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SUOMEN KEMISTISEURAN TIEDONANTOJA

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Tor-Magnus Enari

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Observations on Bacterial Utilization of the Lignin from Brown-Rotted Spruce Wood and of Brauns' Native Lignin.

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Mixed populations consisting entirely of bacteria, proliferating in aerated liquid cultures with finely ground brown-rotted wood as substrate, were shown to cause a decrease of the lignin during 25 days' incubation. These populations could be maintained in numerous subsequent monthly growth passages with isolated lignin as their sole source of energy. The methoxyl content of such cultures decreased during bacterial growth. Pure cultures of *Mycobacterium rhodochrous* and *M. fortuitum* isolated from the mixed cultures on isolated lignin were shown in respirometric experiments to oxidize preparations of lignin extracted with alkaline ethanol from brown-rotted wood, and Brauns' native lignin.

It was shown that lignin is bound non-enzymatically to the bacteria. This adsorption is promoted by an acid reaction. The respiratory activity of bacteria on which lignin had been adsorbed was not affected, as concluded from the unchanged endogenous respiration, and oxidation of glucose and protocatechuic acid.

Introduction

The biological degradation of lignin in plant residues, which takes place mainly in the soil, is a process known to be mainly due to the action of extracellular polyphenoloxidase-producing fungi (Lindeberg, 1955; Flaig, 1964; Haider, 1966; Rösch, 1966). Reports on lignin-utilizing bacteria are sparse and inconsistent. Zobell and Stadler (1940) isolated lignoclastic bacteria from lake waters and deposits. Higuchi *et al.* (1956) reported two *Pseudomonas fluorescens* strains to be capable of oxidizing Brauns' native lignin, ethanol lignin and the acetone-solube fraction of sulphuric acid lignin. Sørensen (1962) isolated from various soil samples *Pseudomonas* and *Flavobacterium* strains which proliferated on Brauns' lignin on silica gel plates. Jaschhof (1964) reported on lignin-utilizing bacteria which were isolated

from lignite. Sundman *et al.* (1964) demonstrated that mixed cultures of bacteria and yeasts are as efficient as hyphomycete-containing soil microfloras in degrading the lignin of brown-rotted wood. Bacteria which actively utilize lignin dimers have repeatedly been isolated from various environments rich in wood residues (Konetzka *et al.*, 1952; Tabak *et al.*, 1959; Sundman, 1964 a, b, 1965).

Though the view has been expressed that the autochthonous bacterial flora of the soil is involved in the decomposition of lignin (Laboreur, 1954; Alexander, 1961), there has not been sufficient information available to support such a view and even in recent reviews on the subject (Schubert, 1965; Hurst and Burges, 1967) the biological degradation of lignin has been treated as a fungal process.

In order to extend our knowledge of the bacterial action on lignin, some experiments have been undertaken in which finely ground brown-rotted spruce wood, lignin preparations isolated from such wood, or Brauns' native lignin from spruce wood were exposed to the activities of mixed or pure cultures of bacteria. The results of these experiments are reported below.

Materials and methods

Origin of bacteria. Enrichment cultures of soil bacteria on brown-rotted wood were set up in the following way: One per cent suspensions (w/v) of soil samples rich in decaying wood residue were beaten for 60 sec. in a homogenizer. The coarse particles were allowed to settle and 1 ml portions of the supernatants were used as inocula in aerated culture vessels containing 250 mg of finely ground, water-extracted, brown-rotted spruce wood in 50 ml of a mineral nutrient solution supplemented with soil extract and with actidion (0.1 mg/ml) to prevent fungal growth in the first growth passages. The composition of the nutrient solution has been published (Sundman *et al.*, 1964). The aerated cultures were subcultured every third week by 0.5 ml transfers. From the third passage on the actidion could be omitted, without the appearance of fungal growth.

The soil samples, designated K, L, M, and O were the same as used for isolation of lignanolytic bacteria by Sundman (1965). They represented decaying sawdust (K), a decaying stump of Norwegian spruce (*Picea abies*, syn. *Picea excelsa*) (L), The uppermost humus horizon of a spruce stand (M), and a decaying pile of brushwood (O).

The brown-rotted wood and the lignin preparations used as energy sources for the bacteria. The brown-rotted wood was a finely ground natural specimen from a rotted stump of Norwegian spruce. It contained on a dry matter basis 68.3 per cent of sulphuric acid lignin, 14.1 per cent of carbohydrate, determined colorimetrically as glucose with anthrone reagent after complete hydrolysis with sulphuric acid. Further details of the brown — rotted wood specimen were given by Sundman *et al.* (1964).

Lignin preparations were isolated from various specimens of brown-rotted spruce wood by digestion with 0.5 N sodium hydroxide in 57 per cent (v/v) ethanol at room temperature according to the method of Phillips as described by Brauns (1952). The crude, acid-precipitated lignin was repeatedly precipitated from dioxan with diethyl ether, and washed with petrol ether as previously described (Sundman, 1964 b). The two preparations used in this work were designated ALDp and ALHp. Their mean content on a dry matter basis of sulphuric acid lignin was 97.4 per cent, of methoxyl 13.7 per cent, and of carbohydrate 0.5 per cent.

Brauns' "native lignin" was isolated from a freshly cut 15-year-old Norwegian spruce according to the method of Brauns (1952).

The lignin preparations were stored under aseptic conditions in a nitrogen atmosphere at room temperature. When intended as energy sources for bacteria the preparations were dissolved in dilute sodium hydroxide to give about 0.2 per cent (w/v) solution, and neutralized with hydrochloric acid to pH 6.

Lignin determinations. Lignin was determined in four ways. *The sulphuric acid method,* as described by Hägglund (1951), was for the present purpose modified by omission of the acetone extraction. It had been shown (Sundman *et al.*, 1964) that the acetone-soluble portion of the brown-rotted wood contained a considerable amount of methoxyl groups (8.2 per cent) and was largely soluble in ethanolic sodium hydroxide (77.5 per cent). It was therefore concluded that part of the biologically enriched and transformed lignin of brown-rotted wood went into solution upon treatment with acetone. The sample to be analysed was reduced from 1 g, as in the original method, to 250 mg. Further, the reaction mixture was diluted with 100 ml of water instead of 320 ml, left over night at room temperature, and digested on a boiling water bath for 2 h, instead of being refluxed for 6 h. It was found that after this modified hydrolysis no carbohydrate remained in the undissolved part of the brown-rotted wood. *The thioglycolic acid method* of Holmberg, as described by Bengtson (1936), was used without modifications to determine lignin in 250 mg samples of brown-rotted wood. *Colorimetric determination with the Folin-Denis reagent,* as described by Enkvist *et al.* (1956) was used without modifications for determination of 0.1—1 mg of lignin. *Spectrophotometric determination:* 4—10 mg of lignin or a corresponding amount of the acid sediment of a lignin culture was dissolved in 10 ml of dioxane. The solution was diluted with water to give a solution of about 2.5 mg of lignin/100 ml. The ultraviolet absorption of this solution was recorded and the lignin content calculated from the absorbance at the maximum around 280 nm, taking the dilution into account.

Determination of methoxyl groups. The method of Vieböck and Schwappach was applied according to the description given by Houben and Weyl (1953). The determinations were made in duplicate or triplicate employing 0.01 N sodium thiosulphate as titrant.

RESULTS

Bacterial utilization of lignin in aerated enrichment cultures on brown-rotted wood.

If the aerated enrichment cultures described above in the paragraph ,,origin of bacteria" were supplied with finely ground brown-rotted wood which had been treated with boiling water, and washed with water at 50° until the wash water gave negative results in tests for phenols and reducing sugars (Sundman *et al.*, 1964), it was on repeated occasions found that upon incubation at 28° the lignin content of the wood decrased by 2 to 4 per cent as compared with sterile control culture vessels. Table 1 shows the results of a statistically reliable experiment. Plating of such enrichment cultures on KYE agar (Sundman, 1965) and on acid glucose agar (Sundman *et al.*, 1964) did not reveal the presence of any other microbes but bacteria. The viable counts were of the order of 10^7 colonies/ml.

Table 1.

Decrease of lignin in finely ground brown-rotted wood on incubation with a mixed bacterial population.

Aerated culture vessels containing 50 ml medium supplied with 250 g air-dry, water-extracted wood were incubated for 25 days at 28° prior to analysis. Lignin was determined in culture deposits with the aid of thioglycolic acid and sulphuric acid methods. The significance of the results was calculated according to Student's t-test, taking Bessel's correction into account (Moroney, 1963)

Culture vessel No.	Treatment	Lignin determination method	Lignin recovered mg	mean	Decrease of lignin mg	per cent
1	inoculated	thioglycolic acid	153.0			
2	»	»	155.6			
3	»	»	153.1	152.9	5.3	3.4**)
4	»	»	150.0			
5	sterile control	»	157.9			
6	»	»	158.2			
7	»	»	160.7	158.2	0	0
8	»	»	159.0			
9	»	»	155.0			
10	inoculated	sulphuric acid	167.4			
11	»	»	167.4			
12	»	»	170.0	168.3	4.4	2.6***)
13	»	»	169.0			
14	»	»	167.6			
15	sterile control	»	173.8			
16	»	»	172.2			
17	»	»	172.3	172.7	0	0
18	»	»	173.3			
19	»	»	171.8			

**) The figure is significant at the P-level 0.02—0.001.

***) The figure is significant at the P-level < 0.001.

The results presented in Table 1 are considered to be proof of slight bacterial participation in the degradation of lignin of water-extracted brown-rotted wood. If the water extraction of the finely ground brown-rotted wood was omitted (13.3 per cent of dry weight was soluble in hot water) or if the wood powder was alternatively extracted with acetone, no decrease of the lignin content could be detected during three weeks incubation with bacterial enrichment cultures.

Development of mixed bacterial cultures in liquid medium where lignin served as sole added source of energy.

Bacterial populations which had developed in aerated enrichment cultures on brown-rotted wood as described above, and which originated from the four soil samples investigated, were subcultured by transfer of 20 μl portions into 10 ml batches of lignin medium contained in 50 ml Erlenmeyer flasks. The lignin medium was composed of equal parts of 0.2 per cent (w/v) lignin solution and a mineral solution with sodium nitrate as nitrogen source (double strength K solution according to Sundman, 1965). Bidistilled water was used for preparation of all solutions. The culture flasks were covered with straight aluminium caps, and weighed after inoculation. The lignin cultures were incubated at 28° for 3—4 weeks. Parallel lignin-free cultures, and non-inoculated lignin medium were incubated as controls. Loss of weight due to evaporation during incubation was substituted by addition of sterile distilled water. The proliferation of the bacteria in the cultures was followed by making repeated plate counts on KYE agar. Only those cultures in which the plate counts exceeded 10^7 colonies/ml were further transferred by 20 μl transfers to fresh lignin medium every 3—4 weeks.

After two growth passages in lignin medium of 18 bacterial populations from aerated enrichment cultures on brown-rotted wood, there remained only six populations which continued to multiply in the lignin medium. Upon growth of the bacteria the originally clear lignin medium turned turbid and a flaky sediment was formed. These six mixed cultures of bacteria continued to proliferate in the lignin medium, with regular monthly 20 μl transfers to 10 ml fresh medium, during two years, after which the experiment was discontinued.

The composition of the bacterial populations which developed in the lignin cultures was continuously determined. For determination of the composition of a population all the colonies developing on a suitable KYE agar plate, or on a sector of it, upon dilution plating, about 30 colonies in all of each population, were transferred to stab cultures in semisolid KYE agar. Each isolate was classified according to the following seven characteristics: 1) pigmentation and morphology of colonies, 2) type of growth in stab culture, 3) results of tests for nitrate reductase, and 4) oxidase, 5) type of glucose metabolism (Hugh and Leifson, 1953), 6) Gram reaction, and 7) morphology of Gram-stained smears of young (24—48 hr) and aged (14 days) cultures. It was found that throughout the growth passages in lignin medium, from the fourth passage on, when the first isolation of bacteria was undertaken, the composition of the populations showed considerable constancy.

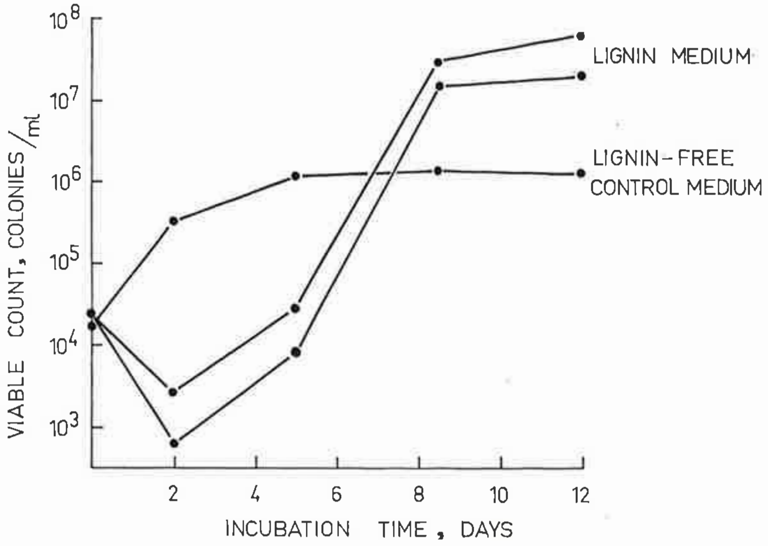

Fig. 1. *Plate counts on KYE agar of mixed bacterial culture „20", when grown in lignin medium and in lignin-free control medium. The culture was dominated by* Mycobacterium rhodochrous.

Each population contained a restricted number (3—5) of recurrent types of bacteria. Two of the populations contained mainly *Mycobacterium*-like organisms, identified as *M. fortuitum**). In two other populations the majority of the isolates resembled corynebacteria. The main organism of one of these populations was identified as *M. rhodochrous**). The remaining two populations contained mainly Gram-negative and Gram-variable single rods, which were not further identified.

Upon incubation of the lignin medium the viable count of the cultures regularly decreased during the first few days of incubation as compared with lignin-free control cultures (Fig. 1). This drop in the viable count of bacterial suspensions upon inoculation into lignin-rich media was also demonstrated by Zobell and Stadler (1940).

During the growth of the bacterial populations in lignin medium, the methoxyl content of the medium decreased by 10 to 30 per cent. All the populations investigated caused a diminution of the methoxyl content, those dominated by *M. fortuitum* usually being the most efficient in this respect (Table 2).

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* Identification of the lignin-utilizing mycobacteria was kindly performed by Dr. Ruth Gordon, New Brunswick, whose help is gratefully acknowledged.

Table 2.

Decrease of methoxyl of soluble lignin during growth of mixed bacterial populations.

Methoxyl determinations of 1 ml samples of lignin cultures which had been incubated for 2 weeks at 28° and 3 ½ months at 20° prior to analysis.

Culture	*Methoxyl recovered*		*Decrease of methoxyl*	
	µg	mean	µg	per cent
Sterile control	123.8 126.9	125.4	0	0
„1”, corynebacteria-dominated	96.4 95.9	96.2	29.2	23.3
„13”, *M. fortuitum*-dominated	95.9 94.9	95.4	30.0	23.9
„14”, *M. fortuitum*-dominated	98.5 95.4	96.9	28.4	22.7
„15”, single rods	102.6 100.0	101.3	24.0	19.2
„19”, single rods	107.3 102.6	105.0	20.4	16.3
„20”, *M.rhodochrous*-dominated	101.1 100.0	100.6	24.8	19.8

With the aid of spectrophotometric lignin determination, however, it was not possible to demonstrate a systematic decrease in the amount of the lignin in the medium during the growth of the bacterial populations, though changes in the shape of the absorption curves of the lignin occurred, as compared with parallel sterile controls of the lignin medium.

Oxidation of lignin in respirometric experiments with washed organisms of pure cultures.

After it had been demonstrated that lignin degradation occurred in aerated cultures of mixed bacterial populations in suspensions of powdered brown-rotted wood, and that such mixed bacterial populations proliferated in numerous subsequent growth passages in a medium in which lignin served as sole source of energy, with a concomitant decrease of the methoxyl content of the lignin, it was considered of interest to investigate the respirometric activity on lignin of pure cultures of bacteria from these mixed populations. The dominating organisms in three of the six mixed bacterial cultures on lignin, *viz. M.rhodochrous* 2001, *M.fortuitum* 1301, and *M.fortuitum* 1401, were taken for such work.

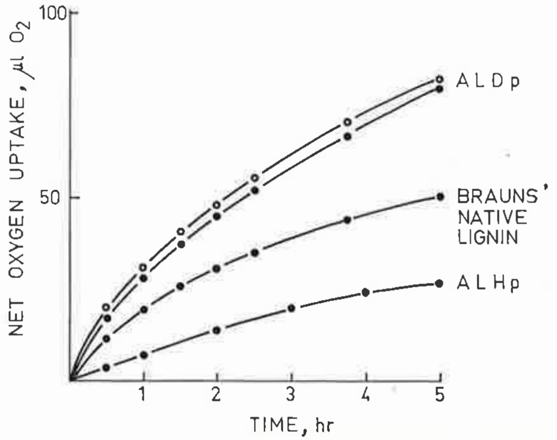

Fig. 2. *Oxidation of various lignin preparations by washed intact lignin-utilizing* Mycobacterium fortuitum *cells.*

The Warburg vessels contained 2 mg of lignin preparation, and 7.5 mg dry weight of bacteria in 1.5 ml M/45 phosphate buffer of pH 6.5, and 0.2 ml of 20 per cent (w/v) potassium hydroxide in centre well.

Legend: ● = bacteria grown for ten growth passages on KYE agar supplemented with 0.01 per cent (w/v) ALDp lignin preparation.

○ = bacteria grown for ten growth passages on KYE agar without lignin.

The bacteria were grown in Roux bottles at 28° for 2 to 4 days on KYE agar supplemented with glucose and yeast extract (Sundman, 1965), or on nutrient agar. The organisms were dislodged with the aid of sterile glass beads and M/15 phosphate buffer of pH 6.5, washed three times, resuspended in the same buffer, and stored in the refrigerator until used in the respirometric experiments.

With such washed bacteria of the *Mycobacterium* strains investigated the oxygen uptake was increased in the presence of Brauns' native lignin and of both lignin preparations isolated from brown — rotted wood. The preparation ALHp caused a markedly smaller oxygen uptake than ALDp or Brauns' lignin, Fig. 2.

Inclusion of lignin in the agar medium on which the bacteria were produced had no influence on the oxygen uptake in the presence of lignin with any one of the three organisms studied. This is illustrated by Fig. 2, where oxidation of lignin preparations ALDp with lignin-adapted and non-adapted bacteria is given.

The oxidation of the lignin preparations was dependent on the age of the bacterial suspension. Though endogenous respiration of the bacteria and their capacity for oxidizing a number of lignin-related monomeric compounds remained unchanged for about 10 days at 5°, the lignin oxidation decreased with age.

Interaction between dense bacterial suspensions and lignin.

The above-reported results of respirometric experiments with pure cultures isolated from mixed bacterial populations which had proliferated through numerous growth passages with lignin as the sole source of energy, strengthened the impression that the lignin preparations supplied were utilized by the bacteria. Since, however, as stated above, no systematic decrease of the lignin could be established during mixed bacterial growth in the lignin medium, an attempt was made to demonstrate utilization of lignin with dense suspensions of pure cultures in the following way. Bacteria were produced as for the respirometric experiments. Washed bacteria equivalent to 30 or 50 mg dry weight, and 4 mg of lignin preparation ALHp in 4 ml M/60 phosphate buffer of pH 5.9 were shaken in 50 ml Erlenmeyer flasks on a reciprocal shaker at 28° for a maximum of 24 hr. After various times, duplicate flasks were taken for analysis. The content was acidified with hydrochloric acid to pH 2, and transferred to a centrifuge tube. The precipitate containing bacteria and lignin was spun down (4400 r/min, 20 min), and the supernatant discarded. With 6 ml of dioxane in three portions the sediment was washed back into the Erlenmeyer flask, which was placed for 3 hr on a shaking machine in order to ensure that the lignin dissolved. The bacteria were spun down in the centrifuge. Erlenmeyer flask and sediment were washed twice with 1.5 ml dioxane. The dioxane supernatants were combined and filled up to 10 ml. Aliquots of this solution were taken for colorimetric and spectrophotometric lignin determinations. The bacterial pellet was quantitatively submitted to colorimetric lignin determination. Parallel control flasks containing lignin but no bacteria, and others containing bacteria but no lignin were submitted to the same analysis, and taken into account for calculation of the results.

With the aid of the above-described analytical procedure it was found that after as little as 5 minutes' contact between bacteria and lignin a considerable part of the lignin could not be recovered. The apparent decrease of lignin had mostly reached a maximum after 1 hr and did not change much during 24 hr. The *M. rhodochrous* strain caused about 25 per cent decrease of lignin and the *M. fortuitum* strains about 30 per cent decrease. Part of the recovered lignin was pertinaceously retained by the bacteria and could not be brought into solution by the shaking with dioxane for 3 hr included in the analytical method (Fig. 3).

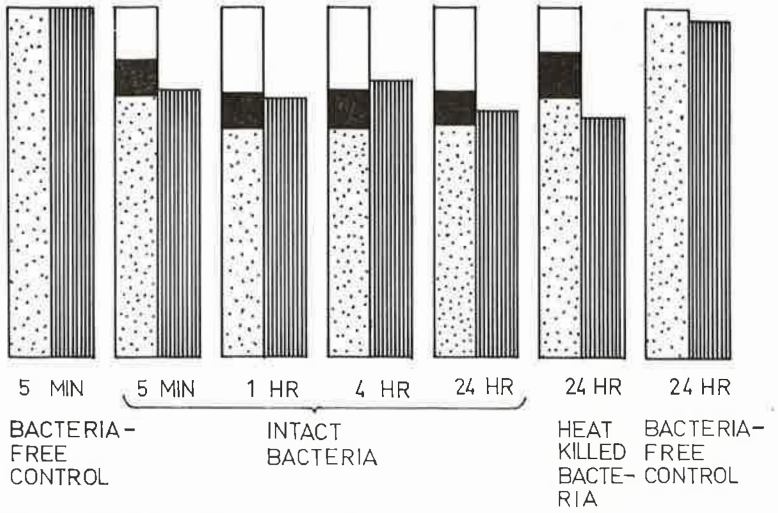

Fig. 3. *Apparent utilization and retention of soluble lignin by bacteria.*

Intact or heat-killed bacteria of *Mycobacterium rhodochrous*, equivalent to 50 mg dry weight, were shaken with 4 mg of lignin in 4 ml buffer for various times. The height of the staples corresponds to the amount of lignin recovered from the sediment of the acidified shaking suspension upon treatment with dioxane.

Legend:
□ apparent utilization of lignin.
■ lignin retained by the bacterial sediment, colorimetric determination.
▥ lignin brough into solution, spectrophotometric determination.
▨ lignin brought into solution, colorimetric determination.

The rapidity of the reaction between the bacteria and lignin, and the similarity of the results obtained with the three lignin-utilizing mycobacteria suggested that the observed decrease of lignin is not due to enzymatic activity of the bacteria, but merely the result of a non-enzymatic reaction between lignin and cell constituents. As shown in Fig. 3, experiment with heat-killed bacteria confirmed this. The non-specific character of the reaction observed was furthermore emphasized by demonstration of the fact that bacteria not known to be concerned with the breakdown of lignin, such as *Escherichia coli*, caused the same changes as the lignin-utilizing mycobacteria. Cyclolignan-utilizing agrobacteria (Sundman, 1965) also gave similar results (Table 3).

Table 3.

Apparent utilization of soluble lignin during 24 hr by dense suspension of various bacteria. The figures indicate percentages of lignin recovered after acidification, and subsequent extraction of the acidified sediment with dioxane, as compared with bacteria-free controls. (In parentheses the percentage of lignin retained by the bacteria. This amount is included in the preceding figure for lignin recovered.)

Batches of 50 mg dry weight of intact or heat-killed bacteria and 4 mg lignin in 4 ml buffer were shaken at 28° for the times indicated in the table. All figures are means of results with duplicate shaking vessels. Lignin was determined colorimetrically with the Folin-Denis reagent.

Organism	*M. rhodochrous* 2001		*M. fortuitum* 1301		*M. fortuitum* 1401	*Agrobacterium sp.* 08	*E. coli*
Experiment No	1	2	3	4	5	6	7
intact bacteria							
5 min	84.4 (9.5)	81.9 (13.0)	70.2 (3.1)	81.2 (3.1)	68.0 (8.3)	78.7 (12.1)	85.6 (2.3)
1 hr	75.0 (9.5)	74.0 (13.0)	68.2 (3.4)	67.2 (2.0)	67.8 (6.1)	80.2 (10.3)	84.5 (2.5)
4 hr	75.9 (10.4)	77.5 (12.7)	66.7 (2.0)	66.6 (2.2)	68.3 (6.8)	82.8 (12.1)	82.1 (2.1)
24 hr	75.4 (8.9)	74.6 (9.9)	71.7 (2.7)	67.2 (2.0)	66.6 (8.0)	79.8 (9.9)	81.3 (4.1)
heat-killed bacteria							
24 hr	87.3 (13.3)		82.7 (8.9)		70.2 (7.8)	81.3 (11.6)	71.9 (1.8)

The amount of lignin retained by the bacteria was characteristic of the bacterial strains investigated, as seen in Table 3, but could not be correlated with known features of the cell surface, such as Gram reaction or acid-fastness.

A retention of lignin on bacterial cells of the kind described above might occur in soil conditions, where soluble fractions of degraded lignin are present. In order to investigate such „tanning" of bacteria with lignin the retention of lignin was studied in greater detail.

It was established that if the acid sediment of bacteria and lignin was treated with M/15 phosphate buffer of pH 7.1, alternatively to dioxane, in order to bring the precipitated lignin into solution, then all the added lignin could be recovered (Table 4). The table shows that after the acid sediment of bacteria and lignin had been treated with dioxane, only 78.8 per cent of the lignin could be recovered, whereas upon treatment with phosphate buffer 101.4 per cent of the lignin was recovered. No explanation could be found for this repeatedly noted phenomenon.

Table 4.

The effect of analytical procedure on apparent utilization of soluble lignin by dense suspensions of bacteria.

Batches of 30 mg dry weight of intact bacteria of lignan-utilizing *Agrobacterium sp.* and 2 ml of lignin solution in a total volume of 4 ml M/30 phosphate buffer of pH 5.9, were shaken for 1 hr at 28°. The analytical procedure included acidification of shaking suspension and thorough treatment of acid sediment with dioxane or or with M/15 phosphate buffer of pH 7.1. All figures are means of four results with duplicate shaking vessels. Lignin was determined colorimetrically with Folin-Denis reagent.

Solvent used to dissolve lignin from acidified sediment	Dioxane		Phosphate buffer of pH 7.1	
	mg	per cent	mg	per cent
Total lignin added according to analysis of bacteria-free control	4.56	100	4.92	100
Lignin recovered from bacterial sediment upon treatment with solvent	0.31	6.8	0.52	10.6
Lignin recovered from solvent solution	3.28	71.9	4.47	90.8
Total lignin recovered	3.59	78.7	4.99	101.4
Apparent utilization of lignin	0.97	21.2	−0.07	−1.4
Total lignin	4.56	100.0	4.92	100.0

Further, it became evident that the "tanning" of bacteria with lignin was accentuated by the acid reaction. Table 5 shows the results obtained with *Agrobacterium sp.* in an experiment where bacteria and lignin was shaken for 1 hr, and part of the shaking batches were acidified prior to analysis. In the non-acidified batches lignin was determined separately in supernatant and sediment after the latter had been washed with phosphate buffer of pH 7.1. In the acidified batches only the acid sediment was taken for analysis, as previously described, using phosphate buffer of pH 7.1 to dissolve the lignin. It is shown in Table 5 that 1.5 per cent of the lignin was retained by the non-acidified cells, whereas 18.5 per cent of the lignin was retained upon acidification. The figure for total recovery of lignin in the non-acidified batches, 96.8 per cent, indicates that there might have been a small fraction of the lignin which was loosely adherent to the bacterial sediment, and which was removed by washing with the phosphate buffer.

Table 5.

The effect of acid environment on the retention of soluble lignin by dense suspensions of bacteria.

Batches of agrobacteria and lignin were shaken for 1 hr at 28° in phosphate buffer of pH 5.9 as described in Table 4. Half of the batches were acidified to pH 2 with hydrochloric acid prior to lignin determination. The lignin was redissolved in phosphate buffer of pH 7.1. For details see text. All figures are means of four results with duplicate shaking vessels. Lignin was determined colorimetrically with Folin-Denis reagent.

Treatment of suspension prior to lignin determination	acidified		not acidified	
	mg	per cent	mg	per cent
Total lignin added according to analysis of bacteria-free control	4.80	100	5.84	100
Lignin recovered from bacterial sediment	0.89	18.5	0.09	1.5
Lignin recovered from buffer solution	4.00	83.4	5.56	95.3
Total lignin recovered	4.89	101.9	5.65	96.8

Physiological activity of bacteria "tanned" with lignin, as compared with untreated bacteria.

In the humus layer of coniferous forests, the pH might be as low as 3.5 (Vézina, 1965; Schalin, 1968). In such soil, lignin-rich wood residues in all stages of decomposition are abundant, and it is possible that the bacteria present are submitted to the abovementioned „tanning" effect of solubilized lignin. Hence it was of interest to learn whether the adsorption of lignin on the bacterial surface has any influence on the physiological capacity of the bacteria, especially as Basaraba (1964) has shown that endogenous respiration and glucose oxidation of a number of soil microorganisms are repressed by treatment with various tannins.

We investigated the effect of treatment with lignin on endogenous respiration, glucose and protocatechuic acid metabolism with intact washed organisms of the lignan-utilizing *Agrobacterium sp.* K 17. The bacteria were grown in a fermentor for 47 hr at 28° in 1200 ml batches of KYE solution (Sundman, 1965) supplemented to contain 0.2 per cent (w/v) each of Bacto yeast extract and separately sterilized glucose. The medium was aerated with 1.5 l air/min. The bacteria were harvested by centrifugation, washed three times, and suspended in M/15 phosphate buffer of pH 5.9 to give suspensions of 10 mg dry weight/ml. Three 10 ml aliquots of this suspension were treated in the following way. *A*. One portion of 10 ml was shaken for 1 hr at 28° with 13 ml of a 0.2 per cent (w/v) solution of lignin preparation ALHp. The suspension was acidified to pH 2 and centrifuged. The acid sediment was washed twice with M/15 phosphate buffer of pH 7.1, and finally suspended in 10 ml of the buffer of pH 5.9 *B*. A second portion was treated similarly, omitting the lignin. *C*. A third portion was submitted to shaking only, omitting both lignin, acidification, and washings of the sediment. According to colorimetric lignin determinations, 10.6 per cent of the lignin added to portion A was retained by the bacteria.

Oxygen uptake due to endogenous respiration and oxidation of glucose was determined with the three above-described portions A, B, and C. The results are shown in Fig. 4. „Tanning" with lignin had no effect on endogenous respiration or glucose metabolism as shown by comparison of curves A with curves B of the figure. Results with protocatechuic acid oxidation gave the same result, *viz.* that the „tanning" with lignin had no effect on the protocatechuic acid-oxidizing capacity of the agrobacteria. On the other hand, acidification of the bacterial suspension to pH 2 with hydrochloric acid for about 40 min, as in the experiment illustrated in Fig. 4, had some bearing on the oxidative capacity of the bacteria (comparison of curves B with curves C).

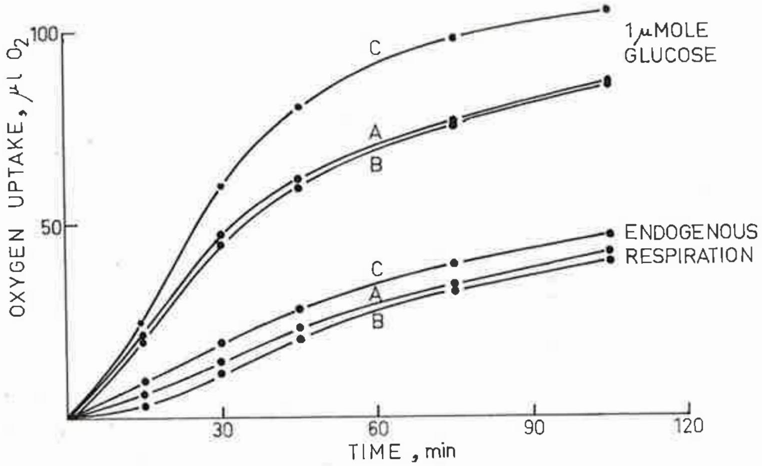

Fig. 4. *Effect of treatment with lignin on endogenous respiration and glucose oxidation by intact washed bacteria of lignan-utilizing* Agrobacterium sp.

The Warburg vessels contained 1 μmole of glucose, and 5 mg dry weight of bacteria in 1.5 ml M/30 phosphate buffer of pH 5.9, and 0.2 ml 20 per cent (w/v) potassium hydroxide in centre well.

Legend:
- A. Bacteria were shaken with lignin and acidified.
- B. Bacteria were shaken without lignin and acidified.
- C. Bacteria were shaken without lignin and not acidified.

DISCUSSION

In the preceding paragraphs evidence is presented for the bacterial utilization of coniferous lignin. The lignin preparations investigated, however, are not representative of the intact protolignin of the coniferous plant, and hence the results cannot give information about the participation of bacteria in the primary splitting of lignin.

A slight diminution of the lignin content of brown-rotted wood was shown to result from bacterial growth in aerated suspensions of the finely powdered wood during 25 days (Table 1). The results obtained with the sulphuric acid lignin determination method, indicating a 2.6 per cent decrease of lignin, should be considered minimal. There is an error, due to presence of protein, inherent in this method, which has not been corrected for. Part of the bacterial protein is hence included in the lignin recovered from the deposit in the inoculated culture vessels, thus diminishing the difference between sterile controls and inoculated cultures. The slight bacterially caused decrease of lignin, which is visualized in Table 1, possibly indicates that only a limited number of sites in the lignin structure are susceptible to the activity of the bacteria, and that no splitting of the lignin molecules occurs.

The mixed bacterial cultures growing on brown-rotted wood caused a decrease of lignin only if the wood had been exhaustively extracted beforehand with water. This has been explained to depend on the preferential utilization by the bacteria of the water-soluble compounds of the wood. On the other hand, brown-rotted wood which had been extracted with acetone showed no decrease of lignin upon exposure to bacterial cultures. Obviously the acetone treatment had not removed all the nutrients easily accessible to the bacteria.

The decrease of methoxyl content during bacterial growth on isolated lignin was well documented and shows that some of the methoxyl groups of the lignin are easily accessible to a variety of bacteria. This conforms with the results of Haider (1966), who, working with enzymatic dehydrogenation polymerizates of variously C^{14}-labelled monomeric lignin building units, found that a lignin-utilizing fungus split the methoxyl groups of the polymerizates more easily than the side-chain structures. Jaschhof (1964), on the other hand, reported that methoxyl content of lignin increased during growth of lignin-utilizing bacteria.

The adsorption on lignosulphonic acids and lignin on the surface of fungal mycelium and of bacteria is known since the works by Gottlieb and Pelczar (1951) and Raynaud *et al.* (1956). The phenomenon has been a pitfall in investigations on bacterial degradation of soluble lignins in those instances where lignin was determined in culture liquids after removal of the bacteria. It was demonstrated in the present work that the adsorption of lignin on bacteria was favoured by an acid reaction. The adsorption might be explained as a reaction between acidic groups (*e.g.* phenolic hydroxyl groups) of the lignin with basic groups of the amphoteric bacterial protein, the basic characteristic of which is accentuated in an acid medium. This fixation of lignin on the bacteria does not seem to affect their activity, as concluded from the unchanged endogenous respiration and oxidations of glucose and protocatechuic acid.

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Studies on the Nutritional Control of Cell Division of *Candida guilliermondii*

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The effect of different nutritional factors on the cell division of *Candida guilliermondii* has been studied. Two strains, a smooth and a rough colony variant were used.

Pyruvate as the sole carbon source causes the yeasts to grow as dividing, yeast-like cells, whereas citrate inhibits the cell division and results in pseudomycelial growth of the yeasts. The inhibiting effect of citrate can be reversed by pyruvate, cysteine, alanine, aspartate, divalent metals or by using enriched CO_2 in the atmosphere.

The results are discussed in the light of earlier studies and a hypothesis based on information from the literature and on the present results is presented.

A number of fungi may grow in two different cellular forms. Depending upon environmental conditions, they may grow in the form of a mycelium or pseudomycelium or in the form of yeast-like cells. This reversible interconversion of yeast and mycelial forms is known as dimorphism, which has been interpreted as an uncoupling of cell division from growth without impairment of growth[1].

Among the fungal species in which the nutrition-dependent dimorphism has been studied are *Sporotrichum schenckii, Histoplasma farciminosum, Histoplasma capsulatum, Candida albicans* and *Mucor rouxii*.

S. schenckii[2] and *H. farciminosum*[3] require CO_2 in the atmosphere (5—20 % CO_2) for the yeast growth. Addition of arginine or glycine to the medium also supported the yeast phase growth in *S. schenckii*[2]. The yeast-like cells of *H. capsulatum* required biotin[4], which had no effect on the mycelial growth of the same species. Furthermore, a sulfide or sulfhydryl group in an amino acid (cystine or cysteine) in the medium was essential for the yeast phase. The necessity of cysteine addition for the maintenance of the yeast phase of *H. capsulatum* was confirmed by Scherr[5] and Pine[6]. It was concluded that the role of -SH groups was to initiate yeast growth because under the growth conditions used, the -SH groups rapidly disappeared from the medium. When *H. capsulatum* was grown on a complex casamino acid medium supplemented with 0.5—2.0 percent citrate, a stimulation of the conversion from mycelial to yeast phase in 6 species of 16 tested was observed[7]. This effect was attributed to the inhibition of the mycelial phase by metal chelation as it could be reversed by addition of calcium or magnesium to the medium.

Extensive filamentation of *C. albicans* was observed, when a poorly utilized carbon source (starch, glycogen or dextrin) was used.[8]. This effect was reversed by addition of cysteine to the medium. It was also found, that in the presence of cobalt, *C. albicans* cells grew as mycelia, but cysteine reversed the effect[9].

The extensive studies on the dimorphism of *C. albicans* were conducted mainly by Nickerson and his colleagues[10,11]. The experimental data have led to a theory, according to which the disulfide bonds in the cell wall mannan-protein complex are reversibly reduced and oxidized by a particle bound enzyme, protein disulfide reductase during cell division. The importance of cysteine in the medium for the yeast-like growth has been attributed to its reducing power, necessary for the reduction of disulfide bridges in the cell wall during division.

In a recent review [12], studies of the physiological control of dimorphism in *M. rouxii* have been discussed. Anaerobiosis and carbon dioxide have been shown to be essential for the yeast-like development in most *Mucor*-species studied. Chelating agents and atmospheric oxygen inhibited yeast-like morphogenesis. On the basis of these results it has been proposed that carbon dioxide condenses with pyruvic acid to form malic acid. This is metabolized through oxaloacetic acid to aspartic acid. The increased aspartic acid content would then stimulate the formation of aspartic-acid-containing mannan-protein complex in the cell wall, necessary for the cell division and yeast-like growth of *M. rouxii*.

During our studies on the physiological effects of iron deficiency on *Candida guilliermondii*, a stable rough colony variant with a tendency towards mycelial growth appeared[13,14]. The present paper gives the results of the studies on the effect of different nutritional factors on the cell division of smooth and rough variants of *Candida guilliermondii*.

Materials and Methods

Organism. Candida guilliermondii (Cast.) Langeron et Guerra was used. The original strain is called smooth ("S") in the present study. The rough ("R") variant was isolated during prolonged growth of the yeast in iron deficiency[13,14]

Growth medium. Solid, synthetic medium was used throughout the study. The basic composition was as follows:

Carbon source	30 g
$(NH_4)_2HPO_4$	3 g
KH_2PO_4	0.2 g
K_2SO_4	1.5 g
$MgSO_4 \cdot 7\,H_2O$	0.25 g
Biotine	10 μg
Agar	15 g
Distilled water ad	1000 ml

The pH was adjusted to 6.0 and the medium sterilized at 121°C for 20 minutes. The medium was poured into sterile petri dishes, allowed to cool and inoculated.

The following carbon sources were used: maltose, glucose, sodium acetate, sodium pyruvate, sodium citrate, sodium succinate, sodium malate, sodium glutamate and cysteine.

As nitrogen sources we used: sodium glutamate, alanine, sodium aspartate, cysteine. When cysteine was used, it was also the sole sulphur source and magnesium was added as $MgCl_2$.

The basic medium contained 0.07 percent N, 0.0025 percent S, 0.07 percent P and 0.05 percent K. When different nitrogen sources or different sulphur sources were used, these percentages were kept constant. The possible nitrogen content in the various carbon sources was not taken into account. When acids were used as carbon source, the added amount (30 g) was calculated as free acid.

Growth in enriched carbon dioxide atmosphere took place with sodium citrate as the carbon source in 5 l wide-neck, airtight bottles as incubators. The desired percentages of carbon dioxide (1.0, 5.0 and 10.0 percent) were obtained by mixing weighed amounts of $CaCO_3$ with lactic acid at the bottom after the bottles with inoculated petri dishes were closed.

Estimation of cell division. After inoculation, the agar plates were incubated for 8 days at 30°C, after which the appearance of the colonies was examined and samples were taken for phase contrast microscopy in a "Thoma" counting chamber. In order to get a representative sample, a whole colony was taken and suitably diluted with 0.9 % NaCl to give a total count of 200—400 cells per 50 squares. Assuming the single or paired cells as yeast-like and cells in groups of three or more as pseudomycelial, both types were counted separately in 50 squares and the total number obtained by addition. The number of pseudomycelial cells was given as percentage of total cells.

From the samples, a stain with ammonium oxalate chrystal violet was also made for the microphotography.

Determination of pyruvic acid. Pyruvic acid was determined by the enzymatic method of Bücher *et al.*[14].

RESULTS AND DISCUSSION.

The effect of different carbon sources on the cell division of the smooth and rough variants of *Candida guilliermondii* is given in Table 1. When the yeasts were grown on citrate, 90 percent inhibition of cell division was observed in both strains.

Microscopical examination revealed bundles of pseudomycelia, as illustrated in Figures 1d and 2d. On the other hand, the growth on pyruvate resulted exclusively in dividing, yeast-like cells (Figs 1c and 2c). The other carbon sources tested gave varying results: maltose and glucose partially inhibited the cell division, but to a lesser degree than citrate; whereas succinate, acetate, glutamate and especially malate supported the growth as dividing cells.

Table 1. The effect of carbon source on the cell division of *Candida guilliermondii*.

Carbon source *	Yeast strain			
	Rough		Smooth	
	Colonial morphology	Pseudomycelial cells %	Colonial morphology	Pseudomycelial cells %
Maltose	R+	75	R±	61
Glucose	R+	68	S+	70
Acetate	R±	26	S+	27
Pyruvate	S+	4	S+	4
Citrate	R+	90	R+	91
Succinate	R±	38	S+	20
Malate	S+	25	S+	4
Glutamate	S+	26	S+	25
Pyruvate + Glucose **	—	51	—	12
Pyruvate + Citrate **	—	33	—	45
Carbon dioxide 1.0 % + Citrate	—	8	—	9
Carbon dioxide 5.0 % + Citrate	—	7	—	8
Carbon dioxide 10.0 % + Citrate	—	9	—	24

*) NH_4-phosphate as nitrogen source.

**) Both added in equivalent amounts to the basic medium.

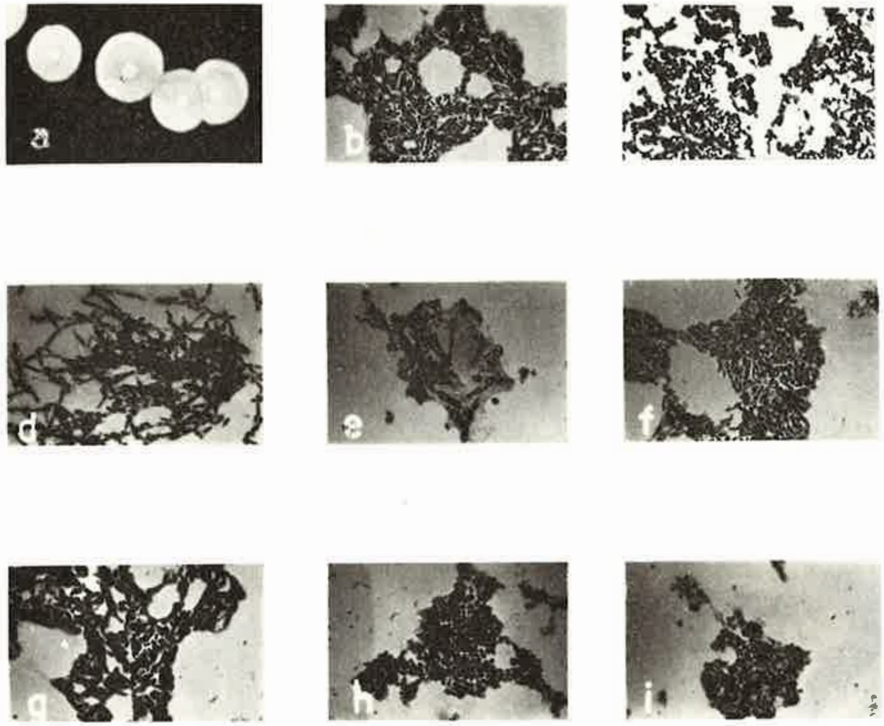


Figure 1. Cell morphology of *Candida guilliermondii* "S" (smooth) grown on different media. Cells stained with ammonium oxalate chrystal violet (enl. 240 ×). a) Colonial appearance on glucose agar.
The microscopical preparations were made from colonies grown on the following media: b) glucose agar, c) pyruvate agar, d) citrate agar, e) citrate+ pyruvate (1:1) agar, f) citrate agar + 1 % CO_2 in the atmosphere, g) citrate agar with cysteine as the sole source of nitrogen and sulphur (medium sterilized by autoclaving), h) citrate + magnesium agar (both added 10 g/l basic salt medium), i) citrate agar with aspartate as the sole nitrogen source.

The possibility of mutation was excluded by the observation, that the effects of citrate and pyruvate on the growth were completely reversible. After two passages on citrate agar the cell division capacity in both variants was fully restored when transferred to pyruvate agar and *vice versa*.

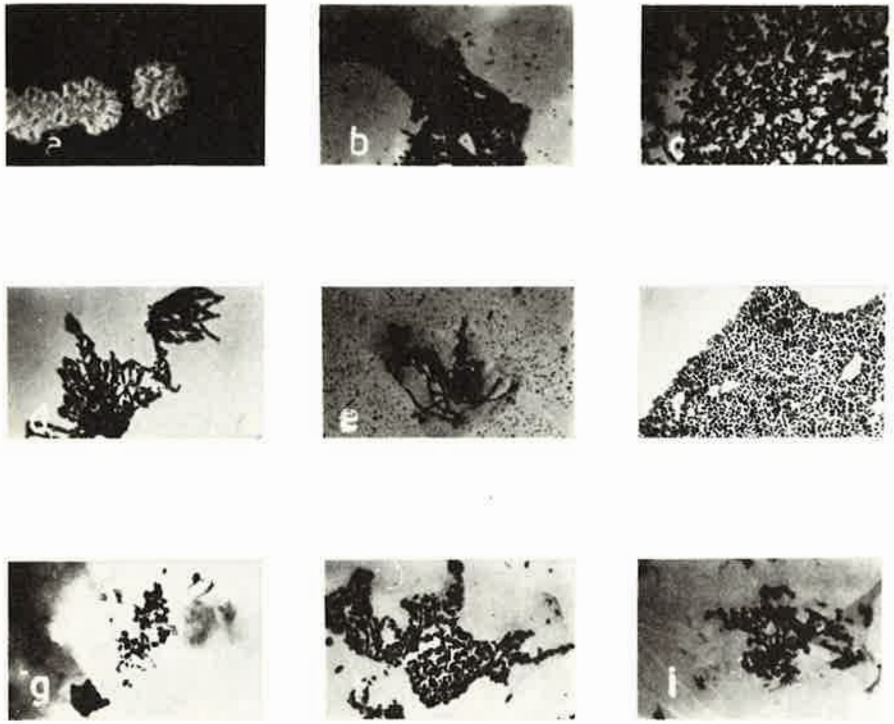


Figure 2. Cell morphology of *Candida guilliermondii* "R" (rough) grown on different media. Cells stained with ammonium oxalate chrystal violet (enl. 240 ×) a) Colonial appearance on glucose agar.
The microscopical preparations were made from colonies grown on the following media: b) glucose agar, c) pyruvate agar, d) citrate agar, e) citrate + pyruvate (1:1) agar, f) citrate agar + 1 % CO_2 in the atmosphere, g) citrate agar with cysteine as the sole source of nitrogen and sulphur (media sterilized by autoclaving), h) citrate + magnesium agar (both added 10 g/l basic salt medium), i) citrate agar with aspartate as the sole nitrogen source.

The tendency towards filamentation was reflected also in colony morphology. Thus colonies formed on citrate or maltose were rough with both variants, whereas growth on pyruvate and malate resulted in production of smooth colony appearance of the yeast strains.

Addition of pyruvate to citrate agar partially restored the cell division capacity of the yeasts (Figs 1e and 2e). A further improvement of the cell division was observed, when the con-

Table 2. The effect of nitrogen source on the citrate inhibited cell division of *Candida guilliermondii.*

Nitrogen source + Carbon source	Yeast strain	
	Rough	Smooth
	Pseudomycelial cells %	Pseudomycelial cells %
Citrate + Alanine	35	57
Citrate + Aspartate	47	33
1) Citrate + Cysteine	56	5
1) Citrate + Cysteine*	61	56

*) Medium sterilized by filtration.

1) Cysteine was used as the sole nitrogen and sulphur source.

centration of the atmospheric carbon dioxide was raised (1—5 %) (Figs 1f and 2f and Table 1). This effect of CO_2 is similar to the phenomenon found in *S. schenckii* [2], *H. capsulatum* [3] and *Mucor*-species[12]. The highest concentration tested (10 % CO_2) resulted partially in the production of anomalous cell forms, in the smooth variant the pseudomycelial growth was to some extent enhanced.

Attempts were also made to reverse the effect of citrate by changing the nitrogen source (Table 2). Both alanine and aspartate stimulated growth as dividing cells, aspartate being slightly more effective (Figs 1i and 2i).

Cell division was considerably promoted, when inorganic nitrogen and sulphur were replaced by equivalent amounts of cysteine. Two types of sterilization procedures of cysteine media were tested. If autoclaved, addition of cysteine caused about 95 percent of the smooth variant cells to grow as dividing cells. Cysteine-citrate medium promoted the growth of yeast-like cells also in the rough strain but to a lesser degree than in the smooth variant (Figs 1g and 2g). When cysteine was sterilized by filtration, the division promoting effect was less pronounced, but still observable.

Table 3. The pyruvate concentration in the cysteine media.

Medium	Sterilization process	Pyruvic acid found mg/100 ml
4 % pyruvate in basic medium, no agar	Autoclaving	4000
1 % cysteine in basic citrate medium, no agar	Autoclaving	220
1 % cysteine in basic citrate medium, with agar	Autoclaving	220
0.5 % cysteine in basic citrate medium, no agar	Membrane filtration	1
0.5 % cysteine in basic citrate medium, with agar	Membrane filtration	1

The effects of cysteine and pyruvate additions to the citrate medium were very similar. It was therefore speculated that cysteine might influence the yeast growth through nonenzymic formation of pyruvate during the media preparation. Table 3 gives the results, when the concentration of pyruvate in the nonincubated growth media were determined after different sterilization procedures. 22 percent of the added cysteine was transformed to pyruvate during autoclaving. Much less pyruvate was found in media sterilized by filtration, but even here a positive reaction was always observed. These results support the idea that cysteine promotes the growth of dividing cells because of transformation to pyruvate and explain also why cysteine medium sterilized by filtration was less effective than the medium sterilized by autoclaving.

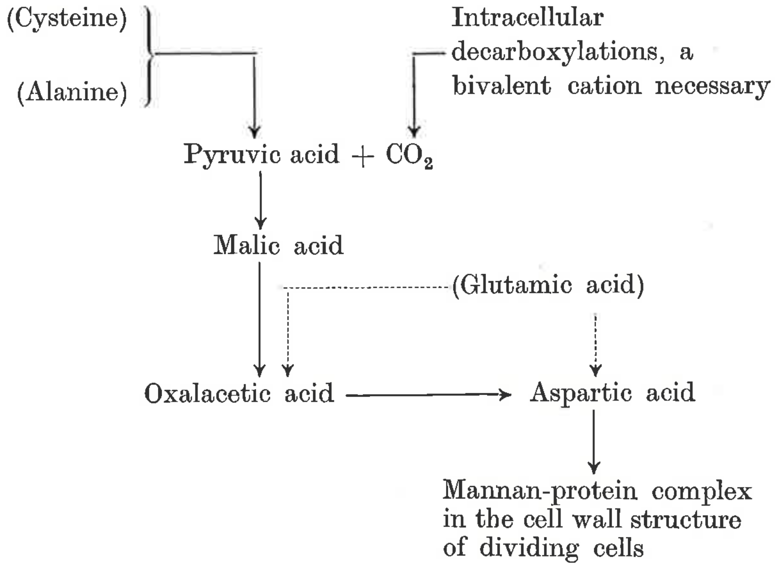
Finally, the effect of metals in reversing the division inhibiting effect of citrate on the yeast cells was tested (Table 4). Iron, zinc or magnesium all had a slight effect in the presence of citrate. When magnesium was added in excess to compete with the metal chelating capacity of added citrate, a complete reversal to yeast-like dividing cells was observed (Figs 1h and 2h). Thus addition of citrate to the medium seems to chelate the divalent cations as also reported for *H. capsulatum*[7]. In *C. guilliermondii*, however, the chelation of metals does not inhibit the mycelial phase but promotes it as in *M. rouxii*[12].

Table 4. The effect of metals on the citrate inhibited cell division of *Candida guilliermondi*.

Carbon source + Metal addition	Yeast strain	
	Rough	Smooth
	Pseudomycelial cells %	Pseudomycelial cells %
Citrate + Iron*	60	53
Citrate + Zinx**	44	72
Citrate + Magnesium***	88	47
Citrate + Magnesium****	26	22

*) 2.5 mg $FeSO_4 \cdot 7\ H_20$/1 basic citrate medium.
**) 0.25 g $ZnCl_2$/1 basic citrate medium.
***) 2.5 g $MgSO_4 \cdot 7\ H_20$/1 basic citrate medium.
****) 10 g $MgCl_2$ + 10 g citrate/1 basic salt medium.

The hypothesis of dimorphism presented on the basis of the studies with *C. albicans*[10, 11] and *M. rouxii*[12] is in agreement with the present results and can be extended to a following scheme:



The growth on pyruvate, malate or glutamate results in dividing cells, because these substrates are converted to aspartic acid within the cell. The differences in the effects are probably due to the different permeability of the substrates. The yeast-like growth on alanine or cysteine depends on the fact, that they can be converted to pyruvate, the former enzymatically and cysteine also during the preparation of the growth media.

The earlier results with other strains indicate, that cysteine -SH groups were necessary for cell division[5,6,8], but in these studies the media were often sterilized by autoclaving, which obviously leads to formation of pyruvate.

We assume that the atmospheric carbon dioxide (0.3 %) is too low for the condensation reaction with pyruvate and must be supplemented with the carbon dioxide formed through decarboxylations. Thus, citrate inhibits the cell division mainly because it chelates metals such as manganese and magnesium, both of which are necessary in different decarboxylations in the cell. This suggestion is supported by the observation that addition of high amounts of magnesium reverses the effect of citrate. High atmospheric carbon dioxide counteracts the inhibitory effect of citrate because it provides the lacking substrate for the condensation reaction.

The effect of pyruvate in promoting the cell division in the presence of citrate can also be interpreted along similar lines. A very high amount of pyruvate would enhance the condensation reaction although only the atmospheric concentration of carbon dioxide (0.3 %) were available. Furthermore, pyruvate decarboxylation requires zinc as the divalent metal, which might not be as easily chelated by citrate as magnesium. Thus pyruvate addition would provide also CO_2 for the condensation reaction.

When aspartate is added to the citrate medium, no extra carbon dioxide is necessary because aspartate is directly incorporated to the cell wall proteins, only its poor permeability might be rate limiting.

It should be noted, however, that although the present scheme can be useful as a working hypothesis, it has not been proven. It does not include all the effects of citrate within the cell nor does it reveal, how the yeasts grow on citrate. It is probable, however, that citrate, when used as a carbon source, is not completely oxidized in the cells, as the oxygen uptake with citrate as a substrate is only half of the uptake when for example succinate is used.[17].

Further studies are in progress to investigate the tenability of the present working hypothesis by measuring the concentrations of the different intermediates on the pathway of aspartate formation. Whether cysteine acts through its -SH groups or through conversion to pyruvate, remains also to be proved.

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On the Anomalous Polarographic Reduction of Certain Anions VIII. The Polarographic Reduction of Trichloroammineplatinate(II).

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The polarographic reduction of $Pt(NH_3)Cl_3^-$ has been studied and the influence of pH and hydrolysis on the current-voltage curves determined. The general polarographic behaviour of this complex was found to be in accordance with results obtained earlier for other platinum (II) complexes.

Introduction.

The polarographic measurements reported here are intended to complement those measurements on platinum (II) complexes reported in an earlier article (1).

Experimental.

The cell, electrodes and measuring equipment were the same as described in reference 1.

The potassiumtrichloroammineplatinate(II) was synthesized from cis-$Pt(NH_3)_2Cl_2$ according to the method described by Elleman et al. (2). Analysis: Pt 53,6 %, H 0,8 %, N 3,7 %. Calcd. for $K[Pt(NH_3)Cl_3]$: Pt 53,55 %, H 0,84 %, N 3,91 %. The UV-spectrum of the $Pt(NH_3)Cl_3^-$ in aqueous solution corresponded to that reported in the literature (3,4).

Results.

Results of the polarographic measurements are shown in figure 1 for a $K[Pt(NH_3)Cl_3]$ concentration of 4×10^{-4} moles/l.

In freshly prepared solutions a small current minimum at about — 1,1 V_{SCE} can be seen and pH has a minor influence on the current-voltage curves.

On addition of gelatine the current minimum increases considerably and the maximum at the beginning of the polarogram disappears (curves 1 & 3, fig. 1).

Current-time curves for $Pt(NH_3)Cl_3^-$ in 1-M $NaClO_4$ were measured and values for the exponent obtained from the curves are tabulated in table 1.

Table 1. Values of the exponent x for the instantaneous i-t relation, $i = kt^x$, as measured in 4×10^{-4} M $K[Pt(NH_3)Cl_3]$ in 1-M $NaClO_4$.

E, V_{SCE}	x
−0,10	0,29
−0,20	Irregular curve
−0,40	»
−0,60	0,27
−1,00	0,30
−1,20	0,26
−1,40	0,33
−1,60	0,23

Discussion.

The polarogram of a freshly prepared solution of the complex $Pt(NH_3)Cl_3^-$ in 1-M $NaClO_4$ (curve 1, fig. 1) is quite similar to that of a hydrolysed solution of $PtCl_4^{2-}$ in the same supporting electrolyte, where the predominant species is the complex $Pt(H_2O)Cl_3^-$ (1). This result is also to be expected because of the very similar constitution of $Pt(H_2O)Cl_3^-$ and $Pt(NH_3)Cl_3^-$.

In a neutral hydrolysed solution of $Pt(NH_3)Cl_3^-$ the predominating species is trans-$Pt(NH_3)(H_2O)Cl_2$ together with some of the corresponding cis-isomer (4). As a result of the substitution of one chloride with an aquo group the reduction process becomes much more difficult as can be seen from curve 2, figure 1.

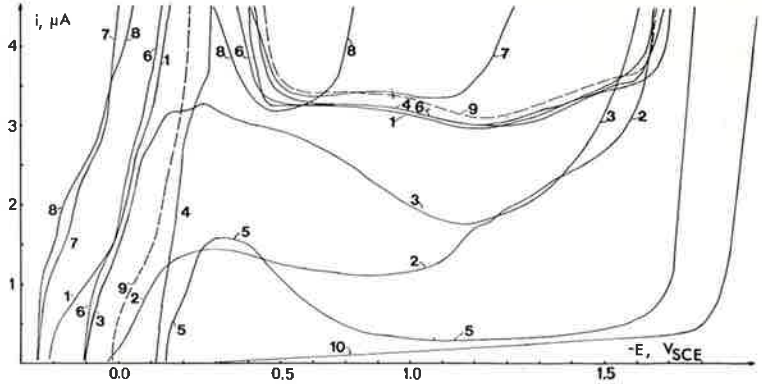

Fig. 1. Polarograms of 4×10^{-4} M $K[Pt(NH_3)Cl_3]$ in different electrolytes and at different pH. The ionic strength of the solutions adjusted to 1,0 with $NaClO_4$.

Curve	Electrolyte	pH	Remarks
1	1-M $NaClO_4$	6	fresh solution
2	»	6	3 days old solution
3	1-M $NaClO_4$ + 0,01 % gelatin	6	fresh solution
4	0,13-M NaOH + 0,87-M $NaClO_4$	12,6	» »
5	» + »	12,6	3 days old solution
6	borate buffer	8,9	fresh solution
7	acetate buffer	4,5	» »
8	1-M $NaClO_4$ + 0,01-M $HClO_4$	1,9	» »
9	1-M NaCl(dashed line)		» »
10	Residual current in 1-M $NaClO_4$		

A more quantitative comparision with the polarographic behaviour of cis- and trans-$Pt(NH_3)_2Cl_2$ is difficult to make because it is not possible to obtain pure solutions of cis- or trans-$Pt(NH_3)(H_2O)Cl_2$. In a hydrolysed alkaline solution, where the two isomers of $Pt(NH_3)(OH)Cl_2^-$ are present (4), the reduction process is still slower than in a hydrolysed neutral solution (curve 5, fig. 1). This is in accordance with the results reported earlier (1), according to which the reduction of a platinum(II) complex is always rendered more difficult when a H_2O group is substituted by a hydroxyl group.

The results of the measurements in 1-M NaCl (curve 9, fig. 1), which are similar to the polarograms obtained by Nikolaeva-Fedorovich et al. (5), show that reduction of $Pt(NH_3)Cl_3^-$ proceeds in the same way in 1-M NaCl as in 1-M $NaClO_4$.

Finally the results of the measurements of *i-t* curves show that the current is not purely diffusion controlled.

During the course of this work an attempt was also made to study the polarographic reduction of the complex $Pt(NH_3)_3Cl^+$. However, the synthesis of $[Pt(NH_3)_3Cl]Cl$ accordning to the method of Tschugaev (6) was not successful. Nevertheless, one can estimate that the reduction of $Pt(NH_3)_3Cl^+$ should be at least as hindered as that of cis-$Pt(NH_3)_2(H_2O)Cl^+$, which is present in a hydrolysed neutral or acid solution of cis-$Pt(NH_3)_2Cl_2$, and which causes the polarographic reduction current to be much lower in a hydrolysed than in a freshly prepared solution of cis-$Pt(NH_3)_2Cl_2$(1).

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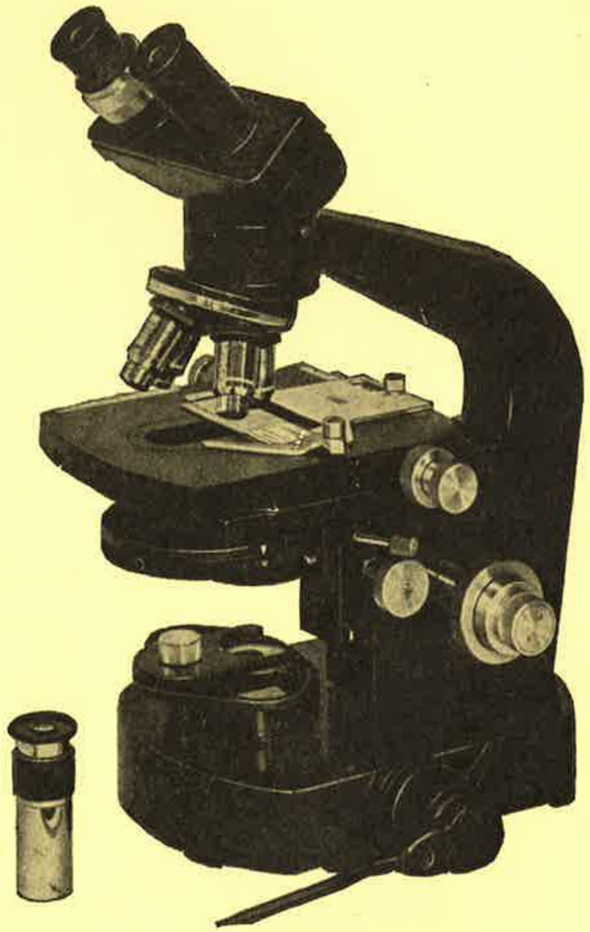

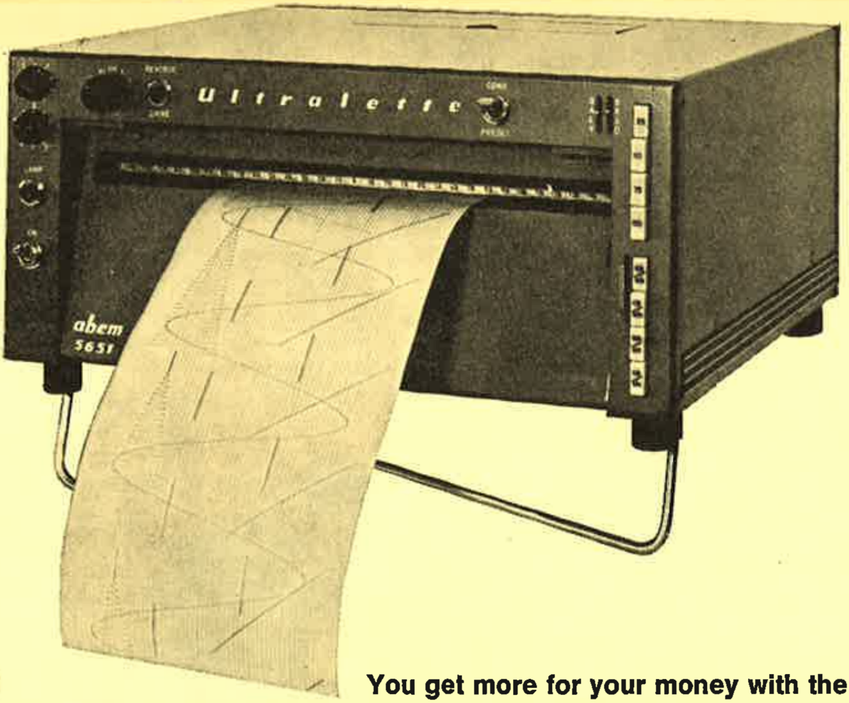

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