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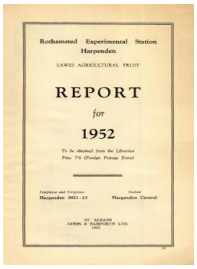

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MICROPREDATORS IN SOIL

By

H. G. THORNTON AND L. M. CRUMP

Introduction

The growth and health of crops is greatly affected by the activities of the soil micropopulation so that the factors that influence the size and composition of this population are a fundamental interest in soil science. The quantity of living matter comprising the soil population is, of course, determined and limited by environmental factors such as the quantity and availability of food material and such variables as pH, moisture, aeration and temperature. But the dominance of certain groups of organisms in the soil population and the prevalence of organisms having specific effects, whether beneficial or harmful, are of far greater importance than is the total size of that population. Here again factors external to the population itself must ultimately determine its biological composition. In this case, however, their action may be indirect, for the varied organisms comprising the micropopulation interact in a very complex manner, so that the effect of an external agent on any one of them may be influenced by its effect on the other organisms. Any attempt therefore to produce a beneficial change in the soil population, whether it be the encouragement of organisms producing desirable biochemical changes or the discouragement of harmful organisms such as pathogens, must involve knowledge of the interaction of such organisms with their associates and competitors in the soil. Competition between organisms in the soil takes place in three principal ways. Firstly, there is a keen competition for the limited supply of available nutrients. Secondly, there are the toxic effects of the products of growth of one organism on others, of which the action of specific antibiotic secretions is an example. This may be quite incidental in that the organism producing them may gain no benefit other than some possibly increased freedom from competition. Thirdly, there are organisms that feed directly upon others as their only or main method of nutrition. These organisms may conveniently be called "micropredators." There are a few organisms remarkable for their modes of nutrition such as fungi that attack nematodes or amœbæ, and the recently described rhizopod protozoa that consume nematodes, but the great majority of micropredators in the soil feed on bacteria. They include protozoa, the active stages of certain myxomycetes and also some Myxobacteria that feed by dissolving bacterial cells and absorbing the products of lysis. There are also some small metazoa such as nematodes that eat bacteria but the work summarized in this report deals only with micropredators having an active unicellular stage.

Interest in soil protozoa has long been maintained at Rothamsted and dates from the work of Russell and Hutchinson on partial sterilization of soil (1909), and their suggestion that, in untreated soil, the numbers of bacteria might be limited by the feeding activities of soil protozoa and that the destruction of the latter might

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account for the observed increase in the number and activity of bacteria in soil after partial sterilization. This theory instigated work at Rothamsted on soil protozoa by T. Goodey from 1910 to 1913, the late Martin and Lewin from 1913 to 1915, Crump who came here in 1915, Cutler who came in 1919 and others who came later. The Protozoology Department was set up in 1919 under the leadership of Ward Cutler originally to study the protozoan fauna of soil, although its scope was later widened. Investigation at Rothamsted and elsewhere showed that soil contained a large and varied protozoan population, amongst which amœbæ and flagellates were predominant, and also that the protozoa existed in an active condition in field soil. This discovery, coupled with the theory of Russell and Hutchinson, made it important to find out whether the numbers of bacteria in soil were controlled by the feeding activities of protozoa. It was therefore necessary to determine what relationship the numbers of bacteria in field soil have to those of the active protozoa.

A technique was devised for the estimation of numbers of protozoa that fed on bacteria, based on a series of soil dilutions (Cutler, 1920), and the plating method for counting soil bacteria was improved. Preliminary counts showed that the numbers both of bacteria and of protozoa changed at short intervals in field soil. In 1920-21 therefore, a series of soil samples was taken from the Barnfield dunged plot at daily intervals for a year and the numbers of bacteria and of the active and encysted individuals of two species of amœbæ and four of flagellates were estimated. (Cutler, Crump and Sandon, 1922.) Marked fluctuations in the numbers of bacteria and protozoa are found ; these were not clearly related to weather conditions but there was a general rise in all groups during the spring and autumn. Changes in bacterial numbers were not related to those of the flagellates. Of the two amœbæ, one, then identified as Dimastigomœba, was much the more abundant in most samples. The frequency of occurrence of high numbers of the active form of this amœba (above 100,000 per gram of soil) was significantly related to that of low bacterial numbers (below 30 millions per gram). This indicated that the amœbae when sufficiently numerous exercised a controlling effect on the changing numbers of bacteria found in the plot by the plating method used. Experiments also showed that amœbæ did keep down the numbers of bacteria when both were inoculated into sterilized soil (Cutler, 1923).

In 1941, on the death of Mr. Ward Cutler, the department was merged with that of Bacteriology to form the present Soil Microbiology Department and it is with the work carried out since then that this summary is mainly concerned.

The selective feeding of soil protozoa

At the time when the surveys of bacterial and protozoan numbers in Rothamsted field soil were made, the quality of the bacterial food supply was not considered, but somewhat later work, both at Rothamsted (Cutler and Crump 1927 and 1935) and elsewhere, showed that soil bacteria differ in their edibility by protozoa. But it was important to discover whether amœbæ, supplied with a mixture of bacterial species, as happens in fresh soil, will feed selec-

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tively and whether they can in consequence change the relative numbers of different bacteria in the soil. B. N. Singh investigated the feeding of amœbæ and of a flagellate on different species of bacteria using an ingenious method in which an inoculum of amœbæ placed at the centre of an agar surface in a petri dish, was presented with a series of radially disposed streaks of different bacterial species whose subsequent rate of consumption could thus be compared. In his first series of experiments (1941a) he tested two species of soil amœbæ on five strains of Aerobacter, and twelve species of soil bacteria; in his second series (1942) two amœbæ and the flagellate Cercomonas were tested on sixteen strains of the nodule organism Rhizobium, twenty other assorted species of soil bacteria and twelve species of plant pathogenic bacteria; in his third series (1945) two amœbæ were tested on sixty-three species of soil and thirty-nine of other bacteria. The species of soil bacteria tested in these experiments differed widely in their characters. About half of them were eaten by the protozoa and showed a range of edibility from some that were readily and completely consumed to others that were but slightly attacked. There were small differences between the two species of amœbæ and rather larger differences between the amœbæ and the flagellate in respect of the particular bacterial species that were eaten. Amœbæ when placed in contact with closely adjacent parallel streaks of readily and less readily eaten species of bacteria usually consumed the whole of the former before making a noticeable inroad on the less edible species. They could, however, be adapted by previous feeding on the less edible species and would then eat both species together. Of eight species of bacteria producing red or pink pigment, seven were not eaten, nor were strains of *Chromobacterium violaceum* or *Pseudomonas æruginosa*. Apart from this relationship to pigment, no clear relationship emerged between the edibility of a bacterial species and any other of its characters, such as gram staining, or slime production.

Species that are eaten differ greatly in their nutritive value as measured by the multiplication rate and by the mean cell size of protozoa fed on them. This was shown, for example, with amœbæ in early work of Cutler and Crump (1927) as more recently for a ciliate by Singh (1941b). Some bacterial species produce secretions highly toxic to amœbæ (Singh 1945). A number of these produce pigments and, in several cases studied, the pigment itself was found to be toxic. This was the case with pyocyanin, prodigiosin and the violet-blue pigment of *Chromobacterium violaceum*.

Not only is the total number of amœbæ affected by the quality of the bacterial food supply but also the percentage of them that is in the active condition. For not only do they tend to form cysts in the presence of unfavourable bacterial food, but Crump has shown that with some species of soil amœbæ, hatching of the cysts is stimulated by the presence of bacteria of which the correct species must be present to ensure maximum excystment (1950).

A highly specific relationship between soil amœbæ and bacteria can thus be demonstrated on laboratory media, and Singh showed that amœbæ are also specific in their consumption of bacteria in soil (1941a). He inoculated samples of sterilized soil with two species of bacteria each in pure culture and with both species together,

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with and without the further addition of soil amœbæ. In the absence of amœbæ, the bacteria fluctuated greatly in numbers in a manner previously observed under similar conditions by Taylor (1936). In the presence of amœbæ, however, one species of bacterium was greatly reduced in numbers and eventually almost extinguished. The other less edible bacterium was little affected and after a month had regained the numbers found without the amœbæ. Thus the amœbæ were able in soil to alter the relative numbers of the two bacterial species.

It seems likely therefore that quality of bacterial flora in differently treated field soils may both influence the numbers of amœbæ and be itself influenced by their selective feeding. That the nutritive quality of the bacterial flora to amœbæ does in fact differ with soil treatment is suggested by counts from field plot samples described below.

Improved method for estimating the numbers of amœbæ in a soil sample

Our knowledge of the soil population is limited by the adequacy of our methods for estimating the numbers of organisms belonging to each of the different groups of which it is composed. Methods for doing this involve making a suspension of the soil sample and, in most cases, diluting this suspension to known degrees. Direct microscope counts are only possible for groups, such as bacteria, present in very large numbers. Otherwise a less direct method must be used. If the organisms to be counted will grow as colonies on a jelly medium, the numbers present at certain known dilutions can then be counted by plating methods.

But important groups such as the protozoa will not do this satisfactorily and here we can only base our estimate on the presence or absence of the organisms in samples from a series of dilutions. Such samples are incubated under conditions ensuring growth of the organisms by which growth their presence is detected. With amœbæ these conditions include the supply of bacteria edible by them. Selective feeding tests showed that strains of Aerobacter were readily eaten by a range of species of amœbæ and other soil protozoa and this knowledge enabled Singh (1946 a and b) greatly to improve the counting method by using as food supply a pure culture of Aerobacter placed in a petri dish on the surface of non-nutrient agar or silica jelly, on which possibly harmful or inedible bacteria from the soil dilution would make little or no growth. The accuracy of estimates by the dilution method is dependent on the number of replicate samples at each dilution that are examined. This was increased by using, for each dilution to be examined, a petri dish in which eight small glass rings were imbedded in the agar or silica jelly, in each of which a sample of the dilution was tested for the growth of amœbæ on Aerobacter.

This improved technique gave consistent results between duplicate samples from field soils, but " recovery " tests from sterilized soil to which known numbers of amœbæ were added showed a consistent loss of about 30 per cent, most of which could be accounted for by non-viability of individual amœbæ in laboratory culture. Thus counts from soil probably represent a systematic underestimate of this order, inherent in any cultural method of counting.

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The numbers of amœbæ and bacteria in differently treated plots

The above technique has been used by Singh to survey the content of active and encysted amæbœ (1949) in plots on Barnfield and Broadbalk and in partially sterilized field plots at Ampthill, Bedfordshire. The samples examined from Barnfield and Broadbalk were taken at nine and six approximately monthly intervals respectively from the plots with no manure (8·0 and 3) farmyard manure (1·0 and 2) and complete artificials (4A and 7) in each field. Over the periods of sampling marked fluctuations in numbers of amœbæ took place. In both fields the numbers of amæbœ, both total and active, were much the lowest in the untreated plots but did not differ appreciably as between the plots treated with farmyard manure or artificials. On the other hand bacterial numbers, determined by both microscope and plate counts, from the same Broadbalk samples by Skinner, Jones and Mollison (1952) were much higher in the farmyard manure plot (2) than in the other two plots (3 and 7), whose bacterial numbers were similar to each other. In other words the ratio of the number of amœbæ to those of bacteria was much higher in plot 7 than in plot 2. This suggests a qualitative difference in food value to amœbæ of the bacterial populations in the two plots.

The setting up by the Chemistry Department of a plot experiment at Ampthill, Bedfordshire, to test the effects of partial sterilization on Sitka spruce nursery beds gave an opportunity to study its action on soil protozoa in the field. An untreated plot and plots whose soil had been partially sterilized with steam and with formalin were sampled at intervals after the treatment and the numbers of bacteria and of amœbæ were estimated by Crump and Singh (1953). Both treatments caused an immediate fall in the numbers of amœbæ and bacteria, the latter estimated by plate counts. After this, in the steamed plot the numbers both of amœbæ and of bacteria rose far above those in the untreated plot. But after formalin treatment the number of bacteria rose well above those in the untreated plot but numbers of amœbæ remained persistently depressed. This result shows that the effects of soil partial sterilization on the micropopulation differ according to the type of treatment used. This conclusion is supported by the different effects produced by steam and formalin on the fungal population of the plots (Mollison 1953).

Classification of soil amœbæ

A difficulty constantly met with in studying the soil protozoa is that of identifying them. Correct identification is of added importance because of the specific reactions which different amœbæ show towards soil bacteria. The taxonomy of small amœbæ was hitherto based to a large extent on characters too uncertain to be of practical value, such as the occasional production of flagella. The type of nuclear division is a more stable character and differs strikingly between different groups of amœbæ, but the difficulty in finding specimens of the different stages of nuclear division has until now limited the usefulness of this character. The discovery of a satisfactory bacterial food supply for cultures of soil amœbæ enabled Singh to devise a beautiful and simple technique in which

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thick cultures of these amœbæ including all stages of nuclear division can be grown on cover slips coated with films of agar supplied with suitable bacterial food (1950). A fortunate habit of the amœbæ to wander through the agar on to the glass surface enables the agar to be removed and the amœbæ to be left adhering to the cover slip, where they can be fixed and stained. With this method he has studied the nuclear division of a number of soil amœbæ and has proposed a classification of amœbæ based on this character (1952).

Giant Rhizopods from soil

The use of a generally edible bacterial food supply for counting amœbæ and for isolating them from soil, resulted in several other types of bacterial predators appearing in cultures from field soil. One of these was a giant multinucleate Rhizopod of the genus Leptomyxa which may attain a diameter of nearly 3 mm. The history of work on this organism is interesting. In 1913 T. Goodey, who was then studying soil protozoa at Rothamsted, found and described three Rhizopods of a type new to the soil fauna and related to the Proteomyxæ. On these he founded the two genera Leptomyxa and Gephyramœbæ (Goodey 1915). Sandon in 1927 found Gephyramœbæ in several soil samples in the course of a survey of protozoa from a range of soils. He however failed to find Leptomyxa although this organism was again found in Australian soil by McLennan in 1930. After this it was not recorded again until Singh (1948a), using Aerobacter as food supply, found that it could be isolated regularly from field soil and obtained it from thirty-eight out of fifty-nine soil samples derived from localities widely scattered over Great Britain and from nine of the plots on Barnfield and Broadbalk. He studied its life-cycle and nuclear division (1948b) and showed that like true amœbæ it was selective in its bacterial food requirements but differed from the amœbæ with which he compared it, in the species of bacteria that it would eat (1948a). A few estimates made by the dilution method from the soil of Barnfield plot 1·0 revealed its presence in dilutions up to 1/1,000.

Soil Acrasieæ

The improved methods of culture used for soil Rhizopods also revealed the abundance and widespread occurrence in soil of a second group of amœboid Protista, the Acrasieæ, particularly the genus Dictyostelium, which was first described by Brefeld in 1869. Singh obtained this organism from soil samples collected from widely scattered localities in Great Britain. He found it in 33 out of 38 arable soils examined but only in 3 out of 29 grassland soils (1947a). He also found it in all the plots from Barnfield and Broadbalk. The Acrasieæ pass through a remarkable life cycle, in one stage existing as amœba-like forms, " myxoamœbæ," which later, under suitable conditions, collect together and form fruiting bodies superficially resembling those of certain fungi. Inside these, spores are formed which are released and from which the amœboid forms are hatched. In the amœboid stage they feed on bacteria and in this stage Dictyostelium, like other predators, was found to be specific in the species of bacteria that it would attack (1947a and b). It

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will also develop and form fruiting bodies when grown in sterilized soil supplied with suitable bacterial food and was then found greatly to reduce the numbers of bacteria in the soil (1947b). The growth of the organism in sterilized soil as judged by the development of fruiting bodies on the soil surface, was found to be dependent on the species of bacteria supplied. The spread of the organism through the soil was dependent on its moisture content. There was little evidence of spread at moisture contents below 25 per cent, and below 15 per cent moisture no fruiting bodies were found even at the point of inoculation, perhaps because the amœboid forms could not assemble in such dry soil. The organism will also pass through its life cycle in fresh unsterilized soil.

Soil Myxobacteria

The Myxobacteria were recognized as a group by Thaxter in 1892 but the group has been comparatively little studied till recently and even now many forms are known only by their fruiting bodies. The more highly developed types of Myxobacteria pass through a life-cycle. In the active stage they consist of thin rods, capable of a sliding motion the mechanism of which is not understood. After a while these rods collect to form swarms each of which may become covered with a coating to form a fruiting body. Inside this the rods turn into the so-called " microcysts " which are usually round or oval bodies but which in some species have the form of short rods. They are eventually released and develop into the active rod stage. Some of the Myxobacteria found in soil do not swarm to produce fruiting bodies. Important amongst them is a group attacking cellulose and placed in the genus Sporocytophaga (Stanier 1942), which was originally found and studied at Rothamsted in 1912 by Hutchinson and Clayton, who mistakenly considered them to be Spirochætes. Another genus, Cytophaga (Winogradsky), even lacks the microcyst stage. Some species in the genus also attack cellulose while others have a more generalized nutrition. One of these that can attack chitin, was isolated by Stanier (1947) during a short visit to Rothamsted. The " higher " Myxobacteria from soil, that have been studied by Singh, belong to the genera Myxococcus, Chondrococcus and Archangium. These organisms are micropredators since they feed readily on true bacteria previously killed and dissolved by their secretions. In a joint investigation Oxford and Singh (1946) found that Myxococcus produced two types of secretion one of which had a toxic effect on a considerable range of bacterial species while the other was a powerful bacteriolytic and proteolytic enzyme that would lyse dead bacteria, though not attacking live ones. Myxobacteria of this predator type are again selective in the bacterial species which they will attack (Singh 1947c). At one time they were regarded as dung inhabiting organisms but they have been found to be widely distributed in British soil and to occur in all the various plots of Barnfield and Broadbalk most of which do not receive dung, so that their status as soil inhabitants is no longer in doubt. Dilution counts from the soil of Barnfield plot 1 ·0 gave numbers of predaceous Myxobacteria ranging from 2,000 to 76,000 per gram.

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Conclusion

There is no means of estimating the effect on the bacterial flora of soil of the micropredator population as distinct from other competitive and antagonistic factors. The daily counts of amœbæ and bacteria from Barnfield made by Cutler, Crump and Sandon (1922) showed evidence of a limitation of bacterial numbers when the number of amœbæ in an active state exceeded 100,000 per gram of soil. In view of the variety of other micropredators now known to inhabit the soil it is not surprising that the effects of any one gronp such as the amœbæ should be distinguishable only when present in exceptionally high numbers. Any assessment of the quantitative effect of the micropredators as a whole would require that the numbers of each type should be estimated from a range of soil samples and compared with bacterial counts. Such a task is at present beyond the capabilities of our counting technique.

But the selective attack on different bacterial species, evidence for which has been found with all groups of micropredators, adds greatly to their interest from the point of view of soil ecology. Singh tested eighty-seven very varied strains of soil bacteria against eight micropredators, comprising a large and a small soil amœba, the giant Rhizopod *Leptomyxa reticulata* Goodey, the myxamœbæ of two species of Acrasieæ (Dictyostelium) and three species of predaceous Myxobacteria (Anscombe and Singh 1948). Any one of these predators was found to attack about half of the bacterial species tested, but owing to the dissimilarity in feeding habits of the various predators there were only seven of the bacterial strains that were not attacked by any of the predators and only twelve were attacted by all of them. Certain groups of bacteria such as the nodule bacteria seem to be generally resistant to attack by micropredators while others such as strains of Aerobacter are attacked by all of them. If it is desired to establish any kinds of bacteria in soil, their resistance to predators should be considered. The presence of micropredators also complicates the unravelling of the effects of soil treatments and especially those like partial sterilization that are liable to check the predators. This was appreciated by Russell and Hutchinson in their original hypothesis although this now appears to us as an over-simplification of the complex perturbations that must occur when the balance of micro-organic life is radically upset by soil treatment.

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LARGE-SCALE PRODUCTION of EDIBLE PROTEIN from FRESH LEAVES

By

N. W. PIRIE

The study of plant viruses is the study of leaf protein; for not only are all the known viruses proteins, but to purify them it is necessary to separate large amounts of normal leaf protein from the preparation. Since 1934 we have been engaged in this work and, during the succeeding years, have become increasingly interested in the proteins of the normal leaf. In part this interest was stimulated by war-time food shortages which made it important to see whether leaf proteins could be used as human food and in part it was the result of a recognition of their intrinsic biochemical importance. The study of animal viruses was preceded by a fairly detailed knowledge of the properties of animal proteins and the behaviour of tissue extracts, but during the early phases of work on plant viruses much more was known about the soluble proteins of the infected tobacco leaf than about those of the normal leaf.

The idea that extracted leaf protein could be of nutritional and industrial importance is not new (c.f. Pirie 1942 a and b) and when work, in collaboration with the Food Investigation Board and Imperial Chemical Industries, started in 1940 there was some past experience to build on. Several patents covering leaf-protein preparation had been taken out, some of them dealing with phenomena that have been well known since the pioneer studies of Rouelle* in 1773, but all the experience had been gained on the laboratory scale. No method had been worked out for handling more than a few pounds of leaf and it seemed likely that the conversion of laboratory-scale extractions and fractionations into a large-scale process would prove difficult. It was easy enough to see what we were trying to do; the only problem was how to do it.

During 1940 and 1941 therefore, a series of extraction tests was made with full-sized mills of many types. At first these tests were based on the crudest empiricism but a few principles soon began to emerge. On the one hand significant amounts of protein are not brought out of the leaf by simple pressure, but on the other hand it is not necessary to open each cell. Fine subdivision is indeed, a disadvantage because it is more difficult to separate leaf fragments from the dispersed protein the smaller the fragments are. Some subdivision, coupled with intimate rubbing and bruising of the leaf, releases much of the protein, and the rubbing is done as well by rubbing leaf on leaf as by rubbing the leaves between two elements of the machine. As in most large-scale operations the process should be continuous both in theory and practice. The distinction

* Those who have worked on leaf protein recently have not been deeply interested in the history of science and have not devoted much attention to Rouelle nor even given his initials. I have in the past erroneously attributed this work to G. F. Rouelle but it was, in fact, done by his younger brother Hilaire Marin who succeeded to the demonstratorship at the Jardin du Roi in Paris on the elder Rouelle's death in 1770.

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is important, for some mills seem to be working continuously when they are, in fact, filling up with resistant pieces of fibre, and this may not be recognized on short runs. The mill must cope with occasional stones for these will inevitably accompany agricultural crops. Power consumption must be kept down both for economy and to avoid over-heating the charge. Unless a wasteful cooling system is to be used, it is clear from first principles that the limit comes at about 50 HP for a grinding rate of 1 ton of wet crop per hour. Preliminary drying or adding large amounts of water to the crop are both to be avoided if possible.

This much became clear by 1942 but then work along these lines was stopped. The reasons for this decision were never made clear; it is not, therefore, possible to express an opinion on their validity. At Rothamsted, however, work continued on the laboratory scale and the results were systematized by Crook (1946) who finally managed to extract 95 per cent of the protein in tobacco leaf by very fine grinding and by maintaining mild alkalinity and low salt concentration. Crook and Holden (1948) and others at Rothamsted, using similar techniques, have separated protein from about thirty different species of leaves in varying yield, and we now have enough experience to be able to tell from the appearance and feel of a leaf what its protein content, and the extractability of that protein, is likely to be. This work was done with the idea of large-scale extraction directly in mind, but much of the other work of the Biochemistry Department also gives information about the separation and fractionation of proteins from the leaf. Work on viruses and on pectase, protease, cellulase, normal nucleoprotein and enzymes concerned in the oxidation of manganese has been described in successive Annual Reports; we have also described the action of commercial proteolytic enzymes on leaf fibre and the effect of fertilizers on the protein content of the leaf.

Protein is held in many different ways in the leaf and when one particular leaf enzyme is being studied selective methods of extraction are an advantage. When, however, a bulk protein preparation is wanted it is an advantage to get all out in one operation. Protein is held in the leaf in three main ways. It may be dissolved in the fluids liberated when the cell structure is damaged by grinding; it may be present in the chloroplasts, nuclei and other microscopically recognizable cell components; it may be in the cell walls. The intensity of grinding will influence the composition of the mixture by varying the extent to which these components are released. The nutritional and physiological state of the leaf will also effect the composition of the isolated protein, because they affect the ratios in which some different enzymes occur (Holden and Tracey 1948) and it is reasonable to assume that the protein is largely made of enzymes.

For practical purposes a protein may be said to be in solution if it does not settle out under gravity in a few hours. Much of the protein in a leaf extract is soluble initially but coagulates after a few hours at room temperature. Many actions are probably involved in this coagulation; some proteins are so associated with enzymes as to be intrinsically unstable (Pirie 1950) some are probably clotted, as milk is, by leaf proteases (Tracey 1948), while some combine

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with tannins and other leaf components and precipitate slowly. Changes of this type probably also go on quickly and may be responsible for some of the readily sedimentable protein in extracts. Chloroplasts and chloroplast fragments are easily separated from the fibre of many leaves and these may make up the greater part of the readily sedimentable protein in some extracts. In some leaves, however, the chloroplasts do not readily separate from the leaf matrix and in some, precipitation by tannins is so rapid that part of the protein remains in the fibre. Holden and Tracey (1950) have discussed the necessity for assuming that any significant amounts of protein are held in the cell walls. They found that the ratio of nitrogen to chlorophyll is nearly the same in isolated chloroplasts and in washed tobacco leaf fibre. There is no reason to think that protein in the cell wall would be associated with chlorophyll. It is probable, therefore, that most of the introgen remaining in the fibre is present as entangled chloroplasts and chloroplast fragments. Suggestions have been made that part of the lignin of the leaf contains nitrogen, but the total amount that is held in this way is small.

In 1948 a grant from the Agricultural Research Council enabled large-scale work to start again and the survey of existing machinery was continued. Ten different designs of swing-hammer mill were tested under varying conditions and the conclusion was reached that this method of grinding was not suitable because it depends on impact between an unsupported particle and the moving hammer. Wet leaves are not shattered by this type of impact. Designs in which a compacted mass is rubbed or has bars forced through it, as in the domestic meat mincer or the screw expeller, are satisfactory on a small scale but, because the ratio of surface to volume changes when the scale increases, they consume excessive amounts of power when the scale is increased. The idea of continuous rubbing however, was attractive, so having found that none of the existing mills would handle the soggy dough-like mass that results when fresh leaves are ground, a mill was designed that cannot clog and is adjustable to the texture of the material being used.

The basis is a Christy and Norris " coir sifter," designed to separate coconut husk from fibre, and is a drum 4 ft. 6 in. long and 3 ft. in diameter with an axial shaft carrying plain rectangular beater arms. It was fed tangentially at one end and discharged radially at the other. Now it is fed axially at one end and discharges tangentially at the other, many more beater arms have been introduced, so that no space inside the drum more than $\frac{3}{4}$ in. wide is left unswept by an arm, and the arms have been modified so that some have propellor-shaped ends and the others U-shaped ends. By varying the ratio of these two types, the rate of movement of the charge through the machine can be controlled to get the correct amount of grinding. There is no obstruction at the exit; material comes out whatever its state of grinding when it has traversed the mill. This is an important distinction from most types of hammer mill because the charge generally has to stay inside until it has been ground fine enough to get through a screen. Ground leaves soon choke a screen.

The primary merit of this machine is that it works and has run for many hundreds of hours at the Grassland Research Station

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without a breakdown. But it is a makeshift and the next one should be designed from the beginning rather than adapted from an existing machine. It should be smaller, it should be so arranged that it can be opened and cleaned easily, and it should be easy to rearrange the distribution of the two types of beater arm. I am confident that the basic principle is sound and, having made and tested 10-15 types of beater arm, that the beaters are the simplest possible. When fed with succulent crops it handles 4-6 tons an hour and takes 10-20 HP, but the rate of working falls and the power consumption rises with drier crops. Good grass goes through at 1 ton an hour and consumes 20 HP, grinding becomes more extravagant than this only with crops that are so dry and mature that protein extraction is unsatisfactory even in the laboratory.

In theory the amount of work that has to be done in grinding a mass of leaves is extremely small, so that in the earlier phases of this work there was always the hope that a much more efficient arrangement might be found. So far nothing has made it seem likely that this will prove possible and all the other arrangements consume more power. Tracey has carried out some (unpublished) experiments in which a weight was dropped on to 20 g. lots of grass and the percentage of protein liberated was measured after different amounts of work had been done. Satisfactory liberation required 6×10^9 ergs. If this could be replicated on a large scale, it would mean that a grinding rate of 1 ton per hour would take a little over 10 HP, which suggests that stamping mills would merit more thorough investigation than they have yet received.

In this connection it is interesting to consider what success animals have had in solving this problem. The bullock grinds grass with its teeth and tongue. Figures for its performance are somewhat approximate, but the ones given have been chosen so as to favour the efficiency of the bullock rather than the reverse. The jaw muscles of an 11 cwt. animal weigh 5 lb. and its tongue also weighs 5 lb. This weight is not all muscle used for chewing, but we will assume that it is and also that its rate of working is 0·01 HP per pound. This is the rate that Gray (1936) found for the strenuous conditions of dogs running on a treadmill and men rowing; it is, therefore, certainly a greater rate than would be compatible with the placid expression of a chewing bullock. We may be sure, therefore, that not more than 0·1 HP is being expended during 8 hours in which it collects grass and chews it roughly and the further 8 in which it chews the cud. After this the mass has about the consistency at which we aim for satisfactory protein extraction. The bullock eats 30 lb. dry matter or 150 lb. of fresh grass during the 16 hours, so that its 0·1 HP machinery is handling material at 9·4 lb. an hour. To get a rate of 1 ton per hour by replicating the same machinery we would need $2{,}240 \times 0{\cdot}1/9{\cdot}4 = 24$ HP. The actual rate of working may be only half this but it would seem that the course of evolution has not produced a mechanism much more efficient than our hasty adaptation.

The problem of pressing the juice from the ground leaf mass does not seem to be so nearly solved. On the laboratory scale it is easy, and on a large scale it is also easy if small molecules are the only valuable components of the extract. But much of the leaf protein

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is present as particles up to 5μ in diameter and such particles are easily held back by tightly compacted masses of fibre. Arrangements that expose large bulks of material to high pressures are not therefore well adapted to our purpose. Moderate success has been achieved with a machine made at the National Institute for Agricultural Engineering. This has a perforated steel drum which is supported on three rollers inside it. Opposite each roller a larger wooden roller presses the drum on the outside, these outside rollers are driven and carry the drum around by friction. Ground leaves are fed on to the outside of the drum and are carried by it under each of the three wooden rollers in turn so that at each nip juice is pressed through the drum and into a tray inside. With this simple arrangement many tons of juice have been made but it is difficult to keep the layer of material on the drum even and the time during which pressure is applied in passage through the nips is too short for the juice to run away effectively.

Juice is so easily pressed by hand from minced leaves enclosed in a cloth and it is so easy, by continual hand pressing, to get a product containing only 65 per cent of water, that it is tempting to underestimate the problem of large-scale juice extraction. The pressure applied by hand is only about 30 lb. per sq. in., but it is maintained for many seconds, the charge is continually being rearranged, so that new parts are brought near the filtration surface by finger action. This is not an action that it would be easy to simulate with a machine, but it should not be needed if the thickness of the layer being pressed is kept small. With this in mind, new designs for a press are being discussed and in them three principles stand out clearly ; the layer, after pressing, should not be more than $\frac{1}{4}$ in. thick; pressure should be maintained for a few seconds ; there should be no movement between the charge under pressure and the filtration surfaces. There are so many ways of achieving these desiderata that we can be confident of success as soon as sustained work on the problem starts.

Any robust press designed to work quickly will allow some leaf fragments to pass through into the juice, so that a further stage of straining is needed before the protein can be separated. This presents no difficulties. The protein is then coagulated by heat or by adding acid. With many batches of juice, acid gives the better yield ; it also gives a purer product but one that is more difficult to handle because it is finely divided. If heat is applied rapidly with live steam, the curd is coarse and easily filtered off. From this stage on the protein is handled by normal chemical engineering methods. So far the substances remaining soluble in water after heating or acid coagulation have been discarded but, as is well known, the leaf at various stages in its growth contains valuable amounts of carbohydrate, and non-protein nitrogen part of which appear in this juice. It is therefore, essential that methods of using it should be explored.

By this sequence of grinding and pressing it is easy to get out in the form of protein a quarter of the nitrogen in leaves containing more than 2·6 per cent of nitrogen and possible, by rewetting the pressed mass and pressing again, to get out a third. The yield is lower than that reached in the laboratory but this is to be expected.

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The remainder of the nitrogen is either soluble or else it remains as unextracted protein in the pressed residue. There would be obvious advantages in getting this out also; the question of how much it is economic to extract depends simply on the costs of extraction compared with those of growing more leaves. Further grinding is an obvious step but as already mentioned it has defects. We have made a fairly through study of the enzymic degradation of leaves (Holden, Pirie and Tracey 1950), mainly because of an interest in the liberation of viruses, but also with the application to protein extraction in mind. The enzymes used were juices or extracts from snails and various fungi ; these would hardly be practical for large-scale use but the work showed that leaf residues were easily digested. In practice it would be easiest to seed the mass of leaf residue with a culture of a cellulase producing micro-organism and to let growth and fibre digestion proceed together. Hitherto cellulase has been an unreasonably neglected enzyme, but during the past few years it has begun to get the attention that its academic and practical interest warrants.

Protein which it is not economic to extract from the fibre will not, however be wasted. Cattle eat the residue readily, both when it is fresh and after drying, and it is very easy to ensile. The idea of drying it as winter feed is particularly attractive because although the nitrogen content is generally only 1 ·5 to 2 per cent, it is economical to dry and is satisfactorily handled by a rotary drier. Normal grass drying is not the unqualified success it was expected to be and one reason is that the protein content of a leaf is approximately proportional to its water content. The more worthwhile it is to make the dried product, therefore, the more water has to be dried off to get it. Thus really good leaves with 4 ·8 per cent of N on the dry matter may contain 93 per cent of water when cut, whereas those with only 2 ·4 per cent may contain only 75 per cent of water; to get a ton of dry matter from the former necessitates drying off 13 tons of water and from the latter 3 tons. The former is an extreme case ; much of the dried " grass " at present being made in Britain is of the low quality of the latter. But if there were that extension in the use of fertilizers and irrigation water that is widely, and rightly, advocated, much more of the material coming to the driers would have such a high nitrogen and water content as to make drying doubtfully economic. Many proposals have been made for resolving the dilemma that the better the technique used in growing a forage crop the more expensive it becomes to dry it. To them we may add the proposal that the crop should first be processed to get out much of the protein and most of the water, so that only the residue containing about 65 per cent of water, would be dried. The proposal is that protein preparation should be a supplement to grass drying. It is easy to make a rough estimate of the protein and water content of a batch of leaves visually, and each load that arrives at a processing station should be sent straight to the drier if it is of low quality but should be used first for protein production if it is of high quality.

One of the difficulties encountered in the introduction of modern agricultural methods into undeveloped areas is the lack of power to run tractors and pumps. The residue of leaves from which protein has been extracted might be a valuable fuel. It could be used either

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directly or after fermentation to give alcohol or methane ; the first course is simpler and more economical and the granular texture of the residue as it comes out of a press make it much more suitable for mechanical handling than the other agricultural wastes with which it is sometimes proposed that furnaces should be stoked. Research is already going on on the design of engines to run on low grade fuel ; it would seem that this is one of the fuels that should be tried.

The advantage of developing these techniques for separating protein from leaves depends on three propositions : That the leaf is the best place to look for further supplies of protein : That the protein and other components of the leaf are of more value to us after they have been separated from each other than they were when they were mixed : That there is no better method of making the separation. There will be little argument about the first, all the terrestrial protein sources now used on a large scale, e.g., beans, meat and milk depend on the leaf. Yeasts and some other micro-organisms can make protein directly from ammonium salts or even from atmospheric nitrogen and fish depend mainly on algæ and unicellular plants, but these sources should be looked on as complements rather than as alternatives to leaf protein. The only unusual feature of leaf protein separation is the intimacy with which the useful and the less useful parts are mixed in the starting material. In principle it is comparable to such well established separations as grain from chaff, oil from oil seeds and sugar beet tops and crowns from sugar beet. These separations are well known to be advantageous because by them the value of at least one of the products is enhanced. If leaves are to be used as a source of protein in the human diet, the only alternative would be to grow leaves with an exceptionally high protein content. Research on the conditions needed for a plant to produce high protein leaves regularly and a search for the species that can be easily made to do this would be both interesting and valuable, but for some time it is likely to be easier to grow a lower quality leaf and then to separate the digestible protein from the indigestible fibre. These are all issues that have been argued at greater length elsewhere (Pirie 1951, 1952, 1953).

Under existing conditions there are two main ways in which the separation is brought about. In the plant, the growth of seeds and tubers entails the translocation of protein, so that the fibre remains in the sere leaf and the protein appears in a digestible form along with fat and carbohydrate. Ruminant animals also separate the protein for us when they feed on leaves. Each process involves waste and the waste is especially great with animals because their value as sources of concentrated protein is a consequence of the fact that they are even more wasteful of carbohydrate than they are of protein. It is this fact that enables a bullock to lay down meat containing 60-70 per cent protein, in terms of dry matter, when fed on a diet containing only 10 per cent. Few will dispute that the products of animal conversion have more gustatory appeal than the products made from leaves are likely to have in the near future, but culinary enterprise can often bring about surprising changes. Furthermore a policy of leaf-protein production would probably not diminish the amount of food available for animals. First, there would be low-grade batches of protein suitable for pig and chicken

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food ; second, there would be the leaf residue suitable for cattle food, and third, leaf protein production depends on the growing of high quality leaf. With leafy crops the improvements of quality by manuring, irrigation and frequent cutting would be accompanied by a total increase in yield both of protein and dry matter. This increase, it is true, could be achieved without the further step of making leaf protein, but the idea has never proved particularly attractive because such intensely cultivated crops tend to have too high a protein content for any but the most productive milking cows.

These advantages have naturally not escaped general notice and several commercial projects for making protein or protein concentrates have been started. Information about these projects is not always easily obtained but they seem to have the common defect that an attempt is made to get out the protein in one operation and to use one machine for all types of leaf. In practice the attempt does not succeed and the crop is passed several times through the same machine. Rollers and oil expellers or modifications of them are most commonly used. There seems to be no advantage in passing the charge through the same machine twice rather than passing it successively through two machines, or even three, each designed for the particular job to be done. *A priori* it is unlikely that a machine which is efficient at grinding leaves would also be efficient at separating the juice from the ground mass. All the evidence from our own work and the work of others suggests that it is better to start with an adjustable mill that can produce an approximately standard product from a wide range of raw materials, and then to feed this product into a press. Research is still needed until a workable unit has been built. This work can with advantage be done by anyone who knows the starting material, knows the aim, and has the enthusiasm to do it. The results will bear the same relationship to the final design that Trevethick's steam engines bear to those used now and the metamorphosis will call for the most expert available engineering skill, but some sort of working unit is a necessary first step.

Besides the work that is needed on the machinery there is also much scope for botanical and agricultural work. First there is the choice of crop. Hitherto a forage crop has had to have a texture and flavour acceptable to stock. A mill is less exacting and opens up wide botanical possibilities. Most of the necessary research to find which plants give the biggest return of dry matter and extractable protein per acre can be done on small scale plots. But once some conclusions have been reached they need confirmation by large-scale extraction, partly to confirm the laboratory results and partly to get enough protein to be sure that it has the expected feeding value. It may be that no crops better than those already in use, either in Britain or overseas, will be found at an early stage in the work but the standard crops offer much scope for variation. The effect of fertilizers and irrigation is already being actively studied at Rothamsted; this work could usefully be supplemented by a study of the extractability and quality of the protein. All the leafy agricultural wastes also need examination as do plants such as bracken and sedges that grow on uncultivated areas.

Work on the large-scale extraction of leaf protein has now been

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going on in an uncertain manner with support from various Government departments for thirteen years. It seems to have got to a stage at which, with little more effort, a conclusion could be reached and machinery designed which would be suitable for use both in Britain and overseas.

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