mercial water-miscible vitamin preparation from a different source gave similar abnormal effects.

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Comparative Bioautography of Vitamin B_{12} and Related Growth Factors Using *Euglena gracilis* and *Lactobacillus leichmannii*. (18248)

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It has been reported by Hutner *et al.*(1) that *Euglena gracilis* var. *bacillaris*, an algal flagellate, exhibits a quantitative growth response to crystalline vit. B_{12}, and that the desoxyriboside thymidine is inactive for this organism. Winsten and Eigen(2), and Cuthbertson and Smith(3,4) have used paper chromatographic and bioautographic procedures to demonstrate that certain *Lactobacillus leichmannii* strains exhibit a growth response to thymidine and other desoxyribosides in addition to vit. B_{12}. The procedures used by these authors demonstrated that the vit. B_{12} was found in the area of the slow-moving factors on the chromatograms while the desoxyribosides studied appeared as the more rapidly moving growth factors. Hutner's group(1) suggested that comparative studies using *E. gracilis* and lactobacilli would be of value in investigating the functions of vit. B_{12} and related growth factors. This communication presents a method for the use of *E. gracilis* as a bioautographic microorganism, and the application of paper chromatography and bioautography to the comparative study of the response of *E. gracilis* var. *bacillaris* and *L. leichmannii* 313 to vit. B_{12} and related growth factors.

Methods and materials. The general method employed in these comparative studies consisted of subjecting to paper chromatography selected samples of various preparations in duplicate, bioautographing one of the strips with *E. gracilis* var. *bacillaris* (ATCC 10616) and the other with *L. leichmannii* 313 (ATCC 7830) and finally comparing the positions of the areas of growth response of the two organisms. Descending chromatograms were prepared essentially as described by Winsten and Eigen(2) using *n*-butanol saturated with water as the solvent. Twenty-five μl of the material to be examined were applied to a 1 x 20 inch strip of Whatman No. 1 filter paper. After the desired distance of solvent flow had been attained, the strips were removed from the chamber and allowed to air dry in a disinfected hood.

The use of *E. gracilis* as a bioautograph organism required additional precautions against contamination because of its long incubation period. For this reason, aseptic technics, as far as practicable, were used. It appeared that *n*-butanol was in most cases an effective antiseptic agent, so that the wet chromatograms, if dried in a disinfected hood and thereafter handled aseptically, would remain essentially sterile. Large pyrex baking dishes with close fitting plate glass covers, used to contain the nutrient agar layers, were sterilized in an oven at 150°C. The medium was that of Hutner *et al.*(1) with 1.5% agar added, and was sterilized in the autoclave at

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121°C for 15 minutes. The culture was maintained and the inocula were prepared as described by these authors. Subsequent operations were carried out in an inoculation room which had been disinfected with phenol and germicidal lamps. The medium was poured in two layers; a base layer, and a seed layer containing 10 ml of a 6- to 7-day-old culture of *E. gracilis* per 100 ml of medium. Since the organism is heat labile, it was necessary to cool the medium used for the seed layer to about 40°C before adding the inoculum, and to pour the medium onto the plate quickly. After solidification of the agar, the paper strips were laid down on the seeded agar surface, and removed in about 5 minutes. The plate was then incubated 4 days at room temperature in the inoculation room under four 18 inch "daylight" fluorescent lights placed about 18 inches above the plate. Visible response of *E. gracilis* to vit. B_{12} began to appear in 2 days, but 4 days of incubation were required for luxuriant growth. The growth response areas were well defined and a dense green, contrasting sharply with the remainder of the seeded agar plate which showed insignificant growth.

The bioautographs using *L. leichmannii* 313 were prepared as described by Winsten and Eigen(2). The medium used was that of Peeler *et al.*(5) with 1.5% agar and 0.025% sodium thioglycolate added. The growth response areas were clearly visible when viewed with oblique light against a dark background. The sensitivity of the bioautographic plates expressed as the least amount of crystalline vit. B_{12} that would give a definite visible growth when applied to the plate on a 1 cm^2 piece of filter paper, was determined. For *E. gracilis* this was approximately 0.01 mμg and for *L. leichmannii* approximately 0.5 mμg of vit. B_{12}. The results of the bioautographs of both organisms were recorded by direct photography and by full-scale line representation on graph paper. The strips illustrated in this paper were prepared by the latter method. The point of application of the sample and the final solvent front are shown on the strips.

In order to prove that strips prepared in parallel were actually comparable, or to confirm the existence or position of various activities, a split strip technic was used. In this procedure a single strip was chromatographed and cut lengthwise; one-half was applied to a *L. leichmannii* plate, and the other half to a *E. gracilis* plate. The quantitative tube method for interpreting chromatograms described by Yacowitz, *et al.*(6) adapted to single strips, was used with both of the test organisms to compare the growth response in liquid medium with that on the agar plates. Quantitative measurements of the activity of the various materials used were obtained by tube assay procedures using both organisms. The tube assay of Hutner *et al.*(1) using *E. gracilis* was modified by using 10 ml of medium, inoculating uniformly and aseptically with a pipetting machine, and reading color-turbidity at 420 mμ in a Coleman Junior Spectrophotometer. The method of Peeler, *et al.*(5) was used for the bacterial tube assay. The assay ranges for these 2 methods were found to be 0.01-0.15 mμg vit. B_{12} per 10 ml of medium for *E. gracilis* and 0.05-0.5 mμg for *L. leichmannii*. Several liver extracts, A.P.F. concentrates, and other materials of known or suspected activity were screened by these procedures. Of these, crystalline vit. B_{12} (Cobione, Merck), a 15 U.S.P. unit liver extract (Lederle), a sample of corn steep liquor (kindly supplied by Dr. L. A. Underkofler, Department of Chemistry, Iowa State College), and desoxyribonucleic acid (Nutritional Biochemicals Corp.) were selected for detailed study because of their representative and clearly defined patterns of activity. The first 3 were diluted with water to the desired concentration. Four preparations of desoxyribonucleic acid (DNA) were examined: (I) *DNA*—1 g of DNA was dissolved in 10 ml of dilute alkali. This prepara-

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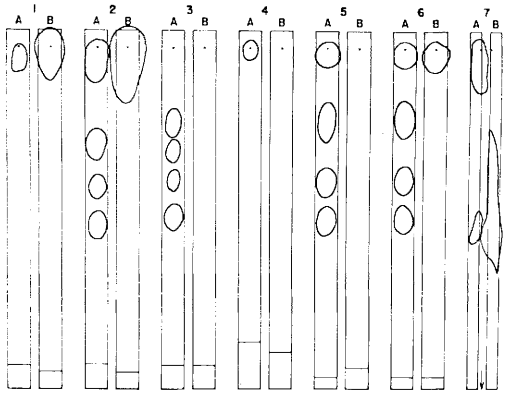

FIG. 1.

Comparative Bioautographs of Vit. B_{12} and Vit. B_{12} Active Preparations.

Strips 1A, 2A, 3A, etc.—*Lactobacillus leichmannii* 313. Strips 1B, 2B, 3B, etc.—*Euglena gracilis* var. *bacillaris*.

Twenty-five μl of the following preparations were applied to the corresponding strips. Vit. B_{12} and the apparent vit. B_{12} activities were measured with *L. leichmannii* 313.

Pair No.

1. Crystalline vit. B_{12} (Cobione); 10 mμg per strip.
2. Liver extr., 25 mμg as vit. B_{12} per strip.
3. Corn steep liquor, diluted 1:5, about 5 mμg as vit. B_{12} per strip.
4. Desoxyribonucleic acid, about 1 mμg as vit. B_{12} per strip.
5. Hydrolized desoxyribonucleic acid about 5 mμg as vit. B_{12} per strip.
6. Same as (5) plus 10 mμg crystalline vit. B_{12} per strip.
7. Same as (6), chromatographed 72 hours, a single strip cut lengthwise.

tion had some growth-promoting activity for *L. leichmannii*; (II) *Hydrolyzed DNA*—1 g of DNA was dissolved in dilute ammonia (final pH 8.5) and autoclaved at 121°C for 3 to 4 hours. The unhydrolyzed DNA was removed by precipitation with acid. This hydrolysis produced an activity *(L. leichmannii)* of 2 to 5 times that of preparation I; (III) *Norited DNA*—10 ml of preparation I were treated with Norite A for 30 minutes and filtered; (IV) *Hydrolyzed Norited DNA*—10 ml of preparation III were subjected to the same hydrolytic procedure described above. All of these preparations were adjusted to pH 5.0 before being applied to the paper strips.

The representations of comparative chromatographic bioautographs of these substances are shown in Fig. 1. The concentrations of the materials applied to the strips as designated in the legend were found in each case to give maximal growth while still retaining sufficient resolution. Since pure preparations of the vit. B_{12}'s and the desoxyribosides were not available, the criteria for evaluating the results of the bioautographs were the growth response to vit. B_{12} as found in Cobione, and the comparison of the R_F values obtained with those reported in the literature.

Results. Both organisms responded to crystalline vit. B_{12} (Fig. 1, Strips 1A and 1B). *E. gracilis* showed a larger area of growth response because of its greater sensitivity to vit. B_{12} and because of the greater diffusion of vit. B_{12} during the longer incubation period.

The response of *E. gracilis* to the liver extract (Strip 2B), differed markedly from that of *L. leichmannii*, showing growth only in the area of the slower moving factors where vit. B_{12} is expected to appear. Even when the concentration of liver extract was increased by 10 times, *E. gracilis* failed to show any response to the faster moving factors. This was confirmed by the chromatograph-tube method, which showed growth in only the first 2 tubes; that is, activity in only the first 2 inches of the strip. *L. leichmannii* on the other hand responded to 3 more rapidly moving factors in addition to the slow-moving factor (Strip 2A). The R_F value of the fastest moving factor indicated that it was thymidine(2,4), and the relative rates of the other moving factors generally correspond to those reported by Winsten and Eigen(2) as shown in Table I. So much liver extract had to be added to give well defined lower areas of growth that the double zone of the slow-moving activity was obscured. The use of less liver extract has shown a definite indication of a double zone of slow-moving activity.

E. gracilis showed no growth response to the chromatographed sample of the corn steep liquor (Strip 3B), while with an identical sample the *L. leichmannii* indicated the existence of 4 moving factors (Strip 3A), the R_F values and relative rates of which paralleled those reported by Winsten and Eigen(2) for their sample of corn steep liquor. Con-

TABLE I. Average Relative Rates and R_F Values for Vit. B_{12} Substitute Growth Factors (Test organism: *Lactobacillus leichmannii* 313)

Preparation	Moving vit. B_{12} substitute growth factors					
	1	2	3	4	5	5
	Relative rates* (2) (terms of Factor 5)					R_F† (Factor 5)
Liver extr.		.51		.78	1	.53
Corn steep liquor		.49	.60	.79	1	.52
Hydrolyzed DNA		.46		.77	1	.48
Values of Winsten & Eigen(2)	.32	.53	.63	.78	1	.52-.67

* Relative rate = Distance from starting point to center of growth zone in question / Distance from starting point to center of fastest moving growth zone

† R_F value = Distance from starting point to center of growth zone / Distance from starting point to solvent front

firmation of these results for both organisms was made with chromatograph-tube studies and direct tube assay.

E. gracilis showed no growth response to the chromatograms of any of the DNA preparations, two of which are illustrated by Strips 4B and 5B. Whenever vit. B_{12} was added prior to solvent flow to a strip containing any of the 4 DNA preparations, growth response with *E. gracilis* occurred, always in the area of slow-moving factors as shown on Strip 6B. The chromatograph-tube method and direct tube assay also indicated that *E. gracilis* was not stimulated by any growth factors in the DNA preparations. *L. leichmannii* demonstrated the existence of a slow-moving factor or factors present in the untreated DNA solutions, (Strip 4A). When this DNA solution was subjected to hydrolysis, the *L. leichmannii* bioautograph (Strip 5A) demonstrated an increased amount of growth in the area of slow-moving factors and the production of 3 rapidly moving growth factors. The *L. leichmannii* bioautographs of the chromatograms of norited DNA (not shown) demonstrated no areas of growth. This norite treatment actually removed very little of the DNA. Hydrolysis of this norited DNA solution resulted in a *L. leichmannii* bioautograph (not shown) which was practically identical with Strip 5A. The slow-moving factors produced by the hydrolysis may or may not have been identical with the slow-moving factors originally present in the DNA solution. The rapidly moving factors, as judged by their R_F values, correspond roughly to the 3 rapidly moving factors found in liver extract. A fourth moving factor, reported by Winsten and Eigen(2), was either not obtained by this type of hydrolysis, or was not resolved on the paper. The addition of vit. B_{12} to the strip prior to chromatography did not change the pattern of the response of *L. leichmannii* to the DNA hydrolyzates, as shown by Strip 6A. Since the slow-moving factors, either originally present or hydrolytically produced in these DNA preparations, were growth promoting for *L. leichmannii* but not for *E. gracilis*, none of these factors can be considered as being identical with crystalline vit. B_{12}.

Further evidence that the slow-moving factors of the DNA preparations and crystalline vit. B_{12} are not identical was obtained by the separation of vit. B_{12} from these slow-moving factors. This separation was accomplished by a long-term (72 hour) solvent flow procedure, the solvent being allowed to drip off the bottom of the strip. A split strip bioautograph of such a chromatograph is shown as Pair 7A and 7B. The more rapidly moving activities moved completely off the paper. The vit. B_{12} moved down a considerable distance, leaving a long trail that was picked up only by the more sensitive *E. gracilis*. The DNA factors remained practically stationary, and again did not promote the growth of *E. gracilis*.

Discussion. The use of *L. leichmannii* alone in the bioautographic examination of chromatograms of vit. B_{12} and vit. B_{12} substitute growth factors has led to the separation and identification of certain desoxyribosides as growth factors for that organism, but has not been particularly informative in the study of the slow-moving factors. These

slow-moving growth factors may include not only vit. B_{12}, B_{12a}(8) and B_{12b}(9); but also nucleic acids, nucleotides(10), and other substances relatively insoluble in the *n*-butanol, that may serve as vit. B_{12} substitute growth factors for the test microorganism. Some resolution of these factors has been accomplished by altering the conditions of chromatography, such as using different solvents(4), treating the paper strips with salts (9), and extending the period of solvent flow.

That the comparative bioautograph technic using *E. gracilis* and *L. leichmannii* as described above gives further insight into the complex nature of the slow-moving group of factors is evident. *E. gracilis* showed a growth response to the vit. B_{12} of Cobione, but did not respond to the one or more unidentified slow-moving factors found in the DNA preparations which were highly growth-promoting for *L. leichmannii*. Additional work is needed, however, on the response of *E. gracilis* to vit. B_{12a} and B_{12b} before further information on the identity of the slower-moving factors can be ascertained by this procedure. Additional evidence that *E. gracilis* does not respond to many of the growth factors that are known to be active for the *L. leichmannii* strains, is apparent in all of the chromatograms presented in Fig. 1. Its lack of response to any of the rapidly moving factors substantiates and supplements the work and suggestion of Hutner *et al.*(1). The comparative bioautograph method may thus serve as a useful tool in the study of the slow-moving factors, as a qualitative method of indicating activities present in preparations which are to be assayed for vit. B_{12}, and as a guide in separation and purification procedures.

E. gracilis as a tube assay organism, although satisfactory for the relatively pure materials used in this investigation, in our hands has not given the desired results in the assay of complex natural materials such as animal tissues and blood.

Summary. 1. A method for the use of *Euglena gracilis* var. *bacillaris* as a bioautograph organism for the paper chromatographic study of vit. B_{12} and related growth factors has been described. 2. Evidence has been presented to indicate the value of comparative bioautographs, using *E. gracilis* and *Lactobacillus leichmannii* 313, in the study of these growth factors.

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A Simple Device For the Growth of Anaerobic Organisms in Liquid Media. (18249)

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The device described herewith (Fig. 1) is essentially a modification of the Theobald Smith fermentation tube(1). It consists of 2 test tubes differing in size which are fitted at the top with screw caps, and which are joined at the bottom with thick-walled capillary tubing. The plastic cap of the small tube is perforated with a drill-hole 2 mm in diameter, and both caps are supplied with solid heavy rubber gaskets so that air-tight seals are formed when they are screwed in place.

For the growth of anaerobic bacteria, the tubes are prepared in the following way: broth of the desired composition (tryptose phosphate; tryptisoy or a fresh meat infusion-

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