In such a case the correct way of expressing protein concentration is g./100 ml. of solvent.

6. The specific volumes of unfractionated rabbit and human serum proteins were found to be 0·729 and 0·732 respectively, and that of the lipids 0·996.

7. It is inferred that the lipids in the serum are at the most in only a loose combination (physical adsorption ?) with the proteins.

It is a pleasure to record our indebtedness to Mr G. S. Adair, F.R.S., for his advice and help given generously in the course of this work; we are also grateful to him and Mrs Adair for the determination of phosphates in the concentrated sera, which were used in the estimation of specific volumes of proteins and lipids. We wish to thank also Dr Evan Jones for the sample of the nephrotic human serum.

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Biochemistry of Nitrification in Soil

1. KINETICS OF, AND THE EFFECTS OF POISONS ON, SOIL NITRIFICATION, AS STUDIED BY A SOIL PERFUSION TECHNIQUE

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Nitrification is the process whereby nitrogen, in the form of the ammonium cation or in organic combination, is converted into the nitrate anion. Nitrification in soil has long been established as a biological process (Schloessing & Muntz, 1877, 1879), but some evidence that, under tropical conditions, a non-biological nitrification in soil may occur has recently accumulated.

The pioneer work of Warington (1878, 1879, 1884) on culture studies of the nitrifying bacteria of soil culminated in the isolation of the nitrate-forming bacteria by Frankland & Frankland (1890) and Warington (1891), and the isolation of both nitrite-forming bacteria and nitrite-oxidizing bacteria by Winogradsky (1890). Winogradsky suggested that the two forms of bacteria which he isolated co-operated in soil to produce the overall effect of nitrate formation from ammonium cation. All these workers found that inorganic media were suitable for culture of the organisms, and there is a fairly general agreement that the nitrifying organisms, while nitrifying, are autotrophic. Whether various forms of organic matter stimulate, inhibit, or leave unaffected, the organisms is a question that now has a vast literature

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which cannot be adequately summarized here. There is a short but good review by Barritt (1933). We would, however, draw attention to the fact that the physical condition of the culture medium may modify the inhibition, if any, brought about by organic substances, e.g. Wimmer (1904) found peptone less inhibitory in sand than in solution cultures. Winogradsky (1931) drew attention to the fact that the data gained from a study of isolated soil bacteria have not been used to elucidate the details of nitrification processes in the soil itself. The apparent lack of interest in the study of nitrification in soil may have been due partly to the assumption that the organic components of soil would exercise a variety of indeterminable effects on nitrification, and partly to the lack of a suitable experimental technique for the study of the biochemical changes in soil.

Stevens & Withers (1910) found that nitrification in soil differed in at least one important particular from nitrification in artificial culture (silica-gel media). Nitrification in soil was far less inhibited by added organic matter than was nitrification in the laboratory media (see also Wimmer, 1904). Nitrification both of cotton-seed meal and of ammonium sulphate took place much more rapidly in soil than in solution (Stevens & Withers, 1909). Soil conditions clearly greatly influence the rate of nitrification.

Desai & Fazal-ud-Din (1937) have obtained evidence from culture experiments that nitrifying organisms can enter into a symbiosis with non-nitrifiers; this may be a factor influencing the behaviour of nitrifiers in soil. Allen & Bonazzi (1915) have shown that soil, even ignited soil, is a better medium than sand for supporting nitrification. It is not clear, however, from their results whether the effect of the ignited soil is due to its acid-neutralizing power. Boulanger & Massol (1903) found that nitrification in solutions spread over cinders was much faster than in a simple layer of solution. Porcelain and pumice were not as effective as cinders; sand and brick were almost inert. A number of workers, e.g. Albrecht & McCalla (1938), Conn & Conn (1940) and ZoBell (1943), have recently shown that the presence of colloids in culture media can influence bacterial behaviour, and doubtless some similar effect occurs in soil. Another factor to be considered in soil nitrification is the effect of variation in strain of the bacteria; for example, Pikorvska (1940) has shown that bacteria isolated from different soils have different nitrifying capacities. Cutler & Mukerji (1931) have obtained from Rothamsted soil four different strains of bacteria, all capable of oxidizing ammonium to nitrite, yet all stimulated by sucrose.

It is to be expected that in soil, with its complex microflora and its special physico-chemical conditions, the kinetics, possibly even the mechanism, of nitrification, will differ materially from what takes place in pure-cultures of the nitrifiers. There can be no question of the great importance of studies of pure cultures of these organisms, especially for the investigation of intermediate metabolic products which are unstable in presence of a medium such as soil. Beesley (1914), for example, has shown the occurrence of hyponitrous acid during nitrification in flask culture, and this has been confirmed by Corbet (1935). But there is as yet no clear evidence of the formation of this substance during nitrification in soil. Meyerhof's early studies (1916, 1917) on the respiratory activities of the nitrifying bacteria throw light on many factors influencing the metabolism of these organisms, and have to be borne in mind in the interpretation of the phenomena of soil nitrification. But for the study of the process of nitrification in soil it is essential to study the course of the events taking place in the soil itself, and to look upon the data obtained from the study of pure cultures as complementary to those found in the natural medium.

There is now an immense literature of field experiments and pot experiments to show the effects of the addition of nitrogenous substances to soil. The literature on the nitrification of organic material was fully reviewed by Whiting (1926) and has since greatly increased. Whiting considers the rapidity of nitrification of the various types of nitrogen (water-soluble, easily hydrolyzable, total) and the influence on this of the carbon content of the nitrogen compounds. This knowledge can be made to be of considerable value to agriculturists who wish to know how quickly fertilizers will supply available nitrogen, but it does not go far in elucidating the processes by which the compounds are in fact nitrified.

The available data on the behaviour of nitrifying bacteria in pure culture are fairly extensive. The relationship of nitrification to pH has been investigated by Meyerhof (1916, 1917) and Winogradsky (1933), both of whom found the optima to lie between pH 7 and 9, with a fairly rapid fall-off on each side. Meek & Lipman (1922) found, however, a far greater spread; they observed nitrifying activity in media with pH as low as 5 and as high as 13. These workers found that the pH-tolerance of the nitrifying bacteria varied with the pH of the soil from which they were isolated. Meyerhof (1916, 1917) investigated the optimum concentrations of ammonium and nitrite for *Nitrosomonas* oxidizing ammonium to nitrite, and for *Nitrobacter* oxidizing nitrite to nitrate. He found the optima to lie at 0·005 N-ammonium for *Nitrosomonas*, and 0·0072 N-nitrite for *Nitrobacter*. Free ammonia and excess nitrite both poisoned *Nitrobacter*. Warington (1879, 1891) and Winogradsky & Omeliansky (1902) had already shown inhibitive effects of ammonia on nitrate production. The optimum temperature for nitrification seems to depend on the prevailing climate, but for

strains isolated in temperate countries it is approximately 25° (Tandon & Dhar, 1934). The effect of organic matter on the nitrifiers is still undecided. Barritt (1933) suggests the nitrifiers may represent a stage in the life cycle of heterotrophs. Winogradsky (1933) continued to maintain that they were strict autotrophs inhibited by organic matter. The general relations between the results obtained with culture experiments and those obtained in soil are obscure. Albrecht & McCalla (1937) summarized the position thus: 'The conditions controlling nitrification in aqueous solution have been studied very specifically. Less definite controls and less refinement in methods have obtained for studies of this process within the soil. The complexity of a sand, silt and clay mixture, as soil, prohibits an accuracy great enough to encompass all the various chemical aspects of so delicate a process as nitrification.'

The present work describes the results obtained with a new and direct technique for the study of nitrification in soil. This technique makes possible the study, within certain defined limits, of the nitrification process as it actually occurs in the soil.

It is proposed in the following series of papers to describe experiments on the mode of transformation of nitrogen compounds into nitrate in soil. The soil is treated throughout the experiments as a biological whole, and the technique is such that metabolic events in the soil may be studied with greater accuracy than has been accomplished hitherto. In essence the attempt is made to study the metabolism of a soil as though it were a living tissue. Emphasis is placed on the changes brought about by the soil *as a whole* under defined experimental conditions, and care is taken that the soil itself is not interfered with throughout the experimental period. It is, of course, certain that many biological and chemical changes occur in the soil during the experiment; but so long as the technique employed gives accurately reproducible results, there is no *a priori* reason why particular aspects of metabolism should not be as amenable to exclusive study in soil as they are in isolated plant or animal tissues. The biological changes taking place in soil are a direct result of the initial chemical stimulus applied to the soil, and are as much a part of the over-all chemical change as the more easily identified metabolic changes themselves. The kinetics of a metabolic process in soil involve not only the kinetics of the catalytic changes taking place but also the kinetics of the biological changes undergone by the responsible cells. Experiments which will be described in this and subsequent papers show with what reproducibility the kinetics of these processes may be studied even in soils of different origins. They indicate that studies of many aspects of soil metabolism yield consistent results so long as there is adherence to the principle of treating soil as a biological whole.

PRELIMINARY INVESTIGATIONS

The method hitherto usually employed for the laboratory study of nitrification in soil is, in one form or another, the pot method.

Quantities of soil are treated with substances under investigation, brought to a suitable water content, and placed in containers (beakers, jars, test-tubes) where nitrification is allowed to proceed. Evaporation of water from the soil is detected by weighing the containers, and compensated by the addition of the requisite quantity of distilled water. Samples of soil are taken at intervals, shaken with some standard extractant, filtered or centrifuged; appropriate quantitative analyses can then be performed on the extract. The extractants in general use are strong salt solutions, often of low pH, which not only dissolve the soluble compounds in the soil but, by base-exchange with the soil colloids, bring into solution the cations (e.g. ammonium ions).

Although this method is of great value for agronomic purposes, it is less suitable for a detailed biochemical study of nitrification in soil.

(1) Only by actual mechanical displacement of soil solution can the cationic equilibrium established between soil solution and base-exchange complex of the soil be determined. The use of an extractant automatically alters this equilibrium, and extract analyses can give only an inferential picture of the changes (if any) in equilibrium ancillary to soil metabolism.

(2) Disturbances of soil by sampling will cause changes in soil aeration, and consequently in nitrification rate.

It is difficult to replicate exactly all physical factors affecting nitrification, e.g. water evaporation, aeration, temperature, moisture content, ratio of soil volume to soil surface.

(3) The physical condition of the soil in the pot is non-uniform and always changing. Water continually evaporates from the top of the soil, which is therefore always drier than the soil at the bottom. Attempts to compensate for this by the addition of water are liable to make the top of the soil temporarily wetter than the bottom. Since the water content of soil is known to affect the rate of nitrification, such changes inevitably introduce some variance into the results. This variance is partly compensated in that it occurs to a roughly similar extent in all containers, but its very existence complicates any attempt to standardize rates of nitrification. So many factors—air currents, atmospheric humidity, temperature, the shape of the container—may influence the evaporation of water from soil, that exact repetition of the conditions is difficult even in parallel experiments in the same laboratory.

These drawbacks inspired a search for a method more suited to the biochemical study of nitrification in soil. One method that had obvious advantages was an adaptation of the 'perfusion technique', which has proved invaluable for the elucidation of the metabolic processes of isolated animal organs. If soil could be intermittently but frequently perfused with an aerated solution of nitrifiable material many of the difficulties inherent in the pot method would be circumvented. The soil would always be at the

same water content (just short of waterlogging); aeration of the whole of the soil would be maximal and automatic; samples of the perfusing fluid could be taken without disturbing the soil; and finally the cation distribution between soil solution and soil colloid could easily be determined by comparing the total quantity of a given cation known to be present in the system (soil and fluid) with the quantity found in the perfusing fluid itself.

The practicability of the whole idea depended, however, on whether adequate nitrification could be obtained at water contents just short of waterlogging. A number of workers, e.g. Schloessing & Muntz (1879), Deherain (1902), Traaen (1916), Stevens & Withers (1909), and Pikorvska (1940), have shown that nitrification in soil is maximal at or near a soil-water content just short of waterlogging; but as a knowledge of the relation between soil-water content and nitrification rate was essential before the practicability of the perfusion technique could be assessed, the relation was established by test-tube experiments. These experiments were carried out as follows:

Equal portions (usually 25 or 10 g.) of sieved air-dried soil were put into a number of boiling tubes. A quantity of ammonium sulphate (sufficient to provide more nitrogen than could be converted to nitrate during the experiment) was added to each tube followed by distilled water to yield the required water content. The tubes were weighed, plugged with cotton-wool, and set aside; loss of weight was compensated every other day and evaporation was thereby kept below 0·5 g. at any time. After 8 days the whole of each tube was extracted with N-K_2SO_4 containing enough H_2SO_4 to bring the pH of the extract to 1·0. The nitrate-N in the extract was determined by reduction. Two trials were conducted. In the first (*a*) 25 g. portions of sieved Rothamsted garden soil (4·0–0·5 mm. fraction) were treated with enough $(NH_4)_2SO_4$ to supply 224 μg. ammonium-N/g. of soil; in the second (*b*) 10 g. portions of the sieved garden soil (2·0–1·0 mm. fraction) were treated with enough $(NH_4)_2SO_4$ to supply 200 μg. ammonium-N/g. of soil. Nitrification was allowed to proceed at room temperature.

Table 1. *The relation between soil-water and nitrification rate*

	Water content of soil (%)	Nitrate-N formed/g. soil (μg.)	Nitrification of $(NH_4)_2SO_4$ (%)
Exp. (*a*)	16	75	33·5
	27	119	53·0
	36	203	90·5
	42*	24	11·0
	48*	29	13·0
	52*	27	12·0
Exp. (*b*)	11	105	52·5
	20	126	63·0
	27	184	92·0
	33	170	85·0
	38*	16	8·0
	43*	2	1·0

* Indicates that the soil was visibly waterlogged.

The results (Table 1) showed fairly clearly that good, even maximal, nitrification rates could be expected at soil-water contents such as would obtain if soil were perfused intermittently with aqueous solutions of nitrifiable materials.

THE PERFUSION TECHNIQUE

Our technique consists in perfusing a column of soil with oxygenated liquid by a circulatory process. The liquid, which contains in solution the substances the metabolism of which is being investigated, is made to percolate through the soil into a flask where it is mixed and aerated and whence it is made to drain again through the same soil. The process is continuous and may be maintained for an indefinite period. The rate of perfusion is such that no waterlogging takes place. The soil is left intact throughout the experiment and analyses are carried out on the soil perfusate.

The process is accomplished in an apparatus, finally devised by one of us (H. L.), of which a full technical description is given in the Addendum to this paper. It has already been briefly described (Lees & Quastel, 1944). The apparatus, as we now use it, consists of a bank of identical units. In each unit a column of sieved soil (usually about 50 g. of the 4·0–1·0 mm. fraction) is held in a vertical glass tube 1 in. in diameter by means of a glass-wool plug placed under the soil column. A small quantity of aerated fluid (containing metabolites) is mechanically taken from a reservoir and run on to the top of the soil column whence it percolates downward through the soil dissolving soluble substances on its way, and eventually returns to the reservoir. When it has all returned a fresh quantity of automatically mixed and aerated reservoir fluid is run on to the top of the soil. Since these operations are automatically performed every few minutes the composition of the fluid in the reservoir is kept in close approximation to the composition of the fluid actually in contact with the soil. Those metabolic activities taking place in the soil, which cause changes in the composition of the fluid in contact with it, are therefore immediately reflected as changes in composition of the reservoir fluid, which can easily be sampled without disturbing the soil. Analyses of the soil perfusate in the reservoir can, therefore, be used to follow the metabolic activities of the undisturbed soil.

The regular downward passage of small quantities of fluid through the soil largely ensures its adequate aeration because each small quantity of fluid drags after it a fresh supply of air. Adequate aeration of the soil is in any case ensured by means of a device that at intervals automatically slightly raises the air pressure above the soil and thus establishes a tendency for air to be driven down through it. By this means excess water is expelled from the soil concomitantly with preventing waterlogging.

This apparatus, which has proved most successful in practice, has made possible a technique offering a number of advantages over the pot method.

(1) The water content of the soil is kept constant and the water is homogeneously distributed in the soil throughout

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p. 807

for **(Andus, 1946)**
read **(cf. Audus, 1946; and Lees (1946), *Nature, Lond.*, 158, 674).**

p. 814
for **Andus** ***read*** **Audus**

the experiment. Variations of temperature within the soil are minimized by the continual waterflow through it.

(2) The soil is undisturbed throughout the experiment.

(3) Maximal aeration of the soil is effected.

(4) Sampling is easy, only the perfusate being examined, and as far as one unit is concerned it suffers from no sampling variance because mixing of the perfusate, or soil solution, is adequately maintained.

(5) Substances can be added to the soil solution in the course of an experiment and at any period corresponding to the known metabolic activity of the soil. Thus it is easy, without disturbing the soil, to add a poison, or bacterial inhibitor, to the soil solution at any phase of the soil metabolism.

(6) Ionic equilibrium between soil and solution is quickly secured and maintained, except in so far as the equilibrium is disturbed by metabolic products of the soil. If the amount of ammonium ion in the system is calculable or known, a perfusate analysis for ammonia can be used for finding the amount of ammonium cation on the soil by simple difference. We have used this method to determine the soil-perfusate distribution of ammonium ion during the course of experiments on nitrification.

(7) Gases entering the apparatus can be controlled. Thus the air may be replaced by any gas or gas mixture.

(8) The soil in the apparatus is capable of being isolated from aerial bacteria, etc. This would enable work to be carried out with control of the bacterial condition.

(9) The soil solution can be replaced at any time by an entirely different solution or fluid, without physical disturbance of the soil.

(10) The soil itself after perfusion for a known time can be examined either by analytical, microbiological, or other technique, the standardization of conditions securing reproducibility of results.

The apparatus is cheap to make, and may be modified for specific purposes. Experiments with it can be carried out in the dark to prevent algal growth. We have used a battery of perfusion units (which can be darkened by black curtains) in a thermostatically maintained room kept at 70° F. The apparatus may obviously be used for the study, under optimal conditions, of aerobic metabolism or of the transformation of any substance, organic or inorganic, incorporated into the soil or perfused through it (Andus, 1946).

The main disadvantages or shortcomings of the apparatus as so far constituted are:

(1) Removal of soil solution for analysis inevitably decreases the total volume of perfusate, and there is no corresponding decrease in amount of soil to compensate for this. The error can be reduced to a low value by removal of as small quantities as possible for analysis, and by having a large initial volume of perfusate. An experiment can be run, however, for any length of time if, in the calculation of the results, due regard be paid to the amounts of fluid removed from the apparatus.

(2) The apparatus is suitable only for studies conducted at a soil-moisture content a little short of waterlogging. Whilst many microbial activities are rapid and optimal at this water content (aeration being also optimal), the impossibility of working at any other water concentration is a drawback.

On balance, the advantages of the perfusion technique for studying metabolism in soil far outweigh its disadvantages, and this technique has been exclusively used in the work to be described.

Preliminary experiments showed that a suitable weight of air-dried soil for each unit was 20–100 g., and that suitable volumes of perfusate were 200–300 ml. of 0·028–0·0071 N-ammonium salt solutions. If more soil was used nitrification was too rapid to be followed accurately, and if lower concentrations of ammonium salts were used the analyses of the perfusates became too inaccurate.

EXPERIMENTAL METHODS

(1) *Determination of ammonium*-N

A sample of 2 ml. containing 20–500 μg. ammonium-N was distilled with 1 ml. of 5 N-NaOH in a Markham (1942) micro-distillation apparatus. The receiver contained 2 ml. of 0·5% boric acid containing 40 ml./l. of Conway & O'Malley's (1942) indicator. After about 10 ml. of distillate had been collected, the contents of the receiver were titrated in a stream of CO_2-free air with N/70 sulphuric acid from a 1·0 ml. burette. The end-point was sharp and estimations were always repeatable to within 0·010 ml. (≡1·0 μg./ml. ammonium-N in the sample).

(2) *Determination of nitrite*-N

A sample (usually 1 ml.) containing 0·5–5·0 μg. nitrite-N was diluted to 11 ml., and 2 ml. mixed Griess-Ilosva reagent were added. After at least ½ hr. the colour was read in a Spekker photoelectric absorptiometer fitted with blue filters (Hilger no. 6), and the nitrite-N content was obtained from the Spekker reading by means of a constantly checked standardization curve.

(3) *Determination of nitrate*-N

(*a*) *Volumetric.* This method is a modification of one suggested by Dr S. G. Heintze (personal communication). To 3 ml. of the sample containing 20–500 μg. inorganic-N in a hard glass test-tube was added 0·15 g. iron and 3 ml. of 8% sulphuric acid; the tube was then placed in a beaker of water maintained at 70°. When the main effervescence had subsided, the tube was tightly closed with a rubber bung and the tube and beaker set aside overnight. The next day, 4 ml. of its contents were distilled in the Markham apparatus. The titration of the distillate was equivalent to the (nitrate + nitrite + ammonium)-N in 2 ml. of the original sample, from which value the nitrate-N could be determined by subtraction of the (ammonium + nitrite)-N. A recovery of 96–102% of added nitrate-N can be relied upon, although complete recovery in presence of large quantities of nitrite-N (200 μg.) cannot always be obtained. As nitrite-N was never more than a few μg. in normal samples, this discrepancy did not usually affect the accuracy of the total-reducible-N determination. When the nitrite-N was sufficiently concentrated to be a possible cause of inaccuracy it was removed from the sample by the addition of a crystal of potassium iodide in presence of sulphuric acid and bubbling with nitrogen. All nitrite-N could thus be removed. The iron was then added, and the mixture reduced in the normal way.

(*b*) *Colorimetric.* Where nitrate concentrations did not exceed about 50 μg./ml. nitrite-N we used almost exclusively, in later experiments, a method based upon the development of colour by treatment with phenoldisulphonic acid. A small sample, say 1–2 ml., of perfusate and an equal volume of 0·02 M-$CuSO_4$ are shaken in a conical centrifuge tube with a spatula-end (0·2–0·4 g.) of a mixture containing 33% solid $Ca(OH)_2$ and 66% $MgCO_3$. The tube is shaken at intervals throughout the day and left to stand overnight. The next day the contents are centrifuged and a measured portion containing 10–100 μg. nitrate-N is evaporated to dryness in a 25 ml. volumetric flask with 0·2 ml. 6% H_2O_2 (A.R.). The evaporation is best done in an electric oven at 110°. After the flasks have cooled, 1 ml. of phenoldisulphonic acid reagent (see Snell & Snell, 1936) is rapidly blown into each and swirled round to cover the deposit therein. The flasks are then set aside for ½ hr., after which about 10 ml. water and excess ammonia (sp.gr. 0·880) are added to each. The colour developed is proportional to (nitrate + nitrite)-N, and may be compared colorimetrically with that of a standard KNO_3 solution similarly treated.

(4) *Calculation of nitrate formation*

The results are usually expressed as μg. nitrate-N (or nitrite-N) formed/g. soil. They are obtained from the sample analyses in the following way.

Suppose 100 g. of soil are perfused with an initial volume of 200 ml. of ammonium sulphate solution and 10 ml. samples yield the following nitrate concentrations at the indicated times:

Duration of experiment in days	1	3	5
Concentration of nitrate-N of perfusate (μg./ml.)	5	25	75

Thus between 1 day and 3 days there is a rise in nitrate-N concentration of 20 μg./ml. occurring in 190 ml. solution (i.e. in the original 200 ml. minus the first 10 ml. sample). Therefore the total nitrate-N formation during this period is (20 × 190) μg. = 38 μg./g. of soil.

Similarly between 3 and 5 days the total nitrate-N formed is (180 × 50) μg. = 90 μg./g. of soil. Therefore between 1 day and 5 days each gram of soil has formed (38 + 90) = 128 μg. nitrate-N.

The figures of the first part of a typical experiment together with the appropriate calculations are given in Table 2. In this, as in all experiments, the nitrification is calculated as from the increase in nitrate concentration over that found on the *first* day. For the experiment detailed in Table 2, 30 g. allotment soil (sieved 1·0–4·0 mm.) were perfused with 200 ml. (initially) N/35 ammonium sulphate solution. Each sample taken from this solution was 10 ml. in volume. The whole unit was weighed before it was sampled, and any slight evaporation that was apparent was made up with distilled water 2 hr. before sampling; this has been a constant practice.

KINETICS OF CONVERSION OF NH_4^+ INTO NO_3^- BY PERFUSION OF AMMONIUM SALTS THROUGH SOIL

Owing to the fact that the nitrate anion is not absorbed upon the soil surface, serial nitrate analyses of the perfusate can yield an accurate estimate of the rate of nitrate formation.

A typical nitrification curve from an experiment in which 50 g. air-dried Rothamsted garden soil (4·0–1·0 mm. fraction) was perfused at 70° F. with 250 ml. N/140 ammonium sulphate solution is shown in Fig. 1. The curve has roughly the character of a

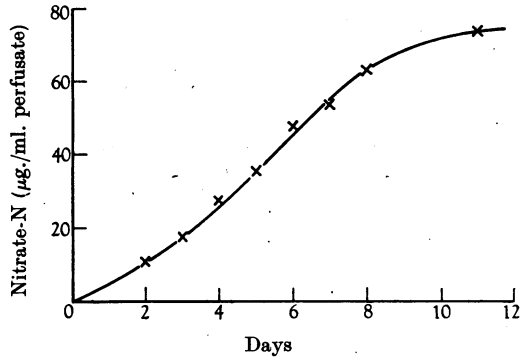

Fig. 1. Variation of nitrate-N (μg./ml. perfusate) with time (in days) of perfusion.

sigmoid curve whose midpoint (representing the most rapid rate of nitrification) falls at the point of half conversion of the total ammonia converted. The curve asymptotically approaches a nitrate value that represents about 80% conversion of ammonia into nitrate. The rest of the NH_4^+ has presumably been

Table 2. *The calculation of results in a typical experiment*

Duration of experiment (days)	1	5	7	9	12
Ammonium-N (μg./ml.) in perfusate	257	235	194	160	111
Nitrite-N (μg./ml.) in perfusate	3	7	1	0	1
Nitrate-N (μg./ml.) in perfusate	5	46	105	164	267
Rise in nitrate-N during intervals between sampling (μg./ml.)	—	41	59	59	103
Volume in which nitrate-N rise was developed (ml.)	—	190	180	170	160
Nitrate-N increase during intervals between sampling (mg.)	—	7·8	10·6	10·0	16·5
Total nitrate-N formed from day 1 (mg.)	—	7·8	18·4	28·4	44·9
Nitrate-N formed (μg./g. soil)	—	260	613	947	1500

synthesized into bacterial matter or insoluble (or strongly adsorbed) N compounds.

The curve is similar to the sigmoid curves encountered in bacterial proliferation studies and is of the kind to be expected if soil nitrification, under our experimental conditions, is brought about by cells that are proliferating. Table 3 shows the results of two perfusion experiments carried out with 30 and 100 g. air-dried Rothamsted garden soil.

Table 3. *Perfusion of air-dried Rothamsted garden soil*

Time after start of perfusion (days)	Ammonium-N in perfusate (μg./ml.)	Nitrate-N in perfusate (μg./ml.)	Nitrate-N (μg./g. soil)
Exp. A. 30 g. soil perfused with 200 ml. N/30 $(NH_4)_2SO_4$			
1	267	4	—
4	267	26	139
6	242	54	309
8	220	98	557
11	186	166	866
13	157	214	1156
Exp. B. 100 g. soil perfused with 250 ml. N/50 $(NH_4)_2SO_4$			
1	100	32	—
4	77	105	175
6	51	157	295
8	22	228	451
11	1	253	503

The results of four experiments on 100 g. soil perfused with N/50 $(NH_4)_2SO_4$ were as follows: nitrate-N (μg./g. soil) at the end of the period of maximum conversion of NH_4^+ to NO_3^-; (1) 503, (2) 537, (3) 530, (4) 521. These results illustrate the reproducibility of end-results obtained by the soil-perfusion technique.

The logistic or autocatalytic sigmoid curve, typical of the course of soil nitrification, shown in Fig. 1, has already been found to apply to nitrification in solution (Pulley & Greaves, 1932; Miyake, 1916). The curve has the equation

$$\log \frac{y}{A-y} = K(t-t_1),$$

where y = nitrate-N produced (μg./g. soil),
A = asymptotic value approach by y,
t = time from start of perfusion (days),
t_1 = time when $y = \frac{1}{2}A$, i.e. time of half completion,
K = constant.

The derivation of the equation is given by Miyake (1916); it is characteristic of an autocatalytic unimolecular reaction, which expresses the fact that the velocity of such a reaction is at any instant proportional to the amount of material undergoing change and to the amount of material already transformed. Miyake applied the equation to the results of Lipman, Blair, Owen & McLean (1912) on soil ammonification and to those of Warington (1879) on nitrification.

Table 4 shows the combined results of four separate experiments in which 100 g. of an air-dried

Table 4. *Course of nitrification*

(Allotment soil (100 g.) perfused with 250 ml. N/50 ammonium sulphate solution. Limiting value for nitrate formation (A) = 570 μg. nitrate-N/g. soil.)

Duration of exp. (t, days)	Nitrate-N formed (y, μg./g. soil)	Duration of exp. (t, days)	Nitrate-N formed (y, μg./g. soil)
3	82	7	233
3	130	7	302
4	175	8	451
4	187	8	495
5	194	10	530
5	185	10	511
6	295	11	503
6	330	11	537

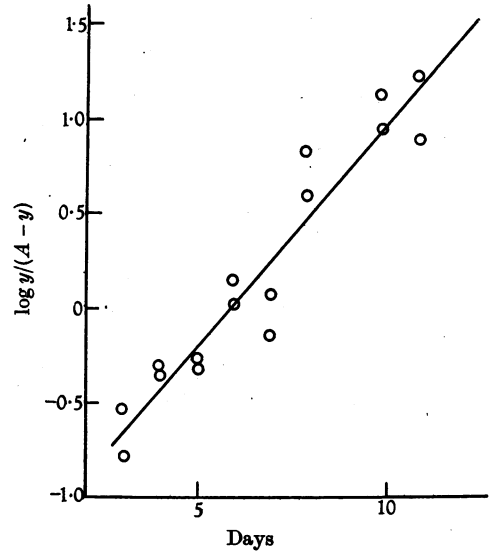

Fig. 2. Variation of $\log y/(A-y)$ with time (in days).

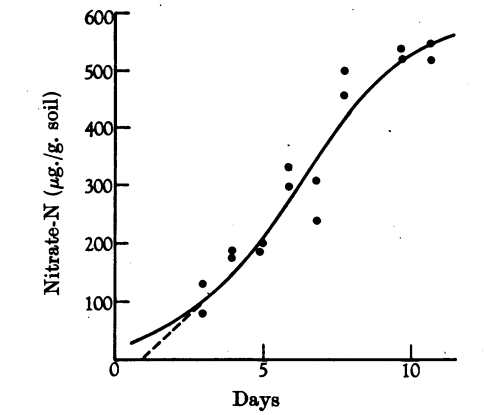

Fig. 3. Variation of nitrate-N formation with time (in days). —— autocatalytic curve; - - - - experimental curve.

Rothamsted garden soil were perfused with 250 ml. N/50 $(NH_4)_2SO_4$ solution. The curve obtained by plotting log $y/(A-y)$ against t is shown in Fig. 2, where the straight line drawn is fitted to the points by the method of least squares. It will be seen that the experimental values are closely distributed about this line. The fit of a logistic curve (having the K and t_1 found for the straight line in Fig. 2) to the unbiased freehand curve drawn to the results given in Table 4 is shown in Fig. 3. The fit is good except at the lower end of the curve, where inaccuracies in the nitrate-N estimations at the start of the perfusion experiment may be expected to occur.

Table 5. *Course of nitrification*

(Allotment soil (30 g.) perfused with 200 ml. N/35 ammonium sulphate solution. Limiting value for nitrate formation (A) = 2200 μg. nitrate-N/g. soil.)

Duration of exp. (t, days)	Nitrate-N formed (y, μg./g. soil)	Duration of exp. (t, days)	Nitrate-N formed (y, μg./g. soil)
4	139	8	645
4	165	9	948
5	260	9	758
6	307	11	866
6	351	11	1120
7	614	12	1497
7	488	13	1156
8	557	13	1430

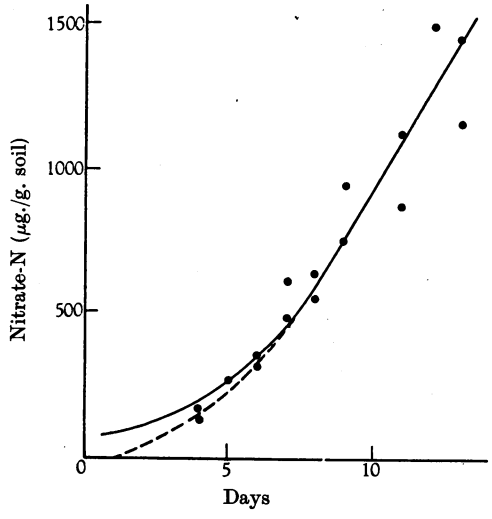

Fig. 4. Variation of nitrate-N formation with time (in days). —— autocatalytic curve; - - - - experimental curve.

An equally good fit to an autocatalytic curve is found for points from experiments in which 30 g. Rothamsted garden soil were perfused with 200 ml. N/35 ammonium sulphate solution. These experiments were run only until the pH of the perfusate had fallen to pH 6·3 from an initial value of pH 7·4, nitrification being then about two-thirds complete. It has been our standard practice to prevent, by suitable arrangement of the quantities of soil and perfusate, too great a pH fall (i.e. exceeding one unit) during the course of an experiment. The combined results of four experiments are shown in Table 5, and the relevant curves are shown in Fig. 4.

The results demonstrate that the course of nitrification in soil under the given experimental conditions is of the kind to be expected on the assumption that nitrification is accomplished by organisms proliferating in the soil.

The fact that these results can be shown to obey an expression deduced on the basis of bacterial multiplication and that this expression is identical with that found in the study of the nitrifying bacteria in pure culture, supports the conclusion that soil nitrification, under the given experimental conditions, is very largely or wholly a microbiological process.

Oxidation of the nitrite ion in soil

Since oxidation of NH_4^+ to NO_3^- in soil is held to be a two-stage process, nitrite being an intermediate product, and since only traces of nitrite are normally found in nitrifying soils, it follows that the conversion of nitrite to nitrate should be appreciably faster than that of NH_4^+ to NO_2^-. It also follows that the speed of oxidation of NH_4^+ to NO_3^- in soil is a measure of the speed of oxidation of NH_4^+ to NO_2^-.

Perfusion experiments with sodium nitrite solution have been carried out, with results which are shown in Table 6 and Fig. 5. In these experiments 200 ml. sodium nitrite solution were perfused through 30 g. air-dried Rothamsted garden soil (4·0–1·0 mm.), the nitrite analyses being made (colorimetrically) on samples of the perfusate. Three different nitrite concentrations were used. The amount of nitrite finally oxidized was less than the amount of nitrite introduced because some nitrite was removed in sampling and some was possibly eliminated by anaerobic denitrification as suggested by Corbet & Wooldridge (1940). Table 6 and Fig. 5 demonstrate that the speed of nitrite oxidation by soil, after a preliminary lag period, is extremely fast, so fast as to make it difficult to obtain an accurate measure of the velocity constant of the process. The speeds of nitrite oxidation after the initial lag periods as seen in Fig. 5 appear to be approximately parallel.

An approximate calculation from the curves given in Fig. 5 for the velocity constant K of the logistic equation $\log [y/(A-y)] = K(t-t_1)$, gives $K = 0{\cdot}60$ for M/560 $NaNO_2$. The velocity constant K for the process of nitrification of NH_4^+ has been found to be 0·145 for M/35 $(NH_4)_2SO_4$ (200 ml. perfused through 30 g. soil) and 0·225 for M/50 $(NH_4)_2SO_4$ (250 ml. perfused through 100 g. soil).

Table 6. *The oxidation of sodium nitrite by 30 g. garden soil*

	Nitrite-N oxidized (μg./g. soil)		
Concentration of sodium nitrite	M/560	M/140	M/35
Nitrite-N added (μg./g. soil)...	167	667	2670
Duration of exp. (days)			
2	0	0	0
3	26	0	0
4	66	0	0
6	163	123	0
7	—	276	0
8	—	425	0
9	—	620	0
11	—	—	122
13	—	—	790
14	—	—	1450
15	—	—	2400

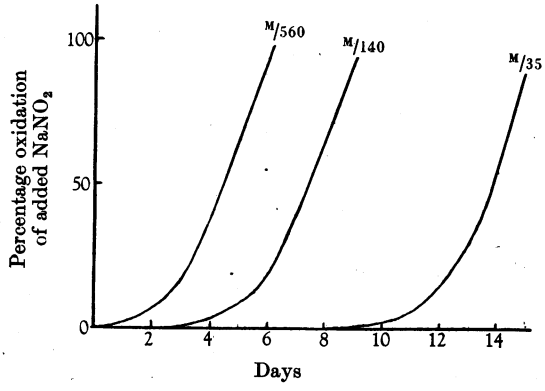

Fig. 5. Variations of rates of nitrite oxidation with time (in days) of perfusion for various concentrations of $NaNO_2$.

It is evident that the velocity of transformation of nitrite into nitrate is much greater than that of ammonium into nitrate under similar experimental conditions.

The kinetics of nitrite oxidation in soil under the given experimental conditions are those to be expected if the oxidation is accomplished by cells that are proliferating, the speed of oxidation being large enough to explain the fact that during normal nitrification of ammonia little or no nitrite appears during the process.

THE EFFECTS OF POISONS ON SOIL NITRIFICATION

The effects of poisons were studied by adding these substances to the perfusion fluid at the start of the experiment and comparing the rate of formation of nitrate with that in a control experiment.

Chloretone. At a concentration of 0·4 % chloretone (trichloro*iso*butanol) causes complete cessation of nitrification (see Table 7), and no nitrite is formed at this concentration. Chloretone is a useful inhibitor of metabolic changes in soil, for it is water soluble (to 0·4 %), of low volatility, contains no nitrogen and does not affect the ionic equilibria between soil and soil solution. Apparently it is only slowly changed in soil, but further experiments are required to be certain of this.

Ethylurethane. At a concentration of 0·1 % (0·011 M) ethylurethane suppresses at least 90 % of the nitrification in soil (Table 7). Meyerhof (1916) has already shown that urethane powerfully inhibits the metabolism of one of the isolated nitrifying bacteria studied in absence of soil. He found that 0·016 M-ethylurethane inhibits the respiration of the nitrite-forming cells by 42 %, whilst 0·11 M-ethylurethane inhibits the respiration of the nitrate formers (*Nitrobacter*) by only 4 %. The inhibitory effect of ethylurethane on soil nitrification may be largely due to this suppression of the metabolism of the nitrite formers. The inhibitive effect of urethane on soil nitrification is reversible and may be removed by washing the soil (Table 8).

Table 7. *The effects of various poisons on nitrification in Rothamsted garden soil*

Exp. (1) 150 g. soil perfused with 250 ml. N/35 $(NH_4)_2SO_4$ for 9 days.
Exp. (2) 100 g. soil perfused with 250 ml. N/50 $(NH_4)_2SO_4$ for 10 days.
Exp. (3) 100 g. soil perfused with 250 ml. N/25 $(NH_4)_2SO_4$ for 14 days.
Exp. (4) 150 g. soil perfused with 250 ml. N/85 $(NH_4)_2SO_4$ for 11 days.

Exp. no.	Poison in perfusate	Rise in nitrate-N concentration of perfusate (μg./ml.)
1	None	266
	0·011 M-Ethyl urethane	14
2	None	230
	M/100-Cadmium sulphate	51
	M/25-Cadmium sulphate	2
3	None	533
	M/250-Quinhydrone	0
	M/250-Catechol	195
	M/250-Sodium β-naphthoquinone-sulphonate	309
4	None	137
	0·4 % Chloretone	0

Quinones. The bacteriostatic action of certain quinones is well known and the inhibitions by hydroquinone, catechol and β-napthoquinone-sulphonate of soil nitrification noted in Table 7 are not unexpected.

Cadmium. Meyerhof (1916) has made an extensive study of the effect of metallic cations on the respiration of isolated nitrifying bacteria growing in a suitable culture media. We have studied the effects of a few cations on soil nitrification and observed the toxic action of cadmium (Table 7).

Chlorate. This substance at a molar concentration of 10^{-5} selectively suppresses the conversion in soil of NO_2^- to NO_3^-, so that when ammonium salts in presence of chlorate are perfused through soil, nitrite instead of nitrate accumulates. A full description of the effects of chlorate has already been given (Lees & Quastel, 1945).

PERSISTENCE OF POISONS IN SOIL

For these experiments 50 g. portions of allotment soil in perfusion tubes were treated with 25 ml. (enough to saturate the soil) of a solution of a bacterial poison and the tubes left overnight. Control tubes were treated with 25 ml. distilled water. Next morning the soil in each tube was washed with 15 separate lots of 25 ml. distilled water added at 5 intervals. Tests on the washings showed that this treatment had removed excess poison from the soil. The tubes, containing the washed poisoned soils, were then fitted into perfusion units and perfused with 200 ml. N/50-ammonium chloride solution. The nitrate accumulation in each perfusate was then followed by the usual analytical procedures.

The results (Table 8) show the nitrate accumulation in each perfusate, measured when the unpoisoned (control) tubes had almost completed nitrification. The toxic effect of urethane on the nitrifying bacteria was eliminated by water-washing of the soil, whereas the toxic effect of quinhydrone was only partially alleviated under similar experimental conditions. The residual effect of cyanide was surprisingly high. Meyerhof (1916) noted that cyanide inhibits the respiration of nitrifiers, but such inhibitions are usually reversible. The residual inhibition of nitrification by formaldehyde is, within experimental error, complete. This result might have been expected from the well-known use of formaldehyde as a soil-sterilizing agent. Hydroxylamine and hydrogen peroxide appear to have little or no residual effect. This negative result may be due to destruction of these substances by non-biological changes in the soil before their irreversible toxic effects could become operative.

Table 8. *The residual effects of poisons on nitrification of ammonium chloride by Rothamsted garden soil*

Duration of experiments, 14 days. Samples of 50 g. soil.

Soil initially treated with 25 ml. of	Rise in nitrate-N of perfusate (μg./ml.)
Water (2 tubes)	257 (mean)
M/10-Sodium cyanide	10
M/250-Quinhydrone	63
1% Formaldehyde	4
M/250-Ethylurethane	257
M/10-Hydroxylamine-HCl	238
1·5% Hydrogen peroxide	212

DISCUSSION

The experimental results which have been quoted, both those bearing on the kinetics of oxidation in soil of NH_4^+ and NO_2^- and those bearing on the effects of biological poisons on soil nitrification, make it clear that under the conditions obtaining in the perfusion apparatus, nitrification in soil is very largely, if not wholly, a biological process. Whilst this conclusion is entirely in accordance with that of previous workers, our results show that with the perfusion technique it is possible to study metabolism in soil with something of the accuracy obtainable in investigations on plant and animal tissues.

The next step is to obtain information on the conditions underlying the process of nitrification in soil. The investigation of these conditions will form the subject of a further paper.

Addendum. A Soil Perfusion Apparatus

By H. LEES

The apparatus described embodies no new principle, but it provides in a cheap and simple form a means of complying with the essential needs. It consists of two parts:

(1) *The reciprocator* (Fig. 6). This is a 300 ml. Buchner flask fitted with a bung and two tubes as shown. Tap water is run in steadily and slowly through the side arm via a capillary tube, so that the flask alternately fills up and then rapidly empties by means of the siphon tube into a drain, as in the Soxhlet apparatus. The fill-up time is adjusted to about 3 min. At each cycle air is first slowly forced out and then rapidly sucked in. This alternating air-flow is used to drive the perfusion unit proper, shown in Fig. 7.

(2) *The perfusion unit* (Fig. 7). Between 20 and 50 g. of sieved soil is held in a glass tube, 10 × 1 in., by means of a glass-wool plug; a further plug on top of the soil minimizes puddling of the soil surface. (Earlier models took up to

200 g. soil, but this capacity was found not to be necessary.) The bottom end of the tube is fitted with a one-hole rubber bung into which is inserted the side arm of a T-piece, the main tube of which is continued vertically upwards for about 10 in. to terminate in a wide mouth, and vertically downwards through the bung of, and almost to the bottom of, a 300 ml. Buchner reservoir. Through the other hole of the reservoir flask bung runs the water delivery or 'lift' tube, a straight piece of glass tube 16 in. long with a widened lower end. The upper end of this tube is jointed by rubber tubing to a bent 4 in. capillary (bore 1 mm.) which passes through one hole in the top bung of the soil tube. The other hole in this bung is occupied by a 6 in. piece of fine capillary communicating with the atmosphere.

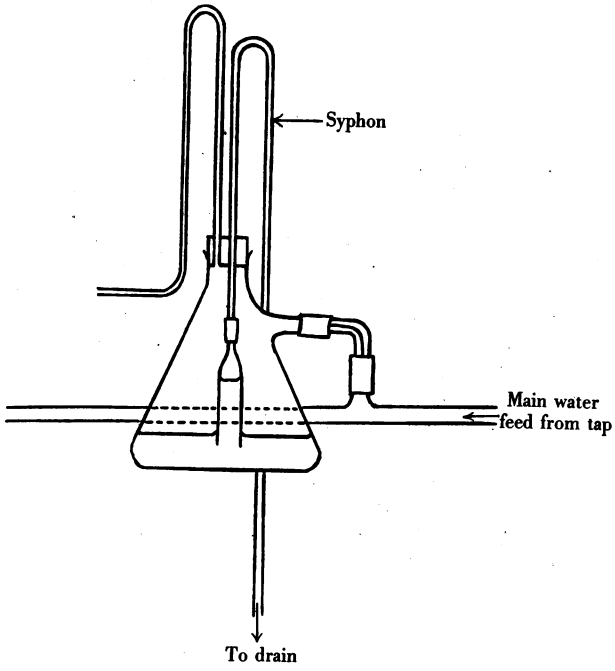

Fig. 6. The reciprocator.

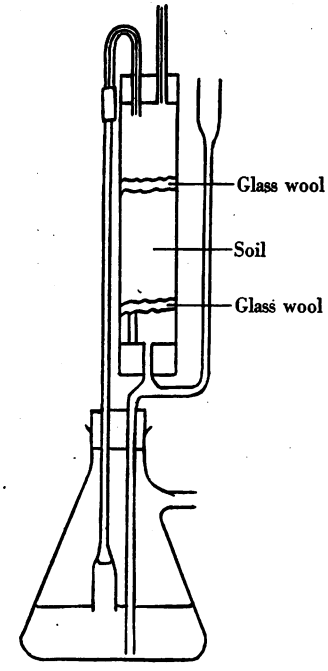

Fig. 7. The perfusion unit.

At the start of an experiment the soil is saturated with part of a measured volume of the solution to be studied and the remainder of the solution (about 200 ml.) is put in the reservoir flask. The lift tube in the flask is then adjusted so that its wide end just touches the reservoir fluid (the rubber joint of the lift tube gives room for this adjustment) and the side-arm of the reservoir flask is connected with the air line of the reciprocator. The action is then as follows:

(i) *During the filling of the reciprocator.* Owing to the inflow of air from the reciprocator the pressure in the reservoir flask rises and fluid is forced up the lift tube. The reservoir level is therefore gradually lowered until the lift tube breaks the surface. The fluid in the wide end of the lift tube immediately falls back into the main body of fluid in the reservoir, but a small column is held by surface tension in the narrow part of the tube. As the pressure is still maintained the small column of fluid in the lift tube is driven upwards on to the top of the soil whence it commences to percolate through the soil and, in time, back into the reservoir flask. Meanwhile as soon as the lift tube is free of fluid, air escapes into the atmosphere. But because both air outlets are capillaries the escape is not instantaneous and for a short time the air above the soil is at a higher pressure than atmospheric while the lower end of the soil column remains at atmospheric pressure. There is thus established above the soil a small transitory pressure of air. This pressure causes air to be gently forced through the soil which is thus cleared of water and aerated simultaneously, and this pressure is maintained until all the pressure built up by the reciprocator is released. Meanwhile fluid has been running through the soil and back into the reservoir flask and eventually enough fluid runs through to enable the bottom of the lift tube to touch the surface again, whereupon the whole cycle is established. Therefore, as long as air is supplied to the reservoir side arm, perfusion of the reservoir fluid takes place.

(ii) *During the emptying of the reciprocator.* When the reciprocator begins to siphon, a suction is applied to the side arm of the reservoir flask whose internal pressure therefore drops below atmospheric. Since the only relatively unhindered passage for outside air into the reservoir flask is through the immersed tube, air is drawn mainly through it, the reservoir fluid being thus mixed and aerated by bubbling. This bubbling and mixing continues until the reciprocator is empty, when the perfusion cycle recommences.

Modification of the apparatus

In order to widen its scope, the apparatus has been somewhat modified so that the incoming air can be freed of any constituent (say CO_2) and any

constituent of the gases evolved by the soil easily measured.

This modification consists in the fusion of the end of the free capillary and the upper end of the external aerator tube (see Fig. 7). At the point of fusion a common out-lead is taken; this out-lead is the only point at which gas is expelled from the apparatus. Air is forced into the apparatus through a bubbler capillary reaching to the bottom of the reservoir flask. A constant air pressure (the operative pressure for the perfusion action) is maintained down this capillary. In effect, therefore, the apparatus works just as the original one did, except that there is no suction phase; the aeration and mixing of the fluid being provided by the constant bubbling caused by the intake of air through the bubbler capillary. In practice air is not actually forced down the bubbler but instead suction is applied (by means of a water pump) to the point of fusion of the external tubes. The suction is reduced to and stabilized at 3 cm. Hg for smooth operation.

As the air flow is now unidirectional, the air entering the apparatus can easily be freed of any constituent and the composition of the outflowing air determined with equal ease. Gaseous products of soil metabolism can therefore be measured.

A further modification at present under test is to use a diaphragm-pump apparatus to circulate gas from the outlet tube to the bubbler-capillary of the form of apparatus just described. It is hoped that by this means pure nitrogen may be circulated and true soil-anaerobiosis conveniently studied.

SUMMARY

1. A perfusion technique is described for the study of metabolism in soil. The advantages and shortcomings of the technique are described.

2. The kinetics of conversion of NH_4^+ to NO_3^- in soil, by the perfusion technique are investigated. The rate of formation of nitrate in soil follows the sigmoid curve given by the equation:

$$\log \frac{y}{A-y} = K(t-t_1),$$

where y = nitrate-N produced/g. soil,
A = asymptotic value reached by y,
t = time (days) from start of perfusion,
t_1 = time when $y = \frac{1}{2}A$,
K = constant.

This expression may be deduced from the hypothesis that soil nitrification is entirely due to the activity of cells that are multiplying.

3. The kinetics of conversion of NO_2^- to NO_3^- have also been studied. This oxidation proceeds much faster than that of NH_4^+ to NO_3^- under similar experimental conditions.

4. A number of poisons, known for their inhibitory effects on cell metabolism, greatly suppress soil nitrification. Chloretone (0·4 %) produces 100 % inhibition. Ethylurethane (0·1 %) produces over 90 % inhibition, the effect being reversible. Quinones are also effective poisons. The inhibitory effects of sodium cyanide, quinhydrone and formaldehyde are not quickly reversed by washing the soils after treatment with the poisons.

5. All the facts support the conclusion that under the given experimental conditions the entire process of conversion of NH_4^+ to NO_3^- in soil is accomplished by micro-organisms. The kinetics of the process in soil may be studied by the perfusion technique with an accuracy approaching that obtained in the study of isolated animal or plant tissues.

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Biochemistry of Nitrification in Soil

2. THE SITE OF SOIL NITRIFICATION

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Chick, in her interesting paper (1906) on the study of nitrification with reference to sewage purification, states that it had been long upheld that a most important preliminary to nitrification both in soil and in sewage filters was an absorption of NH_4^+ or ammonium salts on surfaces of soil particles or filtering medium. In the case of soil, a long controversy had taken place as to whether a physical or chemical process was involved, and the former view on the whole prevailed. Chick, however, concluded against an absorption theory, since nitrification took place in sewage filtering through coke, although no absorption of NH_4^+ took place on the coke.

The problem of the site of nitrification in soil has not been seriously considered for many years, and it has been probably taken for granted that since nitrification takes place in isolated culture media, soil nitrification also occurs at the expense of NH_4^+ held in solution in the soil moisture. Albrecht & McCalla (1937), however, have obtained evidence that NH_4^+ held in the base-exchange complex of clay may be oxidized by bacteria, indicating that NH_4^+ need not be in the aqueous phase in order to be attacked by the nitrifying bacteria. McCalla (1941) has suggested that the bacteria themselves act as base-exchangers and can exchange cations with those taken up in base-exchange complexes (such as clay). This theory is a development of the recent views of Jenny & Overstreet (1940) who postulate a base-exchange between plant roots and soil complexes.

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Waksman, Renszer, Carey, Hotchkiss & Renn (1933), Peele (1936), Rubentschik, Roisin & Bieljinsky (1936), Conn & Conn (1940) and ZoBell (1943) have all either shown that bacteria can be adsorbed on various surfaces or have investigated the effects of surfaces on bacterial activity. Waksman *et al.* (1933), for example, found that nitrifying bacteria of the sea water in the Gulf of Maine were largely adsorbed on to bottom-mud.

Little, however, has been done to determine whether nitrification as it occurs normally in soil takes place wholly or partly at soil surfaces, whether NH_4^+ in soil solution or adsorbed in the base-exchange complexes of soil forms the actual substrate of the nitrifying bacteria in soil, or whether the kinetics of nitrification in soil are dependent on the physico-chemical conditions obtaining in soil.

We have attempted to answer these questions by experiments carried out with the soil-perfusion technique described previously (Lees & Quastel, 1946). The following paper describes the results obtained.

EXPERIMENTAL

Methods

The analytical methods are fully described in our previous paper (Lees & Quastel, 1946).

Soil solution

The evidence shows that in comparison with the nitrification that takes place on the soil little nitrification takes place in the soil solution. This was