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# The Isolation of the Fermentation *Lactobacillus casei* Factor

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On aerobic culture an unidentified organism belonging to the genus *Corynebacterium*[3] produces a compound that is essential for the growth of *Lactobacillus casei* under the conditions described for the detection of the "norite eluate factor."[4,5] The compound, therefore, belongs to the group of substances variously known as the "norite eluate factor,"[4,5] folic acid[6] and vitamin Bc.[7] A preliminary note on the isolation of the fermentation *L. casei* factor has appeared.[8]

The fermentation *L. casei* factor has been isolated as described herein. Before crystallization could be induced the solution had to contain small amounts of electrolytes. If the electrolytes were not present in the crystallizing solvent, gels were invariably formed. Under these specified conditions the compound was crystallized as the free acid, barium salt and methyl ester.

Difficulty has been experienced in obtaining consistent analyses. Therefore, from combustion data no empirical formula could be derived. However, the analyses do serve to distinguish this compound from any related compound previously reported.[7,9]

The ultraviolet absorption spectrum of the fermentation *L. casei* factor is presented in Fig. 1. The compound exhibits the same absorption characteristics as the liver *L. casei* factor.[10] The extinction coefficients are somewhat lower indicating that the fermentation *L. casei* factor is a higher molecular weight compound. The extinction coefficient at 365 mμ in 0.1 *N* sodium hydroxide is 134.

The fermentation compound has the same biological activity for animals as does the liver *L. casei* factor with the proviso that increased amounts are necessary to compensate for the higher molecular weight.[11]

The fermentation *L. casei* factor differs from the liver *L. casei* factor by its relative activity for *Lactobacillus casei* and *Streptococcus faecalis* R. The fermentation compound is 60–80% as active as the liver compound for *L. casei* but only 4–6% as active for *S. faecalis* R. The amounts of the fermentation compound required per ml. for half-maximum growth of *L. casei* and *S. faecalis* R., respectively, are 0.000061 and 0.0042 microgram.

The analyses and biological activity serve to distinguish this compound from any of the com-

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(3) The organism was classified as *Corynebacterium sp.* One of the criteria of classification was the absence of spores. This was determined by heating the organism at 90° for varying lengths of time. The organism was killed even with short periods of heating.

Later studies have shown that the organism is not killed by heating at 85° for periods up to thirty minutes. This evidence introduces the possibility of the presence of spores and, of necessity, questions the validity of the previous classification. Attempts to demonstrate the presence of spores by staining techniques have not been successful. Until the question of the presence or absence of spores is answered, the above classification must be designated as tentative.

Sub-cultures of the organism will be sent on request. All requests should be directed to Dr. B. L. Hutchings, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

The organism can be cultured under aerobic conditions on a medium of the following composition: glucose, 20 g.; glycine, 4.0 g.; dipotassium hydrogen phosphate, 0.5 g.; potassium dihydrogen phosphate, 0.5 g.; ferrous sulfate heptahydrate, 0.04 g.; magnesium sulfate heptahydrate, 0.20 g.; thiamine, 0.40 mg., and tap water to 1 liter. A solid medium can be obtained by the addition of 20 g. of agar to the above medium.

(4) Snell and Peterson, *J. Bact.*, **39**, 273 (1940).

(5) Hutchings, Bohonos and Peterson, *J. Biol. Chem.*, **141**, 521 (1941).

(6) Mitchell, Snell and Williams, THIS JOURNAL, **63**, 2284 (1941).

(7) Pfiffner, *et al.*, *Science*, **97**, 404 (1943).

(8) Hutchings, *et al.*, *ibid.*, **99**, 371 (1944).

(9) Stokstad, *J. Biol. Chem.*, **149**, 573 (1943).

(10) Stokstad, *et al.*, THIS JOURNAL, **70**, 3 (1948).

(11) Hutchings, *et al.*, *J. Biol. Chem.*, **163**, 447 (1946).

pounds previously reported as influencing the growth of *L. casei* or *S. faecalis* R. under conditions of the test.

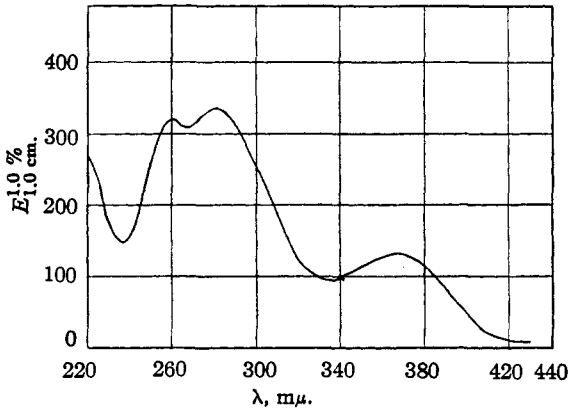


Fig. 1.—Ultraviolet absorption spectrum of fermentation *L. casei* factor in 0.1 *N* sodium hydroxide.

## Experimental

**Adsorption and Elution from Charcoal.**—After removal of the bacterial cells the solution which contained from 3–5 γ of the active compound per ml. was adjusted to *p*H 3.0 and 6 g. of Norite A added per liter of filtrate. After thirty minutes adsorption, the charcoal was filtered off and washed well with water. The charcoal was eluted with 50% ethanol (6 liters per kg. of charcoal). This eluate was discarded. The charcoal was then eluted with 50% ethanol and 10% ammonium hydroxide (by volume) at 70° for one hour (12 liters per kg. of charcoal). The elution was repeated once. The eluates were combined. The recovery approximated 65%.

**Precipitation of the Barium Salt.**—The ammonia–ethanol eluates were adjusted to *p*H 8.0 with concentrated hydrochloric acid and ethanol added to a concentration of 85%. A saturated aqueous solution of barium chloride was added until complete precipitation occurred, meanwhile maintaining the *p*H at 8.0. After cooling to 0–5° the precipitate was centrifuged out and washed with methanol. The active compound was completely precipitated by this procedure.

**Esterification and Extraction with Butanol.**—The barium precipitate was suspended in 0.25 *N* methanol–hydrogen chloride (one-fifteenth the volume of the original filtrate) and esterified at room temperature. At the end of twenty-four hours the esterification mixture was adjusted to *p*H 4.5 by cautious addition of 5 *N* sodium hydroxide, and then concentrated to dryness under reduced pressure. The residue was suspended in a volume of water equivalent to a concentration of 15 micrograms per ml. of the active compound. The water insolubles were centrifuged out and discarded.

The aqueous solution was extracted 3 times with 2 volumes of butanol. Each butanol extract was in turn extracted with one-half volume of water. The combined butanol extracts were concentrated to one-sixth of their volume and extracted with one-half volume of water. This water wash was extracted with 2 volumes of butanol. The butanol extracts were combined and concentrated to dryness under reduced pressure. The esterification and butanol extraction gave a yield of 50–90%.

**Fractionation from Methanol.**—The residue after removal of the butanol was dissolved in the minimum amount of hot methanol. After thorough chilling to −5° the precipitated ester was collected. The precipitate was extracted two times (total volume was one-fifth the volume of methanol necessary to dissolve the residue after removal of butanol) with 0.1 *N* methanol–hydrogen chloride. The extracts were diluted with 2 volumes of methanol and 2 moles of sodium acetate were added per mole of hydrogen chloride. The solution was heated to 60°, centrifuged and the supernatant chilled at −10° for twenty-four hours. The precipitate which formed was centrifuged, then dissolved in hot methanol and sodium chloride was added to a concentration of 0.05 *N*. The solution was centrifuged at 60° and the insoluble fraction discarded. The supernatant was cooled at −10° for twenty-four hours and then centrifuged at 2°. The precipitated ester was obtained in yields approximating 65%.

**Clarification with Florisil.**—The ester was washed free of methanol, suspended in water and 0.1 *N* barium hydroxide was added until the solution was faintly alkaline to phenolphthalein. The hydrolysis of the ester was extremely rapid. The hydrolysate was centrifuged and the supernatant treated with florisil (1 g. per 100 mg. of active compound) for thirty minutes to remove extraneous pigments. The florisil was filtered off and washed with dilute barium hydroxide. The filtrate and washings were combined. Yield was 85–90%.

**Crystallization of the Free Acid.**—One-tenth volume of 1 *N* barium chloride was added to the above filtrate, the solution was cooled to 0–5° and ethanol was added to a concentration of 50%. The solution was chilled overnight, then centrifuged and the precipitate washed with alcohol, ether and dried.

The barium precipitate was extracted with small portions of hot water until all of the active compound was in solution. The resulting extracts were combined and 1 *N* hydrochloric acid was added to *p*H 2.8. The solution was cooled to 5° and the precipitate collected. The precipitate was dissolved in hot water previously adjusted to *p*H 2.8 and containing a small amount of calcium or sodium chloride. On cooling the acid crystallized as very short needles or long threads. The acid could be repeatedly crystallized in this manner.

*Anal.*[12] Found: C, 48.6, 48.0, 47.7; H, 4.8, 4.4, 4.7; N, 16.2, 15.5, 15.5.

**Crystallization of the Barium Salt.**—The amorphous barium salt obtained by precipitation with 50% ethanol was dissolved in hot 0.1 *N* barium chloride. On cooling the compound crystallized as small needles.

**Crystallization of the Methyl Ester.**—The compound was re-esterified by dissolving in 0.1 *N* methanol–hydrogen chloride and allowing to stand at 32° for twelve hours.

The ester crystallized as short needles or long threads from methanol 0.05 *N* with sodium chloride.

TABLE I

SOLUBILITY OF THE FERMENTATION *L. casei* FACTOR

| Compound | Solvent | Temp., °C. | Concn. mg. per ml. |
|---|---|---|---|
| Methyl ester | Methanol | 60 | 5.00 |
| Methyl ester | Methanol | −5 | 0.30 |
| Methyl ester | Methanol–0.05 *N* sodium chloride | 60 | 2.00 |
| Methyl ester | | −5 | 0.11 |
| Methyl ester | Water | 80 | 1.00 |
| Methyl ester | Water | 5 | 0.12 |
| Free acid | Water | 80 | 3.00 |
| Free acid | Water | 5 | 0.10 |

**Solubility.**—The methyl ester and the free acid were soluble under the conditions noted in Table I.

**Microbiological Assays.**—The microbiological activity of the compound was determined for *Lactobacillus casei* and *Streptococcus faecalis* R. according to established procedures.

**Ultraviolet Absorption.**—The ultraviolet absorption spectra of the fermentation *L. casei* factor was determined using a Beckman Spectrophotometer.

**Acknowledgment.**—We are indebted to the Misses E. Boggiano and B. Eames for the micro-

(12) Analytical figures are given for three different lots.

biological assays and the spectrophotometric determinations, and to Mr. L. Brancone and coworkers for the microanalyses.

The authors are especially indebted to Dr. J. H. Williams for his constant interest and counsel and for his efforts in coördinating the work performed in the various laboratories.

## Summary

An isolation procedure for the fermentation *L. casei* factor is outlined. The analyses and biological activity distinguish the fermentation *L. casei* factor from any similar compound previously reported.

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# The Isolation of the *Lactobacillus casei* Factor from Liver

BY E. L. R. STOKSTAD, BRIAN L. HUTCHINGS AND Y. SUBBAROW

Pfiffner, *et al.*,[1] and Stokstad[2] have described compounds isolated from liver which are active in promoting the growth of *L. casei* and *S. faecalis* R. The synthesis of this compound has recently been described by Angier, *et al.*[3] It is the purpose of this communication to describe the isolation of this compound from liver.

## Experimental

Assays for the *L. casei* factor were made by the method of Landy and Dicken.[4] The assay results were expressed originally in terms of an arbitrary standard Solvamin.[5] One-tenth milligram of this constituted one unit and corresponded to approximately the amount required per 10 ml. of medium to give three-fourths maximum growth. The pure liver *L. casei* factor was later found to have an activity very close to 1,000,000 units per mg.

The starting material for this isolation was a commercial preparation of a dried 85% ethanol precipitate of an aqueous extract of liver. Different preparations of liver extract contained between 20,000 and 30,000 units per gram which corresponds to 20 to 30 micrograms of the pure liver *L. casei* factor per gram.

In the isolation of this compound, four essential steps were used. They were: adsorption and elution of the free acid, esterification and extraction of the methyl ester with immiscible solvents, chromatographic adsorption of the ester, and fractional precipitation of the ester from water and methanol. The results of all these isolation steps are presented in Table I.

Eighty kg. of liver extract was dissolved in 1600 liters of water, sodium hydroxide was added to *p*H 8.5, 80 liters of 2 *N* calcium chloride added and the mixture heated to 85° to flocculate the precipitate. The mixture was filtered while hot with the aid of filter cell. The filtrate was cooled to 25° and adjusted to *p*H 3.00. The *L. casei* factor was adsorbed with 16 kg. of Norit A. This adsorbate was washed, first, with 320 liters of neutral 60% ethanol at 25° to remove inert materials and then with two 320-liter portions of 0.5 *N* ammonium hydroxide in 60% ethanol at 70° to remove the activity.

The ammoniacal eluate was concentrated to 80 liters and adjusted to *p*H 3.5 and the precipitate which formed on standing removed by centrifuging. The precipitate was redissolved in 40 liters of water with the aid of sodium hydroxide and reprecipitated at *p*H 3.5. A large amount of inert material was removed by the precipitate but almost half the activity was carried down with it.

This filtrate was adsorbed at *p*H 1.3 on Superfiltrol[6] by

TABLE I

CONCENTRATION OF *L. casei* FACTOR FROM LIVER

| Fraction | Units per gram equiv. liver extr. | Units per mg. solids |
|---|---|---|
| Liver extr: starting material | 20,000 | 20 |
| Norit eluate | 7,000 | ..... |
| Filtrate after *p*H 3 precipitation | 3,500 | ..... |
| Superfiltrol eluate (from Superfiltrol adsorbate of *L. casei* factor free acid) | 3,500 | 340 |
| Barium salt | 2,100 | 420 |
| Esterified mixture of barium salt | 2,000 | |
| Butanol extract of methyl ester | 2,000 | 3,450 |
| Methanol solution of butanol extract. | 2,000 | 3,450 |
| Methanol filtrate from Superfiltrol adsorption of *L. casei* factor methyl ester | 0 | ..... |
| 1st eluate: 92.5% acetone 10 ml. eluant per g. adsorbent | 30 | ..... |
| 2nd eluate: 75% acetone 10 ml. eluant per g. adsorbent | 1,200 | 47,000 |
| 3rd eluate: 75% acetone 10 ml. eluant per g. adsorbent | 325 | 24,000 |
| Precipitate obtained by concentrating 75% acetone eluates to give aqueous solution | 1,000 | 370,000 |
| 1st methanol extract of aqueous precip.: 1 ml. *cold* methanol per kg. equiv. liver | 90 | 35,000 |
| 2nd methanol extract of aqueous precip.: 1 ml. *cold* methanol per kg. equiv. liver | 100 | 380,000 |
| 3rd methanol extract of aqueous precip.: 4 ml. *hot* methanol per kg. equiv. liver | 780 | 950,000 |
| 1st precip. formed by cooling hot methanol extract | 270 | 1,030,000 |
| 2nd precip. formed by concentrating filtrate from 1st methanol precipitate | 313 | 870,000 |

(1) Pfiffner, Binkley, Bloom, Brown, Bird, Emmett, Hogan and O'Dell, *Science*, **97**, 404 (1943).

(2) Stokstad, *J. Biol. Chem.*, **149**, 573 (1943).

(3) Angier, Boothe, Hutchings, Mowat, Semb, Stokstad, Subba-Row, Waller, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels and Smith, *Science*, **103**, 667 (1946).

(4) Landy and Dicken, *J. Lab. Clin. Med.*, **27**, 1086 (1942).

(5) Solvamin, a vitamin concentrate manufactured by Commercial Solvents Corporation.

(6) Superfiltrol, an adsorbent supplied through the coöperation of the Filtrol Corporation, Los Angeles.