BACTERIAL SYNERGISM—THE FORMATION BY *B. TYPHOSUS* OR *B. COLI ANAEROGENES* FROM MANNITOL OF AN INTERMEDIATE SUBSTANCE FROM WHICH MORGAN'S BACILLUS PRODUCES GAS[1].

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In the operations of bacteria under natural conditions it is the general rule to find mixed cultures at work, the variability of the results depending upon many factors. Therefore it has been suggested by Holman (1928) that the general term "bacterial association" be used to cover such processes. When, however, the combined action of two or more micro-organisms effects changes which each by itself is incapable of achieving, the term "synergism" is applied. Synergism appears to be used now in a more restricted sense to describe a particular type of bacterial association, and has been defined by Fiallos (1925) as follows: "two bacilli neither of which causes the production of gas in certain compounds, may do so when artificially mixed together provided one of them is capable of producing acidity (never gas) in these carbon compounds, and the other though inert to these compounds (*i.e.* produces in them neither acid nor gas) is capable of producing gas from glucose." In 1911 Penfold observed the production of gas from a glucose medium in which *B. typhosus* was growing along with a variant non-aerogenic strain of *B. coli communis*, the latter having been derived from a typical gas-producing culture by selective growth on agar containing sodium monochloracetate. Although this organism had lost the power of gas-fermenting glucose it retained the power of gas-fermenting sodium formate. Castellani (1925, 1926 and 1927) states that he noted the phenomenon, which he calls "symbiotic," in 1904 when investigating the fermentation reactions of bakers' yeast, which is not a pure culture but consists generally of two or more species of yeasts together with one or more types of Gram-negative bacilli. The bakers' yeast *in toto* fermented with the production of gas a larger number of sugars than any of the organisms isolated from it. The bacilli isolated from these samples of yeast did not ferment sugars with the formation of gas but caused acidity only. The work of others has multiplied examples of synergic gas formation. Thus Sears and Putnam (1923), using lactose, saccharose and mannitol as the fermentable substances, discovered a

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considerable number of gas-producing pairs of organisms. They did not think that the age of the culture or the numbers of each type of micro-organism added affected the gas production; but they concluded that both bacteria must be living together. These workers did not consider that any information was to be obtained by analysis of the gas produced, since they found that the ratio of hydrogen to carbon dioxide was not constant. Holman and Meekison (1926) investigated synergic gas production from saccharose by *Streptococcus faecalis* and a strain of *B. coli communis.* They pointed out that the hydrogen-ion concentration played an important part, since gas production was finally checked by the increasing acidity of the medium due to the continued growth of the streptococcus. A striking difference was noted by these workers in the composition of the gas formed by synergic action as compared with that formed by the glucose-fermenting member when acting on that sugar. Thus, in saccharose broth with *B. coli* (non-fermenter of saccharose) and *Streptococcus faecalis* (by itself producing from saccharose acid only) the ratio of hydrogen to carbonic acid gas produced was 4 : 1. When *B. coli* was grown alone in glucose the ratio was 1·5 : 1. This was held to be suggestive of a different type of breakdown occurring in the two cases. The authors were unable to substitute for the viable organisms cultures killed by formalin vapour. Holman and Meekison mention a striking example of synergic gas production in a mixed culture of paratyphoid bacilli and *Streptococcus faecalis* in lactose. Gas was formed although the former organism was so greatly in excess of the streptococcus that a film from the mixture showed apparently a pure culture of the bacillus. In this connection the possible fallacy in the examination of cultures of faeces from suspected cases of enteric fever is noted.

As regards the nature of synergic action, it seems to have been generally accepted that for the production of gas the two organisms must be present together in the living state in the fermentable medium (see Silber and Nikolskaja, 1929). The hypothesis is not favoured that an intermediate substance is produced by the one organism and subsequently further broken down by the other with the production of gas. Of course, the intermediate substance might be chemically unstable and incapable of accumulating. Sears and Putnam concluded also that unless the gas-forming organism was present along with the acid-producing organism the intermediate product was at once further degraded by the latter to a substance which did not yield gas. Harden (1930), in reviewing the subject, concludes that an intermediate substance (formic acid) is formed, but no definite experimental proof of this appears to have been published hitherto.

The present work, while it verifies that of others in general respects, supplies direct evidence that in the case studied—the formation of gas from mannitol by Morgan's bacillus along with either *B. typhosus* or *B. coli anaerogenes*—an actual symbiosis of the organisms is not required, since *B. typhosus* forms a relatively stable intermediate substance which is then acted upon with gas formation by *B. morgani.*

METHODS.

Synergic gas production was studied in a medium containing 1 per cent. mannitol in 2 per cent. peptone water. Andrade's indicator was added and the reaction adjusted to a *p*H of 7·2 unless otherwise noted.

In the initial experiments the synergic phenomenon was verified. The following account represents the results obtained from many repetitions of the type of experiment quoted. Durham's fermentation tubes containing 3 c.c. of mannitol medium were used and the cultures were kept for 24 hours at 37° C.: (*a*) those into which *B. typhosus* alone had been introduced showed acid production only; (*b*) those containing *B. morgani* alone showed no change; while (*c*) those containing *a mixture of the two organisms* showed production of acid and gas. Occasionally gas was not seen after 24 hours but appeared after 48 hours. Gas production occurred mainly during the first 48 hours, and ceased under ordinary circumstances after 72 hours. By the end of 4 days' further incubation at 37° C. a very slight shrinkage in the total volume of gas had taken place.

Changes in the H-ion concentration during gas production.

The degree of acid production was estimated by the withdrawal of small samples from the tubes at 24-hour intervals, which were tested with the usual indicators. It was noted that in the *mixture of the two organisms*, during the period of active gas production, the degree of acid formation was always less than with *B. typhosus* in pure culture. Thus in the mannitol medium inoculated with *B. typhosus*, after 24 hours at 37° C. the *p*H had changed from 7·2 to 5·2, and after 72 hours to 4·4. At the end of 240 hours at 37° C. the *p*H was below 4·0, a degree of acidity incompatible with continued viability of the bacillus; indeed, it was occasionally impossible to recover the organism after 72 hours' culture. *B. morgani*, when growing in mannitol medium, produced after 48 hours a slight change to the alkaline side (*p*H between 7·2 and 7·4). On the other hand, when a mixture of the two organisms was introduced into the medium the *p*H after 24 hours' incubation was 5·5, and after 72 hours 5·0.

Growth of the organisms during gas production.

With equal inoculations of both organisms in mannitol medium, after 24 hours' incubation, a loopful of the culture stroked on a Petri plate containing MacConkey's bile salt agar made up with mannitol showed *B. typhosus* (red colonies) to be considerably in excess of *B. morgani* (white colonies), approximately in the proportion of 3 or 5 colonies of the former to 1 of the latter. After 72 hours *B. morgani* was either not recoverable or was extremely scanty, while *B. typhosus*, although recoverable with ease, was much less abundant than after only 24 hours' growth. But when the mixture of organisms was inoculated into a medium whose reaction remained faintly alkaline after growth, *e.g.* 1 per cent. sodium formate in peptone water, *B. morgani* was always recoverable considerably in excess of *B. typhosus*. Accordingly it appeared that the *p*H of the medium might have an important effect on the phenomenon of synergic gas formation. To test this, *B. typhosus* was inoculated into a series of Durham's fermentation tubes containing 1 per cent. mannitol

in 2 per cent. peptone water and incubated for 24 hours at 37° C. The usual inoculum of *B. morgani* was then added to the tubes. Gas production did not take place, *B. morgani* failing to grow. On the other hand, if the *p*H of the medium was adjusted to between 6·5 and 7·2 with sterile 5 per cent. NaOH solution just prior to the addition of *B. morgani*, gas production took place in another 24 hours. Further, by similarly readjusting the H-ion concentration not later than every 72 hours it was possible to maintain gas production in the mixed culture for at least 216 hours, instead of only 72 hours as in the untreated tubes; thus the resulting volume of gas amounted to approximately three times that obtained in the uncorrected medium. If *B. morgani* was introduced into the medium a short time after *B. typhosus*, *e.g.* 3½–7 hours, when acid formation was not yet marked, there was, of course, production of gas. *B. morgani*, which did not produce acid in the medium, could be grown for at least 72 hours prior to the introduction of *B. typhosus* without interfering with the phenomenon.

The question of alteration in biological characters resulting from association of the organisms.

In order to determine whether after their association the two organisms had undergone any change in biological characters the following experiment was carried out. After 48 hours' growth at 37° C. in mannitol medium in which active gas production was taking place, a loopful of the mixed culture was plated on MacConkey's agar made up with 1 per cent. mannitol instead of lactose. At the end of 24 hours' incubation the plate showed red and white colonies in the proportion of about 5 to 1. A red colony and a white one were picked off and each replated on mannitol MacConkey agar and found to give rise to colonies which were consistently all red and all white. A colony of each type was then investigated. The former gave the fermentation reaction of *B. typhosus* and showed agglutination with an antityphoid serum up to titre (1 : 32,000). The latter gave the cultural reactions of *B. morgani* and was not agglutinated by a 1 : 50 dilution of the same antityphoid serum.

Accordingly, the association of the two organisms led to no obvious alteration of the biological characters investigated.

The production of an intermediate substance.

It appeared likely that gas production takes place in two stages, viz. that *B. typhosus* ferments mannitol with the formation of an intermediate substance which, under suitable conditions, *B. morgani* is able to degrade further with gas production. To test this hypothesis experiments were carried out as follows. *B. typhosus* was cultured at 37° C. for 24, 48 and 72 hours in a series of Durham's tubes in peptone water containing 1 per cent. mannitol. At the end of these periods the tubes were placed in a water bath at 56° C. for 45 minutes to kill the cultures. After adjusting the *p*H of each tube to 7·2 with sterile 5 per cent. caustic soda solution *B. morgani* was added and the cultures incubated again at 37° C. In from 24 to 48 hours a large bubble of gas appeared in the tubes in which *B. typhosus* had been grown for 48 hours and also 72 hours. Where *B. typhosus* had grown for only 24 hours, gas production was less marked or absent; but in some experiments the inoculation of 50 c.c. mannitol peptone

water with three loopfuls of a 24 hours' agar culture of *B. typhosus* led after 7 hours at 37° C. to sufficient intermediate substance to be detected by gas formation when, after sterilising by heat, *B. morgani* was subsequently grown in the medium. The procedure was also varied as follows. *B. typhosus* was grown alone in the mannitol medium for 48 hours, the *p*H was then adjusted to 7·2 with caustic soda solution and the culture killed by heat. Subsequent incubation at 37° C. for 72 hours failed to induce gas production. The addition of *B. morgani* at this point resulted in the appearance of gas after 24–48 hours' further incubation at 37° C.

Such experiments indicate that gas production can be ascribed solely to the action of Morgan's bacillus on some intermediate product formed by the prior action of *B. typhosus*. When gas developed the reaction moved toward the alkaline side, from *p*H 7·2 to 8·0. Gas production was almost entirely confined to the first 48 hours. In all cases subcultures made at this time yielded only organisms with the characters of *B. morgani*. It should be noted that in no instance was gas production quite so abundant as when the two organisms were present together in the living state.

A faintly acid medium afforded the optimum condition for gas production by *B. morgani* after the initial growth of *B. typhosus* and sterilisation of the latter by heating; this corresponds with the observation of Grey (1914) that *B. coli* produces gas most abundantly from calcium formate when the reaction of the medium is slightly acid. Thus, at an initial *p*H of 6·8, 3·4 c.c. of gas were produced from approximately 25 c.c. of medium, whereas at a *p*H of 7·2 the yield was 2·3 c.c. of gas, the maximum volume in both cases being reached after 72 hours at 37° C. As will be noted later, the simultaneous inoculation of *B. morgani* and *B. typhosus* yielded under similar conditions from 3 to 5 c.c. of gas in 72 hours, and at least 6·5 c.c. was obtained before gas production ceased—the acid reaction of the medium was not neutralised at any stage in such experiments.

In similar experiments *B. morgani* was grown first of all in the mannitol medium for periods up to 72 hours at 37° C. The cultures were then killed at 56° C. (subcultures on agar being sterile) and thereafter *B. typhosus* was added. In no case did gas production occur, but only acid, after incubation for 72 hours. At this point the *p*H of the tubes was readjusted to 7·2 and *B. morgani* added. Gas and also acid then appeared in from 24 to 48 hours in all instances.

Production of intermediate substance by B. coli anaerogenes. Instead of *B. typhosus* there was also employed a non-aerogenic strain of *B. coli* isolated from an old laboratory culture of genuine *B. coli* type, which fermented the same carbohydrates as the latter but without production of gas. The results with this organism were the same as with *B. typhosus* as regards the synergic phenomenon.

Properties of the intermediate substance.

Thermostability. The intermediate substance produced by *B. typhosus* was found to resist heating at 100° C. for 15 min., since on inoculation of the heated medium with Morgan's bacillus gas was formed. A slightly larger volume of gas appeared to result, however, from a portion of the culture which had been heated at 56° C. for 3 hours.

Filterability of the intermediate substance was demonstrated as follows. *B. typhosus* was grown for 120 hours in 50 c.c. of 1 per cent. mannitol peptone water medium. Then the *p*H of the medium was adjusted to 6·8 and the fluid passed through a Seitz filter. Of the filtrate (which was proved to be sterile by cultural tests) 10 c.c. were pipetted into a fermentation tube which was inoculated with *B. morgani* and incubated at 37° C. Gas production, without acid, took place after 24 hours. Filtrates never yielded so large a volume of gas as was obtained by the other methods; this seemed to be due to the rather less vigorous growth of *B. morgani* in the filtrate.

Failure to produce the intermediate substance by means of killed cultures of B. typhosus.

A heavy 24 hours' growth of typhoid bacilli on agar in a Kolle flask, 4 in. in diameter, was suspended in 2 c.c. of normal saline; 6 c.c. acetone were added and after 50 min. the mixture was centrifuged for 10 min. and the fluid removed. The sediment was resuspended in 8 c.c. saline and centrifuged at high speed for 60 min. The sediment was then suspended in 2 c.c. of normal saline and by way of control 0·5 c.c. of this dense emulsion was added to a tube of peptone water which was kept at 37° C. for 10 days without growth resulting, *i.e.* the method sufficed to kill the *B. typhosus*. (In a preliminary test 30 min. treatment by acetone failed to kill the organism.) To each of three Durham's fermentation tubes containing 4 c.c. of 1 per cent. mannitol peptone water 0·5 c.c. of the killed suspension of *B. typhosus* was added and at the same time a living culture of *B. morgani*. The tubes were then incubated at 37° C. for 72 hours without either acid or gas production, *B. morgani* being alone recovered on subculture. At this point living *B. typhosus* was added to the tubes, when acid and gas formation occurred in 24 hours. An exactly similar experiment was carried out with a dense suspension of *B. morgani* killed by acetone. Living *B. typhosus* was added and acid production only took place. After 72 hours' incubation the *p*H was adjusted to 6·8 and living *B. morgani* added, when gas and acid production occurred.

Additional experiments were carried out on the same lines, in which heat (50 min. at 56° C.) was used to kill the cultures of *B. typhosus*, and in one instance *B. typhosus* was emulsified directly in the mannitol medium, the tube being subsequently kept at 56° C. for 45 min. to kill the organisms. In none was gas formed on inoculating with *B. morgani*, even after 10 days' incubation at 37° C., although the latter organism grew vigorously.

These results indicate that the intermediate product required for gas production by *B. morgani* cannot be formed by even dense suspensions of *B. typhosus* killed by acetone or by heating at 56° C., while killed cultures of Morgan's bacillus cannot replace the living organism.

Composition of the gas as bearing on the nature of the intermediate substance.

Sufficient gas for analysis was obtained as follows. A 50 c.c. burette, graduated from the stop-cock arm, was bent into the form of a U-tube and filled with mannitol medium. The open arm, which was plugged with cotton-wool, served for making inoculations and withdrawing samples for investigation. Thus gas was collected from approximately 25 c.c. of medium in the closed arm. In the experiments described below the *p*H of the medium was not readjusted. The composition of the gas was ascertained as follows. The fluid was rendered highly alkaline by the addition of caustic soda solution, and gas and fluid were then brought into intimate contact by shaking. The resulting diminution in volume measured the amount of carbonic acid gas formed. The nature of the remaining gas was determined by noting whether it was inflammable without explosion; in all cases it burned silently with a bluish flame. Such inflammable gas has been regarded as consisting wholly of hydrogen, and it was ascertained by actual analysis that this is the case (*vide infra*), there being merely a small admixture of oxygen. As Harden (1930) has pointed out, the superior solubility in water of carbon dioxide as compared with hydrogen introduces an inaccuracy into such measurements; but the results obtained under similar conditions are comparable, and the majority of workers have measured the gases over watery media, *e.g.* Theobald Smith (1895).

With the mixed culture of *B. typhosus* and *B. morgani* gas production proceeded for 168 hours, but the greater part was formed in the first 72 hours. In one experiment 6·5 c.c. gas were obtained after 168 hours. The volume had decreased to 6·4 c.c. after 240 hours; analysis of the gas at this time yielded inflammable gas to carbon dioxide in the ratio of 4 to 1. In another experiment the gas, measuring 5·1 c.c., was examined after 72 hours and contained a similar proportion of the two components. Again, *B. typhosus* was cultured alone in the medium for 96 hours; the *p*H which had changed from 7·2 to 4·4 was then adjusted to 6·8 and the apparatus kept at 56° C. for 50 min. to kill the organisms (verified by subculture). *B. morgani* was then added, and at the end of 120 hours' incubation at 37° C. 4·2 c.c. gas had collected in the closed arm—the *p*H being 7·2. The medium was made slightly acid with 20 per cent. sulphuric acid in order to liberate any carbon dioxide which might be held as carbonate in the medium (Ayres and Rupp, 1918); this procedure yielded a total volume of gas of 4·7 c.c. On analysis the ratio of inflammable gas to CO_2 obtained was 3·7 to 1. This approximates closely to the gas ratio in the usual synergic association. When *B. morgani* and the non-aerogenic strain of *B. coli* were grown together in mannitol medium for 96 hours, 6·8 c.c. of gas resulted, the ratio of inflammable gas to carbon dioxide being 4·7 to 1.

The exact composition of the gas produced was determined in experiments in which *B. typhosus* was grown for 24 hours and the medium was then sterilised, the reaction being adjusted to *p*H 6·8, and reinoculated with *B. morgani* which was allowed to grow for 120 hours. The resulting gas (which varied from 2 to 3·8 c.c. in different tests) consisted of hydrogen 88 per cent., carbon dioxide 10 per cent., and oxygen 2 per cent. (It is to be noted that since the medium was not acidulated a proportion of the carbonic acid in the form of carbonate escaped detection*.)

For purposes of comparison a typical strain of *B. coli* was next tested in 1 per cent. mannitol medium. After 72 hours 14·3 c.c. of gas had developed and the *p*H of the medium was 5·2. The gas consisted of inflammable gas to carbon dioxide in the ratio of 2·1 to 1. Again, when *B. morgani* was grown in 1 per cent. glucose medium for 48 hours at 37° C., 8 c.c. of gas was obtained, which consisted of inflammable gas to carbon dioxide in the ratio of 2·6 to 1.

Accordingly, in the gas produced by the associated organisms either when growing together or in succession, there is a considerable excess of inflammable gas as compared with that obtained by the action of *B. morgani* alone acting on glucose, or *B. coli* acting on mannitol. As has been mentioned, a similar result was obtained by Holman and Meekison with a mixture of *Streptococcus faecalis* and *B. coli* in saccharose medium.

Although there may be a relationship between synergic gas production and the capacity of one of the associated organisms to form gas from glucose, it has been shown that glucose does not accumulate as an intermediate product. Thus the medium in which either (*a*) *B. typhosus* alone, or (*b*) the mixture of *B. typhosus* and *B. morgani* had been grown, failed to reduce Benedict's solution. In this connection it was noted that by means of Benedict's solution glucose was detected in the medium in a concentration of 1 : 2000. It was likewise found that *B. morgani* produced gas in appreciable quantity from peptone water containing 1 : 1000 of glucose, but if the concentration was reduced to 1 : 2000 gas production was not apparent. Additional evidence against glucose being an intermediate product is afforded by the different proportions in which the mixture of gases is formed by the mixed culture in mannitol as compared with that formed by *B. morgani* in glucose. Again, it has been noted that *B. morgani* develops gas from the intermediate product produced by *B. typhosus* in the mannitol medium without concurrent acid formation; under the latter condition indeed the reaction changes to the alkaline side.

Several substances are decomposed by organisms with the formation of gas and alkaline products. Sodium formate is broken up in this way by *B. morgani*, *B. coli*, *B. paratyphosus B*, etc. Harden originally (1901) demonstrated that when *B. coli* acted on glucose or mannitol formic acid was produced, and he considered that it was the source of the gas. He found that *B. typhosus* and a non-aerogenic strain of *B. coli* each produced formic acid, but were unable

* I am indebted to Dr S. V. Telfer for these results.

to decompose it with gas formation. Karczag with Móczár (1913) and Schiff (1915), following up the observation of Neuberg and Hildesheimer (1911), showed that pyruvic acid was attacked with gas production by bacteria which are capable of forming gas from sugars. According to these observers, with *B. coli* the gas consisted mainly of hydrogen up to about 90 per cent. and carbon dioxide to about 10 per cent.

With the strain of *B. morgani* used in the present work, after 72 hours' growth at 37° C. in a medium containing 1 per cent. sodium formate, 8·9 c.c. of gas were produced, of which only a trace (0·3 c.c.) was absorbable with caustic soda, the remainder being inflammable; the *p*H moved from 7·2 to between 8·0 and 8·5 during the period of incubation. In a second experiment, after 96 hours' incubation 11·8 c.c. gas were obtained, which on acidulation of the medium was increased to 13·7 c.c.; thus, the ratio of hydrogen to carbon dioxide was 6·2 to 1. When calcium formate was used instead of the sodium salt, as suggested by Grey (1914, 1924), the results were similar. It is to be noted that with a considerable range of concentrations of calcium formate the ratio of hydrogen to carbon dioxide in the gas produced remained practically constant (the medium was previously acidulated in every instance, so as to decompose any carbonates present; controls showed that the sterile medium yielded no gas on acidulation). For example, in an experiment in which peptone water neutral to litmus containing 0·5, 1·0 and 1·5 per cent. of calcium formate respectively was inoculated with *B. morgani* and kept for 96 hours at 37° C. the ratios were 6·5 to 1, 6·8 to 1 and 6 to 1, the total volumes after acidulation being respectively 10·2, 15·6 and 18·8 c.c. With 3 per cent. of calcium formate growth was scanty and the amount of gas too small for measurement. The volume of gas obtained from 0·5 per cent. calcium formate inoculated with *B. morgani* was practically equal to that produced in medium containing 1 per cent. mannitol by the synergic action of *B. morgani* and *B. typhosus*. On the other hand, *B. morgani* in a neutral medium containing 0·25 per cent. sodium pyruvate altered the reaction after 24 hours to the acid side (*p*H between 5·5 and 6) and later to the alkaline side. After 72 hours' incubation, when the *p*H had returned to 7·2, 11·3 c.c. of gas had been produced (an amount exceeding that developed in the synergic reaction, which was about 8 c.c.); this consisted of hydrogen to carbon dioxide in the proportion of 2·14 to 1. The high proportion of hydrogen recorded by Karczag and Móczár as being produced from pyruvic acid was never obtained. Accordingly, with formate alone but not with pyruvate, when decomposed by *B. morgani* a gas ratio was obtained similar to that in the synergic reaction. Further, pyruvic acid could not be demonstrated in the products of synergic growth by the reaction of Alvarez, with a solution of α-naphthol in sulphuric acid. In this connection it may be noted also that in the products of growth of *B. coli* in glucose-containing medium Grey demonstrated only a trace of pyruvic acid.

A number of other organic acids in the form of the sodium salts were also tested for the formation of gas when *B. morgani* and *B. typhosus* were grown

together in the medium containing them, but with negative results in every case, although abundant proliferation of organisms occurred. (Others have found that *B. coli* does not produce gas from them, and this has also been confirmed in the present work.) The acids used were as follows, the concentration in the medium being 1 per cent. unless otherwise stated—only those in italics yielded gas:

Saturated monobasic group: *formic*, acetic, propionic, butyric, caproic (0·25 per cent.), caprylic (0·25 per cent.).

Hydroxy acids: glycollic, lactic.

Keto acids[1]: *pyruvic* (0·25 per cent.), acetoacetic, laevulic.

Saturated dibasic group: oxalic, malonic, succinic, glutaric.

Hydroxy acids: tartronic, malic, tartaric.

Unsaturated dibasic group: fumaric, maleic.

Tribasic hydroxy acid: citric.

Conclusions.

1. As has been found by Penfold and others, certain organisms when growing together exhibit the so-called synergic reaction, *i.e.* the production of gas from a fermentable substance, although none of the organisms alone forms gas from it. (It has been generally held that synergic gas formation occurs only when one of the organisms present is capable of forming gas from glucose or at least from sodium formate.)

2. In the example studied, the synergic action of *B. typhosus* (or a non-aerogenic *B. coli*) together with *B. morgani* on mannitol, it has been shown that by the growth of *B. typhosus* alone in the medium an intermediate substance is formed, which is stable at 100° C. When the *B. typhosus* is killed, *e.g.* by heat, and *B. morgani* is grown in the medium subsequently, this intermediate substance is decomposed with the development of gas. The gas so formed is a mixture similar to that produced when the organisms grow together in the medium containing mannitol.

3. The amount of gas formed by the mixed culture depends greatly on the hydrogen-ion concentration, since acid formation inhibits and finally kills one or both organisms, and so brings to an end gas formation. By adjusting the reaction of the culture at intervals it is possible to maintain both organisms alive and active for a long period; thereby a much greater formation of gas occurs.

4. The mixture of gases formed by synergic action is quantitatively different from that produced by the glucose-fermenting organism when acting by itself on glucose or by *B. coli* acting on mannitol, since in the first case the proportion of hydrogen to carbon dioxide is much higher than in the two latter.

[1] The keto acids, which were sterile, were added directly to sterile peptone water, and the reaction was then rendered neutral to Andrade's indicator by the addition of sterile NaOH solution.

5. The high proportion of hydrogen formed by synergic action corresponds with that produced by the glucose-fermenting organism when acting alone on formates. The gas formed by the latter organism from salts of pyruvic acid does not contain this high proportion of hydrogen. Thus the case of synergic action supports Harden's view as to the part played by formates as the precursor of gaseous products.

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