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

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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.

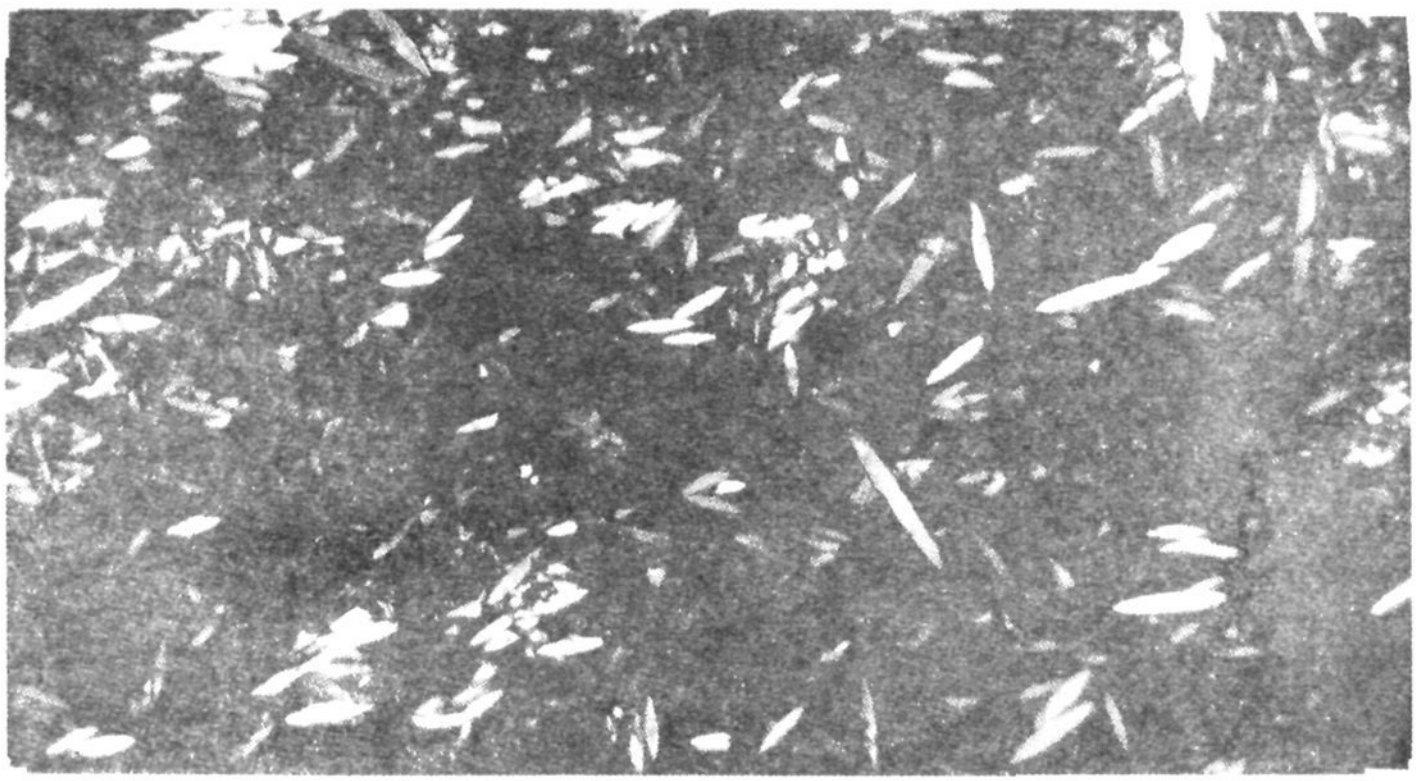

Fig. 1.—Crystals of liver *L. casei* factor.

percolation through a column of the granular adsorbent. Forty liters of the filtrate, equivalent to 40 kg. of liver extract, was percolated through 100 to 200 mesh Superfiltrol in a column 5 inches deep and 8 inches in diameter. The maximum rate of flow was 100 ml. per sq. cm. of filter surface per hour. The column was then eluted by percolation with 20 liters of 1.0 *N* ammonium hydroxide in 60% ethanol. After elution the column was rewashed with 0.1 *N* hydrochloric acid and then reused several times for subsequent adsorptions and elutions. When an adsorbent is used only once a certain amount of activity is adsorbed which cannot be eluted. In subsequent adsorptions and elutions this loss apparently does not occur. After six such cycles no decrease in efficiency of the adsorbent was observed and almost complete recovery of activity was obtained. From the table it can be seen that the Superfiltrol eluate contained 3,500 units per gram equivalent of liver extract and 340 units per mg. of solids. This represents a 17-fold increase in activity with a recovery of 17%.

The barium salt was next prepared by concentrating the superfiltrol eluates and adjusting to *p*H 7.0. The *L. casei* factor was precipitated as the barium salt by adding 9 volumes of ethanol and an excess of barium chloride solution. Little or no increase in activity was achieved but it did convert the material into a form which could be dried, finely ground and then esterified.

It might be noted that while the *L. casei* factor can be precipitated by heavy metals such as lead and silver, and by basic precipitants such as phosphotungstic acid, no large increases in activity could be effected at this stage by the use of these reagents.

The next step consisted in esterification and extraction of the methyl ester with *n*-butanol. Two hundred grams of the finely ground barium salt were esterified in 20 liters of 0.2 *N* hydrochloric acid in methanol for sixteen hours at 25°. Esterification proceeds very rapidly going to completion in one hour at 25° if anhydrous reagents are used. This ester is approximately 10% as active for *L. casei* as the free acid. Part of the activity of the ester may be due to partial hydrolysis of the ester during autoclaving of the sample with the medium as the ester is very rapidly hydrolyzed. Four hours hydrolysis at 100° at *p*H 8.0 produced 75% hydrolysis, and 0.1 *N* sodium hydroxide at 25° gave complete hydrolysis in half an hour. Since the ester possesses only partial activity, assays for potency were preceded by ten minutes saponification with 0.1 *N* sodium hydroxide at 100°.

The esterified mixture was neutralized and evaporated to almost dryness, redissolved in 20 liters of water and adjusted to *p*H 6 to 7. It was then extracted three times with 2 volumes of *n*-butanol. Each butanol extract was washed successively with the same portion of half a volume of water. The distribution coefficient of the *L. casei* factor methyl ester for butanol to water is 3 to 1. The final butanol extract contained 2,000 units per gram equivalent of liver and 3,450 units per mg. of solids.

While chromatographic adsorption of the free acid from aqueous solution proved ineffective, chromatographic adsorption of the ester in organic solvents was highly efficient. Superfiltrol[6] proved the best adsorbent; Brockman alumina and calcium carbonate were much less effective. The *L. casei* factor methyl ester could be adsorbed on Superfiltrol from *n*-butanol, methanol, acetone and water. The only efficient eluant was aqueous acetone. Aqueous methanol, aqueous methyl ethyl ketone, acetone–methanol mixtures, acetone–ethanol mixtures and methyl ethyl ketone–methanol mixtures were ineffective.

Chromatographic separation was carried out as follows: The butanol extract was evaporated to dryness and redissolved in methanol. An aliquot of this methanol solution, equivalent to 0.5 kg. of liver extract, was passed through a 3.5 × 13.0 cm. column containing 75 g. of 200 to 325 mesh Superfiltrol. This was eluted first with 750 ml. of 92.5% acetone and then with three portions of 750-ml. of 75% acetone. The 92.5% acetone elution removed a large amount of highly colored impurities without removing appreciable activity. The 75% acetone rapidly eluted the active factor. The first 75% acetone eluate which had an activity of 47,000 units per mg. was concentrated to 200 ml. during which most of the acetone had been removed. On cooling the aqueous solution most of the activity precipitated out. This contained 1,000 units per gram of original liver extract and 370,000 units per mg. of solids.

This precipitate which formed from aqueous solution was extracted with a small amount of cold methanol using 1 ml. of solvent per kg. of original liver. This removed very little activity but removed most of the dark brown pigment. The *L. casei* factor methyl ester was then dissolved by extraction with a larger amount of boiling methanol (4 ml. per kg. of liver extract). This hot methanol extract was almost colorless and highly active. On cooling the hot methanol extract to 2° the methyl ester separated out in a nearly pure form as a gelatinous precipitate which had an activity of 1,030,000 units per mg. Reprecipitation

from hot methanol yielded a preparation whose activity could not be increased by further purification. The solubility of the methyl ester in methanol is approximately 60 micrograms per ml. at 2°. The results obtained by methanol fractionation are shown in Table I. The analysis of two preparations of the methyl ester as obtained by this method are as follows

	C	H	N (Dumas)
Sample 1	53.0	4.6	21.2
Sample 2	53.1	5.1	20.5

The free acid was prepared by saponifying 2.0 mg. of a nearly pure preparation of the ester with 5.0 ml. of 0.01 *N* sodium hydroxide. The solution was treated with 4 mg. of Darco. On acidifying the filtrate to *p*H 3.0, the free acid precipitated out, which was then redissolved by heating to 90°. On cooling slowly the free acid of the *L. casei* factor crystallized out. The solubility of the free acid at *p*H 3.0 at 2° is approximately 10 micrograms per ml. and more than 0.5 mg. per ml. at 100°. These were recrystallized once from 0.1 *n* sodium chloride and two times from water.

The crystal form of the free acid is shown in Fig. 1.

The extinction coefficients in 0.1 *N* sodium hydroxide were determined with a Beckman spectrophotometer and found to be as follows: $E^{1\%}_{1\ cm.}$ 255 mμ, 565; 282 mμ, 350; 365 mμ, 195.

The amount required per ml. of medium for half maximum growth was 0.00007 microgram for *L. casei* and 0.0003 for *S. faecalis* R. This amount required for half maximum growth is not an accurately reproducible value and varies from one experiment to another.

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Summary

The isolation of the *Lactobacillus casei* factor from liver has been described. This was accomplished by the use of adsorption and elution of the free acid on Norit, esterification and extraction of the methyl ester with *n*-butanol. The methyl ester was chromatographed on Superfiltrol using aqueous acetone as the developer. Final purification of the ester was accomplished by fractional precipitation from water and methanol. The free acid was crystallized from hot water.

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The Degradation of the Fermentation *Lactobacillus casei* Factor. I

BY E. L. R. STOKSTAD, BRIAN L. HUTCHINGS, JOHN H. MOWAT, JAMES H. BOOTHE, COY W. WALLER, ROBERT B. ANGIER, JOSEPH SEMB AND Y. SUBBAROW

The isolation of two *Lactobacillus casei* factors, one from liver[1,2] and one from a fermentation product[3,4] has been described.

These two *L. casei* factors differ in biological activities and physical properties. Extinction coefficients indicated that the fermentation *L. casei* factor has the larger molecular weight. The structure and synthesis of the liver *L. casei* factor have been described in a preliminary communication.[5] The synthetic liver *L. casei* factor has been reported to be the same as vitamin B_c[6] which had previously been isolated from liver and yeast.[7,8]

The relationship of the liver *L. casei* factor to folic acid[9] has not been definitely established although available evidence indicates they are the same.[10]

The liver *L. casei* factor also appears to be the same as the factor designated as the norite eluate factor.[11]

This communication deals with the degradation of the fermentation compound by alkaline hydrolysis under aerobic and anaerobic conditions and by acid hydrolysis.

In experiments on the degradation of the *L. casei* factor from liver and fermentation sources, it was soon observed that hydrolysis with acid or alkali led to the formation of a diazotizable aromatic amine which could be estimated by the method of Bratton and Marshall.[12] The liberation of amine was most rapid in alkaline solutions and it was found that oxygen had a marked effect on the course of the reaction. When the fermentation *L. casei* factor was heated with 1.0 *N* sodium hydroxide at 100° in the presence of oxygen there was a marked change in the absorption spectrum, a diazotizable aromatic amine was produced, a fluorescent pigment was formed and rapid biological inactivation occurred. The *L. casei* factor before hydrolysis has three absorption maxima in 0.1 *N* sodium hydroxide at 257, 282 and 365 mμ. After aerobic alkaline hydrolysis it has two maxima at 263 and 365 mμ.

When the hydrolysis was carried out anaerobically with 1.0 *N* sodium hydroxide at 100° there

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