3-METHOXY-DIHYDROFLUORAN-6-OL-6-PHOSPHATE: A FLUOROGENIC SUBSTRATE FOR THE DETECTION OF ENZYMATIC ACTIVITIES

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I Fluorescence

The remarkable sensitivity and selectivity inherent in fluorometric analyses have been abundantly demonstrated (1,2). A number of other advantages of fluorometry over spectrophotometry makes this technique particularly well suited to the measurement of biological activities of soils. Among these advantages are the relatively fewer manipulations required for assay and the fact that it is a measurement whose magnitude is directly proportional to the concentration of the fluorescent species. The method is restricted, however, to reactions involving the presence of a fluorescent reactant or product, and by the complex interferences of fluorescent inhibitors, light scattering particles and naturally fluorescent backgrounds. These interferences can usually be overcome by a variety of procedures, in which the choice of the fluorescent species plays a major part. Fluorescence assays in dilute solutions are governed by the following relationship (3):

\[ F = 2.3 \ \text{IECD}\ \varnothing \]

Where \( F \) is the total fluorescence flux expressed in quanta per second and integrated over all space, \( I \) is the intensity of exciting energy also in quanta per second, \( E \) is the molar extinction coefficient, \( C \) is the concentration of fluorescent material, \( D \) is the light path length and \( \varnothing \) is the quantum efficiency. While \( I, C \) and \( D \) can be controlled to a great extent by instrumentation, \( E \) and \( \varnothing \) are determined by the choice of the fluorescent species. In this respect, fluorescein has long been known as one of the most efficient fluorescent substances available. Its quantum yield in 0.1M NaOH is 0.92 (4), and both its excitation and fluorescence peaks are located in the visible spectrum at 490 m\( \mu \) and at 520 m\( \mu \) respectively. This eliminates the bulk of the naturally fluorescent background in the near ultra-violet found in most soils. The chemistry of fluorescein, however, is still obscured by a number of uncertainties regarding its structures and designations. Fluorescein is 3,6-fluorandiol (I) and the actual fluorescent species is the dianion of 9-(0-carboxyphenyl)-6-hydroxy-3- isoxanthenone, also known as uranine (II), (5), fluorescein has often been confused with fluorescein, which is its non-fluorescent dihydro derivative, (III).
Appropriate substitution of the hydroxyl hydrogens in structure I prevents the shift of I into II, thus blocking fluorescence. It is the specific hydrolyses at these sites which release II, and provide the means to assay hydrolytic activity through fluorescence. This ingenious concept, elaborated on by B. Rotman (1,2), from earlier work by Boolansky, can be generalized as follows:

\[
AB + H_2O \xrightarrow{\text{catalyst}} AH + BOH
\]

where AB is the fluorogenic substrate and AH is the fluorescent species.

II Soils

Soils are unique media for living organisms, supporting a highly complex population of bacteria, fungi, yeasts, protozoa and algae. Another remarkable property of soils is their ability to absorb and stabilize enzymatic activity outside of a living organism (6,7). Thus, it is not only possible to detect current life, it is also possible to recognize some of the past biological history of the soil.

Phosphatase activity has been given particular attention for the following reasons:
1. It is widespread among terrestrial organisms.
2. It catalyzes a wide range of reactions with moderate specificity
3. It is involved with the unique role of phosphorus in metabolism and energy transfer, which may very well be a universal characteristic of carbon-based aqueous living systems.
4. It is capable of being detected with relatively high sensitivity (8).
III Fluorogens

The phosphate ester derivative of both fluorescein (IV) and its monomethyl ether (V) were prepared according to the methods described by Rotman (9). The rate of spontaneous hydrolysis of these phosphates (sodium salt)

\[
\begin{array}{c}
\text{HO} & \text{P} & \text{O} \\
\text{OH} & \text{P} & \text{O} \\
\text{C} & \text{O} \\
\text{IV} & \text{FDP} \\
\end{array}
\quad
\begin{array}{c}
\text{HO} & \text{P} & \text{O} \\
\text{OH} & \text{P} & \text{O} \\
\text{C} & \text{O} \\
\text{V} & \text{MFP} \\
\end{array}
\]

in Tris-hydroxy methyl amino methane/Maleic acid 0.05M pH = 7.5 buffer at 22°C + 1°C, was measured by the appearance of fluorescence (Turner fluorometer—

Excitation: Wratten 2A + 47B filters)

Fluorescence: Wratten 2A + 12 filters)

<table>
<thead>
<tr>
<th>Structure</th>
<th>Code</th>
<th>Hydrolysis % per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>FDP</td>
<td>0.14</td>
</tr>
<tr>
<td>V</td>
<td>MFP</td>
<td>0.47</td>
</tr>
</tbody>
</table>

It can be seen that the relatively rapid rate of spontaneous hydrolysis precluded the measurement of phosphatase activities at levels where the use of the fluorescence technique is advantageous. These esters are much more stable in acid solutions, but optimum fluorescence was obtained at pH = 8 and above. Above pH = 8, however, these esters were rapidly hydrolyzed; thus pH = 7.5 was chosen as a suitable compromise, with approximately 10% loss in fluorescence yield.
IV Reduced Fluorogens

It is clear that the instability of these substances in alkali results from quinoid tautomerization, at the phosphate ester linkage, with loss of phosphate. Among the several possible solutions to this problem, it appeared likely that the reduced form of fluorescein (III) would form stable phosphate esters.

Reduced fluorescein was prepared according to the method of Liebig (9) and separated from fluorescein impurity by recrystallization from acetic acid. The light straw colored needles gave a m.p. of 125.5 ± 0.5° C. Mixed melting point determination with an authentic sample of dihydro fluorescein (III) showed no lowering of this value. Fluorescein (I) melts at 314-316° C.

Reduced fluorescein (III) was phosphorylated according to the method of Rotman (9), but with the following modifications: after treatment with Dowex 50 (H+), the solution was evaporated to dryness and redissolved in a small volume of acetone-pyridine (98% - 2%) to which was slowly added a fresh solution of potassium iodide in acetone, to the acetone, to the complete precipitation end-point. After exhaustive washing in fresh acetone, the white precipitate of dihydro-fluorescein dishosphate potassium was dried in vacuo and stored in the deep freeze. Purity was determined by fluorescence, after hydrolysis and oxidation, to be 9.3%. Impurities consisted primarily of a variety of stable non-fluorescent phosphate containing derivatives which could not be readily identified and removed. A subsequent improved synthesis devised by J. Westley and involving a reduction of fluorescein diphosphate (VI), abbreviated HFDP, with hydrogen/carbon-palladium yielded a product of at least 90% purity (12).

By the same procedure, the monomethyl ether of fluorescein was phosphorylated and reduced to yield 3-Methoxy-dihydro

\[
\text{VI} \quad \text{HFDP} \quad \text{VII} \quad \text{MHFP}
\]
fluoran-6-Oi-6-phosphate (VII), abbreviated MHFP. Determination of stability proved to be an onerous task. The results obtained for the spontaneous hydrolysis rate of the phosphate varied according to the procedure used in the determination. All determinations were carried out in 0.05M pH + 7.5 tris-hydroxy methyl amino methane/Maleic acid buffer at 22 ° ± 1 ° C. Both the appearance of fluorescence after biological oxidation (to be described) and that of inorganic phosphate were measured in darkness.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Code</th>
<th>By fluorescence</th>
<th>By Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI</td>
<td>HFDP</td>
<td>0.022</td>
<td>0.010</td>
</tr>
<tr>
<td>VII</td>
<td>MHFP</td>
<td>0.091</td>
<td>0.027</td>
</tr>
</tbody>
</table>

A number of factors governed the rate of spontaneous hydrolysis. Among these, the influence of light (incandescent light 100 watt at 50 cm, I.R. filter to maintain temperature at 22 ° ± 1 ° C) increased the observed fluorescence by a factor of nearly 2 as compared to complete darkness, and affected the Pi measurement by a factor of 1.2. Oxidation, as will be described, seemed to be the major factor in the discrepancy observed between the fluorescence and Pi data. The possibility that an organic (i.e. MHFP) or inorganic (P-P) pyrophosphate is accumulated during oxidation, was examined by an assay for the presence of pyrophosphate. This was carried out with the standard procedure of comparing an untreated aliquot with another hydrolyzed for 12 minutes at 100 ° C in 1N NCl, followed by phosphate determinations (13). All conditions of incubation were otherwise maintained unchanged. The results are shown below. (See also Figure 1).

<table>
<thead>
<tr>
<th>Rate of spontaneous hydrolysis (%) under oxidation treatment required to measure fluorescence:</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
</tr>
<tr>
<td>VII</td>
</tr>
</tbody>
</table>

It can be seen from the above table that pyrophosphate accounted for the major portion of the observed discrepancy between the fluorescence and Pi measurements. Thus, the fluorescence assay for the
Twelve Minute Hydrolysis of MHFP with 1N HCl at 100° C

Conditions:
Incubation with peroxidase done before hydrolysis in 0.05M Tris Ac pH 7.5 at 37°C
MHFP concentration used in Lowry-Lopez reaction = 3.3 x 10^-4 M
MHFP concentration used in incubation with peroxidase = 1 x 10^-4 M

Length of time of incubation, with and without peroxidase

Figure 1
spontaneous hydrolysis of MHFP is not a reliable test of its stability in alkaline solution. In fact, MHFP is 17 and 5 times more stable than MFP and FDP respectively under the conditions of the assays. These stability values become considerably more favorable at lower pH's.

V Oxidation of Reduced Fluorogens

The necessity of a prior oxidation of the hydrolyzed, reduced fluorogen is a major disadvantage in the fluorometric determination of phosphatase activity. An understanding of the mechanism and kinetics of oxidation was therefore deemed useful in the development of this soil assay.

MHFP oxidizes spontaneously at a slow rate, yielding MFP which hydrolyzes to MF and Pi at a much higher rate than MHFP. For purposes of measuring fluorescence, however, no adequate method of inorganic oxidation could be developed and resort had to be made to biological catalysts.

For both these reasons, therefore, the mechanism of oxidation was determined as follows.

First, the choice of enzymes was severely limited, not only by availability and stability, but more importantly by the fact that dehydrogenases and oxidases either require phosphopyridine nucleotides, flavin cofactors, or contain a porphyrin residue as well (14). All of these substances or residues exhibit marked fluorescence. The choices, then, restricted themselves to catalase and to horseradish peroxidase. The modes of action of these enzymes are markedly different and served adequately to identify the oxidation pathway of the fluorogenic substrate MHFP.

The generalized mechanism of these two enzymes can be written as follows:

Catalase: \[ 2H_2O_2 \rightarrow H_2O + O_2 \]

Peroxidase: \[ H_2O_2 + RH_2 \rightarrow 2H_2O + R \]

where \( H_2O_2 \) is the oxidant, \( RH_2 \) is the reduced organic substance (here MHFP) and \( R \) is the oxidized substrate.
It is clear that catalase would provide only a source of nascent oxygen to MBFP and it is oxygen which would act as the actual oxidant. Peroxidase, however, would catalyze a transfer of electrons from RH₂ to H₂O₂, yielding the desired product without the intervention of molecular oxygen. For the purpose of an assay, therefore, the second mechanism would be clearly preferable to the first. Conditions and results are shown below.

<table>
<thead>
<tr>
<th>No Enzyme</th>
<th>Catalase</th>
<th>Peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 M Tris/Maleic pH = 7.5 buffer</td>
<td>0.5(*)</td>
<td>0.5(*)</td>
</tr>
<tr>
<td>Catalase 10,000 U ml⁻¹ (*)</td>
<td>---</td>
<td>400 U/ml</td>
</tr>
<tr>
<td>Peroxidase (Horse-Radish) 10,000 U/ml⁻¹ (*)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MHP (*)</td>
<td>0.05μM/ml</td>
<td>0.05μM/ml</td>
</tr>
<tr>
<td>0.01 M Na₂O₂ (buffered)</td>
<td>9.5</td>
<td>9.5</td>
</tr>
<tr>
<td>Fluorescence: Initial</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>1 hour 22° ± 1° C</td>
<td>0.09</td>
<td>0.47</td>
</tr>
<tr>
<td>2 hours 22° ± 1° C</td>
<td>0.15</td>
<td>0.86</td>
</tr>
<tr>
<td>4 hours 22° ± 1° C</td>
<td>0.22</td>
<td>1.09</td>
</tr>
<tr>
<td>Maximum oxidation (calculated)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

(*) Dissolved in buffer, total volume 0.5 ml/10 ml samples.

It can be seen that the oxidation of the reduced fluorogen does not require molecular oxygen since the major pathway involves an electron transfer mechanism. In all observed cases, however, complete oxidation as measured by maximum fluorescence could not be attained, even when fresh H₂O₂ and enzyme was added to replenish the oxidant during incubation. A likely explanation is that horse-radish peroxidase possesses oxidase activity which can degrade the fluorogen (15, 16), by oxidative fission of the structure. The effect would be to lower the fluorescence efficiency and eventually to destroy it entirely. Increasing the concentration of the peroxidase to 0.1 mg/ml yields complete conversion in 15 minutes, during which time no significant destruction of fluorogen occurs (Figure 2).
MHFP: Hydrolysis + Oxidation, rate of oxidation

Conditions:
MHFP- incubated with alkaline phosphatase until hydrolysis complete, then peroxidase + $\text{Na}_2\text{O}_2$ added
.05 M Tris Ac, pH 7.5
22°C
MHFP- conc. on graph = $1 \times 10^{-8} \text{M}$

Length of time of incubation with peroxidase + $\text{Na}_2\text{O}_2$

Figure 2
VI Hydrolysis of MHFP With Phosphatases

Acid and alkaline phosphatases are widespread in animal and plant tissues, including yeasts and microorganisms where they usually occur as a part of the cellular membranes (exoenzymes) (16). These enzymes catalyze the transfer of the phosphoryl radical, $\text{O}_\text{P-OH}$, the synthesis of phosphate esters or their hydrolysis (17) and serve a wide variety of functions, some of them being still obscure. The standard method of assay for phosphatases has been the estimation of inorganic phosphate or other such compounds (18). All of these procedures are relatively insensitive when compared to fluorescence assays.

Alkaline and acid phosphatases are so designated by the pH ranges at which their highest activity is evident, but they are usually still active, albeit at a lower level, over a broad pH spectrum. Thus, acid phosphatases can be assayed at alkaline pH's with relative ease, and vice versa. Several of these enzymes have been highly purified, and the turnover numbers of alkaline phosphatases are usually much higher than those of acid phosphatases (18). Two enzymes were used as standards:

**Alkaline phosphatase** (calf intestine): activity 2,500 µg phosphate liberated per 30 minutes per mg (37°C pH = 5.0)

**Acid phosphatase** (wheat germ): activity 800 µg phosphate liberated per 30 minutes per mg (37°C pH = 9.3)

Only results with alkaline phosphatase are reported since similar data were obtained with acid phosphatase, but at approximately one-third the levels of alkaline phosphatase. Except where otherwise indicated, all results were obtained at pH = 7.5 in 0.05 Tris/Maleic or Tris/Acetate buffer and at 22°C ± 1°C.

Rates of hydrolysis of MHFP as a function of time and enzyme concentration are shown in Figure 3.

It can be seen that the optimal assay conditions are obtained during
MHP Rate of Hydrolysis with Alk. Phosphatase

Conditions:

22°C
.05M Tris Ac pH 7.5
MHFP-W incubation conc. = 1 X 10^-5
MHFP-W conc. on graph = 1 X 10^-8
peroxidase conc. = .1 mg/ml Na_2O_2 conc. = 10^-4M

Incubation with peroxidase = 15 minutes

Length of time of incubation with Alk. phosphatase + 15 minute incubation with peroxidase

Figure 3
relatively short time intervals. The changes of slopes are characteristic of substrate and of the enzyme degradation discussed previously.

A family of curves showing the relation between enzyme concentration and incubation times with fluorescence is presented in Figure 4. The remarkable sensitivity of the assay can be seen from the very steep and linear response up to $3 \times 10^{-2}$ mg/ml enzyme. The minimum concentration measurable at two hours incubation is of the order of $1 \times 10^{-3}$ mg/ml enzyme (15 fluorescence units). This corresponds to the activity of $10^6$ *B. subtilis*/ml in 1 minute incubation, or the activity of $10^4$ *B. subtilis*/ml in 100 minutes (figure 5). *B. subtilis* was grown in minimum phosphate to yield the optimum level of inducible exophosphatase activity. This figure represents a routine sensitivity limited by instrumentation and technique. Experimental results have been obtained with specialized equipment, yielding sensitivities better by at least one additional order of magnitude. With appropriate improvements, it is not unreasonable to suggest that it ought to be readily possible to detect the phosphatase activity of $10^3$ *B. subtilis*/ml in 10 minutes under conditions described.

VII MHFP Fluorogen for the Assay of Soil Enzymatic Activity

The main advantage of MHFP for the assay of enzymatic activity in soils and, by extension, to extraterrestrial soils, resides in its dual requirements for both peroxidatic (and to some extent oxidatic) and phosphatase activity. Tests with a number of soils have shown that both these activities are abundant. Data reported here were obtained with a standard soil LL (top soil neutral, air-dried and ground in agate mortar; collected in the vicinity of Stanford University Medical Center, June 1961, and stored at room temperature) (19).

Incubation with LL soil under standard conditions, without shaking, shows a rapidly increasing fluorescence as a function of time, compared to the inert sterilized soil control (sterilization: dry heat at 200°C for 48 hours). This data was obtained without Na$_2$O$_2$/peroxidase oxidation, demonstrating very substantial oxidative activity in soils. The fact that sterilized soil did not yield these results represents
MHFP: Rate of Hydrolysis with Alk. Phosphatase

Conditions:

25°C
.05 M Tris Ac pH 7.5
MHFP incubation conc. = 1 X 10^-5
MHFP conc. on graph = 1 X 10^-8
peroxidase conc. = 1 mg/ml, Na_2O_2
conc = 10^-4 M
Incubation with peroxidase = 15 min

Figure 4
Hydrolysis of MHPP- with B. subtilis

Conditions:
- 0.5 M Tris Ac, pH 7.5, 25°C
- Peroxidase (-1 mg/ml) + Na₂O₂ (10⁻⁴ M)
- MHPP- incubation conc. = 1 x 10⁻⁵ M
- MHPP-N conc. on graph = 1 x 10⁻⁷ M

Time in Minutes

Figure 5
evidence for the selective biological response of MHFP. (Figure 6 and 7). The sensitivity of the assay for biological activity can be calculated from the minimum observable and reproducible signal. Fluorescence measurements have been made to yield a detectable signal with the Turner Fluorometer Model III, with 1.0 mg. of soil ml\(^{-1}\) in 1 minute. Assuming a linear correlation and instruments of greater sensitivity by only one additional order of magnitude, as little as 0.01 mg of LL soil would yield detectable activity within ten minutes.

Since MHFP in the acid range was shown to be stable to 135° C for 30 minutes without detectable decomposition, this fluorogen would appear to be a useful substrate for the detection of biological activity in soils. The suitability of this substrate in an automated search for extraterrestrial life, however, has not been established.
Hydrolysis of MHFP with Lane Library Soil (no peroxidase added)

Conditions:
0.05 M Tris Ac, pH 7.5, 25°C
Soil not shaken with substrate

1 ml aliquot centrifuged 10 minutes before reading
MHFP Incubation conc. = 1 X 10⁻⁵ M
MHFP conc. on graph = 1 X 10⁻⁶ M

Figure 6
Hydrolysis of MHFP with Lane Library Soil
(no peroxidase added)

Conditions:
0.05 M Tris Ac, pH 7.5, 25°C

10 mg/ml Lane Library Soil; Soil not shaken with substrate (standing incubation)

Figure 7
Acknowledgements:

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REFERENCES

11. Argon sealed-tube measurements.