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ENZYME ACTIVITY IN TERRESTRIAL SOIL IN RELATION TO EXPLORATION OF THE MARTIAN SURFACE

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PREFACE

Our objectives are to explore enzyme activities in soil, including abundance, persistence and localization of these activities, and to develop procedures for detection and assay of enzymes in soils suitable for presumptive tests for life in planetary soils.

We have developed a suitable extraction procedure for soil enzymes and have been measuring activities in extracts in order to study how urease is complexed in soil organic matter. Nearly 30 percent of soil enzymes can be isolated as colloidal, clay-free suspensions.

Mathematical models have been developed, based on enzyme action and microbial growth in soil, for rates of oxidation of nitrogen as nitrogen compounds are moved downward in soil by water flow. These bio-geo-chemical models should be applicable to any percolating system, with suitable modification for special features, such as oxygen concentrations, types of hydrodynamic flow, etc.

The Ubiquity of Some Soil Enzymes and Isolation of Soil Organic Matter with Urease Activity.

Since some enzymes seem to be present in extracellular organic matter in soil, questions arise as to how long their activities may persist and in what state they are preserved. Samples of soil (about 35) were collected fresh and from buried and stored sources, and tested for different activities, the sensitivities of which are given in Table I (Skujins and McLaren 1968). Based on jackbean urease as a reference, as little as circa 2×10^{-14} moles urease per gram of soil can be detected; the other methods are also quite sensitive.

Some representative data for a few of the soils are given in Table II. Aiken (clay), Columbia (fine sandy loam), Dublin (clay loam) and Yolo (silt loam) soils stored 6 to 12 years (not listed) had all five enzyme activities.

Measurable dehydrogenase activities were detected in relatively fresh soils only; these did not reflect microbial numbers or organic matter present but may be correlated with endogenous respiration rates of active microbes.

Urease and phosphatase activities were observed in circa 9000 year-old permafrost peat soil samples whereas in a 32,000 year-old buried silty

soil (3.3% organic C) these were undetectable. With more extensive data than those given in Table II it was possible to conclude that in all three types (permafrost, stored desert, and air-dried) urease activity more closely reflected the organic matter content and not necessarily the microbial count. Such correlations with phosphatase activity were less certain.

During a period of 26 months of storage, a number of latosol clay soils showed changes in urease activity, generally a decrease, but one showed an increase of about 9 percent. Although no activity was found in two high salinity soils, activity was found in twelve other soils ranging in pH from 4.2 to 9.4.

Evidently soil urease in aged soils is not like jackbean urease (the first enzyme to be crystallized) in that no increase in activity was obtained by adding cysteine. Jackbean urease is active only in the reduced state and one would expect that any SH-active forms in soil would be at least partially in an oxidized state (Skujins and McLaren 1969).

The influence of electron-beam sterilization of soils on urease activity was variable: some showed an increase, others a decrease. It is not clear as to whether irradiation leads to a release of some urease from dead soil organisms or to an increase of permeability of cell membranes to the substrate (McLaren et al. 1957; Skujins and McLaren 1969). The subject has been reviewed (Cawse 1973).

Our method of measuring urease activity reveals some activity in a Dublin clay loam at a relative humidity as low as 80 percent, which is below the humidity at which soil microbes can multiply readily. Since the relative humidity on Mars is less than one percent, one would not expect

to find any enzyme activity or microbial growth unless water is localized, for example, at the edge of a melting ice pack (Skujins and McLaren 1971).

Paulson and Kurtz, in a heuristic work, measured the increases in urease activity and of numbers of ureolytic microorganisms in a soil following addition of ammonium sulfate and dextrose. Some of their data are replotted in Figure 1; if the lower urease activity curve is extrapolated to zero population a large ordinate of urease activity remains. After a maximum population has been reached (after about a day and a half) the population declines and so does the whole soil urease activity, as illustrated by the upper curve. Such data have been interpreted to mean that there is an extracellular background "noise level" of enzyme activity attributable either to ureases adsorbed in and on the clay minerals or within the soil organic matter (see, e.g., J. R. Ramirez and A. D. McLaren 1966, for similar work with soil phosphatase). Since adsorption on clays is not a serious impediment to the use of enzyme-proteins as food-stuffs by soil microbes, such as, e.g., by Pseudomonads (Estermann and McLaren 1959), adsorption cannot explain the persistance of urease-proteins in a soil as such a large fraction of measurable urease. Small amounts of soluble urease-active substances have been extracted from soil, e.g. Figure 2, (Briggs and Spedding 1963) but by no means in amounts commensurate with the total activity found in the soil. This leaves soil humus per se as the most likely carrier of the more stable fraction of soil urease activity, as represented by the intercept in Figure 1. This conclusion seems to have been reached by Conrad over 30 years ago (Conrad 1940).

Another method of extraction (Chalvignac and Mayaudon 1971), Figure 2,

successful in a special case (isolation of a tryptophan metabolizing enzyme) gave a product without urease activity but with a trace of tryptic activity (hydrolysing benzoylarginine amide). With these observations in mind we decided to try to extract a significant fraction of soil urease(s) with a combination of physical and chemical steps. Dublin clay-loam, air-dried, was suspended in phosphate buffer at 4^{o}, pH 7, containing urea to break hydrogen bonds in the humus, NaCl to reduce the enzyme denaturing effect of urea, EDTA to reduce cationic cross-linking within the humus and mercaptoethanol to preserve a reduced state of any SH-urease present. Preliminary treatment of a soil suspension by ultrasonic vibration may give better dispersion of the organic matter and chloroform may be added as a biostatic agent.

After a few hours the treated suspension is filtered with the aid of a series of bacteriological, porous filter candles with decreasing pore size to remove microbes and clays. Following dialysis of the filtrate a dark brown precipitate appears. The non-filterable residue may be extracted successively with phosphate solutions of increasing dilution and the additional extracts may be filtered and dialysed to increase the overall yield to between 20 and 40 percent of the original soil activity. By X-ray analysis these precipitates, after dialysis, were free of clays and accounted for over one third of the extracellular urease as estimated from plots of the kind shown in Figure 1. Urease activity in the precipitates was not destroyed by treatment with a broad spectrum mixture of proteolytic enzymes (Pronase). A similar statement may be made for urease activity in the soil per se, Table III (Burns et al. 1972).

A modification of the original method of Burns et al. is shown in

Figure 3. By dialysis of supernatant solutions against EDTA a urease-active sol is obtained instead of an active precipitate (PPT).

It may be safely concluded that soil urease(s) resides in organic colloidal particles with pores large enough for water, urea, ammonium and CO_2 to diffuse but the pores are too small to allow entry of proteolytic enzymes of microbial origin.

This enzyme-active organic matter, i.e. extracted humus, representing such a large fraction of the soil organic carbon, should be a favorable material for general research. Since it retains active enzymes it must be more nearly representative of soil organic matter in situ than are "humic acids" of old.

Incidentally, a mixture of jackbean urease-bentonite and lignin is also resistant to Pronase but adsorption of the enzyme on bentonite does not impart such protection (c.f., Estermann and McLaren 1959).

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Table I

Sensitivity of enzyme assays

Activity	Product	Minimum detectable µM/g soil
Catalase	O_2	0.1
Dehydrogenase	triphenylformazan	0.001
Esterase	phenol	0.005
Phosphatase	beta-naphthol	0.02
Urease	$^{14}CO_2$	0.008

Table II

Enzyme activities in soils

Soil	Age	% organic C	Microbes[1]	P[2]	U[2]	D[2]
Dublin	stored 12 years	2.8	2×10^6	1.4	1.2	0.003
Staten (muck)	fresh	24	2×10^7	0.3	0.5	--
Annanah (silt)	stored 6 years	1.1	8×10^6	0.1	0.08	0.002
Point Barrow	permafrost 9000 years	19	2×10^3	0.8	0.08	0.001
Hilgard (sandy)	stored 60 years	0.16	4×10^4	0.02	0.03	0
Hilgard (sandy)	stored 60 years	0.56	5×10^5	0.1	0.06	trace

[1]Microbes per gram soil. The number for the Point Barrow soil may be contamination.

[2]Activities in μM/g soil/hr substrate reacting in tests for phosphatase (P), urease (U), and dehydrogenase (D) in samples of soil.

Table III

Resistance of dispersed soil urease to the action of Pronase, pH 7.

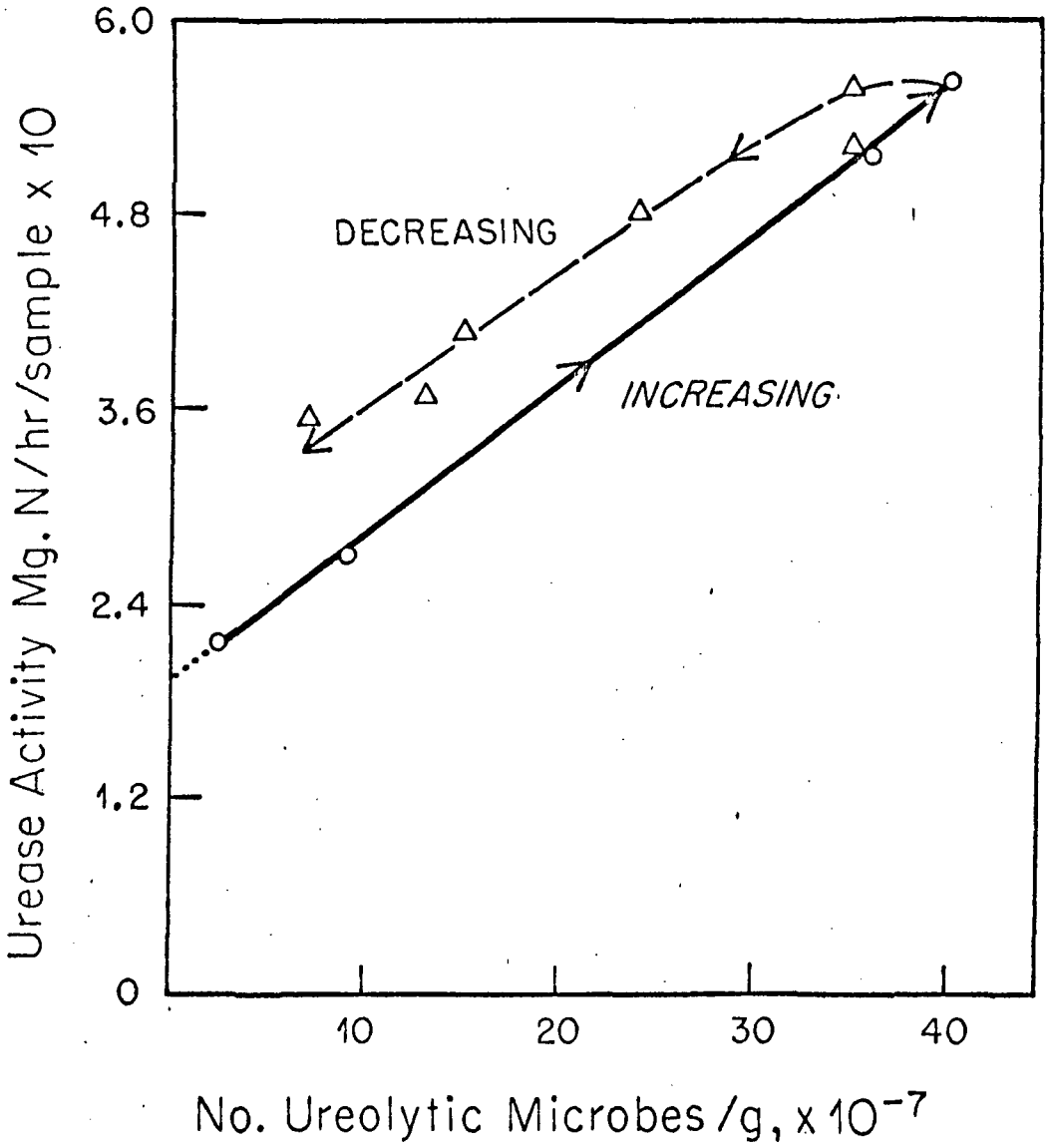
Mixture	μM NH_3 liberated/hr from urea after incubation with pronase
a) precipitate alone 40 mg	1.5
b) precipitate + pronase 40 mg 0.5 mg	2.0
c) precipitate + pronase 40 mg 0.5 mg	0.4 (no urea present)
b) - c)	1.6
d) soil alone	0.3
e) soil + pronase 1 g 0.5 mg	0.6
f) soil + pronase 1 g 0.5 mg	0.3 (no urea present)
e) - f)	0.3

LEGEND TO FIGURES

Fig. 1. Trend in soil urease activity with population changes of ureolytic microorganisms in a Drummer silty clay loam. Adapted from Paulson and Kurtz, 1969.

Fig. 2. Application of the procedure of Briggs and Segal (1962), left side, and of Chalvignac and Mayaudon (1971), right side, to Dublin soil.

Fig. 3. Application of the procedure of Burns et al. (1972) to Dublin soil for extraction of organic matter high in urease activity.



McLaren and Pukite

Figure 1

Figure 2

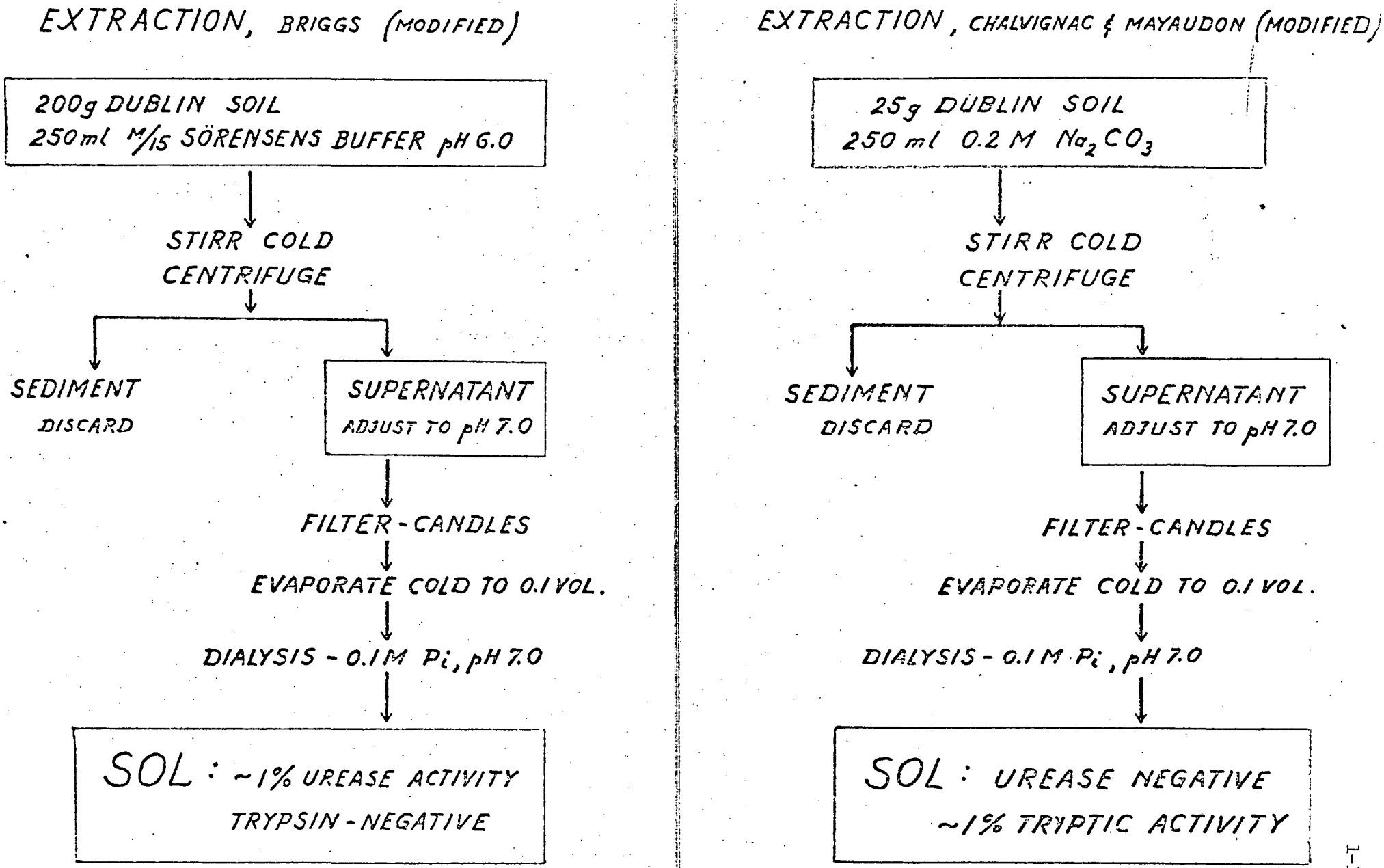
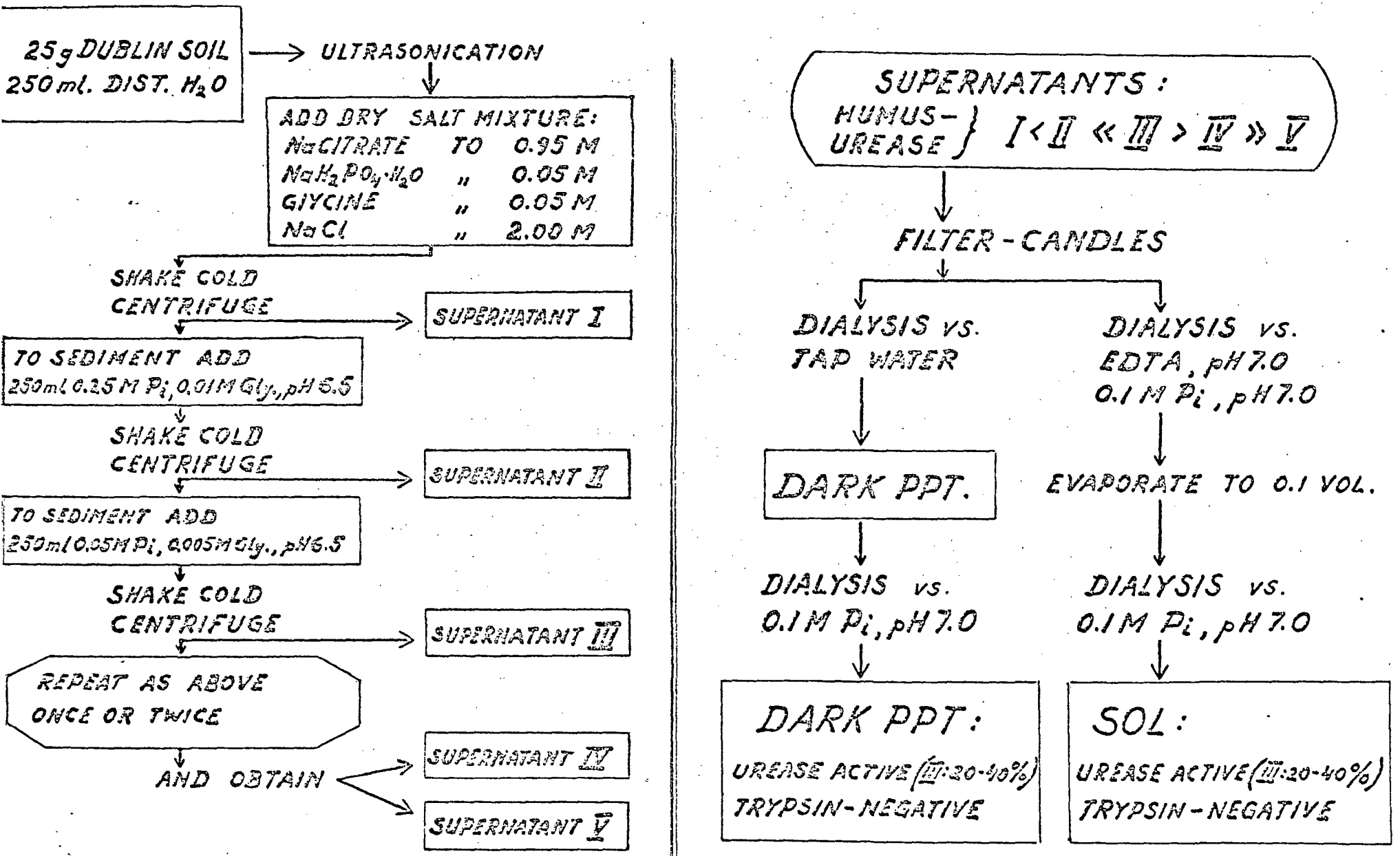

EXTRACTION, BRIGGS (MODIFIED)
200g DUBLIN SOIL
250ml M/15 SÖRENSENS BUFFER pH 6.0
STIRR COLD
CENTRIFUGE
SEDIMENT
DISCARD
SUPERNATANT
ADJUST TO pH 7.0
FILTER-CANDLES
EVAPORATE COLD TO 0.1 VOL.
DIALYSIS - 0.1M Pi, pH 7.0
SOL : ~1% UREASE ACTIVITY
TRYPSIN-NEGATIVE
EXTRACTION, CHALVIGNAC & MAYAUDON (MODIFIED)
25g DUBLIN SOIL
250 ml 0.2 M Na2CO3
STIRR COLD
CENTRIFUGE
SEDIMENT
DISCARD
SUPERNATANT
ADJUST TO pH 7.0
FILTER-CANDLES
EVAPORATE COLD TO 0.1 VOL.
DIALYSIS - 0.1 M Pi, pH 7.0
SOL : UREASE NEGATIVE
~1% TRYPTIC ACTIVITY

Figure 3

EXTRACTION OF HUMUS - BURNS METHOD



A Vector Biochemical Approach to Consecutive Reactions in Soil

Typically, in a laboratory experiment in soil microbiology, both nutrients and excreted products of metabolism and organisms are mixed in space. The system may be open to air but is closed to non-volatile substances. In soil in situ, however, there is a translocation of nutrients and products such that organisms at a lower level do not experience a composition of solutes identical with that to which organisms are exposed at more shallow depths. This applies to series such as

$$H_2S \xrightarrow{1} S \xrightarrow{2} S_2O_3^{=} \xrightarrow{3} SO_4^{=} \quad \text{and} \quad NH_4^{+} \xrightarrow{1} NO_2^{-} \xrightarrow{2} NO_3^{-}$$

Using nitrogen as an example, we may write

$$d(NH_4^{+})/dt \qquad \text{and} \qquad d(NO_2^{-})/dt$$

for changes in concentrations with time at any depth in soil. Integration of concentrations with respect to depth, however, gives vector

quantities $d(NH_4^+)/dX$ and $d(NO_2^-)/dX$ (Rose 1966). At the surface of a soil washed free from soluble nitrogen compounds and to which ammonium is applied, microbes will find no nitrite or nitrate. Microbes at a lower level will be bathed by all three ions in amounts that depend on reaction rates above them and on time required to transport reactants and products to that depth. This problem has already been analyzed in some detail (McLaren 1970).

The rate of oxidation of a nutrient in a given soil volume element (the niche) at a depth X from the surface depends upon the number of cells or biomass. For autotrophic nitrifiers inorganic nitrogen acts as a substrate for growth per se and as a source of energy for growth and maintenance. The overall rate of consumption of ammonium for example can be described by

$$-\frac{d(NH_4^+)}{dt} = (A_1 + B_1)\,\gamma_1 m_1 + \alpha_1 m_1 + k_1' m_1 \qquad [1]$$

where (NH_4^+) is substrate concentration, m_1 is biomass, A_1 is N oxidized for energy for cell growth per unit weight of biomass synthesized, B_1 is the amount of ammonium nitrogen incorporated in cells per unit weight of biomass synthesized, γ_1 is the specific growth rate ($\gamma_1 = d \ln m_1/dt$), α_1 is the N oxidized per unit weight per unit time for maintenance and k_1' is the N oxidized per unit weight of biomass and time in ways that do not lead to growth and maintenance (McLaren 1970).

The last term, $k_1' m_1$, can include oxidation of nitrogen accompanied by heat production and by the synthesis of intracellular polymers such as glycogen, polyphosphate and polyhydroxy butyrate (e.g., see Van Gool et al. 1971) and presumably of extracellular mucus (Winogradsky 1949).

According to Forrest and Walker (1971) maintenance requirement is insignificant compared to energy required for growth, and the results of Erh et al. (1967) and others clearly show that the $k_1'm_1$ term is overwhelmingly greater than the $B_1\gamma_1 m_1$ term. In other words to a close approximation, if one is interested in following the conversions of nitrogen from a biogeochemical point of view as during nitrification and translocation of nitrogen in soil, a good approximation is given by

$$- d(NH_4^+)/dt = (A_1\gamma_1 + \alpha_1 + k_1') m_1 \qquad [2]$$

In a sense NO_2^- is a waste product for all of these but only the last term of equation [1] represents waste metabolism. (The subscript 1 indicates the reaction $NH_4^+ \longrightarrow NO_2^-$ is involved.) Since oxidations of NH_4^+ or NO_2^- are enzymatic, and based on growth studies generally (McLaren 1970, Powell et al. 1967), γ_1 may be set equal to

$$\frac{\gamma_\infty (S)}{K_g + (S)} ,$$

α_1 may be set equal to

$$\frac{\alpha_\infty (S)}{K_a + (S)}$$

and k_1' may be set equal to

$$\frac{k_1''\beta_1 (S)}{K_m + (S)}$$

where S is substrate and γ_∞, α_∞, and k_1'' are maximum values obtainable (McLaren 1970). Here β_1 is the amount of enzyme per unit biomass, k_1'' is a proportionality constant and K_g, K_a, and K_m are saturation constants. The K constants will be considered as equal in so far as the enzyme system is common to reactions represented by the three terms. The relative values of $A_1\gamma_1$, α_1, and k_1'' are not generally known but the ratio $k_1''/(A_1\gamma_1 + \alpha_1)$ may be large for lack of an energy coupling agent (such as a phosphate limitation) (Forrest and Walker 1971) or for a coupling failure due to ineffective use of ATP synthesized as in the absence of CO_2: $(A + B)\, dm/dt = 0$ (N. Walker, private communication).

With the above expressions for γ_1 and k_1'' equation [2] becomes

$$-d\,(NH_4^+)/dt = \frac{(NH_4^+)\, m_1}{K_{m_1} + (NH_4^+)}\,(A_1\gamma_\infty + \alpha_\infty + k_1''\beta_1) = \frac{k_1 m_1\,(NH_4^+)}{K_{m_1} + (NH_4^+)} \qquad [3]$$

where k_1 is the sum of constants. This form of equation [1] can be used to construct a model for the variation of concentrations of nitrification intermediates with depth in soil columns.

By analogy with equation [3] we can write an equation for oxidation of nitrite if this is the only form of inorganic nitrogen supplied, namely

$$-d(NO_2^-)/dt = \frac{k_2 m_2\,(NO_2^-)}{K_{m_2} + (NO_2^-)} \qquad [4]$$

where the subscript 2 applies to the second oxidation step.

If a column of soil is perfused with a solution of nitrite for a few days the population m_2 can increase to a maximum and $m_2 = m_{max_2}$; we define $K_2' = k_2 m_{max_2}$. Of course, when this is true $A_2\gamma_\infty \neq d \ln m_2/dt = 0$. This population is not necessarily the ultimate maximum population: the greatest population reached may be limited by the steady state entering concentration $(NO_2^-)_o$ supplied to the column, be it optimum or less (or in excess with attendant toxicity), by some other limiting nutrient, or if all of these are optimal by spacial considerations (McLaren and Ardakani 1972).

The integral form of equation [4] is

$$(NO_2^-) = K_m \ln \frac{(NO_2^-)_o}{(NO_2^-)} + (NO_2^-)_o - K_2't \qquad [4a]$$

Replacing t by X/tk_o, and ignoring hydrodynamic dispersion, as a first approximation, we have

$$(NO_2^-) = K_m \ln \frac{(NO_2^-)_o}{(NO_2^-)} + (NO_2^-)_o - K_2X \qquad [4b]$$

where $K_2 = K_2'/\varepsilon k_o$; k_o is the entering flow rate of nitrite; εk_o is the rate of flow in the column; and ε is a proportionality constant.

The data in Figure 1 show how nitrite is oxidized in a column X cm in length and containing a maximum population of about 10^5 nitrifiers per cc in the upper 12 cm of a mixture of 90% sand and 10% of a Hanford fine sandy loam. $(NO_2^-)_o$ was chosen as 100 ppm nitrite-N and results with flow rates of 0.36 and 0.73 cm/hr are shown. The lines were calculated from equation [4b] with $K_m = 16$ ppm. The population of nitrifiers was perhaps

only one tenth as great below 12 cm, which may account for the failure of nitrite to fall below a few ppm. The value of K_m for either flow rate falls within the expected range of reported values (McLaren 1970) and the ratio of K_2 for the slower influx to that for the faster is 1.8 as compared to the expected ratio of 0.73/0.36 = 2.

Data are not yet available for ammonium profiles to be obtained in the same way, or for the consecutive reactions 1 and 2, but some predictions are available on how such profiles for consecutive reactions should appear (McLaren 1970). Some thought has also been given to competition of nitrifiers for the same niche (McLaren and Ardakani 1972) and to the vector analysis of systems with growing populations (McLaren 1969).

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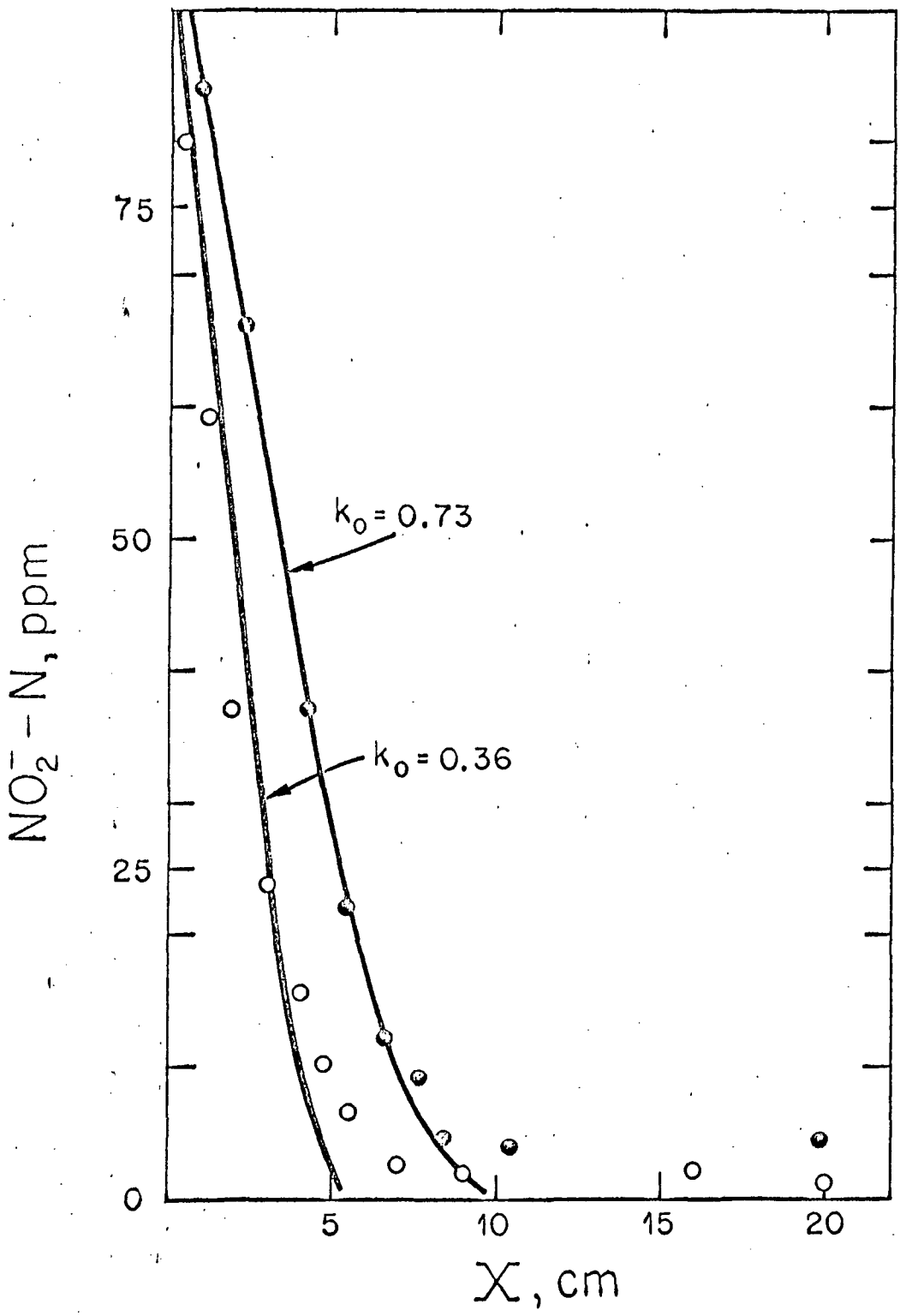
Fig. 1. Influence of two flow rates on nitrification in a sandy soil column. Solid lines are plots of equation [4b]. The cross section of the column was 82 cm^2; the column was sampled during flow by withdrawing small volumes of liquid at the depths indicated by the points shown as circles and dots.


75
50
25
0
5
10
15
20
X, cm
$NO_2^- - N$, ppm
$k_0 = 0.73$
$k_0 = 0.36$