FACTORS AFFECTING THE RATE OF GROWTH OF 
LACTOBACILLUS CASEI

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It has been recognized for some time that Lactobacillus casei develops 
slowly in completely synthetic or semisynthetic media which contain all of 
the absolutely essential vitamins and amino acids. Usually little or no 
growth appears during the first 16 to 24 hours of incubation. Under some 
conditions the addition of asparagine, glutamine, glutamic acid (1), or an 
unknown factor or factors in peptone (2), or a combination of glutamine, 
p-aminobenzoic acid, and pyridoxal (3) will speed up the rate of growth of 
L. casei. Trypsinized preparations of casein and other purified proteins, 
the active principle of which has been named strepogenin (4, 5), also will 
reduce the lag phase of L. casei. Certain synthetic peptides of glutamic 
acid, for example serylglycylglutamic acid, have some strepogenin activity 
(6). Also, evidence has been presented that for optimum growth of L. 
casei during the first 16 hours of incubation glutathione and a factor asso-
ciated with animal products, as well as strepogenin, must be supplied to 
the organism (7). The present paper reports some additional observations 
on the relation of strepogenin and certain other factors to the rate of 
growth of L. casei. The term strepogenin is used to describe the activity 
of trypsinized casein and that of various purified proteins and natural ma-
terials when compared to trypsinized casein as the standard.

Methods

Experiments designed to investigate rate phenomena are technically diffi-
cult to carry out with consistent results, since apparently minor variations 
in the inoculum culture medium and technique may profoundly influence 
the rate of growth (7). In order to obtain reasonably consistent results it 
is necessary to use carefully standardized procedures. Therefore, experi-
mental details are described more fully than would normally be necessary.

Stab cultures of Lactobacillus casei were carried in a medium consisting 
of peptone 0.5 per cent, glucose 1.0 per cent, agar 1.5 per cent, and 0.5 ml. 
each of Salts A and B per 100 ml. of medium (8). Each week a subculture 
was made from a stab culture into 5 ml. of broth of the same composition

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contained in a 15 × 125 mm. tube. The subculture was incubated at 37° for 24 hours. Subsequently, broth to broth transfers were made daily for the remainder of the week. To prepare inoculum, a 24 hour broth culture was centrifuged, and the sedimented cells were washed twice with 5 ml. of sterile distilled water and resuspended in 10 ml. of water. This suspension was diluted with water to give a reading of approximately 95 on the galvanometer of the Evelyn photoelectric colorimeter fitted with a 520 μm filter.

The medium listed in Table I was used for the experiments. Materials to be tested, after appropriate treatment and dilution, were placed in chromic acid-cleaned colorimeter tubes and made up to a volume of 5 ml. with water. 5 ml. of medium were added to each tube, and the tubes were closed with plugs of non-absorbent cotton and autoclaved for 11 minutes at 115°. Higher sterilization temperatures and, to a lesser extent, longer periods of sterilization produced a brown discoloration of the medium which interfered with subsequent turbidimetric readings.

After sterilization, each tube was inoculated with 1 drop of the inoculum suspension described above. It is necessary to use pipettes which will deliver drops of uniform size. 1 ml. Exax pipettes graduated in 0.01 ml. were satisfactory for delivery of the inoculum. Care was taken to allow the drop of inoculum to fall directly into the medium rather than on the walls of the tube. The inoculated tubes were incubated at 37° for 17 hours prior to determining their turbidities, or until a control tube containing 3 mg. of trypsinized casein gave a galvanometer deflection of 40 to 50 in the Evelyn colorimeter with a 520 μm filter. Additional details of procedure will be mentioned later.

### Table I

<table>
<thead>
<tr>
<th>Basal Medium</th>
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<tbody>
<tr>
<td>Acid-hydrolyzed casein</td>
</tr>
<tr>
<td>DL-Tryptophan</td>
</tr>
<tr>
<td>L-Cystine</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Na acetate (anhydrous)</td>
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<tr>
<td>Adenine</td>
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<tr>
<td>Guanine</td>
</tr>
<tr>
<td>Uricil</td>
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<tr>
<td>Pantothenic acid</td>
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<tr>
<td>Riboflavin</td>
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<tr>
<td>Thiamine HCl</td>
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<tr>
<td>Nicotinic acid</td>
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<tr>
<td>Pyridoxamine</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
</tr>
<tr>
<td>Biotin</td>
</tr>
<tr>
<td>Folic acid</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
</tr>
<tr>
<td>L-Asparagine</td>
</tr>
<tr>
<td>Salts A</td>
</tr>
<tr>
<td>K₂HPO₄</td>
</tr>
<tr>
<td>KH₂PO₄</td>
</tr>
<tr>
<td>Salts B</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
</tr>
<tr>
<td>Adjust to pH 6.8</td>
</tr>
<tr>
<td>Add distilled H₂O to 250.0 ml.</td>
</tr>
</tbody>
</table>
Purified proteins and natural materials were trypsinized according to the procedure of Sprince and Woolley (5). Difco trypsin 1:110 was used. The trypsinized casein standard was prepared from S. M. A. vitamin-free casein. To prepare standard curves of the trypsinized casein, the required amount of the lyophilized material was dissolved in water to give a concentration of 1 mg. per ml. This solution could be stored in the refrigerator under toluene for at least 2 weeks without loss of activity.

The hydrolyzed casein used in the basal medium was a sulfuric acid hydrolysate of S. M. A. vitamin-free casein from which the sulfate was removed with barium hydroxide.

**EXPERIMENTAL**

**Inoculum**—The rate of growth of *Lactobacillus casei* under the conditions outlined above is markedly increased by strepogenin and is proportional to strepogenin concentration, generally, within the range of 0 to 1 mg. of casein (Fig. 1). With increase in incubation beyond 16 hours, growth increases in all tubes, including the one with no strepogenin; at 42 hours the stimulatory effect of strepogenin is hardly evident.

Growth response of *Lactobacillus casei* to strepogenin is decreased somewhat if the cells for the inoculum are taken from a 2 day-old broth culture in place of the customary 1 day culture. Cells from 3 day-old broth cultures are only slightly stimulated by strepogenin at 16 hours incubation. Also, growth is proportional to the number of cells in the inoculum. A decrease in the number of cells to one-tenth that normally used decreases the response to strepogenin markedly, whereas a 10-fold increase produces much greater growth of *L. casei* in all tubes. The resulting curves resemble closely those obtained by varying the time of incubation (Fig. 1). Inocula grown in broth enriched with 0.25 mg. of L-asparagine per ml. and in broth in which Wilson's solubilized liver fraction L was substituted for the peptone were stimulated by strepogenin to the same degree as cells taken from the usual peptone medium.

**Activity of Purified Proteins and Natural Materials**—Sprince and Woolley have used solubilized liver extract (Wilson's fraction L) as the standard in measuring the strepogenin activity of trypsinized proteins (4, 5). Comparison of solubilized liver with trypsinized casein indicates that, within the range of 0 to 1 mg. of each, casein is about 4 times as potent as liver extract (Fig. 2). However, if both substances are increased to above 3 mg., there is a change in relative potency. The casein growth curve decreases markedly in slope at the 3 mg. level, whereas that of liver extract does not decrease until the 10 mg. level is reached. Consequently, the two curves cross, and above the 3 mg. level liver extract is much more stimulatory than casein. Acid production was used as the criterion for growth
because the color of the larger amounts of liver extract added interferes with turbidimetric readings. The liver extract may contain a single stimulatory substance which is different from strepogenin or possibly a number of stimulatory substances, one of which may be strepogenin. The greater

![Graph](http://www.jbc.org/)

**Fig. 1.** Response of *Lactobacillus casei* to strepogenin as a function of time

The effect of liver extract as compared to casein does not appear to be due to glutamine, inositol, choline, nicotinamide, thymine, lecithin, oleic acid, cozymase, cocarboxylase, malic acid, succinic acid, fumaric acid, or excess B vitamins (10 times the quantities given in Table I), since addition of 10 mg. each of those substances to tubes containing 2.5 mg. of trypsinized
casein did not appreciably increase growth above that obtained with the casein alone. Other trypsinized natural materials such as yeast extract, tankage, peptone, and distillers' solubles as well as the trypsinized purified proteins, soy bean glycenn, trypsinogen, tobacco mosaic virus, and insulin, when used in amounts of 5 to 50 mg. per tube, failed to give as much growth as liver extract.

![Fig. 2. Growth of *Lactobacillus casei* with liver extract and casein](image1)

![Fig. 3. Inhibition of growth of *Lactobacillus casei* by asparagine](image2)

**Inhibition by Asparagine**—Asparagine consistently inhibited growth. However, the addition of as little as 0.05 mg. of trypsinized casein is sufficient to overcome the asparagine inhibition. The inhibitory effect of asparagine is illustrated in Fig. 3. At 17 hours, maximum inhibition occurred with 0.05 mg. of asparagine per tube, whereas at 24 hours 0.25 mg. of asparagine was required for maximum inhibition. Substitution of amino acids for the hydrolyzed casein in the basal medium (9) did not alter the inhibition by asparagine. In the completely synthetic medium the streptogenin curves were steeper and the blank tubes had consistently less growth.
than in the hydrolyzed casein medium. Glutamine, when substituted for asparagine, was not inhibitory, and tended to neutralize the inhibitory effect of asparagine.

Adaptations of Lactobacillus casei—An attempt was made to "train" Lactobacillus casei to accelerate its rate of synthesis of strepogenin and thus eliminate its requirement for added strepogenin. Serial subcultures were made daily by loop transfer in the basal medium (Table I) in the absence of added strepogenin. Growth was allowed to proceed for 24 hours prior to subculture in order to permit appreciable synthesis of strepogenin to occur, and turbidity readings were made after 16 hours to determine the degree of acceleration of strepogenin synthesis. Tubes inoculated with the usual peptone broth inoculum were included in this experiment as controls. After only five serial transfers, good growth was obtained in the strepogenin-free basal medium within 16 hours. Fig. 4 shows that the accelerated

![Graph showing growth characteristics of Lactobacillus casei](image)

**Fig. 4.** Growth characteristics of Lactobacillus casei serially subcultured in the absence of strepogenin.
culture without added strepogenin grows even more rapidly than the original untrained culture supplied with 3 mg. of trypsinized casein. Interestingly, if asparagine is omitted from the basal medium, the accelerated strain again requires trypsinized casein for rapid growth to the same degree as the original culture. Approximately 0.4 mg. of asparagine per 10 ml. of medium is necessary for abundant growth of the adapted strain. Glutamine can replace asparagine but approximately 100 times more of glutamine than asparagine must be used. These findings again indicate a close metabolic relationship between asparagine and strepogenin.

![Graph](http://www.jbc.org/download)

**Fig. 5.** Growth characteristics of *Lactobacillus casei* serially subcultured in the absence of asparagine and strepogenin.

By using the same technique as described above, except that a medium without strepogenin or asparagine was employed, it was possible to “train” *Lactobacillus casei* to grow fairly well within 16 hours without added asparagine or strepogenin (Fig. 5). However, in sharp contrast to the results with the previously mentioned adapted strain, if asparagine is added to the basal medium, the second adapted strain again requires trypsinized casein for rapid growth, as does the original culture. Thus, the inhibitory effect of asparagine for the original strain has been enhanced.

**Inactivation and Reactivation of Streptogenin**—Trypsinized casein which has been refluxed with hydrochloric acid at pH 1 (glass electrode) for 24 hours is completely inactive in asparagine-free medium, but is fully active
when asparagine is supplied (Fig. 6). Quantitatively, 0.75 mg. to 1.0 mg. of L-asparagine per 10 ml. of medium is required for full restoration of the activity of 3 mg. of acid-inactivated strepogenin. Glutamine is also effective in this respect but approximately 100 times more of glutamine than asparagine is required. Biotin, glutathione, pyridoxal, β-alanine, and aspartic acid are ineffective in restoring activity. Asparagine also restores the activity of acid-refluxed strepogenin in the synthetic amino acid medium (9). Results similar to those with casein were obtained with acid-refluxed solubilized liver extract (Wilson's fraction L).

Trypsinized casein, which is kept at pH 1 at room temperature for 20 hours, shows no loss of activity. Refluxing of trypsinized casein at pH 10 results in complete loss of strepogenin activity, and, in contrast to the results at pH 1, the activity cannot be restored by asparagine. Refluxing of casein and liver extract at pH 7 results in partial loss of activity, which is restored by asparagine.

The adapted Lactobacillus casei culture, which can grow readily without strepogenin but requires asparagine, will not grow when the acid-refluxed casein is substituted for asparagine. This fact plus the fact that the normal L. casei strain requires asparagine for rapid growth with acid-treated casein would appear to indicate that the acid inactivation of trypsinized casein is
due to hydrolysis of the amide group of asparagine, or of other amides which may perform a similar function.

These data emphasize further the close relationship between asparagine and strepogenin. The elucidation of its nature must await the isolation of strepogenin in pure form or at least free of asparagine.

SUMMARY

The effects of size, age, and growth medium on the response of the *Lactobacillus casei* inoculum to strepogenin are described. Trypsinized casein is about 4 times as potent as Wilson's liver fraction L in stimulating growth of *L. casei* when tested within the range of 0 to 1 mg. of each substance per 10 ml. of medium. However, at higher levels the liver fraction consistently supports more rapid growth than casein. Other trypsinized materials did not support as much growth as liver extract. Under the conditions employed, the growth of *L. casei* is markedly inhibited by asparagine, whereas glutamine is not inhibitory.

It was possible by serial subculture in strepogenin-free medium to adapt the strepogenin-requiring strain of *Lactobacillus casei* to grow rapidly without strepogenin. However, it is necessary to add asparagine to the medium for rapid growth of this adapted strain. It was also possible to adapt *L. casei* to grow rapidly without either strepogenin or asparagine. For this second adapted strain asparagine is inhibitory, and the inhibitory effect can be overcome by the addition of trypsinized casein to the medium.

Trypsinized casein which has been refluxed at pH 1 for 24 hours is completely inactive in asparagine-free medium, but is fully active when asparagine is added. Similar results were obtained with acid-refluxed Wilson's liver fraction L. Glutamine, in 100 times the quantity of asparagine, will also restore the strepogenin activity of acid-refluxed casein.

The data presented indicate strongly a close metabolic relationship between strepogenin and asparagine.

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