PHYSIOLOGICAL STUDIES OF ASSOCIATION

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INTRODUCTION

This paper is the second of a series of articles dealing with certain factors which influence the rate of cellulose fermentation (see Sanborn, 1926). In the present work the influence of microbial association is investigated.

One of the most prominent organisms employed in association experiments is *Azotobacter*, significant because of its ability to synthesize the growth-accessory factor, and, with regard to its present application, because of the intimate connection between nitrogen assimilation by *Azotobacter* and cellulose decomposition. Hunter (1923) has shown that *Azotobacter* is capable of synthesizing an accessory factor similar to vitamine B. Mockeridge (1924) noted the growth-promoting effect of *Azotobacter* and *Rhizobium* upon green plants.

The work of Hutchinson and Clayton (1919) indicates that products of cellulose decomposition stimulate nitrogen assimilation by *Azotobacter*. Similar results have been obtained by Pringsheim (1909, 1910).

A cellulose-destroyer, *C. folia*, isolated by the author from decomposing leaves, proved to be a suitable organism upon which to determine the influence of association. The organisms selected for association with *C. folia* were forms which the author has found to be invariably connected with active cellulose destruction in nature, and were isolated from mixed cultures in which cellulose was undergoing rapid decomposition. These organisms are as follows: *Act. colorata*,[1] *Azotobacter*, *B. mycoides*,

[1] *Act. colorata* is a new species, described elsewhere by the author.

B. subtilis, and *B. cereus*. For details in technique and method, reference may be made to the first paper of the series.

EXPERIMENTAL

a. The influence of association with Act. colorata upon the growth and physiological efficiency of C. folia

Investigations by the author showed that hydrogen-ion concentration determinations would serve as a legitimate criterion of the rate of cellulose decomposition by *C. folia* and as a measure of its physiological efficiency. A basic nutrient medium was employed which lent itself readily to the H-ion technique.[2] This medium proved suitable mainly because its "buffer index" is negligible and because it will not support the growth of *C. folia*. For the fermentation experiments raw cotton was added to the nutrient medium. The electrometric method described by Itano (1923) was used throughout the investigation, supplemented by the colorimetric method.

The same general technique was employed as in the experiments previously reported. For the H-ion series, liter Erlenmeyer flasks, containing 200 cc. of the nutrient medium and 3 grams of raw cotton, received inoculations from a twenty-four hour nutrient solution culture of *C. folia*, and from a four-day old culture of the *Actinomyces* in nutrient solution containing raw cotton. Incubation took place at 27°. The results (tables 1 and 2) show the influence of *Act. colorata* upon the growth and physiological efficiency of *C. folia*. Because of the filamentous character of the *Actinomyces* growth and its tendency to break up into segments and form conidia, an accurate plate count, particularly with the *Actinomyces* above, proved impossible.

Tables 1 and 2 indicate that *Act. colorata* exerts a stimulative action upon the growth and physiological efficiency of *C. folia at the expense of the former organism which gradually dies out.* In the fermentation flasks, in five weeks' time no trace of the *Actinomyces* could be found. In order to determine more defi-

[2] The medium is a modification of McBeth's solution, and is described in the first paper of the series.

nitely the nature of the stimulation, an extract was made from the *Actinomyces* cells. Cells from a four-day-old culture growing upon N-free agar were washed in saline solution and then

TABLE 1

The influence of Act. colorata upon the growth of C. folia

DAYS	CONTROL		ASSOCIATION OF C. FOLIA AND ACT. COLORATA IN BASIC NUTRIENT SOLUTION WITH CELLULOSE	
	Act. colorata alone	*C. folia* alone	*C. folia*	*Act. colorata*
1	100*	30†	55†	100*
2	Rapid increase in growth	42	98	66
3	Filaments form segments	54	137	46
4		66	148	30
5	Conidia appear	78	146	22
6	1,000	100	130	20
7	1,000	150	110	18

* Expressed in thousands.

† Expressed in millions.

TABLE 2

The influence of the association of cellulose-destroyers upon the rate of cellulose decomposition

DAYS	C. FOLIA ALONE	ACT. COLORATA ALONE	ASSOCIATION OF C. FOLIA AND ACT. COLORATA
	pH	*pH*	*pH*
Initial	8.40	8.40	8.40
4	7.84	7.56	7.30
8	7.30	7.18	6.20
12	6.96	7.00	5.34
16	6.76	6.90	5.00
20	6.68	6.84	4.84
24	6.66	6.78	4.80
28	6.64	6.70	4.80
42	6.46	6.30	
56	6.16	6.00	
63	6.00	5.82	
70	5.80	5.60	

suspended in nutrient medium. The suspension was acidified to pH 4.6 and boiled for ten minutes on each of two consecutive days.[3] The extract contains the water-soluble substances pres-

[3] The work of Wildiers and others show "Bios" to be unaffected by boiling in acid environment. Method from Thjötta and Avery (1921).

ent in the living *Actinomyces* cells, and inasmuch as the failure of the *Actinomyces* results in the activation of *C. folia*, such an extract should contain the same essential food substances and produce stimulation. The results are given in table 3.

The results of the above experiments show that association with *Actinomyces* causes a stimulation in the growth and cellulose-decomposing ability of *C. folia*. The association may be considered equivalent to the addition of some essential food factor.

TABLE 3

The influence of extracts from cells of Act. colorata upon the growth of C. folia in basic nutrient solution

Expressed in millions

HOURS	EXTRACT FROM ACTINOMYCES CELLS	CONTROL (NO EXTRACT)
Initial	2	2
5	9	3
10	16	3
15	23	4
20	30	5
25	38	6
30	56	7
35	79	8
40	101	9
45	116	9
50	125	10
55	133	11
60	140	10
65	148	8

b. The influence of Azotobacter upon the growth and physiological efficiency of C. folia

One loopful from a five-day-old culture of *Azotobacter* (A4 Jones) on Ashby agar was introduced into 100 cc. of the basic nutrient solution. A uniform suspension was made. A suspension of *C. folia* from a twenty-four-hour nutrient agar culture was also made in 100 cc. of the nutrient solution. One-cubic centimeter portions from this suspension, containing approximately 3,000,000 cells, were used to inoculate test tubes, each containing 8 cc. of nutrient solution.

From the *Azotobacter* suspension dilutions were prepared in nutrient solution. One-cubic centimeter portions from these dilutions were also added to the tubes of nutrient solution. Two hundred *Azotobacter* cells were added in one case; 40,000 in a second; and in the last set, 2,800,000 cells. The living *Azotobacter* cells added were determined by the plate method using Ashby agar. Table 4 gives the results. These figures show that association with *Azotobacter* results in a marked stimulative effect upon the growth of *C. folia.* In order to ascertain more definitely the seat of the factor of stimulation, the influence of *Azotobacter* was investigated more in detail. The following

TABLE 4

The influence of Azotobacter upon the growth of C. folia in nutrient solution

HOURS	AZOTOBACTER, 200 CELLS, + C. FOLIA	AZOTOBACTER, 40,000 CELLS, + C. FOLIA	AZOTOBACTER, 2,800,000 CELLS, + C. FOLIA	CONTROL C. FOLIA ALONE
Initial	3,000,000	3,000,000	3,000,000	3,000,000
6	1,950,000	2,950,000	3,800,000	1,150,000
12	1,300,000	2,850,000	4,350,000	650,000
18	900,000	2,650,000	4,700,000	300,000
24	550,000	2,400,000	4,850,000	100,000
30	300,000	2,000,000	4,450,000	10,000
36	150,000	1,550,000	3,500,000	10,000
42	60,000	1,200,000	1,600,000	10,000−
48	10,000	1,000,000	1,000,000	10,000−

preparations were made from a five-day-old culture of *Azotobacter* cultivated in Ashby solution without $CaCO_3$.

1. Suspension of washed *Azotobacter* cells (living)
2. Extract from *Azotobacter* cells
3. Extract from culture medium

The culture of *Azotobacter* was centrifuged, throwing down the cells and leaving the supernatant liquid clear. The latter was retained for the preparation of the extract from the culture medium. The cells were washed several times with sterile physiological salt solution, after which a cell suspension in 10 cc. of saline solution was prepared.

A similar suspension was used in preparing the extract from the

Azotobacter cells. In this case the saline suspension of washed cells was acidified to pH 4.6 as in the previous experiment, and boiled for ten minutes. The suspension was boiled only once.

From the supernatant liquid obtained after centrifuging the original culture, the extract from the culture medium was made. This liquid was filtered through a sterile porcelain filter and the resulting extract remained sterile. The influence of these preparations upon the growth of *C. folia* was determined as before.

Table 5 indicates that the stimulative effect of *Azotobacter* upon the growth of *C. folia* is caused by association with the living *Azotobacter* cell, and not with extracts from the cell or culture medium.

TABLE 5

The influence of washed Azotobacter cells; of extract prepared from Azotobacter cells; and of extract from the culture medium in which Azotobacter has been growing, upon the growth of C. folia

HOURS	WASHED AZOTOBACTER CELLS	EXTRACT FROM AZOTOBACTER CELLS	EXTRACT FROM CULTURE MEDIUM
6	750,000	100,000	20,000
12	1,020,000	20,000	10,000
18	1,310,000	10,000	5,000
24	1,560,000	10,000	5,000
30	1,670,000	10,000	5,000−
36	1,700,000	10,000	5,000−
42	1,650,000	10,000	5,000−
48	1,460,000	5,000	5,000−

To determine the influence of *Azotobacter* upon the physiological efficiency of *C. folia*, a twenty-four-hour nutrient solution culture of *C. folia* was made, also a suspension of *Azotobacter* in nutrient solution. Approximately equal numbers of each species were used as inocula (500,000,000 cells). The fermentations were carried on as in the previous experiment using 3 grams of raw cotton in 200 cc. of nutrient solution and incubating at 27° (see table 6).

This experiment shows that *Azotobacter*, itself not a cellulose-destroyer, exerts a stimulating action upon *C. folia* both in growth and in cellulose-decomposing ability. This influence is manifest when *C. folia* is in association with living *Azotobacter* cells.

The work was repeated and a similar result obtained using *Azotobacter chroococcum* which was isolated from soil in which active cellulose decomposition was taking place.

c. *The influence of B. mycoides, B. subtilis, and B. cereus, upon the growth and physiological efficiency of C. folia*

Following the same general technique, *B. mycoides*, *B. subtilis*, and *B. cereus* were investigated for their stimulating action. Preliminary experiments based upon visual tests indicated that these organisms, associated with active cellulose fermentation in soil, exerted an accelerating influence upon the rate of cellu-

TABLE 6

The influence of Azotobacter upon the physiological efficiency of C. folia

DAYS	C. FOLIA ALONE	AZOTOBACTER ALONE	CONTROL: CHANGE IN pH OF MEDIUM WITHOUT ORGANISMS	ASSOCIATION OF C. FOLIA AND AZOTOBACTER
	pH	*pH*	*pH*	*pH*
Initial	8.40	8.40	8.40	8.40
2	7.90	8.30	8.34	7.24
4	7.40	8.24	8.28	6.52
6	6.98	8.16	8.22	6.08
8	6.68	8.10	8.16	5.80
10	6.48	8.04	8.12	5.58
12	6.42	7.96	8.06	5.46
14	6.40	7.90	8.00	5.40

lose decomposition. Because of the inability of these organisms to multiply in the basic nutrient medium or to attack cellulose, their numbers rapidly decreased. This decrease could not be traced quantitatively by the plate method because of the similar nutritive proclivities of these organisms and *C. folia*. Microscopic examinations, however, revealed the gradual dying out of the associated bacteria, resulting at the same time in a marked stimulation of *C. folia* in growth and physiological activity. These organisms were cultivated upon nutrient agar (pH 7.60). Aqueous suspensions of the organisms were made, and after washing and centrifuging the cells, extracts were prepared as before by acidifying the washed cell suspension to pH 4.60 and

boiling for ten minutes. In this particular experiment the boiling process was repeated upon three consecutive days. All of the extracts were sterile. The influence of these extracts

TABLE 7

The influence of extracts prepared from the cells of B. mycoides, B. cereus and B. subtilis, upon the growth of C. folia in nutrient solution

Expressed in millions

HOURS	CONTROL (NO EXTRACT)	EXTRACT FROM B. MYCOIDES CELLS	EXTRACT FROM B. CEREUS CELLS	EXTRACT FROM B. SUBTILIS CELLS
Initial	2	2	2	2
5	3	19	13	11
10	3	23	18	22
15	4	28	23	33
20	5	33	28	44
25	6	38	34	56
30	7	49	39	67
35	8	63	45	78
40	9	77	50	89
45	9	84	54	105
50	10	85	56	124
55	11	87	59	142
60	10	88	61	161
65	8	89	64	175

TABLE 8

The influence of B. mycoides, B. subtilis, and B. cereus upon the physiological efficiency of C. folia

DAYS	CONTROL, C. FOLIA ALONE	ASSOCIATION OF B. MYCOIDES WITH C. FOLIA	ASSOCIATION OF B. SUBTILIS WITH C. FOLIA	ASSOCIATION OF B. CEREUS WITH C. FOLIA
Initial	8.40	8.40	8.40	8.40
2	7.73	6.54	6.92	7.10
4	7.24	6.37	6.16	6.66
6	6.96	6.28	6.07	6.48
8	6.80	6.20	5.98	6.42
10	6.77	6.14	5.91	6.32
12	6.76	6.07	5.83	6.24
14	6.74	6.00	5.75	6.15

upon the growth of *C. folia* was tested in tubes of nutrient solution, treated uniformly as in the previous experiments. *C. folia* was plated out at intervals upon nutrient agar (see table 7).

These results show that some essential food factor may be extracted from the cells of these organisms which accelerates the growth of *C. folia* in nutrient solution. To determine the influence of this factor upon the physiological efficiency of the cellulose-destroyer, the fermentation flasks, containing nutrient solution and raw cotton, received inoculations of 300,000,000 cells of *C. folia* and 50,000,000 cells of the associated organism. Table 8 gives the results of the association.

B. mycoides, *B. subtilis*, and *B. cereus* exert a stimulating action upon the growth and physiological efficiency of *C. folia*.

d. The study of microbial associations by means of the China blue-rosolic acid-cellulose medium

In association studies such as those carried on in this investigation, there is often need of a medium which will reveal in detail the results of association. In this experiment such a medium is described. Through its use the influence of association in cellulose fermentation may be observed conveniently and with a fair amount of accuracy. The method is based upon the China blue-rosolic acid reaction described by Bronfenbrenner (1918). The preparation of this "CR-cellulose" medium is as follows:

Basic nutrient solution[4]	1000 cc.
Raw cotton (chemically untreated)	30 grams

The salts are dissolved in 500 cc. of distilled water, and of the remaining 500 cc. a 0.5 per cent agar is prepared. Before sterilization the two portions are mixed and the cotton added, cut into small fragments. With constant stirring, 1 per cent "CR" preparation[5] is introduced. In transferring the medium to

[4] Referred to previously. Described in first paper of series.

[5] "CR" indicator is obtained by mixing equal parts of 0.5 per cent aqueous solution of China blue with 1 per cent solution of rosolic acid in 95 per cent alcohol. On the alkaline side China blue is water clear. Rosolic acid gives on the acid side different shades of pale yellow, which is masked by the deep blue of the China blue and gives sharp color values in media. In alkaline environment, China blue being colorless, the rosolic acid gives a pure red.

petri plates, care is taken to insure a uniform distribution of cotton over the plate. The dishes are then autoclaved. The percentage of agar present proves very satisfactory in offering a smooth homogeneous surface, yet allowing the microbial suspensions added to reach the entire mass of cotton. The pH of the medium prepared in this way is 8.40 and is colored a brilliant red.

After cooling, the surface of the medium is inoculated uniformly with the cellulose-destroyer; one-half of the plate only receives treatment with the associated organism. The indicator has its turning point very close to neutrality, and with the production of acid the medium changes to a deep blue.

TABLE 9

The influence of association upon the rate of cellulose fermentation as determined by the "CR-cellulose" reaction

DAYS	C. FOLIA* ALONE	C. FOLIA IN ASSOCIATION WITH:				
		B. mycoides	*B. cereus*	*B. subtilis*	*Act. colorata*	*Azotobacter*
Initial	Medium red (8.40)	Red	Red	Red	Red	Red
1	Medium red	Red	Red	Red	Red	Red
2	Medium red	Red	Red	Red	Red	Red
		(Slight reduction of indicator observed)				
3	Medium red	Blue ++†	Blue +	Blue ++	Blue ++	Blue +++
4	Medium red	Blue +++	Blue +	Blue ++	Blue ++	Blue +++
5	Medium red	Blue +++	Blue ++	Blue +++	Blue +++	Blue +++
6	Medium red	Blue +++	Blue +++	Blue +++	Blue +++	Blue +++

* *C. folia* alone caused the acid reaction in eight to ten days.

† + = medium slightly bluish (pH 6.8); ++ = blue color definite (pH 6.4–6.6); +++ = intense blue (pH 6.0–6.2).

All of the organisms investigated above were tested out upon this medium in association with *C. folia*. Table 9 shows the influence of association upon the rate of cellulose decomposition as revealed by H-ion concentration changes, using the "CR-cellulose" medium.

The results observed with this medium are quite in accord with the quantitative results recorded above. The greatest stimulation seems to occur when *C. folia* is associated with *Azotobacter*, *B. subtilis*, *B. mycoides*, or *Act. colorata*. In the case of *B. cereus* the initial stimulation is not as marked; in the

course of twenty-five days, however, the acceleration in physiological efficiency caused by association with *B. cereus* even exceeds that caused by association with the other soil bacteria.

The value of the China blue-rosolic acid-cellulose medium lies in the ease of preparation, its adaptability to the H-ion technique, and the accuracy with which the indicator responds to stimulation in the physiological efficiency of cellulose-destroyers. Other advantages of the medium are as follows: The buffer index is negligible; the color change occurs with definiteness at very nearly pH 7.0; the indicator is not affected by heat, shows no evidence of bactericidal action, is comparatively stable; and its constituents are definitely known chemical substances (Bronfenbrenner, 1918).

TENTATIVE CONCLUSIONS

1. Association with *Act. colorata*, *Azotobacter*, *B. subtilis*, *B. mycoides*, and *B. cereus* causes a stimulation in the growth and physiological efficiency of *C. folia*.

2. Stimulation by *Act. colorata* takes place at its own expense. Some essential food substance is apparently furnished by the dead cells.

3. In association with *Azotobacter* the stimulation is brought about by the living cell.

4. *B. subtilis*, *B. mycoides*, and *B. cereus* secrete substances within the cell which function as an essential food factor for *C. folia*.

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