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ANTIBIOTIC PRODUCTION ON ORGANIC MANURES
AND IN THE SOIL IN RELATION TO THE CONTROL
OF PLANT DISEASES

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SECTION I

Introduction

INTRODUCTION

The majority of micro-organisms known to produce antibiotics on the standard laboratory media live normally in the soil. Further, antimicrobial toxins have been isolated from natural or treated soil by various workers. Others have shown that some bacteria and phytopathogenic root fungi tend to die out rapidly when introduced into certain soils. On the other hand, when a soil has been sterilized and becomes reinfected with a pathogen, the progress and virulence of the latter are greatly increased as compared with the behaviour in non-sterilized soil. These observations point to the fact that the soil contains agents inhibitive to certain micro-organisms and thus the question arises whether members of the soil flora have the ability to form antibiotics in their natural habitat, the soil. The problem has to be approached from two angles. First, it is important to establish whether or not antibiotics are formed by the soil flora in the course of their normal metabolism in untreated soil and second, whether by artificial means production could be induced, and enhanced. On the first point, no evidence is as yet available that the majority of the toxins hitherto isolated from the soil were in fact antibiotics in the true sense of the definition. Waksman (1945) defines antibiotics "as chemical substances of microbial origin". The emphasis in this definition should

be placed on the words "of microbial origin". Only indirect evidence is available that such toxins as have been isolated from soil were of microbial origin. The possibility that they were merely intermediates or end-products of a purely chemical decomposition taking place in the soil must not be overlooked, especially as they were frequently general poisons affecting higher plants as well as micro-organisms.

Because of the complexity of soil conditions and the great technical difficulties involved in studying soil processes it appears a difficult undertaking to prove directly that a certain substance extracted from untreated soil is a true antibiotic. At present, the most profitable approach to this problem is the attempt to prove by chemical and biological means that a certain toxin derived from a given non-sterile soil is identical with one of the numerous known antibiotics. This has not yet been achieved.

The difficulties encountered in isolating an antibiotic from non-sterile ^{and untreated} soil could be explained in various ways. First, no antibiotic had been formed either by the indigenous or by the zymogenous flora. Second, one had been formed but was rapidly inactivated by adsorption or destroyed by enzymic action of members of the soil microflora.

On the other hand, it is not certain whether adsorbed antibiotics are to be regarded as being truly inactivated. It is not improbable that by some method of ionic exchange or otherwise certain micro-organisms might have the ability to liberate these adsorbed antibiotics at least temporarily, or in turn adsorb them on their cell walls or allow them to enter in some other manner into their own metabolism. Third, the quantities formed were so small and probably these existed only in local concentrations, that the techniques hitherto employed were not sensitive enough to detect the antibiotic. It is likely that at best, in non-sterile soil where the competition for such materials as are required for antibiotic production is very intense, the antibiotic (provided it was formed and remained stable for some time) could only be present in low concentration. The question arises whether the concentration would be sufficient to affect the equilibrium of the microflora, especially to effect the inhibition of the pathogen.

The situation is different in sterilized soil. If an extract from an autoclaved soil, inoculated with an antibiotic producing organism inhibited the growth of test organisms sensitive to the antibiotic normally produced by the inoculated organism, then it is highly probable that a true antibiotic had been formed in the soil, especially if an extract of the same but not inoculated soil did not display

such inhibitory properties. On the other hand, as many basic antibiotics are readily adsorbed on soil colloids, it does not follow that the failure to extract an antibiotic from soil is conclusive proof that one had not been produced. Equally, there is no conclusive proof that an antibiotic had been formed if an organism cultured in sterile soil inhibits the growth of another micro-organism introduced with it, even if the latter is known to be sensitive to an antibiotic normally produced by the former in the laboratory. It has yet to be shown that microbial antagonism is due solely to the formation of a toxin produced by one organism and injurious or lethal to another.

Intermediate between these two extremes is the case where a toxin is formed in non-sterile soil as the result of a treatment designed to encourage intense microbial activity, such as by organic manuring, deep soil cultivation, liming and other methods of soil management. If this toxin had not been detected previously in the untreated soil, the assumption that it is of microbial origin, i.e. an antibiotic, is well justified.

The possibility of inducing antibiotic formation by artificial means in soil is of major economic importance as it may provide an efficient method of biological control of phytopathogens. It is known that many of these are affected

in-vitro by antibiotics. If therefore members of the soil flora could be induced to form antibiotics in the soil it might be possible to control soil-borne pathogens effectively by inhibiting their further development. Moreover, as some antibiotics exhibit systemic properties, control even of air-borne pathogens may be envisaged, through the uptake of soil-produced antibiotics by the roots and translocation through the host plant.

There are several methods by which antibiotic formation might be induced. First, by the large scale inoculation with organisms known to form antibiotics to which a given pathogen is sensitive. Second, by following up this process of inoculation with a type of soil management, especially manuring, which will not only help the establishment of the freshly introduced organism but enhance antibiotic production. Third, by applying such methods of manuring and soil cultivation in general as will encourage certain members of the soil flora to develop luxuriantly and produce antibiotics. The supply of adequate nutrients in the form of organic manures may exert two effects, possibly simultaneously. It may enhance the germination and rapid multiplication of indigenous organisms which before may have been dormant, also, with the organic matter incorporated with the soil, a new flora, mainly a zymogenous one, would be introduced and this in view

of the heavy supply of nutrients would become established, at least temporarily, at the expense of the indigenous organisms. Such a process could be aided by various methods of soil cultivation, such as aeration, liming and adequate water supply, and by raising the temperature where possible.

It has been shown that among saprophytes there are many antibiotic producing organisms, especially Penicillia, Aspergilli and actinomycetes. It is therefore likely that organic manures may introduce many such organisms, but in view of the considerable strain specificity of certain antibiotic producers it is not certain whether soil conditions would favour the development and antibiotic activities of such strains.

Apart from the introduction and subsequent establishment of a fresh flora, comprising possibly antibiotic organisms, it is essential to modify soil conditions in such a manner as to encourage the particular mode of metabolism which results in the formation of antibiotics. It has been shown repeatedly that while an organism may grow luxuriantly on a certain substrate it will not necessarily produce therein the antibiotic usually associated with it and which it forms on another medium.

It is now an established fact that certain root

pathogens can be inhibited or entirely suppressed by the application of organic manures and it is agreed that this control may be due to the phenomenon of microbial antagonism. Very little is known of the mechanism of this action. Explanations put forward are based on the effects of competition, alteration of pH, accumulation of carbon dioxide resulting from an increased microbial activity, and eventually the production of toxins, i.e. antibiotics, by certain members of the actively growing soil flora.

Very little experimental evidence is available as to the extent of the part played by any one factor. It is quite possible that the observed effect, i.e. suppression of a pathogen, is the result of the interaction of all these factors together with others not yet suspected. On the other hand, organic manuring could also encourage root parasites by supplying food in addition to that provided by the host. Moreover, just as certain micro-organisms produce substances toxic to others they may equally form stimulatory agents or necessary growth substances, or they may supply additional nutrients by breaking down complex materials contained in the organic manures incorporated with the soil.

One aim of the work described in this thesis is the study of the part played by antibiotics in microbial antagonism as a result of organic manuring and the first point to be investigated is the adequacy of the soil as such

as a medium for antibiotic production. In culture work abundant antibiotic production necessitates a high level of nutrition and the use of complex media, and it does not follow that even organisms whose natural habitat is the soil can form antibiotics therein, as it is nutritionally a poor medium. The addition of microbial food would therefore seem necessary.

However, it has been reported recently that even in non-supplemented but sterile soil some antibiotic activity could be detected, thus indicating that even such a poor medium as soil alone could support antibiotic production in some instances.

Apart from an adequate food supply the presence of antibiotic producing organisms is of paramount importance. In non-sterile soil such organisms may be members of the indigenous flora. However, zymogenous organisms are frequently more powerful antibiotic producers and, furthermore, active strains may not be available in a given soil; thus inoculation may be required. The establishment at a sufficiently high level of organisms not occurring in a given soil is normally difficult. The soil micro-flora exists in an equilibrium condition and normally tends to resist the intrusion of fresh organisms. Organic manuring supports establishment, especially of saprophytes by supplying selective foods. Other conditions such as aeration,

soil reaction, temperature and water content, may also determine the composition and activity of the microflora, (apart from the freshly introduced antibiotic organisms) and may influence their survival. Thus, one of the difficulties encountered in antibiotic production in non-sterile soil may be due to the failure of the introduced organisms to become established.

It is also important that the organisms should grow actively and not merely survive in the form of spores. It is also not clear whether an organism exposed to intense microbial competition would preserve its antibiotic properties. Many organisms whether pathogenic or antibiotic lose their characteristic properties when sub-cultured in the laboratory in pure culture. In the soil the conditions are the reverse of pure culture and it has to be seen how this would affect antibiotic activities. On teleological grounds antibiotic properties should be preserved if not improved by sojourn in soil, in view of the fact that the soil is the natural habitat of most antibiotic producers. However, it is not certain that all the active antibiotic strains isolated from soil were in fact actively growing therein. Pathogenic organisms which, as a result of sub-culturing in the laboratory,

have lost their virulence, regain it again when passed through the host. The soil might be regarded as analogous to a host plant and recovery or improvement of antibiotic properties might be expected as the result of passage through the soil. Further, it seems possible that soil conditions may favour the selection of active, antibiotic strains. This leads to the question of the physiological function of antibiotics and whether they may be regarded as defence mechanisms. There is no agreement on this point. If antibiotics were a means for the "survival of the fittest" then passage through non-sterile soil of an organism grown hitherto in pure culture should improve its antibiotic properties; by exposure to keen competition the need for an efficient defence mechanism would arise.

These are but a few of the problems of the complex of interacting factors confronting the student of antibiotic production in the soil. An attempt has been made to investigate them, and the results are reported below.

SCOPE OF THE THESIS

The scope of the work was to study antibiotic production in the soil and to establish whether a correlation may exist between the observed control of certain root parasites by organic manuring and the production of antibiotics as a result of that treatment.

It was not intended to investigate whether antibiotics are present in natural soils and whether they are formed by the indigenous flora in the normal course of their metabolism, i.e. whether they can be regarded as a normal factor in the soil economy. The aim was limited to the study of the possibilities of inducing antibiotic production by artificial means in a given soil, but it was hoped that the observations obtained on the conditions governing antibiotic production in that way might indirectly provide information about antibiotic production in natural-untreated-soil. In particular, it was assumed that with some caution the results might be applied to interpret processes taking place under field conditions.

The method employed to induce antibiotic production in a given soil consisted in the inoculation with specific antagonists simultaneously with the supply of organic manures, mainly carbohydrate sources.

The fungi chosen for this purpose were: A.clavatus, A.terreus and P.patulum because all produce expansine (clavacin, clavatin, claviformin, patulin, penicoidin, anhydro-3-hydroxymethylenetetrahydro-1: 4-pyrone-2-carboxylic acid) in culture media, an antibiotic which is formed by a wide range of soil fungi and shows in addition to its antibacterial properties marked antifungal activity.

In addition two unidentified species of Penicillium and F.javanicum were examined.

Four species of Streptomyces were included in these experiments because actinomycetes are not only powerful antibiotic producers but large numbers and many types are likely to be found in soil supplemented with organic manures.

The conditions which were believed to favour antibiotic production in the soil were examined, the first being the effect of additional nutrients as supplied by organic manuring. Experiments were therefore carried out to show whether crop plants usually used as organic manures in agriculture would by themselves support antibiotic production. In addition materials were tested which could be but hitherto have not been used for this purpose. Antibiotic production by fungi and actinomycetes on these substrates was then examined.

The effects of supplements of glucose, various sources of nitrogen and one of phosphorus on cultures on manures were then examined and an estimate made of the relative effects of nutrients and microbial competition on antibiotic production by P.patulum. For this purpose yields on completely sterilized materials were compared with those obtained on partially sterilized or non-sterile materials, and the

effect of the release of nutrients due to heat treatment was investigated. Once it was established that certain organic manures were adequate substrates for antibiotic production, the problem was studied in the soil, either in autoclaved not amended soil or in autoclaved soil supplemented with materials serving as carbohydrate sources. A parallel series of experiments as carried out on manure cultures was repeated in the soil, comparing antibiotic production by fungi and actinomycetes, the effect of nutrition versus competition, the factor of release of nutrients by heat and so forth. Further, the influence of microbial competition on the maintenance of titre of an antibiotic already formed in the soil was examined. In an attempt to investigate the causes of the difficulty to detect an antibiotic in non-sterile soil the survival of A.clavatus cultures introduced into non-sterile soil was studied, and the changes that occurred in their antibiotic activity as a result of sojourn in non-sterile soil. This led to observations on the mutation of A.clavatus and to a correlation between morphological characters and antibiotic activity.

A SURVEY OF THE RELEVANT LITERATURE

The growth inhibiting properties of certain soils on specific micro-organisms

These have been demonstrated either by the extraction of toxins from untreated or treated soils or by observations that certain micro-organisms disappeared when introduced into certain soils.

Toxic substances in non-sterile soil: The earliest reports of the presence of microbial toxins in the soil came from Greig-Smith in a series of papers on soil fertility (1912-1917). He showed that certain soil treatments such as supplements of organic matter and low moisture content resulted in an increase in toxin production while aeration, drying or treatment with chloroform led to a decrease. He stressed the selective action of the toxins and limited his work to demonstrating soil toxicity against Bacillus prodigiosus only. Hutchinson and Thaysen (1918) stated that they were unable to demonstrate antimicrobial toxicity in the soils they tested. Rayner, in her work between 1934 and 1935, has tried to correlate the failure to establish conifer plantations on Wareham Heath soils in Dorset with the presence of toxins therein. She suggested that these toxins were of microbial origin. This work was carried further by Neilson-Jones (1941). He carefully discriminated between nutritional and purely toxic effects, and postulated that the growth

inhibiting toxins found in Wareham Heath soils were of biological and not of chemical origin. In an attempt to prove this hypothesis he sterilized soil, thereby destroying the toxins which were not heat stable, and added an inoculum of non-sterile soil. A layer of nutrient agar placed over the soil mixture (method initiated by A.G.Morton) became infected with fungal mycelium, but the latter gradually became disintegrated. He inferred that the micro-organisms introduced into the sterilized soil from the inoculum produced toxins which diffused into the agar and gradually destroyed the organisms growing thereon. Neilson-Jones dismissed the possibility of competition for food by the invading soil organisms in view of the fact that the agar layer contained an additional food supply sufficient to satisfy all the nutritional requirements of the infecting organisms. It could, however, be argued that the non-sterile soil inoculum had carried with it a chemically originated toxin which diffused first into the sterilized soil and then eventually into the agar: this would satisfactorily explain the gradual destruction of the fungal mycelium on the agar film. Neilson-Jones suggested that the toxin was hydrogen-sulphide produced by the sulphate reducing bacteria in the soil but he had not analysed it chemically. The question arises, should hydrogen sulphide be regarded as a true antibiotic in the

recognised sense. The author also stressed the fact that such toxins were not characteristic of Wareham Heath soil only but that "they can be related in a general way with degenerative changes taking place in woodland and other organic soils not showing marked symptoms of toxicity." The difference, he holds, is only one of degree of toxicity. Like Greig-Smith, he also found that air drying destroyed the inhibitory agents. Later work on Wareham Heath soils by Brian, Hemming and McGowan, (1945) came closer to an explanation in terms of antibiotics of the toxicity of this soil mycorrhiza. They were able to show that species of Penicillium belonging to the groups of the P.nigricans P.janewewskii series P.jenseni and P.janczewskii Zal. were prevalent in this soil. P.jenseni produces gliotoxin to which mycorrhizal fungi are sensitive on standard media. These workers tentatively suggested therefore that possibly gliotoxin and other antibiotics had been formed in this soil and were inhibiting the mycorrhiza essential for the growth of conifers.

An antifungal substance extracted from a soil in Germany by Winter (1940) inhibited the growth of Ophiobolus graminis. Winter was not concerned with a discussion whether this toxin was of microbial origin or not but endeavoured to show that the observed suppression

of O.graminis under certain conditions was not due to an accumulation of carbon dioxide as postulated by Garrett (1944) but to the action of an antimicrobial, complex poison.

Waksman and Woodruff (1941) prepared extracts from two soils, one fertile and the other infertile. While the water and alcohol extracts showed no inhibitory properties, the ether soluble fraction inhibited the growth of Bacillus subtilis and Sarcina lutea in the same concentrations as the antibiotic actinomycin. Bact.coli was resistant. The infertile soil contained the larger quantity of the inhibitory substance. The authors inferred that "soils contain substances of the actinomycin type, which inhibit the growth of certain bacteria in culture media." These workers did not attempt to prove the microbial origin of the toxin. Further, in the same paper they showed that actinomycin was inactivated to a large extent in soil, and it appears therefore not very probable that the inhibitory action observed was due to the presence of actinomycin. In spite of the similarity of the bacterial spectrum with that of actinomycin other antibiotics or toxins of chemical origin may act similarly. Newman and Norman (1943), in an attempt to elucidate the reasons for the low microbial activity observed in the subsurface layers of soils, came to the conclusion that these contained a population different

from that of surface layers, being less efficient in the decomposition of organic matter supplied. They tried to establish the population of surface layers in the lower soil horizons by simultaneously supplying microbial food, but apparently failed to do so. They explained this failure as being due to the accumulation of antibiotic substance in the subsurface soil and demonstrated that in-vitro alcoholic extracts of one horizon inhibited members of the flora of other soil layers without affecting its own. The same criticism as before applies again here. There is no evidence that the toxins accumulated in subsurface layers were of microbial origin, i.e. true antibiotics. Furthermore the difficulty of establishing a new flora in soils or soil layers must not necessarily be caused by accumulation of antibiotics but may be due to other forms of manifestation of microbial antagonism.

In 1948 and 1952 Grossbard showed that various soils contained water soluble substances which slightly inhibited the growth of Bact.coli and Bacterium carotovorum, while Sarcina lutea was almost completely inhibited. Sinha (1950) made a similar observation with a displaced soil solution, but using different test organisms. Again no evidence was supplied that these substances were of microbial origin.

With the exception of the work of Greig-Smith all the reports quoted so far deal with soil which had not been treated i.e. refer to investigations whether in soil antibiotic or toxin formation is a normal phenomenon, either in a specific soil or in general. Campillo (1947), however, tested a soil which was cropped with lucerne or French beans and proved that the petroleum-ether soluble fraction of the soil extract had a bactericidal action on Rhizobium meliloti. He stated that the formation of this substance was not correlated with the pH, texture or the content of organic matter in the tested soils. He postulated the presence of antibiotic substances in the extract. However, in view of the lack of correlation between formation of toxicity and such soil conditions which normally would influence microbial activity, it is suggestive that he was dealing with chemically rather than biologically originated toxin. On the other hand, Nandi (1948) clearly showed that the supply of carbohydrate food, such as glucose and straw (presumably as an energy source) led to the formation of an inhibitory substance which could be detected in the displaced soil solution. He, like Greig-Smith treated the soil in such a manner as to encourage microbial activity, namely by supplying microbial food and by prolonged incubation; As these treatments resulted in the formation of a toxin, it

appears most likely to be one of microbial origin, i.e. an antibiotic. Sinha (1950) showed that nutrients of different type, such as dried blood, would lead to the formation of antibiotics in non-sterile soil. Probably in this case another group of micro-organisms with different food requirements was forming the inhibitory substance.

Further evidence on antibiotic production in non-sterile soil was provided by Brian et al. (1951). Oat seedlings growing in non-sterile as well as heat sterilized soil enriched with wheat meal, and inoculated with P. griseofulvum, showed the presence of griseofulvin in their tissues. In sterilized soils activity appeared sooner and was frequently greater than in non-sterile soil. The fact that the oat plants have taken up the antibiotic from the soil and translocated it throughout represents the first demonstration of systemic properties in antibiotics. Brian and his co-workers, however, do not stake a definite claim that antibiotic production had occurred in either the non-sterile or sterilized soil. They envisaged the possibility that the antibiotic had been present in the meal inoculum itself and was not formed directly in the soil mixture. A definite claim for antibiotic production in non-sterile as well as in sterilized soil has been made by Gregory et al. (1952) using P. patulum and an unidentified

species of a soil bacillus. In non-sterile soil the antibiotic could only be detected in soil supplemented with 0.5% soy-bean meal, 0.5% glucose and 0.2% corn steep liquor, by means of the soil displacement method. As was to be expected the concentrations were less than those in sterilized soil. The antibiotic was formed after 4 days incubation but disappeared rapidly, probably owing to microbial destruction.

The disappearance or suppression of organisms introduced into non-sterile soil: Another manifestation of the inhibitory properties of certain soils is provided by the numerous observations on the dying out or failure to become established of introduced organisms, which may not be soil organisms at all, such as Bact.coli, or others, indigenous to a great variety of soils but which fail to become established in others. An example may be afforded by the difficulty encountered in introducing active Rhizobium strains into certain soils. Further, certain plant pathogens are less virulent or do not occur at all in some soils, e.g. O.graminis and Dydymella lycopersici (Bewley, 1947).

On the first point numerous reports are available on the dying out of bacteria in non-sterile soil and their ready survival in sterilized soil. This was shown by Martin (1897-1900) and by Smith (1904) for Salmonella typhosa and

by Sedgwick and Winslow (1902) for the former organism and Bact.coli also. Wakeman and Woodruff (1940) gave a detailed account of the decrease in numbers of Bact.coli after addition to non-sterile soil: this was followed by an increase in those soil organisms which are antagonistic to Bact.coli. The rate of its disappearance seemed to be independent of pH and temperature of incubation.

The work of James, Campbell and Gunner (1949) has provided a good illustration of the disappearance of a fungal phyto-pathogen when introduced into non-sterile soil. They found that a saltant of Helminthosporium sativum was no longer present after the first crop when introduced into non-sterile soil, though it had retained the pathogenicity of the parent and remained relatively stable in characteristics when in pure culture. These workers claimed that various soils contained a thermolabile substance inhibiting the growth of the saltant and that it was formed by members of the indigenous flora. This hypothesis was supported by the fact that certain organisms isolated from the soil, such as Aspergillus flavus, when cultured in the laboratory produced a substance which inhibited the growth of H.sativum in vitro. Furthermore, when barley seeds infected with both H.sativum and one of the antagonistic soil isolates were grown in sterile soil, a lower "disease rating" was recorded among the ensuing

seedlings than from the controls which were infected with the pathogen alone. Thus a protective action was apparent in sterile soil. The authors therefore suggested that the disappearance of the saltant of H.sativum is due to the formation of antibiotics in the non-sterile soils. Though this appears very probable the evidence is not quite conclusive. Many soil organisms produce substances in laboratory media which inhibit phytopathogenic fungi. This had been demonstrated extensively in earlier work on O.graminis and other organisms such as Rhizoglyphus solani. It does, however, not follow necessarily that such substances are produced equally in non-sterile soil. Furthermore, the protection of the barley seed was recorded on sterilized soil only where antibiotic production is more likely, as will be shown later. The protective effort may also be due to other factors of microbial antagonism than antibiotic production. However, the hypothesis of James et al. is more strongly supported than similar suggestions of other workers because they have actually demonstrated the dying out of the H.sativum saltant in the soils from which the antagonistic organism had been isolated, and not merely a protective action on the host plant.

Toxic substances in sterilized soil: Such substances were demonstrated by Lewis (1923) , who inoculated sterilized soil amended with either 5% cotton seed meal or fresh horse manure with Pseudomonas fluorescens and later extracted the soil with water or alcohol. While the water extracts had no inhibitory effect on his test organisms, the alcohol extract clearly inhibited them on culture media but not in soil, since when the soil culture was sterilized and inoculated with B.cereus no growth inhibition took place. He also showed that the active principle formed by Ps.fluorescens in culture media was adsorbed on charcoal and field soil. Although Lewis has clearly demonstrated antibiotic formation in the sterile soil he drew the conclusion that free bacterial toxins would probably not accumulate in the soil in sufficient quantities to inhibit other bacteria.

Nickel and Burkholder (1947) found that various actinomycete cultures grown in supplemented soils completely inhibited the growth of Azotobacter vinelandii when it was subsequently introduced. They used autoclaved soil and the supplements were animal manures and cover crop materials. Even in unamended sterile soil cultures of the actinomycetes some reduction of the number of A.vinelandii was reported. The authors succeeded in preparing from some of the actinomycete

soil cultures an aqueous extract which clearly exhibited antibacterial properties. This is the first record in the literature of the demonstration of antibiotic activity in an aqueous extract of an actinomycete culture in soil. Obviously the antibiotic was not adsorbed by the soil colloids. Grossbard (1947) was unable to demonstrate antibiotic production in unsupplemented sterilized soil cultures of P.patulum but showed that additions of small quantities of glucose or other carbon sources gave rise to considerable yields of antibacterial and antifungal material.

All these workers stressed the need for an additional supply of food in sterile soil in order to obtain activity, but Gottlieb and Siminoff (1950) demonstrated growth inhibition of B.subtilis by S.griseus in unamended sterile soil. They postulated that this was not due to the production of an antibiotic but merely to microbial antagonism, especially as a strain of S.griseus which did not produce streptomycin on the standard media also exerted a similar inhibitory action on B.subtilis in the soil. It could, however, be argued that in the different medium provided by sterilized soil even a strain shown to be inactive on laboratory media may be able to produce an antibiotic. Another argument brought forward was that streptomycin is inactivated in the soil, but Sinha (1950) demonstrated considerable persistence.

Hessayon (1951) claimed to have observed trichothecin production in unsupplemented soil, using a method similar to one employed by Neilson-Jones (1941). He placed an agar layer over a sterilized soil culture of Trichothecium roseum and showed that the growth rate of his test organism (Fusarium oxysporum var. cubense) was reduced by 11%. The complete growth inhibition obtained by the previous workers was not achieved. The first instance in which an inhibitory substance formed in sterilized as well as in non-sterilized soil cultures was identified with a known antibiotic was provided by the work of Brian et al. (1951): the presence of griseofulvin was identified by biological methods. Chemical identification was possible for chloromycetin production in sterilized soil by S. venezuelae (Gottlieb and Siminoff, 1952). This antibiotic was successfully extracted, though in small amounts, from unsupplemented soil. However, enrichment with alfalfa hay (lucerne), and better still with tryptone, increased the yield, while carbohydrate sources such as starch or straw were ineffective. The latest report on the production of an antifungal substance in sterilized, supplemented soil comes from Gregory et al. (1952). They used P. patulum, Trichoderma lignorum, and unidentified species of Streptomyces and Bacillus. Their test organism was Pythium debaryanum.

In unsupplemented soil cultures of *P.patulum* mere traces of antibiotic were detected but when organic material was added activity could be observed after only 4 days incubation. T.lignorum yielded only traces even on supplemented soil while the unidentified Streptomyces and Bacillus formed antibiotic only in a mixture of soil with the best liquid medium. However, in that case the Bacillus showed greater yields than when cultured on the liquid medium alone. This is most likely due to the further supply of nutrients coming from the soil and suggests that the failure to detect an antibiotic in soil containing only manures such as straw or alfalfa (lucerne) was due to a large extent to nutritional deficiencies.

Microbial antagonism as a means of suppressing soil-borne phytopathogens

1) IN-CULTURE

There are numerous reports that many soil organisms suppress the growth or altogether destroy soil-borne pathogens in pure culture. It is not possible here to review the unwieldy mass of information on this topic. If one wished to make an analogy to Greig-Smith's statement that there are as many toxins in the soil as there are bacteria, one might venture to say that there are as many antagonists in the soil

as there are pathogens. It is probably truer to say that a number of organisms can antagonise each pathogen or produce stimulatory substances as well. This is well illustrated by the extensive work of Slaggett and Fellows (1947) who surveyed, repeated and amplified the experiments of other workers on microbial associations between O.graminis and other members of the soil flora. This study was initiated by Sanford and Broadfoot (1931), who tried to explain the conflicting variations in virulence of this organism not in terms of nutrition but as the action of the microflora, varying with soil conditions, on the pathogens. These authors, as well as Broadfoot (1933), Brommelhuis (1935), Lal (1939) and Slaggett and Fellows (1947) showed that in pure culture certain soil organisms produced toxins to which O.graminis was sensitive. From this they concluded that under soil conditions similar factors may have been responsible for the observed control of O.graminis in the field by such treatments as in general would encourage microbial activity.

The methods employed to demonstrate microbial antagonism consisted either in growing the pathogen together with a soil isolate on the same agar plate, or by adding a suspension or a culture filtrate, sometimes even autoclaved, to agar plates or culture solutions of the pathogen. Reduction in growth rate or complete inhibition was then observed.

Slagg and Fellows (1947) clearly demonstrated that while a fungus may form an inhibitory substance on one medium, alteration of the composition of the latter, e.g. by variation in the nitrogen source, resulted in the production of materials which stimulated O.graminis. On the other hand, on a given substrate one organism would produce an inhibitory agent and another a stimulatory one. Further, using the same medium and the same soil isolate the stage of its growth would determine whether an inhibitory or a stimulatory substance was formed. An example of this was provided by Rhizopus nigricans. This work illustrates well the complexity of microbial associations. The authors conclude that the suppression of O.graminis in the soil would therefore be determined not only by the composition of the microflora but by nutritional factors as well.

Extensive work initiated by the investigations of Weindling (1932) has been carried out on the microbial associations of Trichoderma lignorum, Weindling demonstrated the disintegration of hyphae of the pathogen Rhizoctonia solani in the presence of the common soil fungus T.lignorum. Later Weindling (1934) and Weindling and Emerson (1936) isolated a lethal principle, the antibiotic gliotoxin, which was shown to cause the hyphal destruction and also affected

other organisms such as Phytophthora parasitica, Sclerotium nolfsii and others. This work was confirmed by Allen and Haensler (1935). Wood (1951) demonstrated antagonism between R.solani and a number of fungi such as F.claviforme, (F.clavariaeforme), T.viride, and S.lavendulae as well as between B.cinerea and the above organisms. A Fusarium species proved to be an effective antagonist as well.

Yang (1951) isolated from the soil a species of Trichoderma and various bacteria which inhibited the development of Ustilago zeae. Pythium de Baryanum and other species of this genus were extensively studied by Van Lujk (1938) who showed that culture filtrates of P.expansum inhibited these pathogens. He was the first worker to isolate an antibiotic identical with patulin, namely expansine. Actinomycetes, foremost among active antibiotic producers, were shown to antagonise P.arrhenomans by Cooper and Chilton (1950) and by Johnson and Leander (1952). Fungi isolated from the Louisiana soils from which the above actinomycetes derived also inhibited this Pythium (Luke, 1952). Other workers engaged on isolating antibiotic producing actinomycetes were Landerkin, Smith and Lochhead (1950). They isolated from soils in Northern Canada 860 cultures of actinomycetes, of which a large percentage showed antagonism against at least

one of the three phytopathogenic fungi H.sativum, F.culmorum and F.linii. It was suggested that the high proportion of antibiotic actinomycetes found in Northern soils may be due to the lower degree of plant growth in those regions.

Darpoux and Faivre-Amiot (1950) found among the numerous antagonists they investigated an unidentified actinomycete (no.105) which antagonised Sclerotinia libertiana, Pseudomonas tabaci and other pathogens. Culture filtrates from an unidentified bacterium exerted a similar action on Alternaria solani.

2) IN SOIL

Control measures against phytopathogenic fungi by microbial antagonism have been mainly confined to soil-borne pathogens, though several reports are available on the attempted control of diseases affecting the aerial parts of plants. The most recent reports are those of Wood (1951) and Newhook (1951) on the control of B.cinerea. By colonizing the moribund tissues of the lower leaves of lettuce with active antagonists, Wood (1951) was able to reduce considerably subsequent infection with B.cinerea on lettuces cultivated in frames where the humid and warm conditions aided the establishment of the saprophytes. However, in field experiments

carried out by Newhook (1951) the spraying of plants with soil suspensions containing a mixture of antagonists or with specific cultures was ineffective.

Three methods are available for controlling soil-borne plant pathogens by microbial antagonism. First, inoculation of the soil with antagonists only, second, supplementation with nutrients simultaneously with the introduction of antagonistic cultures, and third, various forms of soil management, designed to encourage microbial activity, in particular organic manuring but omitting inoculation with specific organisms.

i) The inoculation of the soil with antagonists without any additional supply of nutrients

One of the earliest reports on microbiological control of plant diseases comes from Porter (1924) who achieved a suppression in disease incidence due to F.linii on flax and to H.sativum on wheat by antagonistic organisms inoculated in to sterilized soil infected with the pathogens. Sanford and Broadfoot (1931) obtained an effective control with wheat infected with O.graminis as a result of using either suspensions or culture filtrates of antagonistic soil organisms. The experiments were carried out in sterilized soil. Slagge and Fellows (1947) confirmed these results.

In addition they also used non-sterile soil. Here, although a decrease in disease incidence was obtained the results were not consistent. Allen and Haensler (1935), working with non-sterile soil, reported that seed decay and "damping-off" of cucumbers and garden peas induced by Rhizoctonia and Pythium was greatly reduced as a result of heavy inoculation of the soil with Trichoderma. Garden peas showed less response to the treatment than did the cucumbers.

Utilising their earlier observations on the antagonistic action of Trichoderma against Rhizoctonia, Weindling and Fawcett (1936) obtained good control under greenhouse conditions of "damping-off" of citrus seedlings caused by R.solani when sterile or non-sterile soil was inoculated with Trichoderma and the soil acidified to pH 4 by means of peat or sulphate of ammonia. Although acid conditions improved the degree of control, they were not the only cause of it. In sterilized and acidified soil, disease incidence was not reduced in the absence of Trichoderma. However, in the field an acid reaction alone helped to reduce the disease attack, apparently by encouraging Trichoderma which is to be found in most soils as a member of the indigenous flora, though Wood (1951) was unable to obtain control of R.solani on lettuces by mere acidification of the soil, owing to the plants being damaged. He also found that, while addition of cultures of

antagonists to sterile soil simultaneously with the inoculum of R.solani substantially reduced the "damping-off" of lettuce seedlings, under field conditions this treatment was quite ineffective. This is a general experience, reported by many workers. In non-sterile soil mere inoculation with antagonists was either ineffective or disease incidence was reduced to a lesser extent than in sterilized soil.

However, positive results were reported by Meredith (1946) in Jamaica, in field experiments with non-sterile soils infested with the Panama disease of bananas (F.oxysporum var. cubense). These soils received heavy inocula of a mixture of active actinomycetes. It is remarkable that mulching the plants exerted a deleterious effect in most cases. As was to be expected, assessment of the relative protective action by species of actinomycetes showing varying degrees of activity in pure culture was difficult owing to the great variation within replicates. The criteria for the evaluation of the results were the size of the plants, and disease incidence. An interesting feature of this work is the observation that a given species of actinomycete was more active in agar plates containing an extract prepared from the soil from which this organism was isolated than from other soils. The same observation was made in field experiments.

Darpoux and Faivre-Amiot (1950), working with a soil isolate, designated as actinomycete 105, obtained protection of melon plants attacked in naturally infected soils with a wilting disease. The melon seeds were soaked in a suspension of the antagonist and then planted out. Similar experiments were carried out with tobacco seeds infected artificially with Ps.tabaccae but in this case sterilized soil was used. The authors have not made a comparison of disease reduction in sterilized soil with that in unsterilized soil but have merely claimed a control of the respective diseases.

Tveit (1950) obtained striking protection by the antagonistic action of Chaetomium sp. of oat plants exposed to H.victoriae, in both non-sterile and sterile soil. The antagonist was added to the soil together with the inoculum of the pathogen. The emergence of the seedlings was almost 100% and the disease incidence was considerably reduced. The species of Chaetomium used was isolated from the surface of Brazilian seed. H.victoriae is rarely found in Brazil and Tveit infers that this might be explained by the presence of Chaetomium species on the seeds. Other species of Helminthosporium were also affected by the same antagonist.

There is no doubt that in the work quoted above microbial antagonism was operating. Moreover, the introduced

antagonists became well established and the nutrients present in the un-supplemented soil appeared adequate, not only for the growth of the antagonists but also for a mode of microbial activity which resulted in effective suppression of the pathogens.

That antibiotics might have been formed in the soil was suggested by some of these workers but no evidence was provided.

11) The inoculation of the soil with antagonists, and the simultaneous supply of nutrients in the form of organic matter

Millard and Taylor (1927) made one of the first experiments combining inoculation with an antagonist and organic manuring. In an attempt to explain the observed control of potato scab by green manuring (Millard, 1931) these authors showed that while S.praecox, an antagonist of S.scabies, controlled the pathogen in sterilized soil the addition of green manures enhanced the activity.

Grossbard (1945-1948) conducted a series of experiments designed to control "damping-off" in tomato seedlings due to Ph.cryptogea and Ph.parasitica by supplementing steamed soils with either glucose, sugar beet pulp or wheat straw inoculated with either A.clavatus or P.patulum. The experiments

were carried out in a greenhouse without aseptic precautions, and the soil rapidly became recolonized so that by the time the pathogen was introduced a mixed flora was present, though with a preponderance of the antagonist. The criterion for judging the amount of control was the spread of "damping-off" from a localized inoculation. It was found that the sugar beet pulp almost completely arrested the spread of the diseases. The straw-glucose supplement gave inconsistent results, leading either to a control or to a stimulation of the "damping-off". These results fit in with the observation of Slaggs and Fellows (1947) of the production of stimulatory substances under certain conditions. However, several other factors may have been responsible for the observed increase in virulence.

Wood (1951) has shown that while the addition of antagonistic cultures alone to Rhizoctonia infested soils did not control the pathogen, a combination of soil amendments with inoculation of antagonists resulted in some cases in the protection of lettuce plants, even in the field. One of the antagonists used by Wood, P.claviforme (P.clavariaceforme), was a patulin producer.

111) Organic manuring without the inoculation of specific antagonists

This method has so far yielded the best results in field experiments on the control of soil-borne diseases.

Millard (1923) demonstrated the control of potato scab by heavy manuring with green, organic matter. Millard and Taylor (1927) suggested that this beneficial effect was due to an increase in the actinomycete and probably also the bacterial population following the addition of the green manures, and that members of this population with a greater growth rate than S.scabies in multiplying rapidly eventually 'starved' the pathogen out. Millard and Taylor emphasised that the full effect of green manuring is dependent on other conditions being right for intense microbial growth and activity, such as acidity, optimum moisture content and so forth. There is some disagreement with the results of later workers but an evaluation of the differences is not possible as Millard and Taylor (1927) did not specify what type of grass cuttings they used. Millard (1923) used rye grass mainly. Even if the same material was used, results varied. For instance Millard (1923) obtained a good control with rye grass but Sanford (1926) did not control S.scabies by the use of chopped rye; however, Sanford in his later

work which he reviewed in 1946, did bring about a decrease in disease incidence in the field, as a result of manuring with rye and red clover of 17 and 8% respectively in one test and by 30 and 42% in another. Rouatt and Atkinson (1950) showed that while soy-bean and red clover did increase the numbers of actinomycetes, rye did not have a similar effect. Control of the scab was obtained by soy-bean only.

O.graminis and R.solani are two pathogens which respond well to certain soil treatments designed to modify microbial activity. As regards O.graminis, Garrett, in his extensive studies (reviewed 1938, 1944 and 1950) and Blair (1938) as regards R.solani have clearly shown that survival rate of the pathogens was decreased as the result of organic manuring with materials of a high carbon and low nitrogen content. Slagge and Fellows (1941) have further shown that O.graminis disappeared more rapidly in warm, loose, moist soil where microbial activity was at its best than in a wet, cold, compact soil.

Wood (1951) found that while acidification of the soil had no effect on R.solani the addition of organic supplements, such as fresh lawn cuttings, straw and maize meal, did result in a suppression of the parasite.

In Arizona and other cotton growing areas the serious cotton root rot disease (Phymatotrichum omnivorum) was successfully controlled by placing large quantities of corral manure, hay or cut green field crops in furrows, covering them with soil and watering them well to encourage microbial antagonism (King, 1937).

Thankam (1949) has shown that the survival rate of another parasite of cotton, F.vasinfectum, was considerably reduced in laboratory experiments as the result of soil amendments, high in carbon but low in nitrogen.

Another method of eliminating or suppressing a pathogen by an agricultural practice of soil management consists in a specific programme of rotation. Samuel (1923) has shown that the incidence of 'Take all' in Australia could be reduced by allowing the land to remain fallow for one year between pasture and wheat. In Canada, Broadfoot (1934) obtained a reduction of incidence of H.sativum by alternate crops of oats.

The effect of root exudates in modifying the population of the rhizosphere has been extensively studied by Lochhead and his collaborators. Lochhead, Timonin and West (1939) have shown that the mechanism of inter varietal resistance is based to some extent on the difference in the rhizosphere populations of resistant versus susceptible plants. They

found a more pronounced "rhizosphere effect" in the case of the susceptible than in the case of the resistant varieties studied.

The mechanism of microbial antagonism

These few examples suffice to illustrate that organic manuring may in some cases control soil-borne pathogens, but it is clear from the discrepancies in results and the variation observed that this means of biological control is still somewhat unreliable. No doubt, this is due to lack of understanding of the mechanism of microbial antagonism.

There are in the literature many teleological considerations to explain the mechanism of microbial antagonism but little experimental evidence. Millard and Taylor (1927) have put forth food competition as a possible factor. Rosen and Shaw (1929) showed that the observed antagonism between Sclerotium rolfsii and F.vasinfecum was determined by pH. Millard (1923) and Wakeman (1948) suggested that the beneficial effect of green manuring as observed in the control of S.scabies was due to an acid reaction as a consequence of the manurial treatment.

West and Hildebrand (1941) in an extensive study of strawberry root rot believed that variation in the type of decomposition according to the cover crop used for the control of this disease, was a limiting factor. They used

red clover and soy-beans. While both brought about a shift in the microbial populations, only soy-beans effected control. Glucose and acetic acid acted similarly and from this these workers concluded that the carbohydrate (fermentative) decomposition of the soy-beans was more favourable than the putrefactive processes occurring with red clover. Rouatt and Atkinson (1950), working with potato scab, had similar results. In both cases it was apparent that the bacterial index varied with the supplement and that a high index was associated with a low degree of infection. They therefore showed that the use of the correct type of supplement is essential. This well explains the differences in response of a pathogen to "organic manuring" and stresses the importance of the choice of a specifically suitable material. These workers therefore explained the mechanism of microbial antagonism in terms of nutrition of the microflora.

Organic amendments were found to stimulate the germination of sclerotia of P.gmnivorum, (Mitchell et al., 1941). Fungi, bacteria and actinomycetes, multiplying rapidly, colonized the germinating sclerotia and eventually decomposed them. Garrett, in his extensive studies on the control of O.graminis by organic manuring reviewed in 1938, 1944 and 1950 suggested that accumulation of carbon dioxide

as a result of increased microbial activity in the micro-climate around the roots of the host plant, may explain the observed suppression of this pathogen.

The production of toxins by antagonistic organisms encouraged by the heavy supply of organic matter was suggested by many students of these problems to explain the mechanism of microbial antagonism. The action of such toxins was demonstrated by Weindling (1932, 1934) to consist in the killing of the hyphae. Christensen and Davies (1940) showed that a thermostable toxin produced by B.mesentericus caused sectoring in H.sativum. This gave rise to a number of variants differing in cultural characters, physiology and, to some extent, morphology. Changes in parasitism were most striking and some variants were virtually non-parasitic. The action of the toxin was highly specific, because other fungi were not affected, and even different races or different variants of the same race of H.sativum responded in a different manner to the same concentration of this antibiotic. The occurrence of sectoring seemed to depend on concentration of the antibiotic, on the medium and temperature. It is remarkable that when B.mesentericus was grown in close association with H.sativum sectoring did not occur although the growth of the pathogen was inhibited. The authors stress the fact that their experiments were carried out in laboratory

cultures and that it was not at all certain that B.mesentericus would increase the frequency of mutations also in the soil or on the host. However, in view of common occurrence of B.mesentericus in the soil and on the host plant, 'it seems reasonable to assume that, under certain conditions, the proper association of the two organisms might occur that would induce mutation in Helminthosporium sativum'. Novogrudski (1949) isolated 53 strains of bacteria, mostly of the Pseudomonas group, which displayed mycolytic properties, dissolving the hyphae of 12 different fungi. He showed that the population of mycolytic bacteria was larger in fallow soils or in fields cropped with legumes and demonstrated that mycolysis retarded the development and reduced the numbers and the virulence of Fusarium sp. Earlier Singh (1946) isolated an antibiotic from a species of Myxococcus. This killed bacteria, which subsequently became lysed by exocellular enzymes. These bacteria served as food for the Myxobacteria. A corollary from Singh's work is a suggestion of a possible function of antibiotics in the life cycle of micro-organisms. Wakeman (1950) dismissed the possibility that antibiotics are a tool for providing food or that they are part of a defence mechanism. Brian (1949), referring to defence mechanisms, agreed with Wakeman and

said that there "is much evidence to the contrary". He stated that many antibiotic organisms exist in nature in strains varying in their capacity to produce antibiotic and there is no evidence that high yielding strains are more common in the soil than low yielding ones.

On the other hand, Schiller (1927) has shown a case of forced antagonism. If yeasts, which normally are compatible with certain Staphylococci, are grown in a starvation medium, the former suddenly develop bacteriolytic properties and feed on the latter. Further, Nadson and Zolkiewicz (1922), studying pigment formation by Spicaria purpureogenes, found that pigment was only formed in association with certain yeasts; this pigment eventually caused the death of the latter. However, these examples refer to laboratory cultures only.

SECTION II

Experimental Part

Part II

Materials and Methods

MATERIALS AND METHODS

Organisms : P. patulum Bainier, strain P.189 was used, kindly supplied by Mr. G. Smith. According to Raper and Thom (1949) P. patulum is identical with P. urticae Bainier. Two isolates (a and b) of A. clavatus Desm., kindly supplied by Roche Products Ltd. A. terreus Thom (N.C.T.C.3911) and two unidentified species of Penicillium were also used. The latter were isolated by the author from the rhizospheres of tomato seedlings growing in soil infected with Phytophthora parasitica Dastur, but which nevertheless remained healthy. The two Penicillium species varied greatly in the extent of their antibiotic production in successive sub-cultures. Preliminary experiments were also made with Fusarium javanicum, Bacillus subtilis (subtilin producing strain) both kindly supplied by the Bacteriology Department of the Imperial College of Science, Streptomyces antibioticus, kindly supplied by Professor Waksman, and two other unidentified species of Streptomyces which were isolated from the soil.

Manures. These comprised (a) plant materials, used either in the fresh or decomposed state (b) horse manure (c) sugar beet pulp (d) dried blood (e) glucose.

(a) The plant materials were harvested in July or September and comprised bracken (Pteridium aquilinum Kuhn), red fescue (Festuca rubra L.), Italian rye grass (Lolium multiflorum Lam.), lawn mowings, white mustard (Brassica hirta Moench), red clover

(Trifolium pratense L.), lucerne (Medicago sativa L.), sainfoin (Onobrychis viciaefolia Scop.), timothy grass (Phleum pratense L.), and wheat straw. Most of these crop plants were kindly supplied by district officers of the National Agricultural Advisory Service. Samples of timothy grass and red fescue were kindly supplied by the Welsh Plant Breeding Station. Samples from various localities were obtained and an attempt was made to secure them at the same stage of growth, namely after flowering. The haulms of the "green manures" were still green. In addition samples of fescue and yellow mustard were tested after the haulms had turned straw coloured. They were taken from seed crops and had consequently been left in the field until the end of the season. The manures were used in both the fresh and the decomposed state. The fresh materials were air-dried immediately after harvesting. Usually they were cut into short lengths, but the straw was chaffed ($\frac{1}{2}$ " approx.) or as a meal, and the timothy was also in the form of a meal. The process of decomposition was brought about in two ways. Either "Adco" was sprinkled over a large quantity of the material, which was watered and turned at intervals, or the "Adco" was omitted and decomposition was brought about merely by watering and turning. The materials were left in a greenhouse which was thermostatically controlled (20° C) and were used when they had assumed a brownish, homogeneous appearance.

(b) The horse manure was well decomposed material from animals

bedded on wheat straw. It was stacked in a large heap in the open for two to three months, turned, and restacked at intervals as in the preparation of mushroom compost. Six different lots were tested.

(c) The sugar beet pulp was kindly supplied by the British Sugar Corporation Ltd., It was used either finely shredded as received or in the form of a fine meal.

Cultures on manures. Quantities of 5 or 10g. were put into 150 or 250 ml. Erlenmeyer flasks and 50 or 100 ml. respectively of distilled water were added. In several cases 3.5% of glucose was added prior to sterilization.

Soils. Six different soils were used in this work:

No. 1, from Goff's Oak. It was a medium loam, subsoil, of a type frequently used for mushroom casings, with hardly any undecomposed plant residues.

No. 2, also came from Goff's Oak, but from a different field as it was not possible to obtain any further samples from the field from which soil no. 1 derived. It was a top soil, a heavy loam with a considerable clay content and contained large quantities of undecomposed plant residues. Both soils came from uncultivated land.

No. 3, from Nazeing Common. It was a top soil cut just beneath the turf of a pasture. It was a medium loam containing large

quantities of undecomposed plant residues.

No. 4, also from Nazeing; a medium to light loam taken from a low-lying field, which had not been cultivated, containing a fair quantity of undecomposed plant residue but less than soils 2 or 3.

No. 5, from Chelsea Physic Garden. A light loam top soil, dark-coloured, from a well cultivated garden, probably containing large quantities of soot and undecomposed plant residues.

No. 6, from Harlington. A very heavy clay top soil from a heavily limed plot with hardly any undecomposed plant residue, though the plot had been cropped continuously.

The pH values of these soils (determined by Marconi or Cambridge meters on a mixture of 24g. soil and 60 ml. of distilled water) are given in Table 1, together with the total carbon contents, estimated by the wet oxidation method except in the case of soil no. 1 where the figure is for organic carbon (Walkley and Black method). I am indebted to Dr. Cornfield for the wet oxidation data and to Dr. O. Owen for the organic carbon determination. Before use the soils were air-dried and passed through a 2 mm. sieve, if they were to be steamed or sterilized by propylene oxide. If they were to be autoclaved, they were oven-dried.

Table 1.
pH and carbon content of the soils used.

Soil No.	pH	Total Carbon content [†] % (w/w)
1	5.5	3.23 x
2	5.5	2.65
3	6.5	--
4	5.3	3.12
5	6.6	5.41
6	7.6	2.0

[†] By the wet oxidation method.

x Organic carbon, Walkley and Black method.

Sterilization.

(a) By heat. Steamed materials were heated at 100° for 30 min. Autoclaving was at 15 lbs./sq. in. for 20 min. with the manures and 1 hr. with the soils.

(b) By propylene oxide. (i). Hansen and Snyder (1947) described a method for the gaseous sterilization of biological materials for use as culture media, which was employed here. Last traces of propylene oxide were removed by aeration by a similar technique as has been used commercially for the sterilization of spices by ethylene oxide (Phillips and Kaye, 1949).

The material was placed in a 250 ml. filter flask and lightly sprayed with water from an atomizer. The flask was closed with a rubber bung carrying a glass tube, with a piece of rubber tubing attached and a screw clip. A partial vacuum was created and then 0.5 ml. of propylene oxide was quickly

introduced through a glass tube. The screw clip was tightened and the flasks allowed to stand for 24 hours at room temperature. The propylene was removed by suction, air sterilized by passage through a cotton wool filter was admitted and removed again by suction. This process was repeated twice, and finally a sterile cotton wool plug was substituted for the bung in a sterile chamber. The repeated airing of the material with a stream of sterile air is not essential. However, it was found, especially in the case of the manures that it was sometimes difficult to establish P. patulum on materials sterilized in this manner. It was believed that perhaps traces of propylene oxide were left behind when aeration was carried out merely by replacing the rubber bung by a sterile cotton wool plug.

(ii). A second simpler method was used in later experiments, as described by Ingram and M. and Robinson R.H.M. (1952) for ethylene oxide. Portions of 100g. of air-dried soil were placed in 250 ml. round, screw-capped bottles, 0.5 ml. of propylene oxide introduced and the caps tightened to seal the bottles, which were then placed in a 37° incubator for 24 hr. The caps were then replaced by sterile cotton wool plugs and the bottles re-placed in the incubator to allow the propylene/^{oxide} to escape.

Raising the containers to 60° as recommended by the above authors was omitted as it was desired to avoid high temperatures at all stages.

Cultures in soils. In use the soils were held either at 50% or

100% of saturation, as determined by the method of Keen and Razkowski (1921) on air or oven dried samples as required. When supplemented soils were used the moisture holding capacity was determined with the supplement added to the oven-dried sample at the appropriate rate. The capacity varied according to the nature and concentration of the supplement, and did not necessarily vary directly with the concentration for a given supplement.

Two types of container were used for the soil cultures, namely, 5" pots (employed only in the early stages of the work for qualitative tests, as the apparatus was clumsy and conditions were hard to control), and 250 or 500 ml. Erlenmeyer flasks.

(a) Pot cultures. Five-inch pots were filled with soil and covered with Kraft paper which was firmly secured. The drainage hole was connected to a flask by means of a glass tube and rubber bungs. The whole apparatus was sterilized in sections and rapidly assembled afterwards. After inoculation the cultures were incubated in a greenhouse, with the paper covers still in place. The pots were weighed at intervals and the water lost by evaporation replaced.

(b) Flask cultures. For these, portions equivalent to 100 or 200 g. of soil were placed in 250 or 500 ml. Erlenmeyer flasks and the water content adjusted to 100% of saturation.

Inoculation. The flasks containing manures were each inoculated with a loopful of spores from slope cultures of the fungi one week old and growing on a medium made up in distilled water and containing (all as % w/w) NaNO_3 , 0.3; KH_2PO_4 , 0.1; KCl , 0.03; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; glucose, 4.0; malt extract (Allen & Hanburys Ltd), 0.2; agar, 1.5.

The pots and flasks containing soil were inoculated with 10 ml. of spore suspension in distilled water prepared from the above slope cultures; 10 ml. of water were used for one slope and the initial suspension was further diluted 10 times.

Incubation. Both manure and soil cultures were incubated at 25° except where otherwise stated.

Preparation of fluids for antibiotic assay.

(a) From cultures on manures. There was abundant fluid in these cultures, so samples were pipetted off and usually assayed directly. When negative results were obtained, sometimes the assay was repeated after the fluid had been reduced to one-half or one-quarter of its volume under reduced pressure at 35° .

(b) Extracts from soil cultures. Seven different methods were used.

(i) By leaching from pot cultures. 50 ml. of distilled water were sprayed evenly over the surface of each pot and the soil again

covered with sterile paper. The leachate which collected in the sterile flask attached to the drainage hole of the pot was filtered through No. 2 Whatman filter paper and assayed.

This method was also used for examination of soil from boxes employed in glasshouse experiments. 200g. of soil were placed on a muslin cloth over a funnel, 30 ml. of water was sprayed evenly over the soil and the leachate collected in a flask connected via a rubber bung to the stem of the funnel. The funnel was covered to lessen evaporation.

(ii) By centrifuging. After trying various times and speeds it was found that 45 min. at 2800 r.p.m. (r.c.f. 2260) was adequate. Centrifuging has proved to be the most convenient and efficient method and was therefore used in most experiments where the moisture content was high.

(iii) Shaking and centrifuging. When unheated soil was to be examined, shaking with water was adopted because the soil structure was intact and extraction might have been more difficult. 100g. of soil were shaken with 60 ml. of water on a mechanical shaker for 12 hr., the liquid allowed to settle and centrifuged.

(iv) By pressing. The soil was wrapped in cloth and squeezed by hand or in a mechanical press. The fluids were subsequently filtered on a Buchner funnel.

(v) By perfusion. A soil perfusion apparatus (Lees, 1947) was used, and percolation was with distilled water for 48 hr. The percolate was either sterilized by filtration through an L7 Chamberland candle or left untreated, as desired.

(vi) By hot water. Successive small quantities of boiling water were poured on to the soil in a funnel, the apparatus being in an open hot steamer. Hot water extracts were also prepared by shaking soil with hot water for 10-15 min., the container being wrapped in cotton wool to minimize ~~the~~ cooling. The fluid, after settling, was clarified by centrifuging.

(vii) By organic solvents. 100 g. of soil were shaken for 5-6 hr. on a mechanical shaker with 50 ml. of ether, acetone, or chloroform. The mixture was filtered on a Buchner funnel, the filtrate evaporated to dryness and the residue taken up in water for assay.

Assay of antibiotic activity.

Test organisms : The following bacteria were used. Bacillus mycoides, kindly supplied by Dr. W.J. Dowson. This organism was chosen because of its considerable sensitivity to inhibition and stimulation by the soil extracts and its ability to show^{up}/well partial inhibition.

B. subtilis, a laboratory culture of the Experimental and Research Station, Cheshunt. In later work the strain A.T.C.C.6633

(the streptomycin standard test strain), kindly supplied by Boots Pure Drugs Co., Ltd., was used.

Bacterium carotovorum. The type culture, the phytophthorum, and the aroidese strains were used, all supplied by Dr. W.J. Dowson.

Bact. coli. N.T.C.C.86 kindly supplied by Dr. Heatley.

Sarcina lutea, from the laboratory collection of the Imperial College of Science.

Corynebacterium fascians, kindly supplied by Dr. W.J. Dowson.

The fungi used were :-

Phytophthora cryptogea, Ph. parasitica and Thielaviopsis basicola, all kindly supplied by Mr. P.H. Williams.

The two Phytophthora species were chosen as representatives of phytopathogens which do not spore freely in pure culture, thus necessitating mycelium transfer, and T. basicola as a readily sporing type.

The determination of the most suitable assay media

(a) Assays with bacteria. It was decided to use the cylinder or cup-plate method.

Many tests were made to determine the optimum quantity of agar, using 2, 1.5, 1.15, and 1%. It was found that 1.15%

gave the largest zones, the medium yet being hard enough for cups to be cut satisfactorily with a cork borer. The brand of agar seemed to influence the concentration required.

Four media were compared. They were all made up in distilled water and contained (%w/w):-

Lemco agar.

Lab-Lemco, 0.3; Bacto-peptone, 1.0; NaCl, 0.3; agar 1.15. pH 7.0 before autoclaving. The medium was filtered through paper pulp.

Albumen-treated Lemco agar.

Composition as above, but an attempt was made to clear the medium by the use of a small quantity of egg albumen.

Difco nutrient agar.

Difco beef extract, 0.3; Bacto-peptone, 1.0; NaCl, 0.5; pH 6.8, filtered through paper pulp.

Difco nutrient agar plus yeast extract.

To the above medium 0.3% of Difco yeast extract was added. Final clearing was by paper pulp filtration.

Using pure patulin in pH 7 buffer solution (250 g/ml) and the test organisms B. mycoides, Bact.coli and the three strains of Bact. carotovorum, the results set out in Table 2 were obtained. The zone diameters are means derived from

Table 2a . Results of tests to demonstrate antibiotic production and sensitivity of test organisms.

Antibiotic-producing organism.	Test organism.	Medium	Period of pre-growth (days)	Period of subsequent incubation.	Inhibition.	Illustration.
<u>Streptomyces antibioticus</u>	<u>Colletotrichum atramentarium</u>	p.g.	4 ϕ	7 days	+	Figs. 4 & 5.
	<u>B. mycoides.</u>	d.y.	4	18 hr.	+	_____
	<u>B. subtilis</u>	d.y.	4	18 hr.	+	_____
	<u>Bact. coli</u>	d.y.	4	18 hr.	0	_____
	<u>Bact. carotovorum</u>	d.y.	4	18 hr.	0	_____
<u>S. griseus mutant</u>	<u>Ph. parasitica</u>	p.g.	5 ⁺	21 days	+	Figs. 6a - c
<u>Penicillium species A</u>	<u>Ph. parasitica</u>	p.g.	8 ⁺	5 days	+(lytic)	Fig. 7
<u>Aspergillus clavatus</u> & <u>Penicillium natulum</u>	<u>Ph. parasitica</u>	p.g.	4 ⁺	8 days	+	_____
	<u>C. atramentarium</u>	p.g.	4 ϕ	8 days	0	_____
	<u>Pydimella lycopersici</u>	d.	5 ⁺	14 days	0	_____
	<u>Thielaviopsis basicola</u>	p.g.	5 θ	8 days	+	_____
	<u>B. mycoides</u>	d.y.	4	18 hr.	+	_____
	<u>B. subtilis</u>	d.y.	4	18 hr.	+	_____
	<u>Bact. coli</u>	d.y.	4	18 hr.	+	_____
	<u>Bact. carotovorum</u>	d.y.	4	18 hr.	+	_____

p.g. = Potato glucose agar. d = Dox agar. d.y. Difco nutrient agar plus yeast extract.

+ = pregrowth of Ph. parasitica.

ϕ = pregrowth of C. atramentarium.

⁺ = pregrowth of D. lycopersici.

θ = pregrowth of T. basicola.

10 replicates. Inoculation was at the rate of 1 ml. of an
broth
18 hr./culture to 200 ml. of medium. Evidently, the four
test organisms responded differently on the various media,
in respect of the amount of sharpness of definition of the
zones. Upon the latter depends the ease of measurement.
In general a heavy growth provides contrast and so leads to
clear, well-defined zones. There was no clear indication
that one medium gave consistently larger zones than the
others with all the test organisms, but there were obvious
differences in sharpness of definition. Bact. carotovorum
does not on the whole lend itself to cylinder- or cup-plate
assays because of its relatively poor growth on most media.
However, ~~the~~ on Difco-yeast agar growth was comparatively
heavy and consequently the zones of inhibition were well
defined. On the other hand B. mycoides, while growing equal-
ly luxuriantly on all media did not form well defined zones
on the two Lemco media. Difco agar and Difco-yeast agar a-
gain gave the clearest zones with this organism. Treatment
with egg albumen did not improve the Lemco medium signific-
antly. On the whole the Difco-yeast agar gave the smallest
zones but in spite of this it was adopted as the assay medium
because it gave zones which were particularly easy to meas-
ure, and made possible the use of the Bact. carotovorum
strains. In work which is so closely related to plant path-
ology it was considered important to be able to use as a

test organism a phytopathogen of the importance and wide distribution of Bact. carotovorum. Throughout the work this organism gave reproducible and reliable results on Difco-yeast agar.

(b) Assays with fungi. The serial dilution method was decided on. Carrot broth, malt extract and pea broth were compared as assay media, using Ph. parasitica. It was desired to have a clear medium as this would enable bacterial contamination to be detected and on this account potato dextrose broth was not employed. The media were made up with distilled water and contained (% w/w):-

Carrot broth.

Grated carrots 25. The carrots were boiled for 30 min., pH 7.2.

Malt extract.

Dry malt extract, 1.0; glucose 1.0; KNO_3 , 0.2; KH_2PO_4 , 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1.

Pea broth.

Tinned peas, 14. The peas were brought to the boil, and the supernatant liquid was used and centrifuged several times to clear it.

Rose bengal (0.008%) was added to each of the three

media to minimize bacterial growth. At this low concentration it did not affect the growth of the test fungus.

Pea broth gave the best results. On it growth was fastest and frequently the final results could be read after 5 days. It was interesting to note that the rate of growth varied with the brand of peas used.

Preparation and amount of inoculum for antibacterial assays.

The optimum temperature of B. mycoides is stated to be 37°. The strain used here frequently did not grow at all or grew very poorly at that temperature and had an optimum of 25°. The flocculent sedimentary growth of B. mycoides had to be broken up before an even inoculum could be obtained. A standard method was evolved whereby the flocculum was broken up as thoroughly as possible with a loop and the tube then rotated between the palms of the hands about 12 times. This yielded fairly reproducible results.

Many workers have reported that the size of inoculum of Staph. aureus and other organisms affects antibiotic assay results. Preliminary experiments showed that this applied also to the organisms used in the present work. Rates of inoculation of 1 ml. of 18 hr. broth culture (incubated at the appropriate temperature) to 20, 200, and

2000 ml. of medium were tested. The best results were obtained at the 1/200 rate. The strain of B. mycoides used here was especially sensitive to inoculum size. At 1/20 growth was so heavy that test fluids with low potency gave negative results. At 1/2000 the colonies remained separate and an even film of bacterial growth was not formed.

Final assay technique.

Antibacterial activity.

The cylinder-plate method was used at first and later the cup-plate method. In addition some assays were done by the serial dilution method.

Plate methods.

The medium, usually Difco-yeast agar was bulk seeded from 18 hr. Difco-yeast broth cultures of the test organisms incubated at their optimum temperatures, at the rate of 1 ml. of culture to 200 ml. of medium. Cups were cut with a 9 mm. cork borer (No.5) after the medium had set. Patulin, (generously given by Boots Pure Drug Co. Ltd. and by Roche Products Ltd.) was used as a standard for assessing the activity of the test fluids. It was dissolved and diluted in either phosphate buffer solution, pH 6.5, or the centrifugates from the controls in the soil experiments. The latter diluent was used in order to allow for any nutritional or inhibitory

factors present in the soil centrifugates under test which might affect the activity. A stock solution (2g/l) in pH 6.5 buffer remained stable for many months in the refrigerator, as also did the stock solutions in soil centrifugates. A standard curve was constructed on each occasion assays were performed, from the readings obtained from a range of concentrations, using the same batch of medium and inoculum as was used for the culture fluids. Each assay plate contained two concentrations of the standard. The unknown was tested in six to ten replicates for each organism. The results have usually been expressed in terms of the "patulin equivalent", the quantity of antibiotic, calculated as patulin present in a culture.

Typical standard curves for patulin in pH 6.5 buffer, constructed from data of assays with B. mycoides and Bact. carotovorum, are presented in Fig. 1, where the diameters of the zones of inhibition have been plotted against the logarithms of the concentrations, using the base 2, because the standard was diluted in a twofold series. This mode of plotting gives fairly straight lines. The Bact. carotovorum line runs above that of B. mycoides and its slope is less steep, indicating that the former organism is more sensitive, though the precision of the assay is somewhat less. Most of the results presented in this thesis relate to B. mycoides

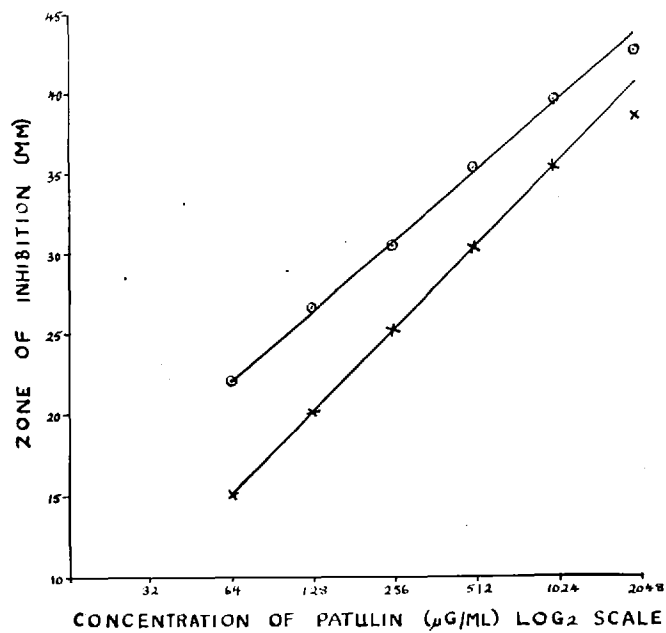


Fig. 1 . Standard curve of patulin in pH 6.5 buffer solution.

O — O Bact. carotovorum

x — x B. mycoides

assays although in every case Bact. carotovorum was also used as a second test organism.

When soil centrifugates were assayed the deviations from the straight line tended to be larger, especially in fluids from glucose-treated soils, but straight lines still represented the data quite satisfactorily. However, as shown in Fig. 2 extracts from glucose-treated soil did depress the activity of patulin, so in assaying the antibiotic in extracts from soil cultures the appropriate standard curve was always used. Nandi (1948) demonstrated the presence of substances in the soil inhibitory to the action of tyrothricin.

Cooper and Woodman (1948) showed that straight lines were obtained in penicillin assays when plotting logarithms of concentration against the squared distance of diffusion; Asher (1951) working with subtilin demonstrated that while the findings of Cooper and Woodman could be applied to zones of inhibition with diameters greater than 25mm., the linearity did not extend to values smaller than that. He showed that in this lower range plotting zone diameters gave the more satisfactory straight lines. Fig. 3 shows the data of Fig. 2 plotted according to Cooper and Woodman's method, and clearly curves fit the points best. However in the upper ranges, the curves approximate to straight lines, so the situation seems to be similar to that encountered by Asher.

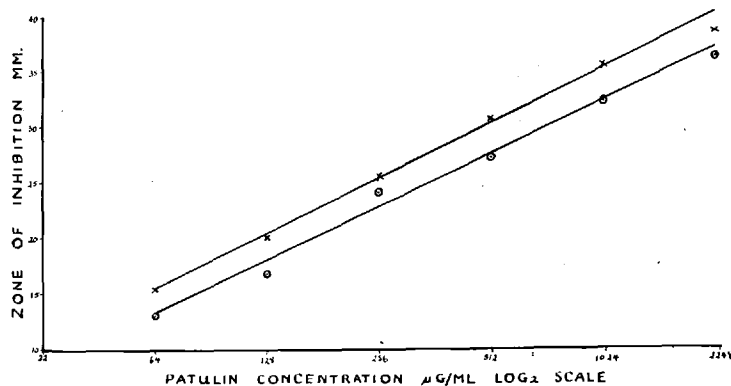


Fig. 2.

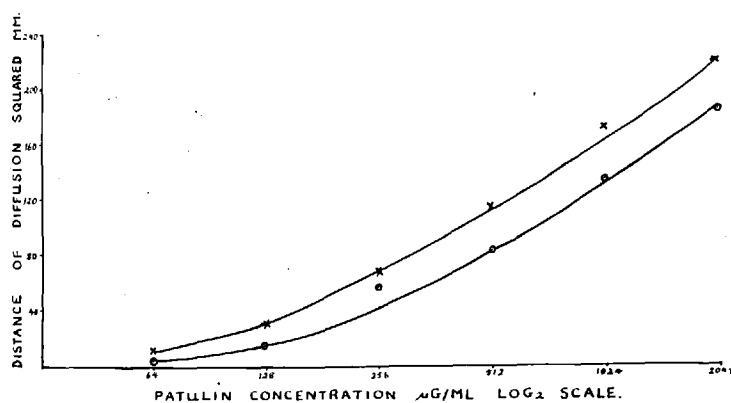


Fig. 3.

A comparison of the Cooper-Woodman method of plotting and that used in this work to obtain the best fit for a straight line.

Fig. 2 . Relationship between $\log_2$ concentration of patulin and zone diameter.

Fig. 3 . Relation between $\log_2$ concentration of patulin and the squares of the distance of diffusion.

○ — ○ Patulin dissolved in extract from glucose-treated soil.
 x — x Patulin dissolved in pH 6.5 buffer.

The serial dilution method.

Dilutions in final volumes of 5 ml. were made up in small test tubes, usually in twofold steps, but where highly potent fluids were concerned intermediate concentrations were tested. The diluent was Difco-yeast extract broth. Lemco broth could not be used for assaying soil extracts as a gelatinous precipitate formed when the two were mixed. The inoculum was again diluted to 1/200.

Antifungal activity.

The serial dilution method in pea broth was used. Tubes (6 x 1 in.) containing 10 ml. of medium including the various concentrations of the test fluids were inoculated with the test organisms. As controls 9 ml. of broth with 1 ml. of centrifugate from control soils, or of buffer solution, were employed.

Inoculation of the Phytophthora species was by means of a 5 mm disc of mycelium, out from the most recent growth of a colony 7 days old on pea agar. The T. basicola inoculum was a loopful of dry spores from a 7 days old slope culture on potato-glucose agar. The tubes were examined at intervals of 5 days but the results presented are those finally obtained after 3 weeks incubation at 25°.

The preparation of soil extracts for use as culture media.

The soils used were : no. 2 from Goff's Oak for fungal

cultures and no. 6 from Harlington for bacterial and actinomycetes, the latter suiting these organisms because of its relatively high pH.

1 Kg. of soil with or without a supplement and having its moisture holding capacity adjusted to 100% of saturation was autoclaved for 1 hr. at 15 lb./sq. in. pressure, and immediately centrifuged. The centrifugate was divided into lots of 40 ml. in 150 ml. flasks and re-sterilized by steaming for 15 min.

Demonstration of antibiotic production and sensitivity
of test organisms

The organisms mentioned in the METHODS AND MATERIALS section are known to form antibiotics in culture media, but it was thought desirable to show that the strains secured were active in this respect, and whether the test organisms were sensitive. A series of tests was made, in which the producing organisms were grown on suitable media in dishes, the test organisms being introduced after an interval or present in the medium at the time of inoculation of the antibiotic producer. The results are shown in Table 2a and Figs. 4, 5, 6 and 7.

It is clear from the above qualitative tests that B.subtilis and B.mycoides were antagonised by S.antibioticus, the S.griseus mutant, P.patulum and A.clavatus; while Bact.coli and Bact.carotovorum only by the two fungi A.clavatus and P.patulum. Ph.parasitica was sensitive to the action of the S.griseus mutant, A.clavatus, P.patulum and Penicillium species A; and C.atramentarium to that of S.antibioticus.

It was decided to use in further work as antibiotic organisms chiefly S.antibioticus, P.patulum and A.clavatus, with B.mycoides, B.subtilis, Bact.coli, Bact.carotovorum, Ph.parasitica and T.basicola as the principal test organisms.

Table 2

A Comparison of the Efficiency of Four Media for the Assay of the Antibacterial Activity of Patulin (250 g/ml).

Assay Medium	T e s t O r g a n i s m s.														
	B. Mycoides.			Bact. Coli			Bact.			Carotovorum			Strains.		
	Zone Diam (MM)	Amount of Growth	Defin ition of Zone.	Zone Diam (MM)	Amount of Growth	Defin ition of Zone.	Type			Phytophthorum			Aroidae		
							Zone Diam (MM)	Amount of Growth	Defin ition of Zone	Zone Diam (MM)	Amount of Growth	Defin ition of Zone	Zone Diam (MM)	Amount of Growth	Defin ition of Zone
Lemco	17.5	Very heavy	Very poor	17.6	Heavy	Good	26.5	Very poor	Very poor	19.7	Good	Very poor	22.0	Poor	Very poor
Albumen treated Lemco	19.5	heavy	Poor	17.2	Very heavy	Less clear than on Lemco alone.	22.6	Very poor	Very poor	22.5	Good	Very poor	21.6	Moderate	Poor
Difco	19.2	Heavy	Excellent	17.3	Heavy	Very good	24.8	Moderate	Good	22.7	Good	Good	22.5	Good	Excellent
Difco plus yeast extract.	17.9	Very heavy	Excellent	16.6	Very heavy	Good	21.9	Good	Excellent	21.3	Very good	Excellent	21.9	Good	Very good

* Means of 10 replicates.

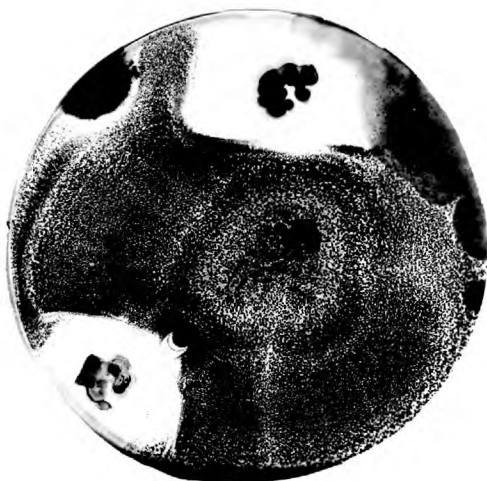


Fig. 4 .



Fig. 5 .

Demonstration of antibiosis I.

Antagonistic effect of S. antibioticus.

Fig. 4 . A nutrient agar was inoculated with Colletotrichum atramentarium. After 4 days 2 unnamed species of bacteria, an unidentified Aspergillus species and S. antibioticus were introduced. Only S. antibioticus exerted an antagonistic effect.

Fig. 5 . Control. Inoculation with S. antibioticus was omitted.



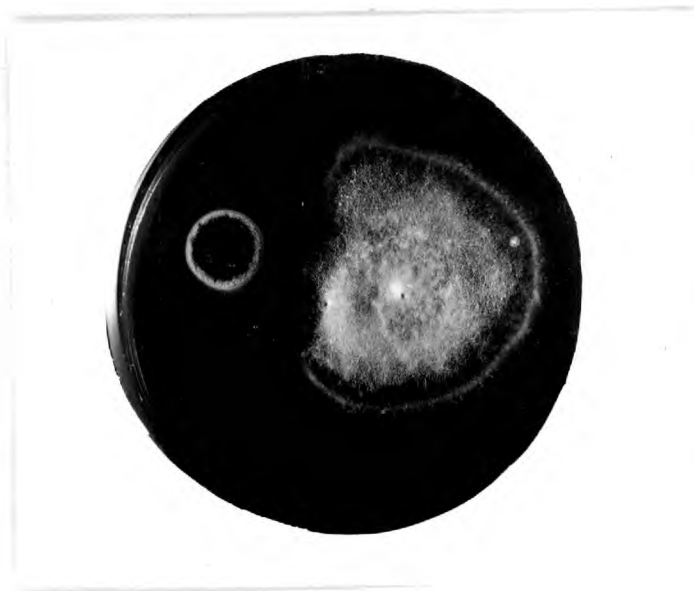
Demonstration of antibiosis II.

Fig. 6 . Antagonistic effect of *S. griseus* (a mutant) on *Phytophthora parasitica*.

a.) As the result of the diffusion of an antibiotic produced by the *Streptomyces*, growth of *Ph. parasitica* is arrested along a straight line. The antagonistic action is not based merely on competition as shown in (b).

b.) Instead of *S. griseus* an unidentified species of *Penicillium* was introduced. In spite of the presence of a competing organism as in (a), the growth of *Ph. parasitica* was not inhibited.

c.) Control.



Demonstration of antibiosis III.

Fig. 7 . Mycolytic effect of Penicillium species A on Phytophthora parasitica. The Penicillium was introduced after P. parasitica had already formed a normal colony. After 5 days disintegration of the hyphae began opposite the Penicillium colony.

Part II

Antibiotic production on manures

The production of an antibiotic substance by *A.clavatus*
on wheat straw

Chaffed wheat straw, obtained from a farm in Broxbourne, was used either fresh or composted. Composting was carried out either by the use of 'Adco' or by merely watering and turning the material at intervals. 4 sets of flasks were prepared.

- 1). Fresh or composted straw ('Adco' treated or otherwise) without any further treatment, to serve as a control.
- 2). As 1) but inoculated with *A.clavatus*.
- 3). As 1) but autoclaved.
- 4). As 2) but autoclaved and then inoculated with *A.clavatus*.

All flasks were incubated for 14 days at 25° when a small portion of the metabolic fluid was pipetted off and assayed against *B.subtilis* and *Bact.coli* (inoculum 1/20) by the cylinder-plate method. The results, presented in Table 3, are expressed as zone diameters, and with data for activity on Czapek-Dox solution for comparison. In Fig.8 an assay plate of *B.subtilis* is shown, and Fig.9 is of a *Bact.coli* assay.

The data in Table 3 show clearly that *A.clavatus* produced an antibiotic substance on fresh, autoclaved wheat straw but that it failed to do so on any of the other

Table 3. The antibacterial activity of 14-day cultures of A. clavatus on non-sterile or autoclaved fresh or composted wheat straw.

Substrate	Sterilization	Zone Diameter mm.	
		Inoculated	Uninoculated
Wheat straw fresh	Untreated	0	0
	Autoclaved	16	0
Wheat straw tested with Adcor	Untreated	0	0
	Autoclaved	0	0
Wheat straw composted	Untreated	0	0
	Autoclaved	0	0
Czapek-Dox	Autoclaved	18	8

Test organism : Bact. coli.

8 = stimulation.

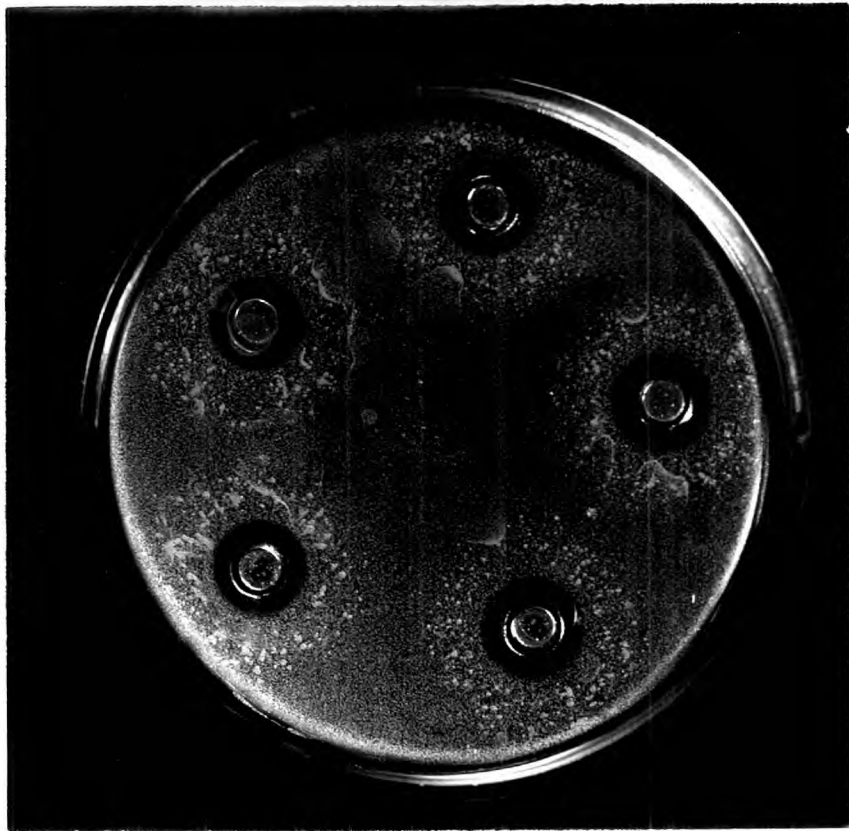


Fig. 8 . Antibacterial activity of Aspergillus clavatus cultures on wheat straw. Note zones of stimulation surrounding area of inhibition. Test organism : Bacillus subtilis.

substrates. In addition to an inhibitory effect a slight stimulation was observed on B.subtilis plates (Fig. 8). This was absent in Bact.coli assays (Fig. 9). The uninoculated materials did not yield an inhibitory substance. Less antibiotic was formed on autoclaved fresh wheat straw than on Czapek-Dox. Although the fungus grew well on the composted, autoclaved straw apparently forming more spores than on the fresh material, no antibiotic was produced, indicating that material permitting luxuriant growth was not necessarily adequate for antibiotic production.

The effect of the addition of glucose to wheat straw cultures of A.clavatus

The above experiment was repeated, but 3.5% of glucose was added to both the fresh and the composted straws, and the results shown in Table 4 and Figs. 9 and 10 were obtained.

By comparing Tables 3 and 4 it can be seen that the addition of 3.5% of glucose to fresh, autoclaved wheat straw led to a higher antibiotic titre. This is clearly shown by the difference in zone diameters in Figs. 9 and 10. In addition glucose induced antibiotic formation also on autoclaved, composted straw while no production occurred on the non-sterilized and uninoculated materials, where slight stimulation of Bact.coli was observed, probably due to

Table 4. The effect of the addition of 3.5% glucose to 14-day wheat straw cultures of A. clavatus.

Wheat straw substrate		Sterilization	Zone diameter mm.	
			Inoculated	Uninoculated
Fresh,	alone	Untreated	0	0
Fresh,	plus 3.5% glucose	Autoclaved	23	S
Treated with 'Adco,'	alone	Untreated	0	0
Treated with 'Adco,'	plus 3.5% glucose	Autoclaved	21	S
Composted,	alone	Untreated	0	0
Composted,	plus 3.5% glucose	Autoclaved	22	S

Test organism : Bact. coli.

S = stimulation.

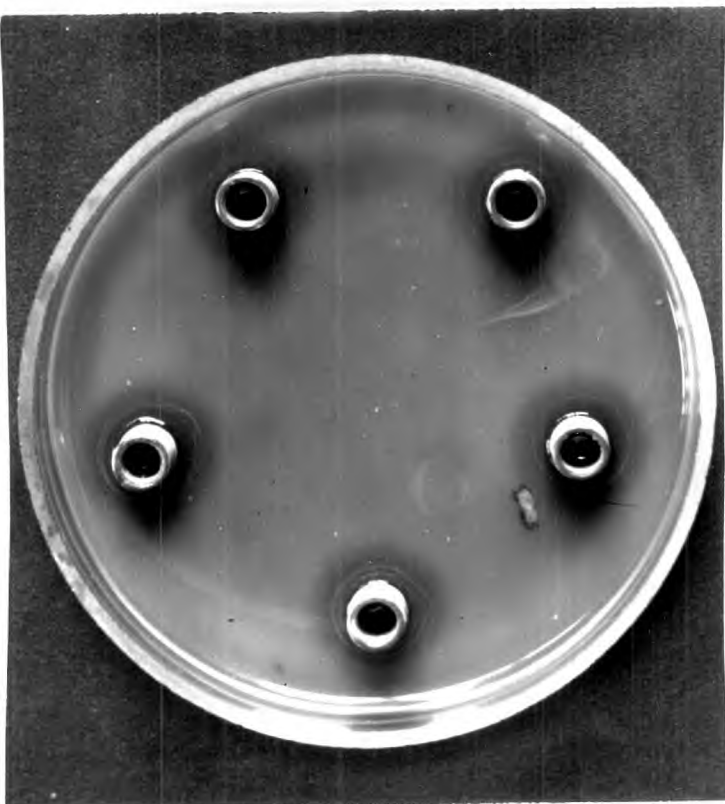


Fig. 9 .

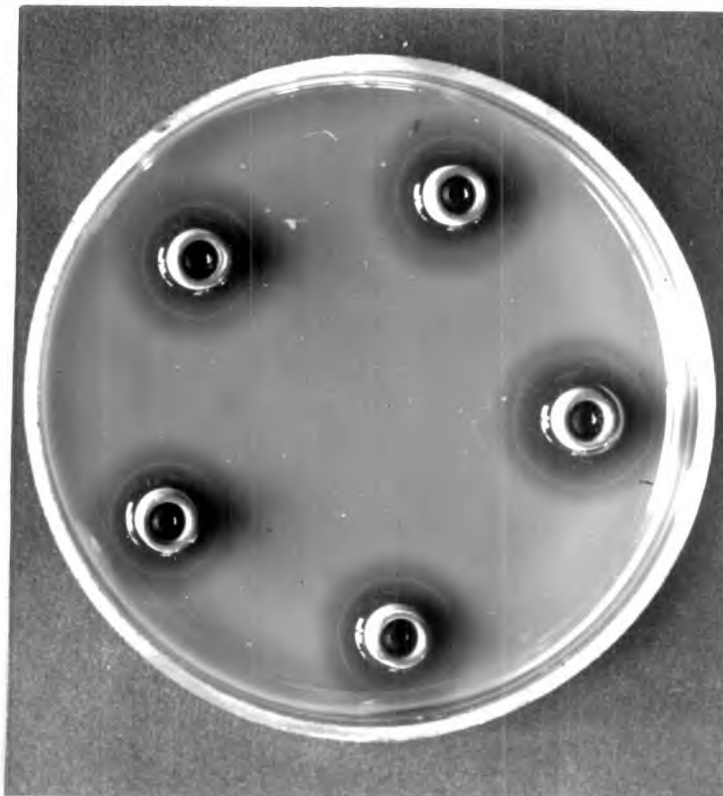


Fig. 10 .

The effect of the addition of 3.5% glucose to wheat straw as a medium for antibiotic production by Aspergillus clavatus. Test organism : Bacterium coli.

Fig. 9 . Assay of culture on wheat straw alone.

Fig. 10 . Glucose was added.

residual glucose. Activity levels on the suitable substrates were very similar and no significant difference was noted between 'Adco' treated or otherwise composted straw. It appears therefore that in the decomposition process only one material essential for antibiotic production had been removed, namely easily available carbohydrate: all the other nutrients were still present at a sufficiently high level.

The production of an antibiotic by *P.patulum* on wheat straw

It has been shown by Anslow, Raistrick and Smith (1943) that *P.patulum* as well as *A.clavatus* can produce patulin in synthetic media. They were working with two strains, P.189 and Ad 77, which were compared with *A.clavatus*, and it was found that *P.patulum* P.189 gave the best result. Therefore in future investigations that strain was used as a rule. The technique for testing antibiotic production in wheat straw cultures of *P.patulum* was identical with that used with *A.clavatus*. The results of Table 5 were obtained. They confirm on the whole those of the experiments with *A.clavatus*; antibiotic was formed in autoclaved wheat straw cultures. The titre was increased by supplementation with 3.5% of glucose. As before, composted wheat straw did not support antibiotic production but on adding 3.5% of

Table 5. A comparison of the persistence of activity of cultures of P. patulum and A. clavatus on fresh or composted wheat straw and the effect of the addition of glucose (3.5%)

Wheat straw substrate	Period before complete disappearance of activity (weeks)	
	P. patulum	A. clavatus
Fresh, alone	28	6
Fresh, plus 3.5% glucose	41	28
'Adco' treated, alone	No antibiotic produced	No antibiotic produced
'Adco' treated, plus 3.5% glucose	32	20
Czapek-dox	-	4

Test organisms : B. mycoides and Bact. coli

glucose an inhibitory substance was formed of a titre close to that produced when fresh straw was used.

The maintenance of antibiotic titre in wheat straw cultures of *P.patulum* and *A.clavatus*

A series of flasks was prepared containing autoclaved fresh or composted wheat straw (Adco method), alone or supplemented with 3.5% of glucose. All flasks were inoculated with *A.clavatus* and *P.patulum* at the same date and incubated at 25°. At intervals small roughly 5 ml. portions of the metabolic fluid were pipetted off and assayed against *Bact.coli* and *B.mycoides*, the first assays being made after one week of incubation. As assays were carried out over a period of ten months, the fluid removed was replaced at intervals by sterile water. The results are given in Table 3a and in the case of *A.clavatus* there is a comparison with Czapek-Dox solution.

Antibiotic activity was recorded in all cases except the non-supplemented composted straw. The persistence of the antibiotic varied with the substrate. Activity disappeared sooner in the fresh straw without glucose, followed by the Adco treated, glucose supplemented straw.

Table 5a. The antibacterial activity of 14-day cultures of *P. patulum* on non-sterile or autoclaved, fresh or composted wheat-straw, and the effect of the addition of 3.5% glucose.

Wheat straw substrate	Sterilization	Zone diameter mm.	
		Inoculated	Uninoculated
Fresh, alone	Untreated	0	0
	Autoclaved	14	0
Fresh, plus 3.5% glucose	Untreated	S	S
	Autoclaved	21	S
Adco treated, alone	Untreated	0	0
	Autoclaved	0	0
Adco treated, plus 3.5% glucose	Untreated	S	S
	Autoclaved	20	S
Composted, alone	Untreated	0	0
	Autoclaved	0	0
Composted, plus 3.5% glucose	Untreated	S	S
	Autoclaved	20	S
Craper Box	Autoclaved	14	S

Test organism: Bact. coli.

S = stimulation.

Remarkable is the degree of persistence for 41 weeks on the fresh, glucose supplemented straw. A.clavatus cultures lost activity sooner than those of P.patulum but persistence was greater even on the unsupplemented fresh straw than on Czapek-Dox medium, which contains 4% of sucrose as sole carbon source. Probably the straw cultures contained a great deal more available carbohydrate and production was consequently continued over a longer period. No data are available for Czapek-Dox cultures of P.patulum but it is thought that for the same reason activity would be most likely to persist longer in straw cultures. When considering maintenance of activity one has to discriminate between continued production and destruction of the antibiotic already formed. In view of the fact that persistence was greater on the supplemented materials it is more probable that this is a case of continued production rather than one of a slower rate of destruction.

Antibiotic production by P.patulum on timothy grass and a comparison of the activities with those of wheat straw cultures

In an attempt to find a plant material which would support a higher level of production and on which activity would be maintained longer than on wheat straw, a comparison was made of the activities of timothy grass and wheat straw

cultures of P.patulum over a period of 7 months. Only fresh materials were tested and the technique employed was identical with that used in the previous experiment except that B.mycooides and Bact.carotovorum were used as test organisms. In Fig. II the patulin equivalents (B.mycooides assays only) are shown plotted on a logarithmic scale (base 2) against time.

Evidently timothy provides an adequate substrate for antibiotic production by P.patulum. In both cases production was rapid, and when the first assays were done at the end of a week, the timothy culture contained more than twice as much antibiotic as the straw. Between the second and the ninth week activity in the straw cultures remained roughly constant, but following this a slow decline set in. The decline in the timothy culture began sooner and was more rapid and after 24 weeks very little could be detected, though the straw culture still contained a moderate amount.

Antibiotic production by P.patulum on various substrates

Further attempts were made to find a medium for antibiotic production by P.patulum superior to either wheat straw or timothy grass. Tests were carried out on red clover, red fescue, white mustard, perennial rye grass, lawn mowings, (fresh and decomposed), lucerne, sainfoin, horse manure, sugar beet pulp (molassed) and peat. Timothy grass and

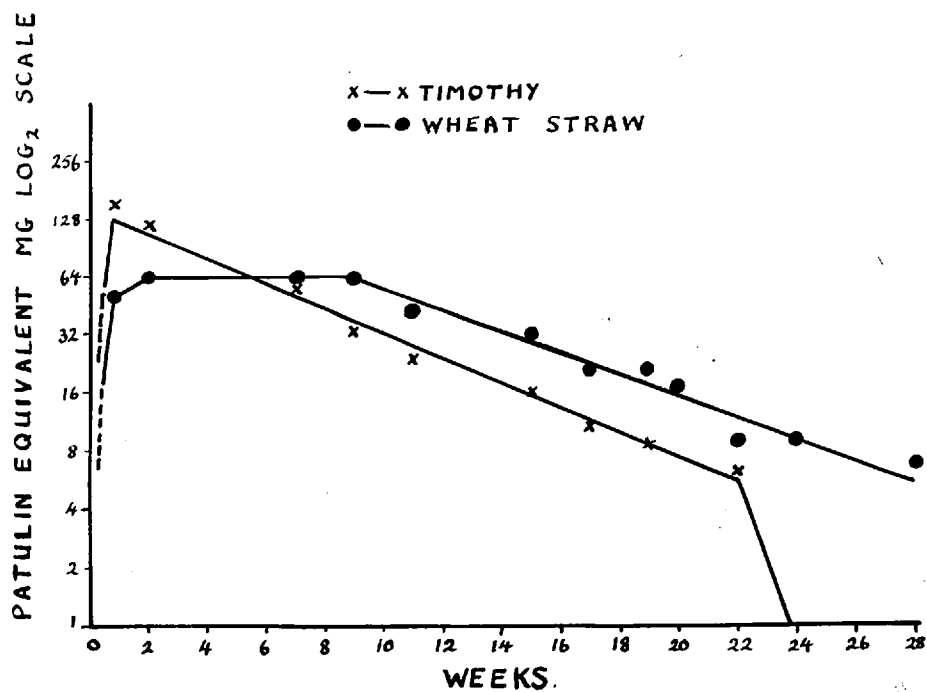


Fig. 11 . The antibacterial activity of cultures of P.patulum on wheat straw and timothy grass.

wheat straw, the latter being used as a standard, were also included. To some of the plant materials 3.5% of glucose was added. Assays were carried out at intervals as before, and the previous finding that maximal concentration was frequently present after about 2 weeks, following which there was usually a decline, was confirmed. Accordingly only the results obtained after 14 days are recorded in Tables 6 and 7, in which the activities are related to that present in a wheat straw culture, taken as one hundred.

It is clear that an antibiotic was produced on a number of materials, the best being sugar beet pulp, while others such as horse manure were quite unsuitable. With unsuitable materials the addition of glucose led to the formation of an antibiotic of considerable titre, indicating that the absence of activity was due to the lack of a suitable carbon source. The addition of glucose to materials already adequate always caused a large increase in the antibiotic titre. It is interesting to note that suitable materials became unsuitable after composting, but became suitable again when supplemented with glucose. It would seem that available sources of carbon were removed by the organisms

Table 6. Relative antibacterial activity in 14-day cultures of P. patulum on various substrates and the effect of the addition of glucose.

Substrate	Relative antibacterial activity (%)	
	Alone	With 3.5% glucose.
Wheat straw, fresh	100 ⁺	426
Wheat straw, composted	0	391
Lawn mowings, fresh	80	400
Lawn mowings, composted	0	380
Cracken	33	274
Timothy grass	200	426
Rye grass	0	-
Mustard	0	174
Red fescue	0	147
Red clover	0	-
Lucerne	0	200
Gainfoin	0	54
Sugar beet pulp	316	-
Horse manure	0	-
Peat	0 [*]	280

* The fungus did not become established.

+ Relative to wheat straw as 100, which here represents an expansive equivalent of 50 mg.

Test organism : B. mycoides.

Table 7. Relative antibacterial activity of 14-day cultures of P. patulum on substrates derived from different districts and harvested at various times, and the effect of the addition of glucose.

Substrate	Sample	Origin and time of harvesting	Relative anti-bacterial activity (%)	
			Alone	With 3.5% glucose
Red clover	A	Water meadows, River Lea, Broxbourne, September 1948	0	-
	B	As A, July 1949.	0	-
	C	Broxbourne Hillside, September 1948.	62	372
	D	Brentwood, July 1951.	0	-
Red fescue	A	Writtle, Nr. Chelmsford, Sept. 1947. Haulms still green.	0	147
	B	Brentwood, September 1949 Haulms still green.	32	348
	C	Brentwood, July 1951. Aberystwyth Strain S.59 Haulms straw coloured after threshing of the seeds.	84	400
	D	Aberystwyth July 1952. Same strain as in C. Aftermath growth after cutting back the panicles.	108	201
Mustard	A	Brentwood, July 1948. Haulms still green.	0	174
	B	Brentwood. September 1948. Haulms straw coloured.	21	300
Rye Grass	A	Brentwood, July 1948	0	-
	B	Broxbourne Hillside, July 1948	83	-
Timothy grass	A	Writtle, Nr. Chelmsford. July 1948.	200	400
	B	Aberystwyth, Sept. 1948.	180	390
	C	Broxbourne, Sept. 1948	200	426
Horae manure		Six samples of different origin.	0	-
Sugar beet pulp mixed " " plain	A	November 1947.	295	-
	B	November 1948.	316.	-
	C	November 1948.	300	-

+ Activity expressed as in Table 1. Test organism : B. mycoides.

causing decomposition of the materials. The leguminous crops were quite unsuitable. None of the crop plants was a more suitable substrate than either timothy grass or wheat straw. In view of the longer period over which activity was maintained in the latter (Fig.11) it should be regarded as the best among plant materials commonly used as manures in agricultural practice for antibiotic production by P.patulum. However, sugar beet pulp was the best of all, giving an activity almost as great as that of glucose supplemented timothy grass and wheat straw. The response to the glucose supplement was not of the same order in all materials. While the composted materials gave almost the same activities as the fresh as a result of the addition of glucose, crop plants such as the legumes, bracken, red fescue and mustard, though producing an antibiotic, gave lower yields. It is interesting to note that the fungus would not grow on non-supplemented peat, but on addition of glucose it not only grew well but produced an antibiotic of a considerable titre.

Differences in antibiotic activity between various samples of the tested materials

Numerous samples of wheat straw deriving from different localities were tested and no significant difference in activity was noted. Nevertheless, it was considered probable that the green manures might show different responses in accordance with the varying balance of nutrients as a result of cultivation under different conditions and harvesting at different stages of growth. Tests were made with a series of plant materials, deriving, as far as was practically possible, from different districts, namely the water meadows of the river Lea in Broxbourne, Herts: the hills of Broxbourne, Herts: Writtle and Brentwood in Essex and Aberystwyth in Wales. They were harvested either in July or September of 1948, 1949 and 1951, and the results are presented in Table 7. In addition two samples of sugar beet pulp from harvests in 1947 and 1948 were compared, and a further comparison was made between molassed and plain sugar beet pulp harvested in the same locality and in the same season.

The figures in Table 7 show that the above assumption was correct and that with some crop plants activities varied

with the district and the time of harvest. This is clearly shown in the case of red clover: a sample from the water meadows of the Lea gave rise to no activity, while another harvested at the same time on the hills of Broxbourne showed a good titre. The same observation was made with rye grass. The effect of the stage of maturity is illustrated by the results with mustard. One sample was harvested in the green stage in July and the other in September of the same year, both being cultivated in the same district. While the first sample did not give rise to an antibiotic, the matured sample did. This is probably due to the fact that the matured material had a greater carbohydrate content. The various samples of timothy grass and sugar beet pulp showed little variation; the same result had already been observed in the case of wheat straw. Probably, if a material contains a large percentage of carbohydrate, any further increase of this proportion which might be found in different samples does not lead to any further response and that probably also explains why there was no marked difference in the behaviour of plain and molassed sugar beet pulp. Numerous tests with different samples of the various crop plants have also shown that some materials consistently gave the same type of response, namely

always some antibiotic activity as with wheat straw or timothy grass, or no antibiotic at all. The latter behaviour was seen mainly with leguminous crop materials, though one sample of red clover out of three did support the formation of a stable antibiotic (Table 7). In the others there was some activity after 4 days, which later gradually disappeared and was seldom detectable after 8-10 days.

No correlation was found between rate of growth and sporulation in the cultures and antibiotic production. Wheat straw cultures showed comparatively slow growth and sporulation but a high titre, while lucerne cultures, though inactive, grew rapidly and sporulated heavily, as also did the active cultures of lucerne plus glucose. The rates of diminution of activity during continued incubation were observed to vary with different substrates, confirming the results shown in Fig. 11. Glucose favoured maintenance of activity for prolonged periods.

The effect of the addition of nitrogenous and phosphatic fertilisers on the activity of 14-day wheat straw cultures of *P.patulum*

The aim of this work was to find a material which when incorporated with the soil would induce maximum antibiotic production therein. The preceding work has shown that the greater the carbohydrate content of the substrate the greater the yield of antibiotic, and this has been particularly well illustrated by the effect of glucose on antibiotic titre. However, supplementing soil with materials rich in carbohydrate alters the C/N ratio, if only temporarily, and this may cause nitrogen starvation of plants growing in such soils. Further, certain crops require the addition of phosphatic fertilisers, especially for good root development. For these reasons the effect of nitrogenous and phosphatic fertilisers on antibiotic production by *P.patulum* on wheat straw was studied.

Several sources of nitrogen were investigated but only one type of phosphatic fertiliser, superphosphate, which is the one most commonly used. The nitrogenous fertilisers were sodium and potassium nitrate, sulphate of ammonia and urea, and they were supplied in quantities calculated to

add 1 g. of nitrogen to 100 g. of straw. The superphosphate was added on the basis of 1.5% of P_2O_5 . In addition mixtures of sodium nitrate plus superphosphate and potassium nitrate plus superphosphate were tried in an endeavour to imitate a 'complete fertiliser' as it is commonly used in agricultural practice. As controls non-supplemented wheat-straw cultures were also used. Assays were made at weekly intervals against B.mycooides and Bact. coli. The pH was determined before and after incubation for 14 days. The results (Table 8) refer to B.mycooides assays of 14-day cultures, and have been expressed as the percentage difference in yield as compared with the controls.

It is evident that all the fertilisers lowered the 14-day titre considerably, the degree of reduction being smaller with superphosphate than with the nitrogenous fertilisers. With the latter, the results varied with the source of nitrogen, the nitrates giving the smaller and sulphate of ammonia and urea the larger degree of suppression. It is possible that had several sources of phosphate been tested similar variations would have been noted. The greatest suppression was shown by the mixtures of nitrates with superphosphates, which suggests an additive effect. There was no correlation between pH and

Table 8. The effect of the addition of nitrogenous and phosphatic fertilisers on the antibacterial activity of 14-day wheat straw cultures of P. patulum.

Fertiliser added	pH		Patulin equivalent	Deficit below control (%)
	Before	After 14 days incubation.		
Nitrate of soda	6.0	6.5	28	60
Potassium nitrate	6.2	6.5	24	66
Sulphate of ammonia	5.5	4.5	10	86
Urea	6.8	7.1	13	81
Superphosphate	4.3	4.2	37	47
Superphosphate & sodium nitrate	4.5	6.3	5	93
Superphosphate & potassium nitrate	4.3	5.9	7	90
No fertiliser added	5.5	5.8	70	0

Test organism : B. mycoides.

activity. The urea treatment, which gave a very low titre, brought about the highest pH while superphosphate, with a pH as low as 4.2, gave the least degree of suppression. With the exception of sulphate of ammonia and superphosphate, the pH values rose slightly during incubation.

The nitrogenous fertilisers also adversely affected the persistence of activity, and the various sources of nitrogen acted in the same order as on yield after 14 days: superphosphate, however, arrested destruction of activity. After 12 weeks the titres in the superphosphate supplemented cultures were greater than in the controls, while at 24 weeks activity had disappeared completely from the latter but was still present in moderate amounts in the former.

Studies on the production of antibiotics on various autoclaved substrates by micro-organisms other than *P.patulum*

Fungi: *A.clavatus* like *P.patulum* produced an antibiotic on wheat straw with or without a glucose supplement.

Kent and Heatley (1945) showed that *A.terreus* can also produce patulin. For this reason a series of experiments was made to investigate antibiotic production by these fungi on various substrates.

The technique was identical with that used for *P.patulum*. The materials tested were wheat straw with and without the addition of glucose, plain sugar beet pulp, timothy, fescue, mustard, lucerne and horse manure, and the results are shown in Table 9 where only the activities after fourteen days incubation are given.

The general picture is similar to that with *P.patulum*, sugar beet pulp, timothy, and wheat straw being adequate materials, in decreasing order of efficiency. The fluids obtained from cultures of *A.terreus* on wheat straw and timothy were active against *B.mycoides* but inactive against *Baet.carotovorum*, whereas *A.clavatus* cultures on all these materials were nearly equally active against both organisms.

Table 9. The antibacterial activity of cultures of A. clavatus and A. terreus growing on different substrates

	A. clavatus		A. terreus	
	Patulin equivalent (mg.)			
	B. mycoides	Bact. carotovorum	B. mycoides	Bact. carotovorum
Fresh wheat straw	28	30	11	0
Fresh wheat straw with 3.5% glucose	110	117	38	6
Sugar beet pulp	49	50	21	3
Timothy grass	40	41	13	0
Fescue	0 [±]	0 [±]	0	0
Mustard	0 [±]	0 [±]	0	0
Lucerne	0	0	0	0
Horse manure	0	0	0	0

± Results were variable. Low antibiotic activity was occasionally observed on these substrates.

It was further observed that the zones of inhibition of B.mycoides with the A.terreus had ragged edges, whereas this organism gives sharp edged zones with patulin solutions (Fig.12). Although A.terreus does form patulin (Kent and Heatley,1945) the above observations suggest that it is not formed on wheat straw or timothy. However, cultures on wheat straw with added glucose, and sugar beet pulp, were both slightly active against Bact.carotovorum and possibly patulin was present therein, suggesting that patulin formation depends even more strongly with A.terreus than with A.clavatus on the presence of easily available carbohydrate.

Actinomycetes; The organisms studied were S.antibioticus and Streptomyces species no.100. The latter was isolated from the rhizosphere of tomato plants and was found to be strongly antagonistic to Ph.parasitica.

The materials used were wheat straw, sugar beet pulp, sainfoin, lucerne, timothy grass and fescue. Of the latter, two samples were tested, one in the early stages of growth and still green, and the other matured and straw coloured, having been left in the field for the production of seeds. Both were grown in the same locality. The wheat straw was

~~Plate~~

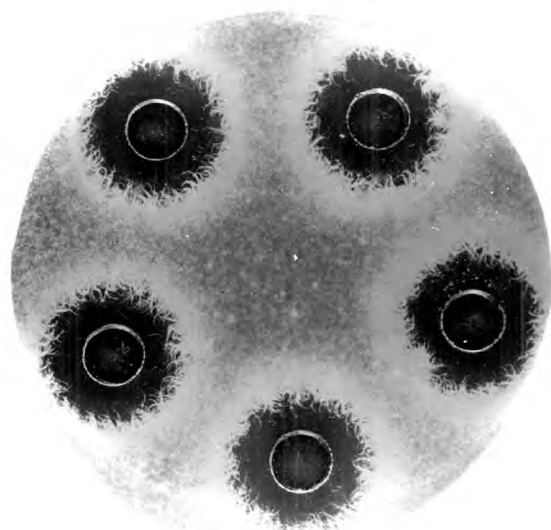


Fig. 12. Assay of the culture fluid of A. terreus growing on wheat straw, using B. mycoides. Note diffused edges of the zones with the rhizoid outgrowths of the colonies protruding into the area of inhibition. This picture does not alter. The areas of inhibition do not become overgrown.

employed with or without a 3.5% glucose supplement.

Inoculation of S. antibioticus was carried out by means of six to ten discs (5 mm) of a 5 day plate culture of the organism on Difco-yeast nutrient agar. The addition of a piece of agar appears to encourage actinomycin production in liquid cultures (Waksman and Woodruff 1942). However, the main reason for using this mode of inoculation was the difficulty in establishing S. antibioticus on manures.

Streptomyces 100 was introduced in the form of a spore suspension. Both organisms grew very slowly and the cultures had to be incubated for at least three weeks at 37°.

Frequently the cultures did not grow at all. Assays were made using B. mycoides, Bact. coli and Bact. carotovorum as test organisms. The strain of B. mycoides used here did not show the usual clear zones of stimulation (Figs. 13 and 14). The culture had been plated out recently as it was thought to be either contaminated or mutating to a smooth strain without the typical rhizoid outgrowths at the edge of the colony. Apparently a fresh mutant was picked out accidentally. Data are also given for cultures of the respective organisms on the usual standard media, i.e. for S. antibioticus in a

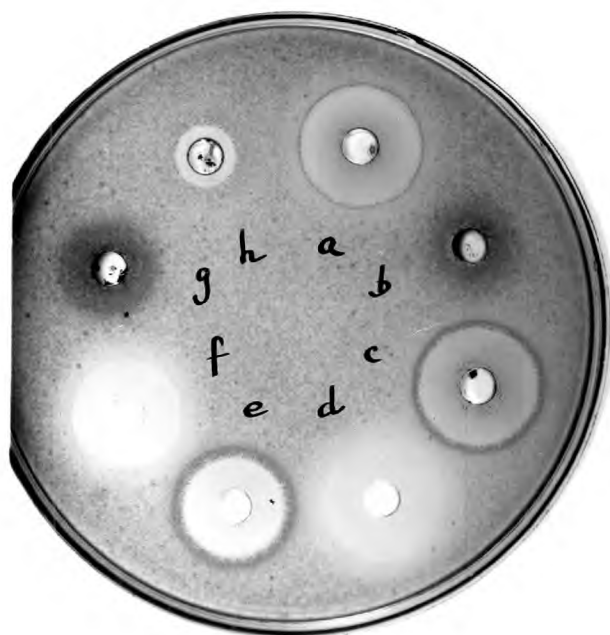


Fig. 13 .

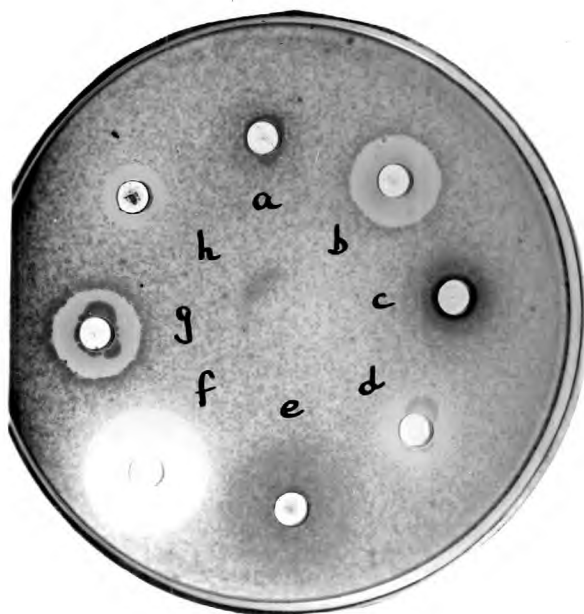


Fig. 14 .

Figs. 13 & 14. A comparison of the antibacterial activity of cultures of Penicillium patulum and Streptomyces antibioticus on various manures. White areas : growth inhibition. Dark areas : diffusion of pigments present in some manure cultures. Test organism : a fresh strain of B. mycoides less sensitive to stimulation.

- a - Timothy grass
- b - lucerne
- c - sugar beet pulp
- d - patulin in buffer solution, 1 mg/ml on Fig. 13 , 0.06 mg/ml on Fig. 14 .
- e - wheat straw (activity was exceptionally low)
- f - patulin in buffer solution, 0.06 mg/ml on Fig. 13 , 1 mg/ml on Fig. 14 .
- g - sainfoin
- h - patulin in buffer solution 0.06 mg/ml.

Fig. 13 . P. patulum cultures

Fig. 14 . S. antibioticus cultures.

nutrient broth as recommended by Waksman and Woodruff (1942) for actinomycin production; for Streptomyces 100 a Difco yeast nutrient broth; for the fungi a Czapek-Dox solution. The results are given, for B.mycoides assays only, in Table 10 and Figs.13 and 14. They are expressed as zone diameters because it was not possible to express activity in terms of the value of the corresponding antibiotics. Actinomycin was not obtainable and the nature of the antibiotic formed by Streptomyces 100 was not known. The results may be compared with those of cultures of the same age of A.clavatus, A.terreus and P.patulum growing on similar substrates.

An antibiotic was produced by the two species of Streptomyces on some materials. S.antibioticus showed larger zone diameters than did Streptomyces 100. In both cases B.mycoides was sensitive but neither Bact.coli nor Bact.carotovorum responded, although Waksman and Woodruff (1942) have demonstrated that actinomycin affects Bact.coli at high concentrations. It is therefore possible that actinomycin was formed in quantities too low to inhibit the Gram-negative bacteria or, alternatively, that an antibiotic different from actinomycin A or B was produced.

The outstanding feature of the results in Table 10 and Figs.13 and 14 is the fact that the two Streptomyces cultures tend to form an antibiotic on substrates on which the fungi did not yield an inhibitory agent, such as sainfoin and lucerne, while on the other hand no antibiotic was formed by the actinomycetes on such excellent fungal substrates as timothy grass and sugar beet pulp. As regards the latter, it was at first believed that the failure to establish the Streptomycetaceae was due to the low pH, but when this was adjusted to 6.5 by adding a buffer solution growth did not take place either. In the experiment of which the data are given in Table 10 and Figs.13 and 14, S. antibioticus did not form an antibiotic on wheat straw but in previous experiments low antibacterial activity with zones 12 to 17 mm.wide had been recorded. Streptomyces 100 wheat straw cultures never yielded an antibiotic.

It is obvious from this work that the actinomycetes utilize different plant materials for antibiotic production than do fungi. It is generally known that the former have different nutritional requirements, and this is further illustrated by the results of cultures on the two samples

Table 10. The antibacterial activity of cultures of actinomycetes and fungi growing on different substrates.

Substrates	S. antibioticus	S.no.100	A. clavatus	A. terreus	P. patulum
	Z o n e D i a m e t e r mm.				
Fresh wheat straw	0	0	23	18	27
Fresh wheat straw plus 3.5% glucose	NG	NG	30	23	34
Sugar beet pulp	NG	NG	29	22	32
Timothy grass	0	0	27	19	31
Fescue : early growth	19	14	0	0	0
: matured	0	0	25	-	27
Sainfoin	18	12	0	0	0
Lucerne	21	15	0	0	0
Standard medium	25 A.M.	19 D.Y.	19 C.D.	-	27 C.D.

Test organism : B. mycoides.

N.G. = the organism did not grow.

A.M. = actinomycin medium.

D.Y. = Difco-yeast broth.

C.D. = Czapek-dox solution.

of fescue. One, which was harvested in the early stages of growth, did not support antibiotic production by the fungi but did so with both species of Streptomyces, while the fungi formed a high titre of antibiotic on the matured grass, on which the Streptomyces species yielded no inhibitory substance at all. It seems that the higher protein and lower carbohydrate content in younger plants is more suitable to antibiotic production by the two Streptomycetaceae tested, while the higher carbohydrate content in mature crop plants was better for the two fungi. In view of this it is, however, inexplicable why timothy grass should consistently give negative results, as it is more likely to have a higher protein content than is straw, on which S. antibioticus formed some antibiotic.

The variation in response to plant materials grown in different localities observed with the fungal antagonists was also noted with the actinomycetes. There is a greater tendency to deviation of results, and activities within replicates varied considerably. It was generally difficult to establish the actinomycetes on manures. Occasionally, even if they grew well, producing a heavy crop of spores, no antibiotic could be detected. As only two species of Streptomyces were tested there is not sufficient evidence

to state with certainty that actinomycetes are not so efficient as fungi as producers of antibiotics on organic manures. However, that this may be the case is strongly suggested by the fact that with S. antibioticus the activity of cultures on the standard nutrient broth is high compared with that on manures. P. patulum for instance produces a higher titre on sugar beet pulp than in Czapek-Dox solution and on straw it is equally as good as in Czapek-Dox solution.

Antibiotic production by P. patulum cultures on plant materials which were not autoclaved

It is evident that P. patulum produced an antibiotic of a varying titre on a number of plant materials provided they had been autoclaved. During this process no doubt various compounds had been broken down and rendered more available to the fungus, in particular the complex polysaccharides present in straw as cellulose and hemicellulose. The question then arose whether this decomposition by autoclaving was an essential prerequisite for antibiotic production, or whether the fungus itself was capable of breaking down the complex materials. Further, autoclaving entirely eliminated the competing microflora and it was considered important to study what effect microbial competition would exert on the

level of production and maintenance of titre. For these reasons the problem was attacked in various ways. First, by using steamed materials; here a moderate release of nutrients would probably take place, and some organisms would survive giving an intermediate set of conditions. Second, by studying antibiotic production in autoclaved materials into which other organisms were later introduced. Here, there would be a large release of nutrients and competition by the invading flora. Third, by using propylene oxide treated substrates; all release of nutrients would be avoided and sterility achieved in most materials. Fourth, by using non-sterile materials there would be no release of nutrients, at least not on the same scale as resulting from heating, though some intermediate break-down by other micro-organisms might take place. There would also be the full impact of a microbial flora.

Steamed materials

Wheat straw, with and without a 3.5% glucose supplement, and sugar beet pulp, were tested, with autoclaved materials as the controls. The glucose supplemented straw and the sugar beet pulp were tested once only but several trials were made of unsupplemented wheat straw. Two samples of this were

used. One, which came from Rothamsted, was tested once only. The materials were steamed for 30 min. For comparison, an experiment was made using supplemented and unsupplemented straw steamed on three consecutive days, so as to eliminate microbial competition entirely. Probably this involved a larger release of nutrients, but this should not have been quite on the same scale as that resulting from autoclaving. Assays were made at intervals but the results in Table 11 refer to 14 day cultures only. They show that the activities on once steamed wheat straw were very variable, and even at the best relatively small. This variability contrasts with the high and uniform development of activity in cultures on autoclaved straw. The cause of this irregularity is not certain. It could have been due either to the development of actively competing organisms which had survived the steaming, or to variations in decomposition of the straw on different occasions, with consequent variation in the level of nutrition. That the lowered level of nutrition is an important factor in the reduced yield of antibiotic is supported by the fact that the addition of glucose increased the titre considerably, as did steaming for three consecutive days. In the latter

Table 11. The antibacterial activities of 14-day cultures of P. patulum on steamed wheat straw and sugar beet pulp.

Substrates	Patulin equivalents (mg) in experiments					
	I	II	III	IV	V ⁺	VI [§]
Fresh wheat straw						
Autoclaved	41	35	47	47	41	47
Steamed	3	3	11	17	3	26
Difference in yield %	93	91	74	66	93	45
Plus 3.5% glucose						
Autoclaved	151	-	-	-	-	132
Steamed	54	-	-	-	-	73
Different in yield	64	-	-	-	-	45
Sugar beet pulp						
Autoclaved	100	-	-	-	-	-
Steamed	54	-	-	-	-	-
Difference in yield	46	-	-	-	-	-

Test organism : B. mycoides.

+ Different sample of wheat straw was used (Rothamsted)

§ Experiment VI. II materials were steamed on three consecutive days, otherwise only 1 steaming.

case, though activity was better than from once steamed straw, it was still lower than on the autoclaved material in spite of the fact that in both cases sterility would have been complete. Further, on steamed sugar beet pulp, which, as shown below, contains adequate nutrients even in the unheated state, activity was at least as high as on autoclaved straw, but it must not be overlooked that even so it was lower than on the autoclaved substrate.

The results presented for wheat straw refer to 14 -day cultures but the maximum yield was not always reached by this time as is normally the case with autoclaved cultures. The optimum patulin equivalents were frequently obtained after five weeks and in one experiment they were at this time as high as those on the autoclaved materials. Probably at this stage a high concentration of antibiotic had accumulated. In general, however, production on steamed straw was considerably less than on autoclaved material.

Contaminated, autoclaved materials

Since the experiments described in the above section had given little information about the relative importance of competing micro-organisms, tests were made on autoclaved materials which were contaminated from the air.

Wheat straw and sugar beet pulp were autoclaved in the normal manner in Erlenmeyer flasks and were then transferred into open non-sterile jars and allowed to stand for four days. Three replicates were made. The materials were then inoculated with P.patulum, incubated at 25°, and assays made at intervals. In Table 12 the patulin equivalents of 14-day cultures are given.

Two of the wheat straw replicates were overgrown with a species of Trichoderma. No antibiotic was detected in one of these while the other contained a very small quantity, but it cannot be said whether this was formed by the Trichoderma or by P.patulum; in the third replicate, in which P.patulum became well established, production took place and a considerable level was reached. However, activity disappeared very quickly in this sample. The experiment was repeated and similar variations between replicates were observed. The sugar beet pulp gave very good yields and the fungus became well established.

Propylene oxide treated materials

Hansen and Snyder's (1947) method of sterilization was used and the materials were well aired by repeatedly passing a stream of sterile air through. Wheat straw, with or without a 3.5% glucose supplement, timothy grass and plain sugar beet

Table 12. The effect of contamination of autoclaved plant material on the level of antibiotic production by P. patulum.

Substrate	No of replicate	Patulin equivalent mg.	
		Contaminated	Control
Wheat straw	1	0	36
	2	4	47
	3	26	49
Sugar beet pulp	1	73	132
	2	87	100
	3	100	123

Test organism : B. mycoides.

Control : Autoclaved materials inoculated immediately with aseptic precautions.

pulp were tested. The glucose added to the straw was supplied in an aqueous solution and was sterilized by filtration, through an L7 Chamberland filter candle. The results of B.mycoides assays are given in Table 13, and show that in most cases antibiotic production took place though at a lower level than on steamed or autoclaved materials. In the presence of glucose the titre on wheat straw was considerably increased. Sugar beet pulp by itself also gave good yields. Both observations agreed with the results obtained with steamed materials. Although the autoclaved substrates gave the highest yields because of the greater release of nutrients, it is clear that P.patulum has some ability to decompose plant materials and to form antibiotic therefrom to some extent. The low yields and variable results on wheat straw may be due to microbial competition. While sugar beet pulp was completely sterilized by the propylene oxide treatment, certain bacteria frequently survived in straw and in some instances it was not even possible to establish P.patulum. Although Hansen and Snyder (1947) claimed that they obtained complete sterility it appears from the observation with wheat straw and with soil (described below) that the sensitivity of micro-organisms to propylene oxide is probably not general but specific and

Table 13. A comparison of the antibacterial activity of 14-day cultures of P. patulum on autoclaved, and propylene oxide treated substrates.

Substrate	Patulin equivalents (mg) in various sterilized substrates and deficits = (%) compared with autoclaving		
	Autoclaved	Propylene Oxide	Deficit in yield %
Wheat straw	45	0 [#]	100
11	48	3	94
111	39	0	100
Wheat straw plus 3.5% glucose	166	113	32
Timothy grass	60	6	90
Sugar beet pulp	86	65	24

Test organism: B. mycoides.

No growth.

therefore disinfection is not complete on some materials, depending on the microflora established thereon. The yield on straw supplemented with glucose was high and uniform. Probably the presence of glucose enabled ready establishment of P.patulum and furthermore enhanced antibiotic production as well.

Non-sterile materials

Samples of wheat straw, unsupplemented or supplemented with 3.5% of glucose, sugar beet pulp and timothy grass were tested. Water was added in the usual proportion, the materials inoculated with 5 ml. of a spore suspension of P.patulum and incubated at 25°. In addition, not inoculated plant materials treated in the same manner were also incubated. Further, samples of the above plant materials, with the water added, were placed in a refrigerator and assayed after 24 hours in order to make sure that the materials themselves did not contain inhibitory substances. The incubated materials were assayed after eight and fourteen days against the two standard test organisms. Several experiments were set up with sugar beet pulp and unsupplemented wheat straw but only one with the supplemented straw. Timothy grass was tested in two trials. Each time three replicates were used. The data obtained are set out in Table 14.

Table 14. The antibacterial activity of cultures of P.patulum on various non-sterile and autoclaved materials.

Substrate	No. of Expt.	No. of Replicate	Patulin equivalent after 8 and 14 days (m.g.)					
			Non-sterile		Autoclaved		* Deficit in yield (%)	
			8	14	8	14	8	14
Wheat straw	1-3	9	No activity		35*	49*	100	100
Wheat straw plus 3.5% glucose	1	1	130	2	152			
		2	130	8	176		25	98
		3	118	2	170			
Timothy grass	1	1	21	4	75	62		
		2	14	7	65	50	66	89
		3	37	7	70	53		
	2	1	8	8	42	60		
		2	9	0	35	40	84	92
		3	3	5	55	55		
Sugar beet pulp	1	1	68	5	90	120		
		2	95	13	90	80	11	92
		3	80	7	90	100		
	2	1	80	26	110	100		
		2	100	4	108	110	9	82
		3	120	30	112	100		
	3	1	42	42	38	70		
		2	22	12	76	76	35	57
		3	62	50	80	96		

* Based on average yields

Table 14a. The antibacterial activities of non-sterile plant materials to which water was added after incubation at 25°.

Plant Material	No. of Replicate	Zone diameters after days (mm)	
		8	14
Wheat straw	1	0	0
	2	0	0
	3	0	0
Wheat straw plus 3.5% glucose	1	12	0
	2	11	0
	3	0	12 P
Timothy grass	1	16	0
	2	12 P	12 P
	3	0	0
Sugar beet pulp	1	32	15
	2	15	20
	3	12 P	0

P = partial inhibition. Activities are expressed as zone diameters because the nature of the antibiotics formed is not known.

It is evident that an antibiotic was formed on the inoculated materials which contained a readily available source of carbohydrate, such as on sugar beet pulp and on glucose supplemented wheat straw. Wheat straw, by itself, always gave negative results, while timothy grass gave rise to an antibiotic of varying activity, in spite of the fact that this material is not regarded as one containing more carbohydrate than wheat straw. Both test organisms were sensitive and the cultures contained a heavy growth of P.patulum. It is therefore highly probable that patulin was formed therein. As regards sugar beet pulp, it is interesting to note that in some replicates yields were almost as great as with the autoclaved substrate. The maximum production was recorded after eight days and activity decreased rapidly thereafter.

An antibiotic was also formed in the non-inoculated substrates which were incubated. The yield varied considerably in each replicate, and the Gram-negative Bact. carotovorum was not always sensitive, suggesting that different antibiotics were formed each time. No doubt, the inhibitory substance formed on materials not inoculated with P.patulum resulted from the metabolic activities of a mixed flora derived from organisms which were present on the materials and which on moistening and incubating began to grow actively. It is not impossible that P.patulum was present

on the materials, because the laboratory in which these experiments were carried out is badly contaminated with this organism.

The materials kept in the refrigerator and assayed after 24 hours did not contain a substance inhibiting the test organisms.

Part III

Antibiotic production in the soil.

ANTIBIOTIC PRODUCTION IN AUTOCLAVED SOIL BY FUNGI

In the preceding section the ability of some fungi and actinomycetes to produce an antibiotic on organic materials which are or could be used as manures has been demonstrated. As the most satisfactory results were obtained with P.patulum and A.clavatus, these two organisms were mainly used at first in studies of ability to form antibiotic in the soil, but later A.terreus, Penicillium species A and B and Fusarium javanicum were also used.

Experimental and results

Preliminary experiments with A.clavatus and P.patulum

A preliminary qualitative experiment on soil No.1 was performed with A.clavatus using pots in the glasshouse, assays being carried out on leachates collected between the 2nd and 3rd week. The soil ^{tested were} ~~was tried~~ both non-sterile ^{or} and autoclaved, and in each case it was either unsupplemented or supplemented with 4% straw or 4% straw plus 2% glucose. Controls were set up, consisting of uninoculated replicates.

Table 15

Antibacterial activities of leachates from
variously treated soils

<u>Substrate</u>	<u>Activity of leachates collected after 2-3weeks</u>			
	<u>Autoclaved soil</u>		<u>Non-sterile soil</u>	
	<u>Inoculated*</u>	<u>Not Inoculated</u>	<u>Inoculated*</u>	<u>Not Inoculated</u>
Soil only	P	P	P	P
Soil plus 4% straw	+	S	P	P
Soil plus 4% straw plus 2% glucose	++	S	P	P

Test organism: Bact.coli

* A.clavatus used

+, ++ - increasing degrees of complete inhibition

P - weak partial inhibition

S - slight stimulation

The results (Table 15) were only qualitative, but demonstrated that inhibitory substances were formed in autoclaved soil which contained a carbon source. Enriched autoclaved but uninoculated soil did not contain inhibitors. Leachates from the uninoculated autoclaved supplemented soil slightly stimulated the growth of Bact.coli while those from unsupplemented autoclaved soil, and from non-sterile soil, whether supplemented or not or inoculated or not,

caused weak partial inhibition. The existence of inhibitory substances in soil is dealt with in more detail below.

In another experiment, also with soil no.1, flask cultures were used. The soil, unsupplemented, was autoclaved, inoculated with P.patulum and incubated for 14 days at 25°, after which the fluid was expressed by hand and assayed against B.mycoides, Bact.coli, Ph.parasitica and Ph.cryptogena. None of the test organisms was inhibited to any extent. B.mycoides was stimulated and the growth of the two species of Phytophthora was faster, the mycelium reaching the surface of the liquid in 10 days as compared with an average of 13 days in the controls. However, Bact.coli was slightly inhibited, there being narrow zones of partial inhibition 11 - 13 mm. in diameter which were difficult to measure. Such slight degrees of inhibition may not be significant, as this effect has been observed with extracts from non-sterile soil as well.

The preliminary experiments having given some positive results, more detailed investigations were then carried out.

Production of antibiotic in supplemented soil by P.patulum

Experiment 1

This was done with autoclaved soil no.1, either alone or supplemented with 2.5, 3.5, and 5% (w/w) of finely ground wheat straw (not straw meal); glucose, 2.5%; wheat straw,

5% plus glucose, 2.5%; finely shredded molassed sugar beet pulp, 2.5 and 5%. In this experiment the water content was slightly higher in the soils containing glucose and in the unsupplemented soils where it was 125% of saturation; the soils containing 2.5% straw or sugar beet pulp were at about 118%, and the other mixtures at about 100%. It was thought that these variations in the moisture content would not materially affect the results, but in later experiments the water content was always adjusted to the same level throughout an experiment. The fluids were expressed by hand after 14 days and assayed by the serial dilution method against B.mycoides, Bact. carotovorum (type culture), a strain of Bact.carotovorum isolated from potatoes (var.phytophthorum) and C.fascians. Antifungal tests were made on certain of the fluids by the same method using Ph.cryptogea and Ph.parasitica. The pH values of the soils were determined by a Marconi pH meter before and after incubation.

The results, presented in Table 16, confirmed those of ^{the} preliminary experiment with A.clavatus, antibiotic production being observed only in extracts from autoclaved soil containing a carbohydrate source. The pH changes

Table 16 . pH values, antibacterial and antifungal activities of fluids expressed from autoclaved soils, (supplemented and unsupplemented) 14 days after inoculation with P. patulum .

Addition to autoclaved soil	pH values		Highest dilution causing complete inhibition of					
	initial	final	<i>B. mycoides</i>	<i>Bact. carotovorum</i>	<i>Bact. carotovorum</i> var. <i>phytophthorum</i>	<i>C. fascians</i>	<i>P. cryptogea</i>	<i>P. parasitica</i>
None	5.25	5.89	S	0	0	0	S	S
2.5% straw	5.03	4.86	P	1/5	GP	0	1/10	1/5
3.5% straw	4.93	5.18	1/5	1/10	1/5	0	1/20	1/10
5% straw	5.02	5.25	1/10	1/20	1/10	1/10	-	-
2.5% glucose	5.63	4.93	1/40	1/40	1/40	1/20	1/100	1/100
5% straw + 2.5% glucose	4.99	4.60	1/80	1/80	1/80	1/40	-	-
2.5% sugar beet pulp	4.77	4.94	1/40	1/40	1/40	1/20	1/80	1/80
5% sugar beet pulp	4.96	4.84	1/40	1/40	1/40	1/40	-	-

S = Growth stimulated

0 = no inhibition

P = Partial inhibition at 1/5

GP = Good partial inhibition at 1/5

during incubation were slight, and the inhibitions observed were not likely to have been due to acidity as such. Wheat straw was the least effective, but its effect increased with rising proportion of straw to soil. An increase in the amount of sugar beet pulp from 2.5 to 4, did not lead to higher titres, except in the C.fascians assay, and the activities induced by sugar beet pulp were of the same order as those obtained with glucose in the soil. However, in later experiments glucose was normally the more efficient carbon source, and the present result is probably to be ascribed to the fact that the serial dilution method is less efficient in showing small differences. The extract from an unsupplemented soil showed no inhibitive effect. It stimulated the growth of B.mycoides.

Bact.carotovorum was the most, and ^{C.fascians} the least sensitive of the four bacterial test organisms. The difference in sensitivity of the two strains of Bact.carotovorum to the fluid from soil-straw mixture is noteworthy.

The data in Table 16 also demonstrate that the fluids had considerable antifungal activity. Although the two fungi tested showed greater sensitivity than the bacteria, the order of effectiveness of the various supplements in inducing antifungal activity is similar to that for antibacterial activity.

Experiment 2

The first experiment having shown that increasing the proportion of carbohydrate material added led to higher activities of the expressed fluids, a further experiment was performed using the same soil and supplements as before, but with closely spaced concentrations in wide ranges of 0.3 to 9% with glucose, 0.6 to 9% with sugar beet pulp and 2.5 to 9% with straw. In addition to the comparisons between inoculated and autoclaved soil, variously supplemented or unsupplemented tests were also done on completely untreated soil, and autoclaved, unsupplemented and uninoculated soil. Further, 4% sugar beet pulp was added to both non-sterile and autoclaved soil, without inoculation. The soils were centrifuged after 14 days and the fluids assayed against B.mycoides and Bact. carotovorum, with Bact.coli as a third test organism in the additional comparisons. The results are presented in Figs. 15, 16 and 17.

The requirements for antibiotic production were found to be that the mixture of soil and supplement must be autoclaved and inoculated, as shown in Figs. 15, 16, 17 e, h, and i. No antibiotic was produced unless these conditions were fulfilled (b, c, d, f, and g). However, B.mycoides was stimulated by materials in the centrifugate from untreated

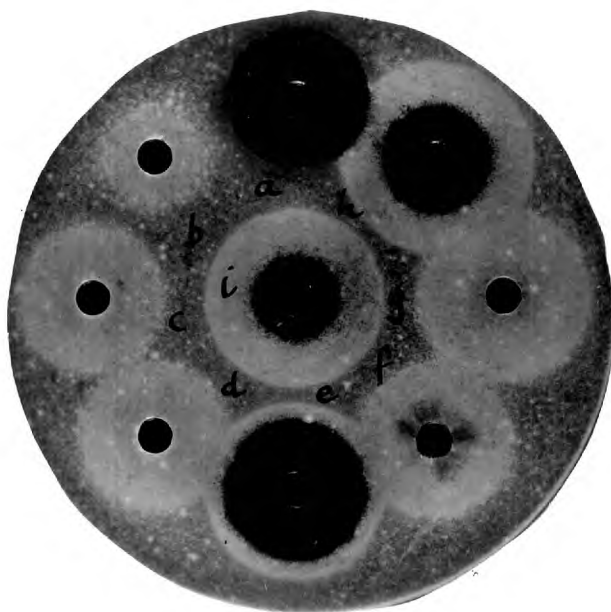


Fig. 15 . Assay of centrifugates from a soil receiving various treatments. Test organism: B. mycoides.

- a - Patulin in a buffer solution, 1 g/l.
- b - Completely untreated. Note stimulation.
- c - Not supplemented, not inoculated, but autoclaved. Note increase in zone of stimulation.
- d - Not supplemented, autoclaved and inoculated with P.patulum. Centrifugate prepared 14 days after incubation at 25° C.
- e - Supplemented with glucose and inoculated as in (d).
- f - Supplemented with sugar beet pulp but not autoclaved or inoculated.
- g - As (f) but autoclaved. Note increase in zone of stimulation.
- h - As (g) but inoculated with P.patulum. Note distinct wide zone of stimulation following the zone of inhibition.
- i - As (h) but supplemented with wheat straw.

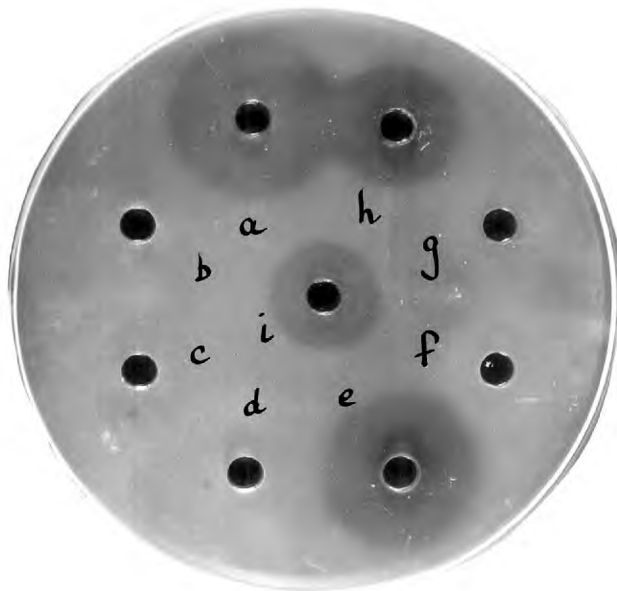


Fig. 16. As ~~P1.~~ Fig. 15, but the test organism is Dact.
carotovorum. Zones of stimulation are generally slight or
 absent. Arrangement of centrifugates as in ~~P1.~~ Fig. 15.

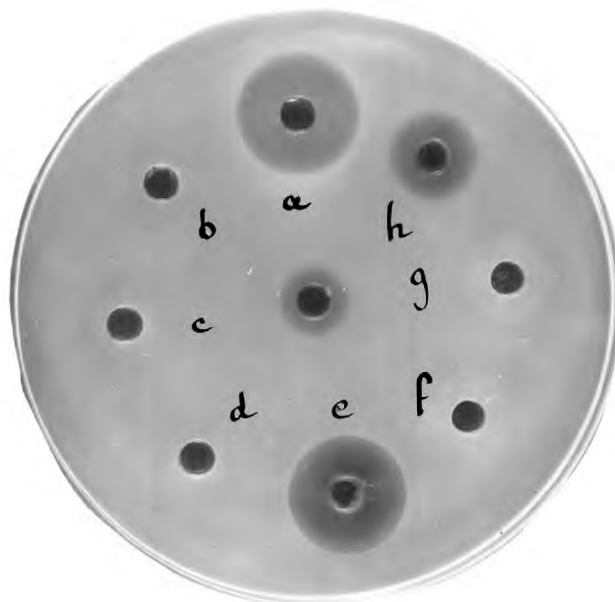


Fig. 17. As ~~Pl.~~ . Fig. 15. but the test organism is Bact. coli. Arrangement of centrifugates as in ~~Pl.~~ . Fig. 15.

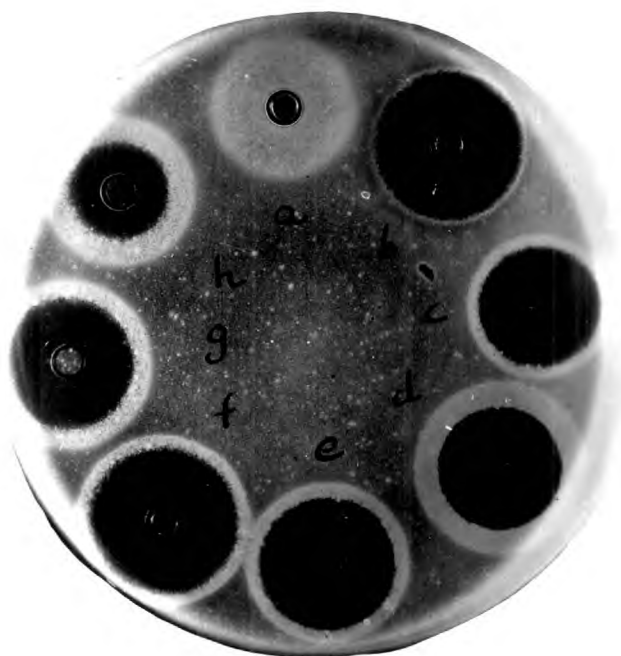


Fig. 18. Assay of centrifugates from an autoclaved soil receiving various supplements. Test organism : B. mycoides.

a - supplement : 2.5% straw but soil not inoculated with P. patulum.

b - patulin in soil extract 1 g/l.

c - h - soils were inoculated with P. patulum.

c - supplement 4.0% straw.

d - supplement 2.5% sugar beet pulp.

e - supplement 2.5% glucose.

f - supplement 4.0% glucose.

g - supplement 4.0% sugar beet pulp.

h - supplement 2.5% straw.

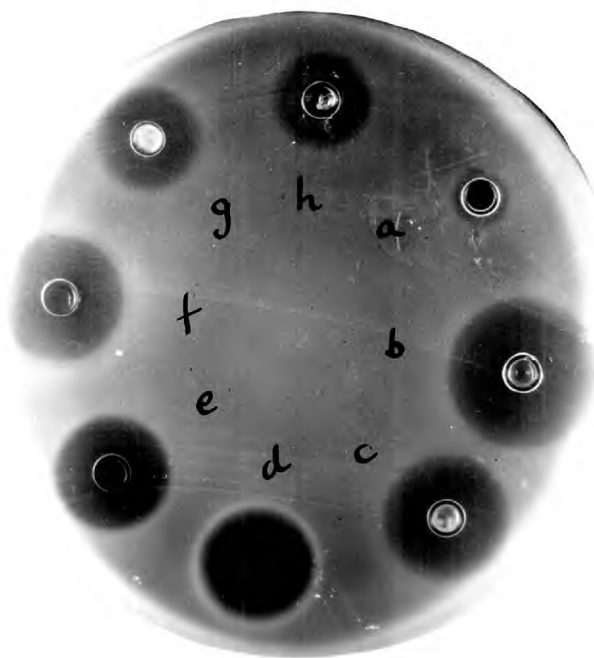


Fig. 19 . As Fig. 18 , but the test organism is BAct.
carotovorum (type culture). Zones of stimulation are
slight. Note narrow zone of partial inhibition in (a).

soil (Fig. /5 b) and this effect was increased when the soil was autoclaved (Fig. /5 c). There was no further response when non-supplemented autoclaved soil was inoculated (Fig. /5 d). When the soil was supplemented with sugar beet pulp but not autoclaved or inoculated a slight lytic effect, as yet unexplained, was observed (Fig. /5 f). This disappeared on autoclaving, but the zone of stimulation was larger (Fig. /5 g). Bact. carotovorum and Bact.coli were not stimulated by the unsupplemented soil centrifugates which stimulated B.mycoides, as shown in Figs. /6 and /7 b,c, and d, but there was some stimulation with sugar beet pulp (Figs. /6, /7 f and g). In Figs. /8 and /9 the effect on B.mycoides and Bact.carotovorum respectively of centrifugates from soils receiving the three supplements at different levels (2.5 and 4%) is compared with that of patulin (1g/l) dissolved in a centrifugate from an autoclaved but unsupplemented soil and with a centrifugate from the straw supplemented but ~~not~~inoculated soil. It is interesting to note even centrifugates which were markedly inhibitory also contained growth stimulants for B.mycoides (Fig. /8 c - h); this stimulation outside the zone of inhibition was not so marked with Bact.carotovorum (Fig. /9 c - h). The straw supplemented soils in particular gave centrifugates lacking in stimulating properties for this organism (Fig. /9 c,h).

Bact.carotovorum was slightly inhibited by the centrifugates from straw supplemented, inoculated soil (Fig. 19a). Fig. 20 shows that an increase in the percentage of glucose added increased the titre of antibiotic up to a maximum at 4.5% after which there was no further effect. The lower curve refers to assay data obtained with B.mycoides and the upper to Bact.carotovorum. Both curves run almost parallel with Bact.carotovorum showing slightly greater "patulin equivalents". This was also shown in later experiments, and the slight discrepancy might have been caused through the Bact.carotovorum zones being less sharp; this may have possibly have led to a biased reading of the zone diameters.

In Figs. 21 and 21a a comparison is given of the ability of sugar beet pulp, wheat straw and glucose to support antibiotic production by P.patulum. In Fig. 21 the patulin equivalents have been plotted against the logarithms of the percentages of material added, while in Fig 21a logarithms to base 2 and base 10 respectively have been used for the patulin equivalents and concentrations of material added. It can be seen that weight for weight glucose was more effective than sugar beet pulp, wheat straw being still less valuable, although

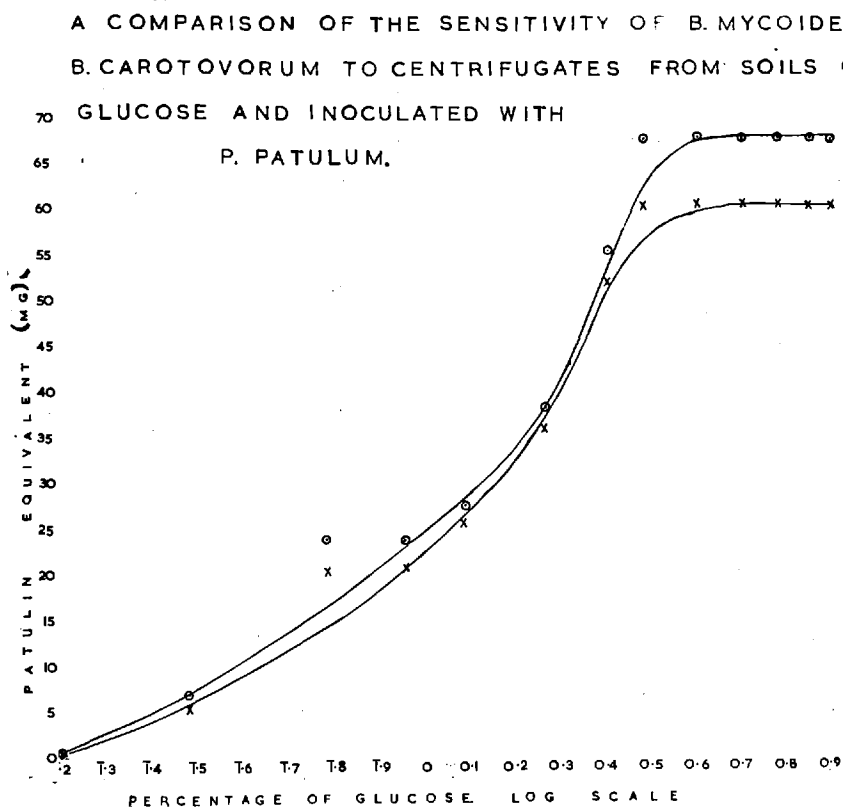


Fig. 20.

THE RELATION BETWEEN PERCENTAGE OF SOURCES
OF CARBOHYDRATE ADDED TO AUTOCLAVED SOIL, INOCULATED
WITH *P. PATULUM*, AND THE ANTIBACTERIAL ACTIVITY OF THE
CENTRIFUGATES OF THESE SOILS.

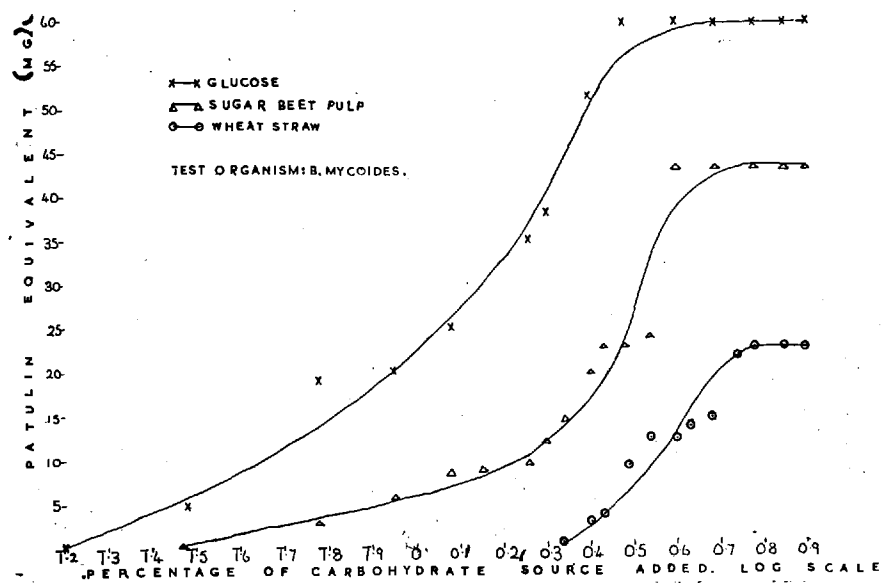


Fig. 21.

OF CARBOHYDRATE ADDED TO AUTOCLAVED SOIL INOCULATED
WITH *P. PATULUM* AND THE ANTIBACTERIAL ACTIVITY OF THE
CENTRIFUGATES OF THESE SOILS.

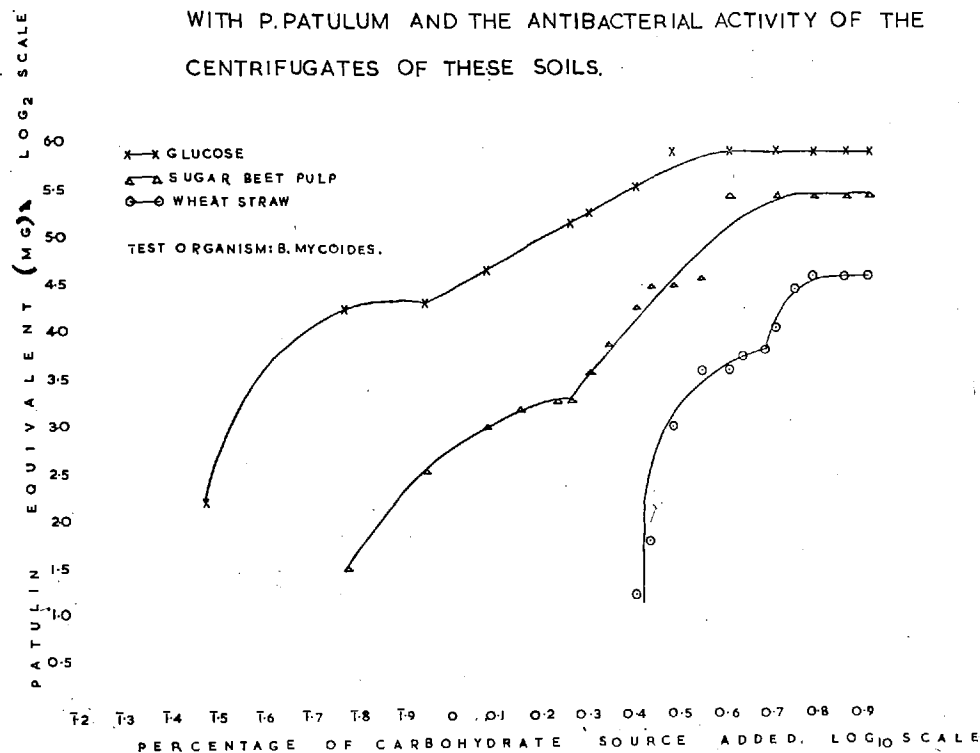


Fig. 21a.

effective if in sufficient amount. Three features of the curves in Fig. 2/a are striking. First, there was a threshold quantity necessary for appreciable antibiotic formation with each material, the amounts varying from 0.3% with glucose to 2.5% with wheat straw. Second, maximum production, which reached different levels in the three cases, was attained over a much narrower range, the order being the same as with the threshold quantities. Third, each of the graphs relating $\log_{10}$ concentration to $\log_2$ "patulin equivalent" (Fig 2/a) shows a "two stage" effect; apparently a first maximum in production was reached with a relatively small amount of each material, with subsequently further stimulation of activity when considerably larger amounts were added. The reason for this is at present obscure. It may be an artefact due to the cycle of increase and decrease in antibiotic (Fig. //) having differed in duration with the different rates of addition of the supplements. If the effect is real, it might be ascribed to the production of two antibiotics at different rates and with different threshold levels and maxima, so that in reality two production curves are superimposed.

Experiment 3

This was designed to show the effect of concentration of supplements on antifungal activity in the centrifugates with a comparison with antibacterial activity. Centrifugates were taken after 14 days from P.patulum inoculated soil no.1, receiving 2.5 and 5% of glucose, sugar beet pulp or wheat straw and tested by the serial dilution method against Ph.parasitica and T.basicola. Cup plate assays were simultaneously carried out with B.mycoides and Bact.carotovorum. The results (Table 17) showed that glucose was the most potent, followed closely by sugar beet pulp with wheat straw least effective. The antifungal activity was higher than the antibacterial, confirming previous results, and Ph.parasitica was somewhat more sensitive than T.basicola. Patulin shows this type of activity. The effect of increased concentration of supplement was to raise the titre with sugar beet pulp and straw, but not with glucose; presumably in the latter case the level was above the maximum shown in Fig.2/a.

Experiment 4

In the previous experiments only soil no.1 had been used, and it was desired to ascertain whether antibiotic production would occur in other soils and whether the yield would vary

Table 17. A comparison of antibacterial and antifungal activities of centrifugates from autoclaved soil receiving 2.5% (w/w) and 5% of various supplements.

Centrifugates from	pH		Highest dilution giving complete inhibition of		<u>B. mycoides</u> ⁺	<u>Bact.</u> <u>Carotovorum</u> ⁺
	Before	After	<u>Ph. parasitica</u> [*]	<u>T. basicola</u> [*]		
Incubation with		<u>P. nautum</u>				
			<u>Stimulation</u> ↓	<u>Stimulation</u> ↓	<u>Stimulation</u> ↓	
Soil only	5.3	5.9				
Soil plus glucose 2.5%	5.6	4.9	1/100	1/100	1/40	1/40
5.0%	5.2	4.6	1/100	1/100	1/40	1/40
Soil plus sugar 2.5%	4.8	4.9	1/80	1/40	1/30	1/30
beet pulp 5.0%	4.9	4.8	1/100	1/50	1/40	1/40
Soil plus straw 2.5%	5.-	4.8	1/10	1/5	∅	1/5
5.0%	5.-	5.2	1/20	1/10	1/10	1/20

* For 3 weeks at 25°

+ For 18 hrs. at 25°

Ø Partial inhibition only at 1/5.

when the same supplements were used in different soils. An experiment was set up using soils nos. 1, 2, 3, 4 and 6, supplemented with 2.5 and 4% of glucose, sugar beet pulp or wheat straw, and ^{a/50} 5% of straw. The mixtures were treated as before, and centrifugates prepared after incubation for 14 days were assayed against B.mycoides and Bact.carotovorum (type culture). The results are tabulated in Table /8.

With none of the uninoculated soils (controls) was there any complete inhibition of B.mycoides. Nevertheless some antibacterial activity was present in soils 2 and 6 with glucose as the supplement as shown in Figs. 22 and 23. These figures show as well that growth stimulating substances for that organism were also present. Bact.carotovorum was more sensitive to the inhibitors in soil 6 than B.mycoides. This is illustrated in Fig. 23, which also shows that stimulation of Bact.carotovorum was only slight. Antibiotic production took place in all 5 inoculated soils, though yields varied somewhat. Soils nos. 1 and 6 gave the best results, while soil no. 3 was the least suitable. In all soils antibiotic production took place with considerable though varying yields when the supplement was glucose or sugar beet pulp. The differences between soils, however, were more marked when wheat straw was used. The threshold

Table 18. A comparison of the antibacterial activities of centrifugates prepared after fourteen days incubation from five different soils, similarly supplemented, and inoculated with P. patulum.

Soil No	Original pH	Patulin equivalents (mg.) with supplements (% w/w) of-						
		Glucose ⁺		Sugar beet pulp ⁺		Straw ⁺		
		2.5	4	2.5	4	2.5	4	5
1	5.5	55	68	22	50	3	12	14
2	5.5	29	51	15	18	0	0	6
3	6.5	29	15	5	11	0	0	0
4	5.3	22	53	14	17	0	0	5
6	7.6	45	46	20	62	0	3	13

Test organism : B. mycoides.

✗ With none of the uninoculated controls was there any complete inhibition of B. mycoides.



Fig.22 .

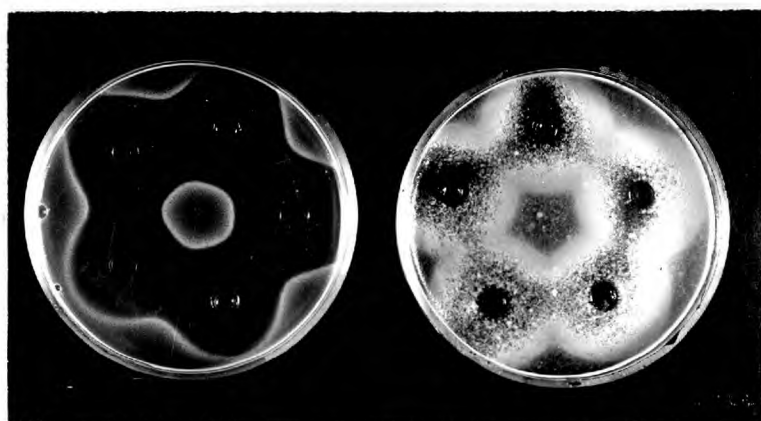


Fig.23 .

The effect on the growth of B. mycoides and Bact. carotovorum of centrifugates from different, autoclaved but not inoculated soils, supplemented with 2.5% glucose.

Fig.22 . Left : no. 2 Goff's Oak soil. Right : no. 4 Nazeing soil. Test organism : B. mycoides.

Fig.23 . Both no. 6 Harlington soil. Test organisms: left, Bact. carotovorum; right, B. mycoides.

Note variation in degree of partial inhibition brought about by the difference in soils supplemented with the same quantity of glucose and triangular areas of partial inhibition on Harlington soil assays.

quantity required for measurable antibiotic activity varied from 2.5% for soil 1 to 4% for soil 6 and 5% for soils 2 and 4.

The response to increases in supplements was not the same in all five soils. Soil no.3 showed a decrease in activity with an increase in glucose supplement. This phenomenon has also occasionally been observed with such active soils as no.6, but it cannot be stated on the evidence available that it is correlated with soil type. It may be accidental. The usual observation is that there is either a greater response with an increase of the glucose supplement or no difference at all. The effect of increases in sugar beet pulp also varied from soil to soil, being considerable for soils 1, 3, and 6, and comparatively small for soils 2 and 4. The two latter soils show a very similar behaviour in most respects. Increase of the straw supplement also gave rise to different yields on the different soils; when the supplement was 5% the yields on soils 1 and 6 were very similar, yet the soils were of a very different type.

Experiment 5

The above experiment was repeated on soils 4,5 and 6. Soil 5 was tested in order to obtain data on a soil with an intermediate pH and a high total carbon content. The

supplements were as before but while glucose and sugar beet pulp were added at 2.5% only, wheat straw was supplied at 2.5, 4, 4.5 and 5%.

In the previous experiment, patulin which was used as a standard had been dissolved in soil extract, in this experiment the solvent was a pH 6.5 buffer solution ~~in an attempt to get a standard yielding more uniform results for comparison.~~

The results are given in Table /9, and they confirm in essentials the results of the previous experiment. Antibiotic production took place in all three soils and the differences were greatest with straw, the threshold quantities varying considerably. Soil 6 again gave the best results. The figures for the glucose supplemented soils are low compared with those in Table /8 while those for sugar beet pulp agree on the whole. The comparatively high readings with straw supplements may be due to the fact that the straw was in the form of a fine meal. It had been well ground in an electric mill for previous experiments, but accidentally it was ground still finer for the present experiment.

It is difficult to explain the low readings for the glucose supplemented soils. A great deal of work has been carried out with no.4 soil supplemented with glucose and variations have been observed, but never had so low a figure

Table 19.

A comparison of the antibacterial activity of the centrifugates, prepared fourteen days after incubation from three different soils, similarly treated, and inoculated with E. patulius.

Soil No.	pH	Total carbon content %	Patulin equivalents (mg) ^m with supplements % (w/w) of :-					
			Glucose 2.5 ⁿ	Sugar beet pulp 2.5 ^o	Wheat straw ^o			
					2.5	4	4.5	5
4	5.3	3.12	15	12	0	0	4	8
5	6.7	5.41	27	24	0	7	16	23
6	7.8	2.0	31	27	0	6	19	20

Test organism : B. mycoides.

^m patulin dissolved in pH 6.5 buffer solution.

^o with none of the uninoculated controls was there any complete inhibition of B. mycoides.

as 15 mg. been recorded. In previous experiments the patulin standard had been dissolved in centrifugates from corresponding but uninoculated soils, a procedure which involved a great deal of labour; therefore in the present experiment a buffer solution standard was used. As can be seen from Fig. 2, this would lead to slightly lower patulin equivalents, but this alone cannot fully explain the low figures with glucose supplements, which were recorded with that supplement only. In an attempt to clarify this point new assays of the centrifugates from the three glucose supplemented soils were made, the standard being patulin dissolved in centrifugates from those glucose supplemented soils. The results confirmed the data in Table 19; in fact the figures were even slightly lower.

The maintenance of antibacterial activity in different soils

The data of the preceding experiments concern fluids obtained after 14 days incubation. In order to discover how well the level of antibiotic activity in various soils was maintained, experiments were carried out on four of the five soils used in experiment 1, namely nos. 1, 2, 3 and 6, supplemented with 2.5% of glucose. A series of flasks was prepared and all flasks were inoculated together. At intervals,

beginning 4 days after the start, three flasks of each soil were selected and the contents assayed. The results of this experiment, which continued for 30 days, are shown in Fig. 24, from which it is clear that activity was retained for a considerable period in all cases but that the antibiotic titre followed a different course in the various soils. Once again soil 3 seemed to be the least efficient, because of the rapid fall in titre after the high level reached in 4 days. Soil 1 reached an equally high titre in 4 days, but the activity continued to rise slowly till a peak was reached at the 20th day, after which a decline set in. This was slow at first, then rapid, but some activity was still present after 30 days. Soils 2 and 6 followed a different pattern, antibiotic activity developing relatively slowly but reaching quite high values after 14 and 12 days respectively, after which an immediate decline set in, which soon became very rapid. Soil 6 had somewhat the higher activity at a given time. These patterns of behaviour account for the relatively poor showing of soil 3 in experiments involving 14 day tests only. If 4 day assays had been the basis of comparison a very different conclusion might have been reached.

In view of the fact, however, that soil 3 showed no response at all to straw supplements, and that activity

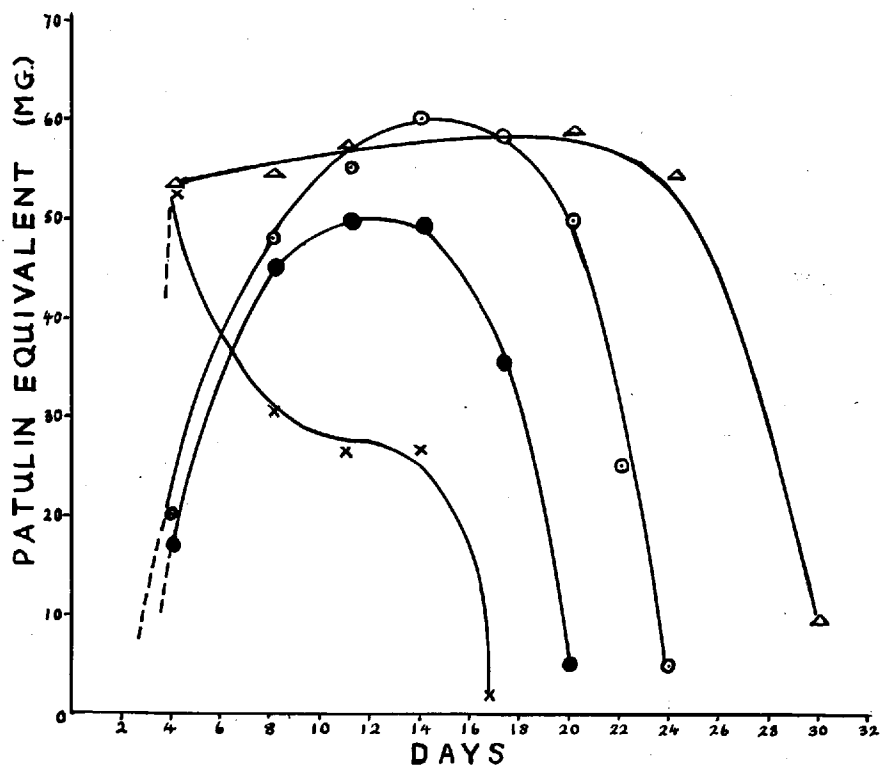


Fig. 24. The antibacterial activity of centrifugates of four different soils receiving 2.5% of glucose and inoculated with *P. patulum*. Test organism : *E. mycoides*.

△—△ Soil no. 1.

⊙—⊙ Soil no. 2.

×—× Soil no. 3.

●—● Soil no. #6.

disappeared rapidly, it should be regarded as the least efficient substrate.

A clear correlation between pH and production level was not possible ~~either~~, though it is true that high figures were obtained with the soils which were nearer to the neutrality point, no.5 with a pH of 6.7 and no.6 with a pH of 7.8. On the other hand soil 1 with a pH of only 5.5 gave very good results and soil no.3 with a pH of 6.5 very low figures. There was some correlation between pH and retention of activity which was better where the pH was lower.

This series of experiments has established two facts clearly; the suitability of a variety of soils for antibiotic production if supplemented and that there is retention of antibiotic activity over varying periods of time.

The effect of various supplements on the pH values of uninoculated soils and of soils inoculated with *P.patulum*.

It was thought that in spite of the buffering action of soil, the pH might be altered as a result of the growth of *P.patulum* in supplemented soil and that the antibiotic activity of soil extracts might therefore be affected, through the test organisms responding to changes in acidity rather than to changes in antibiotic content.

To test the above hypothesis the pH was determined by Marconi meter of cultures of P.patulum in soil no.1 supplemented in various ways; non-inoculated but autoclaved soils similarly enriched were also examined. The soil samples used were those of the experiment in which the effect of additions of glucose on the production of an antibiotic by P.patulum was studied. The concentration range was 0.15 to 6.0% (w/w). There were three replicate samples of each treatment. The mean pH values at the start and after 14 days are shown in Fig.25, plotted against the logarithms of the glucose concentrations together with the antibacterial activities of the centrifugates from the soil cultures.

In general the effect of autoclaving soil was to reduce the pH slightly, but on autoclaving with even the smallest amount of glucose used, the pH rose markedly. With higher glucose concentrations the rise was smaller, and was absent with about 6%. After inoculation and incubation for 14 days the pH was found to have risen above the original values when the glucose concentration ^{not} was _^above 2%, but to have fallen further with higher doses. The curves of activities shows the two stage effect previously observed, and the break occurred at about the same level of concentration as that at which the graph of

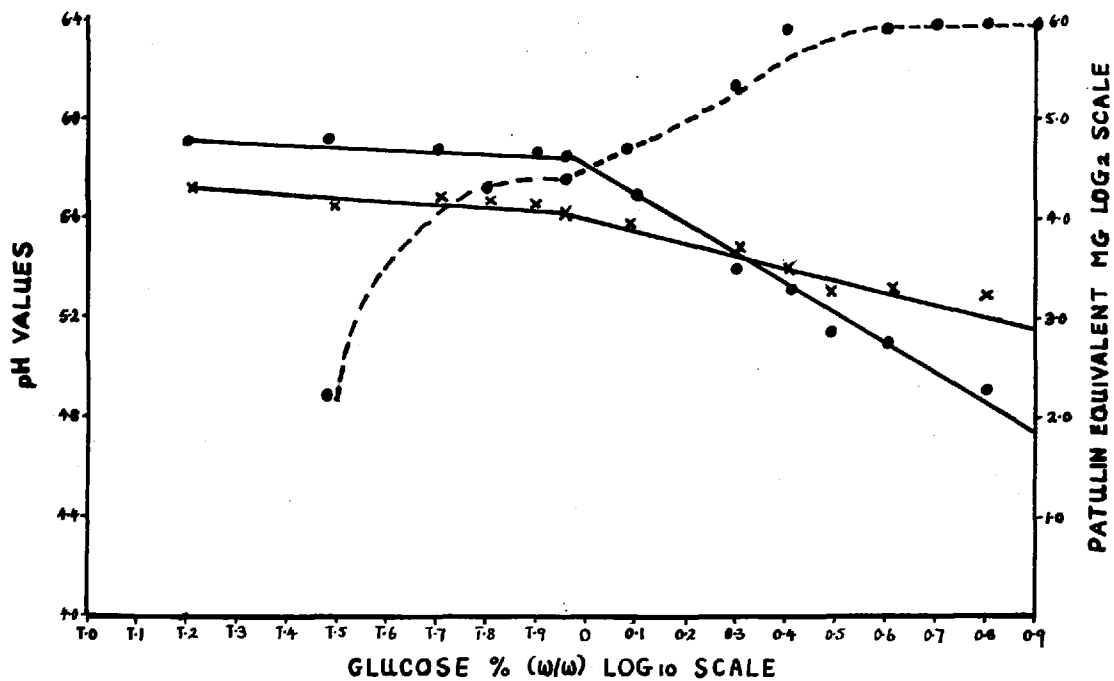


Fig. 25. pH values in soils supplemented with glucose before and after inoculation with *P. patulum* related to antibacterial activity. Test organism : *E. mycoides*.

- x — x Before incubation.
- — ● After incubation for 14 days.
- --- ● Activity of culture.

pH against log concentration became steeper. This correlation of the increase in hydrogen ion concentration with antibacterial activity suggested at first that the response of the test organism was due rather to the increase in acidity than to an increase in antibiotic titre.

Similar experiments were carried out with sugar beet pulp and wheat straw, with glucose for comparison, and the results are shown in Table 20. In this experiment the glucose behaved much as before, except that an increase of pH was observed with 2% whereas this had caused no change before. Autoclaving the glucose with the soil apparently had a rather different action on this occasion, the pH shift being slight, but this was purely the result of the concentration range used. Autoclaving sugar beet pulp in soil tended to depress the pH at the higher doses, the effect being otherwise slight (Table 20) ~~and Fig. —~~. In the inoculated soils after 14 days there was either a slight fall or no change in pH, the effect being rather irregular. With straw, also, there was little change on autoclaving, but in the cultures the pH tended to be high (Table 20). When the activities ^{of the Sugar beet pulp soil mixtures} were plotted against pH values the correlation noted in the glucose experiment was not observed here. ^(Fig. 26) Although the two stage effect occurred again it was not accompanied by a considerable fall in pH and the point

Table 20. The pH changes in soil ^{no.1.} following autoclaving, supplementing and after growth of P. patulum for 14 days.

Supplement	Percentage of supplement added (w/w)									
	2		2.5		3		3.5		4	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
None	5.3	5.8	-	-	-	-	-	-	-	-
Glucose	5.6	5.1	5.4	5.0	5.2	4.9	5.20	4.5	5.2	4.6
Sugar beet pulp	5.2	4.9	5.1	4.9	5.2	5.2	5.1	5.1	4.90	4.7
Straw	5.2	5.5	5.2	5.3	5.1	5.6	5.0	5.5	5.2	5.40

Untreated soil = pH 5.5

(a) Before inoculation.

(b) After inoculation.

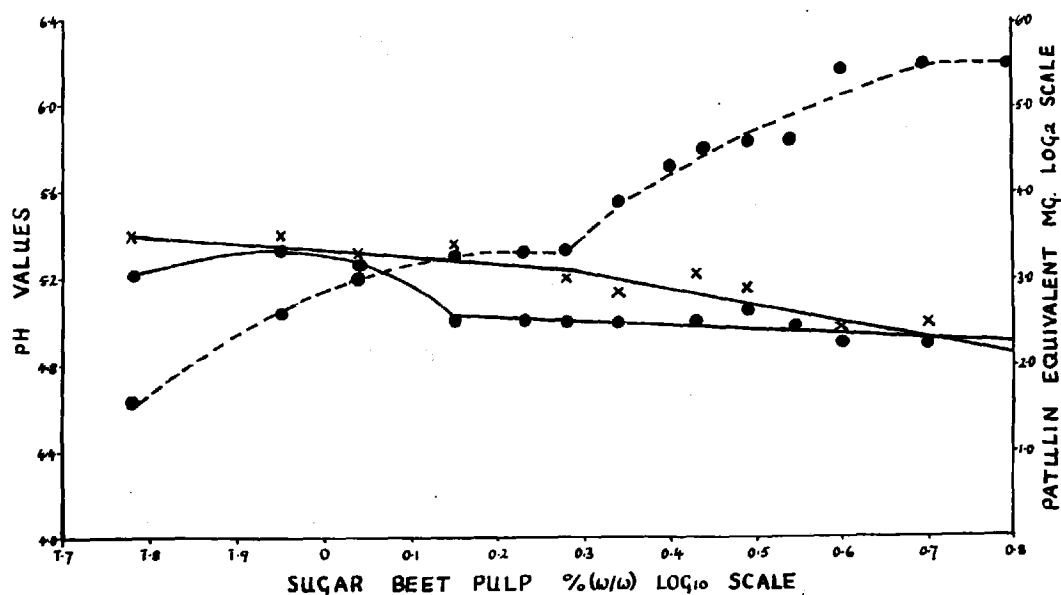


Fig. 26. pH values in soils supplemented with sugar beet pulp, before and after inoculation with P. patulum, related to antibacterial activity. Test organism : B. mycoides.

- x — x Before inoculation
- — ● After incubation for 14 days
- - - - ● Activity of culture

at which the break occurred on the activity line did not coincide with any changes in pH values.

It may therefore be concluded that the greater response of the test organisms was really mainly due to an increase in antibiotic activity and not to the reaction of the soil extract. This confirms an observation made in the section on manures. There, the pH in wheat straw cultures supplemented with sulphate of ammonia was very low, 4.2, nevertheless activity was considerably smaller than in cultures with a higher pH value.

In a further experiment, a comparison was made of the pH drift in three different soil types which were similarly treated. The soils used were nos. 4, 5 and 6. The pH was determined with a pH meter on the untreated soil; the autoclaved soil, with or without supplements and on cultures of P. patulum on supplemented soils after 14 days incubation. An attempt was also made to correlate pH with activity and the results are set out in Table 2/.

In general the pH changes were very small. Autoclaving caused a small decrease in hydrogen ion concentration. This is a well established fact. The addition of glucose brought about a slight rise of pH in the acid soil. This is in agreement with the behaviour of soil no. 1 in the previous

Table 21. The pH changes in three different soils, following autoclaving, supplementing and after growth of P. patulum, for 14 days together with antibacterial activities.

Soil no.	pH Values.																
	Untreated	Autoclaved	Autoclaved and Supplemented.														
		No Supplement	Glucose 2.5%			Sugar beet pulp 2.5%			W h e a t 4%			S t r a w 4.5%			5%		
			(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
4	5.3	5.2	5.4	5.5	15	5.1	5.5	12	5.4	5.4	0	5.3	5.4	4	5.4	5.5	8
5	6.7	6.6	6.5	6.4	27	6.3	6.5	24	6.4	6.3	7	6.4	6.4	16	6.4	6.4	23
6	7.8	7.4	7.0	7.1	31	6.9	7.2	27	7.2	7.2	6	7.2	7.1	19	7.1	7.1	20

Test organism : B. mycoides.

(a) After inoculation, before incubation.

(b) After incubation for 14 days.

(c) Patulin equivalent (mg) after 14 days.

experiment. In the neutral and alkaline soils 5 and 6 all three supplements lowered the pH further during autoclaving, as in the previous experiment also.

The pH rose slightly during the growth of P.patulum with glucose in soils nos.4 and 6 and fell a little in soil no.5, while in the previous experiment soil no.1 showed a very marked decrease.

The soil-sugar beet pulp cultures behaved differently, a distinct rise being noted in all three soils, in contrast with a drop in soil no.1 of the previous trial. Straw, which caused a decrease in acidity in the previous experiment, in this case caused no change. The fall in hydrogen ion concentration was often very marked in the previous experiment while hardly any change occurred in the present experiment.

The irregularity of these results makes interpretation difficult. There was a long interval between the two experiments and it is possible that the P.patulum cultures had changed. It is believed that this explanation is more justified than one based on difference in soil type, because apart from some minor deviations the pattern of changes was very much the same in soils 4,5 and 6 in spite of their distinct differences in type. It must not be overlooked, however, that in many respects soil no.1 behaved very differently.

STUDIES ON THE PRODUCTION OF ANTIBIOTICS
IN AUTOCLAVED SUPPLEMENTED SOIL BY MICRO-
ORGANISMS OTHER THAN P.PATULUM

As it has been established in the section on manures that micro-organisms other than P.patulum also have the property to produce antibiotics, the experiments on amended soil cultures were extended to include A.clavatus, A.terreus, Penicillium species A. and B, Fusarium javanicum, B.subtilis (a subtilin producing strain), Streptomyces antibioticus and two unidentified species of Streptomyces, nos.100 and 400.

The technique was identical to that employed with P.patulum.

No.2 soil was used for the fungal cultures and no.6 for the bacterial and actinomycete cultures as it was alkaline. The results presented in the histogram of Fig.27 are expressed as relative antibacterial activities using the data obtained with P.patulum as a standard.

While F.javanicum, B.subtilis and all the Streptomyces species yielded negative results, the Aspergilli and Penicillia formed antibiotics of varying potency.

The pattern of relative activities varied with the different substances and test organisms used, but A.clavatus and Penicillium species A were on the whole about equally efficient and slightly less active than P.patulum. Next came

THE RELATIVE ANTIBIOTIC ACTIVITY OF CENTRIFUGATES FROM AUTOCLAVED SOILS CONTAINING ONE OF THREE DIFFERENT SOURCES OF CARBOHYDRATE AND INOCULATED WITH 5 DIFFERENT FUNGI

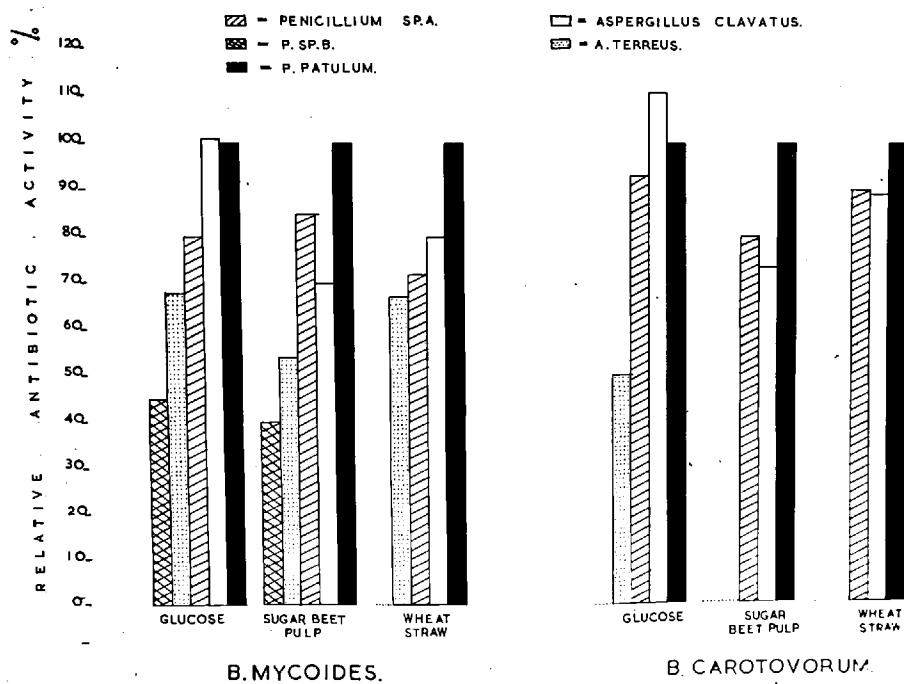


Fig. 27.

A.terreus, and least active was Penicillium species B, which was wholly inactive against B.mycoides on soil plus straw and against Bact.carotovorum on all substrates. In soil supplemented with wheat straw or sugar beet pulp, A.terreus failed to yield an antibiotic completely inhibiting the growth of Bact.carotovorum, though in the latter case good partial inhibition of that organism was obtained. Fig. 27 does not, however, include partial inhibitions. In experiments not involving soil, which were not intended as true comparisons, A.clavatus had proved only a little more than half as active as P.patulum, (Table 9). However, in the present experiment a different isolate (A), which was more active, was used.

The failure to detect an antibiotic in the centrifugates from the Streptomyces, B.subtilis and F.javanicum cultures may have been due to two reasons; an inadequacy of nutrients in the soil or to adsorption and inactivation.

Antibiotic production by *P.patulum* in soil containing
other micro-organisms

Although the work described in the previous sections has shown clearly that various fungi can form considerable quantities of antibiotics when grown in pure cultures in supplemented and autoclaved soil, that is not the condition in which antibiotic production would be required for the control of plant diseases in glasshouses or in the field, where other micro-organisms would be present. The foregoing work cannot be directly related to practical conditions for the further reason that the soil and the supplements were both subjected to autoclaving and this may have resulted in the breakdown of the soil organic matter and of the supplements with possibly enhanced availability of nutrients to the fungi. In glasshouse practice steaming is used and moderate release of nutrients could occur from both the soil and the supplements, while a part of the microflora of the soil would remain to multiply and compete with the antibiotic producer. In the field full competition by the soil flora would be present and there would be no comparable release of nutrients, except by microbial action. Experiments were therefore carried out to study antibiotic production in steamed, and propylene oxide treated, supplemented soil, both inoculated with *P.patulum*.

Steamed soil

The experiments were carried out on soils 1 and 4 and the supplements were 2.5 and 4% of glucose, sugar beet pulp or wheat straw. The results are set out in Table 22, where the patulin equivalents of the centrifugates prepared after 14 days incubation are recorded, together with data from similarly supplemented autoclaved soil for comparison. Clearly, the steamed mixture of soil plus glucose and soil plus sugar beet pulp supported antibiotic production by P.patulum in both cases but the activity was lower than on autoclaved soil. The lowered yield may have been because of competition by the microflora surviving the steaming or the level of nutrients released by steaming being lower than that released by autoclaving. Slightly greater activity was present in the autoclaved no.1 soils with the larger supplement of glucose, but this did not happen in the steamed mixtures, and it is suggested that in these soils other nutrients, possibly nitrogen, may have been a limiting factor, insufficient having been liberated, as compared with autoclaving, to enable the increased carbon supply to be utilised. The fact that soil 4 did not behave like soil 1 may be ascribed to the difference in type. With the sugar beet pulp supplement activity was greater at the higher dosage in both steamed

Table 27 . A comparison of the antibacterial activities of centrifugates of 14-day cultures of P. patulum in either steamed or autoclaved soils of 2 types, supplemented in the same manner.

Soil	Supplement	% (w/w)	Patulin equivalent (mg)					
Centrifugates from			B. mycoides assays.			Bact. carotovorum assays		
			Autoclaved	Steamed	Reduction in yield (%)	Autoclaved	Steamed	Reduction in yield (%)
1	glucose	2.5	52	32	38	55	45	18
4		2.5	55	35	36	57	35	39
1		4.0	60	32	47	74	45	39
4		4.0	56	36	36	57	37	35
1	sugar beet pulp	2.5	23	11	52	22	12	45
4		2.5	30	17	43	35	21	40
1		4.0	44	17	54	50	19	62
4		4.0	50	31	38	50	30	40
1	straw	2.5	7	0	100	9	0	100
4		2.5	4	0	100	5	0	100
1		4.0	18	0	100	20	0	100
4		4.0	12	0	100	13	0	100

Soil 1 = pH 5.5

Soil 4 = pH 5.3

and autoclaved mixtures, of both soils; also the yields in the steamed, ^{sugar beet supplemented} soils were on the whole proportionately smaller than in the glucose soil mixtures. This is consistent with the fact that in sugar beet pulp the carbohydrate is present largely as complex polysaccharides which would decompose more extensively to available carbon compounds on autoclaving than on steaming. This is not surprising in view of the low yields even from the autoclaved mixtures and the markedly smaller yields when steaming was used with sugar beet pulp. The carbohydrate of straw is even less available than that of sugar beet pulp, and steaming is not likely to have broken it down to any appreciable extent.

Propylene oxide treated materials

Soil no.4 was employed for this work. After sterilization with propylene oxide and aeration with sterile air, the glucose content was brought to 2.5% and the moisture to 100% of saturation by adding an aqueous solution, sterilized by filtration through an L 7 filter candle. After inoculation with P.patulum the soils were incubated for 3-4 weeks, the longer period of incubation appearing

desirable because spore formation was much slower than on heat treated soil and comparatively sparse. This may have been due to retention by the soil of traces of propylene oxide or to a lower level of available nutrients because of the absence of heat treatment. It was observed that the spore heads were greener than usual. Once or twice, after spore heads had appeared (in about a week), they disappeared again and a strong acid smell accompanied by the evolution of gas and water developed. This, and the fact that complete sterility was rarely achieved with propylene oxide, suggests that bacterial growth had occurred, leading to lysis of the spore heads. The sterility tests were carried out by placing very small quantities of soil, picked up with a miniature spatula on slopes of Czapek-Dox and Difco nutrient agar. No fungi or actinomyces developed, but a few bacterial colonies regularly appeared, and it seemed that there were only two or three species. One type was consistently seen; it had a thick, raised, cream-coloured colony with a strongly wrinkled, reticulate surface.

As a rule air-dried soil was used for these experiments but by chance oven-dried soil (24 hr. at 100°) was employed on one occasion. After drying, it had been stored for several weeks in a non-sterile but closed tin. During

handling in preparation for the experiment, and prior to treatment with propylene oxide, it was exposed to aerial contamination, and sterility tests, even after propylene oxide treatment, showed bacteria present in three out of four samples.

The results of all these experiments are included in Table 23, which also has data from air-dried soil sterilized by steaming and autoclaving, for comparison.

It can be seen that antibiotic production took place in soil that had not been heat treated but partly sterilized with propylene oxide, but the yield was variable. In the best case, the oven dried soil was about equal to steamed soil, but the propylene oxide sterilized air-dried soils were markedly inferior to steamed soil. Probably lack of other nutrients such as are released by heat treatment was responsible for this.

The importance of the release of nutrients by heat is stressed by the results obtained with oven-dried soil. Oven drying for 24 hours must have led to a considerable release of nutrients and in fact antibiotic production was a little higher than on steamed soil. It should, however, be taken into account that the microbial population was not the same as in steamed soil. There spore forming organisms would survive (in the

Table 23. A comparison of the antibacterial activities of centrifugates from autoclaved, steamed, and propylene oxide treated soil, supplemented with 2.5% of glucose, prepared 3 weeks after inoculation with P. patulum.

Soil treatment.	pH value		Patulin equivalent.
	Before Incubation	After	(%)
Autoclaved	5.2	5.3	55
Steamed	5.1	5.4	35
Propylene oxide			
oven-dried	5.3	5.5	40
* air-dried	5.5	4.6	9
air dried	5.2	5.-	3
Ø air-dried	5.6	5.5	15

Test organism : B. mycoides. Soil No.6.

* Bacterial fermentation observed.

Ø After the method described by Ingram & Robinson (1952).

form of spores), while it might be expected that oven drying at 100° for 24 hours would destroy most spores; any infection that occurred in that soil must have been a secondary one coming from the air, and different in type from that in steamed soil. However, the results show clearly that antibiotic production can occur even without heat treatment.

The bacterial fermentation occasionally observed which sometimes resulted in lysis of spores, did not seem to interfere with antibiotic production. There was little difference in yield between infected and uninfected cultures, and as both Gram-positive and Gram-negative bacteria were sensitive to the centrifugates it is unlikely that another antibiotic was formed. The pH of the centrifugates from infected soils was very low, around 4.6, but it is improbable that growth inhibition was due to acidity.

The experiment was repeated using the method of sterilization described by Ingram and Robinson (1952) but sterility tests showed that not even fungi had been killed. However, when soil which had been treated once was exposed to propylene oxide for a second time only a few species of bacteria survived, and when P.patulum was inoculated it rapidly became established, and bacterial

fermentation did not occur. The activities agreed very well with those given in the first experiments, confirming once more that apart from a source of carbon even unheated soil contains sufficient nutrients for antibiotic production by P.patulum, though in moderate yields.

Maintenance of the antibiotic titre after contamination of cultures of P.patulum in autoclaved soil.

The soil microflora in general must have an important rôle not only in relation to production but also to the maintenance of antibiotic activity. Regarding antibiotic production the work on steamed and propylene oxide treated soil has shown that competition may be important, since at least part of the original microbial flora had survived in these soils.

On the point of persistence, Wakeman (1948) stated that even if antibiotics could be formed in the soil they would not persist owing to inactivation by other micro-organisms. However, Nandi (1948), Sinha (1950) and Nimkar (1950) have shown that certain purified antibiotics, when added to non-sterile soil, do persist for varying periods, during which they exert an influence on the soil microflora. Jefferys (1950) and Gottlieb and Siminoff (1950 a) also found

evidence of antibiotic persistence in soil. These authors used *patulin* in addition to several other antibiotics.

In order to study the effect of a freshly introduced microflora on the titre of the antibiotic in soil the following experiments were performed.

Experiments 1 and 2

A set of Erlenmeyer flasks containing soil no.1 supplemented with 2.5% glucose was autoclaved and all were inoculated simultaneously with *P.patulum*. After incubation for 14 days two replicates at a time were centrifuged and assayed. The remaining flask cultures were then contaminated, either by exposure to the air or by introducing a fresh microflora from soil. For air contamination soil was transferred without any aseptic precautions to open jam jars, which were weighed at intervals to enable the water lost during incubation by evaporation to be replaced by non-sterile tap water.

The other half of the flasks were soil contaminated by adding 20% of non-sterile, non-supplemented soil of the same type. Mixing with the soil of the cultures was effected with aseptic precautions, and subsequently water lost by evaporation was replaced by sterile tap water. Half of each lot was kept at room temperature and the other in a greenhouse. Another set of cultures which was not

contaminated served as a control and was kept in an incubator at 25°. At intervals beginning at 7 days two replicate jars or flasks from each treatment and from the controls were centrifuged and the fluids assayed against the two standard test organisms.

In a repetition of the experiment the same soil and on the whole a similar technique was used, but the cultures were placed under large bell jars lined with damp filter paper, and having wide basins containing water so as to reduce evaporation practically to zero. The first assay was made after four days.

The results are presented in Table 24 in the form of patulin equivalents relative to that present when contamination was carried out, taken as a hundred.

The data indicate that while in the controls anti-biotic activity persisted at a high level, with only a slow rate of decline, in the contaminated soils activity diminished rapidly and was all gone after 13 days at most. In the cultures contaminated with non-sterile soil and kept in the greenhouse activity was lost completely in 7-10 days. It is interesting to note that in the contaminated soils in the first experiment relative activity was higher after 10 than after 7 days, which might indicate that production was

Table 24. Relative antibacterial activities of sterilised soil containing an antibiotic substance, produced by P. patulum, and which was subsequently contaminated.

Experiment	Source of contamination and temperature	Relative activity (%) ^a after exposure to contamination for					
		0 days	4 days	7 days	10 days	13 days	16 days
I	None 25°	100	-	100	86	60	56
	Air, laboratory	100	-	20	59	0	0
	Non-sterile soil, laboratory	100	-	26	36	0	0
	Air, greenhouse	100	-	8	25	0	0
	Non-sterile soil, greenhouse	100	-	0	0	0	0
II	None 25°	100	120	96	100	60	-
	Air, laboratory	100	60	19	3	-	-
	Non-sterile soil, laboratory	100	70	9	3	-	-
	Air, greenhouse	100	100	85	35	-	-
	Non-sterile soil, greenhouse	100	67	3	0	-	-

^a Expressed as a percentage of the maximum patulin equivalents of the controls, obtained after 3 and 2 weeks respectively in experiments I and II.

Test organism: B. mycoides.

continuing. The rapid destruction by soil contamination in greenhouse conditions and the relative slowness under laboratory conditions, seen in the first experiment, was not observed in the second. It had at first been thought that the higher temperature in the greenhouse had caused the decline of potency to be more rapid, the difference being about 10^0 , especially during the day, but the failure to confirm this finding completely in the second experiment, where activity stayed high in the air-contaminated cultures kept in the greenhouse, makes this uncertain. There is, naturally, no guarantee that exposure to aerial contamination would always result in the same type of organisms growing in the cultures. Naturally, the type of microflora introduced, and the numbers, would be expected to influence the result, but it was only at the higher temperature that there was any indication that this did happen. In the second experiment the 4 day assays often showed some loss of potency in the contaminated soils, but it was not great, and this may be regarded as evidence that time is needed for the introduced organisms to multiply and produce their effect. It may be concluded that in soil and in the air there are organisms capable of destroying the antibiotic or of forming inhibitors of its action.

Experiment 3

This was a repetition of experiments 1 and 2, but instead of using a greenhouse to provide the warmer conditions incubation at 25° was employed. All the control flasks were kept at this temperature. Air temperature for half the contaminated flasks fluctuated around 15°. Unfortunately no more of soil no.1 was available and for this experiment soil 2 was used. The same precautions against evaporation as described in the second experiment were taken. For each test three replicates were used, and assays were started five days after contamination. The results are given in Table 25.

After 5 days the control was still at the original level of activity, but subsequently there was a slow fall and after 14 days comparatively little activity was left. Cultures exposed to the air and left at room temperature behaved differently, the activity declining a little after 5 days, but markedly in the next three days and then increasing again to a high level followed by another decrease. The cultures exposed to air and kept at 25° showed a similar pattern of changes but at a lower level as the decline after 5 days was greater, and the antibiotic content fell to zero after 14 days. This pattern of fall and rise and fall in activity was also observed in

Table 25.

Levels of antibacterial activity in sterilised soil containing an antibiotic substance and which was subsequently contaminated.

Source of contamination	Temp. (°C.)	Relative activity (%) ^a after exposure to contamination for				
		0 days	5 days	8 days	11 days	14 days
None	25	100	100	85	74	13
Air	^b 15	100	92	55	85	62
Non-sterile soil	^b 15	100	42	33	20	2
Air	25	100	37	12	29	0
Non-sterile soil	25	100	30	25	0	0

^a Expressed as a percentage of the optimum penicillin equivalent after 14 days incubation under sterile conditions, and by B. mycoides assay.

^b Mean value during the experiment.

experiment 1 and must have some significance as it occurred at approximately the same time in the two different soils. In the present case activity was even higher in the room temperature set than in the controls after 11 and 14 days, but no great significance can be attached to this because at 25^o, as in previous experiments, activity was lost through arial contamination before it disappeared in the controls. Soil contamination, whether at room temperature or at 25^o, caused a large reduction in activity in 5 days, and subsequently there was a further continuous decline in each case, the pattern of fall, rise and fall being absent. At 25^o no activity could be detected after 11 days, but at room temperature the decline was slower and some activity was still present even after 14 days. The more rapid destruction at 25^o confirms the observations on the influence of temperature in the previous experiments.

From these experiments it is clear that even gross contamination with soil did not lead to the immediate destruction of all activity.

Experiments using non-sterile soil

The work on steamed soil showed that antibiotic production was possible not only in pure cultures of P.patulum in soil but also in soils where other micro-organisms were present. As the ultimate object of this work is to achieve the control of plant diseases in the field, i.e. in non-sterile soil, many experiments were done with non-sterile soil, with or without supplements, as a substrate for antibiotic production by P.patulum.

Experimental and Results

Non-sterile and non-supplemented soil

Portions of 100 or 500 g. of soils 1 and 6 in Erlenmeyer flasks were inoculated with 10 ml. of a spore suspension of P.patulum and incubated for 14 days at 25°. As controls similar but uninoculated flasks were set up. Extracts from the 500 g. cultures were obtained by using a soil perfusion apparatus after Lees (1947). The percolate was assayed either untreated or after sterilization by sintered glass filtration, and in both cases half the fluid was concentrated by evaporation under reduced pressure at 35° to a quarter of its original volume. To obtain extracts from the 100 g. cultures, the soil was shaken on a mechanical shaker with 60 ml. of distilled water for

4 hr., left to settle overnight and then centrifuged.

At first soil no.1 was used, with cylinder plate assays and B.mycoides, B.subtilis, Bact.coli and Bact.carotovorum (type culture) as test organisms. Later soil no.6 was tested, using cup plate assays with B.mycoides, Sarcina lutea, Bact.coli and Bact.carotovorum (type culture).

Soil no.1: Inoculated: Unsterilized extracts. There was no difference between the results for the unconcentrated and concentrated extracts, and the following remarks apply in both cases, but the different test organisms varied in their response.

B.subtilis. With this organism no zones of inhibition were observed; occasionally zones of dense bacterial growth, irregular in outline, were recorded. The irregular shape of these zones, and the fact that under the microscope the bacterial colonies appeared unlike B.subtilis suggest the effect was due to contamination with other bacteria rather than to stimulation of B.subtilis. No attempt was made to identify these organisms. Frequently within these zones irregular transparent patches were visible, suggesting antagonistic action against B.subtilis by the contaminants present in the soil extract. It is believed from the position of the patches that the agent causing this

inhibition.was more likely to have been formed on the nutrient agar rather than within the cylinders. Occasionally, the shape of these clear areas suggested bacteriophage action.

B.mycoides. Zones of stimulation 15-17 mm.wide and of regular outline were formed as a rule but occasionally contamination as on the B.subtilis plates occurred.

Bact.coli. Zones of weak partial inhibition were formed varying from 11-13 mm. in width. Occasionally areas of overgrowth due to contamination with clear patches of inhibition as described above were formed.

Bact.carotovorum. (phytophthorum strain). No inhibition or stimulation was observed with this organism: occasionally bacterial overgrowth occurred.

Soil no.1: Inoculated: Sterilized extracts: There was in general no difference in the behaviour of the unconcentrated and concentrated extracts.

B.subtilis. No inhibition and no contamination.

B.mycoides. Stimulation well defined.

Bact.coli. Partial inhibition, zones slightly larger and better defined than with non-sterile extracts.

Bact.carotovorum.(phytophthorum strain). No inhibition.

Soil no.6: Inoculated: Sterilized and not sterilized extracts. The results were similar to those obtained with soil no.1, B.mycoides being stimulated and Bact.coli. slightly inhibited.

Bact.carotovorum. In these assays the type culture was preferred to the phytophthorum strain because it gave more clearly defined zones of inhibition. ~~unlike with extracts.~~ From soil no.1 zones of partial inhibition were obtained. There is sometimes greater variation within assays than within soils or strains. However, it was observed that soil no.6 gave a greater proportion of assays with zones of partial inhibition than did other soils.

Sarcina lutea. This was the most sensitive organism. Consistently, very narrow (11-13 mm) zones of almost complete inhibition with very well defined edges were formed.

Soils 1 and 6; Uninoculated controls. Sterilized and not sterilized extracts. These gave similar results to the inoculated sets, so under the conditions used, and with the extraction methods described, inoculation of the soil with P.patulum appeared to have led to no antibiotic production.

It may be concluded from these experiments that certain soils contain substances slightly inhibitory for some organisms, and stimulatory for others; certain organisms

such as the phytophthorum strain of Bact.carotovorum did not respond in either way.

Non-sterile but supplemented soil

The supplements consisted of glucose, and other carbohydrate materials: they were used untreated, and also autoclaved prior to incorporation with the soil.

Untreated supplements. Four soils were used, nos.1,2, 4 and 6, with supplements of glucose, sugar beet pulp and wheat straw, (2.5 to 4%), water being added to bring the moisture content to 100% of saturation after adding the supplements. The mixtures were then inoculated with P.patulum. Similar uninoculated mixtures served as controls. After 14 days incubation the mixtures were centrifuged and the fluids treated as described in the experiments on unsupplemented soil, the assay organisms being B.mycooides and Bact.carotovorum (type culture). The results revealed no difference between the controls and the inoculated soils. Bact.carotovorum showed no response or slight stimulation. Marked stimulation was observed on the B.mycooides plates; the zones were larger where supplements had been used and varied in size and opacity with the supplements. Usually extracts from soil plus 4% of sugar beet pulp gave the largest zones, 25-30 mm. in diameter.

It was observed in one experiment with soil 6 supplemented with 4% sugar beet pulp, that within the zones of stimulation of B.mycoides produced by an unsterilized extract patches of inhibition of irregular shape appeared (Figs. 28a-c); on further incubation these became larger and clearer. Filtration sterilized extract also gave these patches but they were very small and did not enlarge during further incubation. It was at first believed that bacteriophage action was the cause but if this were so one would have anticipated the same effect with the filtered extract.

Autoclaved supplements: The supplements, sugar beet pulp and wheat straw at 2.5 and 4% were autoclaved with sufficient water to bring the moisture content of the soil with which they were subsequently mixed to 100% of saturation. The mixtures were inoculated with P.patulum, uninoculated portions serving as controls. After 14 days 3 replicates were centrifuged, but in addition further replicates were extracted with ether, acetone and chloroform. In the latter case, 100 g. of soil was shaken with 50 ml. of either ether or acetone, the mixture filtered and the solvent distilled off. The residue was taken up in just enough distilled water to fill the assay cups (about 8 ml.). Subsequently each of these extracted soils was treated with chloroform in a similar manner.



Fig.28a.



Fig.28b.



Fig.28c.

Figs.28a,b,c. Progressive lysis of B. mycoides by an extract from non-sterile soil (No. 6) supplemented with 4% sugar beet pulp.

Fig.28a. After 18hr. incubation.

Fig.28b. After 36hr. incubation.

Fig.28c. After 54hr. incubation.

Assays were conducted with B.mycoides and the phytophthorum strain of Bact.carotovorum.

The centrifugates from these mixtures containing autoclaved supplements gave responses similar to those when untreated supplements were used. B.mycoides was stimulated but not Bact.carotovorum. The organic solvents, however, extracted material which while having no action on Bact.carotovorum caused partial inhibition of the growth of B.mycoides (Table 26). There were variations between the results of replicate assays, except in the case of the chloroform extracts from mixtures previously extracted with acetone. It is interesting that there were no zones of stimulation with the organic solvent extracts on B.mycoides plates.

A repetition of the experiment, using 4% autoclaved sugar beet pulp as the supplement but hot water for extraction gave results identical with those obtained with centrifugates.

Table 26 . The effect on the growth of B. myocides of various extracts, prepared after 14 days of incubation, from non-sterile soil supplemented with 4% autoclaved sugar beet pulp and inoculated with P. patulum.

Extraction method	Effect	
	Stimulation	Inhibition.
Ether	None	Weak partial in 35% of assays
Acetone	None	None
Ether-chloroform	None	Weak partial in 50% of assays
Acetone-chloroform	None	Weak partial in all assays

A comparison of the yield from cultures of *P.patulum* in soil and in soil-centrifugates.

Lochhead, Landerkin and Chase (1946) showed that the addition of soil extract to synthetic media considerably increased the antibiotic titre after one week's incubation in cultures of certain unidentified soil fungi producing patulin.

The following experiments were designed to investigate whether soil extracts by themselves were adequate for antibiotic production by *P.patulum* and other organisms. It was hoped that these experiments might also throw some light on the extent of adsorption by soil colloids of antibiotics produced in the soil. It is well known that antibiotics are readily adsorbed by charcoal, norite etc., and Gottlieb and Siminoff (1952) clearly demonstrated the adsorption of various antibiotics, including patulin, on the soil colloids.

Soil no.1 was supplemented with 2, 2.5 and 2.8% of wheat straw, autoclaved and centrifuged immediately. The centrifugates were resterilized by steaming for 15 min., inoculated with *P.patulum* and after 14 days incubation assays against *B.mycoides* were performed. In addition, soils similarly supplemented and autoclaved were inoculated with *P.patulum* at the same time as the extracts, centrifuged after

14 days and the fluids assayed on the same plate as the centrifugate cultures. The results are expressed in terms of the diameters of the zones of inhibition, as shown in Table 27 .

In each case greater antibacterial activity was obtained when P.patulum was grown in the centrifugates than directly in the soil. In the case of soil with 2% straw no antibiotic was present in the centrifugate while a fair sized zone of complete inhibition was obtained with the corresponding centrifugate culture. This is shown in Fig. 29 . It may be remarked that the threshold quantity of straw required for the demonstration of antibacterial activity is usually 2.5%: nevertheless centrifugate from soil containing 2% straw only supported good antibiotic production. It is possible that the antibiotic had actually been produced in the soil-2% straw mixture but in low yield, so that it had been entirely adsorbed by the soil colloids. Naturally other factors may have played a part, but the results do point to the possibility of partial adsorption even of acid antibiotics of the patulin type.

Similar experiments were carried out using unsupplemented autoclaved soil, but no antibiotic was formed, even in the centrifugate cultures. This supports the view that an additional carbohydrate is necessary even under the most

Table 27 Antibiotic production by *B. pasteurii* in straw supplemented autoclaved soil no. 1. and in centrifugate from similarly treated uninoculated soil

Substrate of culture	Amount of straw (% w/w)	Mean diameter ² of inhibiting zone (mm)	Type of Effect. ²
Straw supplemented soil, autoclaved.	2.0	0.0	Stimulation only (20 mm. zone)
	2.5	12.0	Stimulation and almost complete inhibition.
	2.8	17.0	Stimulation and complete inhibition.
Centrifugate from straw supplemented soil, autoclaved.	2.0	16.5	Stimulation and complete inhibition.
	2.5	17.5	Stimulation and complete inhibition.
	2.8	20.0	Stimulation and complete inhibition.

² Assays against *B. mycoides* after 14 days incubation in each case.

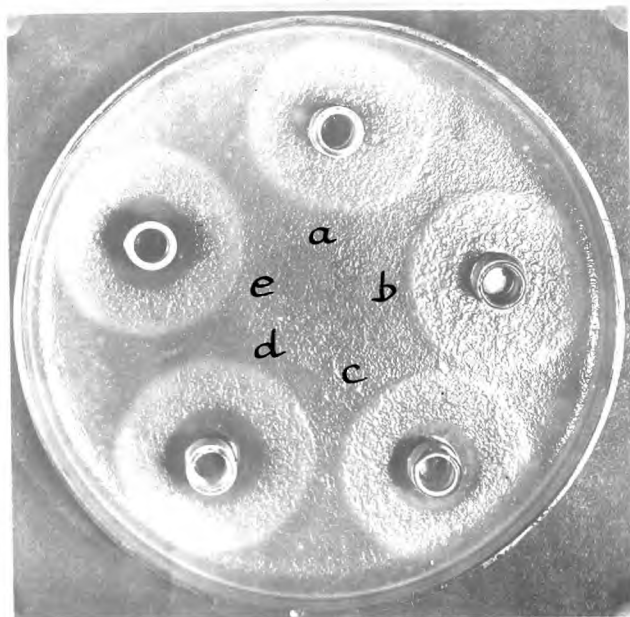


Fig. 29. A comparison of centrifugates prepared from 14-day cultures of P. patulum on soils supplemented with 2% straw, with cultures on centrifugates from a similar but uninoculated soil mixture. Test organism : B. mycoides.

- a - b Centrifugates from soil cultures.
- c - e Cultures on centrifugates.

ideal conditions of culture.

Antibiotic production in soil extracts by other micro-organisms

In previous experiments it was shown that while some micro-organisms produce antibiotics in autoclaved soil supplemented by carbohydrate sources, others apparently failed to do so. This failure was then attributed either to lack of certain essential, selective nutrients or to adsorption, and it was decided to test the ability of these organisms to form antibiotic in cultures in centrifugates from autoclaved supplemented soils where the adsorption factor does not operate to the full extent.

The soils from which the extracts were prepared were no.6 for actinomycetes and bacteria, as it is alkaline in reaction and no.2 for fungal work. They were either used unsupplemented or supplemented with 4% glucose, 4% glucose plus 4% dried blood, 4% sugar beet pulp, 4% wheat straw or 4% dried blood. The organisms used were:- F.javanicum and B.subtilis (the subtilin producing strain), S.antibioticus and two unidentified species of Streptomyces labelled nos.100 and 400.

The test organisms were B.mycoides, B.subtilis (a laboratory culture), Sarcina lutea and Bact.carotovorum.

As controls, uninoculated soil extracts were assayed. The results, referring to the *Streptomyces* cultures, are set out in Table 28 .

B.subtilis and F.javanicum gave negative results with all but one organism, namely Sarcina lutea, with which zones of complete inhibition 12-14 mm. in diameter were formed, but as this organism is frequently inhibited by extracts from untreated soil no great significance can be attached to this, especially since the zone size was the same no matter whether the supplement was sugar beet pulp or straw. It should, however, be mentioned that the degree of inhibition of Sarcina lutea was somewhat greater than with extracts from untreated soil.

S.antibioticus gave negative results throughout, but *Streptomyces* 100 in extracts from soil containing either dried blood or wheat straw clearly showed traces of antibiotic. Streptomyces 400 also did so on extracts from soil containing glucose plus dried blood, but the activity was only against Sarcina lutea. It is interesting that there was in so many cases no partial inhibition of Sarcina lutea and Bact.carotoverum by the *Streptomyces* culture fluids. Normally, any soil extract partially inhibits these bacteria, and the B.subtilis and F.javanicum cultures did show the effect with Sarcina lutea. The result suggests that possibly

Table 28. The antibacterial activity of Streptomyces cultures in extracts prepared from variously supplemented, autoclaved soils.

Supplement.	Effect of <u>Streptomyces</u> 100 and 400 against test organisms.							
	(1)		(2)		(3)		(4)	
	100	400	100	400	100	400	100	400
None	0	0	C [#]	S [#]	0	0	0	0
Dried blood.	C.14	0	P.13	0	0	0	0	0
Dried blood plus glucose	0	0	P [#]	P [#]	0	C.25	P [#]	P [#]
Glucose	0	0	P [#]	P [#]	0	P [#]	P [#]	P [#]
straw.	P.17	0	P.15	S [#]	C.12	0	0	0
Sugar beet pulp.	0	0	0	S [#]	0	0	0	0

Test organisms; (1) B. subtilis. (2) B. typhimurium.

(3) S. lutea. (4) Bact. carotovorum.

C = Complete inhibition } Figures refer to diameter of zone of
P = Partial inhibition } inhibition in mm.

0 = No inhibition.

S = Stimulation.

Results identical with controls, i.e. uninoculated soil extracts.

the *Streptomyces* species may have inactivated the factor responsible for the inhibitory effect, and which should have been present in the soil extract.

The results with the soil-wheat straw mixtures do not agree with the findings from cultures on wheat straw only. On this substrate *S. antibioticus* gave rise to an antibiotic in many cases, though in low yields, while *Streptomyces* 100 cultures invariably gave negative results. In soil mixtures the result is reversed, but *S. antibioticus* is known to be a very variable organism in respect of antibiotic production.

On the whole, the results of the *Streptomyces* and other cultures on soil centrifugates vary little from those grown in the original soil, and it appears therefore that the failure to detect an antibiotic in the soil was due not only to adsorption but to nutritional deficiencies as well.

Part IV

The survival of A. clavatus cultures when introduced into non-sterile soil, and the changes in their antibacterial activity.

The survival of *A. clavatus* cultures when introduced into non-sterile soil, and the changes in their antibacterial activity.

The failure to detect antibiotic formation in non-sterile supplemented soil inoculated with *P. patulum* may be ascribed to various causes. First, the antibiotic may not have been formed at all. Second, it might have been formed in such small quantities that it could not be detected by the methods employed. Third, the antibiotic may have been destroyed, e.g. by microbial enzymes, or inactivated by substances present in the soil.

On the first point, antibiotic may not have been formed because the organism introduced did not become established. Failure even of typical soil micro-organisms to become established in certain soils has frequently been observed; the case of nitrogen fixing bacteria may be cited. It seemed therefore desirable to discover whether a patulin-producing fungus could become established in a soil in which it was not normally present. For this *A. clavatus* was chosen in preference to *P. patulum*, as it is very easily identified by its unmistakable morphology, while the identification of *P. patulum* among the numerous *Penicillia* to be found in the soil would have ~~greatly~~ made the work difficult and laborious.

Further, it was considered important to find out how the indigenous microflora of a given soil, of which A. clavatus was not a member, would affect the antibiotic activities of that organism, and it was thought that possibly an increase in yield might result from passage through soil. It is well known that certain cultures of organisms which can form antibiotics lose this ability gradually and often completely on culture on synthetic media. Penicillium A, isolated by Grossbard in 1948, originally yielded cultures with a patulin equivalent of almost 60 mg. in a culture containing 10 g. of wheat straw, but after being cultivated on Czapek-Dox agar for 4 years it ceased to produce any antibiotic at all either in Czapek-Dox solution or on wheat straw cultures. Culture B of A. clavatus had also seriously degenerated since its isolation in 1945, having formed inactive mutants.

Human and plant pathogenic bacteria often lose that character on prolonged cultivation in the laboratory but recover it during ^{passage} ~~it during passage~~ through an animal or a plant. It was considered, therefore, that in an analagous manner passage through soil might improve antibiotic production by soil fungi. It may well be more than merely fortuitous that the great majority of antibiotic-producing organisms are normal soil inhabitants, and that the property

of forming these inhibitory substances is in some way connected with growth in this habitat.

Barthel (1918) showed that certain bacteria and yeasts preserved their characters well when grown in sterilized soil. Greene and Fred (1934) reported similar observations on fungi and nitrogen-fixing bacteria in which extensive comparisons were carried out with malt extract, potato-glucose, sterilized white bread, and a loam soil rich in organic matter. The loam proved the most satisfactory medium for the maintenance of stock cultures. Miller, Koch and Hildebrand (1947) and Miller (1949) have published similar results with phytopathogenic fungi. In many laboratories cultures of P. notatum and other antibiotic-producing fungi are preserved in soil as a matter of routine, and that ability is successfully maintained. However, all workers in this field used sterilized soil and were concerned with maintenance rather than with regeneration of the character of antibiotic-production.

Materials and Methods.

Soil.

Soil no. 2 from Goff's Oak was chosen. In one set of experiments enough sterile, distilled water was added to 40 g. of air-dried soil to bring the moisture content to

35 % of saturation. In another set the moisture holding capacity was adjusted to 100%.

Organisms.

Two cultures of A. clavatus were used, both from Roche Products Ltd. Culture A received in 1950, was very active in antibiotic production. Culture B, received in 1945, was originally very active but gradually degenerated, and when, in 1951, this experiment was performed its activity was lower than that of culture A. Both cultures were normally maintained on Czapek-Dox agar.

Inoculation procedure.

The moistened soils were inoculated with 1 ml. of a spore suspension of the fungus and allowed to stand on the laboratory bench without any further treatment. At intervals distilled water was added to replace loss by evaporation. Uninoculated controls were also set up. There were several replicates in each case.

Isolation procedure.

Four methods were used.

1.) Warcup's method (1930), with slight modifications. The end of a platinum needle was hammered down to form a miniature spatula. With this tool soil particles were transferred to melted Czapek-Dox agar in a petri-dish and well stirred.

- 2.) Small soil particles were placed on a slippe of Czapek-Dox agar in a 6 x 1" tube.
- 3.) A modified soil dilution method. With a sterile; no. 1 cork borer a column of soil was removed and the lower part put into 10 ml. of sterile, distilled water. The upper part, which contained soil from the top layers, was put separately into another tube of water. The mixtures were well agitated and then a loopful was mixed with 15 ml. of agar and poured. Frequently, when isolations were difficult, 2 ml. of water were used instead of 10ml..
- 4.) Brierley's standard soil dilution method for fungi (1923)

Incubation and examination.

The isolation plates were incubated at 25° for 4-6 days, when typical A. clavatus colonies, which ~~were~~ could easily be recognized with the naked eye were noted. Sub-cultures were made from these and purified by plating 3-5 times. Microscopic examinations were made to confirm the macroscopic observations. However, when dealing with typical colonies of the A. clavatus type culture the macroscopic features are almost sufficient in themselves.

Isolation and testing programme.

Two sets of isolates were made, the first after four months and the second after eight months in the soil. The

cultures isolated after four months were at once compared with the stock cultures for antibiotic-producing abilities. Sub-cultures of these re-isolated cultures were also maintained for another four months in Czapek-Dox agar alongside the original stock cultures, all being transferred monthly, and finally these two sets of organisms and the eight months isolates were tested simultaneously for production of antibiotic. In addition the cultures isolated after eight months were maintained for a further four months in Czapek-Dox agar with monthly transfers, and then retested for activity in comparison with the stock cultures.

The determination of ability to form an antibiotic.

The stock cultures, and cultures re-isolated after sojourn in soil, were tested for antibiotic production in Czapek-Dox solution at 25^o. A loopful of spores from a culture eight days old was inoculated into 40 ml. of Czapek-Dox solution in an Erlenmeyer flask. Assays of activity were made ^{at} weekly intervals by the cup-plate method against both B. mycoides and Bact. carotovorum, with four cups per plate, two of which received fluid from a culture of a soil isolate. The other two contained fluid from a culture of the stock strain. As standard pure patulin in pH 6.5 buffer solution was used, and the results expressed in terms of patulin (μ g/ml.).

RESULTS

The survival of *A. clavatus* in non-sterile soil

Experiments at a moisture content of 35% saturation .

To make sure that *A. clavatus* was not indigenous to the soil employed for this work a sample was examined by Warcup's method and the modified soil dilution method. Also, the uninoculated control soils mentioned above were similarly examined. Although numerous attempts were made, and the plates examined frequently during the period of incubation, which was often nearly a month, on no occasion was a colony of *A. clavatus* observed. It is thus virtually certain that the soil did not contain *A. clavatus*.

On the other hand, on isolation plates prepared by methods 1 and 2 four months later from the inoculated soils typical *A. clavatus* colonies soon appeared. With culture A, colonies appeared at the first attempt and the subsequent purification went smoothly, but with culture B three attempts with the first three methods had to be made before success was achieved (by Warcup's method). Purification, too, involved plating out 5-6 times.

Further attempts at isolation were made after a further period of four months, i.e. after eight months in all, during

which great fluctuations of temperature had occurred. These were deliberately permitted by incubating on the bench, since great temperature fluctuations occur in the field. The results were similar; no A.clavatus was found in the controls, culture A was re-isolated at the first attempt, and culture B was obtained at the second attempt with Warcup's method.

Experiments at a moisture content of 100% of saturation.

As before, no A.clavatus could be detected in the uninoculated soils, but in the wetter conditions attempts at isolation from the inoculated soils, using all four methods, were also negative except that one replicate out of four inoculated with culture A yielded a positive result. The isolations were repeated with the same results. The replicate which yielded A. clavatus at the first attempt did so again.

From these observations it may be concluded that survival of A. clavatus in non-sterile soil is largely governed by the water content.

The changes in the antibacterial activity of A. clavatus cultures A and B after sojourn in non-sterile soil.

The results of the antibacterial activity tests on the freshly isolated cultures are given in Table 29 and Figs. 30

Table 24.

The improvement in ability of A. clavatus to form antibiotic as the result of sojourn in non-sterile soil for 4 and 8 months.

Stock culture	No. of sub-culture	Patulin value ($\mu\text{g/ml.}$) after 14 days					
		Stock culture activity after (months.)			No. of iso-late	Soil isolate activity after (months)	
		0	4	8		4	8
A	1	120	120	134	1	520	329
	2	138	150*	220*	2	1150	295
	Mean	129	135	177		835	312
B	1	85	100	P	1	1100	331
	2	97	110	P	2	708	452
	Mean	91	105	-		904	392

Test organism : B. mycoides.

* Maintained on Czapek-Dox agar.

P $\equiv$ partial inhibition only, corresponding to a patulin equivalent of $< 30 \mu\text{g/ml.}$

* mutation to 'giganteus' strain.

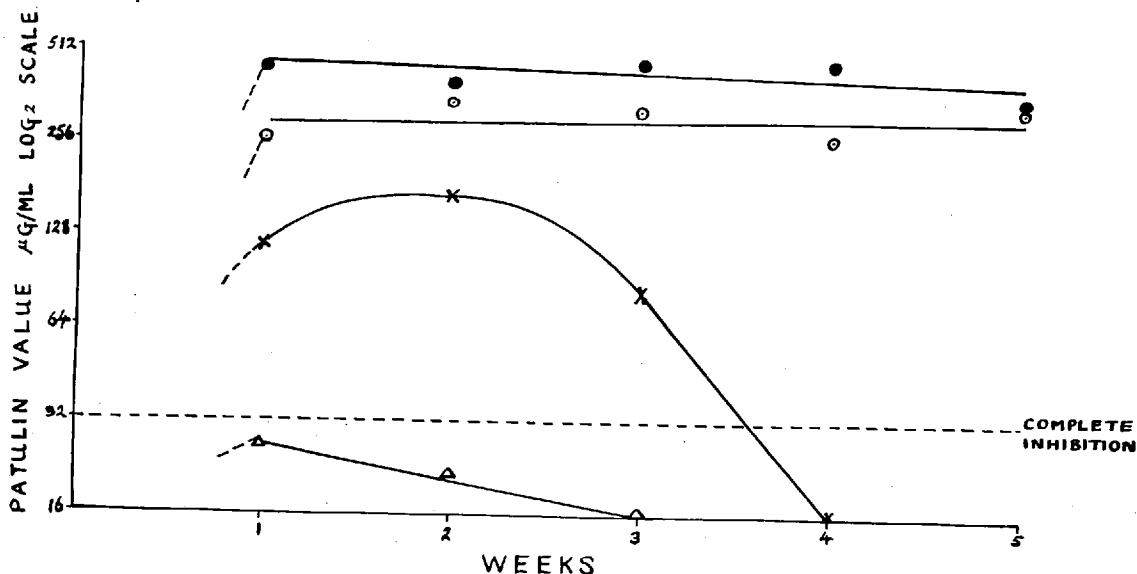


Fig.30 . A comparison of the antibacterial activity of cultures of *A. clavatus* sub-cultured on Czapek-Dox, with those re-isolated after sojourn in non-sterile soil for 8 months.

X — X	}	Active culture (A). Laboratory culture.
○ — ○		
Δ — Δ	}	Inactive culture (B). Laboratory culture.
● — ●		
		After sojourn in soil.

and 3/ and it is evident that sojourn in non-sterile soil caused the antibiotic potency of both cultures to be increased. Further, the mean level of activity of the isolates of culture B was as high, or even higher than that of the re-isolates of culture A, although at the start of this experiment stock culture A was more active than stock culture B. The improvement in potency was greatest in the four months isolates; after eight months in the soil the improvement was less marked.

The activity of the fluids of the cultures of the re-isolated organisms remained at a constant high level for five weeks, whereas the activity of the fluids of the stock cultures rapidly declined. This is shown in Fig.30, in which a comparison of the eight months isolates with the respective stock cultures is given. It is plotted from the means of the patulin values.

As shown in Table 29 the four and eight months isolates lost some of their ^cquired potency when sub-cultured on Czapek-Dox for a further four months, as judged by the 14 day assays, though they were still as a rule more active than the stock cultures, as illustrated in Fig.32. It may be remarked that the estimations of potency made after eight days were in three cases higher than those of the corresponding freshly isolated cultures (Table 30), and it is

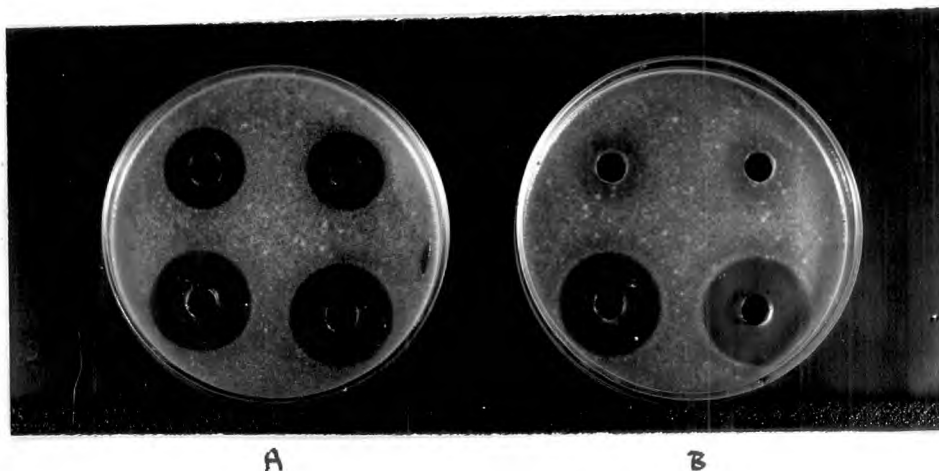


Fig. 31 . A comparison of the antibacterial activity of cultures of *A. clavatus* sub-cultured continuously on Czapek-Dox with those after sojourn in soil for eight months.

A & B upper cups, stock cultures A and B respectively.
A & B lower cups, cultures A and B respectively, after eight months in soil.

All cultures were grown on Czapek-Dox solution for fourteen days.

Table 30

The permanence of the improvement in antibacterial activity of *A. clavatus* induced by sojourn in non-sterile soil for 4 and 8 months, during subsequent sub-culture on Czapek-dox agar for 4 months.

Stock culture	Sojourn in soil (months)	No. of iso-late	At time of isolation				After four months sub-culture on czapek-dox.			
			Patulin values ($\mu\text{g/ml}$) after				Patulin values ($\mu\text{g/ml}$) after			
			8 days		14 days		8 days		14 days	
			Soil iso-late	Mean	Soil iso-late	Mean	Soil iso-late	Mean	Soil iso-late	Mean
A - high activ-ity.	4	1	500	400	520	835	251	456	394	550
	4	2	299		1150		661*		708*	
	8	1	407	309	329	312	280	190	260*	173
	8	2	211		295		100		85*	
B - low activ-ity.	4	1	430	399	1100	904	360	472	580	416
	4	2	367		708		583*		251*	
	8	1	350	473	331	392	123	528	279	284
	8	2	596		452		933*		288*	

Test organism : *B. mycoides*.

* mutating to 'giganteus' type in later stages of culture.

• mutating to 'giganteus' type at once.

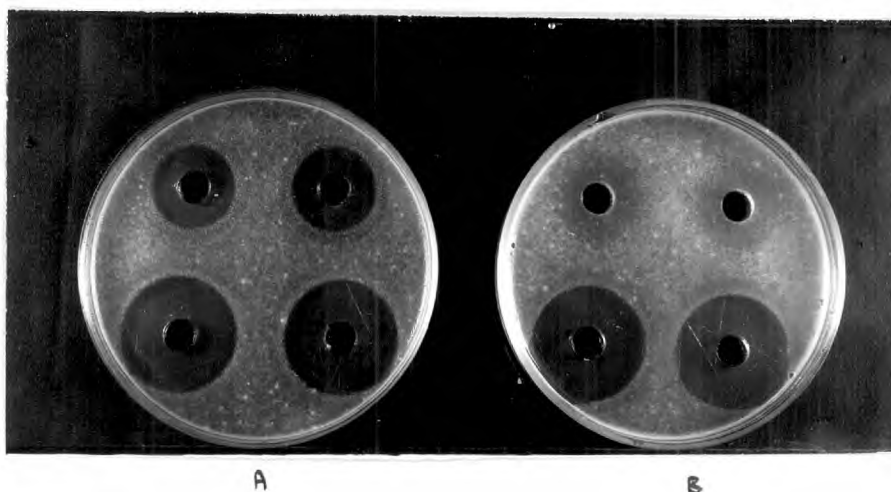


Fig. 32 . A comparison of the antibacterial activity of *A. clavatus* sub-cultured continuously on Czapek-Dox with those after sojourn in non-sterile soil for four months, followed by cultivation on Czapek-Dox for a further four months.

A & B upper cups, stock cultures A and B respectively.

A & B lower cups, cultures A and B respectively, after four months in soil followed by four months on Czapek-Dox.

All cultures were grown on Czapek-Dox solution for fourteen days.

interesting to note that these three cases were those in which the 'giganteus' strain (see below) appeared quickly. The most potent culture at eight days mutated at once.

A test of antibacterial activity was then carried out on the only A.clavatus isolate obtained from soil used in the saturated condition. It was found to be highly active. After 14 days culture in Czapek-Dox solution the patulin value was 540 $\mu\text{g/ml}$, while that of strain 1 separated out from the stock culture A, which was used for comparison, was only 187 $\mu\text{g/ml}$. After 21 days it was noted that the soil isolate began to mutate to the 'giganteus' strain, ~~while the culture of strain 1 remained unchanged.~~

Mutation in A.clavatus.

It was observed that during maintenance in the laboratory culture B had gradually deteriorated. Its morphological characters had altered and the antibiotic titre of its culture fluids had dropped markedly. For example, in 4 months the 'patulin value' had dropped from 105 $\mu\text{g/ml}$. to below 30 $\mu\text{g/ml}$. Another culture (A) maintained its production fairly well during the same period. It was decided to examine these two cultures and attempt to isolate pure line strains. Re-isolated strains after sojourn in soil were also studied.

Experimental

Two methods were used: a dilution method, and hyphal tip

transfer.

Dilution method. A spore suspension was prepared by pipetting 10 ml. of sterile water on to a slope culture on Czapek-Dox agar in a 6 x 1 in. tube and shaking. One loopful of this was transferred to another 10 ml. of sterile water and after shaking one loopful of this was mixed with melted Czapek-Dox agar which was then poured into a plate. After incubation at 25° for three to five days well separated colonies were obtained, from which fresh transfers were made for further purification by a similar procedure.

Hyphal tip transfer. It proved very difficult to separate out single strains showing uniform and stable characters, and it was often difficult to decide whether this was due to contamination e.g. from spores carried from neighbouring colonies. If there were doubts of the purity of a strain, hyphal tip transfers were carried out. After plating by the dilution method on plain agar hyphal tips were cut out from the very young colonies with a very fine glass tube which was bent over at the tip at about 100°, and the end well ground ~~by~~ on carborundum paper. The tube was used like a miniature cork borer and the agar disc containing the hyphal tip was transferred to a plate containing Czapek-Dox agar.

RESULTS.

Several separate strains could be distinguished in stock culture A as well as in stock culture B. The latter contained a group of strains so different from the others that it was at first believed that the cultures had become contaminated.

After thorough purification of the strains they were inoculated into Czapek-Dox solution and their antibacterial activities assayed against B. mycoides and Bact. carotovorum at weekly intervals.

Description of the deviations in morphological characters of various strains from the type culture.

Strain 1. This strain showed the closest agreement with the description given by Thom and Raper (1945). Colonies were ~~plane~~ with a surface mycelial mat and abundant, erect, smooth-walled conidiophores. The conidial heads were clavate, splitting when old into a column of compacted conidial chains. They were evenly distributed and blue-green in colour. The reverse was colourless at first but turned brown after six to eight days at room temperature and somewhat more rapidly at 25°. In this respect it varied from Thom and Raper's description, according to which the reverse turns brown only on ageing. The vesicles were shorter and

wider than those illustrated in Thom and Raper's (1945) monograph. In this ~~rare~~ strain ~~at~~ the ratio of length to breadth was smaller (see Fig. 33) but they were still typically clavate. The sterigmata were in single series and the conidia were smooth and elliptical.

Strains 2 and 3. In these, the colonies tended to be floccose and slightly raised, especially on agar, but frequently they were just like those of strain 1. Sectoring occurred, especially in strain 2, but when transfers were made from these sectors they reverted to the normal type. The sectors frequently contained vegetative mycelium but occasionally almost colourless conidial heads were also present. They were either dirty white or very pale buff.

Strain 4. This closely resembled 1 and was in one way even closer to the type, since the reverse of the colony remained colourless for a long time. At room temperature it remained colourless for two to three weeks or even longer after which there was a gradual change to light brown. At 25^o the change took place after twelve to fourteen days. Eventually the reverse assumed the deep brown colour characteristic of old colonies.

Formation of secondary mycelium and long conidiophores.

Strains 2, 3 and 4 had one important feature in common.



Fig. 33 .



Fig. 34 .

Figs. 33 and 34. Comparison of the vesicles of two strains of *A. clavatus*, derived from stock culture A.

Fig. 33. Strain 1, closest to type culture described by Thom and Raper (1945).

Fig. 34. 'giganteus' strain.

Small, raised, cushion-like areas of white floccose mycelium appeared, which later turned pale yellow or buff. The cushions were well defined and developed either within the sporing felt or just on top of it. In the latter case, it is possible that spores had germinated and formed a new mycelium which in turn produced a second crop of conidia, so that the cushion again assumed the typical appearance of an even blue-green to grey-green sporing felt. In flask and slope cultures, especially when exposed to light, tufts of conidiophores well over 1 cm. long developed in these areas of secondary mycelium (see Fig. 35). They were mostly sterile, but they were surrounded by a zone in which there were conidiophores of intermediate length bearing the typical vesicle of A. giganteus, which is a very long, narrow structure of a slightly lighter green colour (see Figs. 34).

Strain 5. This closely resembled A. giganteus as described by Thom and Raper (1945). It was readily isolated by ordinary mycelium transfer and remained true to type in subsequent sub-cultures. The mycelium was well submerged, especially in liquid cultures but even then an aerial, strongly floccose mycelial felt was formed, snow white at first but turning to light buff later. Fig. 36 shows a colony eight days old. The reverse was colourless, with a tinge of lemon-yellow but it turned a tan colour when old. Sporing, however,



Fig. 35. A liquid culture of strain No. 3, showing tufts of sterile conidiophores, well over 1 cm. in length.



Strain 5.

Fig. 36 An eight-day old colony showing white, aerial,
floccose mycelium.

was not rapid, as described by Thom and Raper(1945), It sometimes took up to fourteen/^{days}for the conidial heads to appear. They were arranged in zones alternating with vegetative areas. The conidiophores were very long and the vesicle long, narrow and light green (see Fig. 34); in fact, they were almost identical in appearance with those formed in the secondary mycelium by strains 2, 3 and 4. However, the tufts of very long conidiophores were never observed in strain 5, either on agar media or in solutions. Plate and flask cultures were exposed for two months to daylight and still there was no formation of these excessively long conidiophores, the length of which is the main criterion for distinguishing A. clavatus from A. giganteus, according to Thom and Raper (1945).

It is remarkable that these tufts of long conidiophores should have appeared in a culture which started off as typical A. clavatus, but that they should not be formed by a strain which was so clearly distinct from its parent culture and closely resembled A. giganteus in all other respects.

Stock culture B.

Strains 6 and 6a. Both these strains appeared to be abnormal. They resembled each other closely, differing only in the colour of the spores, but they were quite unlike the type

culture. The colonies were small, markedly raised and compact, with marked furrowing along radial lines. They remained well separated for a long time. The spores of strain 6 were sky-blue, later becoming buff. Those of strain 6a were buff coloured and did not change on ageing. The appearance of the pellicle in flask cultures was characteristic. Numerous small colonies floated on the liquid and usually after a period of growth they joined to form a crinkled, thick, closely knitted felt with a typically inwardly rolled edge like a dried leaf. The reverse turned dark brown in the first week. In general the macroscopic appearance was greatly reminiscent of Penicillium.

The hyphae were coarse and the septa exceptionally close and well defined. Frequently a bulge formed near the septum giving almost the appearance of a clamp connection. Occasionally the hyphae were distorted and excessively branched. The hyphal tips were rounded and not pointed as in normal strains. The conidiophores and vesicles were of two types but none was the typical structure. Diminutive forms of normal conidiophores and conidial heads were present, having typically clavate vesicles bearing sterigmata in a single series and very short spore chains, (Fig. 37), much as they are in a culture of the normal type in the first stages of development. As in this case they were found in cultures



Fig. 37.



Fig. 38.

Figs. 37 and 38 . Strains 6 and 6a. Abortive forms of conidiophore and conidial head.

Fig. 37 Diminutive form of conidiophore, also showing septum below conidial head.

Fig. 38 Successive conidiophore formation. This is a third conidiophore arising from the sterigma of a secondary one.

one to two months old, they must be regarded as aborted forms. The second type of conidiophore, occasionally arising from the same hyphae as the first type, was very long and coarse, frequently branched, and with a claw-shaped foot cell. At the tip radial branches arose (Fig. 37) each with a clavate apex. Usually they remained sterile but occasionally they bore a small conidiophore with a typical clavate apex with a few sterigmata and chiefly elliptical conidia. These secondary conidiophores were frequently septate (Fig. 39) as were also the small conidiophores of the first type. (Fig. 37). Sometimes a third conidiophore developed from the top of the second, and this in turn bore sterigmata and spore chains. (Fig. 38). Occasionally vesicles were found that either seemed to have split down the middle so as to form a heart-shaped structure, or they were individuals partly fused. These structures sometimes bore sterigmata and spores on each half (see Fig. 40).

It was first believed that this culture was a contaminant and not A. clavatus at all. However, in view of the fact that typical fruiting structures of A. clavatus, though greatly reduced in size, were also present, having arisen apparently from the same hypha as the abnormal structures, and ^{as} the latter occasionally bore clavate conidiophores, it is believed that a distinct mutant of A. clavatus had arisen. Yuill and Yuill (1939) described a most striking A. niger



Fig.39. Strains 6 and 6a. Abnormal branching (fasciation) of the apex of the conidiophore. Note the diminutive conidiophore with a clavate apex on top of the bulbous outgrowths.



Fig. 40 . Strains 6 and 6a showing two aborted conidial heads close to each other, derived either by splitting of the original vesicle or they are individuals partly fused.

variant which they named Cladosarum olivaceum. Instead of forming conidial chains, the secondary sterigmata produced "septate, branching outgrowths of irregular forms in which bulbous and flask-shaped elements are common." The appearance of these branching systems is not unlike that of strains 6 and 6a. Although Yuill and Yuill (1939) referred their organism to a new genus it is not proposed to adopt that course in the present instance. Abnormal formations, especially splitting of the vesicle, has been reported from New Zealand in strains of A. terreus (Dade, 1952), but similar malformations have not yet been reported in A. clavatus. Septate conidiophores are rare and have been observed only in the A. glaucus group (Thom and Raper, 1945). Claw-shaped foot cells, however, have been reported by Klein (1945) in A. clavatus; actually the A and B employed in this work were derived from Klein's cultures.

Strain 7. This strain was intermediate between no. 6 and the type strain. The colonies were exactly like those of strain 6 but the mycelium was small and not abnormally septate. The small conidiophores with normal though small vesicles found in no. 6 were present, but not the split vesicles and branched conidial heads. In addition larger conidiophores were observed with typical conidial heads but somewhat more globose, and in size intermediate between the type strain and the

former diminutive type. The conidia were always blue.

Strain 8. The colony mycelium resembled that of strain 6, but the reverse tended to remain colourless even when old and the pellicle did not become rolled in at the edge. Spore formation was very sparse and the conidia were grass green in colour. The conidiophores were exceptionally short, often ~~branching~~ septate and frequently branched. The system of branching was different from strains 6 and 6a. Short branches each bearing a minute but typical clavate vesicle, short distances apart, were carried at right angles to a modified hypha, a conidiophore like-structure. Sterigmata and spores were few. Sometimes the conidiophores were so short as to make it seem that the conidial heads arose directly from the conidiophore.

This closely resembled the type culture, and

Strain 9. This was isolated from a sub-culture of one used for the original inoculation of the non-sterile soil, and after several transfers it too changed to the gigantes strain after exposure to light, and formed tufts of the typical long but sterile conidiophores.

Antibacterial activities of the various strains isolated from stock cultures A and B.

Each strain was inoculated into three flasks of Czapek-

Dox solution and incubated in the dark at 25°. The fluids were assayed at intervals starting fourteen days after inoculation, the time at which optimum activity was usually obtained with the stock cultures. It was noted that only one of the replicates of strain 5 formed spores in the first week, while the others began to spore between the fourth and fifth weeks. The results are given in Table 3/. Since the three replicates agreed well in all cases except strains 5 and 9, in Table 3/ mean values are given except for these strains.

Results

The antibiotic production varied considerably, from less than 30 to 933 $\mu\text{g/ml}$. in different strains.

Activities of strains isolated from culture A.

As compared with the parent culture, most strains showed higher activity, and on this basis can be classified into three groups.

First, the normal stable strain 1, which formed 148 $\mu\text{g/ml}$ in fourteen days. Second, strains 2, 3 and 4, which mutated to the 'giganteus' type in the later stages of growth. All these were more active than strain 1 and had more or less equal powers of antibiotic production. Third, the giganteus

Table 31.

The relationship between antibiotic production and morphology in various strains of A. clavatus isolated from recent transfers of stock cultures A and B.

Stock culture	No. of strain	Patulin values ($\mu\text{g/ml}$) after (days)							Time (days) for the appearance of		Morphological characters. Shape and size of vesicles.
		14	21	28	35	42	49	56	mutation to 'giganteus' type	sterile conidiophores	
A	A	172	83	0	0	-	-	-	-	-	Normal, stable, closest to type.
	A ₁	148	150	P	0	-	-	-	-	-	
	A ₂	327	275	64	P	0	-	-	13	40	} Normal at first but later mutating to the 'giganteus' type.
	A ₃	251	288	211	P	0	-	-	20	40	
	A ₄	299	596	227	P	0	-	-	7	45	} Typical 'giganteus' type, sporing in 1st week.
	A ₅₁	933	252	80	0	0	-	-	-	-	
	11	35	106	300	80	70	70	70	-	-	} Typical 'giganteus' type. At first vegetative mycelium only. Sporing after 4th - 5th week.
	111	127	172	412	90	70	60	70	-	-	
B	B	P	0	0	-	-	-	-	-	-	Abnormal branching of vesicles, plus diminutive structures.
	B ₆	PW ₁	P ₁	P ₁	0	0	-	-	-	-	
	B ₇	75	P	0	0	-	-	-	-	54+	} Normal structures, intermediate in size. Diminutive structures but normal. Poor sporulation.
	B ₈	P ₁	P ₁	P ₁	0	-	-	-	-	54+	
	B ₉₁	100	90	P	0	-	-	-	-	-	} Normal, like strain 1.
	11	223	150	P	0	-	-	-	13	40	
	111	277	200	P	0	-	-	-	13	40	} Normal at first but later mutating to 'giganteus' type.

Test organism : B. mycoides.

P = partial inhibition only, corresponding to a patulin equivalent of $< 30 \mu\text{g/ml}$.

PW₁ = Partial inhibition only, but zones 22-25 mm. wide.

+ Sterile conidiophores only in one replicate.

strain 5, in which there was much variation between replicates, apparently associated with rapidity of sporulation. The replicates (i) which spored heavily in the first week had formed 933 μ g/ml by the end of the second week, the concentration subsequently falling rapidly. Replicates (ii) and (iii) formed mycelium only at first and the antibiotic titre was very low but when sporing began the titre increased considerably, and activity persisted longest.

The rapid decline of activity after an early high level of production as shown by culture A 5 (i) was also observed with a sub-culture from a soil isolate obtained ~~with~~ from a soil after eight months sojourn, originally inoculated with stock culture B. When isolated, the latter was ^atypical A. clavatus strain but after being sub-cultured for four months on Czapek-Dox agar it suddenly changed to the 'giganteus' form in liquid culture for assay, and after the first week this also had a patulin of 933 g/ml which fell to 283 μ g/ml after the second week (Table 30).

Activities of strains isolated from stock culture B.

Strains 6, 6a and 8 showed abnormal branching of conidiophores and numerous diminutive ~~ponidiophores~~ conidiophores carrying typical clavate but excessively small vesicles. Strain 8 formed fairly normal but very small conidiophores and vesicles.

All three strains produced mere traces of activity. Zones of partial inhibition 22 to 25 mm. wide were formed. This width is unusual. Patulin at concentrations below 30 g/ml gives rise to zones of partial inhibition but these are not larger than 11 to 15 mm. It is therefore possible that another antibiotic had been formed. Strain 7 formed conidiophores and vesicles intermediate in size and was intermediate in activity, with a patulin value of 61 μ g/ml. Strain 9 showed variation between replicates. The first (i) was similar to A 1. Replicates 9 (ii) and 9 (iii) were at first quite normal in appearance but changed to the 'giganteus' strain after thirteen days, the activity increasing a short time after the secondary mycelium of the 'giganteus' type appeared. Formation of tufts of sterile conidiophores more than 1 cm. long were also recorded. This strain had the largest conidiophores and vesicles, and yielded the highest titre of antibiotic of all the strains of stock culture B. These observations point to a possible correlation between shape and size of vesicle on one hand and activity on the other, and this view is further supported by the fact that of the strains of stock culture A the 'giganteus' strain 5 with the very large, narrow and excessively elongated vesicles produced the highest titre. Further, when the typical A. clavatus strains began to mutate to the 'giganteus' type their activity increased almost at once.

DISSEMINATION

SECTION III

DISCUSSION

Antibiotic production on autoclaved manures and in soil mixtures

Although Gottlieb and Siminoff (1950, 1952) have demonstrated antibiotic production in autoclaved, unsupplemented soil by S.griseus and S.venezuelae and Hessayon (1952) by Trichothecium roseum, the author did not succeed in so doing when using centrifugates from autoclaved unsupplemented soils inoculated with P.patulum. The addition of organic material was necessary for antibiotic formation to occur and it no doubt acted by supplying certain nutrients.

It has been clearly demonstrated that materials commonly used in agricultural practice for ploughing-in support antibiotic production by A.clavatus, A.terreus, P.patulum, S.antibioticus and Streptomyces species no.100. The fungi were better able to utilize plant materials for this purpose than the two actinomycetes. For instance P.patulum formed from wheat straw an antibiotic of an activity equal to that of Czapek-Dox cultures, while both species of Streptomyces seemed to prefer the standard laboratory media.

No evidence is available of the existence of a correlation between ability to decompose cellulose and form antibiotic. Norman (1931) has shown that the *Aspergilli* are most efficient in breaking down the hemicelluloses and cellulose in oat straw, but one species of an actinomycete which he tested also had this property. It is, however, interesting to note that the loss in the various straw constituents as a result of the growth of this actinomycete was smaller as compared with for example, *A. terreus*.

The best of the unreinforced substrates for antibiotic production by the three fungi was sugar beet pulp, which is not normally used as an organic manure in this country but it could well be so used in countries where it is cheap and plentiful. Of the common organic manures, wheat straw must be regarded as the best, although yields in 14-day cultures on timothy grass were frequently higher. However, wheat straw cultures maintained good activity for more than 20 weeks, and possibly total production was higher than on timothy grass.

The outstanding feature of the experiments with manures is the effect of glucose on the antibiotic titre of the fungal cultures. Invariably, the titre was higher on

manures supplemented with glucose. As regards P.patulum cultures on materials unsuitable for antibiotic formation by themselves, the addition of 3.5% of glucose not only induced antibiotic production but gave rise to considerable yields though these were variable. Materials which in the fresh state were adequate substrates, became unsuitable when composted, but became suitable once more when supplemented with glucose. All this points to the fact that available carbohydrate, presumably as an energy source, is of prime importance for antibiotic production, and that glucose is an efficient source. Lockhead, Chase and Landerkin (1946) have shown that, of all the carbon sources they tested, glucose was the most suitable for the production of patulin by unidentified species of Penicillium. The results obtained with composted materials point to the conclusion that the micro-organisms engaged in the decomposition depleted the supply of one nutrient only; sufficient of all the other major and minor nutrients (essential substances) was left, because after adding glucose activity was almost as high as on the fresh supplemented materials. However, the response to glucose additions was not the same with all plant materials. For instance, cultures on fresh, glucose supplemented leguminous plant material frequently showed lower activities than those on supplemented composted

straw or lawn mowings, indicating that the former were deficient in other nutrients required for maximum production, apart from carbohydrate.

The importance for antibiotic production of a correct nutritional balance in the substrate is also shown by the fact that yields on the same plant material often varied according to the stage of growth when they were harvested and the district in which they were grown. Samples of fescue harvested in the young stage failed to support antibiotic production while mature growth gave good yields.

The period for which activity was maintained in the cultures also seemed to depend on the nutritional condition of the substrate. In Czapek-Dox cultures of A. clavatus, for instance, activity was lost much more rapidly than in fresh, unsupplemented straw, while glucose supplemented composted straw, and especially supplemented fresh straw, maintained activity still longer.

In considering maintenance of titre two factors have to be distinguished. Continued production may occur with destruction. No evidence has been provided about the interaction of these factors, and it is not possible to say, when dealing with a constant level of activity whether at any point production had actually ceased and destruction had not

yet set in, or whether production was still rapid enough to offset the effect of destruction. Even when a decline in activity was commencing, production might still be going on but at a rate too low to counteract destruction completely.

In the glucose supplemented fresh wheat straw cultures of P.patulum, activity was maintained for 10 months, in spite of the fact that the antibiotic was continually diluted when fluid was removed for assay and replaced by water. Very probably the glucose had all been used up long before activity disappeared and it is therefore not likely that production continued throughout the whole period during which activity was present. It is possible that antibiotic was absorbed or adsorbed by the straw and slowly released into the diluted culture fluid, also it may have been that agents responsible for destruction were removed in the assay samples. That the period of retention of activity was shorter with the unsupplemented fresh straw and the glucose supplemented composted material as compared with the glucose supplemented fresh straw can be explained by assuming that the total production was lower in the composted material owing to a smaller supply of nutrients, other than carbohydrate,

and to an inadequate content of carbohydrate in the fresh straw. This prolonged retention of antibiotic activity, which occurred also in unsupplemented straw cultures may be of prime importance when wheat straw is used as a supplement in the soil, as it would cause the liberation of antibiotic slowly over a long period.

The importance of a correct nutritional balance in the substrate is also manifest from the behaviour of different classes of antibiotic forming organisms. Antibiotic production by the two actinomycetes was better on materials which did not, as a rule, support antibiotic formation by fungi, and vice versa. Leguminous substrates, such as sainfoin or lucerne, never supported antibiotic formation by the three fungi but for the actinomycetes they were the best. Since two species only of Streptomyces were used and three species of fungi, one is not justified in generalising about the whole groups of actinomycetes and fungi, but it is significant that Gottlieb and Siminoff (1932) have reported that while in soil amended with alfalfa hay (lucerne) chloromycetin production was increased, the addition of carbohydrate sources, ^{such as straw or starch} had no such effect. It is known that in ordinary cultures actinomycetes prefer a lower C/N ratio than fungi, and this seems to be true for

manurial substrates as well. Again, the two species of Streptomyces formed an antibiotic on young fescue growth but not on mature material, the latter being preferred by the fungi. This may indicate a preference for a higher content of protein by the actinomycetes. An excess of carbohydrate even seemed in certain cases to prevent the establishment of the actinomycetes, as in the failure to grow on sugar beet pulp, while sometimes growth was not possible even on glucose supplemented lucerne, although lucerne alone was the most favourable substrate, a result not reported in the experimental section.

Although the carbohydrate materials by themselves were inadequate for antibiotic production by the actinomycetes, it was hoped that when they were mixed with soil, other nutrients, especially nitrogen, might be supplied, thereby making them more suitable. This, however, was not the case. Many actinomycetes form basic antibiotics, which are entirely adsorbed on the soil colloids. Wakeman and Woodruff (1942) demonstrated the inactivation of actinomycin by the α -fraction of humus, and Gottlieb and Siminoff (1950) showed adsorption by certain clays. However, adsorption may not have been the only reason for the failure to detect

inhibitory agents in centrifugates from amended soil cultures of actinomycetes, because when sterile centrifugates from similarly supplemented soils were inoculated with three species of actinomycetes the results were also negative, although in this case the impact of the adsorption factor was at least decreased. It cannot, however, be said to have been non-existent, as colloidal clay particles may still have been present. There is thus the possibility that nutritional deficiencies were limiting production; a lucerne or fescue supplemented soil might have supported some antibiotic production. This view is supported by the reports of Gottlieb and Siminoff (1952) on an appreciable increase in chloromycetin production in soil amended with alfalfa hay (lucerne) but on the other hand Gregory et al. (1952) showed that an unidentified species of Streptomyces which they had isolated failed to give an antibiotic in soil mixtures containing alfalfa hay, but when the soil was mixed with the best liquid medium suitable for antibiotic production for this organism, great yields were obtained. On the other hand Skinner (1950) found that a species of Streptomyces exerted a marked antagonistic effect on F. culmorum in a poor medium which in its composition somewhat resembled soil, and he concludes from this that antibiotic production should be possible in so

poor a substrate as provided by soil. It is clear that there are differences in nutritional requirements relating to antibiotic production within species of the same genus.

As regards the fungi, it is evident that the six soils tested contained all the nutrients required for antibiotic production with the exception of an energy source. This could be provided by glucose, sugar beet pulp or wheat straw and other materials as well. The effect of glucose was especially marked in cultures of P.patulum in soil 1, where as little as 0.3% induced antibiotic formation. When a comparison was made between sugar beet pulp and wheat straw, taking glucose as a standard, it was seen that while glucose was the most efficient substrate and wheat straw the least efficient, when the latter was added in large amounts it was fairly effective, especially in view of the long period over which activity was maintained, as shown by the cultures on wheat straw by itself. In soil, especially non-sterile soil, glucose would disappear rapidly; therefore a carbon source which, though only giving low yields, provided a continuing action, might be more valuable. The ideal supplement would probably be straw supplemented with glucose and in fact Grossbard (1946, 1947) showed that this supplement

frequently decreased the spread in the soil of Ph. parasitica, a root pathogen sensitive to the antibiotic in centrifugates from supplemented soil cultures of A.clavatus and P.patulum. Sugar beet pulp, however, was better for this purpose (Grossbard, 1948) and it does contain a good supply of easily available carbohydrate with less available material.

In cultures of P.patulum in soil no.1 there was a threshold quantity of all three materials needed for detectable antibiotic production. This quantity naturally varied with the three supplements since they had different contents of available carbohydrate. Small additions of glucose and sugar beet pulp caused a remarkably big response, but a level was eventually reached above which no further production occurred. Presumably other nutrients then became limiting. The concentration at which maximum production was achieved also varied with the supplements: less was required, weight for weight, of glucose than of the other materials.

The experiments on antibiotic production by P.patulum in various soils have shown clearly that, provided a carbon source is supplied, soils of the very different types studied can support antibiotic production, although the yields varied somewhat from soil to soil.

In general , the variations were not very considerable, in spite of the great difference in soil type. The level of production, the response to increases in the amount of supplement and the period of time over which activity was maintained , all differed in the various soils. Interpretation of the results is somewhat difficult owing to variations between replicates experiments on the same soil sample, but in general it can be said that soil 3 was the least suitable and soil 1 the best, followed by soils 5 and 6, with nos.2 and 4 intermediate.

The soils differed from one another in so many respects that no single factor can be distinguished as that which alone determines the level of antibiotic yield. The chemical composition will be of ~~primary~~ paramount importance as regards nutrients as well as the presence of indigenous toxic substances .For instance the high yields in soil no.6 might be related to some extent to the possible presence of toxins therein , because extracts from this soil, ^{even} ~~even~~ when untreated generally inhibited the growth of Bact.sarotoverum and Sarcina lutea more than those from the other soils , and centrifugates from autoclaved soils no.6 supplemented with glucose

but not inoculated gave rise to almost triangular zones of good partial inhibition (see Figs. 22 and 23). It is therefore possible that these naturally occurring toxins showed an inhibitory effect in addition to the true antibiotics and thus account for the high total activity recorded.

The variation in nutrients other than carbohydrate in the six soils is also apparent because of the differences in response to increases in the supply of carbohydrate.. While soil 3 showed, in 14 day cultures, a decrease in activity when the glucose supplement was raised from 2.5 to 4%, all the other soils either gave increases or no change in activity. Raising the amount of sugar beet pulp always led to higher titres but the actual yields varied with the soil type. Most marked were the differences with wheat straw: soil 3 never formed antibiotic, even at 5%, and with the others the threshold quantities varied. However, at 5% even the very different soils 1 and 6 behaved similarly.

The C/N ratio of the soil, and the ratio after supplementing and autoclaving must undoubtedly influence the results. The total carbon contents of some of the soils were estimated. No correlation between this and suitability for antibiotic production could be discerned, but since the total carbon content does not give any information about the nature of the

carbon compounds present and their availability to the fungus for antibiotic production, a correlation need not exist.

As regards the nitrogen content, the experiments on wheat straw cultures of P.patulum revealed that no matter what source of nitrogen was used to supplement the straw, the antibiotic yield was always smaller. It is therefore possible that in a soil with a high nitrogen content production would be relatively low. The low yields in soil 3 may be explainable on this basis, since it came from a pasture containing a large proportion of clover.

The reaction of the original soils did not seem to be related to yield. The low pH soil 1 gave high yields but so did the nearly neutral soil 5 and the alkaline soil 6, while soil 3, with a high pH, gave very low yields. The alteration in pH due to autoclaving, supplementing and growth of P.patulum again showed no relation to activity.

On the whole the pH drift was very slight due to the buffering effect of the soil. The only drift of any major significance was observed in soil 1 when a gradual increase in the glucose supplement eventually resulted in a pH drop of almost one unit, accompanied by a rise in activity. However, such increases in potency, though at a lower level,

were also observed with sugar beet pulp and wheat straw amendments, and these were not accompanied by drastic drifts in pH. It is therefore safe to conclude that the greater response of the test organisms with increase in supplement was really due to an increase in amount of antibiotic brought about by the larger supply of energy yielding material and not to an increase in the potency of the same amount of antibiotic due to a higher hydrogen ion concentration. This point is important because certain workers explain the effect of organic manuring on disease control in terms of alteration of the soil pH. As regards maintenance of activity, some correlation with acidity is here apparent since activity was maintained longer in acid soils. This is in line with the findings of Jefferys (1950) who, working with several antibiotics, including patulin, showed that destruction of the antibiotic was considerably slowed down in soils with a low pH.

No attempt was made to identify the antibiotics formed by the active organisms. It is highly probable that patulin was formed in the cultures of A.clavatus and P.patulum, because the fluids had anti-bacterial and anti-fungal spectra similar to those of pure patulin. In addition, in work (not

reported in this thesis) on the phytotoxicity of soil centrifugates to dicotyledonous plants it was noted that the symptoms of the phytocidal action closely resembled those caused by patulin. There is, however, a suggestion that in addition to patulin a second antibiotic was produced in cultures of P.patulum on amended soil no.1, because of the "two stage effect" observed. Brian (1951) has shown that P.patulum can also form griseofulvin, but no attempt was made to see whether the "two stage effect" was due to griseofulvin production.

It is evident that A.terreus formed another antibiotic than patulin on manures and in the soil because only Gram-positive organisms were sensitive to it: patulin had probably also been produced in the presence of glucose or on sugar beet pulp. It appears that for patulin production by A.terreus even larger quantities of glucose are required. Nothing is known about the substances formed by the unidentified Penicillium species A and B. While A behaved similarly to P.patulum species B did not even form an antibiotic on some substrates, and in no case were Gram-negative organisms sensitive; thus a substance other than patulin was certainly formed by this organism. It is interesting to note that even the patulin producing fungi

utilized the carbon sources somewhat differently. This is not surprising when it is remembered that variations occurred when the same fungus and the same soil mixtures were used. The antibiotic formed by S. antibioticus may have been actinomycin.

The relative importance of microbial competition and nutritional effects for antibiotic production in mixed cultures

When the micro-organisms were grown on autoclaved substrates with a proper nutritional balance, conditions for antibiotic production were optimal because of absence of microbial competition coupled with the maximum availability of nutrients resulting from autoclaving. These studies, however, were designed to lead up, if possible, to a method of inducing antibiotic formation in non-sterile soil. In this there is no special release of nutrients, only that due to microbial activities and this is probably never comparable with that due to autoclaving; also, there is the full impact of a diverse, actively growing competing microflora. Another object was the encouragement of antibiotic formation in greenhouse soils where partial sterilization is a routine measure, and a moderate release of nutrients occurs due to steaming while a reduced microbial population mainly composed of spore

formers which have survived the steaming is left to compete.

The evidence provided in this thesis shows clearly that both microbial competition and reduction in available nutrients operate to give lower antibiotic yields in cultures of P.patulum on either manures or in soil mixtures which are not autoclaved. It is not yet possible to estimate the relative importance of these limiting factors or how their interaction finally determines the result.

That competition does interfere with initial production as well as with final yields is shown by the lower yields from steamed wheat straw as compared with the autoclaved substrate; or in steamed as compared with autoclaved soil mixtures containing glucose or sugar beet pulp; or in cultures on autoclaved wheat straw which were allowed to become contaminated by exposure to air; also by the inability to detect an antibiotic in non-sterile soil amended with autoclaved manures.

In the steamed substrates and in non-sterile soil competition was not the only factor since the release of nutrients provided by autoclaving was lacking. In the case of the culture on autoclaved wheat straw which was allowed to become contaminated, the composition of the invading micro-flora determined the initiation of antibiotic production.

Of the three replicate cultures of P.patulum only one showed normal production, and it was in this that P.patulum became well established. The others appeared to be overrun by Trochoderma species mainly. On steamed straw there was great variability in the results within replicates in one experiment as well as within repeated experiments. It is possible that the extent of the release of nutrients varied in different steamings, but it is also feasible that the surviving organisms differed and were to some extent responsible for the varying yields. Cultures of P.patulum on non-sterile sugar beet pulp frequently showed activity almost as high as on the autoclaved material, but there was variation and this was undoubtedly due to the flora which, in addition to P.patulum, became established and actively growing during the incubation period. Once maximum production was reached, usually after one week, the titre dropped rapidly, very likely through the activities of the competing flora. However, members of the competing flora may also have formed an antibiotic, because in uninoculated non-sterile sugar beet pulp a weak antibiotic was detected. Its titre varied greatly within replicates and the Gram-negative Bact.carotovorum was sometimes not sensitive, indicating formation of different antibiotics in different experiments.

An antibiotic substance was also produced on non-sterile timothy grass. The greater deficit in yield as compared with the sugar beet pulp was no doubt due to the fact that timothy does not contain as much available carbohydrate as sugar beet pulp. On wheat straw, however, no antibiotic was formed. Although the nutritional factor must not be overlooked it could be argued that perhaps the flora on wheat straw differs from that on the other manures and that with this P.patulum was not a good competitor. In this connection it should be stressed that sterility was rarely achieved in propylene oxide treated straw and it was observed that occasionally P.patulum did not become established.

That microbial competition also interferes with maintenance of activity was clearly shown by the fall in activity in ^{non-sterile} sugar beet pulp cultures after one week. This point is further illustrated by the experiments on the contamination of soil cultures of P.patulum in which antibiotic had already been formed. It is significant that destruction was much more rapid at high temperatures, at which microbial activity is more intense. Further, contamination through mixing with non-sterile soil resulted in a more rapid loss of activity as compared with aerial contamination. This must not be interpreted merely in terms of a difference in

composition of the invading flora, though this must certainly have played a part. Aerial contamination must have been relatively light and also brought about mainly by resting cells or spores which would need a lag period before becoming active, and more time still would be needed before they could multiply to the level present in the soil inoculated cultures, which received organisms in the active condition. This expected lag period is in accordance with the observation that after four or five days exposure destruction was small in the air infected cultures. It is interesting to note that in two experiments these cultures showed a rise in activity, indicating continuing production; or possibly toxic substances responsible for destruction had been removed through the process of aeration brought about when the soils were transferred from the culture flasks into open vessels. Neilson-Jones (1941), for instance, has shown that air drying destroyed certain antifungal toxins. It is also conceivable that organisms introduced from the air were able, after becoming established in the soil, to form a second and more active antibiotic. This idea receives support from the fact that the rise in activity was noted only after 10 days. However, the centrifugates at this point behaved biologically very much

like those prepared at the time of exposure of the cultures, and this is rather against the above suggestion.

Destruction of antibiotics in non-sterile soil has been shown by Nandi (1948), Jefferys (1950), Sinha (1950), Nimkar (1950), Gottlieb and Siminoff (1952) and Monib (1958). It must not, however, be overlooked that disappearance of activity also occurs in pure cultures on laboratory media and in pure cultures in soil, and also that purely chemical inactivation by SH-groups had been recorded by Geiger and Conn (1945) and Cavallito and Haskell (1945).

The mechanism by which microbial competition interferes with initial production, yields and eventual maintenance of titre is based on three factors, acting either singly or together. First, an antibiotic organism introduced into a mixed culture to which it was not indigenous may not be allowed to become established. Second, the organism may become established but antibiotic production is inhibited, perhaps by inability to obtain the nutrients necessary for antibiotic formation in sufficient quantity: this may explain the inability to detect an antibiotic in non-sterile soil, in spite of the fact that most active strains of antibiotic organisms have been isolated from soil and must presumably have been able to grow therein. This failure to detect an

antibiotic in non-sterile soil may perhaps have been due to the use of relatively insensitive techniques rather than to complete absence of production, especially in view of the fact that Gregory et al (1952) have demonstrated antibiotic production by P.patulum in non-sterile soil using the soil solution displacement method. Furthermore, the fact that non-sterile timothy grass and sugar beet pulp supported a high titre of antibiotic in the presence of a mixed flora rather emphasises the likelihood that antibiotics could be formed in non-sterile soil in the vicinity of energy yielding food particles, as suggested by Brian et al. (1951). Third, competition could act through enzymic destruction: this has been demonstrated by Krebs (1944) in relation to patulin, using animal tissues.

While the importance of microbial competition must be stressed there is some indication that the nutritional factor may have an even greater influence. The best illustration of this is the formation of a highly active antibiotic on non-sterile wheat straw supplemented with glucose: yet, the unsupplemented non-sterile wheat straw never showed production and even when steamed, yields were mostly small.

Even in propylene oxide treated straw production was poor and irregular but again competition comes into play here. No doubt in the presence of glucose or an easily available carbon source the organism became rapidly established and thereby virtually sterilized the substrate against other organisms.

The high yields in P.patulum cultures on non-sterile sugar beet pulp emphasises further the paramount importance of the available nutrients and this example would suggest that it overrides microbial competition. Already, work on propylene oxide treated sugar beet pulp has shown clearly that even in the unheated state this material contains all the nutrients required for antibiotic production, but here a competing flora was as a rule absent. It is therefore even more remarkable that such high yields should have been recorded in the presence of other micro-organisms. Production was, in some experiments, even higher than on steamed sugar beet pulp where there may have been a further release of nutrients through the action of heat. This may have been due to random variations, as the differences in either case were not very considerable. Very high yields comparable with those on autoclaved sugar beet pulp have, however, been recorded in repeated experiments and this

fact therefore deserves consideration. It may be that the members of the competing flora were able to break down some of the complex polysaccharides and make them available for P.patulum. Alternatively, it is possible that the mere exposure of P.patulum to competition, and it appears to be a good competitor, resulted in an improvement in its ability to form antibiotic: this property might be used as a defence mechanism. A similar suggestion was made when an improvement in the antibiotic properties of cultures of A.clavatus were observed after a sojourn in non-sterile soil.

The influence of nutrients other than carbohydrate is well illustrated by the work on propylene oxide sterilized soil-glucose mixtures, in which the glucose concentration was approximately the same as in the autoclaved substrate. In fact, it could be argued that it was higher, because autoclaving is generally thought to make glucose less available for the growth of micro-organisms. Frequently, in the preparation of glucose containing media, intermittent sterilization by steaming is carried out. Whether autoclaving glucose makes it less suitable for antibiotic formation is not known, but it is not considered likely that the effect would be large enough

to account for the difference in yields in autoclaved as compared with propylene oxide sterilized substrates. It is true that autoclaving liberates other carbonaceous materials, but 2.5% glucose is such a large concentration that whatever may have come from the soil would not have exerted a very great influence, especially as an increase in the supply of glucose from 2.5 to 4% did not give better activity in some soils. However, in other soils such an increase in glucose did induce a higher yield of antibiotic and this may be due to a greater amount of other nutrients than carbohydrate facilitating the utilization of the larger supply of glucose.

It is probable that nitrogenous substances were made available and that these were primarily responsible for the differences in the level of production observed. This would also explain the lower yields on steamed than in autoclaved soil. Steaming would liberate less of other nutrients than autoclaving but here again interpretation is complicated by the fact that a competing flora was operating. When dealing with steamed sugar beet pulp mixtures a lower availability of carbon is far more important and the deficit in activity is here clearly the result of a combination of a smaller release of carbon as well as of other essential nutrients.

Wheat straw soil mixtures, when steamed, failed to yield antibiotic on soils 1 and 4. Considering that the steaming instead of autoclaving of soil-glucose mixtures led to a difference in production almost as great as the total production on autoclaved wheat straw mixtures, it is hardly surprising that no activity was formed on steamed wheat straw mixtures, especially in view of the fact that cultures of P. patulum on steamed wheat straw alone gave poor results. Again the factor of competition must be considered, but as the addition of glucose to non-sterile straw gave rise to high yields of antibiotic competition appears to be of secondary importance.

A correlation between the production of antibiotics in the soil and the control of plant diseases by organic manuring

That certain root diseases are controlled by the incorporation of organic manures has been shown by many workers. It is not yet clear whether the mechanism of this control is based solely on the nutrient-induced formation of antibiotics active against the pathogens. It is not claimed that the work in this thesis provides direct evidence that this is the case. It is, however, suggested that the experimental evidence provided here points to the likelihood that by incorporating with the soil materials which by

themselves are good substrates for antibiotic production by soil organisms, local concentrations of antibiotics may be formed in the vicinity of such particles of manures, and although microbial competition will eventually destroy such inhibitory agents, even during the period of diminishing activity they may exert some influence on sensitive pathogens. It is significant that damping-off of tomato seedlings caused by Ph.parasitica can be controlled in soils supplemented with sugar beet pulp and inoculated with P.patulum (Grossbard, 1948), and that this material is one of the few which even in the non-sterile state support antibiotic production. Further, the extracts from cultures of P.patulum on soil supplemented with sugar beet pulp completely inhibited the growth of this pathogen in-vitro, even in a dilution of 1/80.

It is also significant that a parallel exists between the requirements essential for antibiotic production by the fungi tested and the conditions necessary for the control of root diseases other than 'damping-off'. The fact that some plant materials were suitable, while others, for example, certain samples of red clover or rye grass, were unsuitable for antibiotic production, fits in with the results of West and Hildebrand (1941) and Rouatt and Atkinson (1950), that while soybeans were effective, red clover and rye grass were

ineffective in controlling certain plant diseases. Further, abundant carbohydrate must be present if an antibiotic is to be produced by the tested fungi. This can be correlated with the findings of Nandi (1948) who succeeded in demonstrating, in non-sterile soil reinforced with glucose, the presence of material with marked antibacterial properties, and with the evidence in the literature (Garrett, 1938; West and Hildebrand, 1941; Grossbard, 1946, 1948, ~~1949~~; Blair, 1943; and Rouxatt and Atkinson, 1950) that soil supplements high in carbohydrate are effective in the control of some root diseases. These effective materials are also low in nitrogen, and it has been shown that raising the nitrogen level reduces antibiotic yields by P.patulum. Thus the results on the controlling effect of carbohydrate manuring on certain soil-borne diseases should not be entirely dissociated from the possibility that this treatment resulted not only in a modification of the composition of the micro-flora but also in the formation of local concentrations of antibiotics, which may exert an inhibitive action on plant pathogens in the neighbourhood of the roots of host plants.

It is, however, of paramount importance to choose the an adequate plant material: the mere incorporation of

'organic matter' does not suffice. Pathogens sensitive to antibiotics formed by actinomycetes will require materials of a different nutritional balance than will those which respond more to fungal antibiotics. In addition, it seems important to introduce with the suitable material a high inoculum of organisms (one or several) which are likely to form an antibiotic to which the pathogen is sensitive and to follow on with such cultural management of the soil, apart from the mere manurial treatment, as will encourage not only the growth of the introduced organism but also the formation of antibiotics. The determination of these conditions will, however, require further studies.

The presence of inhibitory and stimulatory substances in the soil

It was not within the original scope of this thesis to investigate whether untreated soil contains antibiotics. However, evidence was obtained of the presence of inhibitory substances in certain untreated soils. It was observed that their potency varied with different soils, being greatest in soil 6. It is not clear whether these toxins were of microbial origin, i.e. antibiotics, or of chemical origin. Their selective action was apparent, as only Bact.coli, Bact. carotovorum and Sarcina lutea were inhibited, the latter being

the most sensitive organism. These substances might affect the survival of antagonists introduced into the soil to encourage antibiotic formation or act in suppressing pathogens to some extent.

The presence of inhibitory substances in the soil has been observed and reported by many workers, who also recorded that frequently such materials were thermostable, a point which has again been confirmed. Extracts from untreated but autoclaved soil also exerted a similar inhibitory effect on the sensitive test organisms. The addition of glucose seemed to enhance this action. Greig-Smith (1912-17) and Newman and Norman (1943) have reported this already. In this connection it is interesting to note that Sykes et al. (1951) observed that in assays of penicillin and streptomycin the zone diameter increased when certain sugars such as glucose, sucrose, or lactose were added to the solution to be assayed. They were unable to explain this phenomenon. Centrifugates from uninoculated, autoclaved soils containing glucose gave very large zones of good partial inhibition of Bact. carotovorum, and extracts from certain soils similarly treated even slightly inhibited B. mycoides. When dealing with glucose supplemented soil cultures of P. patulum, zones of complete inhibition were formed which were quite distinct in appearance from those formed from extracts from uninoculated glucose

supplemented soil. They were clearly the result of growth inhibition due to antibiotic production and not to glucose introduced toxins. Moreover, the centrifugates were prepared only after 14 days or even 20-28 days growth, and by that time little if any residual glucose should still have been present, although on the other hand toxins formed before the complete exhaustion of glucose might have persisted and exerted an inhibitory effect additional to that of the antibiotic formed by P.patulum.

The selective character of these substances is well illustrated by the fact that B.mycoides reacts entirely differently from for instance B.subtilis or Bact.carotovorum, although all three are primarily soil organisms. B.mycoides was distinctly stimulated by extracts from all the six soils used in these experiments, while B.subtilis was unaffected and Bact.carotovorum was frequently slightly inhibited. Even centrifugates which contained antibiotic also showed growth stimulation outside the zone of inhibition. It is suggestive that most soils contain substances stimulating the growth of B.mycoides. The work of Lochhead and Chase (1943) has shown that some constituents of soil extracts have growth promoting properties for certain organisms. Taylor (1951) stated that the growth promoting properties of soil extract

are due in part to complex nitrogenous substances and in part to calcium, magnesium and strontium ions, while Lochhead and Thexton (1952) provided evidence that a large proportion of the indigenous bacteria depend upon vitamin B₁₂ which can replace soil extract. Furthermore, these authors have demonstrated that various soil bacteria have the ability to synthesise a growth promoting factor similar in action to vitamin B₁₂. It is possible that an analogy could be drawn from these findings to explain the selective growth stimulating action of soil extract on B.mycoides. However, a nutritional factor seems to play a part as well, as the zones of stimulation are markedly increased by autoclaving the soil. When the soil was supplemented the zone diameter was increased further but in this case Bact.coli and Bact.carotovorum were also stimulated, though only very slightly. It could, however, be argued that this stimulation, especially from unsupplemented soil, might have been a manifestation of the presence of antibiotics, or toxins of chemical origin.

That antibiotics give rise to zones of stimulation at the periphery of inhibition zones is well known. Antibiotics seem to have this property in common with other toxins which at low concentrations act as stimulants. It could be argued that the toxins in the soil that inhibited Bact.coli, Bact. carotovorum and S.lutea would have also inhibited B.mycoides were they present in sufficient concentration and that the

difference between B.mycoides and the other test organisms lay merely in the threshold level, i.e. that at which the toxins cease to be inhibitive or lethal and become a stimulant.

The survival of *A. clavatus* in non-sterile soil.

One of the reasons for the failure to detect an antibiotic in non-sterile soil was thought to be the inability of an antibiotic-producing micro-organism to become established at a sufficiently high level when introduced into non-sterile soil.

Experiments have shown that *A. clavatus* could survive in non-sterile soil, though the moisture content was critical, as the fungus survived in soil of a moisture content of 35 % saturation but not in a saturated one. That germination and growth might also have taken place is suggested by the physiological changes the fungus seemed to have undergone in the soil, whereby it became able to form larger amounts of antibiotic and to maintain the titre longer. Counts of survivors were not made because the figures would not give a true picture. Certain identification of *A. clavatus* is only possible when sporing begins but the spores formed would be liable to disperse and yield further colonies. The poor survival in saturated soil suggests that in changing the water content the microbial equilibrium was modified, and that organisms indigenous to the soil and antagonistic to *A. clavatus* multiplied sufficiently to prevent its establishment.

Changes in the antibacterial activity of *A. clavatus* as
a result of sojourn in non-sterile soil.

The improvement in antibiotic potency resulting from passage through non-sterile soil may be explained in various ways, but supporting evidence is as yet lacking.

First, it may be expostulated that certain nutritional stimuli were present in ^{the} soil, perhaps of the nature of growth substances, which enhanced antibiotic production. Lochhead, Chase and Landerkin (1946) demonstrated an increase in antibiotic titre in the first week of growth of unidentified Penicillium species when soil extract was added to a synthetic medium. It is, however, not possible to explain why in this case the increased productivity should be a permanent character, unless a genetical change had taken place. Second, it might be supposed that selection of active mutants was favoured by soil conditions, because the fungus had to survive for a considerable period in contact with the mixed population of micro-organisms present in non-sterile soil. There is some speculation and argument on the question whether antibiotics are a factor in the mechanism of the "survival of the fittest". The increase in antibiotic activity of *A. clavatus* cultures as a result of sojourn in soil would suggest that, in order

to survive, the cultures, hitherto cultivated by themselves, had to further develop their mechanism of defence in order to fight the competition of the indigenous microbial flora; it seems that this was facilitated by dry conditions in the soil. It is interesting to note that after eight months in the soil, activity had decreased again. It could be argued that the longer period had enabled the population of A. clavatus to increase to such an extent that its survival was safeguarded by virtue of numbers rather than by the use of a specific defence apparatus. Alternatively it might be supposed that it was merely by chance that all four isolates after eight months were less active than those obtained after four months. However, the significance of the results may not be great because of the small scale of the experiments, the considerable variations between the soil isolates from each stock culture and to the very active gigantes strain which occurred both in the laboratory stock culture and the sub-cultures of the soil isolates.

Third, it should not be ruled out that sojourn in soil induced a specific kind of mutation and in order to test this possibility experiments are in progress to investigate the effect of soil conditions on the pure line strains isolated above. It is, however, realised that even in such an experiment a further selection of active mutants present in

the pure line strain in a dormant condition may occur and obscure the results.

Fourth, the possibility has to be considered that when the original stock cultures were inoculated into the soil they were not the pure strains they were believed to be. There is no doubt that at the time of inoculation large scale mutation to the 'giganteus' type had not yet taken place. The morphological characters of that type are so ~~marked~~ striking that it could hardly have been overlooked. This also applied to the mutations to strains 6a and 8, but it would be unwise to rule out the possibility that the inoculum contained a small proportion of other strains. On the other hand, the soil isolates were pure line strains, at least when freshly isolated. The question now arises whether the strains isolated from the soil were already present in the inoculum and whether they had remained unmodified, their higher activity due merely to the fact that they were now in pure culture. It is true that some of the strains separated from the stock culture were more active than the latter. The potencies of cultures of cultures of strain 5, as compared with stock culture A, and strain 9, as compared with B, illustrate this point. Further, strain 4 (after three weeks) and strain 5 (after two weeks) were even more active than any of the eight months

soil isolates. There is, however, no evidence that the soil isolates were identical with either of these strains. When first obtained, the soil isolates showed only structures typical of A. clavatus and came closest to strain 1, whose activity was considerably below that of even the weakest eight months soil isolate. Also, the soil isolates differed from the above strains notably in the greater rate and profusion of sporing but also and most strikingly in the ability to maintain activity at a constant level for a long period. All cultures of the separated strains, on the other hand, declined in activity after two to three weeks. Against this it can be said that sub-cultures of the soil isolates on Czapek-Dox agar eventually mutated to the 'giganteus' strain, even occasionally forming tufts of excessively long, sterile conidiophores, and that there exists a correlation between the degree of activity and the time at which this mutation set in. This is clearly shown in one of the eight months soil isolates from culture B which was sub-cultured for four months on Czapek-Dox agar. When grown in liquid culture for assay of activity it at once changed over to the 'giganteus' type forming the typical floccose white mycelium and yielding a patulin value of 933 μ g/ml after two weeks with a decline in the third week to 255 μ g/ml, thus behaving similarly to the quick-sporing replicate of strain 5. On the other hand

the changes to the 'giganteus' type which occurred in the sub-cultures of the soil isolates did not always lead to an increase of activity as it did in the laboratory strains (Table 30). It is likely that the changes to the 'giganteus' type were a result of sub-culturing on Czapek-Dox agar and have been brought about by similar factors that induced strains 2, 3, 4 and 9 to mutate in that direction.

The evidence submitted therefore suggests that the increase in potency of the fresh soil isolates was due to the formation of physiological variants, and that while culture conditions on Czapek-Dox favour the poorer ~~nurtured~~ mutants of the stock cultures, soil conditions select the more potent ones. This suggestion is supported by the fact that the improvement in potency of the soil isolates was lost to some extent on subsequent sub-culturing.

Mutations in A. clavatus.

It is believed that the mutations observed in stock cultures A and B took place to a large extent after the start of the soil experiment and that the proportion of mutants relative to the original type had increased rapidly in the last four months. The changes led to a greatly ~~an~~ decreased activity in culture B while activity of culture A was little affected. The latest assays of culture B

showed merely traces of activity, although it undoubtedly contained strain 9 which, when cultured on its own, showed considerable potency. This strain was separated only with great difficulty. It also had a low viability and frequently did not grow when transferred to fresh Czapek-Dox agar. Supposing it was this strain that was re-isolated from the soil, the low viability would explain the difficulties isolating it from the soil after four months, and it could be said that after eight months its population had increased so that isolation was facilitated. It must be admitted that while the soil isolates were morphologically like the normal strains of culture B, they were unlike it in antibiotic production. The strains separated from culture A had a markedly increased potency but here a complication in interpretation arises from their change to the 'giganteus' strain within seven to twenty days. It appears that the time of occurrence of this mutation affected activity as shown by strain 4 while strain 5, when it bred true to the 'giganteus' type from the start, showed after two weeks a patulin value of 933 μ g/ml, which is almost as high as that of the most active (four months) soil isolates.

Strains 2, 3, 4 and 9 were difficult to maintain; they continued to mutate; old cultures of strain 9 formed 6, 6a

and 8 as variants, while the latter strains did not revert to the typical form. This was also the case with strain 5. Strain 1, on the whole though not invariably, bred true. Strain 9 not only mutated to the inactive form but also to the 'giganteus' type, even forming tufts of excessively long, sterile conidiophores. Strain ~~no~~ 5 was never found in culture B, nor the inactive forms 6, 6a, 7 and 8. It seems that the main difference between the ~~inactive forms~~ Czapek-Dox cultures, A and B, four months and especially eight months after the start of this experiment, was ~~based~~ due to the proportion of the typical strain to the others.

Another point of interest is the suggestion of a possible correlation between shape and size of the vesicle on the one hand and activity on the other. It appears that, shape being constant, the size affected antibiotic activity. The small vesicles of strain 8 were identical in shape with those of strains 7 and 9, but the sizes were different, strain 8 being smallest and strain 9 largest; The activities varied in the same order. Potency seems also to be correlated to shape, size being approximately equal. Strains 1 and 5 both had very large vesicles but the shapes differed and it seems that the longer, narrower vesicle of the 'giganteus' type is associated with enhanced activity. However, it may ~~be~~ actually be that surface area was the important

factor, but no measurements were made and the point awaits clarification. It could be argued that the greater the surface area of the vesicle the greater the number of sterigmata it can carry, and the greater the number of spores that could be formed. In strain 5 there was a suggestion of a correlation between intensity of spore formation and antibiotic activity. However, in the soil isolates the rate and density of spore formation was not correlated with the shape of the vesicles as the latter were characteristic of the type culture.

Philpot (1943) claimed that A. giganteus formed a penicillin-like antibiotic which she named gigantic acid. Florey, Jennings and Philpot (1944) showed that A. giganteus produced patulin. It appears therefore that this species gives rise to two antibiotics, and it is not impossible that one is formed by the vegetative mycelium and the other by the fertile mycelium. The difference in activities of the three replicates of cultures of strain 5 could be explained on this basis. On the other hand, it may also be that the peak production of antibiotic depends on changes in the metabolism associated with sporing and that low initial yields in the late sporing replicates were caused by absence of these conditions, especially as a great increase in activity was noted as soon as sporing began.

There is some disagreement whether A. giganteus should be regarded as a separate species. Thom and Raper (1945) consider it as such and their chief criterion is the conidiophore length. They include under the species name A. giganteus Wehmer all strains which produce conidiophores in excess of 1 cm. in length. According to this definition there is no doubt that the secondary spore crop in strains ~~xxx~~ 2 to 4 was due to A. giganteus and it was thought at first that it was present as a contaminant. However, numerous transfers of the original stock culture were made and always this form appeared in old cultures. Moreover, the sub-cultures of the soil isolates showed the same phenomenon, irrespective of whether they derived from stock culture A or B.

Blochwitz (1929) regarded A. giganteus as a mutation of A. clavatus and the evidence submitted here tends to support his view. Thom and Raper (1945) stated "that while it is true that conditions can be altered so that A. giganteus cultures suggest A. clavatus, no one has yet demonstrated that the reverse can be accomplished." It is believed that the above observations represent such a demonstration of the reverse. It may thus be more accurate to call strains 2 to 5 A. clavatus var. giganteus.

SUMMARY

The production of antibiotic substances by A.clavatus, A.terreus, P.patulum, S.antibioticus and Streptomyces 100 when grown on organic manures has been demonstrated.

Supplements of glucose to the plant materials increased the titre of the fungal but not of the actinomycete cultures. The relative importance of microbial competition and supply of nutrients as factors influencing antibiotic yield was investigated, using unheated and partly sterilized manures.

Antibiotic production in autoclaved supplemented soil by the fungi A.clavatus, A.terreus, P.patulum and two unidentified species of Penicillium was demonstrated; soil cultures of the actinomycetes failed to yield an extractable antibiotic. An investigation of the importance of nutrition, of microbial competition and the factor of release of nutrients by heat was carried out. It was clearly established that P.patulum could form an antibiotic in a partially sterilized soil both with and without release of nutrients by heat, provided a source of readily available carbon was supplied. It is thus clear that the soil as such contains all the nutrients required for antibiotic production by P.patulum except an adequate supply of energy providing material. The level of antibiotic activity was lowered

when soil cultures of P.patulum were contaminated with other organisms derived from soil or from the air, but even with gross contamination with natural soil at 25° activity did not entirely disappear until 11 days had elapsed. Loss of activity was smaller when the temperature was lower. Although it was clearly shown that microbial competition adversely influences level of production and maintenance of activity it is believed that lack of adequate nutrients is still of greater importance in this respect. The type of soil used influenced level of production and maintenance of activity to some extent. No correlation could be established between soil reaction and yield but activity was maintained longer in soils with a low pH.

It was shown that A.clavatus could survive in non-sterile soil, the moisture content being a limiting factor. Sojourn in soil improved the antibacterial activities of that organism. Mutants occurring in the stock cultures were described; they included an abnormal, abortive form. There was a suggestion of a correlation between the morphological characters of the various mutants and their antibiotic activity.

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