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Studies on the stability of 3-*O*-methyl fluorescein phosphate

Monocyclohexylammonium 3-*O*-methyl fluorescein phosphate was first synthesized by ROTMAN, ZDERIC AND EDELSTEIN in 1963 (ref. 1). They suggested that the compound might be used as a substrate for determination of phosphatase activity since the liberated product, 3-*O*-methyl fluorescein, is highly fluorescent. LAND AND JACKIM[2] attempted to develop a phosphatase assay using 3-*O*-methyl fluorescein phosphate but they found it unsuitable because of spontaneous hydrolysis in solution at room temperature. Another disadvantage has been noted that even small numbers of bacteria may attack the compound[3]. We have found that refrigeration will significantly reduce spontaneous hydrolysis of 3-*O*-methyl fluorescein phosphate. Furthermore, if solutions of the compound are prepared using freshly distilled water and are stored at 4° in carefully cleansed glassware, bacterial contamination should be negligible. As an added precaution, however, we recommend the addition of streptomycin sulfate (25 μg/ml) to reduce the probability of bacterial contamination. This can be done without interfering with the measurement of fluorescence.

Glassware was carefully washed with Microsolv detergent (Microbiological Associates, Bethesda, Md.) and rinsed thoroughly in distilled water. Only freshly distilled water and highest purity reagents were used in preparation of solutions. The

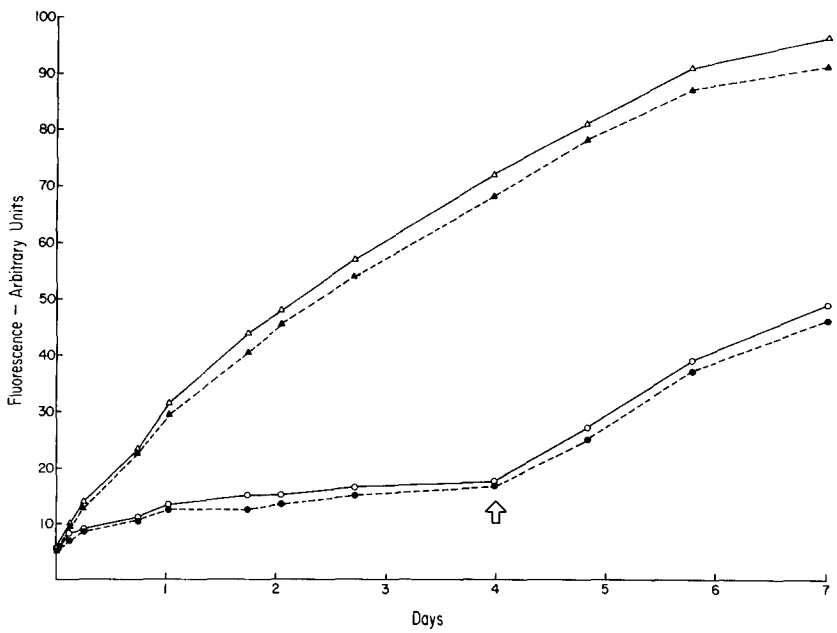

Fig. 1. Increase in relative fluorescence of substrate solutions. △—△, without streptomycin; ▲— — —▲, with streptomycin; both kept at room temperature (25°) for 7 days; ○—○, without streptomycin; ●— — —●, with streptomycin; both kept refrigerated at 4° for 4 days and subsequently removed to room temperature for 3 days. The large arrow indicates when refrigerated samples were transferred to room temperature.

substrate for alkaline phosphatase was prepared by dissolving 3-*O*-methyl fluorescein phosphate monocyclohexylammonium salt (Mann Research Laboratories, New York, N.Y.), 1.0 μg/ml, and $MgCl_2 \cdot 6H_2O$ 0.15 mg/ml, in 0.1 M Tris–HCl buffer (pH 8.7). Fluorescence produced by the liberation of 3-*O*-methyl fluorescein was measured in a Turner Model 111 fluorometer (1 × intensity, no slits) using the buffer as a blank. Activation and fluorescent wavelengths for 3-*O*-methyl fluorescein are 470 mμ and 520 mμ, respectively.

1 l of substrate solution was prepared as described and divided into four 250-ml samples. Two samples, one with and one without streptomycin (25 μg/ml), were kept at room temperature for 7 days. The other two samples, with and without streptomycin (25 μg/ml), were kept refrigerated at 4° for 4 days and then removed to room temperature (25°) for 3 days. Fluorescence was measured on aliquots withdrawn daily from each of the four samples. Results obtained are shown in Fig. 1. Samples kept at room temperature showed a comparatively rapid rate of substrate hydrolysis over the 7-day period. In contrast, samples, once chilled and kept refrigerated, exhibited a minimal rate of hydrolysis. When refrigerated samples were removed to room temperature on the fourth day, however, the rate of hydrolysis was observed to be the same as that in samples kept at room temperature. Streptomycin sulfate added at a concentration of 25 μg/ml was found to reduce fluorescence only slightly. The presence of the antibiotic did not alter the observed rates of substrate hydrolysis either at 4° or at room temperature (25°).

TABLE I

STANDARD PLATE COUNTS BASED ON A 1 ml VOLUME OF SUBSTRATE SOLUTION

Streptomycin sulfate was used in a concentration of 25 μg/ml.

Day	*With streptomycin*		*Without streptomycin*	
	Refrigerated (4°)	*Room temp. (25°)*	*Refrigerated (4°)*	*Room temp. (25°)*
1	32	1	1500	50
3	6	0	500	52
5	2	0	75	26 000
7	0	0	5	890 000
9	0	0	0	1400 000

We also determined that the concentration of streptomycin employed was sufficient to effectively inhibit bacterial growth in substrate solutions. Aliquots were removed under sterile conditions from each sample every 2 days and were serially diluted for bacterial plate culture at 35° and subsequent enumeration. Bacterial colony counts are shown in Table I. There was no significant bacterial growth in either the refrigerated or room temperature sample containing streptomycin sulfate at a concentration of 25 μg/ml. In the refrigerated sample not containing streptomycin a somewhat elevated initial bacterial count gradually decreased, presumably as a result of refrigeration. The room temperature sample which did not contain streptomycin showed considerable bacterial growth after 3 days.

In utilizing 3-*O*-methyl fluorescein phosphate at temperatures required for assay

of alkaline phosphatase, spontaneous hydrolysis will be a critical problem. Any manual procedure will require carefully controlled reaction time and temperature. We are now directing our attention to development of an automated flow procedure in an effort to overcome these difficulties.

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