FURTHER EXPERIMENTS ON VARIABILITY IN THE GAS-FORMING POWER OF INTESTINAL BACTERIA.

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(With 5 Diagrams and 2 Charts.)

In a recent communication (Penfold 1911) I described experiments with three organisms, *B. coli* Escherich, *B. enteritidis* Gaertner and *B. Grünthal*, showing that limitation or complete abolition of certain gas-forming powers normally possessed by these organisms, could be brought about by their growth on chloracetic acid-agar, and further, that in the case of the latter organism, resistance to this medium was associated with such limitation or abolition.

In this paper I desire to give an account of further experiments dealing with the same subject. The same special medium and technique were employed as detailed in the paper cited above. Further experiment had shown that *B. lactis aerogenes* when grown for seven successive generations on chloracetic acid-agar, still gave at the end of the period a good yield of gas on glucose, although it was highly resistant to the chloracetic acid medium; similarly a strain of *B. acidi lactici* gave negative results in spite of long training.

In view of the anomalous behaviour of these organisms it appeared desirable to examine more fully the nature of the selective process in those cases in which non-gas-producing variants had been so easily obtained.

Further experiments with B. coli *Escherich.*

Chloracetic acid-agar plates of this organism show, at suitable concentrations of the salt, mixtures of big and little colonies, that is, the growth of all the organisms is not inhibited equally; at higher concentrations only the big colonies grow. In the successful experiments described in my first paper, the non-gas-producing strains were

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obtained by selecting big colonies in each case. Further work has shown me that the *association of non-gas-production and resistance to chloracetic acid* is in the case of *B. coli* Esch. a very variable one. Some essential connection exists between the two properties, but this connection could be disclosed only by a prolonged statistical enquiry. The following experiment illustrates this point:

A pure culture of normal *B. coli* Esch. was plated out on 12 plates of chloracetic acid-agar. These plates were divided into four groups containing different concentrations of sodium chloracetate, each member of a group of three containing the same concentration of this salt.

The respective concentrations were 0·01 %, 0·05 %, 0·1 % and 0·2 % expressed in terms of the amount of acid added. The three plates of the latter concentration showed only large colonies. Five large colonies from each of the 0·2 % plates were inoculated into glucose-peptone-water in Durham tubes; these tubes were observed daily for five days and the maximum amounts of gas found were as indicated in the following table:

TABLE I.

Colonies from plate	*D*	1/3	1/3	1/3	1/2	5/12
,, ,,	*H*	1/4	nil	1/2	1/2	1/2
,, ,,	*L*	7/12	7/12	5/12	1/2	1/2

These fractions indicate the length of gas tube displaced by gas.

Control tubes of glucose-peptone-water, inoculated from the same original strain of *B. coli* grown on MacConkey plates, gave in the case of twenty different colonies the following volumes of gas:

4 tubes yielded gas equal to 1/2 of the gas tube.
12 „ „ „ „ „ 7/12 „ „ „ „
4 „ „ „ „ „ 2/3 „ „ „ „

The colonies from the chloracetic agar plates, therefore, show one giving no gas, six others below the lowest of the controls, and eight similar to the controls.

The strain from plate *H*, which gave no gas on glucose-peptone-water, yielded, when tested on lactose-peptone-water, 1/12th of a tubeful of gas. It was therefore plated out on a lactose plate and eight of its colonies were tested again on lactose-peptone-water, when seven of these were found to produce no gas while the remaining one showed half-a-tubeful. The large colonies, apparently, do not appear to be homogeneous in their composition.

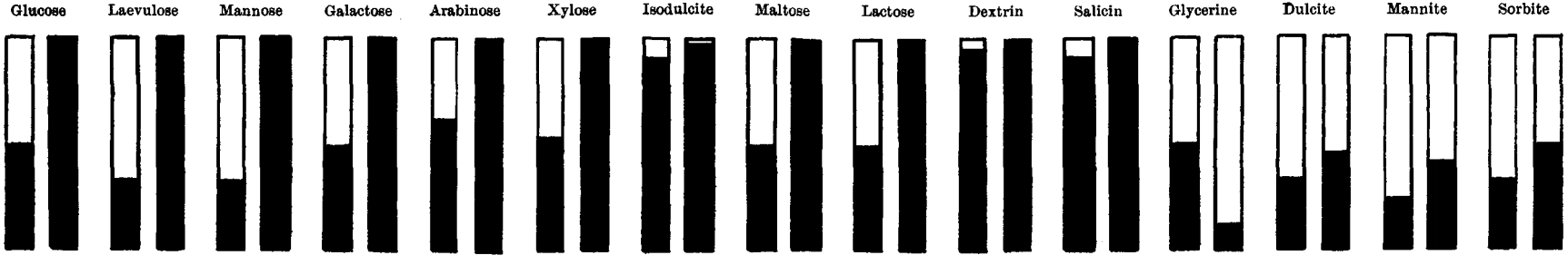

Diagram I. *Bacillus coli communis* Escherich, strain *H*. Left-hand member of couple = normal strain; right-hand member of couple = variant strain. In this diagram the black columns represent gas tubes containing no gas, while in the other columns the white spaces represent the quantities of gas formed. All the tubes were incubated for five days at 37° C., and the amount of gas indicated in the diagram is the *greatest* amount found in the individual tubes during the whole period. The tubes are arranged in couples, the left-hand member representing the normal *Bacillus coli*.

One of the seven lactose broths which gave no gas (denoted by *H*) was subcultured on agar and tested on the usual peptone-waters containing different carbohydrates. Diagram I represents its behaviour in respect of gas-formation on the carbohydrates which it fermented.

This strain "*H*" is a more striking example of the loss of the power to form gas from sugars, than the strain which the writer described in the formerly published paper dealing with gas variants. In the former case the organism still produced traces of gas from galactose, xylose, arabinose, isodulcite, maltose and salicin, whereas this new strain produced only a slight trace of gas in isodulcite, out of nine sugars tested. The essential difference between the selective processes in the two cases was, that in the former strain a large colony was taken off the first chloracetic agar plate haphazard, and replated on the same medium, from which again a single colony was picked off and replated, the process being repeated four times, while in the case of the latter strain, a large number of colonies were tested from the first chloracetic agar plates, with the result that one was found which from the start failed to give any gas on

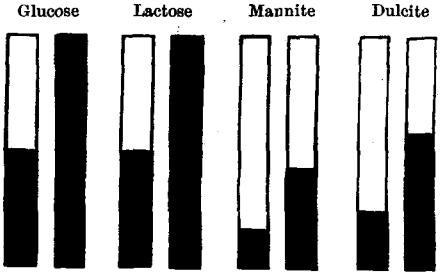

Diagram II. *B. coli* Escherich. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

glucose. In order to make certain that this was not a matter of chance, the same method was repeated again with the same organism. In this case 17 colonies were inoculated into glucose broth, from chloracetic agar plates which had been incubated five days at 37° C. All the broths showed a full acid reaction after 24 hours, but after 48 hours two of the tubes showed no gas. On this date one of the two tubes got broken but the other showed no gas throughout an observation period of six days. This new strain gave the reactions shown on Diagram II. It would appear, therefore, that the easiest and best way to get these variants is by testing a large number of colonies from the first chloracetic agar plates.

The examination of the new variety in Theobald Smith's tubes.

The above-mentioned strain "*H*" was compared with the normal strain from which it was derived, in Smith's tubes, and the results obtained are recorded in Table II. The new strain, it will be seen, gave no gas in glucose but its yield in mannite broth was only 50 % of the normal, a diminution distinctly greater than was found with the Durham tube (see Diagram I).

TABLE II.

Theobald Smith's tubes.

Strain "*H*" *versus* Normal *B. coli* Esch.

	Normal *B. coli.*		Varied *B. coli.*	
	Glucose		Glucose	
	1st tube with 20 divisions	2nd tube with 10 divisions	Tube with 20 divisions	Tube with 10 divisions
Vols. of gas 1st day	10 div.	4·8 div.	nil	nil
,, 2nd ,,	11 ,,	4·8 ,,	nil	nil
,, 3rd ,,	11 ,,	4·8 ,,	nil	nil
,, 5th ,,	10 ,,	4·2 ,,	nil	nil
	Mannite		Mannite	
	Tube with 20 divisions	Tube with 5 divisions	Tube with 20 divisions	Tube with 5 divisions
Vols. of gas 1st day	8·5 div.	2·2 div.	4·5 div.	1·4 div.
,, 2nd ,,	13·5 ,,	3·4 ,,	7·0 ,,	2·1 ,,
,, 3rd ,,	14·5 ,,	4·0 ,,	7·2 ,,	2·1 ,,

Further experiments with B. enteritidis *Gaertner.*

The variant strain of *B. enteritidis* Gaertner described by the author in a former paper, while unable to produce gas from certain pentoses, was still able to produce a little gas from the hexoses. In order, therefore, to obtain strains unable to produce gas from glucose, it was deemed advisable to test a large number of colonies from the first chloracetic agar plates in respect of their action on glucose broth.

A typical strain of *B. enteritidis* was plated on the special medium on a series of plates: the strength of chloracetic acid in these plates varied from 0·1 % to 0·9 %. These plates were grown for three days at 37° C. when colonies of large size were picked off and inoculated into glucose broth in order to test their gas-forming power. The results are given in Table III.

TABLE III.

Strength of chloracetic acid in the plates	Number of large colonies tested	Extreme deviation of gas yield, in fractions of gas tube
0·1 %	4	1/3 to 5/12
0·3 %	4	1/4 to 5/12
0·4 %	4	1/3 to 5/12
0·5 %	4	1/12 to 1/3
0·7 %	4	1/6 to 5/12
0·9 %	4	1/4 to 5/12

Here we have 24 colonies none of which give less than one-twelfth of a tubeful of gas. The same plates were, therefore, further incubated until the tenth day, when the large colonies were again inoculated into glucose-peptone-water and gave the gas yields seen in Table IV.

TABLE IV.

Strength of chloracetic acid in the plates	Number of large colonies tested	Extreme deviation of gas yield, in fractions of gas tube
0·1 %	8	1/12 to 5/12
0·3 %	8	Pin point bubble to 1/12
0·5 %	8	,, ,, ,, 5/12
0·7 %	8	Nil to 5/12
0·9 %	8	Nil to 1/12

It appeared, therefore, that between the third and the tenth day the character of these colonies had altered, the organisms of which they were composed having become distinctly poorer gas producers. The gas-producing elements in the colonies had probably been killed off or outgrown by the non-gas-producing ones. The fact remains that no less than three of the plates showed big colonies able to produce amounts of gas nearly equal to those produced by the normal strains. Out of the total of 40 colonies tested, only three showed themselves unable to produce gas in glucose broth, while no less than six gave an amount of gas just equal to a pinhead bubble. Three tubes out of the 40 showed 5/12ths of a tubeful of gas, *i.e.*, an amount but little different from the normal. It is important to notice, that in spite of these very different gas yields, all the colonies agreed in having grown to a large size on the special medium. Two of the three glucose tubes which gave no gas had subcultures taken from them on to agar slopes. From the latter they were put through the different carbohydrate broths with results shown in Diagram III.

Both these new strains produced no gas on either glucose or maltose but still gave very fair yields from the alcohols; the retention of the slight gas-producing power of xylose is rather curious, and in this respect they differ from the strain of which an account was published in my previous communication.

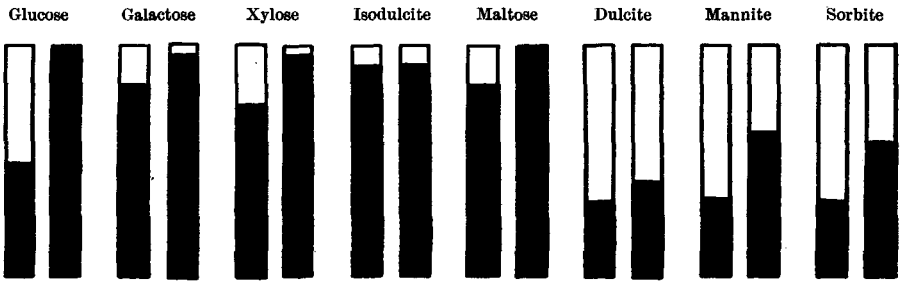

Diagram III. *Bacillus enteritidis* Gaertner. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

Further experiments with B. paratyphosus *B.*

A standard laboratory strain of this organism was plated on chloracetic acid-agar plates, in which the strength of chloracetic acid varied from 0·1 % to 0·9 %. Large and small colonies developed. After three days' growth 14 large colonies from the plates were tested on glucose-peptone-water. All showed some gas production. After seven days' further incubation 20 large colonies were tested on glucose-peptone-water, when no less than 14 of them were found to produce no gas. Five of these were put through various carbohydrates, but none of

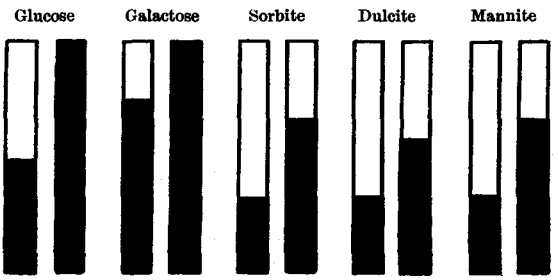

Diagram IV. *B. paratyphosus* B. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

them was able to produce gas from glucose or maltose. One of the strains tested on galactose produced no gas while they all produced gas from the alcohols they fermented. An idea of the amount of gas production on alcohols is given in Diagram IV in the case of one of the strains. The others were very similar.

The occurrence of natural variants of the Gaertner group in respect of gas formation.

I am indebted to Dr Bainbridge for numerous strains belonging to this group, two of which deserve mention in this paper.

1st. A non-gas-producing *B. Aertryck*. This gave all the cultural and serological reactions proper to this variety, except that it failed to produce gas from any sugar.

2nd. A strain of *B. paratyphosus* B. (Murray) which produced very little gas on any medium. This organism was irregular in its action on dulcite broth but apart from this, and its poor gas-producing power, it was a definite strain of *B. paratyphosus* B. Moreover it had been isolated from a definite case of paratyphoid fever. This strain was grown on chloracetic acid-agar with a view to removing its gas-forming power, and the normal and selected strains are compared in this respect in Diagram V.

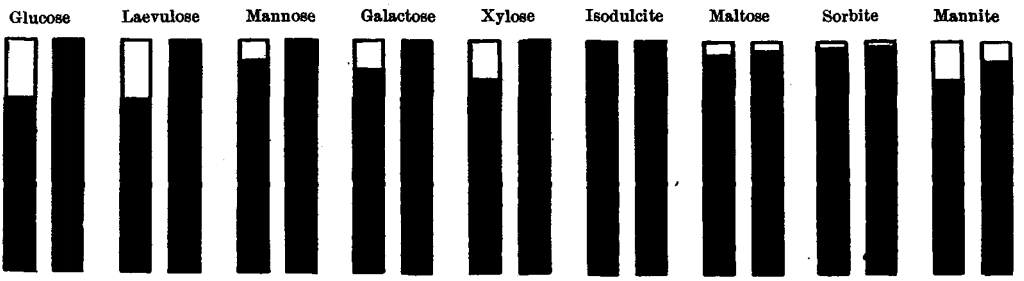

Diagram V. Strain "Murray," *B. paratyphosus* B. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

Here again the power to form gas from sugars disappeared except in the case of maltose; the power to produce gas from mannite and sorbite is retained to a slight extent, but for which fact it would have approached very closely to the naturally occurring non-gas-producing *B. Aertryck* above described. The agglutination characters of this organism were not altered in the process of selection.

Morgan's bacillus No. 1. This organism by growth on chloracetic acid-agar lost the power to produce gas from glucose and laevulose, though it produced a good acid reaction in the medium overnight. On galactose no acid reaction was produced by the new strain until the sixth day, and then, curiously enough, a tenth of a tubeful of gas appeared. This was repeated and both these effects were obtained.

It seems therefore highly probable that this selective process for obtaining gas variants will be fairly generally successful if thoroughly applied. To get successful results, it is advisable to test a large number of colonies in the first instance and to allow the plates to incubate for 7–10 days before testing the colonies.

A comparison of the gas-forming power of the big and little colonies taken from the same chloracetic-agar plates.

B. enteritidis Gaertner was plated out on a series of plates containing ascending concentrations of chloracetic acid. After three days' incubation four big and four little colonies from each plate were subcultured into glucose broth when the average yield of gas was found to be as follows. (The results are expressed as tube divisions filled with gas.)

	Big colonies	Little colonies	Ratio
0·1 % plate	4·8	6·5	0·73
0·3 ,,	4·25	5·6	0·75
0·4 ,,	4·75	5·5	0·86
0·5 ,,	2·4	4·1	0·58
0·7 ,,	3·8	6·0	0·63
0·9 ,,	3·0	7·5	0·4

B. paratyphosus B. treated similarly gave the following figures:

	Big colonies	Little colonies	Ratio
0·1 % plate	2·6	2·5	1·0
0·2 ,,	1·3	2·8	0·46
0·3 ,,	1·0	3·1	0·32
0·7 ,,	0·5	2·6	0·19
0·8 ,,	1·0	3·2	0·3
0·9 ,,	0·5	2·0	0·25

These ratios are plotted in the accompanying Charts I and II.

The figures show that even after three days' growth on the special medium the big colonies are poorer gas producers than the small ones, the latter being in point of fact normal. Twenty-four small colonies of *B. enteritidis* Gaertner gave an average of 5·8 divisions of gas against

five normal controls which average five divisions. The disparity between big and little colonies in respect of gas formation, holds much more strongly when the plates have been incubated for a longer period. The gas yield of the big colonies in the case of *B. enteritidis* Gaertner and *B. paratyphosus* B., after the plates have been incubated ten days, is roughly indicated in an earlier portion of this paper (see pp. 491–3).

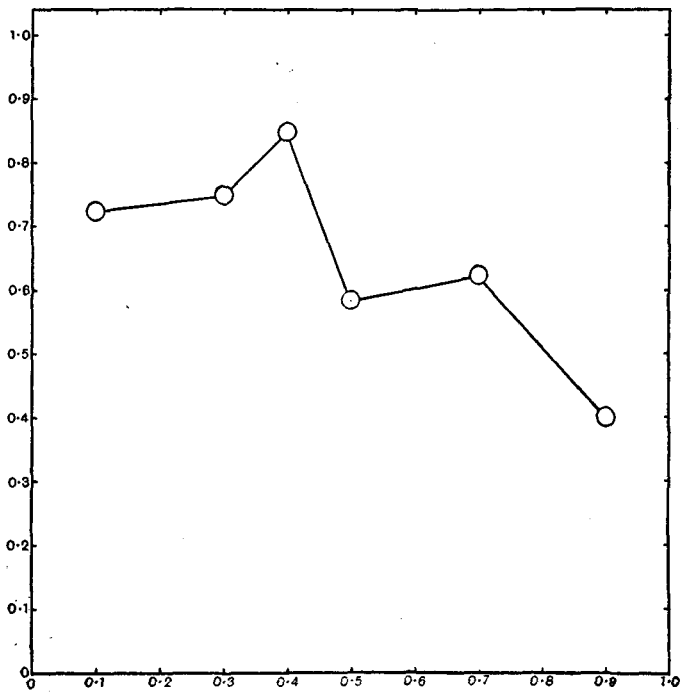

Chart I. *B. enteritidis* Gaertner. Ordinates represent ratios of average volumes of gas developed on glucose by big and little colonies respectively. Abscissae represent strengths of chloracetic acid in the plates.

Increased resistance to the chloracetic-acid medium is indicated by the increased size of some colonies, but in addition, these large colonies, in the later stages of their growth, show secondary colonies composed of organisms which appear to be more resistant to the medium than the average of the colony. In the case of *B. Grünthal*, an examination of some of these showed them to be poorer gas producers than the rest of the colony, and this agreed with the general principle that chloracetic acid resistance and poor gas-producing power are correlated. In the

case of *B. enteritidis* Gaertner, however, the papillae appear to be composed of organisms of similar gas-producing power to those found in the rest of the colony. This statement is based on the examination of about 40 tubes of glucose-peptone-water which had been inoculated, in equal proportions, from papillae and smooth interpapillary areas of colonies respectively. *B. paratyphosus* B. is similar to *B. enteritidis* Gaertner in this respect.

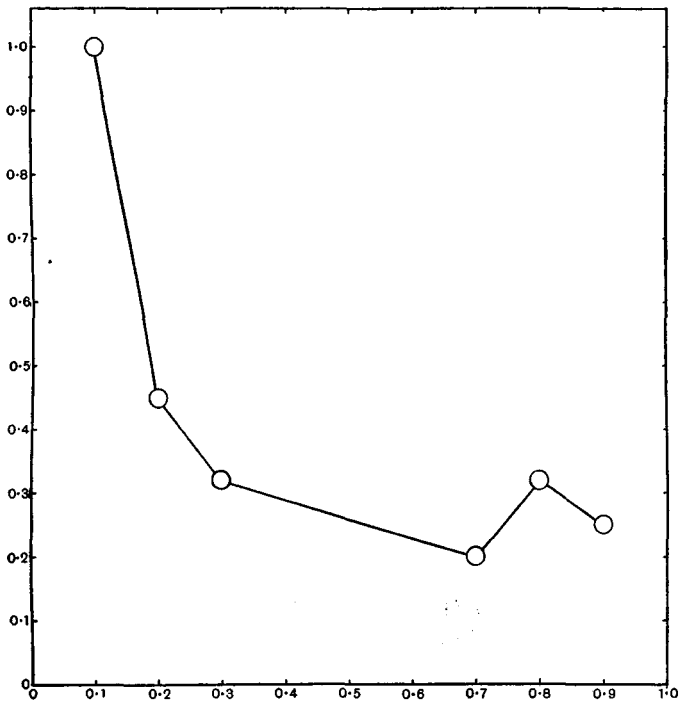

Chart II. *B. paratyphosus* B. Ordinates represent ratios of average volumes of gas developed on glucose by big and little colonies respectively. Abscissae represent strengths of chloracetic acid in the plates.

Summary of foregoing experimental results.

(1) By the method of selection indicated in the paper it has been possible to obtain a variety of *B. coli* Escherich unable to produce gas from any sugar excepting isodulcite. The new strain so obtained was still able to produce gas from all the alcohols it fermented though in diminished amount.

(2) The power of *B. enteritidis* Gaertner to form gas from glucose and maltose has been removed in the same way. *B. paratyphosus* B. and *Morgan's bacillus No.* 1 have similarly given rise to variants not producing gas from the usual sugars.

(3) The easiest method to obtain non-gas-producing variants is by incubating plates of the respective organisms on the special medium for 7–10 days at 37° C. and then testing a fairly large number of large colonies on glucose-peptone-water.

(4) The relationship of chloracetic-acid resistance to non-gas-producing power is undoubted as between the big and little colonies of various strains, but it is of a complex character. The papillae in the case of *B. enteritidis* Gaertner and *B. paratyphosus* B. do not seem to differ materially from the rest of the colony bearing them, in gas-forming power.

Durability of the new character.

In my previous communication dealing with variation of the gas-producing power of these organisms, I showed that an artificially selected strain of *B. coli* Escherich, producing no gas from glucose, evinced no tendency to reversion though subcultured frequently on glucose-peptone-water.

The period was too short, however, to furnish much information in respect of permanency. I have now observed some of the new strains for a considerable period with the following results:

1*st Experiment.* *B. coli* Esch. In December 1910 a new strain of *B. coli* Esch. was selected, which produced no gas from glucose but 7/12ths of a tubeful when grown in mannite-peptone-water. From this period until May 1911 the strain was grown on ordinary agar, being subcultured on it about every fortnight. On May 19th, 1911, *i.e.*, over five months after its selection, it was retested in respect of gas-producing power in glucose- and mannite-peptone-waters with the following result:

	Glucose		Mannite	
Date of observation	Reaction	Gas	Reaction	Gas
20th May	Acid	Nil	Acid	1/8
21st ,,	,,	,,	,,	7/12
22nd ,,	,,	,,	,,	7/12
24th ,,	,,	,,	,,	7/12

2*nd Experiment.* Another strain of *B. coli* Esch. selected out shortly after the above, gave in May a bubble of gas the size of a pin's head when grown on glucose-peptone-water in a Durham tube.

3rd Experiment. An artificial strain of *B. coli* Esch. unable to produce gas from glucose in the Durham tube was grown anaerobically at 37° C. in glucose-peptone-water to which chalk had been added. Under anaerobic conditions it produced a small yield of gas and it was, therefore, thought to be reverting to its natural state. This anaerobic growth was continued for over one month. It was then plated out on lactose agar and three separate colonies were tested on glucose- and mannite-peptone-waters with the following result:

Glucose.

Date of inoculation, March 28.	Colony...	1		2		3	
Observed ,, 29		A.f.	G.n.	A.f.	G.n.	A.f.	G. pinhead bubble
,, 31		,,	,,	,,	,,	,,	,, ,,
April 1		,,	,,	,,	,,	,,	,, ,,
,, 3		,,	,,	,,	,,	,,	G. bubble slightly larger

Mannite.

Date of inoculation, March 28.	Colony...	4		5		6	
Observed ,, 29		A.f.	G. 1/4	A.f.	G. 1/4	A.f.	G. 1/4
,, 31		,,	G. 1/2	,,	G. 1/2	,,	G. 1/2
April 1		,,	G. 7/12	,,	G. 7/12	,,	G. 7/12
,, 3		,,	G. 1/2	,,	G. 1/2	,,	G. 1/2

A.f. = Full acid reaction. G.n. = Gas nil.

The gas appearing in the anaerobic culture was owing therefore only in the slightest degree to reversion; it was probably largely accounted for by the anaerobic growth and the presence of the chalk.

4th Experiment. B. Grünthal, by growth on the special medium and selection from it, had its gas-producing power in respect of lactose reduced from half a tubeful to a pinhead bubble. The strain was grown three months on agar and then plated. Twelve colonies were tested in lactose broth, when all were found to produce the same amount of gas as the strain did when first selected out.

5th Experiment. Strains of *B. paratyphosus* B. occasionally show a tendency to revert, in respect of gas-forming power on glucose, soon after being first selected out, but this tendency does not affect all the new strains. These new varieties have not been long under observation.

Identity.

The demonstration of the identity of the new strains has not been so strictly attended to in this series as in those described in my first communication on this subject where the evidence given was very complete. Since all the new varieties described in this paper show similar qualitative changes to those described in my previous communication it seemed unnecessary to devote so much effort in this direction.

Testing of other chemical substances related to monochloracetic acid in respect of their action on the growth of intestinal bacteria.

Marked variability in the size of colonies on plates, and the development of secondary colonies (*i.e.*, papillae formation), were especially looked for.

The organic acids used were all first neutralised in aqueous solution with saturated sodium carbonate solution, then filtered through a Berkefeld and added to agar plates in the usual way. Many of these bodies are unstable so that their temperature was kept as low as possible throughout the whole work. The chemicals used were all got from Kahlbaum except the potassium chlormalonate for which I was indebted to Miss Smedley of the Lister Institute.

Monobromacetic acid. This preparation inhibited the growth of *B. coli* Esch. and *B. enteritidis* Gaertner in weaker concentrations than monochloracetic acid. In the case of both organisms distinct variability in the size of the colonies was observed in suitable concentrations, but this was not so marked as with monochloracetic acid. Papillae appeared on the colonies, well developed in the case of *B. coli* Esch., but not so well developed in the case of *B. enteritidis* Gaertner. Fifteen large colonies from *B. enteritidis* and *B. coli* plates when tested on glucose showed no loss of gas-forming power. It was not found so easy to produce races highly resistant to this agent as had been the case with monochloracetic acid.

α-Brompropionic acid. In plates containing this agent *B. coli* Esch. produced small papillae on plates having a strength of 0·05 % and 0·1 %. Growth was not obtained on plates having a higher concentration than 0·3 %. No marked variability in the size of the colonies was noted.

B. enteritidis Gaertner and *B. paratyphosus* A. showed, on agar containing this agent, no papillae formation nor marked variability in the size of the colonies.

Colonies from brompropionic acid plates of the above three organisms showed no variations from the normal strains in amount of gas produced from glucose.

Dibromsuccinic acid and the potassium salt of chlormalonic acid failed to select out variants of *B. coli* Esch. or *B. enteritidis* Gaertner. Sodium hippurate and benzoate were tried because of Herter's statement (1909) that they caused *B. coli* to produce less gas. Six concentrations of each salt, in agar plates, were used, from 0·01 % to 2 % in the case of the sodium benzoate, and from 0·02 % to 4 % in the case of the hippurate. In the case of *B. coli* Esch. and *B. Grünthal* no marked variability in the size of the colonies or naked-eye papillae formation was noted. Colonies from the plates of highest concentrations which showed growth were inoculated on the 11th day into glucose-peptone-water and produced normal amounts of gas.

Since the publication of my first communication on this subject Revis (1911) has described the production of an artificial variety from a typical *B. coli*. This variety differed from the normal by being unable to produce gas from any carbohydrate on which it was tested; it was, moreover, unable to clot milk. It was produced by the growth of the normal strain on malachite green media. Revis does not exclude the possibility of contamination arising during the course of the 15 subcultures necessary to produce the new variety. It is precisely because of this difficulty that all points of resemblance between the original and the new strains should be collected, to help us to determine whether the new strain was actually derived from the old strain or not. I made no proviso, as Revis alleges, that before and after variation the agglutination character should remain the same. I believe however that if it does, it helps to establish the origin of the new variety. Of course variations of the agglutination property of the various organisms have been proved to occur, and the proof in this case has been largely furnished by the cultural and other characters, which have meanwhile remained constant. Had Revis proved the identity of the agglutination characters of the old and new organisms, it would have made the case more convincing. When many characters of an organism vary at once it is difficult to convince oneself that a foreign organism did not drop into the test tube during some portion of a long subculturing process and outgrow the strain from which one started. Since there are not

many remaining characters left for identification purposes, one can only produce conviction by repeating the process a number of times with the same result. I feel very little doubt about the variation process described by Revis, as it bears a strong resemblance to the one described in the previous communication I made on this subject, and in that case the identity was determined by serum tests as well as by the standard cultural tests. Moreover other organisms varied similarly in the same environment.

General Conclusions.

1. The results of this research show that organisms of the coli-group can have various gas-forming powers removed by a process of artificial selection on a specially prepared medium. The biochemical properties of the variant strains approach closely those of the naturally occurring anaerogenes-coli class.

2. The power of gas-formation from sugars (always excepting isodulcite) may be lost when gas-formation from alcohols is retained. It is probable, therefore, that two different ferments are engaged in the respective processes.

3. The research raises the question as to the weight to be attached to the power of fermenting glucose and lactose with gas-formation, in recognising *B. coli* in routine examinations of pathological material, water, foods, etc. Hitherto, in all authoritative catalogues of the necessary properties of this organism, this has been included but it probably ought to be regarded as not absolutely essential.

In conclusion I desire to express my indebtedness to Dr Ledingham for valuable help in the course of this research.

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