GROWTH FACTORS FOR BACTERIA

XIV. Growth Requirements of Acetobacter suboxydans

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In recent years a great deal of interest has arisen in the microorganism, *Acetobacter suboxydans*, because of its noteworthy ability to oxidize polyhydric alcohols and aldoses to the corresponding ketosugars and ketoacids.

For example, Fulmer, Dunning, Guymon and Underkofler (1936) developed a laboratory method for the production of sorbose from sorbitol. This process was placed on a semi-commercial basis by Wells, Stubbs, Lockwood and Roe (1937). Sorbose is now produced commercially, and has become an important chemical for the synthesis of ascorbic acid. By means of *A. suboxydans*, Reichstein (1934) produced *l*-adonose from adonitol, Neuberg and Hofmann (1935) and Underkofler and Fulmer (1937) prepared dihydroxyacetone from glycerol, Whistler and Underkofler (1938) made l-erythrulose from erythritol, Tilden (1939) secured perseulose from perseitol, Stubbs, Lockwood, Roe, Tabenkin and Ward (1940) obtained 5-ketogluconic acid from glucose, and Hann, Tilden and Hudson (1938) identified the particular keto compounds formed by the oxidation of a number of common and unusual sugar alcohols.

In most of the investigations with *A. suboxydans*, a water extract of yeast has been employed as the nutrient medium, although Wells, *et al.* (1937) and Stubbs, *et al.* (1940) found that corn-steep liquor supplied all of the growth requirements of the organism. Since no work has been reported regarding the growth substances required by *A. suboxydans*, the present work was undertaken. As a result it has been found that besides a suitable carbon source, organic nitrogen, and mineral salts, the medium must contain pantothenic acid, *p*-aminobenzoic acid, and nicotinic acid.

EXPERIMENTAL

Organism

The culture of *Acetobacter suboxydans* used was obtained originally from the American Type Culture Collection as no. 621. It has been cultivated for a number of years on slants of medium containing 0.5 per cent Difco yeast extract, 5 per cent glycerol and 1.5 per cent agar.

The inoculum was obtained from a 24-hour culture grown in 50-ml. Erlenmeyer flasks in 10 ml. of medium containing 0.5 per cent yeast extract and 5 per cent glycerol. The organism grows rapidly on this medium, forming a tenuous film upon the surface. The culture was shaken, transferred aseptically to a sterile test tube plugged with cotton and centrifuged. The clear liquid was

poured off, the cells were washed twice with 10 ml. of sterile 0.9 per cent saline solution by centrifugation and were finally suspended in 10 ml. of the sterile saline solution. One drop (*ca.* 0.05 ml.) of this suspension was used for inoculating 10 ml. of medium in a 50 ml. Erlenmeyer flask, which was then incubated at 28°C. for 48 hours.

Determination of growth

Growth was determined quantitatively by measuring turbidity. The 10 ml. culture was shaken, transferred to a test tube, and the bacterial cells were uniformly suspended by means of a glass homogenizer. Turbidity was measured by means of an electrophotometer. The instrument was first adjusted to read 100 with the uninoculated medium in the photometer tube and after this medium had been removed, the homogenized culture was transferred to the photometer tube and the percentage of transmitted light read directly. Thus, there is an inverse relation between the photometer reading and the degree of turbidity of the culture. Variation in the turbidity readings of duplicate cultures was never more than one scale division. In the earlier work an Evelyn photoelectric colorimeter was employed with a 660 mμ filter. For the later work a KWSZ photometer was used with a 650 mμ filter. Since test-tube cells, filters, and operation of the two instruments differ, transmission readings for the same bacterial suspension vary somewhat with the two instruments. However, the difference in transmission between controls and test cultures is the important consideration rather than absolute values. No attempts were made to correlate turbidity readings with actual cell counts.

Media

The constituents given in table 1 were present in all media unless otherwise stated. In the early work 0.5 gram of hydrolyzed casein was used but in later experiments this was reduced to 0.3 gram. In all cases 10 ml. portions of medium in 50 ml. Erlenmeyer flasks were employed. In the earlier experiments the media were adjusted to pH = 6.8 $\pm$ 0.1 since it had been shown by Underkofler and Fulmer (1937) that this was a satisfactory value for the production of dihydroxyacetone by *A. suboxydans* in a glycerol yeast-extract medium. Later it was found that in a synthetic medium a distinct optimum existed at pH 6.0 and hence all subsequent media were adjusted to this figure. The growth factors employed were made up in aqueous solution from crystalline compounds unless otherwise stated, and the solutions were stored under toluene in the refrigerator. All media were sterilized for 15 minutes at 15 pounds pressure before use.

Experimental results

Preliminary attempts were made to grow *A. suboxydans* in a comprehensive medium containing all of the available factors which at the time had been reported necessary for various microorganisms. Besides the ingredients listed in table 1, this "shot gun" medium contained riboflavin, pantothenic acid, nicotinic acid, biotin, pyridoxine, thiamine, inositol, pimelic acid, uracil, adenine,

glutathione, glutamine, *l*-asparagine, and solubilized liver extract as a source of the eluate factor reported by Hutchings, Bohonos and Peterson (1941) as necessary for many lactic acid bacteria. Meager growth was obtained with this medium, the photometer reading being 53 per cent as compared with 20 per cent for a culture in glycerol yeast-extract. Growth of the organism, however, continued on successive transfers in this medium.

Many experiments which need not be detailed here showed that the same sparse but continuous growth, could be obtained with only pantothenic acid, nicotinic acid, biotin, pyridoxine, and a small amount (200 micrograms per 10 ml.) of solubilized liver. Since these experiments were inconclusive, tests were made to determine the requirement of the organism for individual growth-substances. The response of the organism to riboflavin was determined on the riboflavin-free yeast medium of Snell and Strong (1939). Not only was riboflavin unnecessary but evidence was obtained that *A. suboxydans* can synthesize

TABLE 1

Constituents regularly used in test media

SUBSTANCE	WEIGHT PER 100 ML. OF MEDIUM
Glycerol	5.0 grams
Hydrolyzed casein*	0.5 or 0.3 gram
Tryptophane	10.0 mgm.
Cystine	7.5 mgm.
Mineral salts A:	
K_2HPO_4	0.05 gram
KH_2PO_4	0.05 gram
Mineral salts B:	
$MgSO_4 \cdot 7H_2O$	0.02 gram
NaCl	0.001 gram
$FeSO_4 \cdot 7H_2O$	0.001 gram
$MnSO_4 \cdot 3H_2O$	0.001 gram

* The casein employed was alcohol extracted and acid hydrolyzed before use.

riboflavin. The amount of the synthesis varied with the extent of growth: a culture reading 38 per cent on the photometer contained 17 micrograms of riboflavin per 10 ml. of medium and one reading 20 per cent contained 85 micrograms.

Experiments were next undertaken to determine whether pantothenic acid was needed in the medium. The tests showed a definite increase in growth either when pantothenic acid was added to a yeast-extract medium that had been treated by the method of Strong, Feeney and Earle (1941), to remove the compound, or when pantothenic acid was added to a medium containing only enough yeast-extract to support slight growth. Since pantothenic acid was needed, the growth-promoting properties of the component parts of the compound were also determined.[1] The (−) α-hydroxy-β,β-dimethyl-γ-butyrolactone possessed quite remarkable growth-promoting properties, the β-alanine practically none.

[1] Synthetic dextro-rotatory calcium pantothenate and (−) α-hydroxy-β, β-dimethyl-γ-butyrolactone were made available for this work through the kindness of Merck and Co., Inc.

Since these preliminary experiments demonstrated the potency of pantothenic acid and its lactone constituent, an experiment was set up to determine the effect of adding graded amounts of the two compounds to the medium. The basal medium (designated B) contained the ingredients listed in table 1 plus 20 mgm. of Difco yeast-extract per 100 ml. of solution. This amount of yeast-extract gave only poor growth but furnished enough of the other required, but at that time unknown, factors to allow excellent growth on addition of pantothenic acid. The results of this experiment are given in table 2. The lactone was more effective in promoting growth than the calcium pantothenate in the larger additions, while the mixture of β-alanine and lactone gave less growth than the equivalent amounts of pantothenic acid or the lactone alone.

TABLE 2

Requirement of A. suboxydans for calcium pantothenate and (−) α-hydroxy-β,-β-dimethyl-γ-butyrolactone

ADDITION (MICROGRAMS PER 100 ML.)	PHOTOMETER READING
None	71
Calcium pantothenate:	
10	48
30	33
50	30
70	26
100	25
α-Hydroxy-β, β-dimethyl-γ-butyrolactone	
10	64
30	32
50	15
70	13
100	7
β-Alanine: 100	71
β-Alanine: 50, α-hydroxy-β, β-dimethyl-γ-butyrolactone: 50	33
Yeast extract control	8

Basal Medium B: per 100 ml., the ingredients listed in table 1 plus 20 mgm. Difco yeast extract; pH = 6.8.

During the course of the work which has been described above, anomalous results appeared occasionally. In certain cases no growth whatever occurred in media which had in previous experiments supported growth to a limited extent. Suspicion arose that the reaction of the medium (pH = 6.8 before sterilization) might not be favorable. Hence a series of media was prepared in which the pH was varied. Basal medium B was employed and the pH adjusted by adding hydrochloric acid or sodium hydroxide. The response to these changes is presented in table 3. It is apparent that a slightly acid pH was definitely favorable, while the pH previously employed was definitely unfavorable. No growth occurred if the pH of the medium was slightly alkaline. In all subsequent work the pH of the media was adjusted to 6.0 ± 0.1 before sterilizing.

After the report by Rubbo and Gillispie (1940) that *p*-aminobenzoic acid is a

growth factor required by *Clostridium acetobutylicum* it was decided to try the effect of this compound upon the growth of *A. suboxydans*. The composition of the basal medium (C) along with the results of this experiment are given in table 4. It is quite evident that *p*-aminobenzoic acid markedly stimulated the growth of the organism. It is probable that the considerable growth obtained in the blank was due to the presence of some *p*-aminobenzoic acid in the casein. This was a new lot of casein and inadvertently was not as carefully purified as in the previous experiments. With a carefully purified casein it is probable that *A. suboxydans* could be used as an assay agent for the quantitative determination of *p*-aminobenzoic acid.

TABLE 3

Effect of pH of the medium upon growth of A. suboxydans

pH OF MEDIUM		PHOTOMETER READING	
Before sterilization	After sterilization	24 hrs.	48 hrs.
5.30	4.55	13.8	8.1
6.10	5.95	9.1	6.2
6.90	6.75	72.7	29.7
7.10	7.18	86.2	92.7
7.75	7.78	93.1	93.1

Basal Medium B plus 80 mgm. Difco yeast extract.

TABLE 4

Requirement of A. suboxydans for p-aminobenzoic acid

p-AMINOBENZOIC ACID (MICROGRAMS PER 100 ML.)	PHOTOMETER READING	
	24 hrs.	48 hrs.
0.0	50.1	45.2
0.01	15.4	12.3
0.1	14.4	10.6
1.0	14.0	10.5
10.0	14.0	10.5

Basal Medium C: per 100 ml., the ingredients listed in table 1 plus 100 micrograms calcium pantothenate and 0.5 micrograms S.M.A. Biotin Concentrate #1000; pH = 6.0.

Experiments were next begun to determine whether biotin was required by *A. suboxydans*. Tests were made with a series of concentrations of S.M.A. Corporation's Biotin Concentrate No. 1000. The composition of the basal medium (D) and the results are presented in table 5. The data demonstrate that the Biotin Concentrate markedly promoted the growth of the organism.

Later, when crystalline biotin became available, attempts were made to substitute the methyl ester and free acid for the Biotin Concentrate, but without success. The experiments were repeated several times with various levels of biotin (from 0.005 to 0.5 microgram per 100 ml.) always with negative response (table 6). It is apparent that some factor other than biotin in the S.M.A.

Biotin Concentrate No. 1000 was responsible for the growth-promoting properties of this material.[2] Additional evidence that *A. suboxydans* does not require biotin was obtained by biotin assay of the yeast extract medium before inoculation (0.0046 microgram of biotin per ml.) and after culturing *A. suboxydans* upon it (0.0143 microgram of biotin per ml.). The data clearly show that *A. suboxydans* can synthesize biotin.

Since it had been found that normal growth of *A. suboxydans* occurred on a medium containing the ingredients of table 1 with addition of calcium pantothenate, *p*-aminobenzoic acid and S.M.A. Biotin Concentrate, it was desirable to determine the specific effect of the various nutrients of table 1. The basal

TABLE 5

Requirement of A. suboxydans for S.M.A. Biotin Concentrate #1000

BIOTIN CONCENTRATE (MICROGRAMS PER 100 ML.)	PHOTOMETER READING	
	24 hrs.	48 hrs.
0.00	97.3	91.5
0.0625	65.3	53.1
0.125	49.2	31.6
0.25	29.1	17.9
0.50	23.2	17.1

Basal Medium D: per 100 ml., the constituents listed in table 1 plus 100 micrograms calcium pantothenate and 1 microgram *p*-aminobenzoic acid; pH = 6.0.

TABLE 6

Requirement of A. suboxydans for biotin

ADDITION TO BASAL MEDIUM (0.5 MICROGRAM PER 100 ML.)	PHOTOMETER READING	
	24 hrs.	48 hrs.
None	81.0	80.2
S.M.A. Biotin Concentrate #1000	17.1	15.4
Crystalline biotin (methyl ester)	77.8	74.8
Crystalline biotin (free acid)	85.2	79.0

Basal Medium D; pH = 6.0.

medium contained, per 100 ml., 5 grams glycerol, 100 micrograms calcium pantothenate, 1 microgram *p*-aminobenzoic acid and 0.5 microgram Biotin Concentrate. Typical data resulting from these experiments are given in table 7. The

[2] As a result of correspondence with Dr. Maurice Landy and Miss Dorothy Dicken, Research Laboratories, S. M. A. Corporation, regarding other possible growth-promoting constituents of Biotin Concentrate No. 1000, they investigated the response of *A. suboxydans* to this product and found that the nicotinic acid contained in it promoted the growth of the organism. Details regarding the response of *A. suboxydans* to nicotinic acid will be given in a separate publication from Research Laboratories, S. M. A. Corporation. We have verified these results and found that for the strain of *A. suboxydans* used, pantothenic acid, *p*-aminobenzoic acid and nicotinic acid are the only growth substances required.

proportions of the materials added to the basal medium were those shown in table 1. Where hydrolyzed casein was used, the amount was 0.3 gram per 100 ml. The mixture of pure amino acids was made up so as to imitate the amino acid composition of casein and 0.3 gram per 100 ml. of solution was added.

It is apparent from the results that nearly normal growth of *A. suboxydans* occurred in a medium containing only glycerol as carbon source, hydrolyzed casein to furnish organic nitrogen, and inorganic salts, calcium pantothenate, *p*-aminobenzoic acid and S.M.A. Biotin Concentrate. The results also show that the hydrolyzed casein was replaceable by a mixture of pure amino acids although the growth was slower and not quite so good with the amino acid mixture as with the hydrolyzed casein.

TABLE 7

Effect of several nutrient materials upon the growth of A. suboxydans

ADDITION TO BASAL MEDIUM*	PHOTOMETER READING	
	24 hrs.	48 hrs.
None	99.9	91.3
Hydrolyzed casein	98.0	89.3
Hydrolyzed casein + salts A	89.1	86.2
Hydrolyzed casein + salts B	98.0	53.6
Hydrolyzed casein + salts A + salts B	23.7	13.1
Tryptophane + salts A + salts B	95.3	69.5
Cystine + salts A + salts B	79.6	54.1
Tryptophane + cystine + salts A + salts B	86.7	57.2
Hydrolyzed casein + tryptophane + cystine + salts A + salts B	22.4	16.8
Amino acid mixture + salts A + salts B	59.3	23.0
Difco yeast extract control	18.6	7.6

* See text for composition of basal medium.

SUMMARY

The growth factor requirements of a strain of *Acetobacter suboxydans* were found to be as follows:

Riboflavin was not required in the medium but was found to be synthesized by the organism.

Pantothenic acid, or one component of this compound, i.e., (−) α-hydroxy-β,β-dimethyl-γ-butyrolactone, was an essential factor for the growth of *A. suboxydans*.

p-Aminobenzoic acid was also required.

S.M.A. Biotin Concentrate No. 1000 furnished another essential factor, not biotin, which was indispensable for the growth of the organism. This factor has been found by other investigators to be nicotinic acid (see footnote p. 188).

Hydrolyzed casein alone furnished adequate organic nitrogen for the nutrition of the organism. Excellent growth of *A. suboxydans* was obtained when a known mixture of amino acids replaced the hydrolyzed casein.

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