DISCOVERY OF A NEW ACETATE-REPLACING FACTOR[1]

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Many lactobacilli fail to grow in a semisynthetic medium devoid of acetate, but adequate with respect to well recognized growth factors (Guirard *et al.*, 1946). Under these conditions, lipoic acid is a growth factor for a number of species (Reed *et al.*, 1951). The extent to which lipoic acid substitutes for acetate in the nutrition of a wide variety of species has not been critically examined. During the course of studies on a "vitamin B_{12}" concentrate, this material was subjected to countercurrent distribution. In a basal medium devoid of acetate, it was observed that the content of certain plates from the distribution was highly active in promoting growth of *Lactobacillus acidophilus* strain ATCC 4963. This activity had been distinctly separated by the distribution from lipoic acid which was also present in the concentrate.

This paper deals with microbiological and chemical studies of an acetate-replacing factor. The significance of this factor is not restricted to an acetate-replacing substance for *L. acidophilus* strain 4963, since our new observations prove that it promotes the growth of a number of other species, including *Lactobacillus bifidus* (Tomarelli *et al.*, 1949), *Lactobacillus bulgaricus* strain 09 (ATCC 10812), *L. bulgaricus* (ATCC 7993 and 799), and *Thermobacterium acidophilus* strain R26 (ATCC 11506).

MATERIALS AND METHODS

Lactobacillus acidophilus strain 4963 was chosen as the organism for assay of the factor since its dose-response curve is more nearly linear. Stock cultures were maintained at 5 C in skim milk medium supplemented with 1 per cent tryptose (Difco). Cultures were transferred every 6 weeks to duplicate tubes, one serving as the new stock, the other being used for weekly transfer. Inocula for daily use were prepared from the latter culture once a week with daily subculture in the milk medium during the interim. All cultures were grown for 24 hr at 37 C. The inoculum was prepared by suspending 0.1 ml of a 24 hr culture in 15 ml of sterile physiological saline followed by a dilution of 0.5 ml to 15 ml of saline. One drop of the second saline suspension was used to inoculate each assay tube.

The assay medium is shown in table 1. Lipoic acid was incorporated in the medium although *L. acidophilus* did not demonstrate a need for it in the presence of either acetate or the new factor. When *L. bifidus* or *T. acidophilus* was

TABLE 1

Basal medium for the microbiological determination of the factor with Lactobacillus acidophilus strain 4963

Ingredient	Per Liter Double Strength Medium
Acid-hydrolyzed, norit-treated, vitamin-free casein (Skeggs *et al.*, 1950)	10 g
Trypsin-digested, norit-treated, vitamin-free casein (Skeggs *et al.*, 1948)	5 g
DL-Tryptophan	0.4 g
L-Cystine	0.2 g
DL-α-Alanine	1 g
Glucose	40 g
Adenine, guanine, xanthine, uracil	10 mg each
Thymine, orotic acid	40 mg each
Salts A (Snell and Wright, 1941)	20 ml
Salts B (Snell and Wright, 1941)	10 ml
"Tween 80"	2 ml
Thiamine, pantothenic acid, riboflavin, nicotinic acid	200 μg each
Folic acid, pyridoxal, *p*-aminobenzoic acid	1 mg each
Pyridoxine	400 μg
Biotin	10 μg
Vitamin B_{12}, lipoic acid	20 μg each
Final pH	6.5–6.6

[1] A part of this material was presented at the Southwest Regional Meeting of the American Chemical Society, in Houston, Texas, 1955.

used as the assay organism, deoxyribonucleic acid (DNA) or a deoxyribose-containing derivative was added to the medium. The assay medium was adequate for growth of the *L. bulgaricus* cultures when supplemented with pantetheine and the pH adjusted to 5.8. An arbitrarily selected distillers' solubles fraction served as a standard in the microbiological assays. One mg of this concentrate was referred to as one unit of the factor. Aliquots of a suitable dilution of natural materials or concentrate were assayed without prior treatment.

Common techniques of microbiological assay in test tubes were employed. Volumes of 10 ml of medium were routinely used. Assay tubes were sterilized at 120 C for 12 to 15 min. The incubation period was 24 hr at 37 C. The extent of bacterial growth was determined turbidimetrically with a Klett-Summerson photoelectric colorimeter.

Lipoic acid determinations were carried out with *Lactobacillus casei* in an acetate-free basal medium adequately supplemented with norit-treated, tryptic digest of vitamin-free casein as a source of "strepogenin" (Sprince and Wooley, 1944).

RESULTS

The growth response of *L. acidophilus* strain 4963 to a number of materials is shown in figure 1. Curves *A* depict the growth responses to a sample of distillers' solubles and to sodium acetate. While sodium acetate is more active on an equal weight basis, the activity of the concentrate is greater than can be accounted for on its probable acetate content. Curves *B* show the activity of a 200-fold concentrate before and after 25 months of storage in 20 per cent ethanol at 5 C. It is apparent that the factor in the relatively crude state was quite stable at refrigeration temperatures. A certain amount of variation in the shape of the dose-response curve and the span of growth was encountered in daily use of the assay. However, results obtained in terms of units per mg of material assayed were remarkably consistent. Occasional checking of results

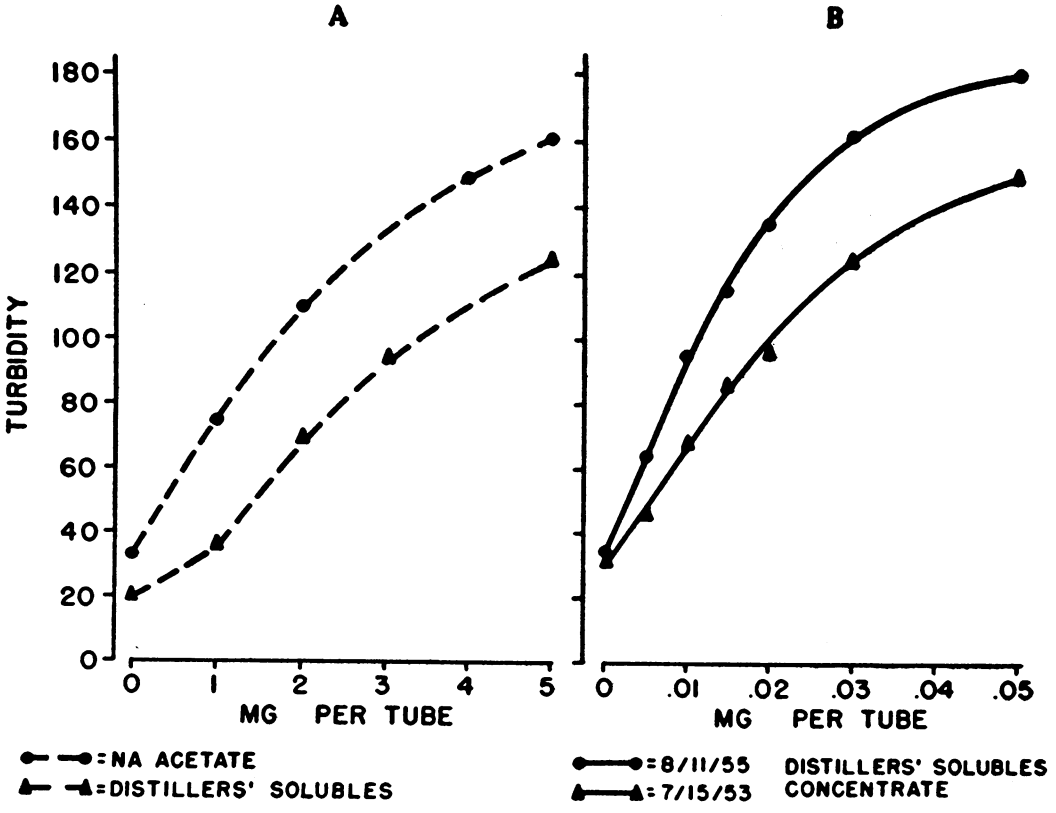

Figure 1. Response of *Lactobacillus acidophilus* strain 4963 to various substances

TABLE 2

Assay data on various materials

Substance	Units
Yeast extracts	3.0/g
(16)	0.8–4.6/g
Malt sprouts	0.4/g
Liver powder L	1.2/g
Crude casein extract	1.5
Whey	2.5
Trypticase soy peptone	0.7
Dried whole liver	0.7
Distillers' solubles	3.5
1 unit liver extract	0.2/ml
15 unit liver extract	0.2/ml
Wine	0.1/ml
Beer	0.2/ml
Urine	0.06/ml

The following materials contained less than 0.001 units/g: tryptone (Difco), tryptose (Difco), peptone (Difco), liver powder S, liver powder 1-20, "cerophyl," wheat germ, hog gastric mucin, dried stomach substance, soy peptone, N-Z-amine.

against all of the organisms responding to the factor gave essentially the same results with each organism. Carrying *L. acidophilus* strain 4963 for too long a period in skim milk reduced its response to the factor. Attempts to standardize the inoculum by subculture in basal medium supplemented with the factor met with little success in reducing the variations encountered. Strict adherence to a 24-hr incubation period also appeared necessary. Considerable "drift" was encountered in assays read at 18 hr. Incubation for 40 hr resulted in an "all or none" effect—no growth in the absence of the factor and nearly complete growth in the presence of minimal amounts. Incubation in excess of 40 hr resulted in irregular growth responses due to gradual synthesis either of the factor or of acetate.

The assay data obtained with various materials are summarized in table 2. Direct assay of certain substances such as blood, eggs, meat was not possible because of interference with turbidimetric determinations by color or precipitates. The residue obtained by ashing potent natural materials was completely inactive.

Over 150 compounds of biological significance were examined for microbiological activity with *L. acidophilus*. No compound, other than acetate, showed consistent activity. Since concentrates are described in this paper with 2,000 times the activity of distillers' solubles or 1,600 times the activity of acetate, it is apparent that a factor more active than acetate exists. Subsequent results provide evidence that the growth factor requirement of *L. acidophilus* strain 4963 under the experimental conditions described is not a multiple one.

A summary of the properties of the factor is presented in table 3. Some of these properties may be influenced by the large amount of other substances in the concentrates.

Of particular significance are experiments demonstrating that the factor is not a combined form of lipoic acid. The contents of each consecutive 10 plates, from the 200-plate distribution of the concentrate from Hiram Walker & Sons, Inc., were combined and subjected to microbiological assay for the factor and for lipoic acid, both before and after hydrolysis with 1 N H_2SO_4 at 120 C for 1 hr. As would be expected from the known occurrence of lipoic acid in a multiplicity of forms, activity as determined by *L. casei* was encountered in a number of regions of the distribution. No increase in activity of any fraction was observed after acid hydrolysis. It may be concluded that no microbiologically unavailable conjugates of lipoic acid that can be cleaved by acid hydrolysis existed in the material examined.

The results of a few fractionation experiments are in order to provide evidence that the factor

TABLE 3

Chemical properties of the factor

Solubility—Soluble in water, methanol, ethanol. Sparingly soluble in chloroform and butanol. May be partitioned into chloroform by prolonged liquid-liquid extraction at acid pH.
Stability—Stable to 0.5 N alkali at 120 C for 1 hr. Some destruction with 0.5 N acid at 120 C for 1 hr. Lability to acid increases with purification.
Adsorption Characteristics—Adsorbed on moderate to large amounts of norit. Not adsorbed by superfiltrol.
Ion-exchange Characteristics—Not retained by strong cation exchange resins Dowex-50 (H^+) or Amberlite IR-120 (H^+). Retained by strong anion exchange resin (Dowex-1) in the OH^- but not the Cl^- form.
Electrophoretic behavior—Migrates on paper curtain electrophoresis like a weak acid. Displacement from vertical line from origin about equal to that of lipoic acid run similarly as a reference compound.

behaved as an entity and was not the resultant effect of a combination of compounds. A concentrate containing 41,600 units of activity was poured onto a column of Dowex-1 (OH^-) and washed liberally with water. The column was eluted with 0.05N formic acid. Activity began to emerge with the first trace of acidity and was contained in 4 fractions as follows: 14,800, 17,600, 7,400 and 1,800 units, respectively. Total recovery was 41,600 units, or 100 per cent of that applied to the column. A concentrate containing 42,500 units of activity was poured onto a column of Dowex-50 (H^+) and washed with water. Fractions were collected from the column and assayed individually for the factor. Activity was encountered in 6 fractions as follows: 3,000, 10,600, 11,700, 8,000, 6,100 and 4,200 units respectively. Total recovery was 43,600 units, or 103 per cent of that applied to the column. A concentrate containing 70,000 units of activity was subjected to a 30-plate countercurrent distribution between the phases resulting from the equilibration of 3 parts chloroform, 4 parts water, and 4 parts ethanol. Activity was encountered in all plates with peak activity in plate 14. Total activity observed in the 30 plates each individually assayed was 69,200 units, or 99 per cent of the activity subjected to countercurrent distribution. The quantitative recoveries of activity obtained in the above three separate fractionation procedures show that the material being fractionated is a single compound. Had the microbiological responses observed been due to a combination of known or unknown compounds, unaccountable losses in activity would have been expected, especially from a procedure with the resolving power of countercurrent distribution.

DISCUSSION

Various studies have been reported which indicate the presence of a growth factor(s) for chicks and for rats in distillers' dried solubles, fish solubles, liver, etc. A purified material was obtained which was apparently active in rats at a level of 10 μg. per day. This material showed an ultraviolet absorption maximum at 2820 A; the factor was tentatively called "vitamin B_{12}" (Novak, Hauge, and Carrick, 1947; Hauge, 1948; Novak and Hauge, 1948*a*, 1948*b*). At one time, it appeared that *Lactobacillus arabinosus* would respond to the factor in distillers' dried solubles (Hauge, 1948). The low biological activity of orotic acid compared to that of concentrates of "vitamin B_{12}" and certain absorption spectra were considered by Manna and Hauge (1953) to indicate that orotic acid might be a decomposition product of "vitamin B_{12}."

The chemical and physical properties described for the concentrates of "vitamin B_{12}" do not necessarily show that the best concentrate was a substantially pure substance. If the active substance in the best concentrate were actually present to the extent of only a few per cent or less, then identification by these properties becomes difficult.

Austin and Boruff (1949) investigated the preparation and properties of concentrates of "vitamin B_{12}" from the distillers' dried grain solubles of Hiram Walker and Sons, Inc. They described a simplified purification procedure and reported a growth response in chicks from their concentrates. Drill (1954) reported that a "vitamin B_{12}" concentrate of unstated source seemed to have a partial lipotropic activity in rats which were fed a high fat diet. Samples of concentrates from Hiram Walker and Sons, Inc. were made available to others for nutritional studies. For example, their concentrate was found by Cunha *et al.* (1950) to benefit growth for the pig during certain periods of development. We also received samples of their concentrate from distillers' dried solubles through the courtesy of Dr. J. M. Van Lanen.

We subjected their concentrate to a 200-plate countercurrent distribution between ether and water with the objective of biologically examining the content of certain plates in various tests with chicks, rats, and microorganisms. The distribution between ether and water turned out to be a particularly fortunate choice, because it distinctly separated lipoic acid from a new acetate-replacing factor, the presence of which was discovered by subsequent experiments on the growth of *Lactobacillus acidophilus* (ATCC 4963).

Since the original concentrates, designated "vitamin B_{12}," were of unknown purity and of unknown lipoic acid content, and since the original culture of *Lactobacillus arabinosus* was not available, one cannot relate this new acetate-replacing factor to the original microbiological studies. Ott *et al.* (*unpublished data*) have found that, under their specific dietary conditions, no significant growth-promoting effect in chicks was observed with concentrates of "vitamin B_{12}."

Thus, it is not yet possible to compare in chicks our acetate-replacing factor and "vitamin B_{12}" concentrates.

Present evidence indicates that our factor is distinct from certain others which have been described previously. Obvious differences in distribution (table 2) or properties (table 3) and in some instances a negative test of the active compound(s) or an appropriate concentrate (over 150 possible compounds and concentrates were examined) serve to differentiate the factor from strepogenin (Sprince and Woolley, 1944), factor 3 (Schwarz, 1952), *Lactobacillus bifidus* factors (Rose *et al.*, 1954, Hendlin *et al.*, 1955) the Pilobolus factor (Hesseltine *et al.*, 1952), the Reiter treponeme factor (Oyama *et al.*, 1953), the lemon juice factor (van Wagtendonk and Conner, 1953), the *Lactobacillus casei* mutant factor (Camien and Dunn, 1953), the ethanol utilization factor (Rao and Stokes, 1953), the aspartic acid deaminase factor (Williams and Christman, 1953), the *Crithidia* factor (Patterson *et al.*, 1955), and coenzyme P (Castelfranco *et al.*, 1955).

A number of other factors stimulating the growth of either microorganisms or higher animals[2] have been described in the literature. Characterization of these factors in general has been very incomplete, and in some instances adequate evidence has not been presented that the effect observed is due to a single component of natural material.

SUMMARY

Evidence is presented for the existence of a new factor required in the absence of acetate for the growth of a number of lactobacilli including *Lactobacillus acidophilus* (ATCC 4963), *L. bifidus* (Tomarelli), *Lactobacillus bulgaricus* strain 09 (ATCC 10812), *L. bulgaricus* (ATCC 799 and 7993), and *Thermobacterium acidophilus* strain R26 (ATCC 11506).

Commercial whey, fish solubles, distillers' solubles, and certain yeast extracts are relatively rich sources of the factor.

Details of a microbiological method for the determination of the factor are described and a procedure by which useful concentrates of the factor may be obtained has been outlined.

[2] For a review of unidentified growth factors r chicks see Nutr. Revs. **14**, 75–79 (1956).

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