A STUDY OF A BACTERIAL ASSOCIATION

I. THE BIOCHEMISTRY OF THE PRODUCTION OF LACTIC ACID

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During the late war large amounts of acetone and butyl alcohol were manufactured in various countries by the fermentation of cereals and other carbohydrates. It was to be expected that the industrial application of such a process would involve numerous difficulties, including those in connection with sterility and the preservation of pure cultures. One of the many interesting observations of scientific interest made during this period was the fact that the majority of unsatisfactory fermentations due to contamination in England, the United States and Canada were essentially alike in their biochemical and bacteriological characteristics (Nathan, 1919, Reilly and others, 1920, Thaysen, 1921, Speakman, 1920). In addition to the anaerobic bacillus responsible for the production of acetone and butyl alcohol the mash in these fermentations invariably contained a small bacterium, which in stained preparations could be seen in the form of short chains (plate I, fig. 1). This organism gave rise to small, circular colonies on aërobic plates or slopes of lactose nutrient agar (plate I, fig. 2).. It has been described in greater detail by Thaysen (1921). At the end of twenty-four hours growth in artificial medium the organism contains numerous large volutin granules, which stain pink in a preparation lightly stained with methylene blue. Lactic acid is formed in cultures containing glucose, maltose or lactose. There is little if any, amyloclastic activity in media containing starch. The organism does not form spores. It was regarded by Thaysen as a new species, and named *Bacterium volutans*.

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The biochemical characteristics of the contaminated fermenters were as follows: After a brisk evolution of gas lasting for about 20 hours after the time of inoculation with the culture of *B. granulobacter-pectinovorum*, the rate of gas production, instead of continuing to rise, fell rapidly to zero. During this period, and for many hours after gas production had ceased, the acidity of the mash steadily increased to very abnormal figures. Reilly and co-workers (1920) have shown that this mash is very rich in lactic acid. The yields of acetone and butyl alcohol from such a fermentation were sometimes nil. In view of the fact that the contaminant is a producer of lactic acid, the first explanation we entertained of these phenomena was the simple and obvious one, namely, that contamination had overcome by more rapid growth the butyl alcohol organism, and utilized any soluble carbohydrate in the medium to form lactic acid. Any toxic action exerted by *B. volutans* we assumed to be due to the general and specific action of the high acidity produced. These conclusions had to be abandoned as soon as we isolated pure cultures of *B. volutans*, and investigated the behavior of this organism growing alone and in association with *B. granulobacter-pectinovorum* in different media. We shall endeavour to show in this communication that the lactic acid produced in mixed cultures is not produced by *B. volutans* but by *B. granulobacter-pectinovorum*, and that this change in the metabolism of the latter organism is due to the action of some metabolic product of *B. volutans* which inhibits partially or completely the majority of its normal biochemical processes.

In the recent literature reports have been made of three bacterial associations similar to the one we are investigating. The association containing *B. coli* and *B. paratyphosus* has been carefully investigated by Smith and Smith (1920–21). They show that *B. coli* produces no gas but the normal amount of acid in lactose broth, which has been previously inoculated with *B. paratyphosus*. The factor which is responsible for the inhibition of gas production increases in power as the time allowed between the two inoculations is increased up to four days. After remaining stationary for several days it gradually loses strength, and

disappears from the medium at the end of three or four weeks. By separating the cells of the *B. paratyphosus* culture from their metabolic products, and submitting these two components to heat and other treatment, the authors have endeavored to determine the nature of the inhibitory agent. They regard the available experimental evidence as inconclusive, but offer the tentative hypothesis that it is some metabolic product of *B. paratyphosus*. Owing to the fact that it is destroyed at temperatures slightly above the thermal death point of the bacteria they conclude that it is possibly an enzyme.

The organism responsible for the characteristic flavor and "eyes" of a Swiss cheese is *B. acidi-propionici*. It has been shown by Sherman and Shaw (1920–21) that the amount of propionic acid produced by this organism in an artificial medium containing lactose is increased six or seven-fold by adding to the same medium a culture of *Streptococcus lacticus* or *Lactobacillus casei*. The possibility of the increased yield of acid being due to the formation of propionic acid from the lactic acid produced by the associated organism has been eliminated by the fact that similar results were obtained by the addition of organisms which do not ferment lactose. The authors did not investigate further the biochemistry of the association.

The third case is discussed in a paper by Fouassier (1921) which we have only been able to review in abstract form. Brief mention is made of the fact, that the production of lactic acid by an organism isolated from milk is stimulated by association with *B. subtilis* and Tyrothrix.

EXPERIMENTAL

Cultures. The cultures of *B. granulobacter-pectinovorum* used in this investigation were derived from the laboratory stock culture. Those of *B. volutans* were originally derived from pure cultures of the organism isolated in 1917 from a typical contaminated fermenter. We found that these cultures, on continued growth in artificial media, lost their toxic properties and so we were compelled to adopt a method for the isolation of a fresh toxic strain.

Three 300 cc. Erlenmeyer flasks containing 200 cc. of 3 per cent maize mash are sterilized in the usual manner. To each flask 5 to 10 grams of unsterilized maize meal is added, and they are then inoculated with 1 or 2 cc. of a vigorous culture of *B. granulobacter-pectinovorum*. The flasks are incubated at 36°C. At this stage they contain the varied flora of the meal in addition to the culture added. The latter soon begins to ferment and produces CO_2 and H_2. The result is that only obligate or facultative anaerobes can possibly develop in the medium, and usually at the end of three or four days the mash contains only *B. granulobacter-pectinovorum* and *B. volutans*. Strains of *B. mesentericus* occasionally persist in small numbers. A 10 cc. sample of the mash in each flask is then titrated with 0.1 N NaOH in order to discover whether the characteristic high acidity of a mixed culture has been formed. *B. volutans* is isolated from the acid mash by the usual bacteriological methods, and cultivated in wort or yeast water.

Experiment I. Three experimental flasks containing 750 cc. of 3 per cent maize mash were sterilized for two hours at 15 pounds steam pressure. They were allowed to cool to room temperature, and inoculated in the following manner with pure cultures of *B. granulobacter-pectinovorum* and *B. volutans:*

Flask A: Inoculated with 10 cc. of *B. granulobacter-pectinovorum.*

Flask B: Inoculated with 10 cc. of *B. granulobacter-pectinovorum* 10 cc. of *B. volutans.*

Flask C: Inoculated with 10 cc. of *B. volutans.*

The *B. volutans* culture used was derived from a contaminated fermenter. The flasks were incubated at 36°C., and periodic determinations of the acidity of 10 cc. samples were made. The pure culture of *B. granulobacter-pectinovorum* alone gave a normal fermentation. In flask B there was a vigorous fermentation for about thirty hours, and then gas production diminished very rapidly. There was a considerable residue of unfermented starch. There was no sign of gas production or starch hydrolysis in flask C. The results from the experiment are given in table 1 and figure 2.

The results from the experiment indicated that it was possible to reproduce in the laboratory under controlled conditions the

TABLE 1

TIME AFTER INOCULATION	ACIDITY*		
	A	B	C
hours	*cc.*	*cc.*	*cc.*
4	0.2	0.2	0.2
12	1.2	1.0	0.3
22	3.5	3.1	0.7
25	3.4	3.6	
29	2.9	4.1	0.9
34	2.0	4.4	0.9
48	1.8	7.1	1.0
58	1.7	8.5	1.1
72		9.4	1.1
97	2.0	9.6	1.2

* The titratable acidity in this and other tables is expressed in terms of 0.1 N acid per 10 cc. of medium.

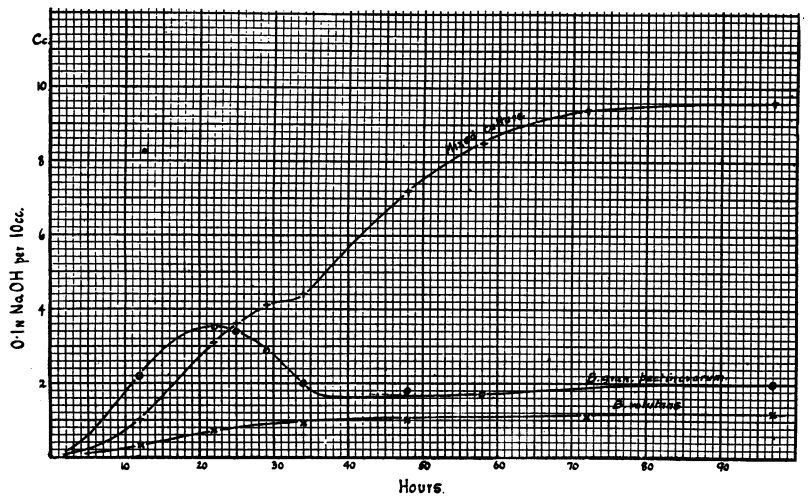

FIG. 1. CURVES SHOWING THE ACIDITIES OF PURE CULTURE FERMENTATIONS BY BOTH ORGANISMS AND OF A MIXED-CULTURE FERMENTATION

contaminated fermentation process of the plants. Much to our surprise we found that the *B. volutans* culture alone did not produce a large amount of acid in maize mash. Still considering that in a mixed culture the lactic acid was produced by this organism, and not by *B. granulobacter-pectinovorum*, we sought

for a possible explanation of the non-production of high yields of lactic acid in flask C. The most logical explanation seemed to be that it was owing to the inability of *B. volutans* to hydrolyse starch. In the mixed culture this operation would be performed by the glucoamylase secreted by *B. granulobacter-pectinovorum*. We therefore studied the behavior of pure cultures of *B. volutans* in media containing lactose or glucose.

Experiment II. Experimental flasks containing different media were sterilized, inoculated and incubated. Samples were withdrawn at intervals from the flasks, and titrated. The information regarding media and inoculum used, and the experimental results are summarized in table 2.

TABLE 2

FLASK	MEDIUM	INOCULATION		ACIDITY AFTER			
		B.v.	B.g.p.	16 hours	37.5 hours	43 hours	60 hours
		cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
A	Broth + 1 per cent maize meal + 2 per cent lactose....	10	0	0.5	0.9	1.0	1.1
B	Same.....	1	10	1.5	3.2	3.5	4.0
C	Broth + peptone + 3 per cent lactose.....	10	0	0.8	1.1	1.1	1.3
D	Same.....	1	10	1.6	2.6	2.8	3.0

We found that in media containing maize and lactose, or peptone and lactose, pure cultures of *B. volutans* do not produce acidities which are at all comparable with those of mixed-culture fermentations. The results obtained from media containing glucose also indicated that the failure to obtain high acidities in maize-mash cultures of *B. volutans* was not due primarily to a shortage of glucose.

Owing to the fact that we still considered *B. volutans* to be the main producer of lactic acid in the contaminated fermentation we carried out experiments to discover whether the products of the *B. granulobacter-pectinovorum* fermentation are used up to form lactic acid. These products would be absent from pure cultures of *B. volutans*, and the low acid production under these circumstances could then be explained. Flasks of maize mash

were fermented to different degrees by *B. granulobacter-pectinovorum*, and then sterilized without loss of volatile products. They were then inoculated with pure cultures of *B. volutans*. The results gave us no encouragement to believe that this was the solution of our problem. We were thus compelled to adopt the hypothesis that the lactic acid formed in a mixed culture was mainly due to the activity of *B. granulobacter-pectinovorum*, and only indirectly to the presence of *B. volutans*. At this time it was known that in pure cultures of *B. granulobacter-pectinovorum* there is a small amount of non-volatile acid, and work was in progress to determine the nature of this acid. In a recent paper (1923) we have shown that in pure cultures of *B. granulobacter-pectinovorum* small amounts of lactic acid are produced. Having adopted the hypothesis that in mixed cultures this particular process is activated in some unknown manner we proceeded to carry out the following experiments.

Experiment III. Four experimental flasks each containing 750 cc. of 5 per cent maize mash were sterilized and inoculated with varying amounts of the two cultures.

Flask A: received 6 cc. of *B. granulobacter-pectinovorum.*

Flask B: received 6 cc. of *B. granulobacter-pectinovorum* + 0.75 cc. of *B. volutans.*

Flask C: received 6 cc. of *B. granulobacter-pectinovorum* + 1.5 cc. of *B. volutans.*

Flask D: received 6 cc. of *B. granulobacter-pectinovorum* + 3.0 cc. of *B. volutans.*

The flasks were incubated together, and acidity determinations made in the usual manner. All the flasks containing a mixed inoculum gave typical acid fermentations. On two occasions the mash in flask D was analyzed by the Duclaux method for volatile and non-volatile acids. The results from the experiment are given in table 3. The curves in figure 2 are based on the results from the control flask A and flask D.

The results given in the first part of table 3 brought to our attention a most suggestive fact, namely, that the highest acidity was not obtained in flask D, which received the largest amount of *B. volutans* culture, but in flask C, which received only one-half of this amount. This matter was investigated further in experiment IV.

TABLE 3

TIME AFTER INOCULATION	ACIDITY			
	Flask A	Flask B	Flask C	Flask D
hours	*cc.*	*cc.*	*cc.*	*cc.*
18.5	3.95	3.20	2.90	3.20
22.0	3.90	4.20	3.80	3.90
29.5	2.40	4.50	4.80	5.20
42.5	3.00		7.30	7.00
80.0	2.90	7.70	9.60	8.70

Duclaux analysis of Flask D

TIME AFTER INOCULATION	ACIDITY DUE TO		
	Acetic acid	Butyric acid	Lactic acid
hours	*cc.*	*cc.*	*cc.*
29.75	0.90	1.50	2.75
80.00	0.85	1.45	6.27

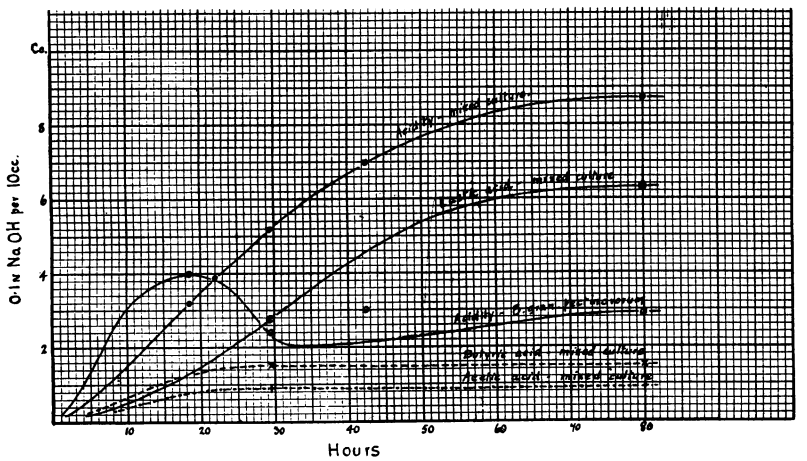

FIG. 2. CURVES SHOWING THE ACIDITY OF A CONTROL, FLASK A, AND OF THE MIXED CULTURE IN FLASK D; ALSO THE DEVELOPMENT OF ACETIC, BUTYRIC AND LACTIC ACIDS IN FLASK D

The results obtained from the Duclaux analyses provide an interesting study when compared with similar results from a fermentation by a pure culture of *B. granulobacter-pectinovorum* (Speakman, 1923, fig. 1). During the first eighteen hours the con-

taminated fermentation differed in the following respects from the normal. There was a reduced production of butyric acid and acetic acid, but an increased production of lactic acid. The most striking differences developed during the period from the twentieth hour to the end of the fermentation. In the contaminated medium gas production fell off rapidly to zero. There was no further production or utilization of volatile acid and consequently no further production of neutral substances. The production of lactic acid continued at an increased rate for about 40 hours, and then gradually fell to zero. The final concentration of lactic acid in the medium was very high, amounting to 6.27 cc. of 0.1N acid per 10 cc. of mash. This amount is considerably higher than that obtained by the cultivation of *B. volutans* in media containing maize, glucose or lactose. It is also greater than the sum of the amounts obtained in separate pure cultures of the two organisms in any of these media. The results obtained indicate that the greater part of the lactic acid was produced by some abnormal physiological type of *B. granulobacter-pectinovorum*. This being the case we considered that there ought to be some definite relationship between the growth of *B. granulobacter-pectinovorum* and the amount of lactic acid produced. Similarly if, by increasing the dosage of *B. volutans* cultures, we could progressively inhibit the growth of *B. granulobacter-pectinovorum*, the yields of lactic acid should vary inversely with the amounts of added *B. volutans* culture. To test the validity of this hypothesis the following experiment was carried out.

Experiment IV. Five flasks of 3 per cent maize mash were sterilized, cooled and inoculated. Each flask received the same volume of *B. granulobacter-pectinovorum* culture, but four of the flasks received different volumes of the *B. volutans* culture. Acidity determinations were made at regular intervals, and the results from the experiment are given in table 4.

The results from this experiment show that as we increased the volume of *B. volutans* in the inoculum, the final acidity of the mash fell. In similar experiments we were able, by further increasing the amount of *B. volutans*, to inhibit to a greater degree

the normal fermentation, and reduce the amount of lactic acid produced. These experiments as a whole afforded additional evidence in favor of our hypothesis that the lactic acid formed is largely produced by *B. granulobacter-pectinovorum.*

Experiment V. We endeavored in this experiment to determine in a more quantitative manner the relationship between the amount of growth and normal activity of *B. granulobacter-pectinovorum,* and the degree to which lactic acid is produced in mixed cultures. The first of these two measurements was made by determining the loss in weight due to gas production. The second measurement was made by titration. Erlenmeyer flasks containing 200 cc. of 3 per cent maize mash

TABLE 4

Effect of increasing quantities of B. volutans in inoculum

FLASK	INOCULUM		ACIDITY OF FLASK AFTER					
	B.g.p.	B.v.	7 hours	12 hours	24.5 hours	31 hours	48 hours	72 hours
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
A	10	0	1.0	2.3	3.6	2.8	2.3	2.4
B	10	1	1.0	1.7	5.0	5.8	7.4	7.7
C	10	2	0.8	1.5	4.3	5.2	6.0	7.0
D	10	3	1.1	1.5	4.1	5.3	6.7	7.4
E	10	5	0.8	1.2	2.7	4.1	5.8	6.6

were sterilized, and cooled. They were then inoculated with varying ratios of the two cultures. Each flask was than closed with a sterilized rubber stopper fitted with an Alwood valve containing H_2SO_4. Before incubation the series of flasks was carefully weighed. At the end of three days they were removed from the incubator and weighed a second time. A 10 cc. sample from each flask was titrated. The results from this experiment are given in table 5 and figure 3.

The results indicate that the fermentations in this group were of three types. Flasks A to D gave normal fermentations, although Flask C was slower than the remainder for some unknown reason. The amounts of *B. volutans* culture added to these flasks had no apparent effect on the metabolism of *B. granulobacter-pectinovorum.* Flasks D to F were all acid fermentations in which only a relatively small amount of carbohydrate was

TABLE 5

Effect of increasing quantities of B. volutans in inoculum

FLASK	INOCULAM		LOSS IN WEIGHT	ACIDITY
	B.g.p.	B.v.		
	cc.	*cc.*	*gm*	*cc.*
A	2.0	0.0	3.605	3.0
B	2.0	0.025	4.069	3.5
C	2.0	0.075	2.422	3.3
D	2.0	0.15	3.711	3.15
E	2.0	0.25	0.461	6.0
F	2.0	0.50	0.454	5.8
G	2.0	1.00	0.387	4.8
H	2.0	3.00	0.183	2.7
I	2.0	5.00	0.205	2.9

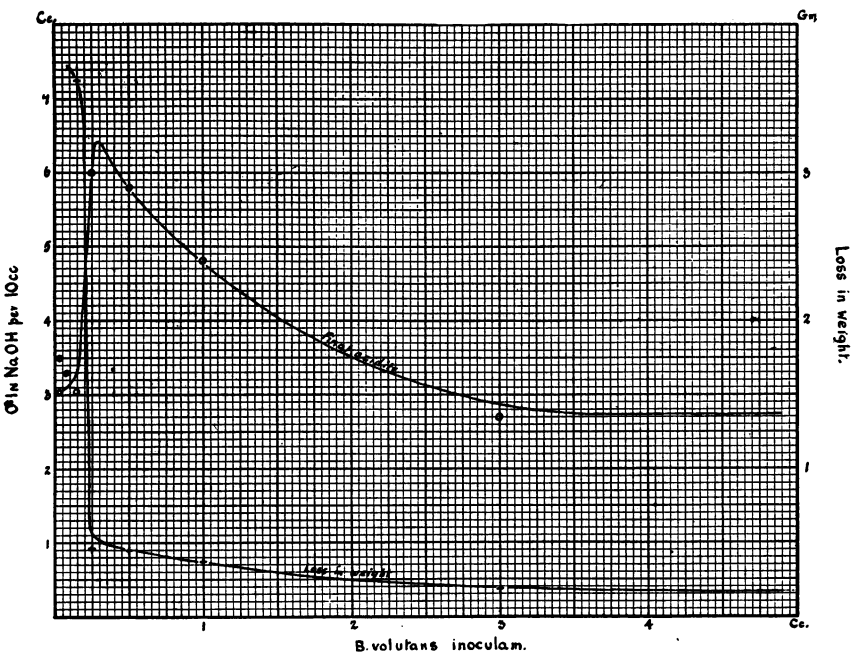

FIG. 3. CURVES SHOWING THE INFLUENCE OF INCREASING AMOUNTS OF *B. volutans* ON THE DEGREE OF NORMAL FERMENTATION BY *B. granulobacter-pectinovorum* AND ON THE FINAL ACIDITY OF THE MEDIUM

utilized. The titration figures show that there is a definite relationship in this group of flasks between, (a) the activity of *B. granulobacter-pectinovorum* as measured by gas production, (b) the amount of *B. volutans* culture added, and (c) the amount of lactic acid produced. The amount of lactic acid produced varies directly with (a) and inversely with (b). These results confirm those obtained in experiment IV. In Flasks H and I there was only a meagre production of gas, and the final acidities were also low. This experiment as a whole was repeated, and the results obtained confirm those which we have given.

DISCUSSION

The results from the experiments which we record show quite clearly that what we regarded during the war-period as a typical contaminated fermentation was only one of several possibilities resulting from the association of these two bacteria. There are fermentations containing both organisms which are indistinguishable chemically from the normal. This type can be reproduced in the laboratory by the addition of small volumes of toxic *B. volutans* culture to the mash, or large volumes of non-toxic *B. volutans* culture obtained by the continued growth of the organism in laboratory media containing sugar. The amount of inhibitory substance added to the mash at the time of inoculation, plus the amount which is developed in the medium, is insufficient to disturb the normal biochemical equilibrium of the cells of *B. granulobacter-pectinovorum*. A second type, represented by flasks H and I in experiment V, is one in which the amount of inhibitory substance in the inoculum is so large that there is hardly any normal growth or activity of *B. granulobacter-pectinovorum* in the mash. The medium contains practically a pure culture of *B. volutans* at the end of three days, and the normal amount of lactic acid is produced by this organism. The third and most interesting type is represented by flasks B to E in experiment IV and flasks E to G in experiment V. The amount of toxic material in the original *B. volutans* culture added to these flasks is not sufficient completely to inhibit the growth and activity of *B. granulobacter-pectinovorum* during the first twenty

hours of the normal fermentation period. Partial inhibition, proportional to the volume of *B. volutans* culture added, does occur however. The result of this relationship is that we find a sub-normal production of gas and volatile acid during this period. At this point, which marks normally the beginning of the most active part of the fermentation, the concentration of the toxic material in the original *B. volutans* inoculum, or more probably this amount plus whatever has been developed in the mash, is sufficient to inhibit completely the following biochemical processes which normally are active in *B. granulobacter-pectinovorum:* (a) the production of volatile acids, (b) the oxidation and reduction of volatile acids, and (c) the oxidation of lactic acid. By each of these processes gas is also produced, and it is possible to follow the progress of the inhibition by observing the rate of gas production. The only normal process which continues in the cells of *B. granulobacter-pectinovorum* is the production of lactic acid. If we interpret the biochemistry of the association in this manner we observe that it resembles the association of *B. coli* and *B. paratyphosus* very closely. There is the same fundamental principle involved, namely, the partial inhibition of one organism by one or more of the metabolic products of the second.

Apart from the experimental evidence contained in this paper for the existence of a type of *B. granulobacter-pectinovorum* which is abnormal in the physiological sense, descriptions exist in the literature of similar mutants from closely allied species. The following passage is taken from the report of Bredemann's exhaustive study of the amylobacter group (1909, p. 446).

> These observations, that at the same time it is possible to transform a strictly anaerobic spore-forming bacillus, which is a vigorous fermenter, into an aerobic, non-spore forming and non-fermenting coccus, seemed so surprising that I have convinced myself of the correctness of this phenomenon with quite unexceptionally pure cultures. The microoidia could not only be observed on one stem, but all stems in so far as I have tested them showed these phenomena in exactly the same manner.

To show the importance of this passage in connection with our problem it is necessary to observe that Bredemann would include *B. granulobacter-pectinovorum* in the amylobacter group. Similar conclusions were recorded in an earlier paper by Winogradsky (1902) who found that it was possible to develop a form of *Clostridium pasteurianum* which differed from the parent stock both morphologically and physiologically. The morphological aspects of this most interesting problem have been discussed recently in great detail by Löhnis (1921). Concerning ourselves only with its physiological aspects, we find that the micro-oidia are produced by heat treatment, or by the accumulation of acid products in the medium owing to the absence of $CaCO_3$. They produce no gas in media containing carbohydrate, but continue to produce acid. It has been shown by Grassberger and Schattenfroh (1907) that from bacilli which normally produce butyric acid, a new bacterium occasionally develops which produces no gas, and although acid production continues, the acid formed is lactic instead of butyric. The literature which we have cited concerning these physiological mutants, confirms our conclusion that in the association which we are investigating, the rapid production of lactic acid is due to a disturbed equilibrium in the physiological processes of *B. granulobacter-pectinovorum*, the change in equilibrium being brought about by the inhibition of certain biochemical processes by some inhibitory factor in the *B. volutans* culture. We are unable to state definitely whether our physiological mutant corresponds morphologically to the micro-oidia of Bredemann. At no stage however in our investigation have we been able to obtain aërobic growth corresponding to that described in the literature.

During the course of our experimental work we have been able to make some observations which are suggestive as to the nature of the inhibitory factor in the *B. volutans* culture. From the original culture of this organism we have obtained a strain which grows luxuriantly in laboratory media with the production of lactic acid, but these cultures do not inhibit or change the physiological behavior of *B. granulobacter-pectinovorum*. It would seem improbable therefore, that the cells of *B. volutans*

or the products of carbohydrate metabolism are the inhibitory agent. We have also found that the production of this factor in cultures of *B. volutans* is dependent upon, (a) a meagre supply of soluble carbohydrate, and (b) a supply of vegetable protein. We have adopted therefore the tentative hypothesis, that the inhibitory agent is a product of the nitrogen metabolism of *B. volutans*.

SUMMARY

1. The biochemistry of the association *B. granulobacter-pectinovorum-B. volutans* has been qualitatively and quanitatively investigated.

2. It has been shown that the characteristic production of large amounts of lactic acid by this association, in media containing carbohydrate, is due to a partial inhibition of the physiological processes of *B. granulobacter-pectinovorum* by some factor produced in *B. volutans* cultures.

3. The inhibitory factor is not characteristic of cultures of *B. volutans* grown for several generations in artificial media containing sugars.

4. The tentative hypothesis has been adopted, that this substance is a product of the nitrogen metabolism of *B. volutans* growing in media containing vegetable protein and only traces of carbohydrate material.

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PLATE 1

FIG. 1. Preparation made with mash from a contaminated fermenter, showing *B. granulobacter-pectinovorum* in the form of faintly stained rods and the more deeply stained chains of *B. volutans*.

FIG. 2. Colonies of *B. volutans* on an aerobic wort-agar plate. The stroke-culture was made from the same mash as the slide of figure 1.

FIG. 3. Preparation made from a pure culture of *B. volutans* in wort. The culture was derived from a single colony fished from the growth in figure 2.

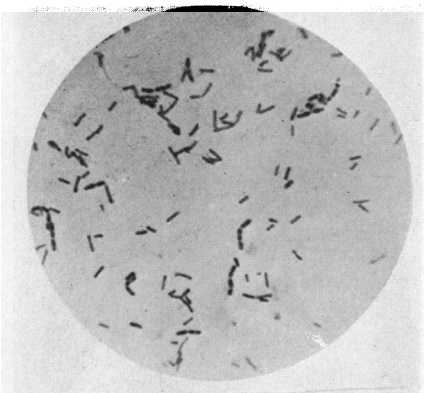

FIG. 1

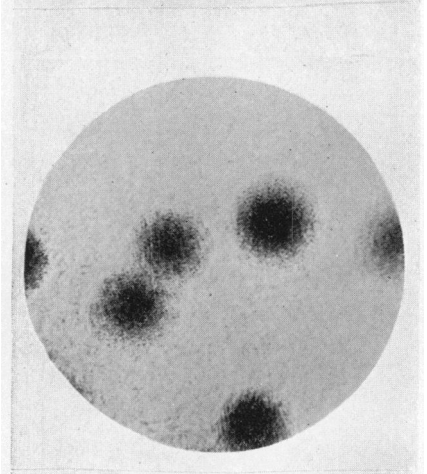

FIG. 2

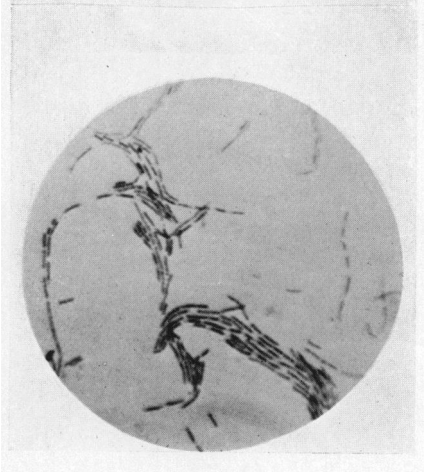

FIG. 3

(Speakman and Phillips: Biochemistry of a bacterial association)