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UNIDENTIFIED GROWTH FACTOR FOR A LACTIC ACID BACTERIUM

DONALD E. WEINMAN, GEORGE K. MORRIS, AND WILLIAM L. WILLIAMS

Department of Chemistry, University of Georgia, Athens, Georgia

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ABSTRACT

WEINMAN, DONALD E. (University of Georgia, Athens), GEORGE K. MORRIS, AND WILLIAM L. WILLIAMS. Unidentified growth factor for a lactic acid bacterium. J. Bacteriol. 87:263-269. 1964.—*Lactobacillus bulgaricus* Georgia strain required an unidentified growth factor, named Georgia bulgaricus factor (GBF), when grown on a semisynthetic basal medium. Aqueous extracts of torula yeast and beef liver were the best sources of GBF. Adenine, ribonucleic acid (RNA), alkaline digests of RNA, and Mg^{++} also stimulated growth, but to a considerably lesser extent than liver and yeast extracts. Several purines, pyrimidines, and related compounds also promoted growth responses in individual experiments, but not consistently. Intact deoxynucleic acid (DNA), deoxyadenylic acid (dAMP), and deoxyguanylic acid (dGMP) inhibited growth. The DNA and dAMP inhibitions were fully reversed by crude sources of GBF, while dGMP inhibition was only partially reversed. RNA reversed DNA inhibition to a small extent. GBF was stable to heat at pH 2.5 to 11 and to prolonged light exposure. It was destroyed by heating at pH 1.0. The GBF activity moved as a single component in paper chromatography. It was firmly adsorbed on charcoal and poorly soluble in organic solvents. A concentrate, 28 times more potent than liver extract, was prepared by prolonged paper chromatography. All known growth factors and biological compounds readily available were assayed for GBF activity, none of which gave a response similar to the crude extracts. Final proof that a new growth factor exists must await definite identification of the active compound.

Nutritional studies conducted on bacteria in defined and semidefined media led to the discovery of many new growth factors, some of which were subsequently identified as vitamins required for other species, including man. An organism that had not been investigated nutritionally, *Lactobacillus bulgaricus* Georgia strain (GS), was observed to grow very slowly in a semidefined medium similar to media considered nutritionally adequate for related lactic acid bacteria in other studies (Kihara and Snell, 1960; Williams, Hoff-Jørgensen, and Snell, 1949; MacLeod and Snell, 1950). Addition of certain crude extracts to the medium promoted rapid growth of the organism.

This paper presents evidence that the organism requires an unidentified growth factor widely distributed in natural materials. Several known substances promoted submaximal growth of the organism but were not identical with the unidentified growth factor. Intact deoxyribonucleic acid (DNA) and purine deoxyribotides inhibited growth, but the inhibition was reversed by crude sources of the unidentified factor. Initial purification and properties of the factor are described.

MATERIALS AND METHODS

Test organism. L. bulgaricus GS was isolated from local dairy products and maintained by weekly transfers on litmus milk containing 1% yeast extract (Difco), 1% glucose, and 0.5% tryptose. Cultures were incubated for 24 hr and stored at 4 C.

Preparation of inoculum. Transfers were made from a litmus milk culture incubated 24 hr to a medium containing (per 5 ml): basal medium, 2.5 ml (Table 1); yeast extract (Difco), 2 mg; malt extract (Difco), 2 mg; liver extract, 3.3 mg; and fresh boiled whey, 0.5 ml. Incubations were at 37 C for 20 to 23 hr. The cells were washed two times with 5 ml of 0.9% NaCl solution, standardized to 20% transmission with a Spectronic-20 colorimeter, and diluted to 100- to 10,000-fold in the same solution. The diluted inoculum was added to the double-strength basal medium at the rate of 1 ml for each 22 ml. The 10,000-fold diluted inoculum was used in most experiments, but some adjustment between 100- and 10,000-fold dilution was necessary to maintain a suitable graded response to liver extract.

Assay procedures. Samples were pipetted into Pyrex tubes (13 by 100 mm), adjusted to a volume of 1.5 ml with distilled water, and covered with aluminum caps. Both samples and double-strength medium were autoclaved at 120 C for 6

min. After autoclaving and cooling to 37 C, the basal medium was inoculated, and 1.5 ml were added aseptically to all tubes. After incubation at 37 C for various periods of time, growth was measured turbidimetrically with a Bausch & Lomb Spectronic-20 colorimeter at 650 mμ. Growth was expressed in dry weight of cells, as determined from a previously prepared graph. For example, per cent transmittancy of 70, 35, and 20 corresponded to 0.45, 1.5, and 2.5 mg of dried cells per tube. Best responses could generally be determined after 22 to 27 hr of incubation. After 48 hr, all tubes usually contained heavy growth.

Paper chromatography. Ascending chromatograms on paper cylinders were developed with NH_4OH-water at pH 10 (Wyatt, 1955). Descending techniques were used with (i) *n*-butanol saturated with water; (ii) isopropanol, 170 ml; concentrated HCl, 41 ml; and water to 250 ml; and (iii) *n*-butanol–acetic acid–water (25:6:10).

Liver extract. Fresh minced beef liver still warm from the animal was mixed with an equal weight of water, homogenized, and autoclaved for 1 hr at 120 C. Insoluble coagulated material was removed by pressing through cheesecloth and by filtration. After centrifugation, the supernatant fluid was stored at −7 C. Total solids of the finished product were 33 mg/ml.

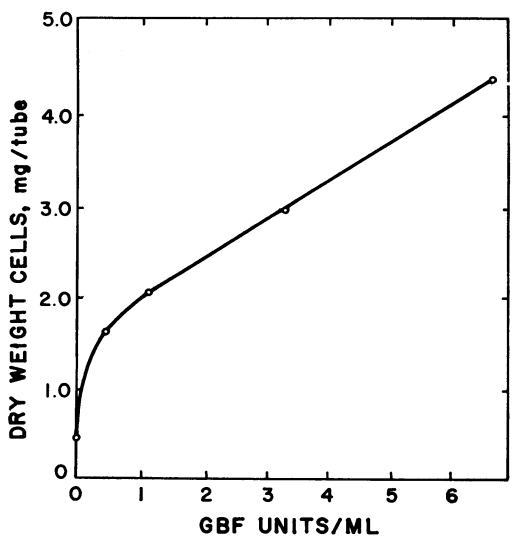

FIG. 1. *Effect of GBF on the growth of Lactobacillus bulgaricus GS grown on a semisynthetic basal medium.*

Liver extract standard. A unit of Georgia bulgaricus factor (GBF) activity was defined as the amount of GBF in 1 mg of liver extract prepared as above. Thus, 1 ml of liver extract contained 33 units. A standard preparation was stored frozen for use throughout this study.

Torula yeast extract. A thick suspension of Torula yeast obtained from Lake States Yeast Co., Rhinelander, Wis., was received semifrozen and refrigerated at 4 C for 1 month. The yeast slurry was mixed with an equal volume of water, steamed at 100 C for 10 min, autoclaved at 121 C for 2 min, and centrifuged at 13,200 × *g* for 10 min; the supernatant fluid was stored at −7 C. Total solids were 63 mg/ml.

Ribonucleic acid (RNA) digest. RNA (yeast nucleic acid, C grade, Calbiochem) was hydrolyzed with 0.3 N KOH for various lengths of time at room temperature, neutralized to pH 6.5 with perchloric acid, and diluted to a concentration of 2 mg/ml.

DNA digest. DNA (40 mg, C grade, Calbiochem) was digested with 10 mg of deoxyribonuclease in 20 ml of 0.03 M $MgSO_4$ for 2 hr at 37 C. The solution was sterilized by filtration through sintered glass or by autoclaving, and was stored at 4 C.

RESULTS

Response of the organism to additions of liver extract in GBF units is shown in Fig. 1. The growth response to GBF on the basal medium was used as a basis for assaying other preparations by comparison with a standard liver extract. Torula yeast extract, having a potency of 2.5 units per mg, was a better source of GBF activity than was the standard liver extract. Growth-promoting activity was found in whey, malt extract, extracts of the sea pansy (*Renilla reniformis*), hens' eggs, dried distillers' solubles, and bovine spleen, all having a potency less than liver extract. Increasing the concentrations of the components used in the basal medium (Table 1) resulted in greater growth with both the salts B (Snell and Wright, 1941) and with the adenine, guanine, and uracil solution. Increasing each of the salts B components indicated that Mg^{++} is the source of activity (Table 2). Specially purified, spectrographically analyzed $MgSO_4$ produced a response identical to that of the reagent grade $MgSO_4$ used in salts B. The ash of liver extract (Table 3) was inactive. Table 4 lists some repre-

TABLE 1. *Composition of the basal medium*

Component	Amt per 1 ml of final medium
	ml
Acid-hydrolyzed casein (10%)*	0.05
Enzymatic digest of casein (5%)†	0.2
	μg
Adenine	10
Guanine	10
Uracil	10
NaCl	10
$FeSO_4 \cdot 7H_2O$	10
$MnSO_4 \cdot 4H_2O$	10
Pyridoxal hydrochloride	4.2
Thiamine	0.5
Calcium pantothenate	0.5
Riboflavine	0.5
Niacin	0.5
p-Aminobenzoic acid	0.5
Pyridoxine	0.5
Inositol	5.0
Niacinamide	0.5
Pantethine	2.0
Pyridoxamine dihydrochloride	3.7
Ascorbic acid	3.7
Orotic acid	3.7
Mevalonic acid	3.7
	mμg
Folic acid	25
Biotin	2.5
Lipoic acid	50
Leucovorin (5-formyl tetrahydrofolic acid)	50
Cyanocobalamin	1
	mg
KH_2PO_4	1
K_2HPO_4	1
$MgSO_4 \cdot 7H_2O$	0.2
Glucose	10
Lactose	10
L-Asparagine	0.1
Cystine	0.1
Cysteine	0.1
Glacial acetic acid	2.5
Tween 80 (polyoxyethylene sorbitan monooleate)	0.1

* Acid hydrolyzed casein was prepared as published (Williams et al., 1949), except "Vitamin Free" casein was used and brought to pH 5.2 with NaOH after filtering.

† Enzymatic digest of casein was prepared as published (Williams et al., 1949), except "Vitamin Free" casein was used, 8 g of USP pancreatin were added, and the solution was readjusted to pH 8.0 after incubation for 24 hr. Before filtration the pH was brought to 6.5 with 4 N HCl, and 15 g of activated charcoal (Darco G-60) were added per liter. The mineral salts were prepared as salts A and B (Snell and Wright, 1941).

TABLE 2. *Response of Lactobacillus bulgaricus GS to increased concentrations of salts contained in salts B solution**

Substance	No. of times concentration increased over that in basal medium				
	1.0	1.2	1.66	3.0	5.0
$MgSO_4$	5.3	6.7	13.3	17.3	19.3
NaCl	5.3	5.3	5.3	5.3	5.3
$FeSO_4$	5.3	6.0	4.0	4.0	4.0
$MnSO_4$	5.3	6.0	5.3	5.3	5.3

* Growth is expressed as per cent dry weight of cells compared with dry weight of cells produced from 7 units of GBF per ml.

sentative results obtained from additions of certain purines, pyrimidines, and their derivatives to the basal medium. The effect on growth of the compounds listed and related compounds varied from inhibition up to about one-half the growth obtained with seven units of GBF per ml. The most consistent responses were from additions of adenine. Uracil, which stimulated growth in earlier experiments, became virtually inactive in later experiments. The solution of adenine, guanine, and uracil gave optimal responses over a range which varied from one to three times that concentration present in the basal medium. Growth responses to the other compounds listed fluctuated between experiments and were unpredictable. Similar fluctuations were noted with 4-amino-5-imidazole carboxamide, cytidylic acid (CMP), guanosine triphosphate (GTP), uridine diphosphate (UDP), 2′- or 3′-uridylic acid (2′-3′-UMP, mixed isomers), cyclic 2′,3′-uridine monophosphate (cyclic 2′,3′-UMP), hypoxanthine, inosine, biopterin, and flavine adenine dinucleotide (FAD). Shown in Table 3 are compounds which were tested and found to be devoid of GBF activity. In addition to the vitamins tabulated, the vitamins of the basal medium were increased 60-fold without promoting growth. Intact RNA was stimulatory at low concentrations where its activity assayed up to 60 units per mg, but concentrations greater than 0.25 mg/ml of RNA failed to promote further responses (Fig. 2). The activity of alkaline hydrolysates of RNA was less than that of the intact RNA. The responses

were variable and showed no relationship to the degree of hydrolysis.

The purine nucleotides, deoxyguanylic acid (dGMP) and deoxyadenylic acid (dAMP), inhibited growth in the presence of GBF (Fig. 3); deoxycytidylic acid and thymidylic acid were without effect. Inhibition by both compounds was partially reversed by doubling the liver extract concentration. Both intact and enzymatically digested DNA were inhibitory and were partially

TABLE 3. *Substances which produced little or no growth response*

Substances	Range tested	Substances	Range tested
	μg/ml		*μg/ml*
n-Acetylglucosamine	6.6–1,000	Histidine	10–200
Cis-aconitic acid	17.7–1,000	DL-Meso-homocystine	10–200
Acid ash of liver extract	≈1.0–50 mg	L-Hydroxyproline	10–200
Adenosine diphosphate	10–200	Inositol	26.6
Adenosine triphosphate	10–200	L-Meso-lanthionine	10–200
β-Alanine	10–200	Lecithin (β-dipalmitoyl)	33–100
Alloxan	10–200	Leucovorin	26.6
p-Aminobenzoic acid	26.6	Levulinate, Ca	33–100
α-Aminobutyric acid	10–200	Lipoic acid	26.6
γ-Aminobutyric acid	10–200	Menadione	500–2,500
β-Aminoisobutyric acid	10–200	6-Mercaptopurine	33–100
2-Aminopurine, nitrate	33–100	DL-1-Methyl histidine	10–200
Aminophylline	33–100	Niacin	26.6
2-Aminopyrimidine	33–100	Niacinamide	26.6
Ash of liver extract	≈1.0–50 mg	DL-Norvaline	10–200
DL-Asparagine hydrate	10–20	Orotic acid	10–200
8-Azaguanine	33–200	Oxalacetic acid	17.7–1,000
Barbituric acid	10–200	Oxalosuccinic acid	17.7–1,000
Betaine	10–20	Pantethine	26.6
Biotin	26.6	DL-Phenylalanine	6.6–400
n-Butanol	2–400 × 10^3	Phosphocholine chloride	33–100
Caffeine	10–200	Purine	33–100
L-Canaverine	10–200	Pyridoxal HCl	26.6
L-Carnosine	10–200	Pyridoxamine	26.6
5′-dGMP	10–200	Ribose-5-phosphate	2–20
Coenzyme Q_{10}	50–1,000	Spermidine trihydrochloride	33–100
Cyanocobalamin	0.5–26.6	Soluble RNA, yeast	10–400
DL, Allo-cystathionine	10–200	Thiamine	10–200
Cytidine	10–200	2-Thiohistidine	10–200
L-Cysteic acid	10–200	Thiouracil	10–200
Cytosine	10–200	Thymidine	10–200
Deoxycytidine	10–100	Thymidylate	10–200
Deoxyguanosine	10–200	Thymine	10–200
Dihydrouracil	33–100	DL-Tryptophan	10–200
Dihydroxyphenylalanine	10–200	Tyrosine	33–333
Ethanol	2–400 × 10^3	Tween 80	66–4,000
Folic acid	26.6	Uridine diphosphate	10–200
Formiminoglycine	33–100	Uridine triphosphate	10–200
Fructose-6-phosphate	17.7–400	DL-Ureidosuccinic acid	33–200
Glucosamine	66–1,000	Uric acid	33–100
Glutamine	120–1,200	Uridine	10–100
Glycocyamine	10–200	2′-3′Uridine monophosphate (mixed isomers)	10–200
Guanosine	10–200	Xanthosine	33–100
2′-3′-Guanosine monophosphate (mixed isomers)	10–200		

TABLE 4. *Effect of known compounds on growth of Lactobacillus bulgaricus GS**

Compound	Amt (mg) added per ml of final medium				Adenine, guanine, and uracil present in basal medium
	0.01	0.033	0.1	0.2	
Adenine	7.4	22	25.9	0	–
Guanine	0	0.033	0	0	–
Uracil	0	0	26.6	30.0	–
Cytosine	3.3	8.7	10	6.6	–
Adenine, guanine, and uracil, each	24.4	35.6	18.5	3.7	–
Adenosine	I	I	0.09	0.18	+
Guanosine	I	I	I	I	+
AMP	5.5	16	28	16	+
GMP	I	0	0	I	+
UMP	5.5	5.5	22	33	+

* Growth is expressed as per cent dry weight of cells compared with that obtained with 7 units of GBF per ml. I = inhibition; 0 = same growth as in blank.

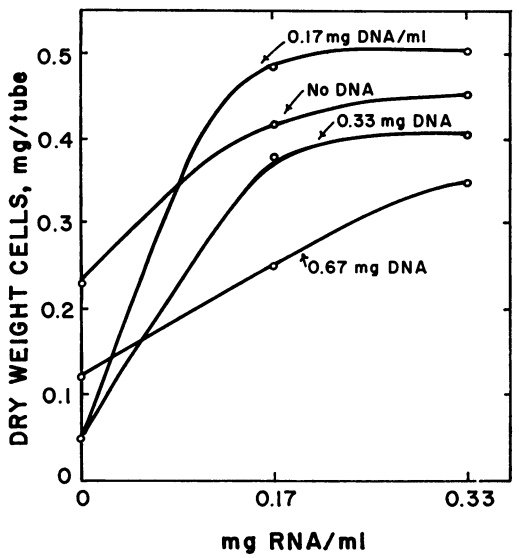

FIG. 2. *Growth response of Lactobacillus bulgaricus GS to RNA and effect of DNA.*

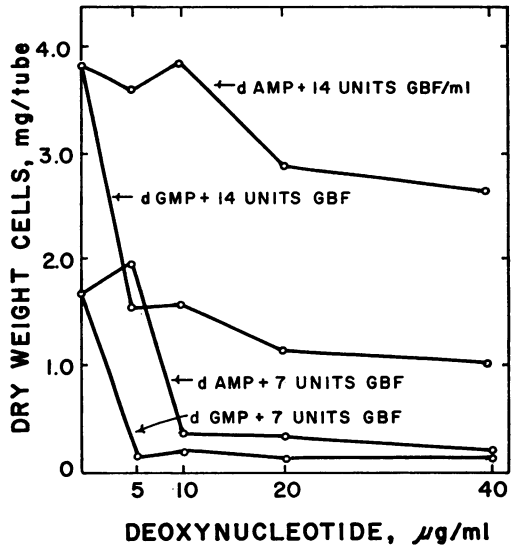

FIG. 3. *Inhibition of growth of Lactobacillus bulgaricus GS by dGMP and dAMP and reversal by GBF.*

reversed by liver extract. Intact DNA at a low concentration (0.17 mg/ml) increased the growth response to intact RNA (Fig. 2). Higher concentrations inhibited the stimulatory effect of RNA additions. The antiprotozooal compound 2-acetylamino-5-nitrothiazole (100 µg/ml) inhibited growth completely, and inhibition was not reversed by liver extract (0.2 ml/ml).

Adenine, thymine, xanthosine, and acid-hydrolyzed casein each increased the GBF content of a pure culture of Torula yeast when added to a synthetic medium (Atkin et al., 1943) upon which the yeast was grown. Adenine was the most effective of added stimulants.

Physical properties of GBF. After autoclaving liver extract for 2 hr at pH 2.5, 6.5, and 11.1, GBF activity remained to the extent of 95, 90, and 92%, respectively. Autoclaving for 30 min at pH 1.0 resulted in complete loss of activity. Exposure of sterile liver extract, contained in a Pyrex tube, to sunlight for two daylight periods resulted in a loss of less than 10% of activity. A similar loss was experienced in the light-protected control.

Bioautographic studies, by the technique of Long and Williams (1951), indicated only one active zone. Active zones were cut from the paper chromatograms and eluted with water by centrifugation. After development of liver extract with NH_4OH/H_2O at pH 10, GBF activity could be demonstrated in an area adjacent to the solvent front. Uridine, cytosine, thymine, 5′-UMP, and 2′-3′-UMP (mixed isomers) were in a similar area on a control sheet. GBF activity remained near the origin after 40 hr of development with *n*-butanol–water. On the control sheet, 2′-3′-UMP (mixed isomers), uridine triphosphate (UTP), adenosine diphosphate (ADP), and guanine were located in the same area. Development with isopropyl alcohol–HCl–water resulted in a loss of GBF activity.

Treatment of liver extract with anionic (triethylaminoethyl) and cationic (Dowex 50 W-X8) resins failed to remove GBF activity. Treatment of liver extract with activated charcoal (Darco G-60) at pH 1.5, 6.0, 11.7 for 30 min resulted in 66, 92, and 98% loss of GBF activity from the filtrate. Elution of the charcoal cake with *n*-butanol gave a fraction with a specific activity of 2.4 units per mg and a yield of 3.2%. Elution with other solvents resulted in lower yields. Attempts to extract and concentrate GBF from liver extract with ethyl ether, ethyl alcohol, ethyl acetate, chloroform, benzene, *n*-butanol, acetone, or 2-propanol were unsuccessful.

With descending paper chromatographs with 3 ml of liver extract per sheet (Whatman 3 MM paper; 168 hr, butanol–acetic acid–water), a fraction was eluted from the origin with a specific activity of 28 units per mg. In all chromatographic experiments, the GBF activity migrated as a single entity.

DISCUSSION

Growth responses to Mg^{++} similar to our observations were reported for *L. arabinosus* and *L. casei* by increasing concentrations of $MgSO_4 \cdot 7H_2O$ above the 0.2 mg/ml used in our basal medium (MacLeod and Snell, 1947). The lack of growth response to the ash of liver extract (Table 3), coupled with submaximal responses to Mg^{++}, eliminates Mg^{++} as the active principle in liver extract.

The purine- and pyrimidine-related compounds, which migrated in the same manner as GBF on paper chromatograms, did not produce growth response comparable to that of GBF. Of the other related compounds tested, adenine produced the most consistent stimulatory responses. The maximal responses to adenine were submaximal to those of liver extract and occurred at a certain optimal concentration which varied between experiments. The variable and submaximal growth responses obtained with the compounds mentioned in Table 4 were in marked contrast to the greater and consistent responses to liver extract. These results, therefore, distinguish GBF from the stimulatory compounds and from those listed in Table 3, which produced little or no growth response. Variable responses were reported by other workers in nutritional studies conducted with a related organism, *L. arabinosus* R26, with regard to its uracil and deoxycytidine requirements (Løvtrup and Shugar, 1961; Jeener and Jeener, 1952).

Rose and Carter (1954) showed that the orotic acid requirement of *L. bulgaricus* O9 could be replaced by cyclic 2′,3′-UMP, 5′-UMP, or by polynucleotides with 2′- or 3′-UMP in monoester linkage. *L. bulgaricus* GS did not respond consistently to the cyclic 2′,3′-UMP or 5′-UMP, and responses to intact RNA or hydrolyzed RNA were much lower than those of liver extract.

It is not possible to determine at this time the relationship between GBF as a growth stimulant and the effects of nucleic acid components upon growth. It is quite possible that the responses seen reflect an effect at two or more different points in the metabolism of *L. bulgaricus* GS. The variable responses to purines, pyrimidines, and related compounds, coupled with a complete loss of response to uracil, support the latter contention; the responses to liver extract were constant throughout the period of investigation. It should be noted that the maximal responses to any single known compound were no greater than half that of liver extract.

Conversely, the partial activity of RNA and adenine suggests that these compounds could be either products of GBF function or precursors of GBF. Adenine stimulation of GBF biosynthesis in Torula yeast grown on a defined medium favors a precursor role for adenine.

The inhibitory effect of intact DNA, dGMP, or dAMP remains unexplained. Similar inhibition was reported for murine leukemia cells (Morris and Fischer, 1963; Morris, Reichard, and Fischer, 1963), in which deoxyadenosine triphosphate,

deoxyguanosine triphosphate, and thymidine triphosphate inhibited the conversion of cytidylic acid to deoxycytidylic acid, restricting the cells' capacity to synthesize DNA. This is not the reaction inhibited in *L. bulgaricus* GS because, in contrast to the results with murine cells, inhibition by dAMP and dGMP was not reversed by deoxycytidine or deoxycytidylic acid (*unpublished data*). With the murine cells, deoxyadenosine and deoxyguanosine were inhibitory, but the phosphorylated forms of these compounds are required to produce growth inhibition of *L. bulgaricus* GS. In contrast to the observed inhibition by dAMP and dGMP, deoxynucleotides have been shown to replace vitamin B_{12} in the nutrition of several species of lactic acid bacteria (Kitay, McNutt, and Snell, 1950).

The physical properties indicate that the growth factor activity is stable to ordinary conditions, but is destroyed at pH 1.0. To establish that a new growth factor was needed for optimal growth, the components of the basal medium were varied in such a manner as to discover any growth-restricting imbalances that might be present. Moreover, the basal medium is of the same general composition used in microbiological assays with closely related organisms. All of the reported growth factors known to us, the components of the basal medium, and those biological compounds readily available were assayed at several concentrations for GBF activity; none of these elicited a growth response similar to that of liver extract. The growth factor activity migrated as a single entity with paper chromatography, and was shown to be widely distributed in nature.

These data thus lend support to our contention that a new growth factor is required, but final proof will depend upon definite identification of the compound.

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