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Growth Stimulating Substances for *Lactobacillus Casei*[1,2,3]

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In the course of an investigation into the possibility of extending the microbiological pantothenic acid assay of Snell, Strong and Peterson[4] to biological materials in general, it was observed that the basal medium which they used was grossly deficient in some substance or substances capable of stimulating the bacterial growth. Although supplementing the medium with a yeast preparation made possible the development of a widely applicable assay method,[5] variations in the effectiveness of successive batches of the supplement necessitated a further study of the growth stimulants present.

Investigation of their chemical nature was at first hampered by the lack of a convenient test because in the growth trials as previously conducted[4,5] the effect of the stimulants was screened by the response from pantothenic acid itself. It was found, however, that if the seventy-two-hour incubation period was shortened to twenty-four hours, little growth occurred on the unsupplemented medium even in the presence of excess pantothenic acid, whereas the addition of the stimulants resulted in greatly increased growth. With the aid of this test a number of substances were discovered which markedly influence the growth of the test organism. The effect of these compounds on the microbiological assays for pantothenic acid[5] and riboflavin[6] was then investigated.

Experimental

Basal Medium and Yeast Supplements.—The basal medium used throughout was that previously described.[4,7] It contained 0.5% alkali-treated peptone, 0.6% sodium acetate, 1% glucose, 0.01% cystine, inorganic salts, and 1γ riboflavin per 10 cc. Except where otherwise indicated, 5γ of synthetic *d*-calcium pantothenate was added to each 10 cc. of this medium for all of the twenty-four-hour growth tests.

The preparation of the lead acetate-precipitated yeast extract, and of the *norited* yeast supplement has been previously described.[5,6]

Bacteriological Technique.—The procedure for handling the bacterial cultures and carrying out the seventy-two-hour fermentations has been explained previously.[5]

In general the same technique was also followed for the twenty-four-hour assays. The diameter of the culture tubes was increased from 14 to 18 mm., because more consistent results were obtained with the wider tubes during the early stages of growth. The tubes were shaken well after inoculation but not during the growth period. The bacterial response was determined either by titration of the acid produced, or by turbidity measurements on an Evelyn photoelectric colorimeter fitted with a 540 mμ filter.[6] For this purpose each tube containing the sterilized and inoculated medium was placed in a special adapter sleeve in the colorimeter, the instrument adjusted to a galvanometer reading of 100, the tube removed, and the resulting galvanometer deflection recorded. This served as a blank reading for each individual tube. To determine the turbidity in an incubated tube, the galvanometer was adjusted to the proper blank value, the tube inserted, and the deflection recorded. This procedure, in which each tube served as its own blank, made it possible to determine growth by turbidity even in the presence of colored materials, and without the use of special tubes. Each tube, however, was marked on one side, and always placed in the instrument in the same relative position.

The twenty-four-hour growth experiments were difficult to reproduce because the bacteria did not always begin to grow at an equally long interval after inoculation. The absolute values for the titrations or turbidity measurements were consequently shifted but the relative results, as deduced from any one experiment, were consistent and reproducible. During the latter part of the work asparagine, glutamic acid, adenine, guanine, and norit eluate factor concentrate[8] were added to the medium in which the inoculum was grown, *i. e.*, the basal plus excess pantothenic acid. The use of this inoculum appeared to minimize the above variations.

Testing of Known Compounds and Mixtures.—Nearly all materials were tested in the form of neutral, aqueous solutions. In the case of a few substances which were soluble only in acid solution, allowance was made for the small amount of acid thereby introduced.

Mixture A was an aqueous solution which contained in each cc. 0.8γ cozymase, 0.004γ biotin, 40γ norit eluate factor concentrate, 60γ vitamin B_6, 60γ vitamin B_1, 4γ cocarboxylase, 360γ riboflavin, 22γ *d*-calcium pantothenate, 40γ muscle adenylic acid, 40γ indoleacetic acid, 400γ pimelic acid, 200γ uracil, 1400γ choline chloride, 300γ nicotinamide, 200γ *i*-inositol, 200γ cholic acid, 40γ

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(3) Presented in part at the Chicago meeting of the American Society of Biological Chemists, April, 1941. Original manuscript received July 17, 1941.

(4) Snell, Strong and Peterson, *J. Bact.*, **38**, 293 (1939).

(5) Strong, Feeney and Earle, *Ind. Eng. Chem., Anal. Ed.*, **13**, 566 (1941).

(6) Snell and Strong, *ibid.*, **11**, 346 (1939).

(7) Snell, Strong and Peterson, *Biochem. J.*, **31**, 1789 (1937).

(8) Snell and Peterson, *J. Bact.*, **39**, 273 (1940). The concentrate of norit eluate factor was kindly supplied by Mr. B. L. Hutchings. The potency of the preparation was such that 0.3γ gave half maximum bacterial response.

taurocholic acid, and 800γ *l*-asparagine monohydrate. It was tested at a level of 1 cc. per 10 cc. of medium.

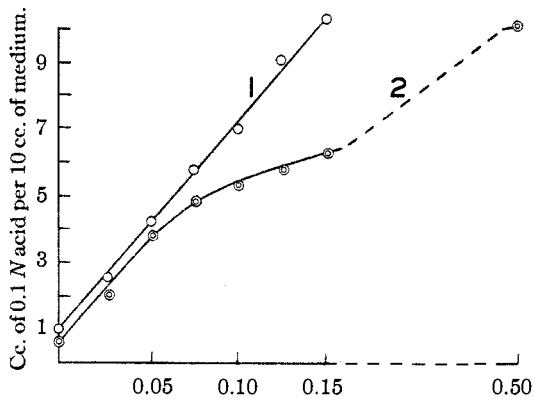

Fig. 1.—Standard curves for pantothenic acid assay: Curve 1, basal medium plus norited yeast supplement equivalent to 25 mg. of yeast extract per 10 cc. of medium; Curve 2, basal medium alone. The cultures were titrated after seventy-two hours of incubation.

Mixture B was a similar solution which contained in each cc. 40γ *l*-histidine, 1000γ *l*-tryptophan, 630γ *d,l*-isoleucine, 760γ *l*-arginine hydrochloride, 670γ *l*-tyrosine, 820γ *l*-proline, 1000γ *l*-valine, 780γ *d,l*-leucine, 880γ *l*-lysine hydrochloride, 2 mg. *d,l*-alanine, 1 mg. glucosamine, 200γ indoleacetic acid, 330γ sarcosine, 1150γ creatine, 310γ creatinine, 500γ pimelic acid, 1 mg. nicotinamide, 100γ thiamin, 100γ pyridoxin, 2γ *p*-aminobenzoic acid, 210γ yeast adenyl-thiomethyl pentose,[9] and 900γ of a yeast nucleic acid preparation (Merck). This solution was tested at a level of 1 cc. per 10 cc. of medium.

A further mixture containing 1 mg. each of *l*-methionine, *i*-inositol, and choline chloride per cc. was tested separately, but was inactive.

Since heating caused rapid inactivation, glutamine solutions were sterilized for the bacterial tests by filtration, and were added to the tubes after they were autoclaved. Natural glutamine was used.

Asparagine.—Natural *l*-asparagine was converted to *l*-aspartic acid by hydrolysis with 10% hydrochloric acid.[10] The product, which caused no stimulation in the bacterial tests, was reconverted to *l*-asparagine over the diamide of aspartic acid.[11] From 11 g. of aspartic acid, 5.2 g. of the crude diamide was obtained and 1.6 g. of this product yielded 1.06 g. of *l*-asparagine monohydrate, $[\alpha]^{22}D$ −5.4°, (*c* 2.16% in water). Equivalent weight by Linderstrøm-Lang titration of the amino group,[12] 150.2, calcd., 150.1. Both the natural and resynthesized asparagine were converted into the corresponding urea derivative by reaction with α-naphthyl isocyanate. Each preparation melted at 200–201°, as did a mixture of the two.

(9) Levene and Sobotka, *J. Biol. Chem.*, **65**, 551 (1925). This sample was obtained from Dr. Levene, and was made available to the authors through the courtesy of Professor C. A. Elvehjem.

(10) Schiff, *Ber.*, **17**, 2929 (1884).

(11) Fischer and Koenig, *ibid.*, **37**, 4585 (1904).

(12) Linderstrøm-Lang, *Compt. rend. trav. lab., Carlsberg, Ser. chim.*, **17**, 4 (1927).

Results

The inadequacy of the basal medium is revealed by the curves in Fig. 1. The norited yeast supplement was essentially free from pantothenic acid but contained some substance or substances which permitted increased acid production at low levels of added pantothenic acid (0.10–0.15γ per 10 cc. of medium). That these substances were merely stimulatory for the organism was indicated by the response on the unsupplemented basal medium in the presence of excess pantothenic acid.

Supplementing the basal medium with an excess of riboflavin, pyridoxin, nicotinic acid, biotin, tryptophan, cystine, alkali-treated peptone or norit eluate factor produced no beneficial effect at all comparable to that of the yeast preparation. These materials have been shown to be required by *L. casei* or to contain essential growth factors. Doubling the concentration of all the ingredients of the basal medium and adding reduced iron were also ineffective.

Shortly after the above growth tests, which were carried out with the seventy-two-hour incubation procedure, were finished, the twenty-four-hour procedure was developed. With the aid of this test some of the properties of the active material in the norited yeast supplement were studied, and a few preliminary attempts at purification were made. At this time a mixture of known compounds was tested (mixture A, Table I) and found to be fairly effective. The active substance in the mixture proved to be *l*-asparagine. Synthetic asparagine was as effective as the natural material. It was then found that glutamine and glutamic acid were also effective, and that a combination of glutamic acid with asparagine showed enhanced activity (B5, Table I). Since the response was not as great as that obtained with whole yeast extract (B7, Table I), a large number of additional compounds (mixture B) was tested, both alone and with the above stimulants. As a result, three additional substances were found which exerted a stimulating action, *viz.*, adenine, guanine, and the norit eluate factor. Although the last had already been tried, its effect was missed because of the absence of asparagine and glutamic acid. The purines were tried because of an effect produced by hydrolyzed nucleic acid and adenyl-thiomethyl pentose.

Many tests were carried out in order to decide which of the individual compounds was effective,

TABLE I

EFFECT OF VARIOUS SUBSTANCES ON ACID PRODUCTION BY *Lactobacillus casei* DURING 24-HOUR INCUBATION[a]

Expt.[b]	Additions per 10 cc. of medium[c]	Titration values,[d] cc. Actual	Titration values,[d] cc. Increase over blank
A1	None	2.8	...
A2	1 cc. Mixture A	5.3	2.5
B1	None	3.8	...
B2	5 mg. asparagine	6.5	2.7
B3	5 mg. glutamine	5.6	1.8
B4	5 mg. glutamic acid	5.1	1.3
B5	No. B2 plus B4	7.3	3.5
B6	5 mg. yeast extract (Difco)	6.0	2.2
B7	15 mg. yeast extract (Difco)	9.3	5.5
C1	None	2.3	...
C2	1 cc. mixture B	3.3	1.0
C3	No. C2 plus 5 mg. each of asparagine, glutamine and glutamic acid	9.5	7.2
D1	None	3.8	...
D2	5 mg. asparagine plus 5 mg. glutamic acid	7.3	3.5
D3	100γ adenine, 100γ guanine, 1γ norit eluate factor concentrate	5.1	1.3
D4	No. D2 plus No. D3	9.3	5.5

[a] The exact time of incubation varied from 22 to 25 hours in different experiments. [b] The tests grouped under any one letter (A, B, etc.) were all performed at the same time, and are therefore strictly comparable to others in the same group. [c] The medium used was the basal medium plus 0.5γ *d*-calcium pantothenate per cc. [d] The volume of 0.1 *N* acid produced per 10 cc. of medium.

how much of each was needed, and what combination of stimulants gave the greatest response. When the purines, norit eluate factor, glutamic acid and asparagine were all added to the basal medium (plus excess pantothenic acid), nearly maximum growth resulted in the twenty-four-hour period (D4, Table I). This mixture of stimulants, therefore, effectively replaced the norited yeast supplement under these particular cultural conditions. Glutamine was also active but did not enhance the effect of this mixture.

Glutamine was effective at the lowest concentration tried, 1γ per 10 cc. of medium giving a detectable response. Larger amounts up to 100–200γ gave progressively greater stimulation. The effect of 1 mg. of glutamic acid was observable, and 5 mg. gave a maximum result. The corresponding values for asparagine were 0.1 and 5 mg., respectively. Adenine and guanine were ineffective at 10γ but completely effective at 100γ.

The following substances in the amounts indicated caused no significant stimulation when tested individually by the twenty-four-hour procedure: 5 mg. α-ketoglutaric acid, 4 mg. succinic acid, 3 mg. β-alanine, 4 mg. succinimide, 2 mg. formamide, 5 mg. urea, 5 mg. ammonium sulfate, and 5 mg. ammonium sulfate plus 4.4 mg. aspartic acid. The latter combination caused a distinct inhibition of growth. A mixture of ammonium sulfate and glutamic acid was no better than the acid alone. The urea derivative obtained from asparagine and α-naphthyl isocyanate was also inert in amounts equivalent to 5 mg. of asparagine.

A comparative study next was made of the effect of glutamine, asparagine, and glutamic acid throughout the twenty-four-hour growth period. The curves in Fig. 2 are based on turbidity measurements made at intervals on the same continuously growing cultures. It is evident that the relative effect of the various supplements varied at different stages of growth, glutamine being distinctive in that its effect was more pronounced early in the growth period. The heaviest growth at the end of twenty-four hours was produced by a combination of asparagine and glutamic acid.

In contrast to the stimulating effect of the above structurally related substances, aspartic acid was strongly inhibitory (Fig. 2). Additional experiments in which turbidity was measured after a twenty-four-hour incubation permitted the following conclusions: (a) the aspartic acid inhibition increased in proportion to the amount added to the basal medium until at a level of 18 mg. per tube practically no growth occurred; (b) aspartic acid nullified the stimulating effect of

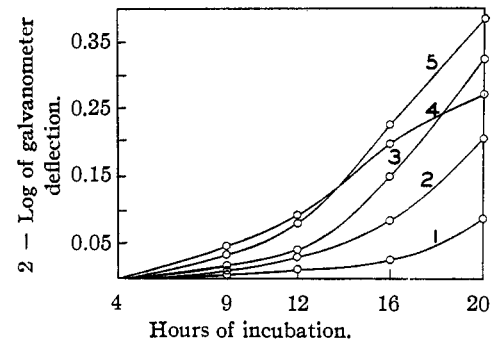

Fig. 2.—Effect of additions to basal medium on growth during twenty hours of incubation: Curve 1, 4.4 mg. of aspartic acid per 10 cc. of medium; 2, no addition; 3, 5.0 mg. of *l*-asparagine monohydrate per 10 cc. of medium; 4, 0.5 mg. of glutamine per 10 cc. of medium; 5, 5.0 mg. of *l*-asparagine monohydrate plus 5.0 mg. of glutamic acid per 10 cc. of medium. The basal medium contained excess pantothenic acid in every case.

asparagine but not that of glutamine or glutamic acid; (c) glutamine was particularly effective in counteracting the inhibition caused by aspartic acid, as little as 1γ per tube giving a very marked response.

The influence of the above substances on the pantothenic acid assay[5] was then studied. It was found that either 5 mg. of glutamic acid, 2.5 mg. of asparagine or 100γ of glutamine was as effective in supplementing the basal medium as a quantity of the norited supplement equivalent to 25 mg. of yeast extract. Adenine and guanine, when added to the complete medium, produced a definite inhibition at a level of 100γ per tube, whereas glutamic acid, uracil, and thymine were neither inhibitory nor stimulatory.

Analogous tests showed that in the riboflavin assay,[6] 100γ levels of adenine and guanine were likewise inhibitory, and that 2.5 mg. of asparagine per tube also produced a definite decrease in the bacterial response. Glutamine, however, caused no inhibition, and aspartic acid, surprisingly, was also inert when tested at a level of 4.4 mg. per tube.

In each case where inhibition was observed the extent of the decrease in growth was such that the presence of the inhibitory substances in the quantities mentioned would cause an error of approximately 20% in the determination of riboflavin or pantothenic acid.

Discussion

Except for one report, the details of which are not at present available,[13] no evidence has previously been presented to show that either glutamine or asparagine possesses growth-promoting properties for any of the lactic acid bacteria. McIlwain[14] reported stimulation of *Streptococcus hemolyticus* by glutamine and glutamic acid. The effective quantities were similar to those found for *L. casei*. A survey of the literature reveals that asparagine, though widely used in bacteriological media, can in practically every case be completely replaced by other substances. Glutamic acid has been found necessary for other lactics by Möller.[15] Snell and Mitchell[16] reported a stimulatory activity of various purines and pyrimidines for related lactic acid bacteria. Since the completion of this work, Stokstad has reported that thymine, adenine, guanine, xanthine, and hypoxanthine influence the growth of *L. casei*.[17]

Aspartic acid was shown strongly to inhibit growth on the basal medium plus excess pantothenic acid and to reduce the stimulation caused by relatively large amounts of asparagine. In contrast, the stimulation by glutamic acid and by small amounts of glutamine was but little affected by rather large amounts of aspartic acid. From these and other observations the following assumptions might be made. (1) Glutamine is the active material and can be synthesized by the organism. (2) Large amounts of glutamic acid increase the synthesis of glutamine. (3) Asparagine in some way replaces glutamine, possibly by donating its amide nitrogen to glutamic acid. (4) Aspartic acid inhibits the replacement of glutamine by asparagine possibly due to its affinity for a common enzyme. (5) Asparagine may have some functions other than glutamine formation.

The effects of these various stimulatory and inhibitory substances on the microbiological assays for riboflavin and pantothenic acid are obtained at such high concentrations that these substances are of no practical importance insofar as their interference with the methods as published is concerned.

Summary

1. Asparagine, glutamine, glutamic acid, adenine and guanine were shown to stimulate the growth of *L. casei* under certain cultural conditions. Glutamine was stimulatory at very low concentrations.

2. Under certain conditions aspartic acid, asparagine, adenine and guanine were inhibitory. The inhibition caused by aspartic acid was counteracted by either asparagine, glutamine or glutamic acid.

3. The relation of these substances to the microbiological assay of pantothenic acid and riboflavin has been investigated.

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(15) Möller, *Z. physiol. Chem.*, **260**, 246 (1939).

(16) Snell and Mitchell, *Proc. Nat. Acad. Sci., U. S.*, **27**, 1 (1941).

(17) Stokstad, *J. Biol. Chem.*, **139**, 475 (1941).