A *Lactobacillus* of Cecal Origin Requiring Oleic Acid

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Introduction

The stimulatory effect of oleic acid on the growth of *Lactobacilli* in the presence of suboptimal amounts of riboflavin has been demonstrated by Strong and Carpenter (1). Bauernfeind *et al.* (2) showed the same effect on pantothenic acid assays. Williams and Fieger (3) reported that oleic acid had a marked stimulatory effect in biotin assays and later found (4) that *L. casei* could be grown in an essentially biotin-free medium, provided oleic acid was present. Guirard *et al.* (5) found several fatty acids, including oleic acid, were capable of replacing the nutritional function of acetate with several *Lactobacilli*. *Corynebacterium diphtheriae* and *Clostridium tetani* have been demonstrated to require oleic acid by Cohen and Mueller (6) and Feeney *et al.* (7), respectively.

In the course of studying the nutrition of cecal *Lactobacilli*, isolated from rats fed on a highly purified diet, a strain was found which was unable to grow on a synthetic medium considered complete for *L. casei* (Table I).[1] None of the known bacterial stimulants available in the laboratory furnished the essential factor.

The method of approach in determining the nature of the missing factor was to find the natural supplements which would permit growth when added to the synthetic media. One of these was fractionated to determine the chemical properties of the essential factor. Graded dilutions of the supplement were used in constructing the standard curve for assaying the potency of the fractions made from it.

[1] The amount of pyridoxine is suboptimal if used without subsequent heating in the presence of amino acids (Snell and Rannefeld, 11). In this case, enough "pseudo-pyridoxine" was produced during autoclaving so that high levels of pyridoxine gave no increased response.

TABLE I

Double Strength Basal Medium

Hydrolyzed casein	2.5	gm.
L-Tryptophan	50.	mg.
L-Cystine hydrochloride	50.	mg.
Adenine sulphate	5.	mg.
Guanine hydrochloride	5.	mg.
Uracil	5.	mg.
Thiamine chloride	100	γ
Pyridoxine	100	γ
d-Ca pantothenate	100	γ
Riboflavin	200	γ
Nicotinic acid	50	γ
p-Aminobenzoic acid	40	γ
Biotin	0.75	γ
Pteroylglutamic acid	10	γ
Inositol	50	γ
Glucose	5	gm.
Sodium acetate	3	gm.
Speakman Salts A [1]	2.5	ml.
Speakman Salts B [2]	2.5	ml.
Distilled water to	250	ml.
pH	6.6–6.8	

[1] Salts A contained: 25 g. of K_2HPO_4 and KH_2PO_4/250 ml. of solution.

[2] Salts B contained: 10 g. $MgSO_4 \cdot 7H_2O$ and 0.5 g. each of $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$ and NaCl/250 ml. of solution.

Experimental

Organism Used

The organism is a gram positive rod, occurring in short chains or singularly, never motile. It is the dominant florum in the cecum of rats fed on a highly purified diet. On Bacto tomato juice agar, colonies are of the X type, *e.g.*, having delicate filament outgrowths, giving a rough wooly appearance. It grows well at 45°C. but not at 50°C. At 30°C. it will not curdle milk in less than 7 days. On lactose-yeast-peptone agar, it tolerates a pH of 7.8, a sodium chloride concentration of 2.5%, and a phenol dilution of 1:300. In litmus milk, the curd is firm and the litmus is slowly reduced. Glucose, sucrose, lactose, and salicin, but not mannitol, are fermented. According to the criteria presented by Curran *et al.* (8) and Sherman and Hodge (9), the organism has the characteristics of *L. acidophilus*.

Basal Media

The basal medium used in all experiments except where specifically stated is listed in Table I.

The purines and pyrimidines were combined in one solution and stored in the refrigerator. The vitamin supplements were stored in brown bottles under toluene with exception of biotin, which was preserved in 25% alcohol.

Testing Procedure

Neutralized supplements were added to 5 ml. of the double strength basal medium and the final volume brought to 10 ml. The testing procedure was essentially that of Snell and Wright (10). The standard curve was constructed by plotting the titrations against graded concentrations of the crude material from which the fractions were made.

Measurement of Growth Response

After 72 hours incubation at 37°C., culture tubes were titrated with 0.1 *N* NaOH using bromthymol blue as the indicator. Potency of the source material was determined by the amount of acid produced. Recovery of activity in the chemical fractions were calculated from the standard curve of the crude substance.

RESULTS

Response to Vitamins and Bacterial Growth Stimulants

No growth occurred when the following were added to the basal medium: thymine, orotic acid, choline, asparagine and glutamine. The following vitamins: thiamine, pyridoxine, calcium pantothenate, riboflavin, nicotinic acid, *p*-aminobenzoic acid, biotin, and pteroylglutamic acid, gave no response when added to the basal medium at five times the level stated in Table I.

Response to Natural Supplements

Soybean protein, crude liver extract, Bacto peptone, yeast extract (Difco), Vitab, Indian molasses, concentrated orange juice, apple

TABLE II

Response to 500 γ of Active Supplements

Material tested	Titration of 72-hour culture (ml. 0.1 *N* NaOH)	Titration of uninoculated sample. (ml. 0.1 *N* NaOH)
Pressed liver cake	5.6	0.8
Bacto heart infusion	4.2	0.9
Asparagus juice	2.1	1.1
Fish solubles	3.6	0.8
Distillers solubles	3.6	0.8

honey, pentanucleotides, corn steep liquor, papain digested liver cake, and purified anti-pernicious anemia extract [2] showed no response at levels of 500 γ/ml.

Table II gives a list of active natural supplements and their activity. Fish solubles was chosen for further study because of its solubility.

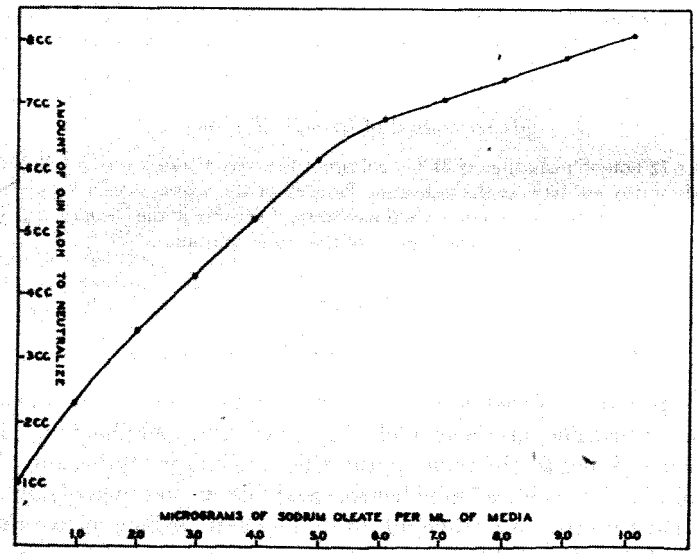

FIG. 1. Response to fish solubles.

Experiments Indicating the Nature of the Active Substance

Ten g. samples of fish solubles were diluted with 20 ml. of water and adjusted to pH 2, 7 and 9. They were then autoclaved at 15 pounds pressure for 2 hours. The samples which were autoclaved at pH 2 and 7 showed no change in activity. However, the sample autoclaved at pH 9 had double the activity of an untreated sample. When the alkali-treated sample was adjusted to pH 2, the active material was readily extracted by petroleum ether. The same results were demonstrated with distiller's solubles.

In addition, a solution of fish solubles was adjusted to pH 4.5 and

[2] Lederle Laboratories 15 unit liver.

filtered. The activity remained in the fatty acid residue on the filter paper.

Data from these experiments are given in Table III.

TABLE III

Response to Fractions of Fish Solubles and Distillers Solubles

Fractions tested (number of times assayed given in parenthesis)	γ Equivalents of fish solubles	Activity (fish soluble equivalents)	
		Range	Average
1. Alkali-treated fish solubles (5)	100–400	1.8 –2.2	1.90
2. Acid pet. ether extract of 1 (5)	200–600	1.1 –1.6	1.44
3. Acid pet. ether residue of 1 (5)	800–1600	.11– .43	0.33
4. pH 4.5 ppt. of fish solubles (3)	100–600	1.06–1.30	1.12
5. pH 4.5 filtrate of fish soluble (2)	600–5000	inactive	0
6. Alkali-treated distillers solubles (2)	100–500	1.70–2.20	1.95
7. Acid pet. ether extract of 6 (2)	100–400	1.60–2.00	1.80
8. Acid pet. ether residue of 6 (2)	800–4000	0 – .31	0.15

Response to Sodium Oleate

The above data indicated the growth factor might be a fatty acid (1). Na oleate was assayed and found to be 275 times as active as fish solubles. Fig. 2 shows the response of the organism to sodium oleate.

Response to Purified Sodium Oleate

To eliminate the possibility that the response was due to some water-soluble impurity in the sodium oleate, the following process was applied. An aqueous solution of the salt was adjusted to pH 2 and extracted with petroleum ether. The oleic acid was re-extracted from the petroleum ether solution with 0.01 *N* NaOH. Full activity was obtained in the purified substance.

Response to Acetate

The sodium acetate in the basal medium was varied from 0.6 to 2.0% with no effect on the response to sodium oleate. Complete substitution of the acetate with a phosphate buffer did not affect the sodium oleate assay.[3]

[3] Growth determined turbidimetrically.

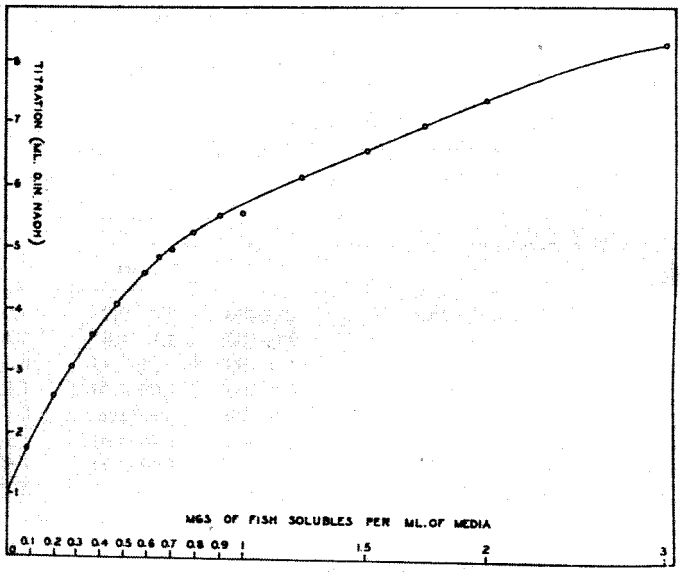

FIG. 2. Response to untreated sodium oleate.

Response to Other Oleic Acid Derivatives

Lecithin was found to have 4% the activity of sodium oleate. Sorbitol monooleate (Tween 80) at 50 γ/ml. level was 10% as active as sodium oleate; at higher levels it proved toxic to the organism. Sorbitol monostearate (Tween 60) was inactive.

Maintenance of the Organism on Basal Medium Containing Sodium Oleate

The *Lactobacillus* described in this paper was passed through 30 daily transfers of the basal synthetic media containing 5 γ of sodium oleate/ml. with no decrease in response. Subcultures into basal medium without sodium oleate never have shown growth.

DISCUSSION

This investigation has shown that oleic acid is an essential factor in the nutrition of the *Lactobacillus* described above. Continuous subculture on a synthetic medium is possible only in the presence of the substance.

The properties of the factor as present in fish solubles are those of a fatty acid. Oleic acid gives maximum growth of the organism when used to replace fish solubles.

Other investigators have demonstrated oleic acid to be a growth stimulant of *Lactobacilli* with suboptimal amounts of riboflavin, pantothenic acid and biotin, but maximum growth could be obtained in its absence. The strain of *Lactobacillus* used in this study, does not grow in the absence of sodium oleate when all of the vitamins are added in a 5-fold excess of what is ordinarily considered sufficient for *Lactobacilli*. Several rich vitamin sources, such as crude liver extracts, yeast and Vitab, fail to induce growth. The sodium oleate was purified to insure that the growth stimulation was not due to a water-soluble impurity.

Guirard *et al.* (5) found evidence that certain *Lactobacilli* may use acetate to synthesize essential lipoidal material, including fatty acids. The *Lactobacillus* used in this investigation did not require acetate, but could not grow in the absence of oleate.

Summary

1. An essential growth factor, present in fish solubles, necessary for the growth of a *Lactobacillus* isolated from the cecum of a rat, was demonstrated to be oleic acid.

2. The *Lactobacillus* could be transferred repeatedly on synthetic media only in the presence of sodium oleate.

3. Its requirement for oleic acid could not be modified by high levels of sodium acetate, biotin, riboflavin, pantothenic acid, or any of the other known factors tried.

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