TABLE III. Difference in Inactivation Values of Virus as Measured in Embryos and Membranes.

Min. of inactivation	Logarithm of titer: Embryos	Logarithm of titer: Membrane preparations	Difference
0	9.5	8	1.5
16	9.2	8	1.2
32	8.2	7.5	.8
48	8.3	5.8	2.5
64	5.5	3.8	1.7

curacy to the embryo technic although appreciably less sensitive. Evidence is submitted to show that both technics measure the same infectious entity.

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A Rapid Pad-Plate Microbiologic Assay for Thymine.* (21166)

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Although several methods(1-4) are available for the microdetermination of thymine, none of these is suitable for the rapid estimation of this material in a variety of biological materials. Williams, Esposito and Pierce(5) have described a microbial plate method for the assay of vit. B_{12}; this makes use of paper pads as originally used in the assay of antibiotics on plates containing a solid assay medium heavily inoculated with microorganisms. As compared with the usual microbiologic methods of assay, this technic affords many advantages, particularly for the assay of large numbers of samples. The present study is concerned with the development of a similar microbial assay method for thymine and thymidine using *Streptococcus faecalis* (ATCC 8043) as the test organism.

Experimental. Materials. Thymine. Material purchased from Nutritional Biochemicals, Inc., Cleveland, Ohio, was further purified by chromatography on Dowex I followed by several recrystallizations from water. *Thymidine* was prepared with the aid of intestinal phosphatase from thymidylic acid; the latter was isolated from calf thymus deoxyribonucleic acid by the method of Hurst, Little and Butler(6). The deoxyriboside was purified by adsorption on Dowex I, hydroxyl form, at pH 11.0; after washing the column thoroughly with 0.01 *N* NaOH followed by water, the thymidine was eluted with 0.04 *M* formic acid. Thymidine was obtained as a dry powder by lyophilization of the formic acid eluant. *Dihydrothymine* was obtained from Dougherty Chemicals, Richmond Hill, N. Y.; *5-methylcytosine* was supplied by G. H. Hitchings, Wellcome Research Laboratories, Tuckahoe, N. Y.; *5-hydroxymethyluracil* and

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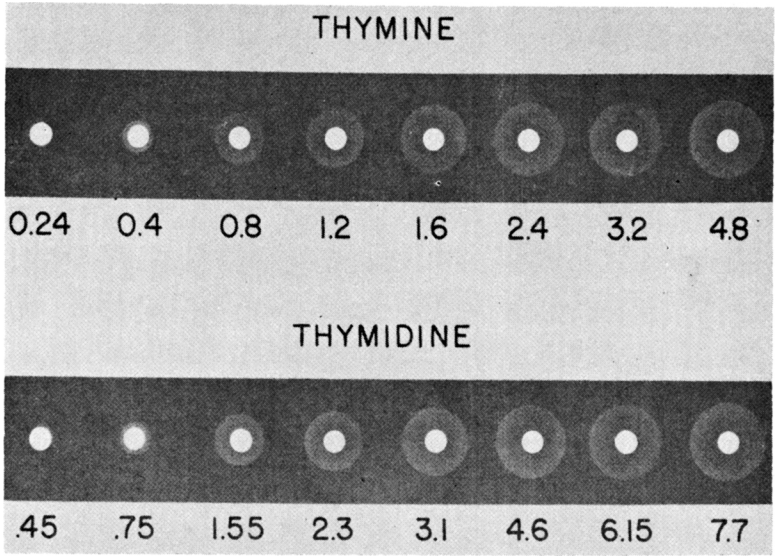

FIG. 1. Growth response of *Streptococcus faecalis* to increasing amounts (μg) of thymine and thymidine.

5-hydroxymethlcytosine were supplied by Sharp and Dohme Division, Merck and Co., West Point, Pa., through the courtesy of C. S. Miller and J. M. Sprague. *Organisms.* Lyophilized cells of *Streptococcus faecalis* (ATCC 8043), used in these studies, were prepared by growing the organisms in 500 ml batches of folic acid assay medium(7) which was supplemented with 1 mg of thymine per liter. After incubation of the inoculated medium for 18-20 hours, the cells were harvested by centrifugation, washed twice with isotonic saline, and finally lyophilized in a lactose suspending medium(8). The dry powder was transferred to small vials and stored in an evacuated desiccator over P_2O_5. The cells of one such preparation have remained viable, when stored in this manner, for a period of more than a year. *Pad Plate Assay.* Solid medium for this assay was prepared as follows: to 125 ml of folic acid assay medium(7) was added lyophilized enzymatic casein hydrolysate (General Biochemicals) (2 mg per ml), aminopterin (5 μg per ml) and agar (1.5%). The agar was dissolved by autoclaving for 10 minutes. After cooling to 50°, 30 mg of 2,3,5-triphenyltetrazolium chloride‡ was added to the medium as a freshly prepared aqueous solution (2 ml). After inoculation of the medium, using the lyophilized organisms suspended in saline (1-2 mg of dry cells were adequate for the inoculation of 125 ml of media), the medium was poured into a suitable tray§ carefully maintained in a horizontal position. The samples to be assayed were placed on small filter paper discs (Schleicher and Schuell No. 740-E, 6.35 mm diameter) with the aid of a Micro-Metric microburette and were allowed to dry at room temperature. These pads, together with pads containing known amounts of thymine or thymidine (which can be prepared previously and stored in small bottles), were placed at suitable intervals on the surface of the agar. The agar was then separated from the edge of the tray with a spatula, and, after covering with plate glass, the tray was placed in a 37° incubator. The zones of growth usually were visible after

‡ It has been found that the addition of 2,3,5-triphenyltetrazolium chloride, which is reduced to an insoluble red compound by the growing cells, improves visibility of areas of faint growth. Blue tetrazolium and neotetrazolium were not as satisfactory as the 2,3,5,-triphenyl derivative.

§ Plexiglass plates (12 x 8 inches) were used. These plates were quite satisfactory if allowed to dry thoroughly under slight pressure for 24 to 48 hours between assays. If this precaution is not observed the plastic becomes hydrated and the plates tend to warp.

5 or 6 hours, however, all recordings were made after an 18-hour period. The diameters of the zones of growth were measured with pointed calipers; the measurements obtained from the standards were used to construct a growth curve from which the amount of thymine on the pads to be assayed could be determined.

Results. The growth response of *S. faecalis* to varying amounts of thymine and thymidine is shown in Fig. 1. It is evident that this method will detect amounts as low as 0.40 and 0.75 μg of thymine and thymidine, respectively. A plot of the diameter of the zones of growth against the amount of compound gives a typical growth response curve.

Specificity. *S. faecalis* does not respond to the following compounds under the conditions of these experiments (the maximum amount of each compound tested is indicated parenthetically after each compound). Thymidylic acid (75 μg), 5-methylcytosine (6.5 μg), 5-hydroxymethyluracil (28 μg) 5-hydroxymethylcytosine (50 μg) and dihydrothymine (50 μg). In addition to the above mentioned compounds several low molecular weight (di- and tri-) polynucleotides,‖ which were known to contain thymine, but the exact structures of which have not been elucidated, did not support the growth of the test organism. Furthermore, there was no growth of *S. faecalis* when various materials, such as tissue homogenates, known to contain folic acid or its biologically active derivatives, were placed on the plates, indicating that aminopterin had effectively inhibited the utilization of these compounds. It was concluded tentatively that this assay is specific for thymine or thymidine, although it cannot differentiate between the two compounds.

The method has been used in connection with studies of the absorption, excretion and metabolic degradation of thymine and thymidine; the results of these studies will be reported elsewhere. It may be stated, however, that following relatively large oral doses of thymine to rats (up to 400 mg) and to men (up to 6 g) absorption was rapid and nearly complete. Less than one-fourth of the amounts administered appeared in the urine. These findings, together with studies using rat liver slices, have shown that thymine is very rapidly catabolized.

Summary. A rapid microbial (*S. faecalis*) pad-plate assay procedure, specific for thymine and thymidine, is described, in which response to folic acid and its derivatives is prevented by aminopterin. The method is sensitive to thymine in amounts ranging from about 0.4 to 5 μg and to thymidine in amounts of 0.75 to 7.5 μg.

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