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Report for 1958

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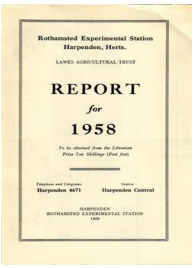

Soil Microbiology Department

P. S. Nutman

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SOIL MICROBIOLOGY DEPARTMENT

P. S. NUTMAN

R. Cooper went in June to spend a year at the Timiryazev Academy, Moscow. Miss G. Lim arrived on 1 September from the University of Malaya, Singapore, to study the influence of soil micro-organisms on the infection of legumes by nodule bacteria. R. M. Jackson has been awarded the Ph.D. degree of London University.

Dr. R. J. Swaby of the Division of Soils, Commonwealth Scientific and Industrial Research Organization, Adelaide, Australia, spent two months in the department working on the red pigment formed in the leaves of certain ineffectively nodulated plants of subterranean clover. The pigment was shown to occur in small quantity in effectively nodulated plants. Chemically it differs from that of the seed testa and from a wide range of naturally occurring anthocyanins and anthocyanidins; it most closely resembles pelargonin. Mr. H. Fritsche of the Berlin Technical University, W. Berlin, helped N. Walker in the synthesis of halogenated aromatic compounds used in his work. The following visitors came for shorter periods: Mr. A. R. Azmi of the Atomic Energy Commission, Karachi, Pakistan; Mr. H. H. Louw of the University of Stellenbosch, South Africa.

Work on the biochemistry of the decomposition of the halogenated phenyoxyacetic acid herbicides, on the survival of bacteriophage of *Rhizobium* in artificial soil mixtures and on certain aspects of rhizosphere microbiology was ended. New lines of work started were on the microbial decomposition of pentachlorophenol, the detergent "Teepol", synthetic oestrogens added to soil, on the changes taking place in the microflora of the gut of three species of earthworm, on the influence of very small additions of nitrates to the earliest stages of nodule formation in clover and on genetic recombination in *Rhizobium trifolii*.

The anaerobic fermentation of cellulose

An anaerobic cellulose-digesting bacterium has now been isolated from soil in pure culture. Its cells are slender, Gram negative rods, 0·3–0·7 μ wide and 2–8 μ long, with terminal spherical spores about 1 μ in diameter. The organism is motile at some stages of growth, and produces acid and gas from several sugars, of which cellobiose is most readily used. Two types of colonies form on solid media; some small and compact, others diffuse and spreading. In deep culture in agar media, the spreading type of growth is the more active, especially in gas production. Spreading growth is suppressed by a low concentration of lithium chloride. Colony type changes readily and reversibly; it has not been possible to separate the two forms.

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Studies on the decomposition of cellulose have been made in a mineral salt solution buffered at pH 7·0 in sealed nitrogen-flushed bottles containing finely divided cellulose, yeast extract and a reducing agent such as cysteine or sodium thioglycollate. At 35° C. bacterial attack on the cellulose particles becomes apparent about a week after inoculation, when coarse aggregates form from the cellulose flocculi which cohere to give a viscid mass. The bacteria are closely associated with the cellulose, so that the supernatant fluid at first remains clear, but becomes turbid when later the cells become motile. Digestion takes several weeks to complete, during which the pH falls from 7·0 to about 5·0, because formic, acetic and malic acids are formed. Hydrogen and carbon dioxide are also produced, but not methane.

Of the reducing sugar present in old cultures, glucose is probably the major constituent, but concentrates of culture filtrates have not yet been sufficiently purified to establish this beyond doubt; only trace quantities of cellobiose are present. *Clostridium cellobioparum* Hungate, a rumen organism, differs in this respect in producing cellobiose but no glucose in cellulose fermentation. However, as the Rothamsted isolate is very similar in other ways to *C. cellobioparum*, it is regarded provisionally as a strain of this species. (Skinner.)

The decomposition of anionic surface active agents by soil bacteria

A bacterium capable of using both primary and secondary alkyl sulphates as carbon sources has been isolated from local soil. The organism is a species of *Pseudomonas* as yet unidentified. In mineral salt solution containing 0·1 ml./l. of " Teepol 530 " (a secondary alkyl sulphate) growth is rapid and the surface tension of the medium rises from about 36 to 68 dynes/cm. in 48 hours. Ability of the liquid to foam on shaking is lost during this period. The organism also grows well in similar mineral medium containing 2.0 ml./l. " Teepol " (corresponding to *c.* 600 p.p.m. of the active constituent). Primary alkyl sulphates with alkyl radicals containing from ten to eighteen carbon atoms are also utilized. A number of detergents of the alkylolamide type also serve as carbon sources for this bacterium, but not tetrapropylene benzene sulphonate, an active constituent of some commercial detergent powders. Indeed, though many soil micro-organisms tolerated this persistent compound, none was found that could use it. (Skinner.)

The microbial decomposition of pentachlorophenol and di-ethyl-stilboestrol

Pentachlorophenol, which is used to proof fabrics against rots, is very resistant to attack by soil micro-organisms; no appreciable loss has so far occurred in enrichment culture maintained for 12 months. Pentachlorophenoxyacetic acid has been prepared, and its stability in soil is also being examined. Di-ethyl-stilboestrol, a synthetic oestrogen administered to cattle and poultry to promote live-weight increase, disappears rather slowly in incubated suspension of soil, presumably by microbial activity, though no isolates have so far been obtained which can use these substances in pure culture. (Walker.)

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The influence of 2 : 4-D on the bacterial flora of Rothamsted allotment soil

The effect of various treatments with 2 : 4-dichlorophenoxyacetic acid on numbers and kinds of organisms occurring in Rothamsted allotment soil has been followed using the viable plate count to follow general changes occurring in the bacterial population and a dilution counting technique to follow changes in the micro-organisms able to break down 2 : 4-D. In the dilution count 2 : 4-D is added to each dilution in small amounts, and after incubation the cress seedling test is used to determine the end point of the disappearance of 2 : 4-D. A small dose of 2 : 4-D (50 p.p.m.) only slightly affects the total count and the number of organisms able to decompose 2 : 4-D, but larger doses (500 p.p.m.) considerably increase the latter. Earlier work with bacteria isolated from soil showed that the breakdown of 2 : 4-D is due to adaptive enzyme formation (*Rep. Rothamst. exp. Sta. for 1955*, 66), and again the adaptation of organisms in soil to breakdown of 2 : 4-D was induced by doses of 2 : 4-D (0·2 p.p.m.), far too small to cause detectable population changes.

Several new organisms able to decompose 2 : 4-D were isolated and classified into three main species: *Bacterium*, *Achromobacter* and *Pseudomonas*. (Steenson.)

The effect of earthworms on soil micro-organisms

Counting methods have been developed for yeasts, fungi, actinomycetes and various groups of bacteria occurring in the intestines of three species of earthworms: *Lumbricus terrestris*, *Allolobophora longa*, *A. caliginosa*. Results show that the numbers of bacteria and actinomycetes in the gut reach densities about 1,000× greater than in the surrounding soil, with maximum numbers occurring in the hind gut. The numbers of yeasts and fungi do not increase significantly. Preliminary investigations on the breakdown of cellulose and chitin in the intestine of *L. terrestris* indicate that enzymes produced by the worm rather than bacteria play a major role in these processes.

Counts of yeasts, fungi, actinomycetes and bacteria have been made on casts freshly produced by *A. longa*. Changes in the number and respiratory activity of micro-organisms have also been followed using casts aged under field conditions. Plate counts show that the numbers of yeasts and fungi in the casts increase rapidly in the first few days, and then remain fairly constant for a considerable time. Numbers of actinomycetes and bacteria do not change significantly. The rate of oxygen uptake by cast material is initially high and declines very slowly; it is still considerably higher than that of soil after 50 days. Total nitrogen in fresh casts is high, usually 5–6 mg./g. dry weight of cast. In freshly voided casts ammonia forms 90–98 per cent of the mineral nitrogen and 4–6 per cent of the total nitrogen. The rate of nitrification of this ammonia is being followed. (Parle.)

Nitrification

Further improvements have been made in the method of growing *Nitrosomonas*, notably by omitting minor elements in the medium

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(Cu is found to be markedly inhibitory at 0·2 p.p.m.), and by determining the temperature and pH range for growth. Cell densities of about 2×10^8/ml. have now been achieved in 5-day cultures and sufficient cell material obtained for a preliminary spectroscopic examination. This has revealed high levels of substances with cytochrome absorption spectra. A continuous culture apparatus is now being installed for this work. Fresh strains of both *Nitrosomonas* and *Nitrobacter* are being isolated. (Walker and Meiklejohn.)

Nitrogen fixation by Azotobacter and Clostridium

Counts of *Azotobacter* on selected plots on Broadbalk were continued. Unless the full examination of the data shows any points on which further information is needed, it is not proposed to continue this study. The results obtained agree in the main with those of former years (*Rep. Rothamst. exp. Sta. for 1956*, 73 and *1957*, 76).

A method for counting nitrogen-fixing anaerobes in soil, adapted from that used by Hart, seems to give valid results, but it is very laborious. The few counts made confirmed earlier results, which showed that nitrogen-fixing anaerobes are more numerous than Azotobacter in the Broadbalk soils. (Meiklejohn.)

Nitrogen fixation by other free-living soil bacteria

Nitrogen-fixing bacteria other than *Azotobacter* and *Clostridium* are frequently found in appreciable numbers on plates of nitrogen-poor media and may play a significant part in the nitrogen cycle of the soil. Some strains were isolated which showed appreciable nitrogen fixation and a bacterium, probably *Aerobacter* sp., was selected for study. Nitrogen fixation by this strain is unaffected by ammonium salts and nitrates, but inhibited by amino acids. When soil, but no other combined nitrogen source is added to the mineral medium, nitrogen fixation is unimpaired, provided a substrate such as glucose is present; with no carbon supply no growth or fixation occurs. A start was also made on investigating the nitrogen-fixing power of these bacteria and of *Azotobacter* in mixed culture, and in the presence of the plant root. So far the results have shown that fixation in mixed culture is sometimes better. Most of the bacterial strains examined can fix as much nitrogen under conditions of reduced oxygen tension as with full aeration. (M. E. Brown.)

Soil fungistasis

Attempts were made to obtain fungistatic extracts from soil with dilute acids and bases, with solutions of salts such as sodium and potassium pyrophosphate, with sodium acetate, citrate and oxalate, and with organic solvents. Inhibitory activity was found with several extractants, but sterilization by passage through Ford Sterimats, sintered glass filters or by exposure to ultra-violet always removed the activity. The inhibition before sterilization is caused by bacteria which are active during the subsequent germination tests and can be eliminated by adding aureomycin to the assay agar.

Bicarbonate is being considered as a possible source of soil

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fungistasis, and a study is being made of the effect of bicarbonate on the germination of the conidia of *Penicillium citrinum*. Concentrations as low as 0·005 per cent of potassium bicarbonate depress germination, but complete inhibition is not obtained until the concentration is increased to 0·5 per cent. Suitable methods for detecting and estimating bicarbonate diffusing into agar in contact with soil are being developed.

An examination of the redox potentials on the surface of agar discs incubated over soil revealed no significant differences from the potentials occurring on control agar discs which support germination. Over the range normally favouring germination, temperature does not influence soil fungistasis significantly.

Following the observation that seedling roots may stimulate germination of inhibited fungal spores in soil, exudates from germinating seeds and seedling roots have been examined for their effect on soil fungistasis and for the presence of substances likely to be responsible for stimulation. Exudates from peas 24 and 48 hours after germination stimulate germination of *Gliocladium roseum* conidia in the presence of soil and contain glucose, fructose and sucrose. Concentrated root exudates from older pea seedlings growing either in nutrient salt solutions or distilled water neither counteract soil fungistasis nor contain detectable quantities of sugars. Preliminary work on the factors influencing chlamydospore formation in a strain of *Fusarium solani*, an unidentified *Fusarium* and *Trichoderma viride* showed that they are formed by *Fusarium solani* much more often in dark than in light, but with the order two light has little effect. (Jackson.)

Phage-induced changes in Rhizobium

The partially effective strain obtained by bacteriophage, from the ineffective strain Coryn, when tested on seeds of late Montgomeryshire red clover gives rise to mixed responses with the tested plants, as previously reported. Numerous further isolations were made from single nodules on either effectively or ineffectively responding plants. These again failed to give a uniform response in symbiosis so that the source of this variation is being sought elsewhere. A test was first made of the effectiveness of a Coryn phage mutant upon fifteen selected families of red clover of very diverse origin obtained from P. S. Nutman. With five of these families a mixed response was obtained, as with commercial red clover. With seven families the response was wholly ineffective, and with three it was uniformly effective, showing that the source of variation lay in the host. Plant material for the full genetic analysis of this variation is being prepared. Work on formal bacterial genetics in *Rhizobium trifolii* has been started using standard methods. Initially this will concern the most convenient properties of the bacterium—phage resistance and antigenic constitution. At a later stage it is hoped to include bacterial characters of symbiotic significance. (J. Kleczkowska.)

The physiology of initial nodulation

Previous work (*Rep. Rothamst. exp. Sta. for 1952*, 62) showed that clover plants grown on agar slopes are stimulated to nodulate

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earlier by preplanting the agar slopes with another clover plant that is removed before the test seedling is sown, and the effect was attributed to some substance secreted by the donor seedling. Further investigation has shown this explanation to be incorrect; the stimulation occurs because the preplant removes from the medium traces of nitrogen present as an impurity in the water supply. With completely nitrogen-free medium, no stimulation by preplanting can be detected in red clover, although small effects are sometimes found with other species; these are being further investigated.

As little as 2·5 p.p.m. of nitrate nitrogen (equivalent to 25 μg. N/seedling) delays nodulation of white clover by 2 days. This effect is also given by nitrite, but not by ammonium salts, asparagine or urea, all of which are assimilated by the host plant at about the same rate. Experiments in which nitrate was added to the medium at intervals showed that inhibition is closely related to uptake of nitrate by the root.

Nitrate has long been known to depress nodulation when used at concentrations normally employed in nutritional work, but at these levels it also occurs with ammonium salts.

The need for controlled environmental conditions has become increasingly urgent as the work on the physiology of nodulation has progressed. The above experiments were done in a small cabinet maintained in a cold room at fixed day and night temperatures and artificially lit with fluorescent and incandescent lamps giving about 1,000 f.c. at the leaf surface. These conditions were wholly satisfactory for red, white and subterranean clover and *Medicago tribuloides* but not for lucerne. Under these conditions of growth much better differentiation was obtained in bacterial strain effectiveness than in the glasshouse. (Gibson.)

Root-hair infection of clover by nodule bacteria

A detailed survey of root-hair infection in twelve small-seeded legumes has been completed. In all species except those very sparsely infected, infection takes place in two distinct stages, as previously reported for *T. repens* and *T. fragiferum* (*Rep. Rothamst. exp. Sta. for 1957*, 77). In both phases infection proceeds at an exponential rate, the initial rate being higher. The change in rate occurs at about the time the first nodules or lateral roots are formed. Very considerable variation has been noted in the site of origin of infection threads in the hair (apical or lateral), their mode of development and morphology; some of these differences are characteristic of host species.

Bacterial strains differ independently of host in the numbers of infection they induce upon the host. Infection threads are often arrested in their development in the root hair. In young seedlings the proportion of arrested infection threads is related to the intensity of infection. This proportion is high on very sparsely infected plants and decreases as the number of infections increases, to a minimum on seedlings infected at about twenty sites; thereafter the proportion of arrested threads increases progressively. The proportion of infection threads arising on lateral outgrowths of root hairs increases as the seedling ages, but is not otherwise related to infection density.

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No infections have been observed in the root hairs of nodulated plants of *Lotus hispida, L. augustissimus* and *Anthyllis vulneraria*. (Nutman.)

The host genetics of symbiosis

Selected material of red and subterranean clover was tested extensively, further selections made and a comprehensive breeding programme completed. In red clover the work is directed towards the separation and genetic analysis of plant material which responds ineffectively with certain normally effective strains of bacteria. In subterranean clover the density of nodule formation on the root is the character under investigation. The results of this study will be reported after the next generation has been examined. (Nutman.)

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