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The Role of Streptomycetes in Decomposition of Chitin in Acidic Soils

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The influence of pH on chitin hydrolysis by streptomycetes from a range of acidic and neutral soils was studied in vitro in an acid soil. On the basis of activity ranges and optima for chitin hydrolysis, acidophilic, acidoduric and neutrophilic categories of streptomycetes were distinguished; these categories were broadly related to the pH requirements for growth. Responses of streptomycetes to chitin amendment of acidic organic and mineral horizons of a pine forest soil were studied. Acidophiles were involved in the decomposition process and the resulting ammonification led subsequently to activity of neutrophiles. This succession was particularly marked in the poorly buffered mineral horizon. Similar, but smaller, responses occurred when the horizons were amended with mycelium from basidiomycete sporocarps. The role of streptomycetes in decomposition of fungal chitin in acidic litters and soils is discussed.

INTRODUCTION

Previous studies of the pH requirements of soil streptomycetes have distinguished between neutrophiles which grow from pH 5·0 to 9·0 with optima close to pH 7·0, and acidophiles which grow from pH 3·5 to 6·5 with optima from pH 4·5 to 5·5 (Jensen, 1928; Williams *et al.*, 1971; Flowers & Williams, 1977). Acidophiles appear to be restricted to acidic soils and litters; neutrophiles are most numerous in soils close to neutrality but also occur in low numbers in acidic soils (Williams & Mayfield, 1971). Acidophiles probably play an important part in decomposition processes in acidic soil and litter. They have a diversity of hydrolytic abilities (Khan & Williams, 1975) and many produce extracellular diastases with optimum activity between pH 4·0 and 4·5 (Williams & Flowers, 1978).

Ability to degrade chitin is one of the most characteristic features of soil streptomycetes. Chitin media are often used to isolate and enumerate streptomycetes (Lingappa & Lockwood, 1962; Hsu & Lockwood, 1975). Extracellular chitinases have been isolated from several laboratory cultures (Jeuniaux, 1955; Berger & Reynolds, 1958; Skujins *et al.*, 1970; Tiunova *et al.*, 1976*a*, *b*). Amendment of soil with chitin leads to a large increase in numbers of streptomycetes, which may result in the suppression of root pathogenic fungi (Mitchell & Alexander, 1962; Sneh *et al.*, 1971). A similar stimulation of streptomycetes occurs when fungal cell-wall components are added to soil (Mitchell, 1963) and it is likely that fungal hyphae provide a major source of chitin for streptomycetes in soil. Another potential source of chitin is the exoskeletons of soil arthropods (Okafor, 1966).

Therefore we decided to study the roles of acidophilic and neutrophilic streptomycetes in the decomposition of pure chitin and fungus mycelium in acidic soil and litter. An initial survey was made of the effects of pH on chitin hydrolysis by streptomycetes in vitro. The responses of the streptomycete populations in a litter and soil horizon of an acidic pine forest soil (Freshfield, Merseyside, U.K.) to amendment with chitin and fungus mycelium were then studied. General aspects of chitin decomposition in this soil were previously examined by Gray & Baxby (1968) and the ecology of the streptomycete population has been intensively studied.

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METHODS

Selection of chitin-hydrolysing soil isolates. A total of 24 streptomycetes previously isolated from a range of acidic and neutral soils were tested for their ability to hydrolyse chitin. Acidophilic and acidoduric isolates were inoculated on to a medium containing (%, w/v): colloidal chitin (prepared by the method of Hsu & Lockwood, 1975), 0·3; $MgSO_4.7H_2O$, 0·05; $FeSO_4.7H_2O$, 0·001; $ZnSO_4.7H_2O$, 0·0001; $MnCl_2.4H_2O$, 0·0001; KH_2PO_4, 0·5; Bacto agar (Difco), 2·0; the final pH was 5·5. Neutrophiles were grown on the same medium but with 0·1% (w/v) K_2HPO_4 substituted for KH_2PO_4; the final pH was 7·0. Spore suspensions of the isolates (stored in 10%, v/v, glycerol at −20 °C; Wellington & Williams, 1978) were used to point-inoculate the medium, each plate receiving five replicate inoculations of 0·01 ml of suspension. Plates were incubated at 25 °C for 14 d and the colony areas and diameters of hydrolysis zones were measured.

Effect of pH on chitin hydrolysis. The 10 strains which produced the largest hydrolysis zones were used. Origins of these strains are given in Table 1. The reaction of the chitin/mineral salts medium was adjusted to cover the range pH 2–11, in increments of 0·5 units. Appropriate buffers and the necessary volumes of 0·1 M-HCl were added after autoclaving. Buffers used were (%, w/v): KH_2PO_4, 0·5 (pH 2·0–4·5); KH_2PO_4, 0·054; K_2HPO_4, 0·035; Na_2HPO_4, 0·028 (pH 5·0–6·0); K_2HPO_4, 0·07; Na_2HPO_4, 0·056 (pH 6·5–8·0); $Na_2B_4O_7.H_2O$, 0·095; NaOH, 0·018 (pH 8·5–11·0).

Plates were point-inoculated, with five replicate inoculations for each isolate at each pH. After incubation at 25 °C for 21 d, the areas of hydrolysis zones and colonies were calculated. The pH of all media was also checked and only slight changes (±0·1 unit) were detected.

The pH at which each isolate produced its largest hydrolysis zone was noted. In order to assess the effect of pH on chitin hydrolysis independently of its effects on streptomycete growth, cylinders of medium (6 mm diam.) free of streptomycete growth were aseptically removed from these hydrolysis zones. These were placed on to the surface of fresh chitin/mineral salts medium previously adjusted to pH 2–11 in 1 unit increments. Five replicate cylinders were transferred to the medium at each pH. Plates were incubated at 25 °C for 14 d, after which the diameter of hydrolysis zones around the discs was measured. The cylinders rapidly equilibrated to the pH of the plates on which they were placed.

Effects of chitin and fungal mycelium amendments on streptomycetes in an acidic soil

(*i*) *Selection and properties of soil.* The soil selected was a developing podzol under *Pinus nigra* at Freshfield, Merseyside, U.K. Previous studies of the pH requirements of streptomycetes in this soil showed that mixed populations of acidophiles and neutrophiles occurred in the adjacent F_2–H litter and A_1 horizons (Williams *et al.*, 1971). As they also provided a contrast between a totally organic and a primarily mineral horizon, they were selected for this study.

The humic acid content of each horizon was estimated, as humus is the most potent buffering agent in soil (Russell, 1973). A 2 g sample of air-dried, sieved soil was added to 20 ml 0·5 M-NaOH in a 100 ml conical flask. The suspension was shaken orbitally (190 rev. min^{-1}) for 24 h at room temperature. The supernatant was collected after centrifugation at 3000 ***g*** for 10 min, the whole procedure being repeated until the supernatant was clear. Combined extracts were acidified with 1·0 M-HCl, resulting in precipitation of the humic acid fraction, which was then sedimented by centrifugation. The sediment was washed three times with distilled water, dried and weighed. Five replicate determinations were made for each horizon.

The buffering capacity of each horizon was estimated. Samples of fresh soil (10 g) were placed into vials, and from 1 to 19 ml 0·04 M-NaOH (in 1 ml increments) was added to the vials, the volume being made up to 20 ml with distilled water. Distilled water alone was added to control vials. The same procedure was applied to acid-washed sand and 1·0 M-citrate buffer which served as examples of poorly and well buffered systems, respectively. After 30 min equilibration, the pH of all suspensions was determined with a glass electrode.

An indication of the natural occurrence of chitin in both horizons was obtained by determination of their hexosamine content (Stevenson, 1965).

(*ii*) *Effects of chitin and fungal mycelium amendments.* Samples (8 g) of freshly collected F_2–H litter and A_1 soil were placed in sterile McCartney vials. Sterile flaked chitin (BDH) was added to half the vials (2·5%, w/w) and mixed well; the unamended vials served as controls. Tops were lightly screwed and the vials were incubated at 25 °C for 40 d, their moisture content being maintained by regular additions of sterile deionized water.

At the beginning of the experiment and at 10 d intervals, the pH and ammonium-N concentration were determined. Three replicate vials were used for each determination. The pH was determined by placing a glass electrode into a paste of soil plus deionized water (1 : 1, by vol.) which had been allowed to equilibrate for 30 min. The ammonium-N concentration in 2 M-KCl extracts of soil or litter was measured using the indophenol blue method of Kempers (1974), a modification of that of Weatherburn (1967).

Numbers of streptomycetes were estimated at the beginning and end of the experimental period by the dilution plate procedure, using starch/casein medium (Küster & Williams, 1964) at pH 4·5 and 7·0, and chitin/mineral salts medium adjusted to the pH of the material in the vials at the time of sampling. All media contained the antifungal antibiotics nystatin (Squibb, Twickenham, Middlesex) and cycloheximide (Koch-Light, Colnbrook,

Table 1. *Origins of streptomycete isolates used to study effect of pH on chitin hydrolysis*

Streptomyces strain	Soil type	Soil pH
SW9	Podzol	3·5
SW26	*Juncus* litter	4·0
MR2	Podzol	3·8
HF3	Podzol	3·9
SW72	Coal spoil	4·5
H	Podzol	3·5
C1	Sand dune	7·8
C5	Sand dune	7·8
F70	Sand	7·5
M1	Brown earth	5·5

Bucks.) each at a concentration of 50 μg ml^{-1}. After incubation at 25 °C for 14 d, the colonies on all media at appropriate dilutions were counted. The number of colonies producing hydrolysis zones on the chitin medium was also noted.

The proportions of acidophilic, acidoduric and neutrophilic strains were estimated. Twenty colonies were randomly picked off from plates of each medium/treatment combination. Each colony was divided and inoculated into a tube of broth at pH 4·5 and at pH 7·0. The medium contained (%, w/v): glucose, 1·0; L-asparagine, 0·05; $MgSO_4.7H_2O$, 0·05; $FeSO_4.7H_2O$, 0·001; $ZnSO_4.7H_2O$, 0·0001; $MnCl_2.4H_2O$, 0·0001. The reaction was adjusted after sterilization by addition of either KH_2PO_4 (pH 4·5) or K_2HPO_4 (pH 7·0) to a final concentration of 0·5% (w/w). Presence or absence of growth was noted after 7 d at 25 °C. Acidophiles were recognized by growth at pH 4·5 only, acidodurics by growth at pH 4·5 and 7·0, and neutrophiles by growth at pH 7·0 only.

The same procedures were used in a more intensive study of streptomycetes in chitin-amended A_1 soil. The experimental period was reduced to 20 d and streptomycete populations were studied at 5 d intervals.

Changes in pH, ammonium-N, and streptomycete populations in both horizons amended with fungus mycelium were also studied. Fungus material was obtained from the fruit bodies of *Lactarius rufus*, which were numerous in the pine forest. Fresh fruit bodies were cut into small pieces, air-dried and ground to pass through a 500 μm mesh sieve. The resulting powder was added to soil or litter (2·5%, w/w) and determinations were carried out as with the chitin amendments.

RESULTS

Effect of pH on chitin hydrolysis in vitro

Measurement of the colony areas of streptomycetes on chitin medium at different pH values indicated that three categories could be distinguished. Acidophiles grew between pH 4·0 and 6·0, with an optimum around pH 4·5; acidoduric isolates grew from pH 4·5 to 8·5, with an optimum near pH 6·5; neutrophiles grew from pH 5·0 to 8·5, with an optimum near pH 7·0.

These categories were therefore employed in the study of the effect of pH on chitin hydrolysis by enzymes in agar cylinders free of streptomycete growth (Table 2). Although the ranges of activity overlapped, the patterns of the three groups were readily distinguished and generally reflected their pH requirements for growth. However, the ranges for chitinolysis were broader than those for growth. Optimum hydrolysis by acidophiles was usually at pH 4·0, while that of neutrophiles varied between pH 5·0 and 8·0. Acidoduric isolates produced the largest zones between pH 4·0 and 6·0.

Properties of the F_2–H and A_1 horizons

Relevant properties of the horizons selected for study of chitin decomposition are given in Table 3. The organic nature of the F_2–H horizon contrasted with the mainly mineral A_1 and this was reflected in the large differences in their nitrogen and humic acid contents. Both horizons were acidic but their buffering capacities were markedly different. The reaction of the F_2–H was similar to that of citrate buffer, which increased from pH 3·7 to 4·1 after addition

Table 2. *Effect of pH on chitin hydrolysis by chitinases in agar cylinders free of streptomycete growth*

Results are expressed as the mean diameter of hydrolysis zones (mm); standard errors are shown in parentheses.

Source of enzymes	pH of medium 3·0	4·0	5·0	6·0	7·0	8·0	9·0	10·0
Acidophiles								
SW9	20·0 (1·0)	50·0 (1·5)	50·0 (0)	50·0 (0)	0	0	0	0
SW26	95·7 (9·8)	133·3 (11·8)	107·7 (12·7)	84·3 (5·3)	0	0	0	0
MR2	125·0 (4·9)	154·4 (7·0)	133·2 (6·5)	80·0 (10·2)	0	0	0	0
HF3	99·0 (6·4)	98·6 (3·6)	61·2 (2·8)	57·0 (4·4)	0	0	0	0
Acidodurics								
SW72	0	212·0 (9·9)	213·0 (15·2)	220·5 (6·5)	100·0 (8·2)	50·0 (0)	41·2 (2·2)	0
H	0	147·0 (7·0)	213·6 (19·8)	187·8 (9·8)	86·0 (8·3)	41·2 (2·2)	41·2 (2·2)	0
Neutrophiles								
C1	0	0	50·0 (0)	69·0 (5·0)	58·4 (3·4)	52·8 (2·8)	50·0 (0)	50·0 (0)
C5	0	0	82·2 (3·2)	100·0 (8·2)	85·4 (3·9)	82·4 (5·8)	79·5 (15·5)	55·6 (3·4)
F70	0	0	178·3 (17·2)	185·7 (7·7)	202·2 (11·0)	222·8 (12·4)	160·0 (11·0)	137·6 (10·0)
M1	0	57·0 (4·0)	95·5 (6·9)	95·5 (6·0)	83·0 (4·0)	57·0 (4·0)	47·3 (2·8)	0

Table 3. *Some properties of the F_2–H and A_1 horizons of the Freshfield pine forest soil*

	pH	pH after addition of 0·04 M-KOH	Humic acid (%, w/w)	Hexosamine (%, w/w)	Total nitrogen* (%, w/w)	Organic matter* (%, w/w)
F_2–H horizon	3·8	4·8	30·4	0·39	1·2–0·95	100
A_1 horizon	4·4	9·2	0·6	0·04	0·02	4·9

* Figures from Goodfellow *et al.* (1968).

of 0·04 M-KOH. The A_1 had little more buffering capacity than acid-washed sand, which increased from pH 5·3 to 11·8. The high buffering capacity of the F_2–H horizon was due to its high humus content. The higher hexosamine content of the F_2–H was probably primarily a reflection of a larger biomass of fungi, other microbes and arthropods in this horizon.

Chitin decomposition in soil

Decomposition of chitin by actinomycetes, bacteria and fungi results in ammonification with consequent effects on soil pH. In the A_1 horizon the pH increased steadily from 3·8 to 7·5 over 20 d, while the increase in the F_2–H was only about 1 pH unit (Fig. 1). Nevertheless, the rate of chitin decomposition, as indicated by release of ammonium-N, was greater in the

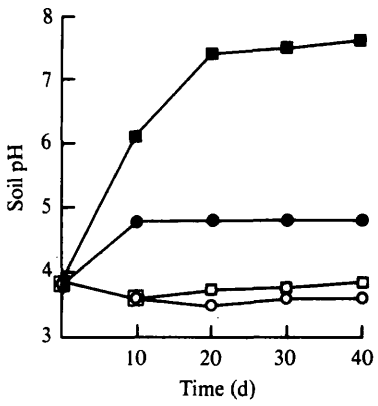

Fig. 1

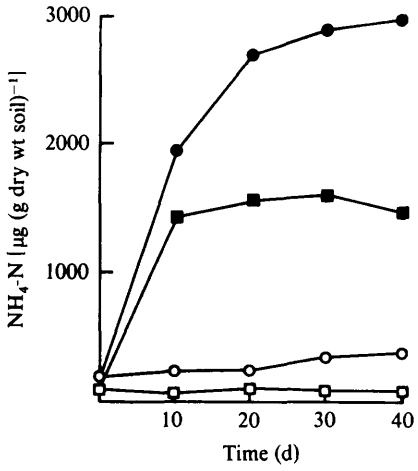

Fig. 2

Fig. 1. Changes in pH in the F_2–H horizon control (○), the F_2–H plus 2·5% (w/w) chitin (●), the A_1 horizon control (□), and the A_1 plus 2·5% chitin (■).

Fig. 2. Release of ammonium-N from the F_2–H horizon control (○), the F_2–H plus 2·5% (w/w) chitin (●), the A_1 horizon control (□), and the A_1 plus 2·5% chitin (■).

Table 4. *Effects of chitin and fungus mycelium (2·5%, w/w) amendment on the pH and streptomycete populations of the F_2–H and A_1 horizons after incubation for 40 d*

	F_2–H horizon			A_1 horizon		
	Control	Plus chitin	Plus fungus mycelium	Control	Plus chitin	Plus fungus mycelium
Final soil pH	3·6*	4·8†	4·3†	3·8*	7·6†	5·6†
Changes in counts of chitinolytic streptomycetes‡	0	+2·7†	+2·2†	+0·3	+3·1†	+1·4†
Changes in counts of acidophilic streptomycetes‡	+0·8	+1·9†	+1·9†	+1·0	−5·2†	+1·3
Changes in counts of neutrophilic streptomycetes‡	+0·2	+1·6†	+0·5	+0·4	+4·8†	+0·6

* No significant change in pH during incubation period.
† Figures significantly different from control ($P = 0·05$).
‡ Counts expressed as $\log_{10}$[no. (g dry wt soil)$^{-1}$].

F_2–H; final concentrations were twice those in the A_1 (Fig. 2). This was obviously a reflection of the greater buffering capacity of the F_2–H horizon.

The streptomycete populations of both horizons were clearly involved in chitin decomposition, amendment leading to significant increases in counts in all cases (Table 4). Numbers of chitinolytic streptomycetes increased more or less in parallel with the total population; the proportion of chitinolytic isolates in unamended soil was high (usually > 70%) and did not increase in amended systems.

Responses of acidophilic, acidoduric and neutrophilic streptomycetes during chitin decomposition reflected changes in soil pH. Differences noted were largely between acidophiles and neutrophiles, as very few acidoduric strains were detected in these soils. In the F_2–H horizon where the bulk pH reached 4·8, both acidophiles and neutrophiles increased (Table 4); the latter were probably able to grow in micro-sites of higher pH (Williams &

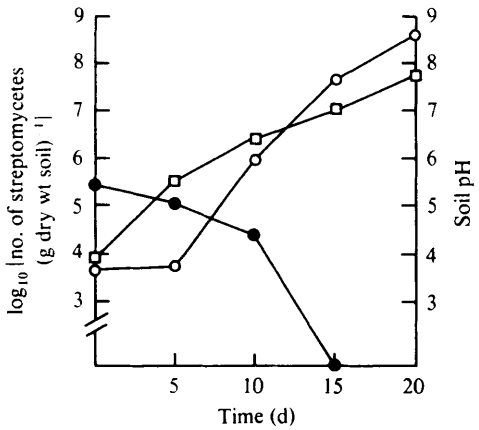

Fig. 3. Changes in numbers of neutrophilic (○) and acidophilic (●) streptomycetes, and in soil pH (□), in the A_1 horizon plus 2·5% (w/w) chitin.

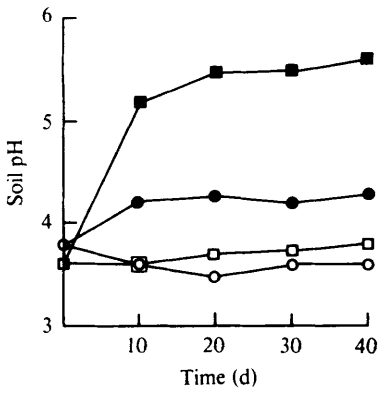

Fig. 4

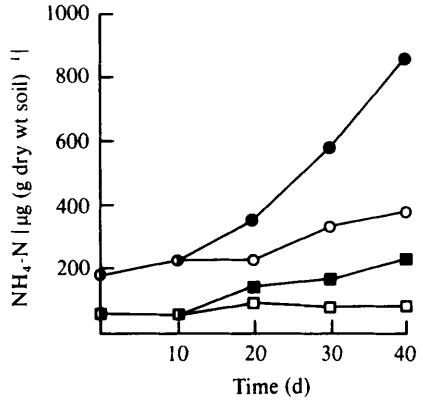

Fig. 5

Fig. 4. Changes in pH in the F_2–H horizon control (○), the F_2–H plus 2·5% (w/w) fungus mycelium (●), the A_1 horizon control (□), and the A_1 plus 2·5% fungus mycelium (■).

Fig. 5. Release of ammonium-N from the F_2–H horizon control (○), the F_2–H plus 2·5% (w/w) fungus mycelium (●), the A_1 horizon control (□), and the A_1 plus 2·5% fungus mycelium (■).

Mayfield, 1971). Microscopic examination of amended F_2–H treated with various pH indicators suggested spatial variation from pH 4·4 to 6·0. The reaction of the A_1 horizon reached pH 7·6 and this resulted in a sharp increase in neutrophiles along with a decrease of acidophiles. Changes in the A_1 horizon were studied in more detail (Fig. 3). The pH rose above 5·5 after 5 d, initiating the increase of neutrophiles and the decline of acidophiles; the latter were undetectable at 15 d.

Fungal mycelium decomposition in soil

Amendment of both horizons with fungus mycelium also resulted in pH changes; the increases were smaller than those with chitin, the A_1 reaching pH 5·6 and the F_2–H, pH 4·3 (Fig. 4). Release of ammonium-N was similarly reduced, with more ammonification again occurring in the F_2–H horizon (Fig. 5). There was, indeed, little evidence of decomposition in the A_1 horizon.

Numbers of chitinolytic streptomycetes were significantly increased by amendment with fungus mycelium in both horizons (Table 4), as were total numbers. In the F_2–H horizon the small increase in pH was reflected by a stimulation of acidophiles but little increase in neutrophiles. Neither group showed significant increases in the A_1, where there was little evidence of decomposition. In contrast to the response seen after chitin amendment of the A_1 horizon, acid-tolerant streptomycetes and other soil microbes were unable to initiate decomposition to an extent sufficient to induce neutrophile activity.

DISCUSSION

Streptomycetes isolated from a wide range of soils were able to hydrolyse colloidal chitin. The pH optima for this process were broadly related to those for growth and to the pH of the soil from which isolates came. Distinctions between the activity ranges and optima of acidophiles and neutrophiles were similar to those found for starch hydrolysis by Williams & Flowers (1978). Studies on purified chitinases from streptomycetes have indicated optimum activity at pH 7·5 (Reynolds, 1954), pH 4·2–5·0 (Skujins *et al.*, 1970) and pH 4·2 (Skujins *et al.*, 1974), but the growth requirements of the producing strains were not given.

Stimulation of streptomycetes by chitin amendment of soils with a reaction near to neutrality is well known and may persist for long periods. Vruggink (1970) detected 30-fold increases 10 months after addition of 1·5% (w/w) chitin. Streptomycetes clearly also play a role in chitin decomposition in acidic soil and litter, where fungi have been regarded as the most important colonizers of chitin (Gray *et al.*, 1968). Acidophilic and acidoduric streptomycetes are also involved. Release of ammonia by the deacetylation and deamination of *N*-acetylglucosamine residues may ultimately raise the pH sufficiently for activity of neutrophiles also. The behaviour of streptomycete populations in the poorly buffered A_1 horizon provided a good example of a microbial succession caused by environmental rather than nutritional changes during decomposition. Such gross increases in pH will rarely occur naturally in acid soil, but in localized sites of decomposition there may be an opportunity for limited growth of neutrophiles. This may account for their routine occurrence in acid soils in low numbers (Williams & Mayfield, 1971). Ammonification in chitin-amended soils may also be of underestimated significance in the control of plant pathogenic fungi. Hora & Baker (1972) claimed that ammonia was an important fungistatic factor in chitin-amended soil.

Addition of fungus mycelium to the two horizons induced similar but less marked changes. Ammonification, and therefore probably decomposition, was significantly less than with chitin. The major components of the walls of basidiomycetes and many other fungi are microfibrillar chitin and amorphous glucans (Bartnicki-Garcia, 1968). Figures available for other basidiomycete sporocarps indicate that those of *Lactarius rufus* would contain around 30–40% (w/w) chitin. This is one factor contributing to the smaller degree of ammonification. Another is that initiation of decomposition requires both glucanases and chitinases, although it has been claimed that streptomycetes often produce both (Skujins *et al.*, 1965).

Fungal hyphae are probably the major source of chitin for streptomycetes in soil. This may not be restricted to dead hyphae, as there is evidence that streptomycetes can lyse live mycelium (Lloyd *et al.*, 1965; Lloyd & Lockwood, 1966). Glucosamine is the predominant hexosamine in soil hydrolysates, and this is particularly so in podzols under coniferous litter (Sowden, 1959). As fungi predominate in such acidic soils, it is likely that most of the glucosamine is derived from chitin in their walls. The hexosamine content of the F_2–H horizon in the present study was similar to that of other soils examined (Stevenson, 1957; Sowden, 1959), while that of the A_1 was lower.

Therefore, streptomycetes are likely to play a major role in the decomposition of fungal material in acid litters and soils. Such materal constitutes an important substrate for microbes in these environments. The productivity of fungi within litter and soil is difficult to estimate, but sporocarps in one pine forest studied produced 30 kg dry wt ha^{-1} annually (Richardson, 1970).

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