Plant and Soil 77, 285–293 (1984). Ms. 5523
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Effect of substrate concentration, inorganic nitrogen, O_2 concentration, temperature and pH on dehydrogenase activity in soil

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Received 3 June 1983. Revised September 1983

Key words Dehydrogenase Electron transport system Nitrogen Oxygen pH Substrate Temperature

Summary Dehydrogenase activity was measured in a sandy loam soil under a variety of incubation conditions using the reduction of 2-(p-iodophenyl-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to iodonitrotetrazolium formazan (INT-formazan). There was a high positive correlation between dehydrogenase activity and substrate concentration, incubation temperature, and soil pH. Dehydrogenase activity also displayed a high negative correlation with O_2 concentrations. Ammonium sulfate at concentrations from 40 to 120 μg/g soil had no significant effect on dehydrogenase activity. However, at concentrations of 160 and 200 μg/g, dehydrogenase activity was significantly reduced. Potassium nitrate at concentrations ranging from 40 to 200 μg/g had no significant effect on soil dehydrogenase activity, whereas sodium nitrite significantly inhibited activity at concentrations of 120 and 160 μg/g soil.

Introduction

Trevors *et al.*[12] recently reported on the use of 2-(p-iodophenyl-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) for the measurement of dehydrogenase or electron transport system (ETS) activity in soil. The INT is reduced to iodonitrotetrazolium formazan (INT-formazan) by soil enzymes and microorganisms. The INT-formazan can be easily extracted with methanol and quantified spectrophotometrically. The INT method offered the advantage of increased sensitivity over the TTC procedure. At the present time, little information is available on the physical, chemical and biological factors that affect the INT assay. Most research in the past has focused on the factors that affect dehydrogenase activity measurements using 2,3,5-triphenyl tetrazolium chloride (TTC).

Any assay of soil microbial activity may be influenced by numerous conditions which fluctuate to varying degrees in the same soil. The effects of substrate concentration, O_2 concentrations, temperature and pH on microbial dehydrogenase activity in soil are important because they may significantly affect the outcome of ETS activity measurements. Bremner and Zantua[3] reported on the effect of pH

on enzyme stability in soils. The pH stability of soil enzymes was dependent on the soil being assayed[6]. Dehydrogenase or ETS activity was reported to be higher in the presence of buffers at pH 7.6 than in soil amended with either calcium carbonate or water[9]. The optimum pH range for ETS activity has been reported to be between 7.4 and 8.5[8]. However, many soils types may not be included in this range.

The effects of environmental temperatures on microorganisms have been extensively reviewed by Baross and Morita[1] and Tansey and Brock[10]. Incubation of soil samples at 37°C increased soil dehydrogenase activity above the values normally observed at lower temperatures[4]. Trevors[13] reported that ETS activity was 58-fold higher at 20°C than at 4°C in the same sediment sample, whereas an increase in the incubation temperature from 4 to 10°C brought about a 4-fold increase in ETS activity[13]. It also has been reported that urease, phosphatase, and sulfatase enzyme activity was detected in soils − 10 and − 20°C[2]. However, no data was reported for ETS activity at these subzero temperatures.

Oxygen concentrations and diffusion rates significantly affect the growth and respiration of soil bacteria[8]. They may have pronounced effects on the specific growth rate, maximum growth rate, respiration, numbers and types of bacteria isolated from soil[8]. It has been reported that dehydrogenase activity is higher in anaerobically incubated soil than aerobically incubated soil when assayed using TTC[5].

The present report describes the effects of substrate concentration, inorganic nitrogen, oxygen concentration, incubation temperature, and pH on dehydrogenase activity in soil, measured using the INT method described by Trevors *et al.*[12].

Materials and methods

Soil samples

Sandy loam soil was collected from the top 10 cm of an agricultural field at the Cambridge Research Station, University of Guelph, Ontario, Canada. The soil was passed through a 2-mm sieve and used in soil dehydrogenase activity measurements. Various characteristics of the soil were measured as previously described[11] and are presented in Table 1.

Soil dehydrogenase activity

A 1.0 g sample of soil was placed in a 16 × 150 mm glass test tube, and 0.20 ml of a 0.4% (w/v) filter-sterilized aqueous solution of INT (Aldrich Chemical Co., Milwaukee, WI) was added to each tube. The tubes were loosely capped and incubated for 48 h at the appropriate temperature. Sterile controls were prepared by autoclaving test tubes containing 1.0 g of soil for 30 min at 121°C on two consecutive days. After 48 h, each tube received 10 ml of methanol, and were then mixed with a vortex mixer for 1 min. Each methanol extract containing the INT-formazan was filtered through Whatman No. 5 filter paper. The INT-formazan in the methanolic extract was measured spectrophotometrically at 480 nm against a blank of a methanol extract of soil minus INT. The absorbance values obtained were converted

Table 1. Characteristics of the soil

Characteristic	
pH (H_2O)	7.7
Organic matter (%)	3.24
Sand (%)	78
Silt (%)	10
Clay (%)	12
Water holding capacity	0.49 ml/g
No. Heterotrophs	1.57×10^6 /g

Values are expressed on a per g dry weight of soil basis, where applicable.

to μg/ml INT-formazan using a standard curve of INT-formazan (Sigma Chemical Co., St. Louis, Mo.). All trials were carried out in triplicate, and are expressed per gram dry weight of soil. Dry weight determinations were calculated after drying soil samples for 24 h at 105°C.

Effect of INT substrate concentration on soil dehydrogenase activity

Soil samples used in this part of the research were partially air dried to allow a larger volume of INT solution to be added without adjusting the water holding capacity above the 60% level, which was used throughout the experiments. Because of the slight drying and remoistening of the soil, the ETS activity increased substantially in this experiment only.

The INT substrate was added over a range of concentrations from 200 to 1200 μg/g soil by varying the volume of 0.4% (w/v) INT added. Soil samples that received less than the maximum 0.3 ml (1200 μg/ml) of INT were supplemented with the appropriate volume of sterile distilled water. The total volume of solutions added to each tube maintained the soil sample at 60% of its water holding capacity. The soil samples were incubated for 48 h at 20°C in the dark, at which time the INT-formazan concentration was determined. For the remainder of the experiments, a 0.2 ml volume of 0.4% (w/v) INT was used. The 0.4% (w/v) concentration was used as it is the maximum solubility for INT in H_2O. The 1200 μg INT/g soil gave a higher measure of ETS activity than the lower concentrations, indicating that a relationship existed between the INT substrate concentration and dehydrogenase activity. If a larger volume of INT was added, water-logged anaerobic conditions were created in the soil sample. This also has a pronounced effect on the outcome of the assay.

Effect of O_2 concentrations on soil dehydrogenase activity

Soil samples were prepared as previously described and closed with serum stoppers. The tubes were evacuated and the appropriate volume of sterile air was injected with a 50 ml syringe to readjust the initial O_2 concentration to 0, 5, 10, 15 and 20%. The balance of the gas phase was maintained by injecting the appropriate volume of purified helium to adjust the gas phase of the test tube to 1 atm. The O_2 concentrations were also quantified using the gas chromatography procedure described by Tam and Trevors[11] and Trevors *et al.*[12] to ensure that the initial concentrations of O_2 were correct. The soil samples then received 0.2 ml of 0.4% (w/v) INT, and were incubated for 48 h, at which time the INT-formazan concentration was determined. The initial O_2 concentrations decreased due to microbial respiration. Therefore, dehydrogenase activity would be influenced by the decreasing concentrations of O_2 and increasing concentrations of CO_2. It is impossible to maintain the same O_2 concentration for the 48 h period. The only alternative would be to decrease the incubation period to several hours instead of 48 h.

Effect of temperature on dehydrogenase activity in soil

Soil samples were prepared as previously described and incubated over a range of temperatures from 5 to 70°C. Temperatures were maintained to within ± 0.5°C of the desired temperature. ETS activity was determined after 48 h of incubation in the dark.

Effect of pH on soil dehydrogenase activity

Soil samples (1.0 g) were placed in glass tubes and the pH adjusted over a range from pH 3.9 to 10.6 using either 10 *N* H_2SO_4 or 10 *N* NaOH. The milliequivalents of acid or base added adjusted the soil pH to the values given in Table 5. Since the pH adjustment of the soil represented a sudden change from the normal pH (Table 1), a second series of soil samples was allowed 12 days preincubation to allow microbial populations to readapt prior to analysis. The dehydrogenase activity in soil samples allowed the 12-day adjustment period were compared to the soil samples whose pH was adjusted and immediately assayed for ETS activity.

Effect of inorganic nitrogen levels on soil dehydrogenase activity

A 1.0 g amount of sieved soil was placed in a 16 × 150 mm glass test tube and amended with either ammonium sulphate, potassium nitrate or sodium nitrite. The inorganic N supplements were added as 0.1 ml volumes over a range of concentrations from 40 to 200 μg/g. The controls received 0.1 ml of sterile distilled water. Each tube then received 0.20 ml of an aqueous sterile solution of INT. The total volume of the solutions added to each soil adjusted it to 60% of its water holding capacity. All soil samples were incubated at 20°C in the dark for 24 h at which time the INT-formazan was extracted and quantified as previously described.

Statistical analysis

Curve fitting and correlation coefficients were computed on an Apple II Plus microcomputer using a CMA statistics pack (Charles Mann and Associates, Santa Fe Trail, California, USA).

Results and discussion

The high positive correlation (0.996) between the INT substrate concentration and dehydrogenase activity indicated that the outcome of the assay was highly dependent upon the initial INT concentration (Table 2). It also suggested that the increasing INT concentrations were not inhibitory to soil dehydrogenase activity. For the remainder of the experiments, a 0.2 ml volume (equal to 800 μg/g soil) of INT was used. This represented the maximum volume of INT solution that could be added to each soil sample without adjusting the water holding capacity above 60%.

Initial O_2 concentrations ranging from 0 to 20% displayed a high negative correlation of −0.868 when plotted against soil dehydrogenase activity (Table 3). Soil samples incubated at temperatures ranging from 5 to 70°C showed a high positive correlation (0.993) between temperature and soil dehydrogenase activity. Activity increased in a linear manner over the entire temperature range (Table 4). For example, the dehydrogenase activity at 70°C was 8.5-fold higher than at 5°C. The INT technique for determining dehydrogenase activity is a useful method for measuring activity as it is very sensitive at low temperatures often found in the natural environment.

Soil pH can significantly affect microbial activities in soil. Dehydrogenase activity was decreased when soil samples were adjusted to a lower pH value from the initial soil pH of 7.7. Soil dehydrogenase

Table 2. Effect of substrate concentration on dehydrogenase activity in soil

INT substrate (μg/g soil)	ETS activity* (μg INT-formazan/g)
200	32.4 ± 1.35
400	62.2 ± 2.90
600	82.1 ± 2.22
800	102.i ± 1.02
1000	140.2 ± 4.83
1200	162.8 ± 8.44
Line of best fit.	Y = 0.129 X + 6.366
Correlation coefficient	0.996

* Mean ± S.E.M. (n = 3)
Sterile controls of the above treatments displayed no activity.

Table 3. Effect of O_2 concentration on dehydrogenase activity in soil

O_2 Concentration (%)	ETS activity* (μg INT-formazan/g)
0	62.8 ± 9.46
5.0	47.5 ± 1.07
10.0	48.8 ± 0.68
15.0	44.5 ± 0.29
20.0	41.3 ± 1.56
Line of best fit	Y = − 0.920 X + 57.580
correlation coefficient	− 0.868

* Mean ± S.E.M. (n = 3)
Sterile controls of the above treatments displayed no activity.

Table 4. Effect of temperature on dehydrogenase activity in soil

Temperature (°C)	ETS activity* (μg INT-formazan/g)
5	16.3 ± 0.85
10	22.7 ± 0.67
20	43.0 ± 0.97
30	69.0 ± 1.22
40	85.9 ± 2.81
50	89.5 ± 2.60
60	124.1 ± 7.83
70	138.1 ± 7.58
Line of best fit	Y = 1.884 X + 6.467
Correlation coefficient	0.993

* Mean ± S.E.M. (n = 3)
Sterile controls of the above treatments displayed no activity.

Table 5. Effect of pH on dehydrogenase activity in soil immediately following adjustment with acid or base

pH	ETS activity* (μg INT-formazan/g)
3.9	6.93 ± 1.49
5.4	15.23 ± 4.31
6.2	22.67 ± 0.29
7.7	38.5 ± 1.05
9.1	54.0 ± 1.88
9.8	55.3 ± 2.69
10.6	54.6 ± 5.88
Line of best fit	$Y = -25.867 X + 8.127$
Correlation coefficient	0.982

* Mean ± S.E.M. (n = 3)
Sterile controls of the above treatments displayed no activity

Table 6. Effect of pH on dehydrogenase activity in soil adjusted with acid or base and allowed 12 days incubation

pH	ETS activity* (μg INT-formazan/g)
5.8	12.9 ± 5.13
6.6	34.0 ± 4.16
6.8	31.2 ± 2.26
7.7	38.4 ± 3.24
9.1	38.8 ± 4.17
9.5	23.5 ± 2.02
9.6	17.1 ± 0.12

* Mean ± S.E.M. (n = 3)
Sterile controls of the above treatments displayed no activity

activity increased in a linear manner when plotted against pH. The correlation coefficient was 0.982 for the relationship. Table 6 summarizes the affect of pH adjustment on ETS activity when the soil samples were allowed 12 days preincubation prior to the assay of activity. The pH measured immediately after the addition of acid or alkali changed during the 12 day preincubation to give different pH values. Very little activity was observed below pH 6.6 and above a pH of 9.5. It is also interesting to note that the soil samples initially adjusted to a pH of 3.9, increased to a pH of 5.8 after 12 days. The general trend was for the adjusted soil to slowly return towards the unadjusted pH of 7.7. This trend was more pronounced in the acidified soil samples than in the soil samples adjusted with alkali.

Table 7 shows the effects of various concentrations of inorganic N on the INT assay for dehydrogenase activity in a sandy loam soil. Ammonium sulfate at concentrations ranging from 40 to 120 μg/g had no effect on dehydrogenase activity. However, at concentrations of 160 and

Table 7. Effect of nitrogen amendments on dehydrogenase activity in soil

Soil amendment (μg/g)	ETS activity* (μg INT-formazan/g)
NH_4	
0	82.6 ± 0.37
40	79.0 ± 2.37
80	79.5 ± 2.14
120	82.7 ± 4.88
160	71.2 ± 2.05**
200	74.1 ± 2.09
NO_3^-	
0	90.7 ± 7.82
40	88.7 ± 6.20
80	81.4 ± 2.74
120	82.4 ± 8.29
160	89.7 ± 1.19
200	87.3 ± 1.79
NO_2^-	
0	104.2 ± 3.16
40	93.5 ± 7.27
80	93.5 ± 5.97
120	95.7 ± 6.21**
160	81.8 ± 7.84**
200	95.4 ± 7.84

* Mean ± S.E.M. (n = 3)

** Significantly different from the control at the 95% level.

200 μg/g, activity was significantly reduced 13.8 and 10.3%, respectively. Nitrate concentration from 40 to 200 μg/g had no stimulatory or inhibitory effect on soil dehydrogenase activity. Nitrite concentrations of 120 and 160 μg/g, significantly reduced activity. However, 200 μg/g of nitrite produced no significant reduction.

Bremner and Tabatabai[2] reported that the TTC method of assaying soil dehydrogenase activity was influenced by nitrate and other inorganic soil constituents. It was reported that nitrate, nitrite and Fe^{3+} reduced dehydrogenase activity over a 24 h period. The present study suggests that nitrite and ammonium ions may reduce ETS activity, whereas nitrate appears to have no significant effect on ETS activity. There appears to be no linear relationship between nitrite concentration and inhibition of activity, since 200 μg/g of nitrite did not reduce activity, and lower concentrations did cause an inhibition.

It is interesting to note that although the inhibitory concentrations of added N are statistically significant at the 95% level, the actual percent reduction in dehydrogenase activity is rather small, and if tested in numerous soil types, may not be a significant factor affecting dehydrogenase activity determinations using the INT assay. The inhibitory effects

were not observed below 120 μg/g ammonium and 80 μg/g nitrite per g soil. Both of these concentrations are relatively high. Also, the INT assay for measuring the activity in soil may be a more useful measure than the TTC assay. This is especially true if the assay is not affected by a wide range of N concentrations.

The exact reason for increased dehydrogenase activity in the presence of increasing INT concentrations is not known (Table 2). There is the possibility that higher INT concentrations acted as a more competitive hydrogen acceptor, or was more permeable to the microbial cells. Decreasing the O_2 concentrations below 5% increased soil dehydrogenase activity. This may be due to the lack of competition between O_2 and the INT as the final hydrogen acceptor. Regardless of the O_2 concentration, a good measure of dehydrogenase activity can still be obtained. The TTC assay is more dependent on O_2 concentrations, providing a less sensitive assay of activity. Incubation temperatures are critical in the standardization of any assay. The linear increase in activity at temperatures from 5 to 70°C indicated the need for accurate temperature control. Since soil temperatures rarely reach high temperatures, the assay should be carried out at temperatures normally found in the natural environment.

The INT assay for determining soil dehydrogenase acrivity is a relatively new technique[12]. Since this assay is a more sensitive measure of activity than the TTC method, the factors that affect the outcome of the assay must be described. The high correlation coefficients clearly showed the excellent relationships between dehydrogenase activity and substrate concentration, O_2 concentration, temperature and pH. It is suggested that these parameters, as well as others (such as soil amendments) are carefully controlled during the activity assay. Soil samples from different locations may display significant differences in microbial populations, pH, or have different diffusion rates for O_2, which could all affect the outcome of the dehydrogenase assay. Because of this, it is difficult to attribute differences in soil dehydrogenase activity to a single characteristic of the soil.

Acknowledgements This research was supported by a Canada NSERC grant, and a University of Guelph, Research Advisory Board grant. The research and technical expertise of Mr R. Merrick is gratefully acknowledged.

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