FACTORS NECESSARY FOR MAXIMUM GROWTH OF CLOSTRIDIUM BIFERMENTANS

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The nutritional requirements of some members of the genus *Clostridium* are, as yet, incompletely known. Not only is maximum growth in some species, such as *Clostridium parabotulinum* (Elberg and Meyer, 1939), dependent upon some unidentified substance, but in others, such as *Clostridium tetani* (Mueller and Miller, 1948) and *Clostridium perfringens* (Adams, Hendee, and Pappenheimer, 1947), high yields of toxin or exocellular enzymes are produced only in the presence of unknown substances, although luxuriant growth can be obtained in their absence.

The results of the present study indicate that in addition to biotin, nicotinic acid, pantothenic acid, and pyridoxamine, some unidentified material is necessary for the maximum growth of *Clostridium bifermentans* in a synthetic medium.

MATERIALS AND METHODS

The strains of *C. bifermentans* used in this study were obtained (Smith and George, 1946) from cases of clostridial myositis. All of these organisms were gram-positive rods, were motile, had central to subterminal oval spores, were obligately anaerobic, liquefied gelatin, digested and blackened iron milk, produced indole, gave a negative test for "vanillin violet," and fermented glucose and maltose but not lactose, sucrose, or salicin. Only strain 6 did not produce lecithinase.

In the study of growth factor requirements, spore suspensions were used for inocula rather than suspensions of vegetative cells. The greater resistance of the spores made it possible to wash them repeatedly to free them of the medium in which they had been grown. Such spore suspensions were prepared by inoculating brain-heart infusion broth (Difco) containing 1 per cent tryptone, incubating at 35 C for 3 to 7 days, heating at 65 C for 15 minutes, and centrifuging. The sedimented spores were washed 4 times with M/60 phosphate buffer, pH 6.9. The spores were finally suspended in a quantity of phosphate buffer equal to the volume of broth in which they had been grown. Five-hundredths ml of spore suspension were used to inoculate each 5-ml tube of experimental medium.

In experiments in which vitamin-free casein hydrolyzate (General Biochemicals, Inc.) was used as a nitrogen source, it was used at a concentration of 0.5 per cent. To this medium were added 0.5 per cent K_2HPO_4, 0.02 per cent of a salt mixture ($MgSO_4 \cdot 7H_2O$, 4 g; NaCl, 0.2 g; $MnSO_4 \cdot 4H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.2 g), and 0.01 per cent tryptophan; the pH was adjusted to 7.5. Glucose was autoclaved separately as a 10 per cent solution in distilled water and was added

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in this form to the sterile medium to give a final concentration of 1 per cent glucose.

When amino acids were used as the nitrogen source, the following amounts were used per 100 ml of medium: L-tryptophan, 0.02 g; L-cystine, 0.002 g; L-glycine, 0.002 g; DL-alanine, 0.01 g; DL-leucine, 0.001 g; DL-isoleucine, 0.05 g; DL-serine, 0.08 g; DL-phenylalanine, 0.02 g; DL-lysine, 0.03 g; DL-methionine, 0.002 g; L-glutamic acid, 0.15 g; DL-valine, 0.04 g; DL-aspartic acid, 0.01 g; L-arginine, 0.01 g; L-histidine·HCl, 0.01 g; L-tyrosine, 0.03 g; and L-proline, 0.04 g.

The other constituents of the medium were the same as those used for the "vitamin-free" casein hydrolyzate. An amino acid mixture similar to the one utilized by Boyd, Logan, and Tytell (1948) in their study of the growth requirements of *C. perfringens* was also used in certain experiments.

Before tubes of medium were inoculated, they were heated for 10 minutes in a bath of boiling water and cooled to room temperature. They were then inoculated and incubated in Brewer anaerobic jars in an atmosphere of 95 per cent hydrogen and 5 per cent carbon dioxide. Turbidity determinations were made in a modified Lange photoelectric colorimeter, with 1-cm cuvettes, and the results are expressed in percentage of light absorption.

The quantitative representation of the growth of *C. bifermentans* is complicated by the fact that growth in an unbuffered medium is limited by the production of acid and that growth in a buffered medium is soon followed by spore formation and autolysis of the vegetative portion of the cells with a consequent decrease in turbidity; and, consequently, a constant level of turbidity is not attained.

Treatment of tryptic digests with chloramine-T (Van Slyke *et al.*, 1941) to inactivate *alpha*-amino acids was done by adding 3 ml of 10 per cent chloramine-T in water to 0.5 ml of 0.5 M acetate buffer, pH 4.12, and 3 ml of tryptic digest solution containing 30 mg of digested protein. The mixture was incubated at 40 C for 15 minutes, and 2.0 ml of 10 per cent ammonium chloride solution were added to inactivate the excess chloramine-T. This mixture was allowed to stand at room temperature for several hours and was then filtered and heated to boiling for 10 minutes.

EXPERIMENTAL RESULTS

The results of preliminary experiments indicated that the strains of *C. bifermentans* could be grown in a vitamin-free casein hydrolyzate glucose medium fortified with biotin, nicotinamide, pantothenic acid, and pyridoxamine. The results of a "drop-out" experiment, summarized in table 1, indicate that the maximum growth of all strains was dependent upon nicotinamide and pantothenate, that the growth of 5 of the 12 strains was independent of added biotin, and that the growth of 3 of the strains was independent of added pyridoxamine. Pyridoxal could be substituted for pyridoxamine. Similar results were obtained in a "drop-out" experiment using pyridoxine in place of pyridoxamine except that the stimulation brought about by pyridoxamine was more marked than that brought about by pyridoxine. Nicotinic acid could be substituted for nicotinamide.

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The growth of the biotin-independent strains was not inhibited by avidin, in the form of 1 per cent raw egg white, but the growth of the biotin-dependent strains was. Sodium oleate, in a concentration of 1 μg per ml could not be substituted for biotin for strain 14.

When these experiments were repeated with various mixtures of amino acids in place of the "vitamin-free" casein hydrolyzate as the nitrogen source, little growth was obtained. This small amount of growth could be increased somewhat by doubling the concentration of amino acids or by using the amino acid mixture of Boyd, Logan, and Tytell (1948). The growth was barely perceptible with most strains, although there was variation from one strain to another with regard to the amount of growth obtained. In all cases, however, the growth was markedly increased by the addition of a small amount (0.01 to 0.05 per cent) of peptone.

TABLE 1

Effect of omission of growth factors

STRAIN	FACTOR OMITTED					
	All	Biotin	Nicotinamide	Pantothenate	Pyridoxamine	None
1	6.0*	12.8	8.8	3.2	19.3	21.9
2	5.2	5.1	16.1	4.7	22.3	45.0
5	6.4	31.1	16.0	12.8	25.1	40.3
7	1.8	53.2	18.7	0	26.0	51.2
11	1.8	32.6	16.6	3.4	30.2	24.0
13	1.4	6.1	15.0	1.0	28.0	44.0
14	6.7	15.0	14.9	10.9	15.0	63.1
16	5.0	6.5	11.3	5.8	18.8	38.2
26	5.0	50.3	9.2	6.7	13.2	35.8
27	6.0	52.0	11.0	6.3	10.6	36.4
28	3.4	22.9	2.1	0	10.0	15.1
32	0	6.0	4.5	0.3	26.7	22.9

* Turbidity expressed in percentage of light absorption.

The following experiments were conducted with strain 14, which did not grow appreciably in the amino acid medium to which peptone had not been added.

Since growth was obtained in the casein hydrolyzate medium, but not in the amino acid medium, it appeared that the former contained some substance necessary for the optimal growth of *C. bifermentans*. The effect of this unknown substance or substances was not duplicated by that of adenine, guanine, xanthine, uracil, choline, riboflavin, *para*-aminobenzoic acid, thiamine, sodium oleate, nicotinamide, calcium pantothenate, pyridoxamine (each in a concentration of 0.5 μg per ml), folic acid (0.05 μg per ml), vitamin B_{12} (0.002 μg per ml), and biotin (0.0005 μg per ml) either singly or together. When these factors were used together, no growth was obtained even when the medium was further enriched by the addition of 100 μg per ml of threonine, asparagine, inositol, glutamine, glucosamine, or yeast nucleic acid, or by 0.25 per cent sodium acetate, or by 10 μg per ml of glutathione.

A number of substances commonly used in microbiological media were then

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added to the basal medium in a concentration of 0.01 per cent. The results, given in table 2, indicate that the only substances that were active in promoting growth were those that resulted from the breakdown of protein, with the exception of human plasma. This specimen had been obtained from plasma kept sterilely in the cold for several months. A freshly obtained specimen did not support appreciable growth.

Various natural materials were suspended in 10 times their weight of water and autoclaved, the supernatant fluids were then added to separate tubes of amino acid medium in a concentration of 1 per cent. Extracts of liver, kidney, fish muscle, lettuce, carrot, and banana were without activity. Growth was stimulated, however, by extracts of whole egg or yeast.

TABLE 2

Effect of various ingredients of culture media on the growth of C. bifermentans in amino acid medium

SUBSTANCE ADDED	TURBIDITY
None	0.0
Dried skim milk	0.5
Witte peptone	1.5
Meat extract	0.8
Egg albumen (crystalline)	1.2
Gelatin	0.8
Powdered liver (after aqueous extraction)	0.8
Malt extract	1.0
Casein	1.9
Proteose peptone	17.4
Neopeptone	15.6
Peptone (Difco)	15.8
Tryptone	24.6
Brain-heart infusion broth (Difco)	24.8
Human plasma (1 per cent)	26.4
Yeast extract	19.9
Peptonized milk (Difco)	10.7

Since most of the peptones stimulated the growth of *C. bifermentans*, it seemed worth while to test the hypothesis that the unknown factor or factors were a protein degradation product. To 50 ml of 1 per cent solution of casein (Baker, cp) were added 50 mg of trypsin (Difco). This was thoroughly mixed, and a specimen to serve as a "trypsin-casein" control was immediately removed and heated in a boiling water bath for 10 minutes. The remainder of the mixture was adjusted to pH 8.5 with N NaOH and was incubated at 35 C for 20 hours in the presence of chloroform to inhibit bacterial growth. From time to time the pH was readjusted to 8.5. The digest was sterilized by autoclaving, and measured amounts were added to tubes of the amino acid medium. Tryptone and "vitamin-free" casein hydrolyzate were included in this experiment as positive controls. The results, shown in table 3, indicate that the trypsin and the casein had no

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growth-promoting activity in the concentrations used, that the "vitamin-free" casein hydrolyzate possessed appreciable activity, and that the digestion of casein by trypsin gave rise to material that was quite active in promoting growth.

Thrice-crystallized egg albumen and purified pigskin gelatin were also digested with trypsin, and the products of these digestions were likewise active in stimulating growth, as the data in table 4 indicate. Although it seemed unlikely that an amino acid freed from the proteins by the action of trypsin could be responsible for the stimulation of growth, it appeared that further evidence on this point might be obtained by treating the digested albumen with chloramine-T. Such

TABLE 3

Effect of tryptic digests of casein on the growth of C. bifermentans in an amino acid medium

SUBSTANCE ADDED	TURBIDITY
None	2.3
Trypsin-casein control (50 μg/ml)	2.3
Trypsin-casein control (100 μg/ml)	2.7
Trypsin-digested casein (50 μg/ml)	6.1
Trypsin-digested casein (100 μg/ml)	15.6
"Vitamin-free" casein hydrolyzate (200 μg/ml)	15.6
Tryptone (50 μg/ml)	14.9
Tryptone (100 μg/ml)	22.0

treatment was found by Van Slyke *et al.* (1941) to result in the conversion of *alpha*-amino acids into nitriles, but to affect peptides only slightly, if at all. As the data in table 4 show, the ability to stimulate growth was not greatly affected by this treatment. It is probable, therefore, that the material necessary for the growth of *C. bifermentans* in amino acid medium is a peptide.

No increase in the amount of growth of *C. bifermentans* was found when the concentrations of arginine and tyrosine were raised to 0.25 per cent. Shull, Thoma, and Peterson (1949) reported that such concentrations of these amino acids served markedly to increase the amount of growth of *Clostridium sporogenes* in an amino acid medium in the absence of a certain peptidelike material.

It seems unlikely that the factor necessary for the growth of *C. bifermentans*

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is identical with "strepogenin," a peptide found necessary for the optimal growth of hemolytic streptococci by Sprince and Woolley (1945). As can be seen from the data in table 4, tryptic digests of egg albumen and gelatin were active in promoting the growth of *C. bifermentans*, whereas Sprince and Woolley found that tryptic digests of these materials did not contain "strepogenin."

TABLE 4

Effect of tryptic digests of albumen and gelatin on the growth of C. bifermentans in an amino acid medium

SUBSTANCE ADDED	TURBIDITY
None	2.0
Gelatin-trypsin control (100 μg/ml)	4.3
Trypsin-digested gelatin (100 μg/ml)	21.0
Albumen-trypsin control (100 μg/ml)	6.0
Trypsin-digested albumen (50 μg/ml)	17.6
Trypsin-digested albumen (100 μg/ml)	26.9
Trypsin-digested albumen (200 μg/ml)	40.4
Chloramine-T-treated-digested albumen (65 μg/ml)	15.1
Chloramine-T-treated-digested albumen (200 μg/ml)	37.4
Tryptone (100 μg/ml)	21.8

Several attempts to concentrate the active material from tryptone did not meet with success. The active material was found to pass through cellophane dialysis tubing, to be insoluble in ether, chloroform, and acetone, and to be only slightly soluble in methanol, ethanol, and phenol. Fractionation experiments were conducted with ethanol and acetone and yielded fractions only 5 times more active than the tryptone used as starting material. In general, the growth-promoting activity was associated with the material precipitated from water solutions of tryptone by high concentrations of ethanol or acetone.

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SUMMARY

The maximum growth of *Clostridium bifermentans* in a medium composed of "vitamin-free" acid-hydrolyzed casein, glucose, and inorganic salts was found to be dependent upon added biotin, nicotinic acid or amide, pantothenic acid, and pyridoxal or pyridoxamine. Some strains did not require biotin and some strains did not require pyridoxamine.

The maximum growth of *C. bifermentans* in an amino acid medium was found to be dependent upon some unknown substance, probably a peptide.

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