II. THE EFFECTS OF ACIDS, ALKALIES, AND SUGARS ON THE GROWTH AND INDOLE FORMATION OF *BACILLUS COLI.*

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The Effect of Alkalies on the Growth of *Bacillus Coli.*

In an earlier paper [Wyeth, 1918] it was shown that the final reaction produced by *B. coli* grown in 2 % glucose peptone was dependent upon the initial reaction of the medium, and was not a "physiological constant" as had been suggested by Michaelis and Marcora [1912].

The results obtained by the use of different media and acids were recorded for various initial reactions lying between $P_H = 4{\cdot}23$ and absolute neutrality ($P_H = 7{\cdot}00$). Since the introduction of the hydrogen electrode as a means of measuring the reaction (P_H) of liquids few results of the investigations of the growth of *B. coli* in media initially more alkaline than $P_H = 7{\cdot}00$ have been recorded. It was thought desirable, therefore, to supplement the former research by making an examination of the behaviour of *B. coli* when grown in 2 % glucose peptone made alkaline by the addition of *N* sodium hydrate: an endeavour also being made to determine a "limiting value" of alkalinity, above which the growth of the organism is inhibited.

Material and Experimental Methods.

Pure cultures of *B. coli* obtained from human faeces were used. Experiments described in a former paper indicate that no differences in behaviour exist between strains of human and bovine origin. In this connection it may be noted that Murray [1916] made a comparative study of *B. coli* isolated from the faeces of man, horse, and cow. He agrees that all the strains prepared exhibited remarkable similarity of behaviour, and especially as regards their acid production.

A sterile 4 % glucose peptone medium was prepared by the method described in the former paper. It was then rendered alkaline by the addition

of *N* sodium hydroxide[1]. If the alkali be added before sterilisation, caramelisation,—with consequent production of acids,—occurs and the medium does not attain the desired degree of alkalinity. The following procedure was therefore adopted. A number of flasks each containing 125 cc. of 4 % glucose peptone were prepared. To the contents of each flask the required volume, viz. $(125 - x)$ cc., of distilled water was added. The media were then sterilised by heating in an autoclave for 1 hour at 120°, allowed to cool, and finally the necessary volume (x cc.) of sterile *N* NaOH was added to the contents of each flask, which finally contained 250 cc. of sterile 2 % glucose peptone rendered alkaline with x cc. of *N* NaOH. In the series of experiments performed, the value of x was varied from 0 to 7·0 cc. of *N*/10 NaOH per 10 cc. of medium, and the initial reactions of the media ranged from $P_H = 7{\cdot}0$ to $P_H = 11{\cdot}0$. Inoculation was performed by adding to each 250 cc. of medium 0·5 cc. of a pure culture of *B. coli* grown for 18 hours in 2 % glucose peptone. The inoculated media were then incubated at 37° for 9 days, previous experiments having shown that the fermentation, as measured by change of final reaction (P_H) was practically complete at the end of the eighth day.

Results of Inoculation Experiments.

In the data given below H_1, H_2, etc. refer to strains of *B. coli* obtained from human faeces. Final P_H is the lowest value recorded during a period of 216 hours, and the + sign shown in column 5 of the table indicates that a positive result was obtained as regards fermentation, etc., while "0" indicates that a negative result was obtained.

Table I. B. coli *grown in* 2 % *glucose peptone rendered alkaline by the addition of N* NaOH.

Strain of *B. coli* used, H_4.
Time of incubation at 37°, 216 hours.
Temperature of experiment, 20°.

No. of flask	Cc. of *N*/10 NaOH* per 10 cc. of medium	Initial reaction P_H	Final reaction P_H	Fermentation	Cc. of *N*/10 NH_3 produced per 10 cc. of medium	Cc. of *N*/10 volatile acids produced per 10 cc. medium
1	7·0	10·80	—	0	—	—
2	6·0	10·50	—	0	—	—
3	5·0	10·28	—	0	—	—
4	4·0	9·82	4·82	+	0·02	2·65
5	3·5	9·72	4·81	+	0·03	2·78
6	3·0	9·51	4·81	+	nil	2·80
7	2·5	9·40	4·79	+	nil	2·68
8	2·0	8·97	4·72	+	nil	2·65
9	1·0	8·51	4·64	+	0·02	2·75

* Equivalent volumes of *N* NaOH were used.

[1] *N* NaOH was used since a higher P_H was required than could have been obtained by the use of *N*/10 NaOH, the approximate reaction of which is $P_H = 10{\cdot}00$. For ease of comparison the equivalent amounts of *N*/10 NaOH are shown in the tables.

Consideration of Experimental Data.

The above results are typical of a large number obtained in several series of experiments, and of these the final P_H values are represented graphically in Fig. 1, Curve I. It is obvious that, as in the case of *B. coli* grown in a 2 % glucose peptone medium possessing an initial reaction less than $P_H = 7{\cdot}0$, the final reaction produced by the organism when grown in alkaline glucose peptone (*i.e.* whose initial reaction is more alkaline than $P_H = 7{\cdot}0$) is dependent upon the initial reaction (P_H) of the medium, although it varies within narrow limits. It was found that growth is permitted by a solution the initial reaction of which is $P_H = 9{\cdot}82$, but that a medium of initial reaction $P_H = 10{\cdot}28$ inhibits growth. There is therefore a "limiting value" for the initial reaction of alkaline glucose peptone which lies somewhere between these two values. The final acid reaction reached by a culture of *B. coli* in alkaline glucose peptone of the highest permissible degree of alkalinity (*i.e.* initial reaction $P_H = 9{\cdot}82$) is such that its $P_H = 4{\cdot}82$ which is identical with that determined by Clark [1915] as the final acid reaction of "human" *B. coli* grown in a medium containing 1 % Witte peptone + 1 % glucose.

In the "reaction resultant" curve for *B. coli* in 2 % glucose peptone shown in Fig. 1 (I) the initial reactions plotted on the 120° axis are so chosen as to cover the whole range of acidity and alkalinity within which growth of the organism in this medium is permitted. The form of the curve representing the final reactions (P_H) recorded shows that they form an ordered series lying between the acid limit of (approximately) $P_H = 4{\cdot}27$, and the alkaline limit of (approximately) $P_H = 4{\cdot}82$. Further, the connection existing between the initial reaction of the medium and the final reaction of the resulting culture is such that a change in the reaction of the former, whether it be in the acid or in the alkaline direction, produces a corresponding, but much smaller, change in the latter. The form of the reaction resultant curve in Fig. 1 (I) shows also that the resultant reactions obtained throughout the whole range of bacterial activity are due to the production of constant amounts of acid. In addition to measuring the final reactions of the cultures a quantitative determination of the principal products of fermentation was made, some of the results of which are shown in Table I, columns 6 and 7. It was found that practically no ammonia is formed during the fermentation, and that, although considerable volumes of volatile and fixed acids are formed, the actual amounts of each are approximately constant throughout the whole range of experiment, thus confirming the inference drawn from the form of the reaction resultant curve (Fig. 1 [I]). It is evident that the fermentation of *B. coli* in glucose peptone is to all intents and purposes exclusively saccharolytic. In order to find whether proteolysis supervened after saccharolytic fermentation was complete a number of cultures were allowed to ferment for more than 9 days. In a number of instances a rise of E.M.F. equal to 1–2 millivolts was observed, and this was sufficiently constant in occurrence to prohibit the assumption

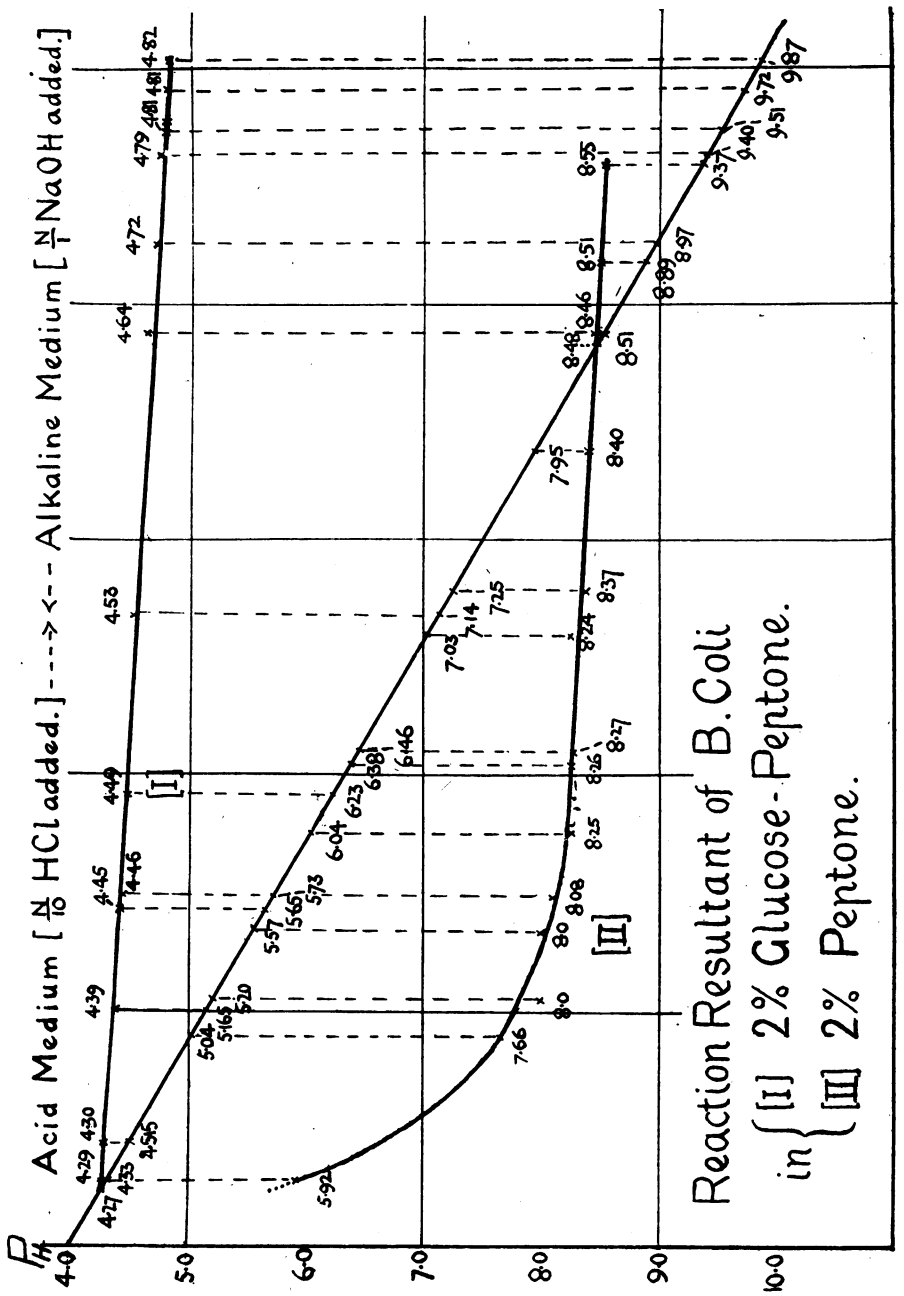
PH
Acid Medium [N/10 HCl added.] ---> <--- Alkaline Medium [N/1 NaOH added.]
Reaction Resultant of B. Coli
in {[I] 2% Glucose-Peptone.
[III] 2% Peptone.
[I]
[II]
4·0
5·0
6·0
7·0
8·0
9·0
10·0

Fig. 1.

that it was always due to experimental error, although it must be conceded that such a variation in any individual culture certainly does fall within the limits of experimental error. That so small a reversion in the alkaline direction is found only in a certain proportion of the cultures proves conclusively that no appreciable amount of proteolysis occurs.

By constructing a titration curve for acetic acid in 2 % glucose peptone and using this in conjunction with the initial reactions of the cultures subjected to quantitative examination, and also with the amounts of acid formed therein, a curve was constructed showing what would be the reaction resultants theoretically produced by the formation of the volumes of acid actually found as a result of these experiments. This curve was of the same shape as that resulting from the final reactions (P_H) actually found, and given in Fig. 1 [I]. The conclusions arrived at after an examination of the initial and final reactions (P_H) of the cultures, and after a quantitative determination of the products of fermentation are therefore found to be in absolute agreement. It must be concluded that neither in the acid, nor in the alkaline range of initial reactions (P_H) is the final acid reaction attained by cultures of *B. coli* in 2 % glucose peptone a physiological constant. It has already been shown [Wyeth, 1918] that the degree of acidity necessary to inhibit the fermentation of *B. coli* in a given medium is subject to very slight variations when different acids are added to produce the initial acid reaction of the medium, and it is possible, therefore, that similar small variations of the alkaline inhibition-point may be caused by varying the alkali used to produce the initial alkaline reaction of the medium. Similar—but larger—variations may be expected to result from the use of different media, as has already been demonstrated for glucose peptone and glucose phthalate media whose initial reactions lie between P_H 4·0 and 7·0. It may be permissible, perhaps, to point out that in certain parts of the reaction resultant curve of *B. coli* grown in 2 % glucose peptone, the final reaction resultant values present so little variation that it is only by the comparison of a large number of cultures, the initial reactions (P_H) of all of which have been accurately measured by means of the H-electrode, that it is possible to detect this ordered relationship between the initial and final reactions of the cultures. As will be shown below, this applies to a less extent to the curve for cultures of the same organism in 2 % peptone. The very narrow limits between which the final reactions produced by *B. coli* grown in 2 % glucose peptone (or similar media) can vary, appears to have led a number of earlier investigators,—few or none of whom appear to have subjected the initial reactions of their media to small and accurately-measured variations,—to postulate a physiological constant for the final reaction of *B. coli* grown in the media with which they experimented.

The results recorded in an earlier paper, together with those now presented, conclusively disprove the existence of such a constant for *B. coli*, and Wolf and Harris [1917] arrive at the same conclusion as regards *B. sporogenes* and *B. perfringens*.

INDOLE PRODUCTION BY *B. COLI* GROWN IN 2 % PEPTONE.

Since the indole test is so frequently employed for diagnostic purposes, and as a proof of the presence of faecal *coli* in water and other media, it was thought desirable to determine whether indole production ran parallel with the growth of the organism, as measured by the change of P_H occurring in the medium employed, or whether fermentation unaccompanied by indole formation could be demonstrated.

The influence exerted by acids and carbohydrates upon the production of indole in cultures of *B. coli* has already engaged the attention of a number of investigators, but few exact measurements of the true acidity of such cultures appear to have been made. Although the primary object of the experiments described below was to determine the effect, if any, of acids and alkalies upon indole production it was found impossible entirely to separate this question from the much wider one of the influence of carbohydrates upon indole formation, since, as will be shown, the statements made by certain investigators regarding this latter phenomenon are by no means concordant. It was therefore considered desirable to undertake a number of experiments involving the use of peptone containing various carbohydrates.

Peckham [1897] found that the indole production of *B. coli* may be taken as an approximate measure of the amount of protein digestion due to the organism.

Theobald-Smith [1897] found that on the third or fourth day of incubation *B. coli* cultures in bouillon gave the indole reaction when the acidity was equal to $N/100$ (by phenolphthalein), or when litmus was coloured faintly blue by the liquid, while with Dunham's solution a well-marked violet-red colour was developed at the end of three days. He suggests that *B. coli* and other facultative anaerobes can produce indole only when they are in contact with oxygen.

Marshall [1907] showed that *B. coli* grown for five days at 37° in peptonised beef broth containing 2·0 % lactose failed to produce indole.

Glenn [1911] added considerably to our knowledge of the influence of carbohydrates on the production of indole by *B. coli*. His chief results are shown below.

Medium	Gas production	% acid produced in 4 days	Indole production hours		
			24	48	72...216
1 % peptone	–	0	–	+	+...+
1 % peptone + 1 % glucose	+	2·2	–	–	–...–
1 % peptone + 1 % saccharose	+	1·2	–	–	–...–
1 % peptone + 1 % lactose	+	2·0	–	–	–...–
1 % peptone + 1 % mannitol	+	2·0	–	–	–...–
1 % peptone + 1 % starch	–	0	+	+	+...+

He found that, in the case of *B. coli*, both glucose and lactose inhibit the production of indole, but, with *Proteus vulgaris*, lactose does not exert an inhibitory effect. He considers that his results "do not disprove the conclusion that the production of acid inhibits the formation of indole," and he concludes

also that the presence of 1 % or more than 1 % of glucose always inhibits the production of indole by *B. coli* grown in peptone[1]. With *Proteus vulgaris* he found that the addition of lactic acid sufficient to produce an acidity equal to that of 0·5 % lactic acid inhibits indole production but that less than 0·5 % of the acid does not produce an appreciable retardation of the process.

Fischer [1915] found, however, that of the sugars, lactose, maltose, galactose, fructose, and glucose, only the last named completely retards the formation of indole. Total inhibition occurs after 43 hours in a medium containing from 1·80 to 2·25 % of glucose. He considers that the acids formed play no role in causing the retardation and that neither the H-ion concentration nor the concentration of the undissociated acids can be taken as the reason of the retardation. He concludes that the acid curves he constructed as well as the retardation experiments he performed with a mixture of galactose and glucose, make it appear possible that lactose is *not* first hydrolysed by *B. coli* but is fermented as such. The cause of the retardation, he suggests, depends upon a peculiar property of the glucose which enables it to inactivate the proteolytic enzyme produced by the *B. coli*.

Homer [1916] suggests that the lessened indole formation resulting from the activity of *B. coli* in glucose-containing media is due to the formation of a glucose-tryptophan complex which is less easily attacked than is tryptophan itself. This complex she regards as chemically unsuitable for bacterial decomposition and hence a lessened growth of *B. coli* ensues.

Zunz and György [1916] found it possible to grow *B. coli* in "l'eau physiologique" and in "l'eau physiologique + 1 % peptone" and that in these media indole formation occurred in less than 36 hours and 16 hours respectively. They conclude that indole production by *B. coli* depends upon the medium being rich in tryptophan and that the presence of certain carbohydrates inhibits the reaction. Unlike Glenn, they found that a medium containing peptone water + 1 % saccharose permits growth of *B. coli* together with indole formation within 96 hours—but not within 24 hours—after inoculation. Fischer does not appear to have experimented with saccharose, but, like Zunz and György—and unlike Glenn—he found that neither 1 % lactose nor 1 % maltose added to a peptone medium sufficed to inhibit the formation of indole by *B. coli*.

Material and Experimental Methods.

A number of media containing 2 % peptone were prepared and inoculated by methods similar to those previously described. The initial reactions of the tubed media were produced by the addition of either $N/10$ HCl or N NaOH in such quantities as to produce 20 different media whose initial reactions ranged from $P_H = 4{\cdot}0$ to $P_H = 10{\cdot}0$. In addition a number of tubes of 2 %

[1] Wolf has recently examined thirteen strains of *B. proteus* isolated from wounds, and has found that none of them produced indole. It is possible that Glenn was working with impure strains of this organism.

peptone of approximate neutral initial reaction ($P_H = 7{\cdot}0$) were prepared each containing 2 % of one of the following carbohydrates: glucose, maltose, lactose, saccharose, starch and of mannitol.

In order to determine the influence, if any, exerted by acids or alkalies upon indole formation in 2 % peptone a number of sets of the tubes of this medium first mentioned above were inoculated with *B. coli* and incubated at 37°. They were tested for the presence of indole and the P_H of the cultures recorded at the end of 0, 4, 8, 24 hours after inoculation and then at intervals of 24 hours to the end of the ninth day of incubation. The rosindole and vanillin-hydrochloric acid tests were employed in determining the presence or absence of indole in the cultures. A number of the results obtained are embodied in Tables II and III, and on p. 19. The *B. coli* used were of faecal origin, and no differences were exhibited between those of human and those of bovine origin.

The results obtained with a number of cultures of *B. coli* grown in 2 % peptone, containing no glucose but having been made of different initial reactions (P_H) may be summarised as follows:

(i) Whether the medium be rendered acid (initial reaction P_H less than 7·0) by the addition of *N*/10 HCl, or alkaline (initial reaction P_H greater than 7·0) by the addition of *N* NaOH, indole production always occurs if the initial reaction of the medium be such as permits fermentation to take place.

(ii) If the initial reaction (P_H) of the medium approximate to either the acid ($P_H = 4{\cdot}30$) or the alkaline ($P_H = 9{\cdot}37$) limiting value for the growth of *B. coli* in 2 % peptone, the indole formation is subject to well marked retardation, in some cases being delayed for 144 hours, whereas over the greater part of the range of the initial reactions employed indole is formed within 4 to 8 hours after inoculation.

(iii) No matter what may have been the initial reaction (P_H) of the medium, indole was never detected in the culture unless and until its reaction reached a value lying between $P_H = 4{\cdot}70$ and $P_H = 9{\cdot}20$. Thus in a culture, the initial reaction of which was $P_H = 4{\cdot}33$ and of which the acidity ultimately fell to $P_H = 5{\cdot}92$, the production of indole was not observed until the expiration of 144 hours after inoculation, the reaction of the culture then being in the neighbourhood of $P_H = 4{\cdot}80$. Similarly, in a culture of initial reaction $P_H = 9{\cdot}37$, the alkalinity of which eventually diminished to $P_H = 8{\cdot}55$, indole was first detected 96 hours after inoculation when the reaction of the culture was found to be $P_H = 8{\cdot}80$. On the other hand with cultures whose initial reactions lay between $P_H = 8{\cdot}89$ and $P_H = 5{\cdot}20$ indole invariably was found in less than 24 hours after inoculation.

In no case was the presence of skatole detected.

Consideration of Results of Experimental Data.

It would appear that the presence of indole in a culture may safely be taken as an indication of the presence of *B. coli* of faecal origin, but that its

production—like the growth of the organism itself—is retarded by the presence in the medium of an excess of free acid or alkali. The inhibition of indole formation may, however, be one result of a lowered vitality of the *B. coli* consequent upon the presence of this excessive amount of acid or alkali, and it may even be a precursor of the death of the organism, brought about by the action of these substances. The results of these experiments appear to confirm Fischer's statement that the acids and alkalies formed as a result of fermentation by *B. coli* do not cause an inhibition of indole production. On this point Glenn comes to no definite conclusion although he appears to lean towards the opinion that the inhibition of indole formation *may* be due to the presence of acids formed during fermentation.

In experiments with *B. coli* grown in 2 % peptone, had the indole formation occurred *only* in the most acid media of the series under examination it might be argued from a comparison of Curves I and II of Fig. 1 that the inhibitory effect produced by the addition of glucose to a peptone medium might be due solely to the production of acids as a result of saccharolytic fermentation. This would, at first sight, appear to be more probable since the final acid reactions produced by *B. coli* fermenting in 2 % glucose peptone lie between $P_H = 4{\cdot}27$ and $P_H = 4{\cdot}82$. Indole formation is completely inhibited in cultures of *B. coli* in 2 % glucose peptone throughout this range of reaction, and also in 2 % peptone media so long as their reactions remain more acid than $P_H = 4{\cdot}70$. Since in the latter cultures the inhibition is obviously the result of added acid, and the two ranges of reaction are practically identical, there is at hand an obvious explanation of the inhibitory action due to the presence of glucose in the former class of cultures. This explanation, however, cannot be correct for the following reasons:

(i) At no period during the fermentation of a 2 % glucose peptone medium does *B. coli* produce indole even when in the initial stages of the process the reaction of the culture is much less acid than $P_H = 4{\cdot}82$ or $P_H = 4{\cdot}70$.

(ii) The retardation of indole formation by *B. coli* fermenting in 2 % peptone occurs not only when excess of acid is present but also when there is excess of alkali present in the medium. Inhibition persists so long as the reaction of the culture is more alkaline than $P_H = 9{\cdot}20$. It appears probable therefore that the inhibition of indole formation in cultures of *B. coli* in peptone containing excess of either acid or alkali is due to a cause entirely different from that which operates when the inhibition results from the presence of glucose in the medium. In the former case the inhibition may be due to a lowered vitality of the organism, produced by the action of the free hydrogen or hydroxyl ions present, while in the latter instance the cause of the inhibition may be attributed to a peculiar property of the glucose, which enables it to inactivate the proteolytic enzyme of the *B. coli*.

The Influence of Certain Carbohydrates and Allied Compounds upon the Production of Indole in Peptone Media.

For these experiments the tubed media containing 2 % peptone together with either 1 % or 2 % of added carbohydrate were used (p. 16). The media were inoculated with different strains of *B. coli*. The cultures were incubated at 37° and examined at the end of periods of x hours as recorded in the subjoined tables.

Medium inoculated with *B. coli*	Indole formation at the end of hours x = 4	8	24	48	96	Gas production
2 % peptone + 1 % glucose	0	0	0	0	0	+
2 % peptone + 1 % lactose	0	0	+	+	+	+
2 % peptone + 1 % maltose	0	0	+	+	+	+
2 % peptone + 1 % mannitol	0	(+?)	+	+	+	+
2 % peptone + 1 % saccharose	0	0	0	(+?)	+	+
2 % peptone + 1 % starch	+	+	+	+	+	0

Medium inoculated with *B. coli*	Indole formation at hrs after inoculation x = 4	8	24	48	96	Gas production
2 % peptone	+	+	+	+	+	0
2 % peptone + 2 % glucose	0	0	0	0	0	+
2 % peptone + 2 % lactose	0	0	0	0	(+?)	+
2 % peptone + 2 % maltose	0	0	0	0	(+?)	+
2 % peptone + 2 % mannitol	0	0	0	(+?)	+	+
2 % peptone + 2 % saccharose	0	0	0	0	(+?)	+
2 % peptone + 2 % starch	+	+	+	+	+	0

From the above results it appears that the retardation produced by either 1 % or 2 % glucose in 2 % peptone may be regarded as absolute. The same percentages of starch produce no retardation, while that produced by mannitol is comparatively slight. Lactose and maltose appear to possess less retarding power than does glucose since 1 % of maltose or lactose produces but little effect while 2 % of either of these sugars produces a retardation approximating to the absolute inhibition resulting from the action of glucose. Since the initial reaction of all these cultures approximated to $P_H = 7{\cdot}03$ it may be concluded that where retardation was observed it could not be ascribed to the initial presence (or to the ultimate production) of alkali or of acid, but must be the result of some specific action of the added carbohydrate. It must also be noted that where partial retardation occurred, the production of indole was observed at precisely the time when,—if inhibition were due to resultant acidity,—it should begin to be inhibited.

The Effects of Acids and Alkalies on the Growth of *B. coli* in 2 % Peptone.

Material and Experimental Methods.

A number of flasks and tubes of 2 % peptone medium of various initial reactions were prepared as described above. As, in the case of the experiments with glucose peptone, the acid used was $N/10$ HCl and the alkali

employed was *N* NaOH. Some twenty sets of media whose initial reactions ranged from $P_H = 3{\cdot}50$ to $P_H = 10{\cdot}50$ were prepared. Inoculation and incubation were performed as in the preceding experiments.

The tubed cultures were examined for changes of reaction (P_H) and for the production of indole, while the flask cultures were submitted to the same tests, and in addition, a quantitative determination of the products of fermentation was made.

The results obtained in the two series of experiments are shown in the subjoined Tables II and III and a reaction resultant curve for *B. coli* grown in 2 % peptone was constructed, and is shown in Fig. 1 (II).

Table II. B. coli *grown in* 2 % *peptone rendered acid by the addition of N*/10 *hydrochloric acid.*

Strain of *B. coli* used, H_8.
Time of Incubation at 37°, 216 hours.
Temperature of Experiment, 20°.

No. of flask	No. of cc. of *N*/10 HCl added per 10 cc. of medium	Initial reaction P_H	Final reaction P_H	Fermentation	Cc. of *N*/10 NH_3 produced per 10 cc. of medium	Cc. of *N*/10 volatile acids produced per 10 cc. of medium	Indole formation
1	3·0	3·54	—	0	—	—	0
2	2·5	3·93	—	0	—	—	0
3	2·0	4·33	5·92	+	0·64	0·49	+ at 144 hrs
4	1·0	5·20	8·00	+	1·84	1·12	+ at 12 hrs + +at 72 hrs
5	0·5	6·46	8·27	+	2·52	1·37	+ at 12 hrs + +at 24 hrs
6	0·0	7·25	8·37	+	3·00	1·60	+ +at 12 hrs

Table III. B. coli *grown in* 2 % *peptone rendered alkaline by the addition of N* NaOH.

Strain of *B. coli* used, H_8.
Time of Incubation at 37°, 216 hours.
Temperature of Experiment, 20°.

No. of flask	Cc. of *N*/10 NaOH added per 10 cc. of medium*	Initial reaction P_H	Final reaction P_H	Fermentation	Cc. of *N*/10 NH_3 produced per 10 cc. of medium	Cc. of *N*/10 volatile acids produced per 10 cc. of medium	Indole formation
1	5·0	10·23	—	0	—	—	0
2	4·0	9·87	—	0	—	—	0
3	3·0	9·37	8·55	+	4·5	3·95	+ at 96 hrs + +at 168 hrs
4	2·0	8·89	8·51	+	4·1	3·34	+ at 24 hrs + +at 48 hrs
5	1·0	8·51	8·46	+	3·5	2·75	+ at 12 hrs + +at 24 hrs
6	0·5	7·95	8·40	+	3·2	2·21	+ +at 12 hrs

* Equivalent volumes of *N* NaOH were used.

Consideration of Experimental Data.

The salient fact revealed by a study of the values of the initial and final reactions (P_H) recorded in Tables II and III (columns 3 and 4) is that there is for *B. coli* grown in 2 % peptone,—as for the same organism in 2 % glucose peptone,—an obvious connexion between the initial reaction of the medium and the final reaction of the culture. This is represented graphically by the reaction resultant curve (II) in Fig. 1. Next it is observed that there is an "acid" and an "alkaline limiting value" of the initial reactions between which growth is permitted but beyond which the activity of the organism is inhibited. The limiting value in the acid range of initial reactions lies between $P_H = 4{\cdot}33$, which permits, and $P_H = 3{\cdot}93$ which inhibits the fermentation of *B. coli* in 2 % peptone. The degree of alkalinity which serves to inhibit fermentation lies between an initial reaction of $P_H = 9{\cdot}37$ which permits and $P_H = 9{\cdot}87$ which inhibits the process. The activity of *B. coli* in 2 % peptone is thus found to be determined by almost the same initial conditions of acid and alkaline reaction as is the case with the same organism fermenting in 2 % glucose peptone.

There are however striking differences between the behaviour of *B. coli* when grown in the two media, as may be seen by comparing Table I with Tables II and III, and also Fig. 1 (I) and Fig. 1 (II).

In both media the final reactions attained by a culture of the bacillus occupy positions on an unbroken curve, and in both media a diminution of the initial acidity (P_H) of the medium results in a diminution of the resultant acid reaction of the culture but beyond these general resemblances the similarity does not extend.

Whereas the range of initial reactions within which growth is possible has been shown to be practically identical for *B. coli* grown in the two media there is a remarkable difference between the range of final reactions attained in them. In 2 % glucose peptone the final reactions vary between the very narrow limits of $P_H = 4{\cdot}27$ and $P_H = 4{\cdot}82$ but in 2 % peptone the final reactions produced by the organism are as widely separated as $P_H = 5{\cdot}92$ and $P_H = 8{\cdot}55$ while it is quite possible that further experiments may lower the former of these two values to $P_H = 5{\cdot}70$, as will be seen by an inspection of Curve II, Fig. 1.

It is obvious that in the case of *B. coli* grown in 2 % peptone any suggestion of a "physiological constant" for the final reaction values cannot be entertained. It will be noted that the curve (Fig. 1 [II]) representing the reaction resultant of *B. coli* grown in 2 % peptone cuts the axis on which the initial reactions of the medium are plotted at a point representing a reaction of $P_H = 8{\cdot}48$. It is evident that if it were possible to prepare a 2 % peptone medium of which the initial reaction were exactly $P_H = 8{\cdot}48$ the fermentation of *B. coli* in this medium should be unaccompanied by any change of reaction. Several attempts to prepare a medium of this initial reaction were made, but

without success. The medium most closely approximating to that desired had an initial reaction of $P_H = 8{\cdot}51$. In it, fermentation was very active, indole, ammonia, volatile and fixed acids being formed in large quantities while the final reaction became slightly less alkaline—the ultimate value being $P_H = 8{\cdot}46$.

Passing from this point to the acid "death-point" of the organism it is observed that for cultures in a medium of which the initial reaction is acid (P_H less than 7·0) or possessing an alkalinity of less than $P_H = 8{\cdot}48$, the activity of the bacillus results in a change of the reaction of the culture in an alkaline direction. The inference which naturally would be drawn from an inspection of this region of the reaction resultant curve, viz. that the fermentation of *B. coli* in 2 % peptone the initial reaction of which is less alkaline than $P_H = 8{\cdot}48$ results in the formation of an excess of alkaline products, the reaction of the culture thereby becoming more alkaline than $P_H = 8{\cdot}48$, is confirmed by the results of the quantitative examination of the products of bacterial activity (Tables II and III, cols. 6 and 7).

In the absence of sugar, the action of *B. coli* on peptone is frankly proteolytic, the higher nitrogenous complexes being decomposed, with the consequent formation of ammonia, acids and indole.

When, however, *B. coli* ferments an alkaline 2 % peptone, the initial reaction of which is more alkaline than $P_H = 8{\cdot}48$ the activity of the organism results in a diminished alkaline reaction in the culture (*e.g.* a medium whose initial reaction is $P_H = 8{\cdot}89$, reaches a final reaction of $P_H = 8{\cdot}51$). Reference to the results of quantitative examination of the fermentation products shows that this diminished alkalinity is due to a rapid increase in the amount of acid produced. In general it may be observed that, as the initial reaction of the 2 % peptone in which *B. coli* ferments is varied from $P_H = 4{\cdot}33$ to $P_H = 9{\cdot}37$, the production of alkali is at first more marked than that of acid, while in the latest stages the increase of acid production is greater than that of alkali formation. This accounts for the "reversion" of the course of the reaction when the critical point $P_H = 8{\cdot}48$ is passed. It must be emphasised that the reversion does not proceed far enough to interrupt the regular path of the reaction resultant curve (Fig. 1 [II]) since any given culture eventually reaches a final reaction which is more alkaline than that attained by the next lower (more acid) member of the series.

A complete and accurate estimation of the fixed acids produced in the reactions was not performed, but sufficient data were obtained to show that in the neighbourhood of the "alkaline limiting value" ($P_H = 9{\cdot}37$) the total acids formed were in excess of the alkaline products, whereas in cultures the initial reactions of which were acid, neutral, or slightly alkaline (initial $P_H = 4{\cdot}33$ to 8·00) the ammonia production was greater than the formation of acids. It is evident that the theoretical yield of product of a culture whose initial reaction is $P_H = 8{\cdot}48$ (and is therefore constant during fermentation), should consist of equivalent quantities of acid and alkali. With the aid of

titration curves for acetic acid and ammonia added to alkaline and acid 2 % peptone respectively a curve was plotted which represented the effect produced by the addition to 2 % peptone of the amounts of ammonia and acids found in the quantitative determinations performed.

This was done by taking in turn the initial reaction of each culture and finding from the acetic and titration curve what would be the effect upon the initial reaction if the amount of acid actually found by experiment were added to the medium. To the new reaction value thus obtained is then applied the correction representing the effect produced by the amount of ammonia the presence of which had been determined by the experiment, this correction having been found from the ammonia titration curve. The final resultant obtained in each case represents the theoretical "reaction resultant" of the fermentation. The whole series of values thus obtained was plotted as a reaction resultant curve, which was found to be similar to, but not quite coincident with, that shown in Fig. 1 (II). This indicates that the curve representing the observed final reactions is such as would result had it been plotted from a series of final reaction resultant values produced by the formation of increasing amounts of acids and ammonia in a series of media of increasing alkaline reaction, the amounts of these acids and ammonia being of the same order as those found in the foregoing experiments.

Summary.

1. Growth of *B. coli* is possible in peptone media of certain well-defined initial reactions, the limiting values of which are but slightly, if at all, affected by the presence or absence of sugars.

2. The approximate limits of initial reaction are $P_H = 4{\cdot}27$ to $9{\cdot}87$.

3. A change of the initial reaction of the medium results in a change, similar in direction, but smaller in magnitude, in the final reaction of the culture.

4. In the case of *B. coli* grown in 2 % glucose peptone, while the initial reactions of the media vary from $P_H = 4{\cdot}30$ to $P_H = 9{\cdot}82$ the final reactions attained vary only between the very narrow limits of $P_H = 4{\cdot}27$ and $4{\cdot}82$.

5. In the case of the organism grown in 2 % peptone, when the initial reaction of the medium is varied from $P_H = 4{\cdot}30$ to $P_H = 9{\cdot}37$, the final reactions of the cultures vary from $P_H = 5{\cdot}92$ ($5{\cdot}70$?) to $P_H = 8{\cdot}55$.

6. The saccharolytic fermentation resulting from the growth of *B. coli* in 2 % glucose peptone renders the culture more acid than the original medium.

7. The proteolytic fermentation resulting from the growth of *B. coli* in 2 % peptone causes an increase of final alkalinity in the resulting culture unless the initial reaction lies between the alkaline limiting value ($P_H = 9{\cdot}37$ and $P_H = 8{\cdot}48$ in which case the final reaction of the culture is less alkaline than the initial reaction of the medium.

8. The saccharolytic fermentation of *B. coli* in 2 % glucose peptone media of different initial reaction produces approximately constant amounts of acids and no appreciable amount of ammonia.

9. The proteolytic fermentation of *B. coli* in 2 % peptone results in the formation of acids and ammonia, the amounts of both of which increase as the initial reaction of the medium is varied in the direction of increased alkalinity. Near the alkaline limiting value the production of acids is greater than that of ammonia.

10. The formation of indole is retarded by the presence of free alkali or acid in the medium.

11. The presence of certain sugars causes inactivity of the proteolytic enzyme produced by the bacillus and thus inhibits the formation of indole.

12. Different carbohydrates exhibit different degrees of indole-inhibiting power. The addition of 2 % glucose to peptone media produces complete inhibition of indole formation; 2 % lactose or 2 % maltose produce almost complete inhibition, while that produced by 2 % saccharose or 2 % mannite is only partial. 2 % starch possesses no inhibitory power.

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