INTRODUCTION

Biological oxidation of soil organic compounds is generally a dehydrogenation process carried out by specific dehydrogenases involved in the oxidative energy transfer of microbial cells. This activity is a measure of microbial metabolism and thus of the oxidative microbial activity in soils. A good correlation has been shown between microbial biomass and soil dehydrogenase activity (4). Determination of soil dehydrogenase activity is generally done adding alternative electron acceptors to soil samples. Water-soluble tetrazolium salts are the preferred oxidants because they form water-insoluble coloured formazans which can be measured spectrophotometrically.

TTC (3, 14) and INT (8, 9, 13, 15) have mainly been used as electron acceptors. However, several disadvantages have been described for these compounds. TTC is toxic for microorganism, it has low reactivity requiring long incubation times and is reduced by different microorganisms at different rates. Moreover, TTC reduction is inhibited by O₂ and only a small percentage of the transferred electrons are recorded (2, 10). On the other hand, the resulting triphenylformazan is poorly extracted. The use of INT partly avoids these disadvantages because it is reduced more rapidly, it is less inhibited by oxigen and it is less toxic to microorganisms (9, 13, 15). However, INT is less water-soluble than TTC and its reduction is sensitive to soil composition (11). Besides, the extraction of both derived formazans requires the use of toxic organic solvents.

In this study we present a simple and sensitive method for measuring soil dehydrogenase activity, using NADH as electron donor, which avoids the extraction of soil with organic solvents. The results obtained with NADH and INT in samples from different soils are compared.
MATERIALS AND METHODS

NADH, INT, Tris, calcium chloride and tetrahydrofuran were obtained from Sigma (St. Louis, Mo). The rest of chemicals were from Panreac (Barcelona, Spain).

Soil samples
Five salt-affected and transformed for cultivation soils with different organic content and three samples of a biological culture of Calendula officinalis from the Central-Mediterranean region of Spain (Valencia) were used. All soil samples were taken from the surface layer (0-10 cm). In the case of the biological culture the three samples were collected at 0-10 cm, 10-50 cm and >50 cm from the plant roots, respectively, and sieved through a 2-cm mesh. The organic soils were cleared from plant debris and rocks, freeze-dried and, after grinding, passed through a 0.1-cm sieve. All samples were kept at 4°C in watertight polyethylene botles for about one week.

NADH oxidation
0.02g and 0.04g of freeze-dried soil or 0.2g and 0.4g of sifted biological culture soil were placed in separate test tubes (16x100 mm) and 2 ml of NADH-TRIS buffer (0.15 M Tris-HCl pH 7.5 containing 20 mM NADH) were added. The tubes were sealed with plastic stoppers and parafilm and incubated at 37°C for 0 min, 15 min and 30 min with horizontal shaking. NADH oxidation was stopped by adding 5 ml of TRIS-Carbonate buffer (0.1 M sodium carbonate buffered to pH 11.5 with Tris). In the case of incubation for 0 min the TRIS-Carbonate buffer was added previous to the NADH-TRIS buffer. After mixing for a few seconds 0.5 ml of 0.5 M CaCl$_2$ were added and the suspensions were centrifuged at 1500g for 10 min at 4°C. The supernatants were filtered through a 0.22 µm nylon filter (Magna nylon, msi, Ma) and their absorbances measured at 340 nm. The differences in the absorbance between the NADH present at the beginning and that remaining after 15 min or 30 min of incubation were converted in mg or µmol of NADH using a calibration curve and dehydrogenase activity was expressed as mg or µmol of NADH oxidized by 1 g of soil during 1h of incubation.

INT reduction
The INT reduction was determined according to Spothelfer-Magaña and Thalmann (13) as modified by Friedel et al. (8). Briefly, soil samples were placed in test tubes (16x100 mm) and 2.5 ml of INT-TRIS buffer (0.1 M Tris-HCl pH 7.9 containing 2% INT) were added. The tubes were flushed with N$_2$ for 2 min, sealed with plastic stoppers and parafilm and incubated at 46°C in the dark for 30 min and 60 min. The formazan formed was extracted by shaking with 10 ml ot tetrahydrofuran for 1 h in the dark. After filtration and dilution of the filtrates at a ratio of 1:7 with acetone, the absorbance was measured at 487 nm. The amount of INT reduced was calculated from INT formazan calibration curves.

Organic carbon determination
Organic carbon (organic C) content of the soil was measured by the method of Walkley (16). The soil was ground and passed through a 0.5-mm sieve. A weighed sample containing 10 to 25 mg of organic carbon was transferred into a 500 ml Erlenmeyer flask. 10 ml of 1 N potassium dichromate (K$_2$Cr$_2$O$_7$) were added and, after swirling gently, 20 ml of concentrated sulfuric acid poured directly into the soil suspension. The mixture was stirred
gently and then more vigorously for a total of 1 min. After diluting with 200 ml of water, the suspension was filtered, some drops of o-phenantrolin were added and the filtrate was titrated with 0.5 N ferrous sulfate (FeSO₄). Organic carbon content was calculated according to the following formula:

\[
\text{ml FeSO}_4 \text{ blank solution} \quad 100
\]

\[
\text{ml FeSO}_4 \text{ test solution} \quad \text{g soil}
\]

\[
\text{Organic C (meq/100 g)} = 10 (1 - \frac{\text{ml FeSO}_4 \text{ blank solution}}{\text{ml FeSO}_4 \text{ test solution}}) \times \frac{\text{ml FeSO}_4 \text{ blank solution}}{\text{g soil}}
\]

RESULTS AND DISCUSSION

The method proposed for measuring soil dehydrogenase activity is based on the use of NADH as electron donor for the oxidative systems of soil microorganisms. After a short period of incubation of soil samples with an NADH buffered solution the reaction was stopped by diluting and increasing the pH of the medium and the insoluble material cleared by centrifugation and filtration. CaCl₂ was previously added to avoid the extraction of clays and organic matter. Dehydrogenase activity of soil is evaluated measuring the decrease in the absorbance at 340 nm of the filtrates. As there exists the possibility of calcium hydroxide flocculation absorbance measurement should be done as soon as possible. As can be seen in Fig. 1, NADH oxidation by soil was dependent on NADH concentration and showed saturation kinetics. Under our assay conditions the oxidation rate was practically constant when NADH concentrations higher than 15 mM were used. Therefore a concentration of 20 mM was chosen for determining the dehydrogenase activity of the different soils analysed. The use of an electron donor instead of an acceptor avoids the inhibition by O₂ occurring in the reduction methods (8) and the short incubation times minimizes the probability of a shift in the microbial population.

The dehydrogenase activity of several soils was measured using NADH as an electron donor and the results were compared with those obtained by the INT reduction method (Table 1). This method was chosen as reference because, as described by Friedel et al. (8), presents advantages with regard to the TTC reduction. The soil S1 had the lowest activity of the different peats studied probably due to its smaller water content which, as has been already described (12), strongly influences the biological activity of the soil. On the contrary, the soil S4 showed the highest dehydrogenase activity in agreement with its high organic C content (Table 1). Regarding the biologically cultivated soil, as expected from the fact that higher microroganism numbers are found in the root vicinity (1), the region near the plant roots presented greater activity than the more distant ones. However, the oxidative activity of this soil was smaller than that of the peats. With the exception of soil S4, the dehydrogenase activities measured by the NADH and INT methods in the different soils had very good agreement (Fig. 2). In the case of soil S4 the dehydrogenase activity was lower when measured by the INT method.

The dehydrogenase activity determined by the NADH method presented also a positive correlation with the organic C content of soils as indicated by the correlation coefficient (r) of the linear regression (Fig. 3). This correlation was not so clear when the INT reduction method was used. These results are in concordance with those obtained by others showing a correlation between the soil organic C content and the activities of different enzymes. In this sense, Deng and Tabatabai described correlation coefficients for linear regressions varying from 0.7 to 0.9 for several amidohydrolases (5), from 0.78 to 0.93 for several glycosidases (6) and from 0.71 to 0.92 for alkaline phosphatase,
phosphodiesterase, inorganic phosphatase and arylsulfatase (7). However, they did not find correlation for amidase (5). Our results with dehydrogenase activity support the suggestion that organic matter plays an important protective role in maintaining soil enzymes in their active forms and the hypothesis that enzymes are immobilized in a three dimensional network of clay and humus complexes and/or associated with a larger microbial population resulting from the increase in soil organic C (7).

CONCLUSIONS

NADH oxidation rate by soil showed saturation kinetics. With our assay conditions, the reaction rate becomes practically independent on the NADH concentration at values higher than 15 mM.

NADH oxidation compared to INT reduction has the advantages of a more easily determined soil oxidative activity, shorter times of incubation doing an increase of microbial population during the incubation period very unlikely, no inhibition by O₂ and greater sensitivity allowing the detection of lower levels of dehydrogenase activity and the use of smaller amounts of samples and reagents.

The dehydrogenase activity measured with the NADH method showed a better correlation with the organic C content of the soils studied than with the INT method.

NADH oxidation method is therefore a good alternative to INT reduction for measuring soil microbial activity.

Acknowledgements. This work has been supported by IMPIVA, Pla Tecnològic, Conselleria d’Industria, Comerç i turisme, Generalitat Valenciana, Grant 1195.

REFERENCES


Keywords : Dehydrogenase, enzymatic activity.
Mots clés : déshydrogénase, activité enzymatique
$y = -0,1176x^2 + 4,3604x - 0,1386$

$R^2 = 0,9993$