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Enzymatic Characterization of Soil Evidence

"You have come up from the south-west, I see."
"Yes, from Horsham."
"That clay and chalk mixture which I see upon your toe caps is quite distinctive."
Sherlock Holmes in "The Five Orange Pips"
Arthur Conan Doyle

Soils is most assuredly an important type of physical evidence; if one can establish that soil on the shoes or clothing of a suspect matches that of a crime scene it will lend support to a hypothesis that the suspect had, in fact, visited the scene. Likewise, comparison of soil from the tires or the undercarriage of an automobile with the soil from a particular location may establish with greater or lesser certainty that the automobile was at one time present at the location. Furthermore, soil evidence is more likely to be encountered in those offenses against persons or property which society deems most severe. Equally important is that soil evidence, properly examined and properly interpreted, may serve an exclusionary purpose and exonerate the innocent.

Methods currently utilized in crime laboratories for comparison of soil are concerned almost exclusively with *inorganic* components of soil. Sir John Russell, a prominent soil scientist, commented [*1*] that "a clod of earth seems at first sight to be the embodiment of the stillness of death," but continued on to show that it is in fact a highly organized physical, chemical, and biological complex. A modern view of soil [*2*] is that it is a tripartite system composed of (1) finely divided minerals and amorphous inorganic solids; (2) animal, plant, and microbial residues in various stages of decay; and (3) a living and metabolizing microbiota. Forensic interest has, in essence, been confined to the first of these three elements. There has been no suggestion in the forensic literature that materials in the *biochemical* domain might be of value in the characterization of a soil, and yet it is *these* materials, arising from very highly specialized and stylized life processes, that might ultimately impart the highest degree of uniqueness to a given soil.

A vast number of organisms live in the soil. By far the greater proportion belong to the plant kingdom, although the animal kingdom is well represented also. It is estimated that half of the total biomass on Earth resides in the bacteria. The numbers of bacteria in soil are very high, often reaching one billion per gram of soil and occasionally reaching three billion [*3*]. Actinomycetes are also exceedingly numerous, perhaps as many as several hundred million per gram of soil [*4*]. The number of fungi vary widely with soil conditions, but a normal population is on the order of 10 to 20 million per gram of soil [*5*]. The algae vary considerably also, with between 10,000 and three million per gram of

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soil being reported [6]. Protozoa may exist in soil up to a maximum of one million per gram of soil [7], and nematodes up to about 50 per gram of soil [8].

This constellation of microbial life in the soil results in a number of products that reflect the biochemical activity at that moment in time. Products of this activity may eventually be preserved and manifested in the organic matter accumulating in the soil. This would apply both to humic substances and to those materials retaining some measure of their biochemical integrity, for example, enzymes. The biochemical activity of a particular soil, then, is a gestalt of perhaps countless generations of microorganisms, each with its own ecological raison d'etre. The vast number of ecological niches produced by specific fitness traits of microorganisms, together with a multitude of abiotic factors, suggest that the biochemical composition of soils could vary widely, even in soils which are genetically similar from a pedogenic standpoint.

With the exception of soil enzymes, there are few classes of organic compounds which appear to hold promise as geographical markers for forensic purposes. This is primarily due to low concentrations of low molecular weight compounds in the soil. Given a large sample of soil, many compounds may be extracted and isolated, but the quantity in a small sample, such as that often encountered in forensic situations, may be below the threshold of detectability.

The enzyme activity of a soil, however, would seem attractive as a means of characterizing soil for forensic purposes, since the ability of the enzyme to amplify its presence by a turning over of large amounts of substrate enables assay techniques to be applied to the small samples of soils which are often submitted to a crime laboratory. Soil enzymology is also rather attractive from the forensic standpoint because the enzymes may serve as biological markers that depict the history and career of a particular soil. All biochemical action is dependent on, or related to, the presence of enzymes, and organisms in the soil synthesize a wide variety of enzymes in order to exploit the resources of their environment.

Most soils contain many enzymes which are involved in biochemical processes taking place in the soil, or which accumulated during processes which are no longer extant in the particular soil. Certain enzymes are ubiquitous, and may be found in virtually all soils (urease, catalase) [9]. Others may be locally restricted, and indicate special situations (malathion phosphoesterase) [10].

Kuprevich and Shcherbakova state that "The most essential index of biological activity in soil is its enzymatic activity, and a study of this enzymatic activity may provide an objective view of the processes taking place in soil" [9]. They conclude:

> The differences in level of enzymatic activity are caused primarily by the fact that every soil type, depending on its origin and developmental conditions, is distinct from every other in its content of organic matter, in the composition and activity of living organisms inhabiting it, and consequently, in the intensity of biological processes. Obviously, *it is probable that each type of soil has its own inherent level of enzymatic activity* [italics added].

Other soil scientists have recognized the potential of soil enzymology in characterizing soil for agronomic purposes. Galstyan and Tatevosyan state [11]:

> ...the fermental [enzyme] activity reflects some genetical soil peculiarities and can be considered a diagnostic characteristic of different soil types.

The significance of such statements to a forensic biochemical characterization of soil is patent.

Enzymes which have been demonstrated in soil have been reviewed by Skujins [12], Kuprevich and Shcherbakova [13], Hofmann and Hoffman [14], Durand [15], and Voets and Dedeken [16]. Table 1 represents an array of soil enzymes.

The question of the comparative enzymatic activity in similar soils (that is, soils which have shared the same pedogenic factors or which have shared the same restricted geo-

TABLE 1—*Enzyme activities demonstrated in soil* [12].

Enzyme	Reaction Catalyzed
Oxidoreductases	
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$
Catechol oxidase (tyrosinase)	*o*-diphenol + $\frac{1}{2}O_2 \rightarrow$ *o*-quinone + H_2O
Dehydrogenase	$XH_2 + A \rightarrow X + AH_2$
Diphenol oxidase	*p*-diphenol + $\frac{1}{2}O_2 \rightarrow$ *p*-quinone + H_2O
Glucose oxidase	glucose + $O_2 \rightarrow$ gluconic acid + H_2O_2
Peroxidase and polyphenol oxidase	A + $H_2O_2 \rightarrow$ oxidized A + H_2O
Urate oxidase (uricase)	uric acid + $O_2 \rightarrow$ allantoin, CO_2
Transferases	
Transaminase	$R_1R_2\text{-CH-N}^+H_3 + R_3R_4CO \rightarrow R_3R_4\text{-CH-N}^+H_3 + R_1R_2CO$
Transglycosylase and levansucrase	$nC_{12}H_{22}O_{11} + ROH \rightarrow H(C_6H_{10}O_5)nOR + nC_6H_{12}O_6$
Hydrolases	
Acetylesterase	acetic ester + $H_2O \rightarrow$ alcohol + acetic acid
α- and β-amylase	hydrolysis of 1,4-glucosidic bonds
Asparaginase	asparagine + $H_2O \rightarrow$ aspartate + NH_3
Cellulase	hydrolysis of β-1,4-glucan bonds
Deamidase	carboxylic acid amide + $H_2O \rightarrow$ carboxylic acid + NH_3
β-Fructofuranosidase (invertase, sucrase, saccharase)	β-fructofuranoside + $H_2O \rightarrow$ ROH + fructose
α- and β-galactosidase	galactoside + $H_2O \rightarrow$ ROH + galactose
α- and β-glucosidase	glucoside + $H_2O \rightarrow$ ROH + glucose
Inulase	hydrolysis of β-1,2-fructan bonds
Lichenase	hydrolysis of β-1,3-cellotriose bonds
Lipase	triglyceride + $3H_2O \rightarrow$ glycerol + 3 fatty acids
Metaphosphatase	metaphosphate $\rightarrow$ orthophosphate
Nucleotidase	dephosphorylation of nucleotides
Phosphatase	phosphate ester + $H_2O \rightarrow$ ROH + phosphate
Phytase	inositol hexaphosphate + $6H_2O \rightarrow$ inositol + 6 phosphate
Protease	proteins $\rightarrow$ peptides and amino acids
Pyrophosphatase	pyrophosphate + $H_2O \rightarrow$ 2 orthophosphate
Urease	urea $\rightarrow 2NH_3 + CO_2$

graphical location) has not been addressed in the soil science literature. However, there has been considerable work conducted on comparative enzymatic activity in diverse types of soil. The subject has been reviewed by Kuprevich and Shcherbakova [*9*]. Enzyme activity varies among soils. As a rule, it correlates with the quantity or organic matter, the intensity of soil "respiration," microfloral activity, root system activity of higher plants, and in some cases with the abundance of surface colonies of algae and lichens [*13*]. Certain types of enzyme activity, however, are more closely correlated with specific forms of biological activity. Invertase is most closely correlated with the activity of higher plant root systems and with the activity of yeasts [*17*]. α-amylase is produced by both fungi and bacteria, while glucoamylase is produced primarily by fungi [*12*]. Urease is ubiquitous in nature, and the presence of this enzyme may be expected in all soils, with the possible exception of strongly alkaline soils with low organic matter content [*12*]. Other enzymes are quite widely distributed in soil, including phosphatase, catalase, and peptide hydrolases of various specific activities. Trehalase, on the other hand, is correlated exclusively with fungal activity [*18*]. Xylanase is likewise correlated with streptomycetes [*19*]. As a general tendency, carbohydrases are more closely correlated with fungi than with bacteria, which is in accord with the fact that more cellulose is decomposed by fungi than by bacteria [*18*]. Dehydrogenase activity in the soil appears to be related to

the quantity of organic substances which can be decomposed by soil microorganisms, and follows the extant soil biomass more closely than other enzymes [*20*].

Although it is a step removed from the teleological significance of the occurrence of soil enzymes, various attempts have been made to correlate enzyme activity with different crops. It is difficult to draw conclusions from such studies, as most have been carried out on different soils and in different seasons. One legitimate generalization that can be made, however, is that virgin lands present a separate case from other vegetative soil ecosystems [*9*]. Other considerations, including the soil structure, physical properties, organic matter content, seasonal variations, and soil treatment, appear to have a greater predicating influence upon the presence and abundance of soil enzymes.

Some changes in enzyme activity can be expected during drying and storage. The extent of these changes, in some instances, is the subject of conflicting statements in the soil science literature. It does appear that a diminution of activity upon drying and storage is dependent upon the particular enzyme in question. As a general rule, the greatest loss in enzyme activity is immediately upon drying. Once the drying is completed, the activity declines slowly over long periods [*12*].

It is well recognized that enzyme activity in soil is not static, but fluctuates with biotic and abiotic factors. As a consequence, the complexion of soil enzymes in a given sample will shift with time. This would suggest that an evidence and exemplar soil must be collected at approximately the same period of time in order to be matched by enzymatic means, but it also suggests that the addition of the dimension of time will impart a much higher degree of discrimination, and in specific instances will enhance the value of the technique for forensic purposes. If an evidence soil and an exemplar soil are shown to possess a high degree of similarity with respect to the level of enzyme activity, it may be postulated that the soils originated from the same point in time, as well as the same point in space.

Soil Samples

Three soil series were chosen for somewhat different purposes. The soils consisted of samples from three distinct profiles: (1) Dublin adobe clay loam, (2) Columbia sandy loam, and (3) Hanford sandy loam.

Dublin Adobe Clay Loam

The first series chosen for examination consisted of a single generic series, a Dublin adobe clay loam. Four samples were collected in February 1973, and were allowed to air-dry at room temperature. Areas of the soil profile were identified by means of a detailed soil survey map. The samples consist of middle miocene marine sediments which, from a pedogenic standpoint, are quite similar. The locations of the Dublin soils are indicated in Fig. 1.

Samples were again taken from Dublin Sites I–IV in August 1973 for the purpose of determining any variation in K_m from the samples collected from the same location in February 1973.

Columbia Sandy Loam

The second series collected consisted of samples of a Columbia sandy loam, collected from northwestern Sacramento County in February 1973. The area was selected because of the uniformity of the soil with respect to color, texture, and organic matter content. An ancillary reason for the selection of this plot was the relatively low organic matter content of the soil, the reasoning being that if a series of enzyme assays could be

accomplished successfully on this soil, then the techniques could be applied to other soils with significantly greater content of organic matter. In this respect, the Columbia series represents a type of "worst case" situation. Figure 2 illustrates the manner in which the 28 samples were collected.

Hanford Sandy Loam

The third series examined consisted of Hanford sandy loam, collected in April 1973 at the Kearny Agricultural Field Station at Reedly, Fresno County. This site was selected because of the exceptional uniformity of the soil with respect to texture and color. The samples were collected according to the sketch in Fig. 3. Samples 38 and 46 were collected from points approximately 50 feet from Sample 48.

The soil samples from all three series (Dublin, Columbia, and Hanford) were sieved through an 80-mesh sieve prior to testing. This represents a somewhat finer fraction than that utilized for enzyme studies by some other workers, but eliminates the fragments of plant roots, stalks, and leaves which are very much in evidence in a 2-mm fraction. Additionally, enzyme activity is not particularly associated with a sand fraction, and the presence of coarse sand in a sample could only contribute to physical heterogeneity of the sample.

Enzyme Assays

Phosphatase-Colorimetric Determination

The colorimetric determination of phosphatase activity was the technique of Tabatabai and Bremner [*21*]. The method is outlined as follows.

Reagents

Modified universal buffer—A buffer series ranging from pH 3.0 to pH 12.0 was made up in 0.5-pH increments. The preparation of this buffer is as described by Skujins et al [*22*].

tris (hydroxymethyl) aminomethane	3.025 g
maleic acid	2.90 g
citric acid	3.50 g
boric acid	1.57 g
1*N* NaOH	122 ml
H_2O (to make final volume of 250 ml)	

To 20 ml of this stock solution are added 10 ml of 0.1*N* HCl. The solution is then brought to the desired pH with 3*N* HCl, and the volume is brought to 100 ml with water.

Toluene—Reagent.

*Sodium hydroxide, 0.5*M—Dissolve 20 g NaOH in water and dilute to 1 litre.

*Calcium chloride, 0.5*M—Dissolve 73.5 g of $CaCl_2 \cdot 2H_2O$ in water and dilute to 1 litre.

p-*nitrophenylphosphate, 0.115*M—Dissolve 0.964 g of disodium *p*-nitrophenylphosphate tetrahydrate in water and dilute the solution to 25 ml. The solution is stored in the refrigerator and is protected from light.

p-*nitrophenol*—The standard stock solution of *p*-nitrophenol is prepared by dissolving 100 mg in water and diluting to 100 ml. The solution is stored in the refrigerator.

Procedure—One gram of soil is placed in a 50-ml Erlenmeyer flask with 4 ml of buffer, 0.25 ml of toluene, and 1.0 ml of *p*-nitrophenylphosphate. The flask is stoppered and swirled to thoroughly mix the contents, and then incubated at 37°C for one hour without shaking. After one hour the flask is removed from the incubating oven and 4 ml of 0.5M NaOH and 1.0 ml of 0.5M $CaCl_2$ are added. The purpose of the NaOH is

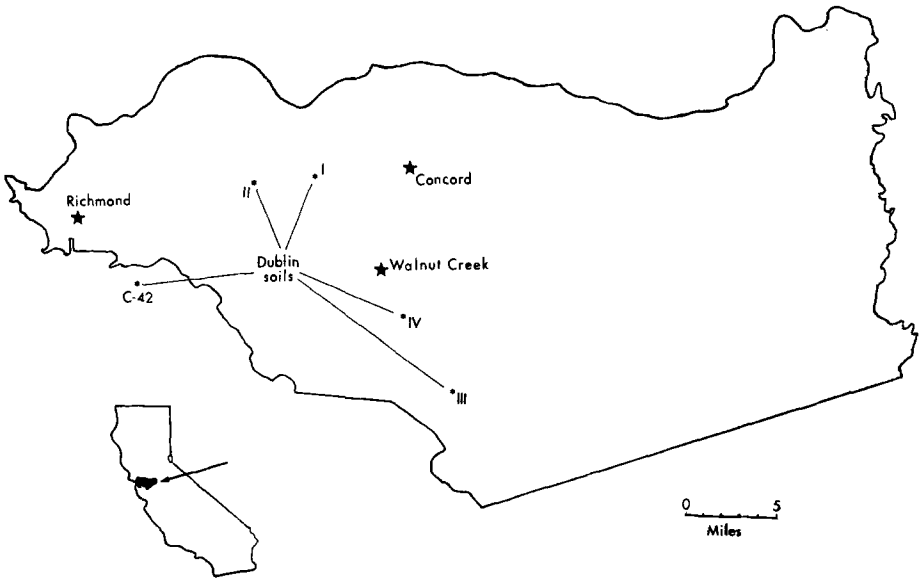

FIG. 1—*Sampling sites of Dublin soils: Contra Costa and Alameda Counties, Calif.*

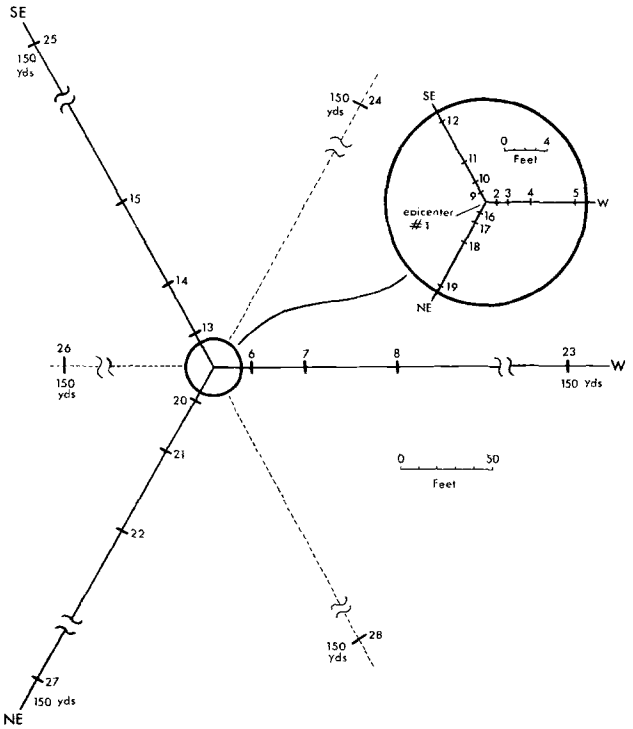

FIG. 2—*Sampling grid for the collection of the Columbia sandy loam soils, Sacramento County, Calif.*

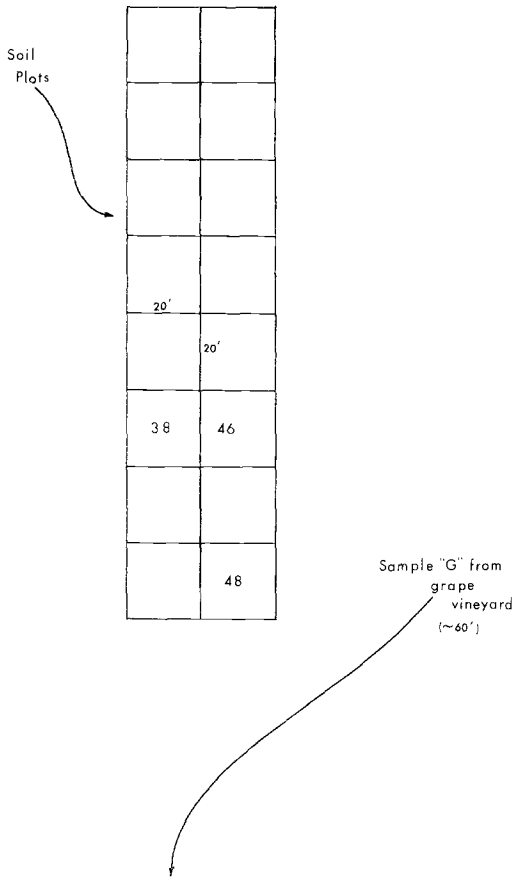

FIG. 3—*Collection of Hanford sandy loam soils: Fresno County, Calif.*

to arrest further enzyme activity and to develop the yellow color of the phenolate ion; the purpose of the $CaCl_2$ is to prevent the dispersion of clay and to prevent the extraction of the soil organic matter by the NaOH which would otherwise interfere with the subsequent colorimetric determination. The mixture is shaken and transferred to a 10-ml centrifuge tube and centrifuged at 2500 rpm for 10 min. The supernatant liquid is then drawn off and transferred to a colorimeter tube; the optical density of the liquid is determined at 420 nm in a AO Spectronic 20® (American Optical Co.) colorimeter against a "no soil" blank. If the optical density of the solution is off scale, the solution is diluted with water until the colorimeter reading falls within the limits of the calibration graph. The calibration graph is constructed from the optical densities obtained with standards containing 0, 10, 25, and 50 ug of *p*-nitrophenol, as obtained by an appropriate dilution of the stock solution. In addition to the "no soil" control against which the test solution is measured, a "no substrate" control is run in which 1 ml of buffer is substituted for the substrate. The purpose of this control is to eliminate the contribution of any extraneous material extracted from the soil by the alkali to the absorbance near 420 nm.

Arylsulfatase—Colorimetric Determination

The method used for the assay of arylsulfatase activity was that of Tabatabai and

Bremner [*23*], the one modification made being the substitution of the modified universal buffer (MUB), as described by Skujins et al [*22*], for the acetate buffer used by Tabatabai and Bremner. This modification was made so that there would be no discontinuity in buffers between pH 3 and pH 12. The assay is conducted in precisely the same manner as that described above for phosphatase activity, but with *p*-nitrophenylsulfate as the substrate.

Reagents

p-*nitrophenylsulfate, 0.005*M—Dissolve 0.129 g of potassium *p*-nitrophenylsulfate in water and dilute the solution to 100 ml. The solution is stored in the refrigerator and protected from light.

Other reagents are as described above for phosphatase determinations.

Procedure—The procedure is identical to that described above for phosphatase determinations.

Urease—Titrametric Determination

The method used for the assay of urease activity was a modification [*24*] of the method of McLaren et al [*25*]. Ammonia is liberated by the action of urease on urea in Conway diffusion dish, and is determined by titration.

Reagents

Urea—Reagent.

Mixed indicators solution—Twenty-two millilitres of an ethanolic solution of 0.084% bromo-cresol-green and 0.016% methyl red is added to 1 litre of a 2% boric acid solution.

Modified universal buffer—Prepared as described above in the phosphatase determination.

Potassium carbonate, 45%—Dissolve 45 g of K_2CO_3 in water, add 25 μl of tergitol (for a final concentration of 0.025%), and dilute to 100 ml.

*HCl, 0.004*N

Procedure—In the outer well of a Conway diffusion dish are placed 0.5 g of soil, 0.18 g of urea, and 2 ml of buffer. In the center well is placed 1 ml of the boric acid-mixed indicator solution. The diffusion dish is allowed to incubate for 1 h at room temperature. One ml of 45% potassium carbonate is then added to the outer well, the dish resealed, and allowed to stand overnight. The boric acid-mixed indicator solution is then titrated with 0.004*N* HCl. A "buffer blank" (no soil blank) control and a "urea blank" (no substrate) control are run along with the soil-substrate test. The urease activity, in μmoles NH_3/g soil/h, is then calculated according to

$$[\text{ml } 0.004N \text{ HCl (buffer blank + urea blank)}] \times 4 \times 2 = \mu\text{moles } NH_3/\text{g soil/h}$$

Tryptic Activity—Titrametric Determination

Tryptic activity is defined as that enzymatic activity hydrolyzing the amide group from benzoylargininеamide. The procedure used is that of McLaren et al [*25*] and is identical to the urease determination, except for the substrate used and the time of incubation.

Reagents

N-*benzoyl*-L-*arginine amide*—Three μmoles of substrate are used per diffusion dish.

This represents 0.9954 mg of solid substrate, an amount too small for accurate weighing. As an alternative, 99.54 mg of substrate are dissolved in 100 ml of the modified universal buffer; 1 ml of this solution will then deliver 3 μmoles of benzoylarginineamide.

The other reagents are as described above for the urease determination.

Procedure—In the outer well of a Conway diffusion dish are placed 0.5 g of soil, and 2.0 ml of buffer containing 3 μmoles of substrate. In the center well is placed 1 ml of the boric acid-mixed indicator solution. The diffusion dish is allowed to incubate at room temperature for 72 h. One millilitre of 45% potassium carbonate is then added to the outer well, the dish resealed, and allowed to stand overnight. The boric acid-mixed indicator solution is then titrated with 0.004*N* HCl and the amount of ammonia produced by the action of the enzyme calculated in the same manner as described above for the urease determination.

Invertase—Colorimetric Determination

Invertase was determined by means of a linked system adapted from the method of Washko and Rice [*26*] and Solomon and Johnson [*27*]. Sucrose is offered to the soil. Any glucose produced by the action of invertase on the sucrose is determined by converting the glucose to gluconic acid and H_2O_2 by means of glucose oxidase. The H_2O_2 reacts with a reduced chromogen in the presence of horseradish peroxidase to give an oxidized chromogen, which is determined spectrophotometrically.

Reagents

Sucrose, 8%—Eight grams of sucrose are dissolved in modified universal buffer (MUB) and the volume brought to 100 ml.

Composite reagent—Seven hundred milligrams of *0*-tolidine dihydrochloride are dissolved in about 150 ml of water. Two hundred milligrams of glucose oxidase and 15 mg of horseradish peroxidase are added and the volume is brought to 300 ml.

Procedure—One gram of soil is incubated with 9 ml of 8% sucrose in MUB at 37 °C for 1 h in a screw-capped test tube. The mixture is boiled for 2 min to arrest the enzyme. The tube is centrifuged at 2500 rpm for 10 min. Two millilitres of the supernatant solution are then mixed with 2 ml of the composite glucose oxidase-peroxidase reagent and incubated at 37 °C for 30 min. The color formed is read spectrophotometrically at 400 nm against a "no soil" blank treated in the same manner as the test.

Enzyme Kinetics

Certain considerations related to the kinetics of the phosphatases and arylsulfatase reactions were examined. The Michaelis constant (K_m) and the maximal velocity (V_{max}) are fundamental concepts in enzyme chemistry, and any study which describes enzyme activity should consider the effect of substrate concentration on velocity in order to determine if substrate concentration is a limiting factor. The same experiments will give rise to the K_m. This term is most attractive from the standpoint of the forensic comparison of soil, since it is independent of sample size and its potential usefulness is thereby enhanced. While V_{max} and K_m are constants of an enzyme, they may vary independently [*28*].

The Michaelis constant is calculated from the Michaelis-Menten equation:

$$V = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

where V is the initial enzyme reaction velocity and S is the substrate concentration. Experimentally, the constant is generally determined graphically by means of a linear transformation of the Michaelis-Menten equation or, more precisely, a linear transform of the equation

$$V = V_m - K_m(V/S)$$

The method used to determine the Michaelis constant and V_{max} in this study was the transformation of Augustinsson [*29*], often attributed to Hofstee, in which V/S is plotted on the abscissa, and V is plotted on the ordinate. V_m is then given by the y intercept, and the slope is equal to $-K_m$. The x intercept is equal to V_{max}/K_m.

This particular linear transform is not as frequently encountered as the method of Woolf, or of Lineweaver and Burk, but is greatly favored by Mounter and Turner [*30*] on the basis that it has finite intercepts on each axis and that the points are more evenly distributed than with the Lineweaver and Burk transformation. In addition, this transform facilitates treatment by regression analysis, which gives a weighted straight line of closest fit.

Dilution Plate Counts of Bacteria, Actinomycetes, and Fungi

Plate counts of bacteria, actinomycetes, and filamentous fungi were conducted on the Hanford soil series by the methods of Allen [*31*].

Reagents

Soil Extract Agar—One thousand grams of soil in 1 litre of water are autoclaved for 30 min at 15 psi (120°C), 0.5 g of $CaCl_2$ is added, and the suspension is filtered through filter paper. One hundred millilitres of this extract, 1.0 g of glucose, 0.5 g of K_2HPO_4, and 0.1 g of KNO_3 are added and the volume brought to 1 l. The pH is adjusted to 7.0, 15 g of agar are added, and the solution is autoclaved at 15 psi for 15 min at 120°C. It is poured into Petri dishes when it has cooled to approximately 55°C.

Starch-Casein Agar—This is a defined medium comprised of 10.0 g of starch, 0.3 g of casein, 2.0 g of KNO_3, 2.0 g of NaCl, 2.0 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCO_3$, 0.1 g of $FeSO_4 \cdot 7H_2O$, and 18 g of agar in 1 l of water. The pH is adjusted to 7.0 and the solution is autoclaved at 15 psi for 15 minutes. It is poured into Petri dishes when it has cooled to approximately 55°C.

Potato Dextrose Agar—ST—This is a defined medium comprised of 39 g of potato dextrose agar (comprised in turn of 200 g of potatoes, 20 g of dextrose, and 17 g of agar), 200 mg of streptomycin sulfate, and 1 ml of Tergitol-NPX® in 1 l of water. The solution is autoclaved at 15 psi for 15 min and poured into Petri dishes when it has cooled to approximately 55°C.

Procedure

A 1:10 dilution of soil is prepared by suspending 10 g of soil in 90 ml of distilled H_2O. Two millilitres of this suspension are added to 18 ml of distilled H_2O for a $1:10^2$ dilution. This dilution procedure is continued until a tenfold dilution series from 1:10 to $1:10^7$ is obtained. The Petri plates are inoculated with 1 ml of the appropriate dilution of the soil suspension. For each soil, plating the fungi requires three plates of a 10^{-2} dilution, five plates of a 10^{-3} dilution, and two plates of a 10^{-4} dilution. The actinomycetes require two plates of a 10^{-4} dilution, five plates of a 10^{-6} dilution, and three plates of a 10^{-7} dilution.

After inoculation, the plates are incubated with the lids down at room temperature.

The fungal and bacterial plates are counted after two to seven days, depending upon the growth, and the actinomycetes are counted after seven to ten days. Numbers of colonies are counted and averaged for a particular dilution. The average number of fungi, actinomycetes, and bacteria is reported as per gram of dry soil.

Statistical Treatment of Data

It was found that values for enzyme activity, maximal velocity, and Michaelis constants were conveniently handled by means of a simple algorithm, the Canberra metric similarity coefficient, with similarity expressed by means of a dendrogram. This work is described in greater detail elsewhere [*32*].

Results and Discussion

The three soil series considered in this investigation (Columbia sandy loam, Hanford sandy loam, and Dublin adobe clay loam) will be commented upon separately with respect to characterization as to geographical source.

The Dublin soils examined are quite similar with respect to pedogenic considerations. Samples I, III, and IV are exceedingly close in color, and key out in the Munsell Soil Color Chart as 10YR/4/2, "Dark Grayish Brown." Density gradient tests performed on the Dublin soils could not distinguish Soils III and IV, as illustrated in Fig. 4, nor could these two soils be distinguished by X-ray diffraction. Considering the techniques ordinarily employed in forensic laboratories, it is likely that Soils III and IV would, when examined, be regarded by the examiner as "consistent with having shared a common geographical location." In fact, however, they were collected from areas several miles apart.

Enzyme determinations were initially carried out at a number of pH values to determine the optimum pH for the particular soil in question and for the enzyme under considera-

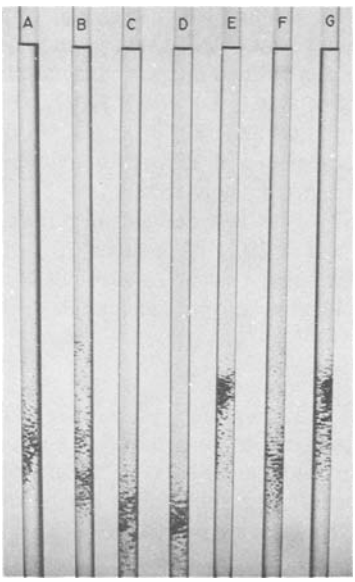

FIG. 4—*Density gradient distribution of the Dublin adobe clay loam soils: Tube A, Dublin Sample I; Tube B, Dublin Sample II; Tube C, Dublin Sample III; Tube D, Dublin Sample IV; Tube E, Dublin Sample C-42; Tube F, replicate of Tube B; and Tube G, replicate of Tube E.*

ion. This is particularly necessary for the determination of the Michaelis constant. The Dublin soil series displayed considerable uniformity in pH optima for the assay of phosphatase, arylsulfatase, and urease enzymes. The pH optimum for phosphatase was 7.0 ± 0.5; the optimum for urease was 6.5 ± 0.5. Figure 5 illustrates the variation of phosphatase, arylsulfatase, and urease activity with pH.

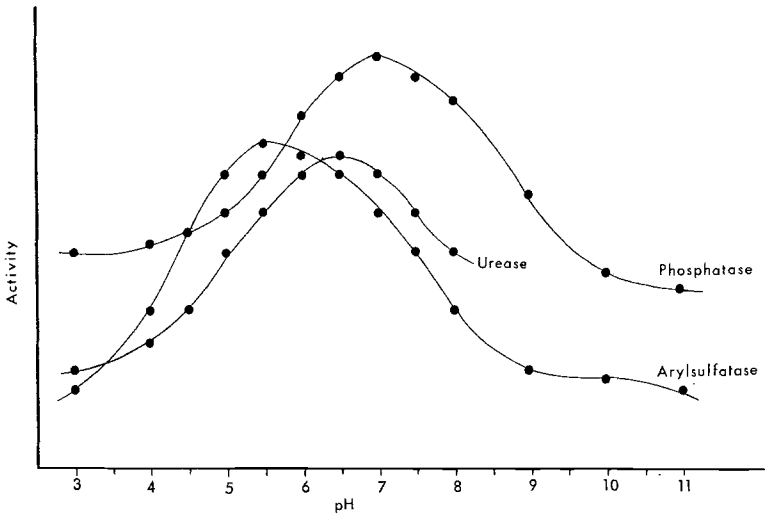

FIG. 5—*Influence of pH upon enzyme activity for Dublin soils. (The ordinate differs for each enzyme.)*

Figure 6 illustrates the absolute activities of the five Dublin soils with respect to the following enzymes: phosphatase, arylsulfatase, urease, trypsin (more correctly, *tryptic* activity), and invertase. Michaelis constants and maximal velocities were also determined for the five soils.

Reference to Fig. 6 illustrates that all of the five Dublin soils can now be distinguished, including Samples III and IV, which cannot be distinguished by conventional forensic techniques. While the results suggest that this approach to the forensic characterization of soils may have merit, the question of the variation of enzyme activity as a function of distance is not addressed by this experiment. This question could be formulated as follows: Although experiments performed on the Dublin soils suggest that it is possible to distinguish soils on the basis of enzyme activities, could soils from only a few inches apart exhibit variation of equivalent magnitude? The Columbia series was specifically used to answer this question.

The Columbia series was represented by a two-acre sampling site, with increasing sampling density toward the epicenter. All 28 samples possessed the identical Munsell Soil Color Code, 10YR/5/4, "Yellowish Brown." Samples representing the center and apices of the site were found to be indistinguishable by X-ray diffraction analysis and density gradient distribution. Phosphatase, arylsulfatase, urease, trypsin, and invertase activities were determined for each of the 28 sampling sites in this series. Table 2 depicts the activities of the 28 sites for each of these enzymes, and Table 3 illustrates values of K_m and V_{max} determined for phosphatase and arylsulfatase enzymes on the epicenter of the site (Sample 1) and with the samples representing the apices of the site (Samples 23–28).

The Hanford series represented an exceedingly uniform site with a known history. Figure 7 illustrates the uniformity of the soils with respect to the density gradient technique, and Fig. 8 illustrates the elemental analyses of the four soils tested. The

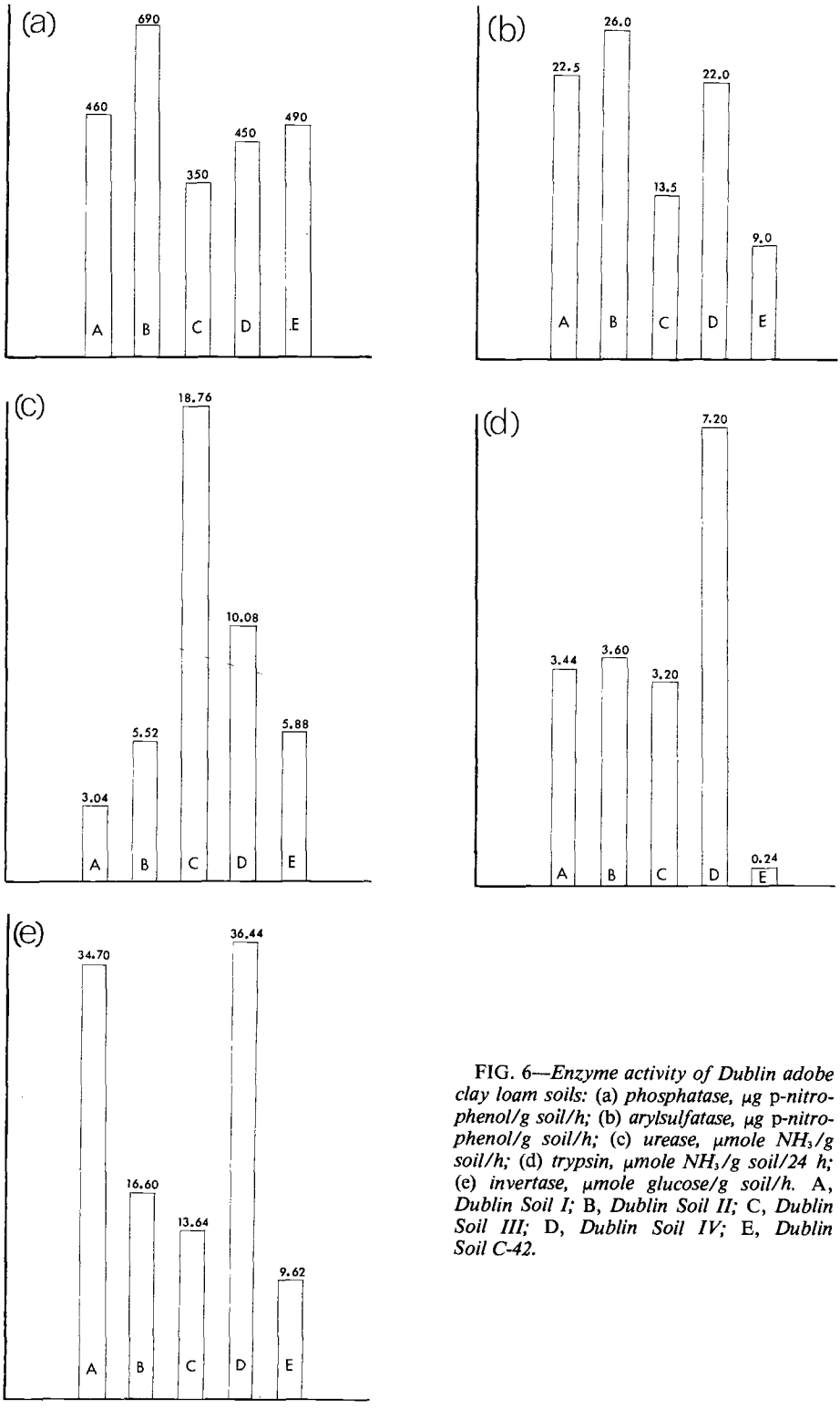

FIG. 6—*Enzyme activity of Dublin adobe clay loam soils:* (a) *phosphatase,* μg p-*nitrophenol/g soil/h;* (b) *arylsulfatase,* μg p-*nitrophenol/g soil/h;* (c) *urease,* μ*mole* NH_3*/g soil/h;* (d) *trypsin,* μ*mole* NH_3*/g soil/24 h;* (e) *invertase,* μ*mole glucose/g soil/h.* A, *Dublin Soil I;* B, *Dublin Soil II;* C, *Dublin Soil III;* D, *Dublin Soil IV;* E, *Dublin Soil C-42.*

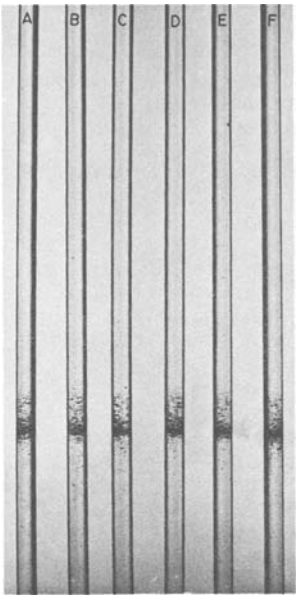

FIG. 7—*Density gradient distribution of Hanford sandy loam soils: Tube A, Hanford Sample 38; Tube B, Hanford Sample 46; Tube C, Hanford Sample 48; Tube D, Hanford Sample "G"; Tube E, Hanford Sample 46 treated with H_2O_2; and Tube F, Hanford Sample 46 heated to 600°C for 12 h.*

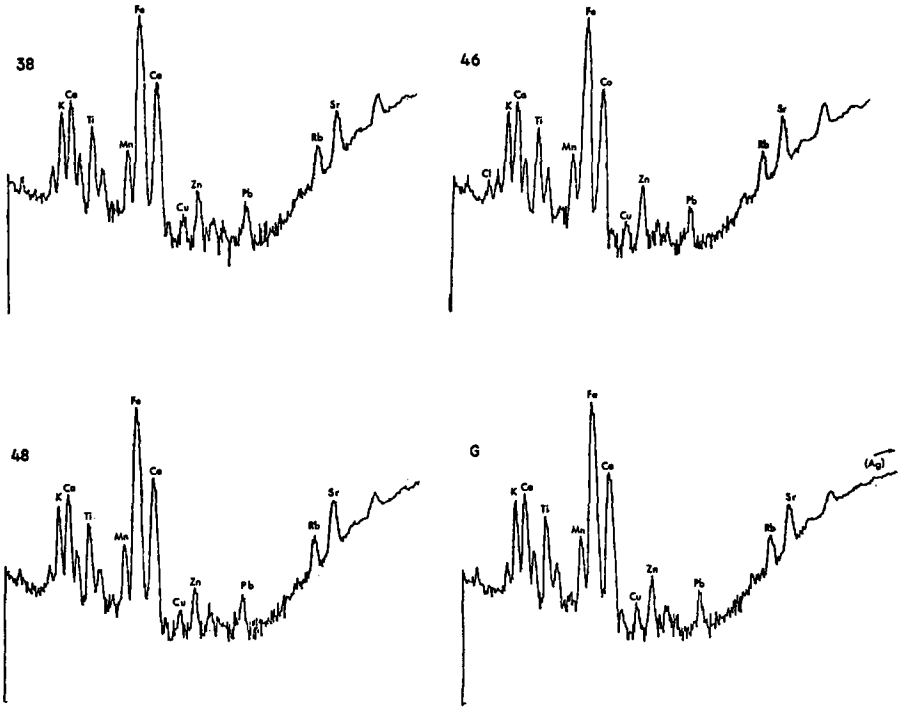

FIG. 8—*X-ray fluorescence spectroscopy of Hanford soils. The elements were identified principally by Kα radiation and confirmed by their Kβ radiation. With the exception of a significant amount of silver noted in Sample "G," the soils are indistinguishable by this technique.*

TABLE 2—*Raw data matrix for the 28-site Columbia series: enzyme activity.*

Site	Phosphatase, μg *p*-nitrophenol/g soil/h	Arylsulfatase, μg *p*-nitrophenol/g soil/h	Urease, μmoles NH_3/g soil/h	Trypsin, μmoles NH_3/g soil/48 h	Invertase, μg glucose/g soil/h
1	95.00	6.50	11.04	1.28	1.65
2	95.00	6.50	10.56	1.28	1.68
3	75.00	6.50	11.28	1.28	1.68
4	65.00	7.50	12.24	0.80	1.97
5	70.00	9.50	9.60	0.80	2.20
6	60.00	6.50	10.08	1.28	1.73
7	60.00	6.00	6.72	2.24	1.93
8	65.00	9.50	10.56	2.96	2.40
9	95.00	6.50	10.80	1.04	1.93
10	75.00	9.00	11.28	1.76	1.73
11	60.00	10.00	13.20	1.04	1.68
12	95.00	11.50	12.95	0.80	1.45
13	110.00	11.50	4.80	2.72	2.58
14	110.00	9.50	7.20	3.68	1.87
15	93.00	11.50	7.68	0.80	1.45
16	95.00	6.50	11.28	1.28	1.65
17	95.00	7.50	12.88	2.24	1.65
18	90.00	7.50	13.68	2.96	1.65
19	115.00	9.50	11.04	1.76	1.70
20	110.00	10.00	9.60	1.76	1.65
21	90.00	7.50	6.72	0.80	1.45
22	80.00	7.00	7.20	2.24	1.54
23	200.00	14.00	18.24	4.64	3.36
24	135.00	9.00	4.80	2.72	2.27
25	150.00	10.00	10.44	3.20	3.36
26	135.00	5.50	13.92	2.72	2.70
27	170.00	14.00	12.00	5.60	3.24
28	225.00	15.00	13.44	7.52	3.36

TABLE 3—K_m *and* V_{max} *of Columbia soils.*

	Phosphatase		Arylsulfatase	
Soil	K_m	V_{max}, μg *p*-nitrophenol/g soil/h	K_m	V_{max}, μg *p*-nitrophenol/g soil/h
1	$2.02 \times 10^3 M$	270	$3.14 \times 10^4 M$	172
23	$2.83 \times 10^3 M$	610	$4.12 \times 10^4 M$	540
24	$3.37 \times 10^3 M$	432	$3.04 \times 10^4 M$	235
25	$2.66 \times 10^3 M$	333	$2.86 \times 10^4 M$	325
26	$3.42 \times 10^3 M$	384	$1.88 \times 10^4 M$	146
27	$4.18 \times 10^3 M$	466	$2.25 \times 10^4 M$	210
28	$2.39 \times 10^3 M$	512	$2.86 \times 10^4 M$	605

variation among soils as determined by the X-ray spectroscopy is within the instrumental precision of the techniques. As with the Columbia series, the Hanford soils cannot be readily distinguished by the comparison of evidence and exemplar soil samples. The complexion of enzyme activity (phosphatase, arylsulfatase, invertase, urease, and tryptic activity) clearly distinguishes the soils, however. Table 4 illustrates the variation in phosphatase and arylsulfatase K_m and V_{max} for these samples.

TABLE 4—*Michaelis constant and maximal velocity for the Hanford soil series.*

Site	Phosphatase		Arylsulfatase	
	K_m	V_{max}, μg *p*-nitrophenol/g soil/h	K_m	V_{max}, μg *p*-nitrophenol/g soil/h
38	$4.24 \times 10^{3}M$	240	$3.23 \times 10^{3}M$	194
46	$4.02 \times 10^{3}M$	285	$3.86 \times 10^{3}M$	265
48	$4.38 \times 10^{3}M$	318	$3.14 \times 10^{3}M$	162
"G"	$2.34 \times 10^{3}M$	416	$3.44 \times 10^{3}M$	310

TABLE 5—*Dilution plate counts of bacteria, fungi, and actinomycetes for the Hanford Soil Series.*

Soil	Number per g Dry Soil		
	Bacteria	Fungi	Actinomycetes
Hanford 38	1.7×10^{7}	4.7×10^{4}	1.7×10^{6}
Hanford 46	2.0×10^{7}	4.5×10^{4}	1.7×10^{6}
Hanford 48	1.7×10^{7}	5.0×10^{4}	1.8×10^{5}
Hanford "G"	5.0×10^{7}	11.0×10^{4}	2.0×10^{5}

To determine if a correlation exists between enzyme activity and microbial populations in the Hanford series, dilution plate counts were performed for bacteria, actinomycetes, and fungi. Table 5 illustrates the results of this experiment. It is interesting to note that sample "G," with the highest enzyme activity, is also highest in numbers of bacteria and fungi.

Although a number of accounts have appeared in the soil science literature describing the fluctuation of enzyme activity with time, these accounts have not included studies of the Michaelis constant and maximal velocity. A brief experiment was conducted to determine if these properties exhibit important variations with time. Samples of the Dublin soils from Sites I–IV were collected at a time interval of six months (first collection 1 Feb. 1973; second collection 10 Aug. 1973).

Figure 9 illustrates a typical plot of V_{max} and K_m. The plot is that of the phosphatase

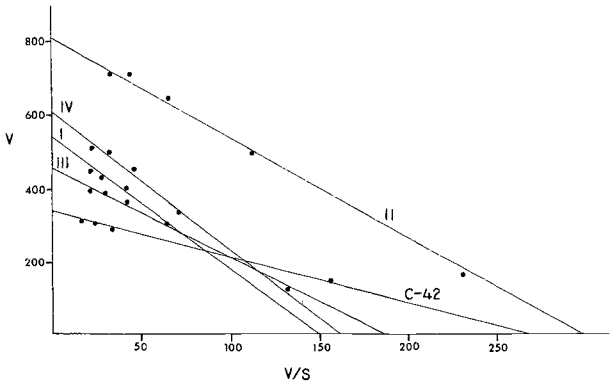

FIG. 9—*Plot of phosphatase* V *versus* (V/S) *for the Dublin soil series. The Michaelis constant is given by the slope of the line; that is, the slope is equal to* $-K_m$.

enzyme for the first sampling of Dublin soils. Table 6 illustrates the V_{max} and K_m of the four soils for the two sampling periods. It is noted that the V_{max} and K_m vary little between the two sampling periods, and that these properties maintain their position relative to one another; for example, Site II possesses the highest V_{max} in both sampling periods.

TABLE 6—*Test for stability of Michaelis constant and maximal velocity for phosphatase enzyme.*

Soil	V_{max}, μg *p*-nitrophenol/g soil/h	K_m
Dublin I		
2/1/73	540	$3.65 \times 10^3 M$
8/10/73	510	$3.28 \times 10^3 M$
Dublin II		
2/1/73	810	$2.75 \times 10^3 M$
8/10/73	800	$2.86 \times 10^3 M$
Dublin III		
2/1/73	455	$2.47 \times 10^3 M$
8/10/73	470	$2.68 \times 10^3 M$
Dublin IV		
2/1/73	605	$3.78 \times 10^3 M$
8/10/73	580	$3.32 \times 10^3 M$

Considerable attention was given to an objective means of assessing similarity (or lack thereof) in sampling sites on the basis of enzymological data. A successful technique was found in the form of the Canberra metric similarity coefficient, with the extent of similarity expressed graphically by means of a dendrogram. This technique, together with a discussion of its application in other areas of physical evidence, is reported in detail in a separate report [*32*]. A dendrogram of the 28-site Columbia soil series is shown in Fig. 10. Inspection of the dendrogram shows that Samples 1 and 16, separated by only a foot, are the most similar. Sample 2, again separated from Sample 1 by only a foot, is the next most similar. The samples at the apices of the sampling plot, some 150 yards from the epicenter of the plot, are the most dissimilar. It is the view of the authors that the algorithm of the Canberra metric similarity coefficient, together with the use of a dendrogram, represents an objective means of estimating similarity in sampling sites.

Summary and Conclusions

Soil is by no means an inert material, but rather it supports enormous numbers of microorganisms, as well as the root systems of higher plants. The living organisms in the soil (primarily the bacteria, actinomycetes, and fungi) manufacture countless different biochemicals. These biochemical compounds are excreted into the soil environment, or escape into the soil environment upon the death of the organism.

Of all the biochemicals encountered in the soil, the enzymes are the most easily demonstrated, principally because of their ability to turn over large amounts of substrate and thereby reveal their presence. In other words, it is not the enzyme that is measured per se, but its presence is detected by an amplification technique based on the fact that many substrate molecules can react and the products be measured after a given time of incubation. With some enzymes, sensitivities can be increased enormously by use of radioactive [*33*] or fluorescent [*34*] substrates. The tests may also be run in tandem, with

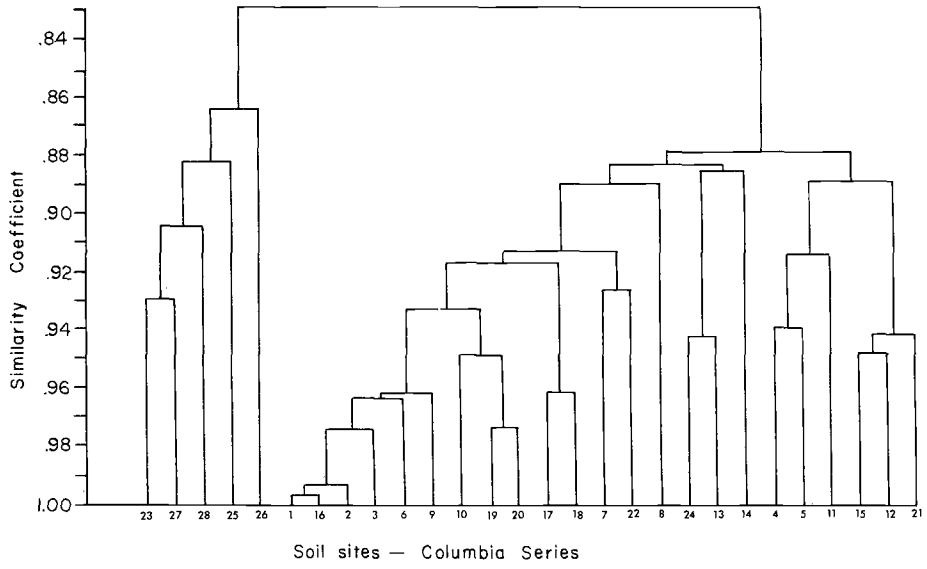

FIG. 10—*Dendrogram illustrating similarity of the 28 Columbia soil samples.*

the soil being washed free of one substrate before another is offered. Enzymes present in soil are quite varied, since the microorganisms in soil are varied; the concept of the ecological niche and the microhabitat imparts an exceedingly high degree of individuality to the soil microbiota, and this is reflected to a greater or lesser extent in the complexion of the enzymes in the soil.

It was found in the present study that the levels of enzyme activity in soil serve to characterize a soil as having originated from a given location. The enzymes considered were phosphatase, arylsulfatase, urease, invertase, and trypsin (more properly, tryptic activity). It was found that soils which were collected from sites within close proximity could be distinguished by their enzyme patterns. In addition to the absolute enzyme activity, the Michaelis constant is considered to possess particular significance in the forensic comparison of soils, since it is independent of sample size. Similarities in sampling sites are assessed by means of an algorithm, the Canberra metric similarity coefficient.

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Errata

Maes, D. and Pate, B. D., "The Spatial Distribution of Copper in Individual Human Hairs," *Journal of Forensic Sciences,* JFSCA, Vol. 21, No. 1, January 1976, pp. 127–149. On p. 129, line 10, the sentence should read, "In addition, a 1-cm segment ..." not "a 1-mm segment." On p. 147, lines 4 and 5 should read "... given growth rates within the reported [*20*] range of 0.1 to 0.4 mm per day. Thus the experiments with radiotracer support those with inactive Cu tracer by giving very similar results." On p. 147, under Discussion, third paragraph, line 6, the sentence should begin "In the case of Subject G" instead of "Subject E."

Thornton, J. I. and McLaren, A. D., "Enzyme Characterization of Soil Evidence," *Journal of Forensic Sciences,* JFSCA, Vol. 20, No. 4, October 1975, pp. 674–692. The manufacturer of Spectronic® 20 was incorrectly identified; Spectronic® 20 is a registered trademark of Bausch & Lomb, Inc.

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