml. of lignin suspension were mixed in sterile test tubes and poured on the surface of the solidified silica gel in the Petri dishes. When the mixture had solidified the lids of the dishes were removed and replaced by pieces of sterilized filter paper for 2–3 hr. until the surface was dry. Only accidental contaminations occurred on plates prepared in this way.

Measurement of lignin decomposition in soil

Carbon dioxide production. The method described by Petersen (1926) was used but on a smaller scale, with 10 g. portions of soil and 250 ml. bottles. Air-dry loam soil (pH 7·2) was thoroughly mixed with 0·1 and 0·25 % (w/w) of lignin in a mortar; 18 % (v/w) of water containing 0·1 % (w/v) NH_4NO_3 was added afterwards.

The number of lignin-decomposing organisms. Portions of air-dry soil which had passed through a 2 mm. sieve were mixed thoroughly in a mortar with different carbon sources and transferred to 250 ml. bottles; 16–18 % (v/w) of water containing 0·1 % (w/v) NH_4NO_3 was added. The bottles were closed with perforated cork stoppers and incubated at 25°. Ten g. portions of soil were removed from the bottles after about 2 weeks of incubation and shaken in 100 ml. portions of sterile water; 0·5 ml. of a suitable dilution, in most cases 1/5000, was added to the surface of a silica gel + lignin plate and spread by means of a glass rod. The lids of the Petri dishes were replaced by pieces of filter paper until the surface was dry. Transparent spots became visible on the plates after incubation at 25° for about 2 weeks. The spots were counted after incubation for a month.

Isolation of lignin-decomposing bacteria

Lignin-decomposing organisms picked from a transparent spot on a silica gel + lignin plate, inoculated with a soil suspension as described above, were enriched by one or two replatings on the same medium. A spreading was finally performed on agar medium GN. All types of organisms which appeared on this plate after incubation for 2–3 weeks were isolated and tested separately by streaking on silica gel + lignin medium. Several bacteria able to produce a transparency on the lignin medium were isolated in this way. The purity of the isolates was checked by cultivation on nutrient agar, nutrient broth and medium GP alternating with cultivation on silica gel + lignin medium.

Microscopic examinations

The lignin particles. A small square of the surface layer of the silica gel + lignin medium was removed and placed in a mixture of equal parts 6 N-HCl and 2% (w/v) phloroglucinol in ethanol. The square was removed from the solution after a few minutes, when the lignin particles were intensively stained. After a short drying on a piece of filter paper the square was placed in a small drop of paraffin oil on a coverslip. This was mounted on a slide with a circular concavity filled with paraffin oil so that the square was hanging down immersed in the oil. The coverslip was sealed to the slide with paraffin and the preparation examined under the microscope.

The microbial growth. Squares of the silica gel + lignin layer were removed and placed in phenol aniline blue (Jones & Mollison, 1948) for 1 hr., then in water for 15 min. The squares were dried and embedded in paraffin oil as described above.

Quantitative determination of the decomposition of lignin

Silica gel + lignin plates were inoculated with bacteria isolated as described above. A suspension (0·2 ml.) of the bacteria in water was spread over the surface by a glass rod. Inoculation with a mixed culture was performed with 0·5 ml. of suspension of surface material from transparent silica gel + lignin plates. The plates were incubated at 25° for 6–8 weeks. Uninoculated control plates were incubated in the same way. At the end of the period of incubation all material was removed from the dishes, air-dried, ground thoroughly in an agate mortar and extracted for 24 hr. with 96% (v/v) ethanol in water in a Soxhlet apparatus. The ethanol was evaporated from the extracts in a current of air and replaced by water which was left for 24 hr. Material insoluble in water was filtered off in an ignited and weighed filter crucible (A1), washed thoroughly with water, dried at 80° and weighed. This material was undecomposed lignin or residual lignin (RL). Before the chemical investigations residual lignin was dissolved in dioxan and precipitated by adding ether.

The silicate material which had been extracted with ethanol was dried in air and extracted with water at room temperature as long as the extract was brown coloured, then with 0·1 N-sodium hydroxide in the same way. The extracts were combined, 2 N-HCl was added to get an acid concentration of about 0·1 N. The resulting brown precipitate was removed by centrifugation and washed with 0·01 N-HCl. The material was again dissolved in 0·2 N-NaOH, reprecipitated and washed as described, then dried *in vacuo* and finally at 80°, and weighed. This material was the oxidized lignin precipitable (OLP).

The supernatant fluids from the precipitations, which were more or less brown coloured, were combined and neutralized with N-NaOH. Charcoal (British Drug Houses Ltd. for decolorizing purposes, thoroughly washed with deionized water) was added, 3 g. to 100 ml., and left for about 2 hr. with periodical stirring. The charcoal was then filtered off and washed with water. Acetone containing 10% (v/v) water was added and a brown coloured material was displaced from the charcoal. The brown acetone extract was evaporated to dryness in air, the residue dried at 80° and weighed. This material, which was a brown powder, formed the oxidized lignin non-precipitable (OLNP).

Analytical methods

Hydrolysis of lignin and its decomposition products with 6N-HCl. The material (5–15 mg.) was allowed to swell with 0·2–0·4 ml. 6N-HCl at room temperature in a glass tube. Hard particles were disintegrated with a glass rod 1·0 ml. of 6N-NCl was then added and the tube was sealed and heated at 105° for 18 hr. Undissolved material was separated from the hydrolysate by centrifugation, washed thoroughly with water, dried at 100°, weighed and total nitrogen determined. The combined hydrolysate and washings were evaporated to dryness *in vacuo*, 0·5 ml. water added and the evaporation repeated. A suitable amount of water was added and α-amino nitrogen determined in a sample; the remainder was used for paper chromatography.

Determination of Klason lignin. This was carried out by treatment with 72% (w/w) H_2SO_4 as described by Björkman (1957).

Total nitrogen was determined by a micro-Kjeldahl method with $SeO_2 + CuSO_4 . 5H_2O + K_2SO_4$ (1+1+8) as catalyst.

α-Amino acid nitrogen was determined by the titrimetric ninhydrin method of Van Slyke, MacFadyen & Hamilton (1941).

Methoxyl (—OCH_3) was determined by the semimicro method of Vieböck & Schwappach as described by Brauns (1952, p. 744).

Ash. Analytical figures are not corrected for ash content, this was not determined because of the small amounts of material available.

Amino acids. These were detected by paper chromatography on Whatman paper no. 1 with the descending technique. The chromatograms were run in one direction with *n*-butanol + acetic acid + water (4+1+5, v/v) and in the other direction with phenol + water (80+20). The dried chromatograms were sprayed with 0·5% (w/v) ninhydrin dissolved in water-saturated *n*-butanol containing a little acetic acid. The ninhydrin-positive spots were identified by comparison with spots produced by pure amino acids.

Phenols. These were observed on paper chromatograms developed in one direction with *n*-butanol + acetic acid + water (4+1+5, v/v). The dried chromatograms were examined in ultraviolet light and fluorescent areas encircled with a pencil, then sprayed with an aqueous solution of 1% (w/v) $FeCl_3$ + 1% (w/v) $K_3Fe(CN)_6$ (Coulson, Davies & Lewis, 1960).

Sugars. These were detected on paper chromatograms developed in one direction with *n*-butanol + acetic acid + water (4+1+5, v/v). The dried chromatograms were sprayed with aniline phthalate or dipped in alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950).

RESULTS

Decomposition of lignin in soil

Carbon dioxide production. Addition of lignin to soil resulted in an increased production of CO_2 (see Fig. 1). Addition of 0·25% (w/w) lignin caused an increase of 1·6 mg. CO_2/g. dry soil during a period of 74 days. This corresponds to about 29% of the carbon added as lignin.

Number of lignin-decomposing micro-organisms. Inoculation of silica gel + lignin plates with suspensions of soil resulted in a large number of transparent spots when lignin or lignin-containing plant material had been added to the soil. Plate 1,

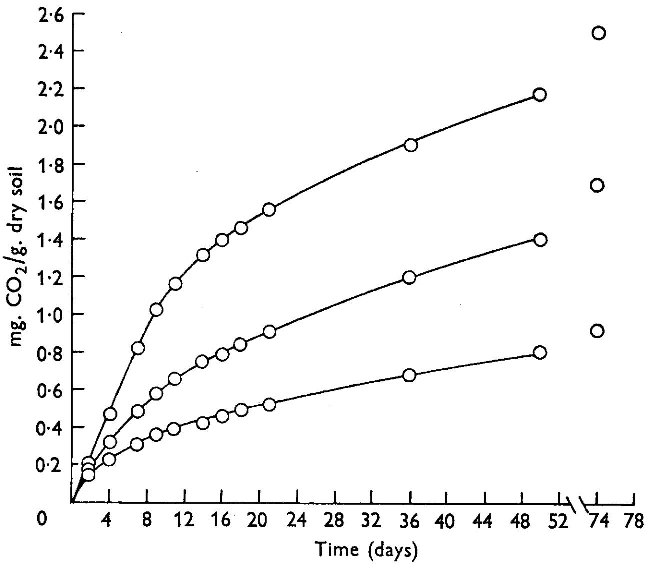

Fig. 1. Carbon dioxide production from soil after addition of 0·25 % (w/w) lignin (upper curve); 0·1 % (w/w) lignin (middle curve); no addition (lower curve).

fig. 2, shows a plate inoculated with 0·5 ml. of a suspension of soil (diluted 1/5000) enriched with 1 % (w/w) powdered wheat straw. Table 1 gives the number of spots observed on plates inoculated with suspensions of soil enriched with a variety of organic materials. The xylan was prepared as described by Sørensen (1957) and the cellulose as described by Norman & Jenkins (1933).

Table 1. *Number of transparent spots which appeared on silica gel + lignin plates after inoculation with suspensions of soil enriched with various carbon sources.*

Inoculation with 0·5 ml. soil suspension diluted 1/5000 except for lignin, where the dilution was 1/25000.

Carbon source	No. transparent spots
Lignin	65
Wheat straw	108
Wood flour	100
Cellulose from wood flour	5
Xylan from wood flour	0
Starch	5
Glucose	14
Xylose	1
Tannin	0
Vanillin	0

A xylan preparation from wheat straw induced a number of transparent spots. This preparation contained, however, 8–10 % lignin-like material not hydrolysed during treatment with 72 % (w/w) sulphuric acid at room temperature followed by boiling under reflux for 5 hr. with 0·5 N-H_2SO_4. It is probably this part of the material which was responsible for the enrichment of the micro-organisms producing the transparent spots.

Decomposition of lignin by pure cultures of bacteria

From transparent spots on silica gel + lignin plates a number of bacteria were isolated which in pure culture produced a similar transparency. Plate 1, fig. 1, shows the transparency produced by a mixed culture (L-15) and a pure culture (*Pseudomonas*-14).

Addition of phloroglucinol + HCl to such plates resulted in a weak coloration of the transparent areas, whereas the other parts of the plate yielded the purple coloration, typical for lignin. This indicated that part of the lignin inside the transparency had disappeared or was unable to yield the colour. Microscopic examination of the surface layer inside and outside the transparent areas showed that only the larger lignin particles were present in the transparent areas, most of the small particles having disappeared. Plate 1, figs. 3 and 4, shows photomicrographs of the surface layer outside and inside a transparent spot.

The surface layer of the silica gel + lignin medium was examined under the microscope after staining with phenol + aniline blue which stained the bacteria but not the lignin particles. Plate 1, fig. 6, shows the fringe of a transparent spot produced by *Pseudomonas*-14. The dark blots are lignin particles surrounded by bacteria. Pl. 1, fig. 7, shows *Pseudomonas*-14 surrounding a lignin particle.

The first indication of a transparency became visible 3–4 days after inoculation and extended then slowly until the medium became too dry. The silica gel + lignin plates seen on Pl. 1, fig. 1, had been incubated for about 1 month after inoculation.

The lignin-decomposing micro-organisms isolated during this work were in all cases bacteria. Fungi and actinomycetes occurred, however, on the lignin medium after inoculation with soil suspensions. Several such organisms were isolated and tested separately on silica gel + lignin medium but none of these isolates produced any transparency; the growth of the isolates on this medium was very feeble.

Pl. 1, fig. 5, shows micro-organisms from silica gel + lignin medium inoculated with the mixed culture L-15. This culture had been replated on lignin medium for about a year. The organisms seen on this photo are considered to be typical for the whole plate.

Description of some lignin-decomposing bacteria

The lignin-decomposing bacteria isolated in pure culture during this work were of two types: (1) non-endospore forming straight rods, 1·3–1·5 × 0·5μ, motile with one or two polar flagella, Gram-negative, aerobic; (2) non-endospore forming straight rods, 1·0–1·2 × 0·6μ, non-motile, Gram-negative, aerobic.

Some cultural, nutritional and biochemical characters. The tests were performed in 5 ml. portions of media containing the respective carbon sources in 0·5% (w/v) concentration and nitrogen sources in 0·1% (w/v) concentration besides the basal mineral solution. Soil extract (25%, v/v) was added in some cases. Incubation was at 25° and observation was over a period of a month.

Growth on nutrient and GP agar. Colonies greyish white (group 1) or yellow (group 2), circular, convex, edge regular, surface smooth and shining, diameter after 1 month 1·0–1·5 mm. Growth nonspreading. No water soluble pigment.

Nutrient broth and GP broth. Turbidity visible 1–2 days after inoculation, growth homogeneous, no soluble pigment, no odour. A deposit of cells 3–4 weeks after inoculation.

Micro-aerophilic conditions. Nutrient broth and GP broth boiled before inoculation and covered by a layer of paraffin oil, a faint turbidity was visible 3–4 days after inoculation; it did not increase during prolonged incubation.

Optimal temperature for growth. Best growth at 30°; no growth at 37°.

Effect of initial pH *of the medium.* No difference in growth rate was observed within the range pH 5·3–7·8.

Growth in defined medium. Basal mineral solution + ammonium nitrate and sodium citrate or sodium acetate: no growth. Addition of soil extract had no effect.

Utilization of carbohydrates. Slight acid formation but no gas from glucose, mannose, xylose, arabinose and salicin. Best growth on glucose and mannose; no or very slight growth on galactose, levulose, sucrose, maltose, lactose, mannitol, glycerol. The sugar solutions were sterilized by filtration. The nitrogen source was peptone.

Utilization of polysaccharides. Strips of filter paper partly immersed in basal mineral solution + soil extract, peptone or ammonium nitrate: no utilization. Starch (soluble), mannan and xylan (Sørensen, 1957) added to GP-agar instead of the glucose: no or very slight utilization.

Potato. Faint growth of a brownish colour (group 1), yellow growth (group 2).

Utilization of nitrogen sources. Peptone and glutamic acid yielded the best growth, ammonium and asparagine medium growth, slow growth with nitrate.

Catalase. Positive.

Gelatine. No or very slight liquefaction.

Milk. No changes of the milk visible.

Formation of nitrite from nitrate. Negative.

Formation of ammonia from peptone. Positive, moderate.

According to *Bergey's Manual* (1957) group 1 belongs to the genus *Pseudomonas* and group 2 to the genus *Flavobacterium.* A further classification according to the descriptions given by *Bergey's Manual* was not possible. The bacteria were isolated from loam soil (pH 6·5–7·5) representing agricultural, garden and forest soils. Six isolates of the *Pseudomonas* sp., three isolates of the *Flavobacterium* sp. The bacteria belonging to the last genus gradually lost in pure culture the ability to decompose lignin.

Quantitative determination of the decomposition of lignin

Tables 2 and 3 summarize the results of the quantitative determinations of the lignin decomposition and the analyses carried out on the extracted material. The nitrogen sources added to the silica gel + lignin medium were peptone (Table 2) or nitrate (Table 3). The figures in the upper part of the two tables indicate the % of the lignin originally present which was recovered as residual lignin (RL) or as oxidized lignin (OLP, OLNP). A sample of lignin from a water suspension, 2 months old, was mixed with quartz sand and air-dried and finally subjected to the extraction procedure used for the silica gel + lignin samples. It is seen (Table 3) that almost 97 % of the lignin was recovered by this procedure.

The silica gel + lignin plates were incubated for 6–8 weeks at 25°. The biological decomposition took place during the first 3–4 weeks as judged by the increasing transparency of the medium. Uninoculated sterile control plates were analysed shortly after preparation or incubated in the same way as the inoculated plates. The later stages of the incubation when the medium dried out more or less were characterized by the development of a brown colour both in inoculated and uninoculated plates. This coloration is considered to be the result of an autoxidation of a part of the lignin. The products were soluble in water but insoluble in ethanol, precipitable (OLP) or non-precipitable (OLNP) on addition of acid. A part of the non-precipitable fraction was able to cross a dialysis membrane.

Table 2. *Material recovered from inoculated and uninoculated silica gel + lignin plates and the results of the chemical analyses performed on this material*

0·05 % (w/v) peptone was added as a nitrogen source.

	Mixed population L-15		*Pseudomonas*-14		Uninoculated*		Uninoculated†	
Recovered as lignin (RL), %	21·6		26·8		52·9		61·4	
Recovered as oxidized lignin (OLP), % ...	26·9		30·7		29·1		22·5	
Recovered total, %	48·5		57·5		82·0		83·9	
	RL	OLP	RL	OLP	RL	OLP	RL	OLP
Nitrogen, %	0·25	2·05	0·26	2·53	0·32	2·54	0·31	2·65
Insoluble residue from hydrolysis with 6 N-HCl, %	84·4	70·0	90·4	70·5	87·3	80·5	85·7	76·9
Nitrogen in residue as % of original matter	0·16	0·57	0·16	0·56	0·19	0·51	0·20	0·59
Nitrogen in residue, %	0·19	0·81	0·17	0·80	0·22	0·63	0·23	0·76
Nitrogen hydrolysed, %	36·5	72·2	39·5	77·7	39·0	80·1	36·5	77·8
α-Amino acid nitrogen as % of original matter	0·08	1·01	0·08	1·03	0·05	1·19	0·05	1·39
α-Amino acid nitrogen as % of total nitrogen	32·0	49·5	30·8	40·6	15·6	47·0	16·1	52·5
α-Amino acid nitrogen as % of hydrolysed nitrogen	89·0	68·0	80·0	52·3	38·5	58·5	45·5	67·5

* Incubated in the same way as the inoculated plates.

† The medium was dried immediately after preparation.

It is seen that about the same % of oxidized lignin was recovered from the sterile plates as from the inoculated ones indicating that the transformation of the lignin to the dark-coloured compounds was accomplished by non-biological means. Similar amounts were recovered from plates dried immediately after preparation as from sterile plates allowed to dry slowly during an incubation period of 6–8 weeks (Table 2). This indicated that the transformation occurred during the drying process. No oxidation occurred in the suspension of lignin in water. Ammonia appeared in some way to protect the lignin against the autoxidation. Larger amounts of lignin and less oxidized lignin were recovered when 0·1 % (w/v) NH_4Cl was added to the medium. This was not investigated further, but nitrate or peptone were added as nitrogen source in experiments where a quantitative determination of lignin and oxidized lignin was performed.

The biological decomposition of lignin. A comparison between the figures indicating the % of material recovered totally and as residual lignin from inoculated and

Table 3. *Material recovered from inoculated and uninoculated silica gel + lignin plates and the results of the chemical analyses performed on this material*

0·1% (w/v) KNO_3 added as nitrogen source.

	Mixed population			*Pseudomonas*-14			Uninoculated			Original lignin	
										A*	B*
Recovered as lignin (RL), % ...	25·0			33·4			59·1			96·1	—
Recovered as oxidized lignin (OLP), % ...	17·0			19·6			13·2			0·6	—
Recovered as oxidized lignin (OLNP), % ...	7·8			9·6			7·5			0·0	—
Recovered total, % ...	49·8			62·6			79·8			96·7	—
	RL	OLP	OLNP	RL	OLP	OLNP	RL	OLP	OLNP	RL	L
Nitrogen, %	0·25	0·62	0·55	0·21	0·58	0·64	0·11	0·39	0·69	0·18	0·17
Insoluble residue from hydrolysis with 6 N-HCl, %	88·9	84·9	40·0	87·0	86·5	17·4	86·7	89·4	14·8	85·3	89·3
Nitrogen in residue as % of original matter	0·14	0·31	0·15	0·12	0·31	0·12	0·07	0·26	0·09	0·12	0·11
Nitrogen in residue, %	0·16	0·37	0·37	0·14	0·35	0·67	0·08	0·27	0·61	0·15	0·13
Nitrogen hydrolysed, %	43·7	49·6	72·8	44·3	47·7	81·6	38·7	31·7	87·0	36·3	33·5
α-Amino acid nitrogen as % of original matter	0·05	0·18	0·19	0·04	0·16	0·12	0·01	0·07	0·12	0·03	0·03
α-Amino acid nitrogen as % of total nitrogen	20·0	29·0	34·6	19·1	27·6	18·8	9·1	18·0	17·4	16·7	17·6
α-Amino acid nitrogen as % of hydrolysed nitrogen	45·5	58·0	47·5	44·5	59·2	23·1	25·0	54·0	20·0	50·0	50·0
$—OCH_3$, %	11·1	10·0	—	12·5	9·8	—	13·9	11·3	—	12·6	12·9

* A. An amount of original lignin from a suspension in water dried on quartz sand and extracted with ethanol etc.; B, Analytical figures for original lignin.

sterile plates respectively, showed a difference which is ascribed to a biological decomposition. Table 3 shows that 49·8 % was recovered totally from plates inoculated with a mixed population, 62·6 % from plates inoculated with *Pseudomonas*-14 and 79·8 % from the sterile control plates. The figures indicating recovered lignin are 25·0 %, 33·4 % and 59·1 %, respectively. This indicates that about 30 % of the lignin was decomposed by the mixed population and about 20 % by the *Pseudomonas* sp. The figures given in Table 2 for medium containing peptone as nitrogen source indicate a degree of decomposition of the same order.

Chemical analyses of lignin and oxidized lignin

The residual lignin and oxidized lignin extracted from the silica gel + lignin medium during the determination of the decomposition of lignin were subjected to various analyses. The results are summarized in Tables 2 and 3.

Nitrogen. Residual lignin was only slightly influenced by the nitrogen source present in the medium from which it was extracted. The content of total nitrogen varied from 0·11 % in a sample from uninoculated nitrate-containing medium to 0·32 % in a sample from uninoculated peptone-containing medium.

The nitrogen content of oxidized lignin was, however, strongly influenced by the nature of the nitrogen compounds present. The total content varied from 0·39 % in a sample from uninoculated nitrate-containing medium to 2·65 % in a sample from uninoculated peptone-containing medium. The total nitrogen content of the insoluble residue from the same samples varied from 0·27 to 0·76 %. The α-amino acid nitrogen content varied from 0·07 to 1·39 %. The nitrogen content of the non-precipitable fraction of oxidized lignin was of the same order as that of the precipitable fraction, but a larger percentage was susceptible to hydrolysis. These figures indicate that oxydized lignin readily complexes with nitrogenous compounds present in peptone especially amino acids. Difco Bacto peptone contains 16·2 % total-N, 15·4 % peptone-N and 3·2 % free amino-N (Sykes, 1956).

Amino acids. Hydrolysis with 6 N-HCl released a number of amino acids from lignin and oxidized lignin. Fifteen to sixteen ninhydrin-positive spots appeared on the paper chromatograms. The most intense of these spots were identified as alanine, glycine, leucine, valine, serine, threonine, glutamic acid, aspartic acid, lysine, arginine, proline. The qualitative amino acid composition appeared to be identical in the hydrolysates of the various fractions but the quantitative composition appeared to differ; in particular the oxidized lignin extracted from peptone-containing medium was different from the other preparations.

Phenols. The chromatograms of the hydrolysates showed under ultraviolet radiation several fluorescent spots with R_F-values ranging from 0·07 to 0·9. Spraying with the ferric chloride + ferricyanide reagent resulted in green or blue spots mainly identical with the fluorescent spots. Hydrolysates of original and residual lignin and of precipitable and non-precipitable oxidized lignin showed almost the same fluorescent spots on the paper chromatograms. Identification of the spots with known compounds was not attempted.

Carbohydrates. The original lignin contained some carbohydrate material; glucose, arabinose, xylose and uronic acids were released during treatment with 72 % sulphuric acid as described by Björkman (1957).

The bacterial decomposition of lignin was not a selective utilization of the carbohydrate impurities. This was shown by hydrolysis of residual lignin from a medium where a bacterial decomposition had taken place. The constituents mentioned above were present in such hydrolysates and, judging by the intensity of the spots, were in the same relative concentrations.

Methoxyl. The —OCH_3 content of residual lignin and oxidized lignin extracted from the nitrate-containing medium is given in Table 3. It is seen that residual lignin from the sterile medium had a —OCH_3 content larger than that of the original lignin. This might be the result of a removal of impurities. The methoxyl content of the oxidized lignin was 10–20 % lower than that of residual lignin.

DISCUSSION

The results of this work indicated that isolated lignin can be decomposed by soil bacteria. Addition of lignin-containing plant material to soil samples resulted in the enrichment of a special population of micro-organisms able to attack the isolated lignin. This indicated that the preparation represented a natural part of plant material and not an artefact produced during the extraction and purification. The preparation contained, however, some protein and carbohydrate material but the bacterial decomposition was not a selective utilization of these constituents, as shown by the chemical analyses of the residual material.

The non-biological transformation of lignin into brown coloured water soluble substances might be catalysed by the presence of silicate. Autoxidations appear to be strongly influenced by the environment; Pratt & Trapasso (1960) observed an autoxidation of a variety of organic solids (e.g. anthracene) by passing air through a mixture of the powdered compound and alumina. Ziechmann (1959) reported that the oxidation of polyhydroxybenzenes proceeded slowly at a neutral reaction in the presence of silicic acid. The chemical analyses of the brown coloured substances indicated a similarity with the lignin and they might represent slightly altered constituents of the lignin molecule. Their ability to react with organic nitrogen compounds was, however, much larger than that of the lignin proper. The complexes which resulted from this reaction had a certain resemblance to the soil humic acid investigated by Bremner (1955) in so far as the nitrogen content, the % of amino acid nitrogen and the amino acids detected in the hydrolysates were similar. This result seems to support the view that soil humic acid complexes result from a reaction between more or less altered lignin originating from the plant material and nitrogen compounds originating from the protein synthesized by the soil micro-organisms. This point of view has been put forward by Gillam (1940) who found evidence that the non-nitrogenous fraction of humic acid consisted of a modified lignin. Mattson & Koutler-Andersson (1943) observed a relation between autoxidation of lignin and fixation of ammonia and they assumed that the humus complexes of soil were derived from lignin by such processes. Handley (1954) found that protein became resistant to microbial attack after reaction with substances, probably tannins, leaching from the litter on top of the soil; such a process was considered to be an important factor in the formation of mor.

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EXPLANATION OF PLATE 1

Fig. 1. Transparency on silica gel + lignin plates after inoculation with lignin-decomposing microorganisms. The left-hand plate inoculated with a mixed culture (L-15); the right-hand plate inoculated with a pure culture (*Pseudomonas-14*). Incubation for a month.

Fig. 2. Transparent spots on a silica gel + lignin plate inoculated with 0·5 ml. of a suspension (diluted 1/5000) of soil enriched with 1 % (w/w) powdered wheat straw. Incubation for a month.

Fig. 3. Photomicrograph showing the lignin particles in the surface layer of an uninoculated silica gel + lignin plate stained with phloroglucinol + HCl. × 400.

Fig. 4. Photomicrograph showing the lignin particles in the surface layer of a silica gel + lignin plate rendered transparent by the growth of *Pseudomonas*-14. Staining with phloroglucinol + HCl. × 400.

Fig. 5. Photomicrograph of the surface of a silica gel + lignin plate inoculated with a mixed culture (L-15). Staining with phenol + aniline blue. × 1000.

Fig. 6. Photomicrograph of the fringe of a transparent spot on a silica gel + lignin plate inoculated with *Pseudomonas*-14. Staining with phenol + aniline blue which stain the bacteria but not the lignin particles. The blots are lignin particles surrounded by bacteria. × 640.

Fig. 7. Photomicrograph showing *Pseudomonas*-14 surrounding a lignin particle. Staining with phenol + aniline blue. × 1000.

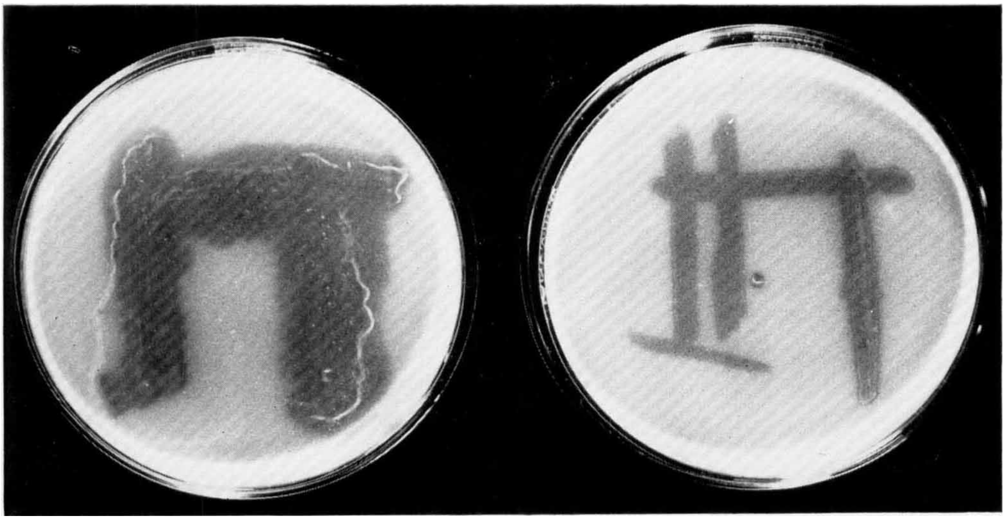


Fig. 1

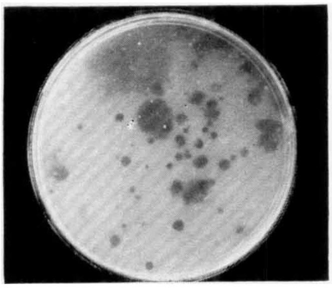


Fig. 2

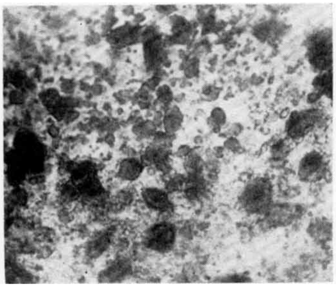
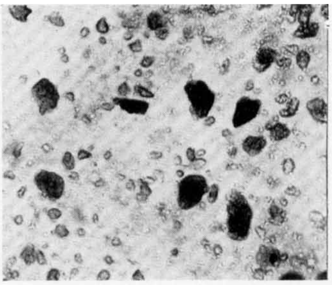


Fig. 3



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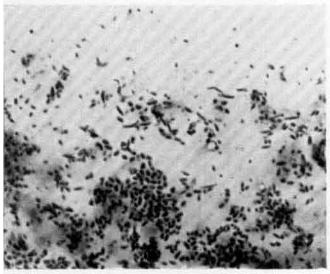

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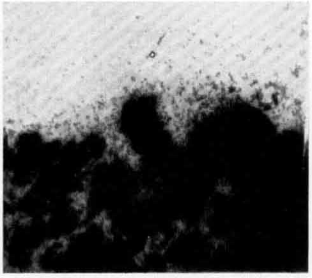

Fig. 6

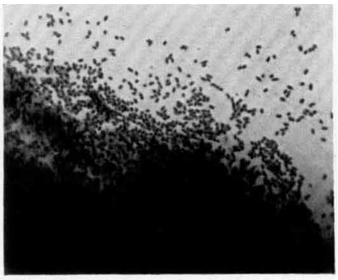

Fig. 7

H. SØRENSEN (*Facing p.* 34)