17195. An Improved Method for the Microbiological Assay of Growth Factors on Paper Chromatograms.*

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The use of paper partition chromatography in the separation of microbial growth factors has recently been reported by Winsten and Eigen[1,2] and Cuthbertson and Smith.[3] These workers placed the developed chromatogram strips on nutrient agar seeded with the test organism. After incubation, zones of growth of the organism were noted along the site of each strip, indicating the presence of various growth factors. The relative concentration of these factors was estimated by the size of the zones.

The method of Winsten and Eigen[1] has been modified so that the chromatogram may be assayed both qualitatively and quantitatively for microbial growth factors using an improved technic. The improved method involves a direct test-tube microbiological assay of the developed chromatogram rather than the more tedious agar plate procedure. A description of the improved method, as used in the microbiological assay of paper chromatograms on which crystalline vitamin B_{12},† thymidine,† and injectable liver extract† were placed, is given in this report. *Lactobacillus leichmannii* (ATCC 4797) was used as the assay organism.

According to Shive, Ravel and Harding,[4] vitamin B_{12} and thymidine are interchangeable growth factors for *L. leichmannii* (ATCC 4797). Shive, Eakin, Harding, Ravel and Sutherland[5] have reported that thymidine may be present in amounts as high as 1% in some experimental injectable liver extracts. The presence of thymidine, therefore, may produce erroneous results in assaying liver preparations and other materials for vitamin B_{12}. By applying the procedure described in this paper, thymidine interference may be avoided.

Method. Whatman No. 1 filter paper (46 cm × 57 cm) was used in a chromatogram chamber similar to that described by Consden, Gordon and Martin.[6] A line was drawn across the paper 10 cm from the top edge. Along this line were placed small dots 3 cm apart. A known volume of the solution to be tested was placed on each dot with a microliter pipette using graded levels of from 1 to 10 microliters. In general each level was placed on from 2 to 4 dots. Evidence that the results could be duplicated was obtained by this means.

In no case was the spot of liquid allowed to spread more than 1 cm in diameter. Spreading was prevented by applying the liquid to the paper in portions of 3 microliters or less and allowing the liquid to evaporate between

* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D.C., and was aided by grants from the Nutrition Foundation, New York City, the Cerophyl Laboratories, Kansas City, Mo.; Commercial Solvents Corporation, New York City; the Cooperative G.L.F. Exchange, Ithaca, N. Y., and the Western Condensing Co., San Francisco. The technical assistance of Betty F. Brown and Diana M. Cameron is acknowledged.

1 Winsten, W. A., and Eigen, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 513.

2 Winsten, W. A., and Eigen, E., *J. Biol. Chem.*, 1949, **177**, 989.

3 Cuthbertson, W. F. J., and Smith, E. Lester, *Biochem. J.*, 1949, **44**, No. 2 (*Proc. Biochem. Soc.*, v).

† The crystalline vitamin B_{12} used in this work was kindly supplied by Dr. E. Lester Smith of Glaxo Laboratories, England, and the thymidine by Dr. William Shive of the University of Texas. The injectable liver extract was Lilly's extract containing 15 U.S.P. units per ml.

4 Shive, W., Ravel, J. M., and Harding, W. M., *J. Biol. Chem.*, 1948, **176**, 991.

5 Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Amer. Chem. Soc.*, 1948, **70**, 2299.

6 Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

TABLE I.
Results of Assay of Paper Chromatograms Containing Injectable Liver Extract, Vitamin B_{12} and Thymidine.

Substance assayed	Amt. placed on paper	Location of growth by number of 2 cm section	Distance to solvent front in cm	Recovery in growth zone in %
Crystalline vitamin B_{12}	0.1 mγ	1	44.5	98.3
	0.2 "	1	37.0	104.4
	0.3 "	1	42.5	98.8
Liver extract, 15 units/ml	0.00015 unit	1	44.2	106.7
	0.0003 "	1	43.5	100.9
	0.0015 "	1	37.0	100.0
	0.0030 "	1	37.0	100.0
Thymidine	0.5 γ	4–7	35.4	—*
	1.0 γ	4–8	35.4	—
	1.4 γ	4–8	35.4	—
	2.1 γ	4–8	34.8	—
Vitamin B_{12}	0.3 mγ	1	43.4	100.0
+ thymidine	0.2 γ	5–7		—
Liver extract	0.00015 unit	1	35.4	103.3
+ thymidine	0.5 γ	4–7		—
Liver extract	0.0045 unit	1	35.4	96.0
+ thymidine	1.4 γ	3–8		—

* Determination was omitted because sufficient thymidine was not available to prepare standard curves.

applications. An electric fan facilitated rapid evaporation. The spots were air dried and the paper was then placed in the chromatogram chamber with the upper edge immersed in the solvent trough.

The solvent used was n-butyl alcohol saturated with water. This was placed in the trough and also in each of two beakers at the bottom of the chamber to insure complete saturation of the chamber with the solvent vapors. The chromatographic procedure was carried out at room temperature. After 24 hours, or when the solvent had advanced approximately 40 cm, the paper was removed and suspended in a hood in front of a fan until all traces of solvent had disappeared.

The paper was then cut lengthwise in strips 3 cm wide so that each strip contained the material from one spot. These strips were then cut crosswise into sections 2 cm wide. The small sections of filter paper were each placed in test tubes, 10 ml of a newly developed medium[7] added and the tubes steamed 15 minutes. This was followed by inoculation with *L. leichmannii* (ATCC 4797) using a drop inoculator. Drop inoculation was found necessary since loop inoculation resulted in fraying of the filter paper which caused increased turbidity. After 16 hours incubation, growth was measured turbidimetrically in a Coleman Spectrophotometer set at 650 millimicrons.

When carrying out quantitative assays, the values for the standard curves were obtained by placing graded amounts of the standard solution of crystalline vitamin B_{12} on 2 x 3 cm sections of dried, butanol-treated filter paper using a microliter pipette and assaying these along with the developed chromatogram.

The presence of filter paper had no significant effect upon the growth of the organism. Pieces of filter paper ranging in size from 1 x 3 cm to 6 x 3 cm were tested and no appreciable differences in growth noted.

Results and Discussion. In Table I are given some typical results using the improved procedure in the assay of chromatograms for vitamin B_{12} and thymidine. The method was

[7] Peeler, H. T., Yacowitz, Harold, and Norris, L. C., unpublished results.

found to be sensitive to 0.1 millimicrograms of vitamin B_{12} present in the developed chromatogram. No measureable destruction of vitamin B_{12} was noted in the chromatographic procedure.

In the assay of the liver extract and crystalline vitamin B_{12} all the growth was confined to the first 2 cm section of the chromatograms. The remaining sections of the chromatogram failed to promote any growth response, indicating that the liver extract used in our experiments contained little, or no, thymidine or other growth factors. Interference from thymidine, therefore, did not occur when assaying the liver extract at a level of 0.0045 unit, or less, per tube.

In preliminary work it is necessary to assay each cross sectional piece of filter paper in the entire length of the chromatogram. Once the range of R_f‡ values of any substance has been accurately determined, it is only necessary to test the section known to contain that particular substance. For example, after separating thymidine from vitamin B_{12}, only the first 2 cm section of the chromatogram needs to be tested to obtain the vitamin B_{12} content of the substance being assayed.

The R_f value of vitamin B_{12} was found to be less than 0.04 while the large thymidine zone had an average R_f value of 0.33 and varied between 0.31 and 0.37. This thymidine R_f value differs from that reported by Hotchkiss[8] who obtained an R_f value of 0.51 using the same solvent but in the presence of gaseous ammonia. Winsten and Eigen[2] reported a double zone of growth for thymidine with the principal zone having an R_f value of 0.54 and a minor zone with an R_f value of 0.41. These latter workers were not able to state unequivocally which of these zones was due to thymidine.

The improved procedure has also been used successfully with the ascending solvent technic described by Williams and Kirby.[9] It appears applicable to the study of unidentified growth factors required by other microorganisms as well as *L. leichmannii* (ATCC 4797) and in the study of growth inhibitors and antibiotics.

Summary. A simple, quantitative method for the microbiological assay of filter paper chromatograms has been described. Some possible applications have also been pointed out.

‡R_f (rate of flow) = Distance from starting line to center of growth zone / Distance from starting line to solvent front

[8] Hotchkiss, R. D., *J. Biol. Chem.*, 1948, **175**, 315.

[9] Williams, R. J., and Kirby, H., *Science*, 1948, **107**, 481.

Received June 6, 1949. P.S.E.B.M., 1949, **71**.

17196. Duodenal Ulcers Produced on a Diet Deficient in Pantothenic Acid.*

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It is well known that various nutritional essentials are concerned with the maintenance of normal epithelial tissue. As part of a program concerning nutritional deficiencies and gastrointestinal epithelium, we have reported the finding of marked antral gastritis on calcium deficient diets.[1] Similar, less marked and less constant lesions were also reported on thiamine deficiency. Under various conditions of inanition the fundic mucosa will show rather typical areas of hemorrhage.[2] Previously, many papers have appeared on the

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[1] Zucker, T. F., Berg, B. N., and Zucker, L. M., *J. Nutrition*, 1945, **30**, 301.

[2] Zucker, T. F., Berg, B. N., and Zucker, L. M., *J. Nutrition*, 1945, **30**, 319.