Reprinted from
THE JOURNAL OF INFECTIOUS DISEASES, Vol. 32, No. 4, April, 1923, pp. 270-279

GAS PRODUCTION BY BACTERIA IN SYMBIOSIS

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There seem to have been few instances observed of the production by bacteria in association of substances which none of the associated organisms alone can produce from the same medium. Nencki [1] mentions the formation from glucose of normal butyl alcohol by mixtures of B. paralactici and B. chauvœi, neither of which in pure culture produces this substance. Burri and Stutzer [2] observed the production of free nitrogen from sodium nitrate by mixtures of B. coli and B. denitrificans. In pure culture neither of these organisms can reduce nitrates to free nitrogen. Recently Knorr [3] reported that certain foul odors are produced in liver broth by the symbiotic action of Fusobacterium and a streptococcus, while pure cultures of each organism on the same medium were entirely odorless. This author also observed the formation of gas in the liver medium by the combined action of Fusobacterium, Sp. sputigenum and streptococci. When streptococci were absent, no gas was formed.

Through some observations made in connection with another research, we had been led to believe that the production of gas in carbohydrate mediums might be brought about by the joint action of two or more organisms not by themselves capable of producing gas on the sugar used. Accordingly, a number of tests were made with mixtures of the ordinary laboratory strains, using lactose, saccharose and mannite as the fermentable substances. The result was that a considerable number of gas-forming pairs were discovered. Table 1 gives the positive results of these tests. On account of a generalization which we were able to formulate from our experiments, it is not considered necessary to record the negative findings.

The medium used had a meat extract peptone base and contained 1% of the fermentable substance. The reaction was adjusted to P_H 7. Pfannstiehl sugars were employed in all tests, and proper controls were made to rule out possible impurities or partial decomposition of

Received for publication, Jan. 5, 1923.

[1] Centralbl. f. Bakteriol., 1892, 11, pp. 225.
[2] Ibid., 1894, 16, p. 815.
[3] Ibid., 1922, I., O., 87, p. 536.

the sugars in the process of sterilization. Five inch Smith fermentation tubes were used, and inoculation was made by transferring one loopful of each species from a 24-hour pure culture in the same medium.

Numerous repetitions of the tests of table 1 were made at different times and with different batches of medium, and it was found that the gas percentages obtained varied within wide limits. The figures given are from one test only. Though in some cases the amount of gas produced by a given pair of organisms ran very low, at no time did

TABLE 1

GAS PRODUCTION BY PAIRS OF ORGANISMS INCUBATED TOGETHER IN SUGAR PEPTONE BROTH

Organisms Inoculated			Sugar	% Gas in 5 Days at 37 C.
B. prodigiosus	+	B. cholerae suis	Lactose	12
Staphylococcus aureus	+	B. paratyph. B	Lactose	5
Streptococcus fecalis	+	B. cholerae suis	Lactose	5
Strep. fecalis	+	B. paratyph. B	Lactose	30
Strep. fecalis	−	B. pneumoniae	Lactose	24
Staph. aureus	+	B. proteus vulg.	Lactose	5
Strep. fecalis	+	B. morgani	Lactose	5
B. butyricus	+	B. proteus vulg.	Lactose	10
Vibrio proteus	+	B. proteus vulg.	Lactose	5
B. lautus	+	B. proteus vulg.	Lactose	10
B. prausnitzii	+	B. proteus vulg.	Lactose	10
Staph. aureus	+	B. coli communis	Saccharose	10
Strep. fecalis	+	B. coli communis	Saccharose	12
B. butyricus	+	B. coli communis	Saccharose	22
Sp. metchnikovi	+	B. paratyph. B	Saccharose	10
Sp. cholerae	+	B. paratyph. B	Saccharose	9
Staph. aureus	+	B. icteroides	Saccharose	25
B. viscosum	+	B. cholerae suis	Saccharose	10
B. prodigiosus	+	B. cholerae suis	Saccharose	10
Strep. fecalis	+	B. acidi lactici	Saccharose	15
Staph. aureus	+	B. proteus vulg.	Mannite	12
Strep. fecalis	+	B. proteus vulg.	Mannite	16
Strep. hemolyticus	+	B. proteus vulg.	Mannite	26
Strep. viridans	+	B. proteus vulg.	Mannite	50
B. dysenteriae, Flexner	+	B. proteus vulg.	Mannite	20
B. dysenteriae Y	+	B. proteus vulg.	Mannite	12
B. typhosus	+	B. proteus vulg.	Mannite	24
B. centrosporus	+	B. proteus vulg.	Mannite	9

we find that a pair giving gas on one occasion would completely fail to produce gas on other occasions, provided the conditions were kept as nearly the same as possible. The ability to produce gas from a given sugar seemed to be a constant quality of the combination.

The experiments described were carried out using a single strain of each species. The question then naturally arose whether the ability to form a gas-producing complex with any given organism is a characteristic of a species or whether there are individual variations in this quality. The data in table 2 answer this question in part. It will be observed that all strains reacted regularly, except B. paratyphosus A 152. In the case of this organism in combination with streptococcus

G3, repeated trials failed to elicit any trace of gas formation. So far as our experiments go, therefore, we can say only that an occasional strain is encountered which fails to react in the same way as the majority.

All the streptococcus strains of table 2 were Streptococcus fecalis according to Holman's classification. The paratyphoid strains and proteus strains 6 and 213 were collection strains of unknown origin. All other proteus strains were recently isolated from infant stools. All cultures were proved to be morphologically and culturally typical, and the paratyphoid strains were known to be serologically true to type.

TABLE 2

GAS PRODUCTION BY PAIRS OF DIFFERENT BACTERIAL STRAINS

Organisms Inoculated	Sugar	% Gas in 5 Days
Strep. G_3 + B. paratyph. B 162	Lactose	5
Strep. G_3 + B. paratyph. B 163	Lactose	5
Strep. G_3 + B. paratyph. B 164	Lactose	35
Strep. G_3 + B. paratyph. B 165	Lactose	40
Strep. G_3 + B. paratyph. A 152	Lactose	None
Strep. G_3 + B. paratyph. A 153	Lactose	30
Strep. G_3 + B. paratyph. A 154	Lactose	25
Strep. N_1 + B. proteus A_3	Mannite	20
Strep. N_1 + B. proteus I_3	Mannite	25
Strep. N_1 + B. proteus 213	Mannite	25
Strep. N_1 + B. proteus L_2	Mannite	20
Strep. N_1 + B. proteus 6	Mannite	15
Strep. N_1 + B. proteus L_2	Fructose	10
Strep. G_3 + B. proteus L_2	Fructose	9
Strep. V_1 + B. proteus L_2	Fructose	12
Strep. O_1 + B. proteus L_2	Fructose	4

CONDITIONS FAVORING GAS FORMATION BY A SYMBIOTIC PAIR OF ORGANISMS

For the study of the effect of the conditions of inoculation and composition of the medium on the rapidity and amount of gas formation, a single symbiotic pair was chosen, Streptococcus fecalis N_1 and B. proteus-vulgaris L_2. Both of these strains had been isolated recently from infant stools. Mannite was used as the fermentable substance. The proteus strain was incapable of attacking this substance alone. The streptococcus fermented it readily with acid formation. The progressive change in the reaction of the medium due to the growth of each organism separately and to that of the two together is shown in table 3. The method used here was to inoculate about 50 c.c. of the medium in small flasks with the specified organisms and then withdraw at intervals a small quantity, 5 c.c., and determine the hydrogen-ion

concentration by the method of Medalia.[4] It is seen that the streptococcus rapidly produces a high acidity and that the proteus strain produces an initial increase of the hydrogen-ion concentration followed by a gradual lowering until a decidedly alkaline reaction is reached. This P_H curve is characteristic of B. proteus in a medium in which no fermentable substance is present. The change in reaction in the medium

TABLE 3

CHANGE IN HYDROGEN-ION CONCENTRATION IN 1% MANNITE BROTH

Culture Inoculated	P_H after Incubation for							% Gas after 1 Week
	4 Hrs.	6 Hrs.	8 Hrs.	10 Hrs.	27 Hrs.	33 hrs.	1 Week	
Strep. N_1	6.9	6.8	6.0	5.4	4.7	4.6	4.4	None
Proteus L_2	6.9	6.6	6.4	6.6	7.3	7.4	8.1	None
Strep. N_1 + Proteus L_2	6.9	6.5	6.2	5.7	6.0	6.0	5.1	20%

TABLE 4

INFLUENCE OF THE RELATIVE NUMBERS INOCULATED ON THE AMOUNT OF GAS PRODUCED BY TWO ORGANISMS IN SYMBIOSIS

Tube No.	Estimated Number of Organisms Inoculated		Ratio	% Gas in 10 Days
	Streptococcus N_1	B. proteus L_2		
1	100	300,000,000	1 to 3,000,000	57
2	1,000	300,000,000	1 to 300,000	45
3	10,000	300,000,000	1 to 30,000	38
4	100,000	300,000,000	1 to 3,000	52
5	1,000,000	300,000,000	1 to 300	45
6	10,000,000	300,000,000	1 to 30	25
7	100,000,000	300,000,000	1 to 3	12
8	1,000,000,000	300,000,000	3 to 1	22
9	10,000,000,000	300,000,000	33 to 1	20
10	10,000,000,000	30,000,000	333 to 1	17
11	10,000,000,000	3,000,000	3,333 to 1	13
12	10,000,000,000	300,000	33,333 to 1	35
13	10,000,000,000	30,000	333,333 to 1	30
14	10,000,000,000	3,000	3,333,333 to 1	35
15	10,000,000,000	300	33,333,333 to 1	39
16	10,000,000,000	30	333,333,333 to 1	22
17	10,000,000,000	3	3,333,333,333 to 1	21

inoculated with both organisms is somewhat more similar to that produced by the streptococcus alone than to the change due to B. proteus alone. The final concentration of hydrogen ions, however, does not reach the high point characteristic of the streptococcus.

It might be expected that the changes produced in any medium by a given symbiotic group would vary in some degree with a variation in the quantitative proportion of the two organisms in the original inoculum. This was found not to be the case, as will be seen in tables 4

[4] Jour. Bacteriol., 1920, 5, p. 441.

and 5. The technic of the tests of table 4 was to prepare suspensions of the 2 organisms and make a series of dilutions of each. A series of large Smith fermentation tubes of mannite broth were then inoculated with 0.1 c c. of the original streptococcus suspension, then each of these tubes in turn with 0.1 c c. of a different dilution of the proteus suspension. Another series of tubes was inoculated with 0.1 c c. of the original proteus suspension and the same amount of the different dilutions of the streptococcus suspension. To obtain the actual numbers inoculated in each case, 0.1 c c. of several of the different dilutions of the 2 original suspensions were plated out and the number of each species inoculated into each tube calculated. The tubes were incubated and daily gas readings taken. Copious gas formation took

TABLE 5

INFLUENCE ON GAS PRODUCTION BY A SYMBIOTIC PAIR WHEN ONE ORGANISM IS INCUBATED FOR A SHORT TIME BEFORE INOCULATING THE OTHER

Time of Inoculating Streptococcus N_1	Time of Inoculating B. proteus L_2	Time Interval in Hours	% Gas in 10 Days
8:30	8:30	0	30
8:30	9:30	1	21
8:30	10:30	2	20
8:30	11:30	3	18
8:30	12:30	4	20
8:30	1:30	5	16
9:30	8:30	1	24
10:30	8:30	2	20
11:30	8:30	3	27
12:30	8:30	4	18
1:30	8:30	5	26

place in all tubes, but the amount had no relation to the numerical ratio of the 2 organisms in the original inoculum. While there was some variation in the amount of gas formed, this variation was not greater than was found in duplicate or triplicate tubes inoculated with constant amounts of both organisms. The plan of the experiments of table 5 was to inoculate equal numbers of the 2 organisms, but to allow one a short incubation period before inoculating the other. The results were the same as in the experiments just described, but show, in addition, that a slight accumulation of the decomposition products of one organism has no effect on gas production by the symbiotic complex.

Another observation which leads us to assume that gas production in mannite or lactose broth is a quality of this symbiotic pair of organisms quite independent of quantity relations as well as of some other factors, such as temperature, is that when daily transfers were made from one tube (mannite or lactose broth) in which the 2 had been

grown to another tube of the same medium, and from the second, after 24 hour's growth, to a third, and so on; there was no tendency for gas production to diminish or for one organism to disappear from the mixture. These transfers were made over a period of 40 days and gas production following the last transfer was just as vigorous as in the first tube. At room temperature the results were the same except for a slowing down of gas production, due, presumably, to a less rapid multiplication.

The effect of the composition of the medium on gas production was tested especially with regard to the concentration of peptone and the

TABLE 6

THE EFFECT OF VARYING THE CONCENTRATIONS OF PEPTONE ON SYMBIOTIC GAS PRODUCTION FROM MANNITE BY B. PROTEUS L_2 AND STREPTOCOCCUS N 1

Percentage of Peptone in Medium	% Gas. Average of 5 Tubes								P_H after 18 Days Incubation
	1 Day	2 Days	3 Days	4 Days	5 Days	11 Days	13 Days	18 Days	
0.1	2–5	16	25	27	29	33	34	35	6.3
0.25	2–5	14	25	31	34	43	42	40	6 4
0.5	2–5	6	11	15	18	32	32	..	6.4
1.0	2–5	6	13	16	23	41	41	..	6.4
2.0	2–5	3	10	18	32	52	51	..	6.6

TABLE 7

EFFECT OF INCREASING BUFFER CONTENT OF MEDIUM

Balanced Phosphate Mixture per Liter	% Gas. Average from 4 Tubes						
	1 Day	2 Days	3 Days	4 Days	6 Days	7 Days	12 Days
1 gm.	5	11	16	19	23	25	27
2 gm.	5	18	27	27	33	31	32
3 gm.	5	16	22	26	23	23	..
4 gm.	5	18	23	26	29	30	..
5 gm.	5	24	28	29	27	27	..

amount of buffer present. In table 6 are given data showing the influence in the peptone concentration. Table 7 gives the effect of variation in the amount of buffer. The basic medium was the same in each case. It consisted of a broth containing 3 gm. of meat extract per liter and having a hydrogen-ion concentration of P_H 6.8. In the case of the medium of table 6, 4 gm. per liter of a balanced phosphate mixture were added, and the amount of peptone varied as indicated. In the other experiment, 0.2% of peptone was added, and the amount of buffer varied. The gas percentages of table 6 are the averages from 5 different tubes. Those of table 7 are the averages of 4 tubes. It will be seen from these tables that the effect of increasing the peptone is to

slow down gas production, copious gas formation taking place earlier in those tubes containing the least peptone. We seem to have here the reverse of a protein sparing action; that is, carbohydrate metabolism appears to be lessened by an increase in the available nitrogenous constituent of the medium. The effect on the final hydrogen-ion concentration of varying the concentration of peptone was practically nil. Likewise the variation in the quantity of buffer seems to have no influence on the amount of gas produced.

THE PROBABLE EXPLANATION OF SYMBIOTIC GAS PRODUCTION

So far as their ability to attack the carbohydrates, alcohols, glucosides and other fermentable substances is concerned, bacteria are divided into 3 groups, the nonfermenters, the acid formers and the gas formers. In the first class, we place organisms such as B. fecalis-alkaligenes and B. pyocyaneus which are incapable of breaking down any of the commonly used fermentable materials. In the second group fall such organisms as B. typhosus, the streptococci and staphylococci which are capable of fermenting certain of the carbohydrates, alcohols, etc., with acid formation but without the production of gas. The third group comprises a large list of species which break down fermentable compounds always with the production of gas as well as acid. If now we examine the organisms of table 1 with regard to their arrangement into these 3 groups, we find that the first group is not represented at all but that each symbiotic pair consists of an acid former capable of fermenting the sugar used in the experiment and a gas former. A large number of combinations not formed in this way were tested for gas formation, but with negative results. It is understood, of course, that in the tabulated tests only those gas formers were selected which are incapable alone of attacking the sugar used.

Considering these qualities of the gas producing pairs of organisms, a progressive action seems to be clearly indicated leading to the formation of gas in these cases. The degradation of the sugar in question is begun by the acid former and, in the course of this decomposition, substances are formed which are utilizable by the gas former, and gas production results. As to the actual chemistry of the process, we can only make certain deductions.

In the first place, it is evident that the substance attacked by the gas-forming member of the symbiotic pair, that is, the actual mother substance of the gas, is not an end product of the action of the acid former. We repeatedly failed in attempts to produce gas by inocu-

lating a gas-forming organism into a medium on which an acid former had been grown, but which had subsequently been sterilized. Sterilization was accomplished both by filtration and by heat. Gas production never resulted for us except when both organisms were growing simultaneously in the medium. If the proposed theory of gas formation be the true one, we must assume that the mother substance of gas is an intermediary product rather than an end product of the acid fermentation of the sugar, and that this intermediary product, even in pure cultures of the acid former, is decomposed as fast as formed. We have the situation, then, in which one organism of a symbiotic pair gives rise to a substance which both organisms can decompose but with the formation of different end products. In the one case, acids only are the end products; in the other, gas and, presumably, acids also.

This type of symbiotic action differs from the one described by Burri and Stutzer.[2] In the latter, the reaction takes place in 2 distinct and separable stages. B. coli breaks down the nitrate to nitrite, which becomes the mother substance from which nitrogen gas is produced by B. denitrificans. The nitrite is an end product of the action of B. coli, therefore simultaneous action of the 2 organisms is not necessary to the attainment of the desired end. The symbiotic relationship in this case is a loose one.

Following this reasoning, we can make certain deductions from our experiments as to the chemical nature of the hypothetical compound which we have assumed to be the precursor of gas in our tests. In our first experiments, which were made with lactose and saccharose, we suspected that inversion might be the first step in the decomposition of these sugars and that the monosaccharides thus formed might be the mother substances from which gas was derived. Glucose, especially, was suspected as being the one sugar attacked by all fermenting organisms. When mannite was added to the list, however, and indeed, proved to give gas in greater abundance than any other substance tested, the chemistry seemed too involved to assume a preliminary transformation of this alcohol to glucose. Later results giving symbiotic gas formation from such compounds as fructose, salicin, and, in one or two cases, from glycerol, led us to believe that we must look to a much simpler substance than glucose for our immediate precursor of gas. Frequent chemical tests for glucose in sugar mediums in which an acid former had grown were all negative.

It is probable that the chemical compound which acts as the mother substance of gas in these symbiotic reactions is the same as when the

gas formers are grown in pure culture on a sugar they are capable of fermenting. It was thought that the H_2/CO_2 ratios might throw some light on this question, but this value was found to vary so greatly in repeated tests that no conclusions could be drawn from it. Experiments are now in progress by which it is hoped some information may be gained on this problem through the application of symbiotic phenomena.

It may be suggestive in connection with these observations on the nature of the mother substance of gas to note that König,[5] Pakes and Jollymann,[6] Omelianski,[7] Loew [8] and Patrouillard [9] reported the formation by bacteria of CO_2 and H_2 from the salts of the lower fatty acids.

DISCUSSION AND CONCLUSIONS

This paper deals with a new phenomenon, namely, the gaseous fermentation by two organisms growing in symbiosis of a substance from which neither organism acting alone can produce gas. From published observations and many others which it did not seem necessary to more than mention, it is concluded that this phenomenon occurs commonly and that therefore a full understanding of it may be of much practical value. It is possible that this type of gas formation often may give rise to false impressions in the case of the presumptive test for B. coli in water and sewage analysis. There are many combinations that could give gas from lactose. The general law governing this phenomenon which is deduced, namely, that a combination of an acid former capable of fermenting the given sugar with a gas former not capable of fermenting this sugar in pure culture may be expected to give gas, should serve to explain in some cases our inability to isolate B. coli from lactose tubes showing considerable quantities of gas. It is not unlikely also that many cases of gas formation in canned goods may be due to symbiotic reactions of this kind. Recently an interesting practical example of symbiotic gas formation was encountered. A culture of Morgan's bacillus was received which when tested on the sugars was found to give gas on lactose. It was about to be discarded as B. coli, but on more careful examination it was found that it was a contaminated culture of B. morgani, the associated organism being a gram-negative bacillus of similar morphology which fermented

[5] Ber. d. deutsch. chem. Gesellsch., 1881, 1, p. 211.
[6] Proc. Chem. Soc., 1901, 17, p. 29.
[7] Centralbl. f. Bakteriol., 1904, 11, 2, pp. 177, 256 and 317.
[8] Ibid., 1879, 12, p. 462.
[9] Compt. rend. Acad. d. sc., 1877, 84, p. 553.

lactose with acid formation but without gas. It appears, then, from these experiments that when inoculation of a mixture of organisms is made into a medium containing a fermentable substance, the appearance of gas in this medium cannot be taken as prima facie evidence that an organism is present in the mixture which produces gas on the substance used.

That the qualitative rather than the quantitative composition of a symbiotic group of organisms is likely to be the factor which determines the nature of the chemical changes produced is implied by our results with the complex, B. proteus-vulgaris and Streptococcus fecalis. It is fully realized, however, that what is true of this pair may not be true of other groups. Nevertheless, we are led to hope that the problems of symbiosis may prove not to be greatly complicated by quantitative relationships.

It is believed, also, that some slight contribution is made by this study to a better understanding of the chemistry of bacterial fermentation by showing that the immediate precursor of gas is a simple substance which is often an intermediary product of the decomposition of sugars by those organisms which produce acid but not gas.