

THE ACTION OF DILUTE ACIDS UPON BACTERIAL GROWTH IN OPTIMUM HYDROGEN-ION CONCENTRATION.*

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THE use of culture media of definite P_H concentration has suggested enquiries into the limiting and optimum zones for each bacterium.

So far as the initial reaction of the medium is concerned, this has been a simple matter. But when it becomes necessary to determine the final reaction of the medium, to consider the composition of varying media, and to allow for intervening conditions which favour or hinder growths, the problem presents many new aspects. Not the least among these, is the appearance of certain acids or salts as a direct result of the metabolism of the inseeded organisms.

In order to study the effects of these substances, it was thought worth while, therefore, to add minute quantities of different acids to certain media, so as to observe the early rate of growth of bacteria, under conditions which usually obtain only at a later stage. To this end, an optimum P_H concentration was determined for each strain in the presence of added acids. That such a series of observations would yield but crude findings was fully appreciated: yet it seemed possible that some of the unknown, and at present uncontrolled factors might be more clearly defined and openings be found for additional enquiry.

After due consideration of the mass of work upon the phase of lag, etc., and an extended experience with the measurement of growth by "opacity" methods, it was decided to approach the subject from the standpoint of total, or hourly, generations at the first maximal period of growth.

The Initial Reaction of Medium and the Maximum Growth.

Prior to the determination of the behaviour of bacteria under the conditions just laid down, it was evident that a preliminary survey should be made of the growth from the standpoint of the initial reaction of the culture medium.

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Some of the pathogenic organisms had already been examined in this way (1919 to 1921¹), but the media varied in composition.

As it is recognised that such differences in the composition of the medium employed are associated with variations in the intermediary and final P_H constants, it was thought well to re-examine these in our own culture media, and to supplement the existent findings with estimations hitherto not included. The results are summarised in Table I. They afford an indication of the general capacity for growth manifested by groups of micro-organisms under strict P_H conditions. They do not, however, allow for the lesser or wider characteristics of individual strains.

TABLE I.

Limits of Hydrogen-ion Concentration permitting Growth of Organisms.

| COCCI. | P_H Zone. | | BACILLI. | P_H Zone. | |
|---------------------------|-------------|---------|-------------------------|-------------|---------|
| | Acid. | Alkali. | | Acid. | Alkali. |
| Staphylococci . | 2·6 | 10·0 | B. coli . . . | 2·6 | 9·5 |
| Streptococcus— | | | B. suipestifer . . | 2·6 | 9·5 |
| Hæmolyticus . . . | 4·5 | 8·0 | B. gærtner . . . | 2·6 | 9·0 |
| Viridans . . . | | | B. pestis . . . | 2·6 | 9·0 |
| Fæcal . . . | 4·5 | 10·0 | B. typhosus . . . | 4·0 | 8·6 |
| Bovine . . . | | | B. para- α . . . | 4·0 | 10·0 |
| Pneumococcus* . | 5·0 | 8·0 | B. para- β . . . | 4·0 | 10·0 |
| Meningococcus* . | 7·4 | 7·8 | B. anthracis . . . | 4·0 | 9·5 |
| Gonococcus* . | 7·0 | 8·0 | B. dys. (Shiga) . . | 4·5 | 10·0 |
| | | | Flexner . . . | 4·5 | 10·0 |
| | | | (Y) . . . | 4·5 | 10·0 |
| | | | V. cholerae . . . | 5·6 | 10·0 |
| | | | B. melitensis . . . | 6·3 | 9·6 |
| | | | B. influenzae . . . | 7·6 | 8·2 |
| | | | B. diphtheriae . . | 6·0 | 8·2 |
| * In nut nasgar broth. | | | | | |

We have already reported upon the group differences which occur in staphylococci obtained from different sources, and we have accumulated data which indicate that the sub-groups or types of pneumococci and streptococci exhibit also some variability in their limiting and optimum zones (1918²). Similarly, Cole and Lloyd (1917¹) have drawn attention to the widening of the range which takes place in the culture of gonococci when hormones are added to the media employed.

The Growth of Bacteria when the Initial P_H Concentration is obtained in the Presence of Dilute Acids.

In an earlier paper we suggested that nutrient media ought to be constructed in such a manner that we may not only direct the biochemical activities of the micro-organism, but possess a means of varying its antigenic and agglutinogenic properties, and even

confront it *in vitro* with the various difficulties which it has to overcome while seeking its food in animal tissues (1918²). Also that, for purposes of immunity, bacterial growth should be incited under conditions approximating to the lytic and physical changes which accompany vital response to an irritant. Some of these features have been borne in mind in the present investigation.

Methods.—The object in view was to determine the rate of growth of various organisms in media of definite P_H concentration in the presence of varying acids. Remembering the complexity of ordinary media and the differing dissociation constant of the several acids, it was evident that as simple a medium as possible should be devised, and that a preliminary survey of the conditions induced should precede any defined plan concerned with specific nutrients.

Hence the medium employed throughout was a peptone solution prepared from pure casein, adjusted to a content of 20 *n*/10 amino-acid (90 per cent. mon-amino acids). This was made neutral to phenolphthalein with NaOH, and then neutralised to P_H 7.6 by the addition of *n*/HCl, HNO₃, etc. The amount of the acid employed was calculated, so that the quantity present in each culture-tube was known.

The organism to be tested was grown in the same medium by daily transference for three days. At first it was deemed sufficient to inoculate tubes of different P_H intensity, and to read the results twenty-four hours later by the "opacity" method. This was soon abandoned in favour of a valuation of the rate of growth during the period of logarithmic increase, and within that average period which precedes any P_H variation. Since, however, the factors that influence the inhibition period are not fully worked out, it was necessary to limit the possible influences as far as possible. Accordingly the period of maximal increase was determined for each organism, the enumerations were made on three daily successive batches, and the P_H narrowed to the range associated with the smallest P_H alteration. To the counts obtained during this period the equation used by Cohen and Clark was applied (1919³):—

$$b = a \times 2^n$$

b = Number of bacteria at end of period T.

a = Number at beginning of the period.

n = Number of generations during T.

n/T = Number of generations per hour.

On the whole, the average of the successive tests based on the percentage value of the day's batch, yielded approximate results. But in some instances there were inhibitions quite out of proportion to the usual experimental error associated with bacterial enumerations. For the moment, these are reserved for further observations.

Standard loopfuls were employed for the inocula, since Graham Smith has found that the variations of numbers encountered in emulsions made by using standard loops are not likely to affect materially the results in different experiments of the same kind (1920¹²).

Into 10 c.c. of the warmed broth a standard loopful of an organism at its maximal period of growth was placed. After thorough shaking, a loopful was planted on a warmed agar plate of the same medium, and counted after twenty-four and forty-eight hours. At the determined maximal period, not over four hours, except with some streptococci, a standard loopful was transferred from the growing broth culture to warmed plates, and counts made as before. As in previous work, therefore, the technic was adapted to allow for errors associated with the age of the organism, the amount of inoculum, and the precise stage of bacterial growth (1918²).

The Differences in Growth in Varying Acid Solutions.

With the experience gained during the scrutiny of the method employed, we now proceeded to determine the effect of the dilute acid solutions upon the growth of pathogenic organisms. Taking the preparation of our medium into consideration, the final amount of sodium chloride approximates to 1·12 per cent. Hence the addition of very dilute acids would not bring about the formation of any sodium salts, and the acids would act at the upper limits of their dissociation capacities. Any salting-out effect would be practically negligible. The control of the colorimetric neutralisations in the presence of these acids, with electrical estimations, and the relations of the dissociation constants, will form the subject of a further communication.

Table II. shows the variations obtained in the actual generations per hour in successive experiments. It reveals the extent of experimental error and permits an assessment of the results to be submitted. All the bacteria were subjected to similar determinations.

TABLE II.

The Variations of successive Daily Experiments.

ACTUAL GENERATIONS PER HOUR.

| Acids. | Dilutions. n/l Solutions. | <i>Streptococci.</i> | | | <i>B. typhosus.</i> | | | <i>B. dysent.</i> (Flexner). | | | <i>B. dysent.</i> (Shiga). | | | <i>B. coli.</i> | | |
|--------------|---------------------------------|----------------------|------|------|---------------------|------|------|---------------------------------|------|------|-------------------------------|------|------|-----------------|------|------|
| | | 1. | 2. | 3. | 1. | 2. | 3. | 1. | 2. | 3. | 1. | 2. | 3. | 1. | 2. | 3. |
| Acetic . | 1/150 | 0·49 | 0·44 | 0·65 | 0·69 | 0·75 | 0·63 | 0·42 | 0·91 | 0·25 | 0·06 | 0·69 | 0·60 | 2·01 | 1·33 | 1·46 |
| Butyric . | 1/166 | 0·49 | 0·42 | 0·69 | 0·13 | 0·46 | 0·70 | 0·05 | 1·16 | 0·67 | 0·47 | 0·77 | 0·81 | 1·29 | 1·10 | 1·30 |
| Citric . | 1/120 | 0·38 | 0·25 | 0·58 | 1·01 | 0·54 | 0·63 | 0·52 | 0·75 | 0·32 | 0·48 | 0·39 | 0·48 | 1·34 | 1·23 | 1·30 |
| Lactic . | 1/166 | 0·46 | 0·41 | 0·74 | 1·24 | 0·95 | 0·76 | 0·69 | 0·90 | 0·70 | 0·26 | 0·87 | 0·78 | 1·38 | 1·30 | 1·39 |
| Nitric . | 1/166 | 0·97 | 0·52 | 0·93 | 1·04 | 1·02 | 0·83 | 0·37 | 0·73 | 0·67 | 0·66 | 0·65 | 0·77 | 1·89 | 1·31 | 1·41 |
| Oxalic . | 1/133 | 0·45 | 0·39 | 0·54 | 0·12 | 0·44 | 0·65 | 0·50 | 0·77 | 0·27 | 0·40 | 0·42 | 0·53 | 1·73 | 1·36 | 1·32 |
| Phosphoric | 1/90 | 0·34 | 0·36 | 0·49 | 1·07 | 0·86 | 0·64 | 0·17 | 0·93 | 0·06 | 0·24 | 0·37 | 0·58 | 1·73 | 1·23 | 1·36 |
| Salicylic | 1/180 | 0·54 | 0·43 | 0·66 | 0·91 | 0·97 | 0·93 | 0·45 | 0·85 | 0·41 | 0·21 | 0·40 | 0·40 | 1·72 | 1·19 | 1·25 |
| Tartaric | 1/181 | 0·37 | 0·38 | 0·48 | 0·62 | 0·83 | 0·73 | 0·59 | 0·67 | 0·49 | 0·27 | 0·39 | 0·47 | 1·68 | 1·17 | 1·19 |
| Sulphuric | 1/210 | 0·58 | 0·55 | 0·47 | 0·75 | 0·90 | 0·75 | 0·54 | 0·70 | 0·36 | 0·69 | 0·67 | 0·77 | 1·89 | 1·30 | 1·38 |
| Hydrochloric | 1/166 | 0·23 | 0·34 | 0·37 | 0·63 | 0·71 | 0·81 | 0·53 | 0·78 | 0·39 | 0·53 | 0·78 | 0·65 | 1·75 | 1·15 | 1·27 |

Calculating the number of generations per hour in terms of percentage of the average daily growth, and taking an average of the three sets of experiments for each acid and each bacterium, the results of the survey are summarised in Table III.

These findings show a consistent action on the part of some of the acids on the one hand, and the predilection of certain bacteria for particular acids on the other. Table IV. indicates the more prominent results in shortened summary,—the intermediaries being omitted.

TABLE III.

The percentage average of Three Successive Growths. P_H 7.6.

GENERATIONS PER HOUR.

| Acids. | Dilutions. n/1 Solutions. | <i>Staphylococci</i> . | <i>Streptococci</i> . | <i>B. coli</i> . | <i>B. typh.</i> | <i>B. paratyph. a.</i> | <i>B. paratyph. β.</i> | <i>B. dysent.</i> (Shiga). | <i>B. dysent.</i> (Flexner). | <i>B. suispestifer.</i> |
|------------------------|---------------------------------|------------------------|-----------------------|------------------|-----------------|------------------------|------------------------|-------------------------------|---------------------------------|-------------------------|
| Acetic | 1/150 | 1.06 | 1.07 | 1.11 | 0.91 | 0.74 | 0.83 | 0.74 | 1.11 | 1.05 |
| Butyric | 1/166 | 0.94 | 1.07 | 0.84 | 0.59 | 0.93 | 1.35 | 1.20 | 0.76 | 0.99 |
| Citric | 1/120 | 1.16 | 0.80 | 0.95 | 0.97 | 0.90 | 0.88 | 1.21 | 1.04 | 0.99 |
| Lactic | 1/166 | 1.13 | 1.03 | 1.04 | 1.32 | 1.33 | 1.29 | 1.11 | 1.19 | 1.09 |
| Nitric | 1/166 | 1.07 | 1.63 | 1.07 | 1.29 | 1.04 | 1.32 | 1.35 | 0.89 | 1.04 |
| Oxalic | 1/133 | 1.03 | 0.95 | 1.03 | 0.53 | 1.12 | 0.98 | 1.03 | 1.07 | 0.97 |
| Phosphoric | 1/90 | 0.98 | 0.81 | 1.01 | 1.14 | 1.09 | 0.50 | 0.79 | 0.67 | 0.87 |
| Salicylic | 1/130 | 0.71 | 1.09 | 0.98 | 1.21 | 0.63 | 0.75 | 0.62 | 1.10 | 0.93 |
| Tartaric | 1/181 | 1.02 | 0.85 | 0.94 | 0.97 | 1.21 | 1.36 | 0.68 | 1.08 | 1.06 |
| Sulphuric | 1/210 | 1.12 | 1.13 | 1.07 | 1.06 | 0.95 | 0.80 | 1.39 | 1.13 | 1.01 |
| Hydrochloric | 1/166 | 1.12 | 0.64 | 0.97 | 0.95 | 1.19 | 0.83 | 1.25 | 0.64 | 1.04 |

TABLE IV.

| | Acceleration. | | Delay. | |
|---------------------------------------|--------------------|--------------------------|------------------------|-------------|
| <i>Staphylococci</i> | Lactic | Citric | Salicylic | Butyric. |
| <i>Streptococci</i> | Nitric | Sulphuric | Hydrochloric | Citric. |
| <i>B. paratyph. -α</i> | Lactic | Hydrochloric | Salicylic | Acetic. |
| <i>B. paratyph. -β</i> | Tartaric | Butyric | Salicylic | Phosphoric. |
| <i>B. typhosus</i> | Nitric | Lactic | Oxalic | Butyric. |
| <i>B. dysent.</i> (Shiga) | Nitric | Sulphuric | Salicylic | Tartaric. |
| <i>B. dysent.</i> (Flexner) | Lactic | Acetic | Butyric | Phosphoric. |
| <i>B. coli</i> | Acetic | (Nitric) | Butyric | Tartaric. |
| <i>B. suispestifer</i> | Lactic | (Hydrochloric) | Phosphoric | Oxalic. |
| | | Sulphuric | | |

We may perhaps point out that these figures were obtained by the use of one medium alone, and that of a pure casein origin. Others might have been obtained had a less defined medium such as meat-broth or meat-extract been employed. The outstanding uniform acceleration with lactic, nitric and sulphuric acids recall the contents of Uschinsky's medium with its high percentage of ammonium lactate. They also suggest that the heavy growths obtained in veal broths may, in part, be due to a higher lactic acid content, since, pound for pound, the flesh of young animals is said to be richer in protein than that of old animals.

They call also to mind the widespread distribution of *D*-lactic acid in muscle and other tissues, and the fact that lactic acid in dilutions of 1 in 500 repels leucocytes (1907⁴). It may be that the increase of lactic acid in the tissues during fatigue, plays a part in the lowered resistance to infection. Similarly, the abnormal amount of tissue lactic acid in anæmia, uræmia, eclampsia, and liver lesions, may be

related to the ease of bacterial invasion in these conditions. Blair Bell points out that lactic acid is commonly present in the vagina, but that gonococci are indifferent to this acid, and therefore proliferate in spite of it (1921⁵).

It may be noted that with the nine strains of bacteria employed, lactic acid acts as an accelerant in six, nitric in five, and sulphuric in three, while in these dilute solutions, phosphoric and butyric appear to retard in three, and oxalic and tartaric in two instances. Poynton and Paine (1900¹⁴) and Beattie (1904¹⁵) in their work on the ætiology of rheumatic fever state that the isolated organisms grew best in milk or bouillon media, "rendered slightly acid with lactic acid." Taylor found that in simple broth there was inhibition of streptococcal growth with higher dilutions of butyric than other acids, and that tartaric behaved similarly with staphylococci (1917⁶).

Graham Smith observed that lactic acid in quantities up to 0.75 c.c. of a $n/10$ solution did not inhibit growth of staphylococcus in a meat extract agar minus salt, while butyric acid in similar strength did reduce the number of colonies. On the other hand, in the presence of salt, lactic and butyric acids caused inhibition of growth in smaller quantities than when salt was absent (1920¹²). The conditions in these experiments differed considerably from those we adopted, but in their general aspects the results are not conflicting.

From the standpoint of the prevention of bacterial growth by the action of acids, Paul and Kronig pointed out that strong acids act generally in relation to their H-ions, but that there was also a specific action of the particular anion, or of the undissociated acid (1898⁷). Winslow and Lochridge found that with weak acids the effect was due to the molecules rather than to the H-ions (1906⁸). While Paul and Reuss considered the toxic action of H-ions of weak organic acids was catalyzed by anions (1910⁹).

TABLE V.

| | Media. | Concentrations in terms of Strong Acids. | | | | | | |
|------------------------------|-----------------------|--|----------------------------------|----------------------------------|--------------|-------------|-----------------|------------------|
| | | HCl. | H ₂ SO ₄ . | H ₃ PO ₄ . | Acetic. | Butyric. | Propionic. | |
| Bial ¹¹ | 1.5% glucose | c.c. 0.005 | c.c. 0.005 | c.c. 0.01 | c.c. 0.06 | c.c. ... | c.c. ... | Yeasts. |
| Taylor ⁶ | Broth | 0.21 | 0.16 | ... | 0.31 | 0.15 | 0.11 | Streptococci. |
| Taylor | Broth | 0.22 | 0.23 | ... | 0.29 | 0.24 | 0.12 | Staphylococci. |
| Cohen and Clark ³ | Peptone glucose broth | 2.0* | ... | ... | 7.5* | ... | Formic. 2.0* | <i>B. coli</i> . |

* $n/1$ solutions.

Wyeth (1918¹⁰, Bial, 1902¹¹) working with 2 per cent. lait-proto-peptone and 2 per cent. glucose and buffered media in which the final titration was adjusted by varying acids, shows that the final P_H of the

media bears a definite relationship to the type of acid employed. For instance—

| | |
|--------------------------|-----------------------------------|
| with HCl adjustment | <i>B. coli</i> yields P_H 4.30. |
| „ Lactic acid adjustment | „ P_H 4.52. |
| „ Acetic acid adjustment | „ P_H 4.57. |

There is also a distinct quantitative difference in the effect of varying acids in inhibiting growth (Table V.).

SUMMARY.

Using a P_H 7.6 casein medium containing dilute amounts of various acids, the period of logarithmic increase may be altered.

Individual bacteria vary in their response to the accelerating or retarding influences, but the presence of lactic or nitric acids in dilutions of 1 in 166 acts generally as a stimulant to growth, while salicylic, butyric and phosphoric delay the period. The influences of these factors upon the antigenic properties of the organisms are under investigation.

The application of these observations to the routine carrying out of blood cultures has yielded promising results, and the details of the technique are being worked out.

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