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

Elie A. Shneour

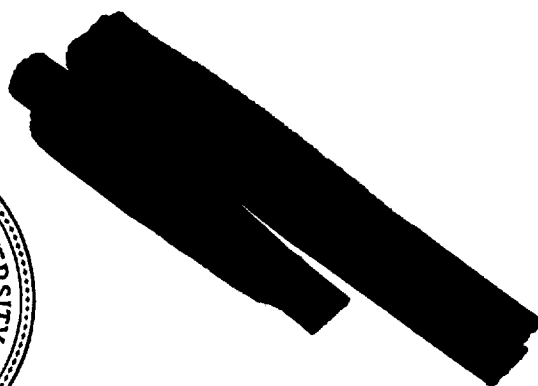Instrumentation Research Laboratory, Department of Genetics
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SUBSTRATE FOR THE DETECTION OF ENZYMATIC ACTIVITIES

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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):

$$F = 2.3 \ IECD \ \phi$$

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 ϕ is the quantum efficiency. While I, C and D can be controlled to a great extent by instrumentation, E and ϕ 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 mμ and at 520 mμ 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 fluorescin, which is its non-fluorescent dihydro derivative, (III).

1.
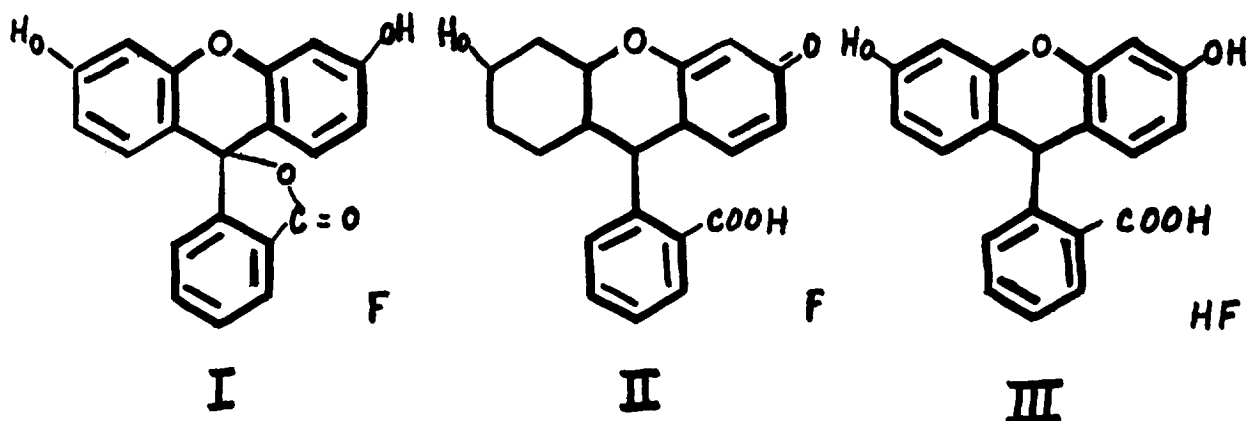
Appropriate substitution of the hydroxyl hydrogens in structure I pre-
vents the shift of I into II, thus blocking fluorescence. It is the
specific hydrolyses at these sites which release II, and provide the
means to assay hydrolytic activity through fluorescence. This ingen-
ious concept, elaborated on by B. Rotman (1,2), from earlier work by
Boolansky, can be generalized as follows:

$$AB + H_2O \xrightarrow{\text{catalyst}} AH + BOH$$
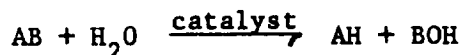
where AB is the fluorogenic substrate and AH is the fluorescent species.

II Soils

Soils are unique media for living organisms, supporting a highly
complex population of bacteria, fungi, yeasts, protozoa and algae. An-
other remarkable property of soils is their ability to absorb and stabi-
lize enzymatic activity outside of a living organism (6,7). Thus, it is
not only possible to detect current life, it is also possible to recog-
nize 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 mono-
methyl ether (V) were prepared according to the methods described by
Rotman (9). The rate of spontaneous hydrolysis of these phosphates (sodi-
um salt)

FDP

IV

MFP

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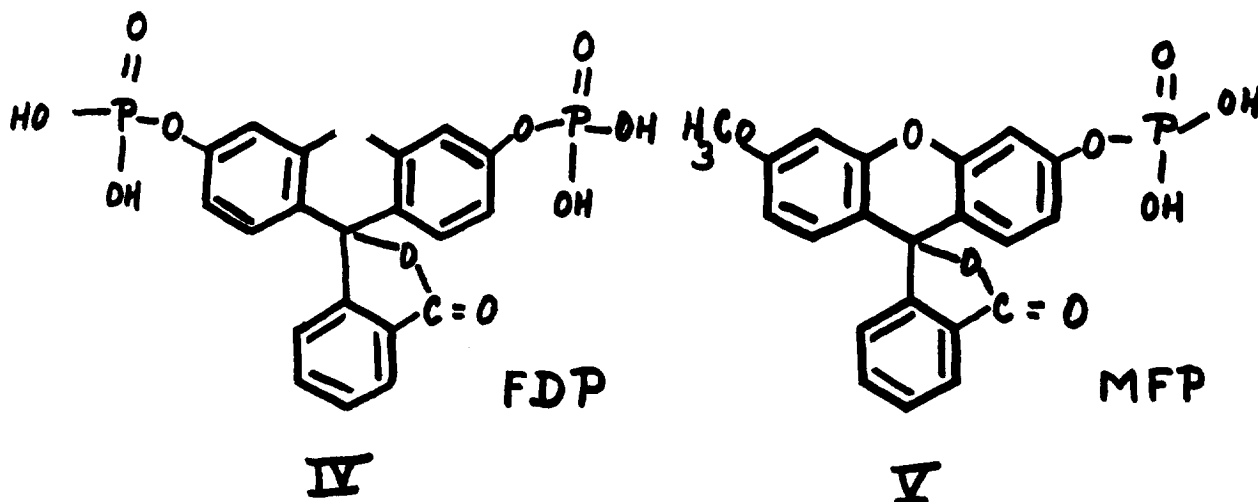
in Tris-hydroxy methyl amino methane/Maleic acid 0.05M pH = 7.5 buffer
at 22° C + 1° C, was measured by the appearance of fluorescence (Turner
fluorometer--

Excitation: Wratten 2A + 47B filters)
Fluorescence: Wratten 2A + 12 filters)

Structure	Code	Hydrolysis % per hour
IV	FDP	0.14
V	MFP	0.47

It can be seen that the relatively rapid rate of spontaneous hydroly-
sis 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 hy-
drolyzed; thus pH = 7.5 was chosen as a suitable compromise, with approxi-
mately 10% loss in fluorescence yield.

IV Reduced Fluorogens

It is clear that the instability of these substances in alkali results from quininoid 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 $\pm$ 0.5° C. Mixed melting point determination with an authentic sample of dihydro fluorescin (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 $\underline{\text{in}}$ $\underline{\text{vacuo}}$ 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
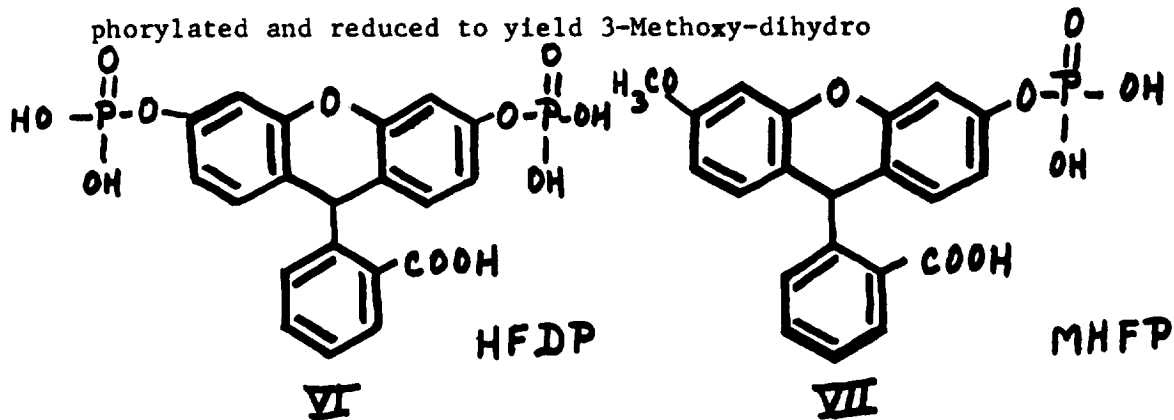
fluoran-6-01-6-phosphate (VII), abbreviated MHFP. Determination of
stability proved to be an onerous task. The results obtained for the
spontaneous hydrolysis rate of the phosphate varied according to the
procedure used in the determination. All determinations were carried
out in 0.05M pH + 7.5 tris-hydroxy methyl amino methane/Maleic acid
buffer at 22° + 1° C. Both the appearance of fluorescence after bio-
logical oxidation (to be described) and that of inorganic phosphate$_{Pi}$
were measured in darkness.

| Structure	Code	Rate of Spontaneous Hydrolysis (%) (in darkness)	
		By fluorescence	By Pi
VI	HFDP	0.022	0.010
VII	MHFP	0.091	0.027

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 com-
plete 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 fluroescence and Pi data. The possi-
bility that an organic (i.e. MHFPP) 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 re-
sults are shown below. (See also Figure 1).

Rate of spontaneous hydrolysis (%) under oxidation treatment re-
quired to measure fluorescence:

		Pi	Pyrophosphate	Total Phosphate
VII	MHFP	0.027	0.044	0.071

It can be seen from the above table that pyrophosphate accounted
for the major portion of the observed discrepancy between the fluor-
escence and Pi measurements. Thus, the fluorescence assay for the

Twelve Minute Hydrolysis of MHFP with 1N HCl at 100° C
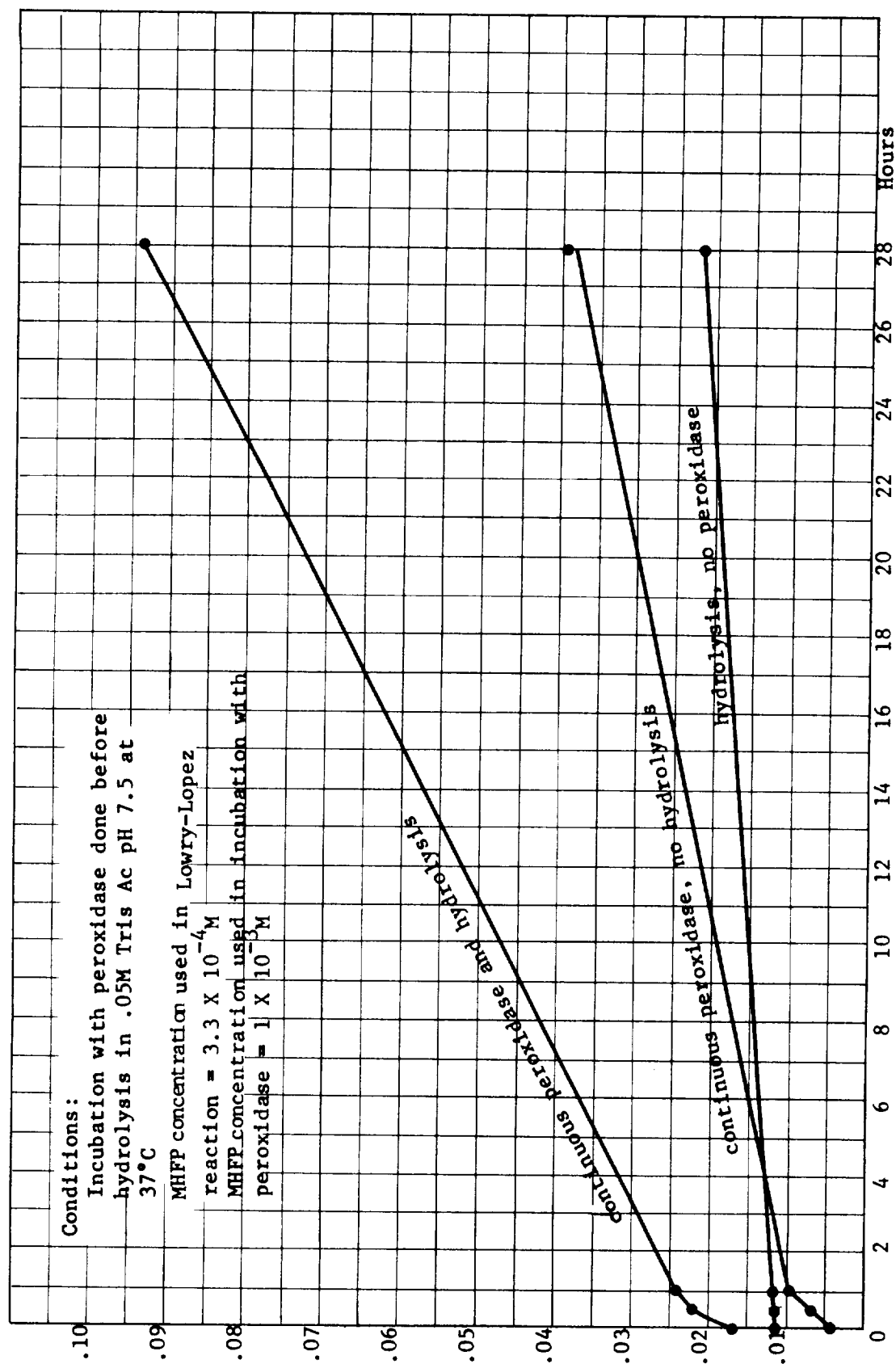
Figure 1

Length of time of incubation, with and without peroxidase

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.
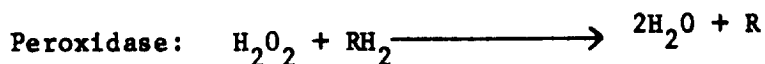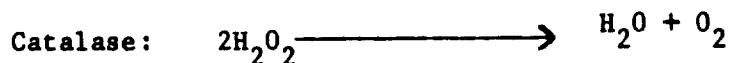

V   Oxidation of Reduced Fluorogens

The necessity of a prior oxidation of the hydrolyzed, reduced flu-
orogen 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 hy-
drolyzes to MF and Pi at a much higher rate than MHFP. For purposes
of measuring fluorescence, however, no adequate method of inorganic oxi-
dation could be developed and resort had to be made to biological cat-
alysts.

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 dehy-
drogenases 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 sub-
strate MHFP.

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

$$\text{Catalase:} \quad 2H_2O_2 \xrightarrow{\hspace{3cm}} H_2O + O_2$$

$$\text{Peroxidase:} \quad H_2O_2 + RH_2 \xrightarrow{\hspace{3cm}} 2H_2O + R$$

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 MHFP and it is oxygen which would act as the actual oxidant. Peroxidase, however, would catalyze a transfer of electrons from RH_2 to H_2O_2, 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.

	No Enzyme	+ Catalase	+ Peroxidase
1.0 M Tris/Maleic pH = 7.5 buffer	0.5[(*)]	0.5[(*)]	0.5[(*)]
Catalase 10,000 U ml^{-1} (*)	---	400 U/ml	---
Peroxidase (Horse-Radish) 10,000 U/ml^{-1} (*)	---	---	400 U/ml
MHF[(*)]	0.05μM/ml	0.05μM/ml	0.05μM/ml
0.01 M Na_2O_2 (buffered)	9.5	9.5	9.5
Fluorescence: Initial	0.02	0.05	0.03
1 hour 22° $\pm$ 1° C	0.09	0.47	78.6
2 hours 22° $\pm$ 1° C	0.15	0.86	84.2
4 hours 22° C $\pm$ 1° C	0.22	1.09	86.1
Maximum oxidation (calculated)	100	100	100

(*) 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 H_2O_2 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).

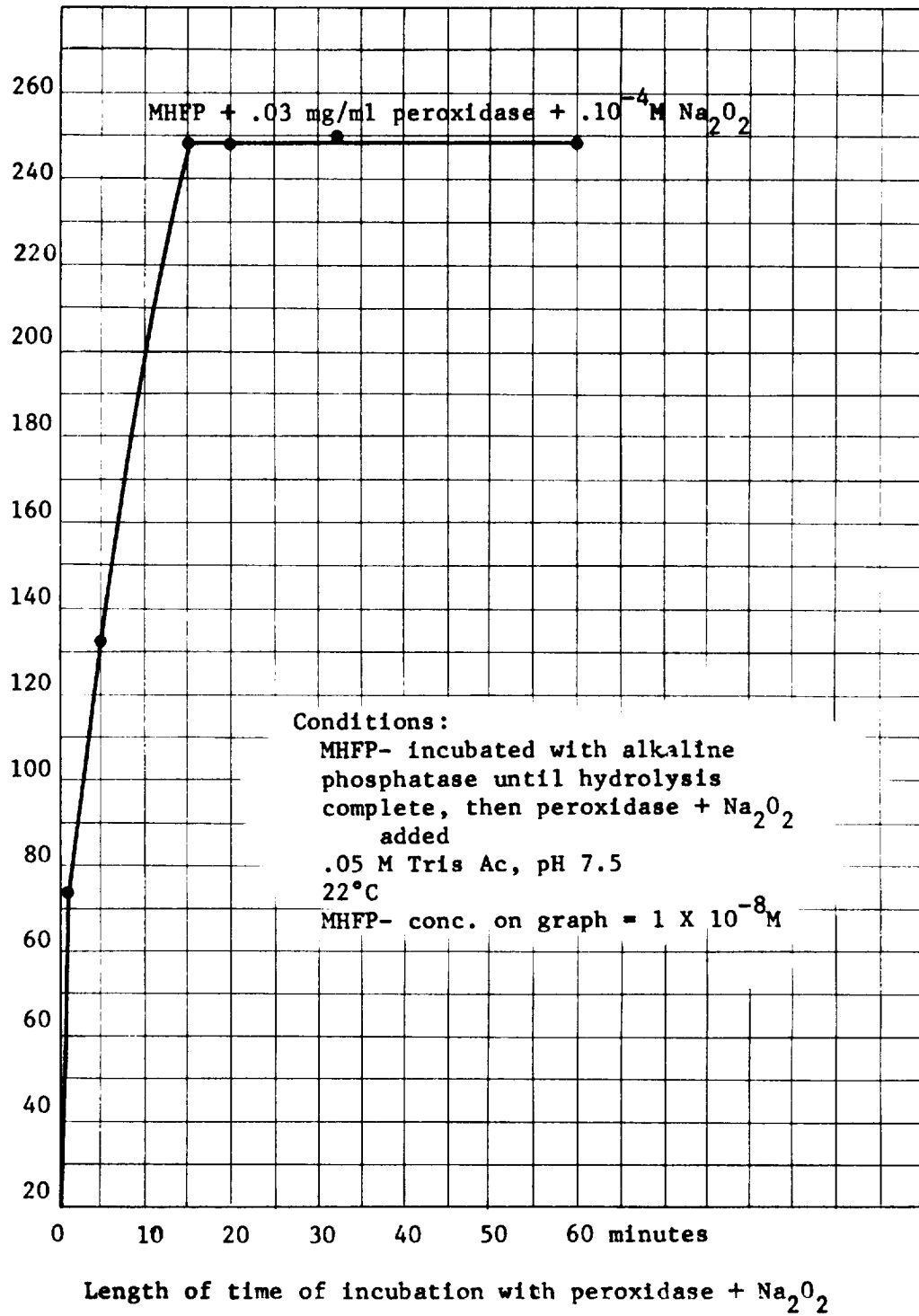MHFP: Hydrolysis + Oxidation, rate of oxidation

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, $\begin{smallmatrix} O \\ \| \\ -P-OH \\ | \\ OH \end{smallmatrix}$, the synthesis
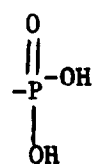
of phosphate esters or their hydrolysis (17) and serve a wide variety of functions, some of them being still obscure. The standard method of assay for phosphatases has been the estimation of inorganic phosphate or other such compounds (18). All of these procedures are relatively insensitive when compared 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 $\pm$ 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. = 1×10^{-5}
    MHFP-W conc.on graph   = 1×10^{-8}
    peroxidase conc. = .1 mg/ml Na_2O_2 conc. = 10^{-4}M

    Incubation with peroxidase = 15 minutes

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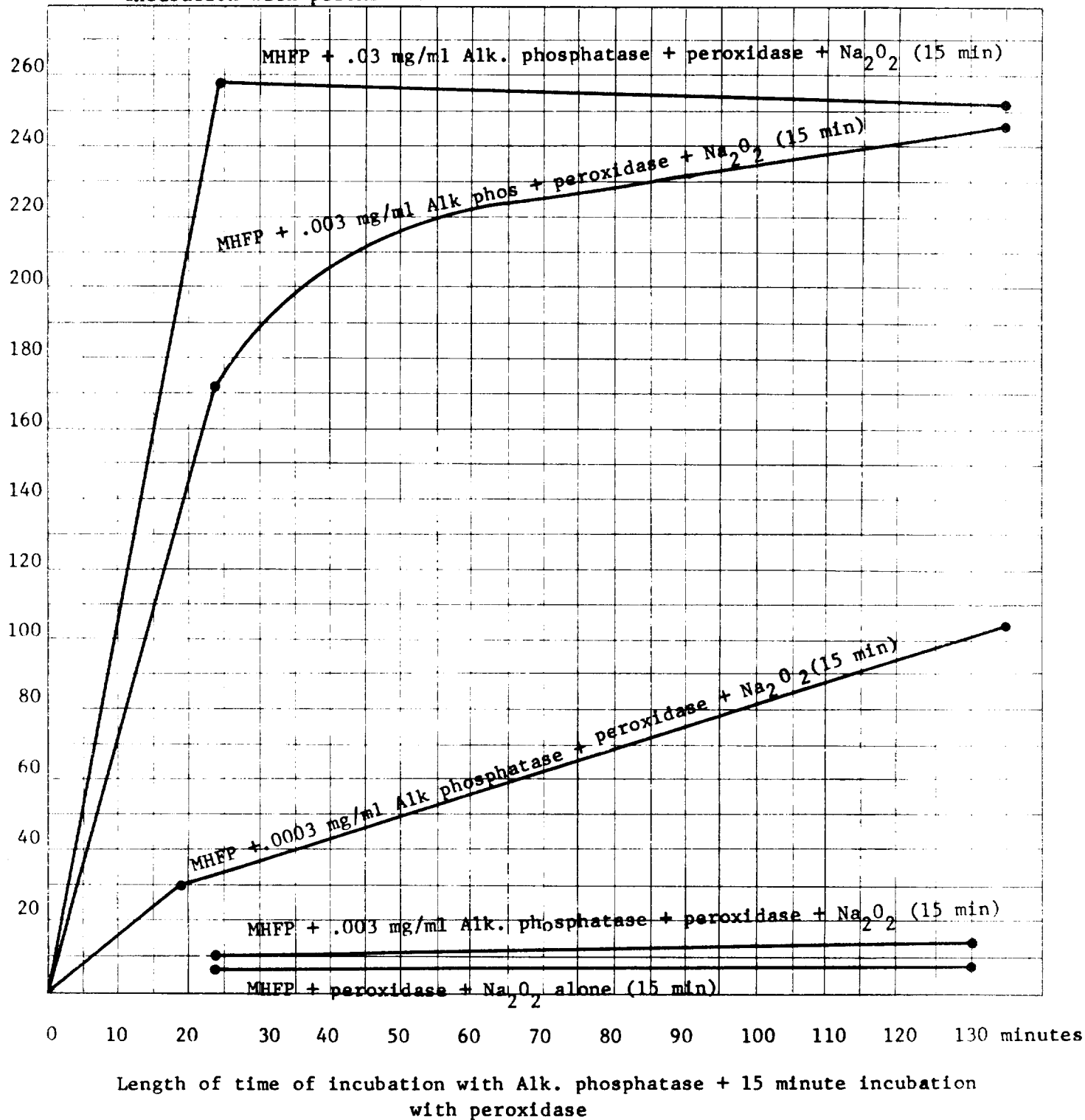with peroxidase

Figure 3

relatively short time intervals. The changes of slopes are character-istic of substrate and of the enzyme degradation discussed previously.

A family of curves showing the relation between enzyme concentra-tion 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 3×10^{-2} mg/ml enzyme. The minimum concentration measurable at two hours incubation is of the order of 1×10^{-3} mg/ml enzyme (15 fluorescence units). This corresponds to the activity of 10^6 B. subtilis/ml in 1 minute incubation, or the ac-tivity 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 bet-ter 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 ob-tained with a standard soil LL (top soil neutral, air-dried and ground in agate mortar; collected in the vicinity of Stanford Univers-ity 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, compar-ed to the inert sterilized soil control (sterilization: dry heat at 200° C for 48 hours). This data was obtained without Na_2O_2/peroxidase oxidation, demonstrating very substantial oxidative activity in soils. The fact that sterilized soil did not yield these results represents
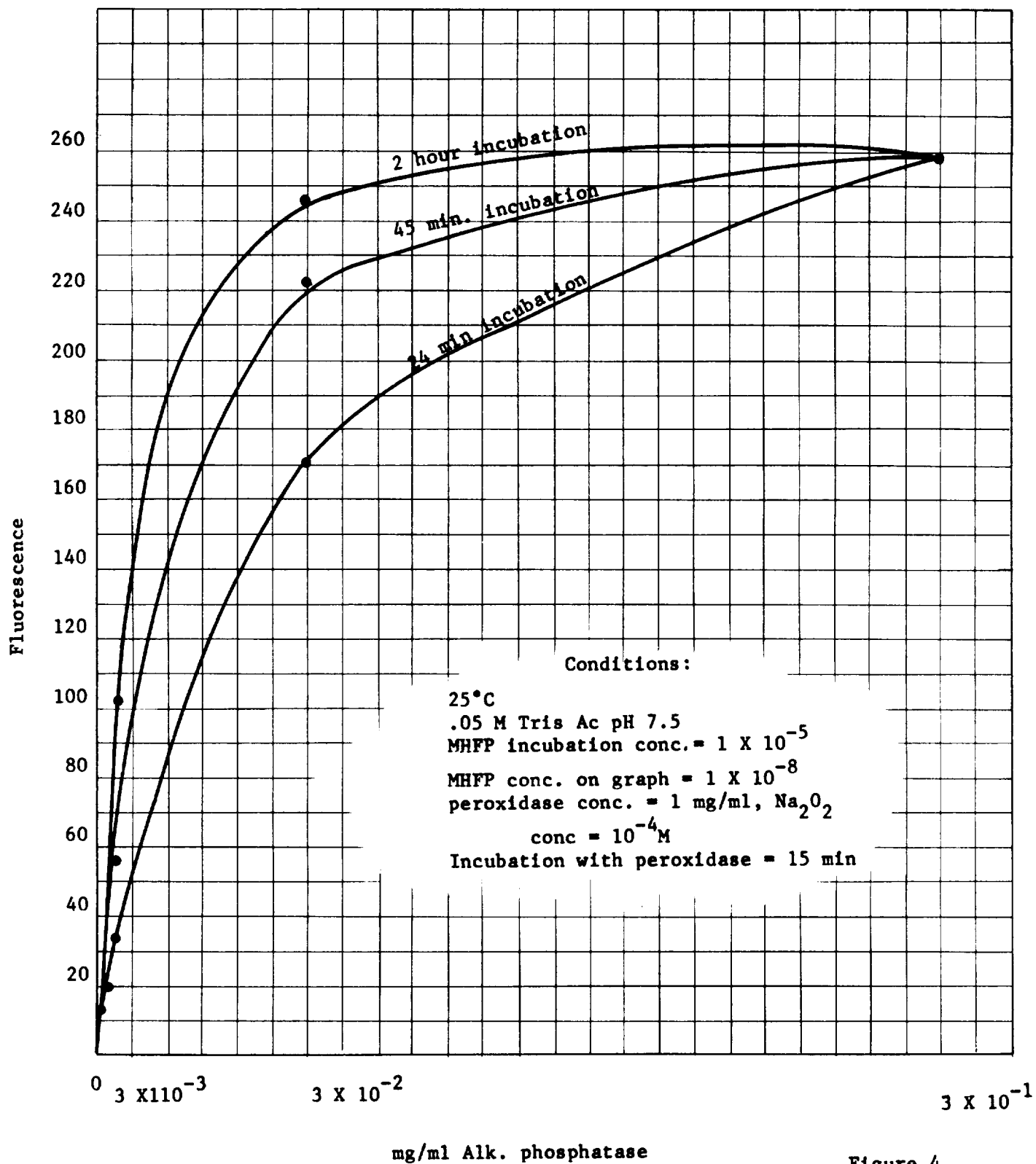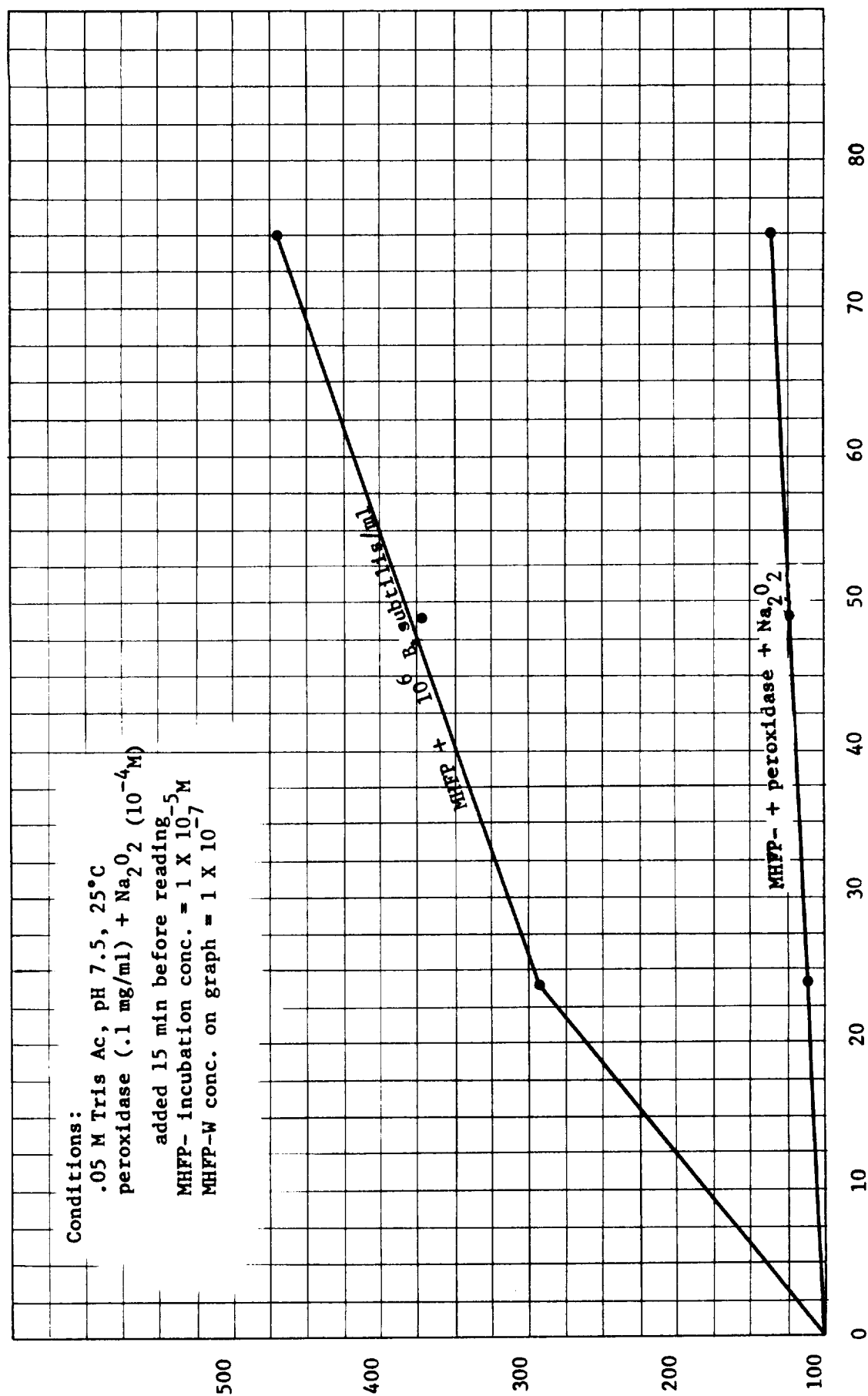
9.

MHFP: Rate of Hydrolysis with Alk. Phosphatase

Fluorescence (y-axis)

260
240
220
200
180
160
140
120
100
80
60
40
20

2 hour incubation

45 min. incubation

24 min incubation

Conditions:

25°C
.05 M Tris Ac pH 7.5
MHFP incubation conc. = 1×10^{-5}

MHFP conc. on graph = 1×10^{-8}
peroxidase conc. = 1 mg/ml, Na_2O_2

        conc = 10^{-4}M
Incubation with peroxidase = 15 min

0   3×110^{-3} 3×10^{-2} 3×10^{-1}

mg/ml Alk. phosphatase

Figure 4

Hydrolysis of MHFP- with B. subtilis

Conditions:
.05 M Tris Ac, pH 7.5, 25°C
peroxidase (.1 mg/ml) + Na_2O_2 (10^{-4}M)
added 15 min before reading
MHFP- incubation conc. = 1 X 10^{-5}M
MHFP-W conc. on graph = 1 X 10^{-7}

MHFP + 10^6 B. subtilis/ml

MHFP- + peroxidase + Na_2O_2

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 111, 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.
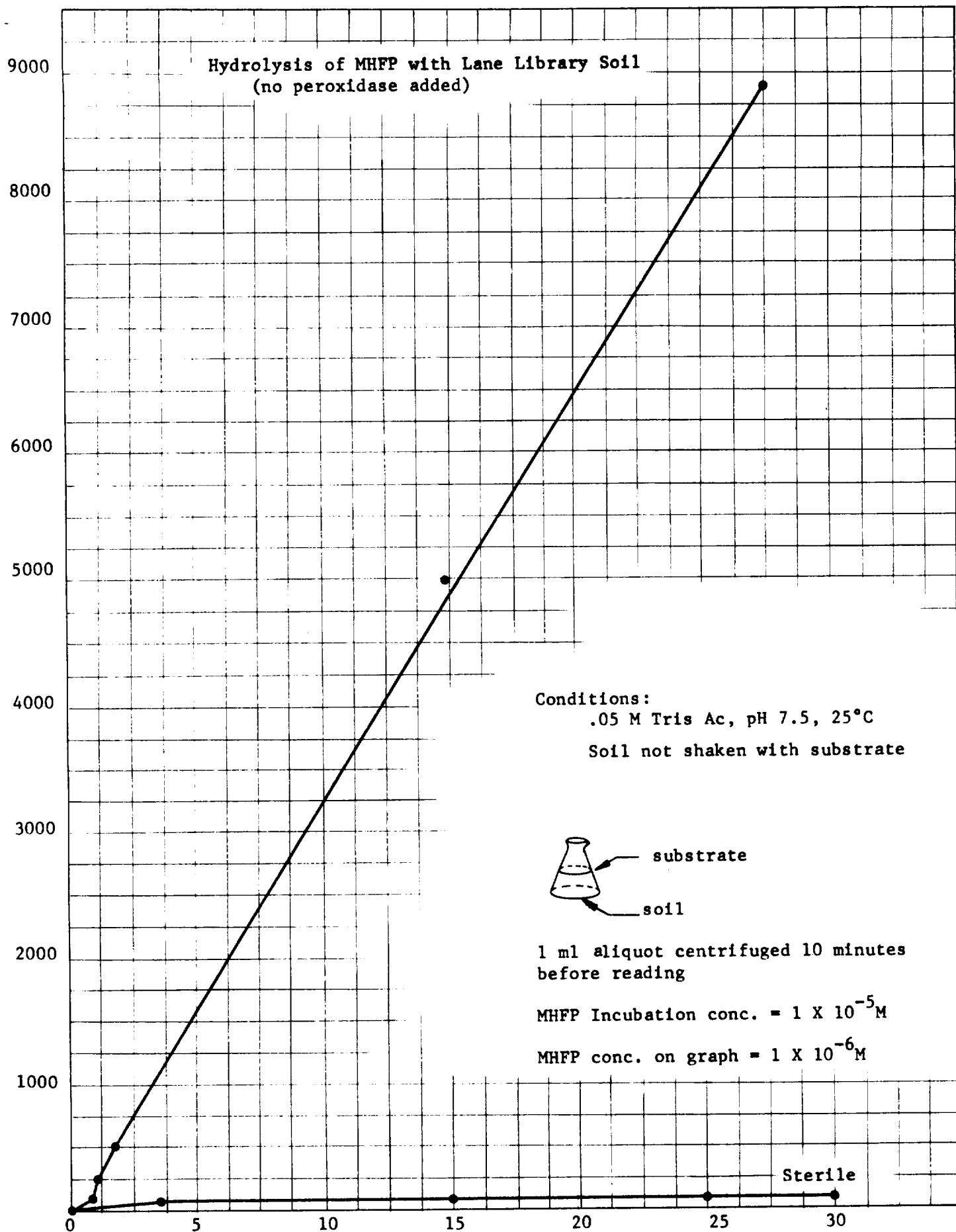
Hydrolysis of MHFP with Lane Library Soil
(no peroxidase added)

Conditions:
   .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

Sterile

Figure 6

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

Conditions:
.05 M Tris Ac, pH 7.5, 25°C

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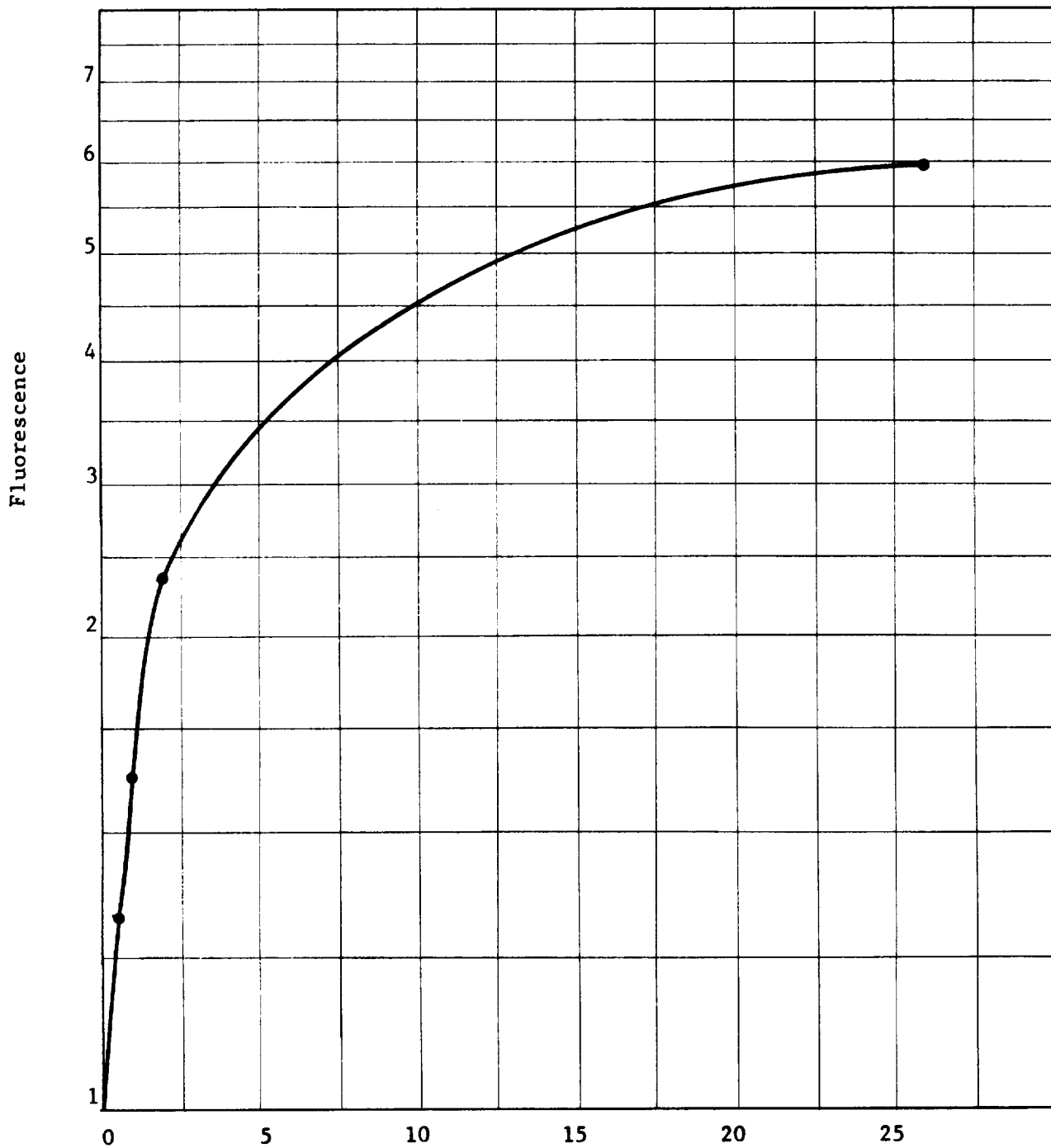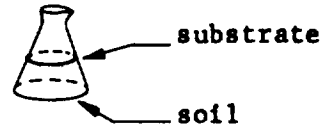shaken with substrate (standing incubation)

Figure 7

Acknowledgements:

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