AN UNIDENTIFIED GROWTH FACTOR IN THE NUTRITION OF LACTOBACILLUS CASEI

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The complexity of the nutritive requirements of *Lactobacillus casei* is known. The organism develops slowly in synthetic or semisynthetic media such as the one described by Landy and Dicken (1942). Assay media for this organism therefore include crude supplements like peptone (Teply and Elvehjem, 1945) or trypsinized casein (Roberts and Snell, 1946). The active principle responsible for the shortening of lag phase of growth supplied by these materials was held to be strepogenin (Sprince and Woolley, 1945). Scott *et al.* (1946) reported requirement of glutathione and another unidentified factor essential for strepogenin action and for producing a further growth stimulation. Other growth factors were suggested later by additional workers (Daniel *et al.*, 1948, 1949; Peeler *et al.*, 1949).

During studies with this organism using the medium of Teply and Elvehjem (1945) slightly modified, it was observed that 24 hour growth measured turbidimetrically was more than three times the normal growth in the presence of an aqueous extract of an animal feed supplement (U. S. Industrial Chemicals, Inc.) of fermentation origin derived from *Aerobacter aerogenes* cultures. The active principle(s) was not a growth essential as the organism could grow on the unsupplemented basal medium; it was found to shorten the growth lag considerably. The properties of this factor are different from those of the known growth essentials and other unidentified factors reported hitherto.

METHODS

The organism employed was *Lactobacillus casei* (ATCC 7469) carried with fortnightly transfer on stabs of the composition: Yeast extract (Difco), 1 per cent; glucose, 1 per cent; sodium acetate, 1 per cent; proteolysed liver, 0.5 per cent; and agar agar, 2 per cent.

Basal medium was that of Teply and Elvehjem (1945) modified by the omission of peptone, alanine, asparagine, and *p*-aminobenzoic acid which were not essential for growth. The medium contained 10 mμg pteroylglutamic acid (Lederle) per tube containing 10 ml final volume. A 24 hour growth in this medium, diluted 100-fold was used dropwise to inoculate each tube. Incubation temperature was 37 C.

Growth response was measured turbidimetrically in a Klett-Summerson photoelectric colorimeter at 660 mμ or acidimetrically by titrating against 0.1 N NaOH with bromthymol blue as indicator.

RESULTS

Growth response of the organism to graded quantities of the aqueous extract of the feed material, equivalent to 20 mg original material per ml, is shown in table 1. Turbidity was measured at the end of 16 and 24 hours of incubation, and acid production, at 72 hours.

Assay of activity. In an attempt to duplicate this stimulation, the effects on growth of certain known supplements were studied using the basic medium. It was observed that glutamine, oleic acid, "tween-80", and higher levels of the B vitamins as recommended by Roberts and Snell (1946) caused an increased initial rate of growth. Glutathione, glutamic acid, and an increased concentration of manganese salts also promoted some growth enhancement. Oleic acid and "tween-80" were interchangeable in their effect. These observations were used in devising an improved medium for *L. casei* (table 2). Strepogenin concentrate was prepared according to Scott *et al.* (1946) and was treated carefully with active charcoal to remove any possible contamination of other unidentified stimulants. An assay curve for the active material using this medium is given in figure 1.

A comparison of growth rates of the organism on the basic and improved media and the effect of supplementation of the latter medium with one ml of active extract equivalent to 20 mg material are shown in figure 2.

TABLE 1

Growth response of Lactobacillus casei to aqueous extract of active material

EXTRACT (2 PER CENT) PER TUBE	TURBIDITY (KLETT READINGS)		0.1 N ACID 72 HR
	16 hr	24 hr	
ml			
0.0	33	52	12.9
0.05	41	60	12.9
0.1	52	73	13.1
0.2	71	94	13.8
0.4	94	118	14.5
0.6	114	136	15.0
0.8	122	142	15.3
1.0	125	148	15.4

TABLE 2

Improved assay medium for Lactobacillus casei

	AMOUNT PER 100 ML DOUBLE STRENGTH MEDIUM
Vitamin-free, acid hydrolyzed casein	1 g
L-Cystine	40 mg
DL-Tryptophan	40 mg
DL-Glutamic acid	100 mg
Adenine sulfate	2 mg
Guanine hydrochloride	2 mg
Xanthine	2 mg
Uracil	2 mg
Thiamin hydrochloride	200 μg
Riboflavin	200 μg
Nicotinic acid	200 μg
Calcium pantothenate	200 μg
Pyridoxine	400 μg
Biotin	2 μg
Pteroylglutamic acid	200 mμg
Glucose, anhydrous	4 g
Sodium acetate	5 g
K_2HPO_4	100 mg
KH_2PO_4	100 mg
$MgSO_4 \cdot 7H_2O$	40 mg
$MnSO_4 \cdot 7H_2O$	5 mg
$FeSO_4 \cdot 7H_2O$	2 mg
NaCl	2 mg
Tween-80	100 mg
Strepogenin concentrate	0.25 g

Some properties of the active principle(s). The activity was remarkably resistant to changes in pH and temperature. At room temperature it was stable for at least 48 hours at all pH values ranging between 1 N acid and 1 N alkali. Autoclaving at 15 lb steam pressure with N-HCl and N-NaOH resulted respectively in 12 and 21 per cent loss in activity; the corresponding losses at the end of two hours were 22 and 32 per cent. Between pH 4 and 9 the activity was stable to these treatments. On ashing the activity was lost completely.

Treatment with alkaline $KMnO_4$ (1 N), H_2O_2 (30 per cent), and saturated bromine water at room temperature resulted in complete loss of activity. Reducing conditions such as zinc and dilute HCl, autoclaving for 15 min at 15 lb steam pressure with one per cent sodium bisulfite,

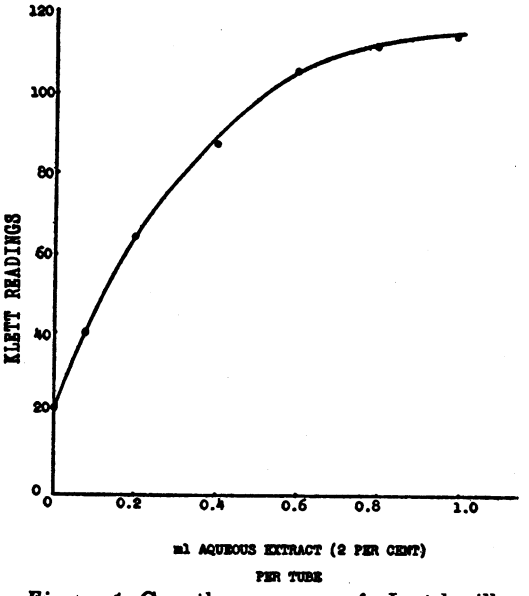

Figure 1. Growth response of *Lactobacillus casei* to active factor (16 hour turbidimetric).

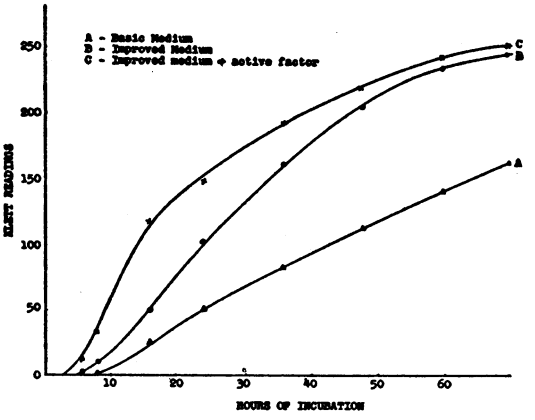

Figure 2. Growth kinetics of *Lactobacillus casei.*

ascorbic acid (10 mg per ml), and thioglycolic acid (5 mg per ml) effected only a very slight inactivation.

Activity could be extracted from the feed material with water, *n*-butanol, and acetone. The factor was completely soluble in 25 per cent aqueous ethanol and 77 per cent soluble in 95 per cent ethanol. It was insoluble in ether and chloroform.

From the aqueous extract the activity was not extractable with chloroform but was partially transferable to *n*-butanol phase. With equal volumes of *n*-butanol and water at pH 2.8, activity in the butanol phase was 2.5 times that in the aqueous phase; it was distributed equally between the two solvents at pH 6.8 and 8.6. Kline and Barker (1950) have reported a growth factor for *Butyribacterium rettgeri* which is soluble in organic fat solvents after treatment with 0.1 N acid. However, in the present case, acid treatment made no difference in solubility.

When various protein precipitating agents were tried on the aqueous extract of the active material, it was found that 17 per cent of activity was removed in lead acetate precipitate, 26 per cent in phosphotungstic acid precipitate, and only 6 per cent in the precipitate on saturation with ammonium sulfate.

The active principle was adsorbed easily on activated charcoal ("norite", British Drug House) but was not taken up by fullers' earth, alumina, or heavy magnesia at natural pH of the active extract (6.7). From the charcoal adsorbate, elution was tried with different solvents (table 3).

TABLE 3

Elution of active principle from charcoal

ELUENT	PER CENT ACTIVITY ELUTED
n-Butanol	85
n-Butanol-acetic acid	74
n-Butanol-ammonia	98
Acetone	69
Acetone-acetic acid	60
Acetone-ammonia	76
Ethanol-water	65
Ethanol-water-acetic acid	65
Ethanol-water-ammonia	80
Ammonia (dilute)	7
Acetic acid 0.1 N	Nil

n-Butanol containing 10 per cent dilute ammonia could elute the activity quantitatively.

Attempts at replacing with known substances. The improved medium (table 2) was used in these experiments without the strepogenin concentrate. Supplements tried were ribonucleic acid, desoxyribonucleic acid, hydrolyzates prepared from these by autoclaving for four hours at 15 lb steam pressure with ammonia to pH 10.0, leucovorin (Lederle), vitamin B_{12} (Merck), choline, inositol, asparagine, aspartic acid, DL-alanine, ascorbic acid, DL-serine, pyridoxal, pyridoxamine, *p*-aminobenzoic acid, fumaric acid, succinic acid, malic acid, and lactic acid. None of these could stimulate growth. Only the strepogenin concentrate added equivalent to 25 mg casein was stimulatory though the extent of stimulation was not comparable with that with the active principle.

Distinction from reported growth factors. Scott *et al.* (1946) have described a factor for *L. casei*, associated with animal proteins and distinguishable from strepogenin by charcoal adsorbability and solubility in 95 per cent ethanol. Unlike strepogenin this factor was stable to oxidizing agents. As the active principle reported in the present study was both charcoal-adsorbable and labile to mild oxidation, there was a possibility of its being a mixture of these two factors. The activity of the charcoal-filtrate and $KMnO_4$ treated extract in combination was studied therefore (table 4). According to Scott and co-workers (1946), the two factors exert their effect only when present together. The combination tried was as inactive as the individual preparations. The observation thus would indicate that the activity adsorbed on charcoal is identical with the one destroyed by $KMnO_4$ treatment.

TABLE 4

Further characterization of the active principle

SUPPLEMENT	GROWTH 16 HOURS KLETT READINGS
None	23
Active extract	105
Charcoal-filtrate	25
$KMnO_4$ treated extract	29
Charcoal-filtrate + $KMnO_4$ treated extract	28

TABLE 5

Distribution of active factor

MATERIAL	POTENCY UNITS PER G
APF supplement (U. S. Ind. Chem.)	260
APF-5 (8108-65) (Lederle)	Nil
Yeast extract (Difco)	150
Peptone	120
Peptone (charcoal treated)	25
Liver fraction L (Wilson)	280
Reticulogen (Lily)	80 per ml
Trypsin digested casein	190
Tomato juice	15 per g fresh fruit
Tomato juice-charcoal-clarified	Nil
Sprouted mung bean (*Phaseolus mungo*) extract	150

Distribution of activity. Several natural materials were assayed by the method described. The medium contained a concentrate of strepogenin. Activity is expressed in units, one unit being equivalent to the amount required to produce half-maximal growth stimulation. In table 5 are given potencies of the materials tested in units per gram dry weight unless stated otherwise.

The APF supplement (Lederle) was a *Streptomyces aureofaciens* fermentation residue. That the inactivity in promoting *L. casei* growth was not due to aureomycin present in the product was ascertained by an addition of an equivalent concentration (4 mg per g) of crystalline aureomycin (Lederle) to the active extract. No change was observed in the growth promoting activity. Heat-lability of aureomycin also suggested that the lack of activity of the Lederle product was not due to the antibiotic. Presumably the actinomycetes do not elaborate the factor.

Several bacterial cultures were tested qualitatively for activity. These included *L. casei*, *Streptococcus faecalis*, strain R, *Lactobacillus arabinosus*, *Escherichia coli*, and *Bacillus subtilis*, all grown on defined media without any crude supplement. Tests with culture filtrates as well as with hot water extracts of crushed cells revealed that the factor was present only in the cells. Cells of *L. casei* grown on the basal medium contained the factor, indicating that the organism can synthesize it.

Tests with *S. faecalis*, strain R, and *L. arabinosus* using the improved medium indicated that 16 hour growth of these organisms, measured turbidimetrically, was enhanced also by the supplement though not to the same extent as with *L. casei*.

Preparation of a concentrate. The foregoing properties were utilized to concentrate the active principle. The following procedure was adopted.

One hundred grams of the crude sample were extracted with water first by autoclaving with 500 ml at 15 lb pressure for 15 minutes and then twice successively by shaking half an hour each time with 200 ml. The extracts were combined. Inert proteinaceous material in the extract was precipitated with a 10 per cent solution of lead acetate, added drop by drop till no more precipitate was formed. The precipitate was removed by centrifugation and washed with a little water in the centrifuge; the washings were added to the centrifugate. Dissolved lead in the centrifugate was removed by passing H_2S and filtering out the lead sulfide formed; concentration under reduced pressure helped in removing H_2S.

The concentrate was treated now with fullers' earth as adsorbent for impurities. Treatment was given with 5 per cent w/v adsorbent and continous agitation on a mechanical shaker. The adsorbent was filtered out, washed with a little water on the filter, and rejected.

The filtrate was treated with norite at 5 per cent w/v level and the treatment continued for one hour on the shaker. The adsorbent was filtered and washed with some water on the shaker again. The charcoal was dried, first by suction and subsequently in an oven at 90 C.

Elution was carried out by stirring the adsorbate with 120 ml acetone containing dilute ammonia, thrice successively. The combined eluates were evaporated on water-bath to small

TABLE 6

Concentration of activity

STATE	TOTAL SOLIDS	ACTIVITY	RECOVERY
	mg/ml	*units/mg*	*per cent*
Starting material	—	0.25	—
Aqueous extract	29	0.9	100
Lead filtrate concentrated	13.4	2.0	80
Fullers' earth filtrate	7.6	2.6	62
Charcoal eluate	0.8	10.2	48
Concentrate	4.8	10.1	47

volume. The activity was determined at intermediate stages during the procedure (table 6).

DISCUSSION

On a medium containing all known growth essentials, early growth of *L. casei* is accelerated markedly by extracts of certain natural materials. The observation first was made with a commercial APF supplement derived from *Aerobacter aerogenes* cultures. Although the factor is concerned with initiation and acceleration of early growth, the organism is able to synthesize it endocellularly. An external supply is thus not essential but serves to shorten the lag phase of growth.

The factor could not be replaced by any known compound. Solubility in 95 per cent ethanol and ready adsorption on charcoal could distinguish it from strepogenin. The factor markedly enhances growth even in the presence of excess strepogenin.

Inactivity of a combination of $KMnO_4$ treated extract and charcoal filtrate of the material to reproduce the growth promoting action would rule out identity of the factor with that reported by Scott and co-workers (1946). These workers subsequently have observed (Daniel *et al.*, 1948, 1949; Peeler *et al.*, 1949) several other unidentified growth stimulants, but the properties of these are undescribed.

Acetate replacing factor of Guirard *et al.* (1946) is not known to stimulate growth in a medium containing acetate, being concerned in oxidative decarboxylation of pyruvate (α-keto acids) in microorganisms (Reed and DeBusk, 1952). The acetate replacing factor is, moreover, soluble in fat solvents, and lability to oxidizing conditions is not absolute. Protogen, required by *Tetrahymena geleii* (Stokstad *et al.*, 1949), and factors reported for *S. faecalis* by Colio and Babb (1948) and Cooperman *et al.* (1946) are related to the acetate replacing factor (Snell and Broquist, 1949). Properties of *B. rettgeri* factor (Kline and Barker, 1950) indicate that it also belongs to the same group.

Comparison with other growth factors reported for lactobacilli has not been possible for lack of sufficient information. These factors are as follows: a vegetable juice factor stimulating acid production by lactobacilli (Metcalf *et al.*, 1946), charcoal adsorbable factor from tomato juice enhancing growth of *L. arabinosus* (Kuiken *et al.*, 1943), and other factors required by *Lactobacillus lactis* (Shorb, 1947) and *Lactobacillus leichmannii* (Peeler and Norris, 1951).

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SUMMARY

Requirement of *Lactobacillus casei* for a factor from natural sources which is concerned with growth initiation and acceleration is reported.

The factor is distinct from others reported and is also not replaceable by known substances.

The factor is synthesized by bacteria endocellularly and is distributed widely in nature.

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