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Quantitative and Qualitative Studies of Phylloplane Bacteria from *Lolium perenne*

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SUMMARY

Populations of heterotrophic bacteria occurring on healthy green leaves of *Lolium perenne* were estimated using a dilution technique. Bacterial populations in spring and summer were relatively low compared with counts obtained in September when the highest maximum and monthly mean air temperature was recorded. The area of leaf surface colonized rose from about 0·0001 % in May to approximately 0·1 % in September. Over 600 isolates were examined and most found to be Gram-negative and chromogenic. The bacterial populations consisted of only a few taxa and the variations in total numbers were due to changes in the relative abundance of these. The predominant flora were identified to the genera *Chromobacterium*, *Corynebacterium*, *Pseudomonas* and *Xanthomonas*. Leaf impression and overpour techniques gave some indication of the micro-ecology of bacteria on the leaf, and showed the presence of actinomycetes which were rarely observed on dilution plates.

INTRODUCTION

The dearth of comprehensive studies on phylloplane bacteria has seriously hindered work on leaf surface microbial interactions. In contrast to the rhizosphere, little is known about the development, maintenance or activity of bacterial populations on the phylloplane. Many of the workers who have considered the bacterial flora of the phylloplane have used methods designed for the study of soil andl eaf litter micro-organisms. However, leaf surfaces have their own chemical and physical characteristics which need modified techniques for the examination of their bacterial populations.

Studies of phylloplane bacteria have largely depended on the dilution technique (Voznyakovskaya & Khudyakov, 1960; Leben, 1961; Crosse, 1965; Last & Deighton, 1965; Vasantharajan & Bhat, 1968), though leaf characteristics have rarely been considered in relation to this method. Other cultural approaches which have been used include impression techniques (Leben, 1961) and maceration procedures (Last & Deighton, 1965; Chakravarti, Leben & Daft, 1972). Direct observation of phylloplane bacteria *in situ* has been attempted using light (Daft & Leben, 1966; Leben & Daft, 1967) and scanning electron microscopy (Leben, 1969).

Such studies have revealed seasonal fluctuations in bacterial numbers on various plants, with maximum numbers on temperate crops in the summer months. Bacterial counts have varied from 10^5 to 10^7/cm^2, or 10^7 to 10^9/g fresh weight, depending upon the plant species and the habitat examined (Crosse, 1965; Last & Deighton, 1965; Vasantharajan & Bhat, 1968; Jensen, 1971). Direct observation studies have suggested that bacteria occur in depressions between epidermal cells (Leben, 1965) and are distributed singly or in small groups (Leben & Daft, 1967).

Certain groups of phylloplane bacteria have been considered to have a wide host range on leaf surfaces (Last & Deighton, 1965) but comparisons between the various kinds of bacteria occurring on the phylloplane of different higher plants are hampered by the difficulties involved in classification and identification. Most classifications which have been used have been artificial and have been based on small numbers of morphological, nutritional (Jensen, 1971) or pigmentation (Stout, 1964) characters.

This paper describes the numbers and kinds of aerobic heterotrophic bacteria on healthy green leaves of *Lolium perenne* L. growing in a grazed pasture. Quantitative aspects of the flora have been assessed by dilution, leaf impression and overpoured leaf techniques. Isolates from the dilution plates were provisionally identified using conventional dichotomous keys and diagnostic tables.

METHODS

Habitat. Lolium perenne S24 (perennial rye grass) was collected from a moderately grazed field (Long Riggs East) at Cockle Park Experimental Farm, Morpeth, Northumberland (Grid Reference NT217917). The farm is situated 32 km north of Newcastle upon Tyne at 99 m above sea-level. Long Riggs East was seeded in 1968, and by 1973 the dominant grass was *L. perenne* S24 with a uniform distribution of *Phleum pratense* and scattered clumps of *Dactylis glomerata*, *L. perenne* S23, *L. perenne* New Zealand Grasslands Manawa rye grass, and *Poa annua*. *Trifolium repens* was scarce. The field, approximately 5×10^4 m^2, was bordered at its northern end by deciduous woodland. There was a distinct slope in the centre of the field but the southern half was flat and ideal for sampling.

Collection of samples. An 8 week sampling cycle was adopted but was modified if >6 mm rain was recorded during the 48 h before an intended sampling date (Table 1). *Lolium perenne* S24 was distinguished from the other rye grasses by its relative size, tillering performance and the stage in the flowering process which had been reached. When sampling, six 0·2 m^2 turfs from areas dominated by *L. perenne* S24 were dug up and placed in open plastic trays. Dung pats and accelerated growth points attributable to urine deposits were avoided.

Sampling of material. Analyses were commenced within an hour of collection and sterile procedures were used where appropriate. Individual healthy, main tillers, free from insects and visible soil particles, were selected and their second and third leaves, counting from the base of the tiller, removed. In October, the fifth and sixth leaves were sampled because the older leaves had become senescent. Each group of leaves was bulked separately. A 25 mm length of lamina was cut with spaced razor blades on a sterile surface. The fresh weight of the lengths was determined, and after analysis the total superficial surface area of the lengths calculated and their dry weight (80 °C) determined.

Microbiological methods. Samples (20 ml) of several media, at pH 7·0, were inoculated with a bacterial suspension obtained by washing Lolium leaves in quarter-strength Ringers buffer (Oxoid BR 52). Triplicate plates were counted after 9 days' incubation at 25 °C. The largest number of bacteria (Table 2) grew on glucose–yeast extract agar (GYEA), containing D-glucose (0·5 %, w/v), yeast extract (Oxoid L 2; 0·1 %, w/v), ferric phosphate (trace), and agar agar (Oxoid No. 1; 1 %, w/v). Using this medium a number of diluents based on Straka & Stokes (1957), all at pH 7·0, were tested following the procedure described below. The highest counts were obtained by using quarter-strength Ringers buffer (Table 3). Further tests at a range of pH values from 5·5 to 8·0 showed that using GYEA and quarter-strength Ringers buffer, the maximum number of colonies was isolated at pH 7·0. With the quantities of leaves and diluent volumes specified below, 20 min shaking was required for the maximum number of bacteria to be detached from the leaves, and

Table 1. *Meteorological data recorded at Cockle Park Experimental Farm at about 0·5 km from the sampling area*

Date (1973)	Temperature (°C) Monthly average	Max.*	Min.*	Grass min.*	Sunshine† (h)	Rainfall (mm/day), on day:‡ 7	6	5	4	3	2	1
May	9·5	14·5	1·7	−2·1	6·1	1·8	1·0	0·3	0·5	0·2	Tr	0·0
June	13·4	—	—	—	—	—	—	—	—	—	—	—
July	14·4	21·0	6·5	4·1	4·3	0·1	0·0	13·8	8·1	0·1	0·0	5·2
August	13·6	—	—	—	—	—	—	—	—	—	—	—
September	13·6	22·7	1·5	−2·0	4·3	2·0	1·3	14·1	Tr	0·0	0·0	0·0
October	8·1	13·4	1·5	−2·0	1·4	0·0	0·0	0·0	0·0	0·5	Tr	0·0

Tr, trace.
* Daily average for 7 days before sampling, 16 May, 11 July, 5 September, 31 October.
† Mean for day before sampling.
‡ Days before sampling on day 0.

Table 2. *Media evaluated for dilution plate technique*

Quarter-strength Ringer buffer was used as diluent, media were prepared with distilled water and solidified with agar agar (Oxoid No. 1, 1 %, w/v). Colonies were counted after incubation for 9 days at 25 °C; standard deviations are also included in the Table.

	10^{-3} × No. of colonies/ g fresh wt		10^{-3} × No. of colonies/ g fresh wt
GYEA	44±1	Grass washings*	27±2
Grass washings–yeast extract (1 %, w/v)	38±3	Yeast extract (1 %, w/v) Glucose (1 %, w/v)	26±2 21±2
Peptone (0·5 %, w/v)–yeast extract (1 %, w/v)	38±3	Tomato juice agar (Oxoid, CM113)	21±2
Sucrose (1 %, w/v)–MC†	36±3	Nutrient agar (Oxoid, C173)	16±1
Glucose–MC†	35±3	Sucrose (1 %, w/v)	10±1
Sugars‡–MC†	33±3		

* Lolium clippings (125 g fresh wt, in distilled water); washed for 3 h and Seitz filtered.
† Mineral salts (0·02 %, w/v) containing K_2HPO_4 (0·01 %, w/v), KNO_3 (0·05 %, w/v), $MgSO_4.7H_2O$ (0·02 %, w/v), $CaCl_2$ (0·01 %, w/v), NaCl (0·01 %, w/v) and $FeCl_3$ (trace), and casein hydrolysate (Difco, 0·1 %, w/v).
‡ Glucose (0·1 %, w/v), fructose (0·1 %, w/v), raffinose (0·1 %, w/v) and sucrose (0·1 %, w/v).

9 days' incubation at 25 °C permitted the growth of the highest number of colonies. Using the shaker described below, the addition of ballotini beads to the buffer did not increase the counts obtained.

Dilution technique. Samples of 50 leaf lengths were added to 100 ml portions of buffer in 250 ml Erlenmeyer flasks fitted with rubber bungs, and agitated for 20 min on a Griffin flask shaker (Griffin and George Ltd, Manchester; 580 throws of 2 cm/min). Mycostatin (2 mg/ml) was incorporated into the initial diluent to suppress fungal growth, following the recommendation of Williams & Davies (1965). Dilutions were prepared to 10^{-3} with the buffer, and 1 ml portions of each dilution were pipetted (Jensen, 1968) into 9 cm polystyrene Petri dishes and overpoured with 20 ml of cooled GYEA. For each dilution, five replicate dishes were incubated aerobically, and five anaerobically in a Fildes and McIntosh jar, for 9 days at 25 °C.

Leaf impressions. Each surface of a 25 mm leaf length, cut as described above, was placed

Table 3. *Diluents evaluated for dilution plate technique*

GYEA was the medium used. Distilled water was used to prepare these diluents except where otherwise specified. Colonies were counted after incubation for 9 days at 25 °C; standard deviations were never > 0·01.

	10^{-6} × No. of colonies/ g fresh wt		10^{-6} × No. of colonies/ g fresh wt
Ringers buffer (quarter strength)	1·17	Trisodium citrate (0·1 %, w/v)	0·29
Peptone (0·1 %, w/v)	1·11	Ammonium sulphate (0·1 %, w/v)	0·27
Yeast extract (0·1 %, w/v)	1·10	Grass washings (see Table 2)	0·22
L-Glutamic acid (0·1 %, w/v)	1·03	Tween 80 (0·1 %, w/v)	0·19
Glucose (0·1 %, w/v)	0·83	Tap water	0·02
Distilled water	0·49		

in turn on to the surface of GYEA (containing 0·4 mg mycostatin/ml) for 5 min (Leben, 1961). Fifty replicate leaf lengths were examined. Leaf lengths washed for the dilution technique were similarly examined after shaking off excess buffer. Dishes were incubated under aerobic conditions for 9 days at 25 °C.

Leaf overpour. An apical leaf length, 60 mm long, was slowly overpoured with 20 ml of cooled GYEA (containing 0·4 mg mycostatin/ml) in a 9 cm polystyrene Petri dish. When the agar gelled, the leaf was approximately 1 mm below the surface of the medium. Ten replicate dishes were prepared from second and third leaves (fifth and sixth leaves in October) and incubated as before.

Purification of isolates. From the dishes used to estimate the bacterial numbers, 650 bacteria were subcultured using a technique which ensured random selection. Bacteria from dishes showing between 30 and 200 colonies were streaked on to GYEA and incubated at 25 °C for 7 days. Streaked cultures were then stained using Hucker's modification of the Gram stain (Hucker & Conn, 1923) and examined microscopically. If the cultures were considered to be pure, they were inoculated on to GYEA slopes, but if they were suspected to be impure they were successively streaked until pure colonies were prepared. Stock cultures were maintained on GYEA slopes at 5 °C and subcultured every eight weeks.

Tests. Micromorphological characters were taken from bacteria grown for 24 to 48 h on GYEA and stained as before, and flagella were examined by the modified method of Cesares-Gil (Plimmer & Paine, 1921). Haemolysis was recorded after 3 days' incubation on nutrient agar amended with 5 % horse blood (Oxoid SR 50), and levan production after 7 days' incubation on GYEA containing 5 % (w/v) sucrose. The ability of isolates to grow on GYEA at 37 and 5 °C was recorded subjectively, after 3 and 14 days' incubation respectively. The remaining tests were incubated at 25 °C. Cowan & Steel's (1965) methods were used to detect catalase activity (method 1), citrate utilization (method 1), gelatin hydrolysis (method 1), indole production (method 2), motility (methods 1 and 2), urea hydrolysis (method 1), oxidation or fermentation of glucose, oxidase activity, nitrate reduction and starch hydrolysis. The formation of pigment on skimmed milk (Billing & Baker, 1963), diffusible pigment production (King, Ward & Raney, 1954), arginine dihydrolase, lysine and ornithine decarboxylase activity (Taylor, 1961) and growth in MacConkey agar (Oxoid CM 7) were all recorded after 5 days' incubation.

Identification of the isolates. Isolates were identified to the genus level or beyond using the diagnostic tables of Cowan & Steel (1965). Cultures classified as *Erwinia* or *Xanthomonas* were identified further, using the keys in Dye (1969) and Hendrie, Mitchell & Shewan (1968).

Table 4. *Numbers of bacteria on the phylloplane of L. perenne on different dates*

Standard deviations are also given.

	10^{-3} × No. of colonies/ cm^2 leaf surface		10^{-3} × No. of colonies/ g fresh wt		10^{-3} × No. of colonies/ g dry wt	
Leaf number* ...	2	3	2	3	5	6
16 May 1973	2±1	1±1	120	34	410	120
11 July 1973	13±3	5±1	840	360	4300	2000
5 September 1973	250±1	150±1	11000	7200	63000	41000
31 October 1973	3±1	10±1	2000	700	10000	3000

* This refers to the position of the leaf, counting the first leaf formed on the tiller as no. 1.

RESULTS

Numbers of bacteria on the phylloplane

There was a fluctuating relationship between the population values expressed on an area or weight basis (Table 4) and this reflects complex variations in weight per unit area throughout the life of the leaf. This was in part due to the continuing growth of the lamina, which on average doubled in length between May and July and increased by half as much again between July and September. The October and May samples were similar to one another in size. In spite of the inherent problems in assessing the exact area washed, further consideration will be given only to the data based on this measurement as it would seem most appropriate for studying a habitat based on surface features.

There was a general increase in the bacterial population on the phylloplane from May to September, but lower numbers were observed on the leaves sampled in October, which were younger than the previous samples (Table 4). A small increase in the bacterial numbers was observed from May to July, and a much larger one between July and September when the leaves were still apparently healthy and without visible signs of senescence. Higher values were observed, on a surface area basis, on the older, second leaves than on the younger, third leaves. The older leaves again yielded a higher count than the younger ones in October, although the numbers on both were significantly lower than on the corresponding second and third leaves in September. These results may reflect the extra time that the older leaves had been exposed on the tiller.

Few colonies developed on dilution plates incubated anaerobically, indicating a population of approximately 4 anaerobes/cm^2. These colonies were of white, Gram-positive rods and were not studied further.

Identification of the phylloplane isolates

Of the 621 isolates successfully studied, 80 % were classified into seven taxa (Table 5). Most were in the genera *Chromobacterium*, *Corynebacterium*, *Pseudomonas* and *Xanthomonas*, with the numerically predominant *Pseudomonas fluorescens* forming 23 % of the bacteria sampled. Corynebacteria, chromobacteria and xanthomonads were present at all sample dates, the latter being particularly evident on the leaves collected in September and October. *Erwinia herbicola* and *Flavobacterium* spp. constituted only a small fraction of the total bacterial flora.

The population increase recorded in September resulted from a quantitative increase in many of the taxa present. However, the largest number of taxa was recorded in October, when the total count was well below that for September. The low count may have been due

Table 5. *Temporal distribution of bacterial isolates from L. perenne*

Numbers on each sampling occasion are given as a percentage of the total for that date. Colony pigmentation was recorded after 3 days on GYEA incorporating 10% (w/v) skimmed milk (Oxoid L31).

	Sampling dates				Percentage of total isolates
	16 May	11 July	5 September	31 October	
Chromobacterium spp.	32	4	7	4	9·2
Corynebacterium spp.	6	6	30	29	16·3
Erwinia herbicola	0	1	2	2	1·3
Flavobacterium spp.	2	0	0	6	1·4
Pseudomonas fluorescens	0	55	0	1	23·1
Pseudomonas spp.	0	0	2	7	2·0
Xanthomonas spp.	28	14	40	5	19·8
Unidentified group (orange pigmented)	5	4	3	12	5·5
Unidentified organisms	27	16	16	34	20·4
Total no. of colonies sampled	100	249	150	122	
Total no. of taxa	10	13	16	20	
Pigment recorded (% of total isolates on each occasion)					
Cream/white	16	9	42	13	
Orange	9	10	9	16	
Pink	32	4	2	4	
Purple	0	0	0	1	
Yellow (deep)	38	14	21	52	
Yellow (pale)	5	62	25	14	

to the fifth and sixth leaves not having been exposed very long on the tiller, or conditions may not have favoured the accumulation and multiplication of bacteria. The phylloplane, therefore, appeared to possess a dynamic bacterial population, with the occurrence and increase of particular taxa coinciding with the decline of others.

Pigmentation

Pigmentation has been considered to be an important property of phylloplane bacteria (Stout, 1960; Klincáre, Krèslina & Mishke, 1971). The majority (81 %) of the isolates from *L. perenne* were chromogenic (Table 5), and the yellow colonies, which predominated, together with the white and cream isolates, were classified in several taxa. The pink isolates, with few exceptions, were classified in the genus *Chromobacterium*. Orange colonies were grouped in several taxa, including xanthomonads, and the purple ones were identified as pseudomonads.

Impression technique

Actinomycetes, rarely seen on the dilution plates, were observed using both impression and overpour techniques. Impression dishes prepared from unwashed leaves showed the presence of a few actinomycete colonies between May and September, but they were comparatively abundant in October (Table 6). Washed leaf pieces produced similar results on impression plates. Actinomycetes were also isolated from impressions prepared using *Agrostis tenuis*, *Deschampsia caespitosa*, *Festuca ovina*, *Holcus lanata* and *Poa pratensis* leaves obtained from several sites in Northumberland.

Table 6. *Actinomycete colonies recorded on impression plates of L. perenne*

The figures in parentheses are estimates of the no./cm.²

	No. recorded on 50 replicates							
	16 May		11 July		5 September		31 October	
Leaf number ...	2	3	2	3	2	3	5	6
Untreated	0	0	11 (1)	0	0	0	63 (2)	132 (4)
Washed in buffer	0	0	0	0	10 (1)	0	61 (2)	123 (4)

Overpour technique

Bacterial colonies developed in the agar along the lamina and margin of the embedded leaves, with numbers increasing towards the meristematic leaf base. In May, the apical portion (approximately 15 mm long) of both the second and third leaves appeared to be sterile. In July and September 60 and 40 % of the leaves, respectively, also had bacteria-free tips. The remaining leaves produced bacterial growth up to the apical 2 to 3 mm. In October the fifth and sixth leaves showed bacterial growth covering the entire length of the leaves. The bacteria which developed were culturally and morphologically similar to those isolated using the dilution technique. Actinomycete colonies also developed in quantities similar to those on the impression dishes.

DISCUSSION

The progressive increase observed in the populations from May to September may have reflected alterations in the characteristics of the leaves and sources and activity of the phylloplane micro-organisms. In particular, the consistent differences between the second and third leaves, the sterility of the leaf tips in May and July, and the very small populations on the fifth and sixth leaves in October, may reflect aspects of the development of the leaves themselves. The data given here do not indicate the exact significance of such factors as solute exudation (Tukey, 1971), erosion of epicuticular wax (Holloway, 1971) and the accumulation of allochthonous materials (Fokkema, 1968), but the influence of pollen appears to have been insignificant as *L. perenne* and several other grasses flower before May when low bacterial populations were recorded. The large increase in populations recorded in September occurred on leaves which had had a long period of exposure, so that allochthonous nutrients might have accumulated and more nutrients may have diffused to the leaf surface.

Meteorological data (Table 1) may explain major fluctuations in the phylloplane populations but minor changes will require studies of the micro-environment at the leaf surface. The large populations in September coincided with the highest maximum and monthly mean air temperatures. In spite of the continuing extension of the lamina from May to September, the total increase in bacterial populations did not suggest any notable arrivals of bacteria via wind or rain, or from adjacent herbage or grazing animals. Similarly, the small populations observed in October on leaves which had been exposed for up to six weeks indicated a very slow rate of colonization, assuming that rain was not a major influence in removing phylloplane inhabitants.

Substantially fewer bacteria were isolated from *L. perenne* than from leaves of *Cucumis sativus* (Leben, 1961), various tropical plants (Ruinen, 1961), *Morus indica* (Vasantharajan & Bhat, 1968) or *Festuca novae* (Stout, 1960). Clark & Paul (1970) noted that there are more

bacteria on legume and vegetable crop leaves than on grasses. Seasonal fluctuations of phylloplane bacteria, with maximum numbers occurring in late summer, have been described for *L. perenne* and *Trifolium repens* (Stout, 1960), and the populations were also found to be greater on younger leaves of these plants. In the present study the increased density of bacterial growth observed towards the leaf meristem of *L. perenne* agreed with Stout's findings.

The estimated area of Lolium leaf surface colonized by bacteria is about 0·0001 % in May, rising to approximately 0·1 % in September. These values have been calculated from counts and measurements of bacteria and leaf surface area assessments corrected for the topography of epidermal cells, and they assume that there were equal numbers of bacteria on adaxial and abaxial surfaces. These figures for the phylloplane are low compared with the 7·7 % estimated for the rhizoplane of *L. perenne* by Rovira *et al.* (1974). With such sparse coverage, extensive direct observations would have to be made if bacteria were to be observed *in situ* on the phylloplane. Such observations are made more difficult by the need to distinguish bacteria from the numerous other small particles found on the leaf surface. Leben (1969, 1974) observed phylloplane bacteria *in situ* on *Glycine max* at comparatively low magnification using the scanning electron microscope, but attempts to repeat this with *L. perenne* were relatively unsuccessful.

Little is known about the species composition of bacterial populations on leaf surfaces. However, there is evidence that the phylloplane bacteria of many temperate plants are mainly chromogenic (Stout, 1960; Jensen, 1971; Klincáre *et al.* 1971), with yellow forms much in evidence. Stout (1958, 1960, 1961) isolated large numbers of flavobacteria from Pinus needles and grass leaves, but in the present study most of the yellow bacteria were classified as *Xanthomonas* spp. High populations of xanthomonads have also been found on the leaves of *L. perenne* and *Trifolium repens* by Stout (1960). In contrast, Last & Warren (1972) found strains of *Erwinia herbicola* to be abundant on the leaves of various Gramineae and Leguminosae. In this study we recorded only a rather limited range of bacterial taxa. This could have been due to the selective effects of the cultural methods used, but it may well be a true result as the phylloplane fungal flora is similarly limited in species composition (Dickinson, 1973).

Most classifications of phylloplane bacteria are monothetic, and unsatisfactory for identifying isolates which give the same response to a few key characters but differ widely in other properties. This problem is particularly relevant in the case of poorly-defined taxa such as *Corynebacterium*, *Flavobacterium*, *Pseudomonas* and *Xanthomonas*. To try and obtain a better classification, phylloplane isolates are being subjected to a numerical taxonomic study. Numerical taxonomy has not yet been applied in phylloplane studies but has been successful in distinguishing between different bacteria occurring in large mixed populations in the rhizosphere (Graham, 1964), forest litter (Hissett & Gray, 1974) and soil (Lowe & Gray, 1972). Goodfellow (1969) showed that numerical taxonomic techniques can distinguish between taxa that would otherwise be lumped together because of current weaknesses in classification.

It is not yet established whether actinomycetes form a significant component of phylloplane populations. Neither Stout (1960), nor Leben (1961) isolated actinomycetes from the leaves they studied, but Jensen (1971) and Dickinson & Wallace (1975) recorded large numbers of Streptomycetes on Fagus and Triticum leaves respectively. The data reported here suggest that the technique employed may be a significant factor as to whether these organisms are isolated from the habitat.

The significance, if any, of the phylloplane bacterial flora to the plant is not known.

Certain taxa, such as corynebacteria or xanthomonads, may represent a reservoir of potential plant pathogens, whereas others may be antagonistic to saprophytic and pathogenic fungi. Representative strains of phylloplane bacteria are now being studied in an attempt to control net blotch disease of *L. perenne* caused by *Drechslera dictyoides* (Drechsler) Shoemaker.

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