THE RELATION OF THE REACTION OF THE CULTURE MEDIUM TO THE PRODUCTION OF HAEMOLYSIN.

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1 Chart in text.

Ever since artificial media have been used for the cultivation of micro-organisms, the question as to what is the best reaction—that is the degree of acidity or alkalinity—has been a moot point. It has been assumed gratuitously that there is an optimum reaction which is the same for all the common bacteria met with in the laboratory. In recent years however some attempts have been made to settle the question definitely, notably by Eyre and Deelemann. On enquiring what reaction of medium was adopted in six different London laboratories, Eyre found that very divergent standards were used, ranging from 20 units of acidity to 10 units of alkalinity on his scale. (20 units of acidity implies that 20 c.c. of normal alkali must be added to each litre to make it neutral to phenolphthalein. In the case of alkalinity the indicated number of c.c. of normal acid must be added.) The importance of having a definite reaction had until recently not impressed itself upon the majority of bacteriologists. The possibility that although many organisms may grow on media of comparatively widely differing reactions, yet only one of these reactions may be suitable for the growth of a more delicate kind, had been overlooked. Consequently they failed to grow bacteria which by careful adjusting of the reaction of the medium could be readily cultivated. Martin (1910), for example, has lately pointed out the ease with which gonococcus can be made to grow

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on serum agar if the reaction is carefully adjusted. In later years the subject has assumed still more importance in connection with the production of bacterial toxins, which have been submitted to a large amount of investigation. In the production of diphtheria toxin it was soon recognised that bouillon containing more alkali than is usually employed for ordinary bacteriological work, yielded the most toxin, and even various devices were invented to avoid the initial production of acid which usually takes place as the consequence of bacterial growth in a sugar-containing medium. Madsen (1896) has made a thorough examination of the factors governing the production of diphtheria toxin. By following the reaction of a growing culture from day to day he found two different types of growth occur,—one in which the medium increases in acidity without abatement, and such cultures are atoxic; in the other the primary increase in acidity is followed by a decrease, and subsequent increase in alkalinity. These cultures are sometimes toxic. The two conditions seemed to have relation to the amount of alkali added in the preparation of the bouillon, in that a strongly alkaline medium gave an alkaline end reaction while a less alkaline medium gave sometimes an acid and sometimes an alkaline end reaction.

In recent years many different toxins have had to be prepared for experimental purposes and the inconstant amount of toxin produced on different occasions has been a source of trouble to many workers. The following experiments have been undertaken to see what part the reaction of the medium plays in the production of a toxin, whether, for example, there is a sharply marked optimum reaction, and if so where it lies, or whether the production is about the same in any medium in which the bacterium grows. The organism with which I have worked is *Vibrio nasik* from which a considerable yield of haemolysin can be obtained and which is susceptible of fairly exact measurement. At the outset it was necessary to define its limits of growth in media of increasing alkalinity and acidity, and at the same time I have compared it with several other organisms commonly met with.

I. *Determination of the Limits of Growth.*

The bouillon was made from fresh meat and contained $1\frac{1}{2}$ % pepton. After filtration NaOH was added until the reaction was just alkaline to phenolphthalein. It was then autoclaved for 15 minutes at 115° C. and filtered *after cooling*, as more precipitate separates out at a lower temperature. The reaction was now tested and more NaOH added if

necessary to make it slightly alkaline. It was autoclaved again and usually a slight precipitate appeared which was removed. The reaction was then adjusted so as to be exactly neutral to phenolphthalein by adding HCl to neutralise excess of NaOH. Test tubes were filled with 8 c.c. of this bouillon. The required reactions were obtained by adding quantities of normal solutions of NaOH or HCl, as the case might be, to the tubes. The tubes were then sterilised. This method was adopted as being the one most commonly used in laboratories when preparing bouillon. The final reaction of the bouillon to indicators after sterilising was not taken into account, as it is probably not significant when a known amount of alkali or acid has been added. The number and nature of the substances in bouillon acting upon indicators make the estimation of the alkalinity or acidity of various samples of doubtful importance, as we do not know to what substances the total alkalinity or acidity is due. It seems a more rational proceeding to start from a fixed point which can be determined with a fair amount of accuracy—the neutral point to phenolphthalein—and vary the quantities of the single constituents, alkali or acid. If the bouillon is always prepared in the same way it is then easy to get it uniform each time. The tubes containing the greatest quantities of alkali and also of acid showed slight precipitates which were neglected. They partly dissolved at the temperature of incubation, 37°. It is interesting to note that they occurred in both alkaline and acid reactions. The tubes were inoculated with young cultures of the organisms investigated, 24–48 hours old, and placed in an incubator at 37°. They were examined after 24 hours and again after 5 days' incubation. The tubes were examined with the naked eye for turbidity, the microscope being employed in doubtful cases, for example with streptococcus. The whole series of organisms was tested at the *same* time and on the *same* preparation of bouillon, and the different members are therefore comparable. The results are set forth in Table I. The double cross + +, indicates the tubes in which growth was visible in 24 hours. The single cross + marks the supplementary tubes which showed growth after 5 days. The former have been enclosed by a dark line to bring into relief those tubes in which growth took place most easily. The numbers indicate the content in NaOH or HCl at the rate of c.c. of normal solutions to a litre.

It will be noticed that the extent of growth varies considerably with the different bacteria, but there seems to be a common optimum roughly about the neutral point, judging from the results of the first day's growth. This is of importance as it shows that bouillon of one standard reaction

may be used for general laboratory purposes, although in certain cases it may be advisable to modify the reaction somewhat. Deelemann (1897) working by a different method also finds that the optimum of growth does not vary greatly for different bacteria. Starting with the blue-litmus neutral point he added different quantities of alkali to gelatin medium and counted the colonies under the microscope. He found the optimum growth usually occurs when from 0·17 c.c. to 0·34 c.c. % $\frac{N}{1}$ NaOH is added. This corresponds to about 21 to 23 acidity units on Eyre's scale, and seems to be an unusually acid medium. But the

TABLE I.

	Alkaline								Neutral	Acid				
	80	70	60	50	40	30	20	10	0	10	20	30	40	50
Vibrio nasik					+	+	++	++	++	++				
V. cholerae	+	++	++	++	++	++	++	++	++	++	++			
B. pyocyaneus		+	+	+	++	++	++	++	++	++	++	++		
Staphylococcus aureus		+	++	++	++	++	++	++	++	++	++	++		
B. anthracis						+	++	++	++	+	+			
B. prodigiosus				+	+	++	++	++	++	++	++	++		
B. typhosus				+	++	++	++	++	++	++	++	++		
Proteus vulgaris				++	++	++	++	++	++	++	++	+		
B. dysenteriae (Flexner)					+	++	++	++	++	++	++	++		
B. subtilis		+	+	+	++	++	++	++	++	++	++			
B. acidi lactici		+	+	++	++	++	++	++	++	++	++	++		
B. mesentericus	+	+	+	+	++	++	++	++	++	++	++	+		
M. melitensis		+	+	++	++	++	++	++	++	++	++	++		
B. coli communis			+	+	+	++	++	++	++	++	++	++		
B. diphtheriae								++	++	++	++			
B. megatherium							+	+	++	+				
Streptococcus (Joint)						+	+	++	++	++	++			
Streptococcus (Blood)								++	++	++				

The numbers at the heads of the columns represent the number of cubic centimetres of $\frac{N}{1}$ NaOH, or $\frac{N}{1}$ HCl as the case may be, which have been added to a litre of bouillon neutral to phenolphthalein.

The double cross represents visible growth in 24 hours. The single cross marks the supplementary tubes in which visible growth took place after five days.

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neutral point to litmus is not determinable with anything like the degree of accuracy that the neutral point to phenolphthalein is and two readings may differ widely.

TABLE II.

Day of Incubation	Quantities giving equal haemolysis				
	Alkaline		Neutral	Acid	
	15 c.c.	5 c.c.	0 c.c.	5 c.c.	10 c.c.
4	0·022	0·02	0·02	0·04	0·065
5	0·017	0·017	0·017	0·022	0·04
6	0·017	0·017	0·025	0·017	0·017
7	0·017	0·02	0·04	0·017	0·017
9	0·13	0·08	Trace in 2·0	0·017	0·025
10	2·0	Nil	—	0·025	0·04
11	—	—	—	0·04	0·13
12	—	—	—	0·17	0·8
13	—	—	—	0·65	0·8
16	—	—	—	0·5	Nil

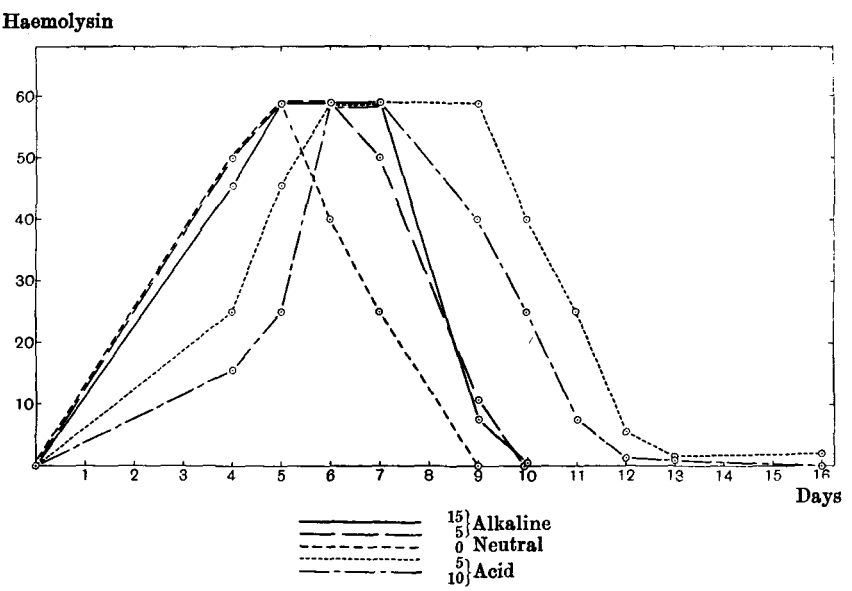
In my experiments in almost every case the limit of growth was extended after the first day and in the majority this took place at the alkaline end. This may be taken to indicate that a degree of alkalinity which partially inhibits growth can be overcome if not too excessive. This may be brought about by a slight growth forming a small amount of acid from the sugars present which neutralises some of the alkali and so brings the reaction of the medium gradually within the limits of vigorous growth, more rapid multiplication then taking place. This view is confirmed by an incident I have met with in the case of *Vibrio nasik*. Having found its alkaline and acid limits of growth in the above manner, I have inoculated flasks of bouillon of 300–500 c.c. with reactions covering this range and have invariably found that the flasks close to the alkaline limit, as indicated in test tubes of 8 c.c. fluid, did not grow, the reason presumably being that the larger amount of alkali was too great for the bacteria to overcome in the manner suggested.

II. *Determination of the Optimum Reaction for Haemolysin Production.*

The haemolysin was that produced by *Vibrio nasik*. Five litre flasks of bouillon containing 275 c.c. of varying reactions were inoculated with young cultures, and small samples were removed from time to time and shaken with toluol. They were all kept in an ice safe until the end of the experiment and all tested on the same blood on the same day

employing the technique instituted by Madsen. In estimating the haemolysin in a given sample, varying amounts of the fluid were put into test tubes and made up to 2 c.c. with 0·9 % NaCl solution, and then 8 c.c. of a 1 % suspension of washed sheep red corpuscles forcibly introduced with a syringe to ensure immediate mixing. The tubes were then shaken, heated for 2½ hours at 37°, again shaken and placed in an ice safe until next day. One tube showing about 30–40 % haemolysis was chosen and matched with others of similar tint throughout the different series. The reciprocals of the quantities giving equal haemolysis were then charted.

From an examination of the chart several points of interest are discovered.



Haemolysin production of *Vibrio nasik*. Bouillon of different reactions.

The production of haemolysin in the case of *Vibrio nasik* is practically co-extensive with its range of growth in media of varying reactions. It is probably a function of the organism's vigorous growth and is only absent in cultures when this is partially inhibited, that is in the most alkaline and most acid media in which multiplication is just possible. Moreover it will be seen that each curve has attained the same height although at different times and lasting for different periods of time. Thus the power of producing haemolysin is not altered at all

by changing the reaction of the bouillon; its appearance and disappearance only are modified.

The production of haemolysin in the alkaline and neutral bouillon cultures took place distinctly earlier than in the acid ones. It seems reasonable to suppose that this is due to the excess of alkali quickly neutralising the acid formed from the decomposition of sugars, whereas in acid media the organisms have themselves to form the necessary alkali from the pepton. Bacterial growths in bouillon if left long enough nearly always become alkaline from decomposition of the pepton, and anything which favours this process would be likely to encourage the speedier appearance of lysins which are derivatives of the nitrogenous bodies. We know that proteids are attacked sooner in sugar-free media, as indol is produced earlier in such a case.

When the amount of haemolysin has attained its greatest height it remains stationary for a short period. A curious flat-topped curve is thus produced. Walbum has found that this is dependent upon an insufficiency of pepton at the point where the flat top begins. His hypothesis is that the bacteria produce a prolysin not haemolytic in itself, but by further action upon pepton giving rise to true haemolysin. This view is supported by the fact that if a sample is taken from any point between the two bounding points of the flat top and pepton added, an increase in the amount of haemolysin always takes place. Upon what the duration of this flat top depends in different experiments it is difficult to say. I believe however that it has some relation to the volume of the culture fluid, for when using larger amounts of bouillon (500 c.c.) than in the present instance I have noticed that the condition lasts longer. In the present experiment all the curves show a flat top except the "neutral bouillon" one. This curve has a fastigium of such short duration that if daily samples had not been taken it would have been entirely missed, and it shows on the chart as a point. The descending limbs of the curves can be well compared in this case as they fall from the same altitude. The point to notice is that the curve representing the most alkaline bouillon has the steepest gradient probably due to the destroying action of the excess of alkali present. Famulener and Madsen (1908) have investigated the increase in the rate of destruction of vibriolysin by heat, caused by alkalis and acids. To vibriolysin, the reaction of which corresponded to one acid unit on Eyre's scale, they added increasing amounts of NaOH and HCl, and compared their rates of destruction at a fixed temperature. The alkaline samples quickly showed an increased rate; and the acid only after a considerable

amount had been added well outside the limit of growth of *nasik* in acid media. Then however the rapidity of increase was greater than in the case of alkali. My experiments are in unison with this result, in that the most alkaline bouillon shows an increased rate of destruction whereas the most acid bouillon does not. It would seem from an inspection of the curves that the acid culture bearing the number five holds its haemolysin for the longest period of time, which would imply that at this point it is least affected by the alkaline or acid content. Thus although there is no optimum of quantity as regards the production of haemolysin in bouillon of different reactions, yet there is an optimum of duration of haemolysin which seems to be about the point where the bouillon requires 5 c.c. of normal NaOH per litre to neutralise it to phenolphthalein.

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Conclusions.

1. Many bacteria have an extensive range of growth in bouillon of varying reactions; some have a much more restricted range, as the streptococci obtained from the blood stream.

2. There seems to be roughly a common optimum of growth when the reaction of the bouillon is neutral to phenolphthalein, provided that the medium is always prepared in the same way as described above. Although small differences may be desirable for individual bacteria, this reaction can be recommended for ordinary laboratory routine work.

3. Most micro-organisms have some power of overcoming the inhibitory effect of excess of NaOH. This is much commoner than the capacity for overcoming excess of HCl which was observed in a few instances.

4. The haemolysin production of *Vibrio nasik* is not limited to an optimum reaction, but is practically co-extensive with its range of growth in media of varying reactions. In a series of bouillon cultures containing different quantities of alkali and acid, the amount of haemolysin produced was about the same in each case, but the *time* at which the maximum was reached was influenced by the reaction. This was modified

in such a way that the most alkaline cultures produced haemolysin soonest and lost it again before the most acid ones. There seemed to be an optimum as regards *length of duration* of haemolysin in the culture in the case of the sample bearing the reaction, acid 5.

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