BEHAVIOUR OF DIPHTHERIA BACILLI IN ACIDIFIED MEDIA, ESPECIALLY WITH REFERENCE TO VIRULENCE TESTS[1].

By I. WALKER HALL, M.D.

Professor of Pathology, etc., Bristol University.

A CONSIDERABLE amount of attention has been directed recently to the provision of optimum conditions for the growth of diphtheria bacilli and the formation of toxin. Anything which tends to accelerate growth not only facilitates the identification of the bacillus in the routine examination of throat swabs, but lessens the time required for the completion of virulence tests: while an increase of toxin yield plays an important part in the production of potent antitoxin.

Walker Hall and Fraser (1922) pointed out the selective action of various acids for the acceleration or retardation of the lag phase in bacterial growth.

Davis and Perry (1916) investigated the action of individual amino-acids and other substances on the growth of toxic strains of diphtheria bacilli. They found that toxin content was increased by the addition of cystin or tryptophane to ordinary bouillon.

Bunker (1919) and Dernby (1921) compared the values of different peptones and determined the most favourable strength of each type.

Davis (1920) demonstrated the importance of accurate removal of every trace of fat from the culture medium so as to avoid any depression of surface tension. Walker Hall (1922) observed the inhibitory influence of some of the soluble fatty acids.

For purposes of rapid growth it has been customary to increase the carbohydrate content of media by adding up to 1 per cent. of glucose, but the presence of even minute traces of sugar has been considered a drawback for toxin formation. Davis (1920) opposes this view. He contends that the removal of muscle sugar by fermentation with *B. coli* or yeasts, gives rise to decomposition products, which may prove harmful upon injection of the toxin into animal tissues.

The necessity of preparing culture media in such a way that there remains a sufficiency of vitaminic, or accessory substances, is emphasised by Davis (1920), who defines toxin as "a catabolic substance elaborated by the bacillus in the presence of certain amino-acids and accessory factors of a vitaminic character."

[1] The expenses of this work were met by contributions from the Medical Research Council and the Bristol University Research Society.

I have much pleasure in acknowledging the careful assistance I have received from Mr E. Guise.

The reaction of the medium has been fully worked out by Bunker and Dernby. They determined the limits of the initial reaction, indicated the changes which occur usually during growth, and showed that there was a definite relation between reaction phase and toxin yield. Davis is not fully in accord with their conclusions.

These observations have been carried out mainly with well-known toxicogenic strains of diphtheria bacilli. How far they may be confirmed under other conditions is a matter for enquiry. In routine virulence tests, the strains under examination differ widely in their toxicogenic properties. They are derived from convalescents, or from carriers, and their inherent capacities may not be apparent until they are transferred to a more suitable soil. It is of importance, therefore, to employ a technique which does not retard their toxic capacities. It has been the purport of the work which is now communicated to develop a method which will correlate the results of earlier studies, and more especially to determine whether the addition of certain dilute acids to various culture media may improve their nutritional and toxin yielding properties. The acids selected comprise some that rapidly, and almost fully, dissociate when added to complex media, and some that dissociate slowly and weakly. The undissociated portions furnish a reservoir for cleavage as bacterial action proceeds. The dissociated cations and anions exert accelerating or inhibiting influences somewhat quickly. They may also enter into combination with substances present in the medium, and provide the bacteria with more easily assimilable food on the one hand, or, on the other, make the available material less easy to utilise.

Methods adopted and Discussion of Previous Work.

Strains of diphtheria bacilli. Ten toxic and two non-toxic.

These were obtained from town and country centres during the 1921 epidemic. They were isolated and subcultured upon serum media and grown

Table I. *Strains of Klebs-Löffler bacilli employed.*

Cultures in veal broth and peptone, *p*H 7·4. Subcutaneous injections of 0·5 c.c. when culture reaction = *p*H 8·0, into 250 grm. guinea-pigs.

Non-toxic:	Case	Origin	Guinea-pig death, days	Local lesion	Time to attain *p*H 8·0, days
D 2	—	Ear	0	0	1
D 14	—	Throat	0	0	1
Slightly toxic:					
D 4	Mild	,,	7	Induration + +	5
D 6	Fatal	,,	7	,, +	3
D 10	Mild	,,	7	,, +	5
D 12	,,	,,	7	,, + +	5
Strongly toxic:					
D 1	Mild	,,	1	Oedema, etc.	3
D 5	Fatal	,,	1	,,	3
D 7	Mild	,,	1	,,	1
D 8	,,	,,	1	,,	3
D 9	,,	,,	1½	,,	7
D 11	,,	,,	1½	,,	7

https://doi.org/10.1017/S0022172400031399 Published online by Cambridge University Press

in *p*H 7·3 veal bouillon and peptone for virulence determinations. When the reaction of the culture medium reached *p*H 8·0, 0·5 c.c. of the unfiltered material was injected subcutaneously into guinea-pigs, weighing 250 grms. The details are stated in Table I.

Culture media.

In view of the existing divergent opinions, it was evident that more than one type of medium should be employed and that the points of difference should be re-examined.

(*a*) *Inclusion or exclusion of fermentable carbohydrates.*

Davis does not remove muscle sugar from beef or veal infusions, but adds sodium hydrate to the medium to bring it to *p*H 8·0. Bunker and Dernby remove the sugar and adjust the bouillon to *p*H 7·2. In the former instance, the promoting influence of the readily assimilable sugar is retained, and the action of the fermentation products is avoided: but the alkaline change is slightly retarded, and the "ripeness" of the toxin is delayed. In the latter, the reaction of the medium favourable for toxin formation is more quickly attained. Both methods have been applied to each of the selected strains.

(*b*) *The addition of peptone to the culture media.*

The differences in the several varieties of peptone and the differing composition of successive batches from the same source, made it necessary to select a useful sample, and to provide a quantity sufficient for the whole of the work. It required to be well buffered between *p*H 6·0 and 8·0, and to possess toxin yielding constituents. After extended trial of a number of peptones from beef, casein, silk and some purified silk and other peptones prepared personally in 1914 (Walker Hall), a delivery of bactopeptone proved to be most suitable. The term "peptone" used henceforth refers to this variety only.

(*c*) *Initial reaction of media.*

The conclusions of Dernby, Bunker and Davis made it necessary to compare the results obtained with media whose initial reactions were different, and to take into consideration the buffer contents of each batch. For the *p*H adjustments with HCl it is accepted that colorimetric estimations are accurate. But as it was intended to use acids other than hydrochloric, it was necessary first to enquire into the possibility of interactions between the indicators and the acid solutions. The findings of this enquiry are recorded elsewhere (Walker Hall, 1922). They show that with the dilutions of acids and the media here employed, colorimetric determinations suffice. However, the *p*H of practically all the media prepared for this work has been checked by electrical measurements.

While the diphtheria bacillus will grow and form toxin within *p*H limits of 5·0–8·3, it is customary for the initial optimum reaction to be taken as

*p*H 7·2–7·6. This has been observed in the ordinary media, but as the action of added acids is more evident at *p*H 6·8, and this zone does not alter, materially, the growth of the bacillus, this point has been taken for some of the experiments.

(*d*) *Acid solutions.*

Normal solutions of typical mineral, monobasic and dibasic fatty acids were prepared by the Cooper laboratory... Their dissociation coefficients and the dilutions employed are stated in Table III. Every precaution was taken to maintain sterility.

(*e*) *Media.*

Fermented and unfermented beef and veal infusions plus peptone were prepared according to Davis, Bunker and Dernby.

An additional sugar-free medium was made from pure casein. It was digested with fresh pancreas extract (Walker Hall, 1918). The resultant broth was also used with the addition of 2 per cent. peptone. After sterilisation it was titrated to a formol titration of 40 *n*/NaOH per litre. Then, following Eyre's scale, it was made neutral to phenolphthalein (*p*H 8·0). The whole brew was next placed in 12 one-litre flasks, and to each there was added the required quantity of one of the selected acids, in order to bring the reaction to *p*H 5·0, 6·8 or 7·5, etc. 20 c.c. of 1/1000 phenol red, or other indicator, was now placed in each litre flask. This quantity proved to be harmless to the growing bacilli, and facilitated the readings of the *p*H changes during culture.

(*f*) *Vitaminic or accessory substances.*

Care was taken during the preparation of media to avoid undue heating, so as to preserve the substances which Davis considers to be present in his medium. The work of McLeod and Wyon (1921) suggests, however, that the growth-promoting power of substances rich in vitamines does not bear any ratio to the known vitamine contents. These authors suggest that the growth-promoting power may be phenomena of the colloid state, and may assist the growth of bacteria by abstracting from them certain by-products which tend to produce automatic inhibition of growth. They point out that a substance devoid of vitamine, namely charcoal, has quite an appreciable growth-promoting effect. Walker Hall and Fraser (1922) observed that certain acids acted similarly.

The Initial, Progressive and Limiting *p*H Reactions for Diphtheria Bacilli.

During the growth of diphtheria bacilli in a suitable culture medium, the reaction of the fluid undergoes a series of changes. Following the stationary period of lag, there is first a change to the acid side, and later to the alkaline. This constitutes a normal curve. A consideration of the details of the published experiments suggests, however, that this curve varies with the initial

reaction and with the constitution of the culture medium employed. This will be more evident upon a study of the results of an experiment recorded in Table II. Here the *p*H changes produced by a definite toxic strain are compared with those of a non-toxic strain, both growing under equal and varied conditions The initial *p*H was adjusted with HCl.

Table II. *p*H *changes produced by diphtheria bacilli in unfermented and fermented bouillons.*

	Initial *p*H	Toxic strain *p*H days					Non-toxic strain *p*H days				
		1	3	5	8	14	1	3	5	8	14
Unfermented veal broth	6·8	6·6	6·4	6·0	5·6	5·4	6·8	6·6	7·5	8·0	8·2
,, ,,	7·4	7·0	7·0	6·0	5·9	5·6	7·0	7·6	8·0	8·6	9·3
,, ,,	8·1	7·7	7·5	7·4	7·0	6·0	8·0	8·1	8·4	8·6	9·0
Unfermented veal broth plus 2 % peptone	6·8	6·6	6·4	7·0	7·2	7·5	6·8	6·5	7·3	8·0	8·3
,, ,,	7·5	7·2	7·1	7·0	7·4	7·6	7·4	7·6	7·8	8·0	8·1
,, ,,	8·1	7·7	7·6	7·6	7·9	8·2	8·1	7·9	8·5	9·0	9·0
Fermented veal broth plus 2 % peptone	6·8	6·7	7·6	7·9	8·1	8·3	6·8	8·2	9·0	9·0	9·3
,, ,,	7·5	6·9	7·8	8·2	8·2	8·3	7·4	8·0	8·8	9·0	9·3
Peptone 2 %	6·8	6·8	6·9	7·0	7·3	7·6	6·8	7·1	7·5	7·6	7·8
,,	7·6	7·6	7·6	7·8	8·2	8·3	7·4	8·3	8·4	8·8	9·2

The limiting concentration also depends upon the associated factors. Bunker (1919) found the acid limit to be *p*H 5·7 in fermented veal broth, but states that when growth is started above this point, the acidity may be increased without harm to the bacteria. Dernby (1921) obtained growth in unfermented buffered veal broth at *p*H 6·0, and Walker Hall and Fraser (1922) record the same figure in casein digest.

The following experiments were devised to demonstrate the behaviour of a number of strains growing in casein digest adjusted with various acids.

Table III. *Limiting p*H *values for Klebs-Löffler bacilli in differing acid media.*

Casein broth. Formol titration = 40 *n*/NaOH per litre. Buffer index = 2·9.

Broth adjusted with	Dissociation *n*/10 15°–25°	Non-toxic	Slightly toxic		Toxic	
		D 2	D 4	D 6	D 1	D 5
Mineral acids:						
HNO_3	94	5·3	5·3	5·4	5·4	5·0
HCl	91	5·0	4·9	5·2	4·8	4·8
H_2SO_4	62	4·9	5·1	4·9	5·1	4·9
Phosphoric	13·9	4·8	4·9	5·0	4·7	4·7
Sat. monobasic:						
Formic	4·5	5·2	5·1	5·3	5·1	4·7
Acetic	1·3	5·3	5·2	5·4	5·2	5·1
Propionic	1·2	5·0	5·5	5·6	5·5	4·9
Butyric	1·19	5·2	5·1	5·2	5·1	5·1
Sat. dibasic:						
Oxalic	9·05	5·3	5·2	5·3	5·3	5·2
Monobasic di-hydroxy:						
Lactic	3·6	5·0	5·2	5·2	5·2	4·8
Dibasic di-hydroxy:						
Tartaric	13·9	4·8	5·0	4·9	5·0	4·9
Tribasic hydroxy:						
Citric	15·2	4·6	4·9	4·5	4·3	4·2

https://doi.org/10.1017/S0022172400031399 Published online by Cambridge University Press

A standard loopful of an 18 hours' growth of each strain was transferred to a series of tubes containing 10 c.c. of an acid broth. The *p*H content varied from 5·0, 5·2 up to 7·2. The cultures were incubated and examined 18 hours later, and growth determined by the opacity and pellicle produced. The *p*H of the tube in which growth just failed to appear, was taken as the limiting concentration. The result, therefore, was the prevention of growth by a specific substance, and not the effect of mixed acids and other inhibitory by-products arising from bacterial metabolism.

Table III contains the record of five such experiments. On the whole, the results approximate, but the bacilli appear to tolerate a larger proportion of citric than of other acids. The toxic strains do not in this regard differ from those that are non-toxic.

Action of Acids on Growth of Diphtheria Bacilli in Relation to Limiting and Optimum Hydrogen Ion Concentrations.

One of the aims of this investigation was the replacement of muscle sugar by a substance that neither directly nor indirectly interfered with the prolonged growth of diphtheria bacilli.

For such a purpose, it was necessary to use a sugar-free broth. A pure casein digest was therefore employed. Standard loopsful of 18 hours' veal broth cultures were added to standardised test tubes containing 10 c.c. of broth adjusted to the required *p*H concentrations by different acids. Following incubation at 37° C. the opacities were determined by the nephelometer after 18 hours and seven days. Each reading was checked by three observers. There was thus obtained for each strain a comparison of its growth in varying acidifications of the same nutrient medium. To quote an example:

The addition of the selected acids to change the reaction from *p*H 8·0 to 7·6 produced the following differences in rate of growth after 18 hours. The numbers correspond to an arbitrary scale based on the nephelometric readings:

Strain D 2. Eighteen hours growth. Initial *p*H 7·6.

Good growths		Weak growths	
With acetic acid	12	With sulphuric acid	6
„ nitric acid	11	„ phosphoric acid	5
„ lactic acid	10	„ oxalic acid	4
„ tartaric acid	9	„ propionic acid	3
„ citric acid	8	„ butyric acid	2
„ hydrochloric acid	7	„ formic acid	1

The regularity with which some of the acids headed and others tailed the lists, suggested that either directly through the dissociated cations, or otherwise through the anions, there were accelerating and inhibiting influences at work. Tabulation of these results in the form set out in Table IV showed a consistent stimulation effect when acetic, nitric, lactic, phosphoric and tartaric acids were employed, and a delayed growth when butyric, formic, and propionic acids were present. But it was apparent also, that the action had some relation to the reaction of the medium, that is to say, to the quantity

of dissociated ions, or the reservoir amount of unaltered acid. For instance, at *p*H 5·0 lactic acid was the more intense activator, at *p*H 6·8 the effect of nitric acid was most marked, and at *p*H 7·6 the effect of acetic acid became evident. Similarly in delaying growth, butyric acid was most active at *p*H 5·0, and formic acid at *p*H 6·8 and 7·6.

Table IV. *Rate of growth of diphtheria bacilli in acidified media.*

Casein digest broth. Buffer index = 2·9. Formol titration 40 *n*/NaOH per litre. Eighteen hours' growth. Opacity index of growth determined by nephelometer.

	Accelerated growth			Delayed growth		
Strains	*p*H 5·0	6·8	7·6	5·0	6·8	7·6
Non-toxic:						
D 2	Lactic	Nitric	Acetic	Butyric	Butyric	Formic
D 14	Phosphoric	Lactic	,,	,,	Propionic	Hydrochloric
Slightly toxic:						
D 4	Lactic	Nitric	Nitric	,,	Formic	Formic
D 6	,,	,,	,,	,,	,,	,,
D 10	Acetic	Phosphoric	,,	,,	,,	,,
D 12	Lactic	Lactic	Acetic	,,	,,	Butyric
Toxic:						
D 1	Phosphoric	Nitric	Nitric	,,	,,	Propionic
D 5	Lactic	,,	,,	,,	,,	,,
D 7	Phosphoric	,,	,,	,,	,,	Formic
D 8	,,	,,	,,	,,	,,	,,
D 9	Nitric	,,	,,	,,	,,	Citric
D 11	Acetic	Lactic	Acetic	,,	,,	Hydrochloric

THE ACTION OF ACIDS UPON THE REACTION CHANGES DURING THE GROWTH OF DIPHTHERIA BACILLI.

Having demonstrated the varying effects of dilute acid solutions upon the rate of growth of diphtheria bacilli, experiments were now devised to determine whether the metabolic activities of the organisms could be influenced in like manner.

During cultivation in bouillon, toxic strains produce less acid than non-toxic strains, and more alkali. Alkalinity is favourable for the formation of toxin up to a certain point: beyond that, the toxin is destroyed. There is thus an alkaline zone which must be reached and may not be exceeded if a rich toxin yield is to be obtained. The alkaline phase is, however, only a contributory factor: its extent cannot be taken as an index of toxicity. Yet it is an important feature of the process.

The alkalinity production was therefore considered as the next step in the enquiry. For this purpose, the reaction changes in various media were compared. The readings from a typical experiment are given in Table V. They show the actual *p*H changes produced by each strain of diphtheria bacilli in equal quantities of differently acidified bouillon after seven days' incubation at 37° C. It is evident that the non-toxic strains are less affected by the presence of the selected acids than those that are toxic. Also, that the toxic

strains exhibit individual variations in the production of alkali and in their response to the action of the several acids. To record in detail the whole of the experiments would be rather confusing. A clearer view of the matter may be obtained by a discussion of the average results, more especially since any method that may be evolved from the enquiry must be applicable to general rather than to individual strains and conditions. The entire findings, of which Table V is a single detail, have been condensed by taking the average of twelve such experiments, and stating the averages of the non-toxic and toxic strains. The final figures are recorded in Table VI.

Table V. *Actual pH alkaline change during culture of diphtheria bacilli in fluid media.*

Casein digest plus 1 per cent. peptone. *p*H = 6·8 adjusted with mentioned acids. Formol titration = 40. Standard loopsful sowings from 18 hour cultures. Buffer index = 1·8. Reserve alkalinity = 1·05. Growth, 7 days. 37° C.

	Non-toxic strains		Toxic strains									
Acids	D 2	D 14	D 1	D 4	D 5	D 6	D 7	D 8	D 9	D 10	D 11	D 12
Nitric	1·4	1·7	1·4	1·4	1·2	0·4	1·6	1·4	1·6	1·4	1·0	0·6
Hydrochloric	1·3	1·4	1·3	1·0	0·7	0·5	1·1	1·1	0·6	1·0	0·9	0·1
Sulphuric	1·4	1·3	1·4	1·1	1·0	0·5	1·0	0·6	1·0	1·0	0·8	0·0
Phosphoric	0·8	1·2	0·8	1·0	0·6	0·3	1·0	0·6	0·9	0·8	0·6	0·1
Formic	0·6	1·7	0·6	0·9	1·5	0·8	1·1	1·5	1·0	1·0	1·0	0·8
Acetic	1·3	1·7	1·3	1·6	1·5	1·4	1·5	1·5	1·5	1·6	1·1	0·5
Propionic	0·8	1·6	0·8	0·6	0·9	0·1	1·3	0·9	1·3	0·8	0·7	0·1
Butyric	1·2	1·6	1·2	1·1	1·4	0·2	1·2	1·4	1·4	1·1	0·6	0·0
Oxalic	1·3	1·7	1·3	1·0	0·5	0·4	0·9	1·1	1·3	1·0	1·4	0·7
Lactic	1·2	1·8	1·2	1·2	1·4	0·8	1·4	1·0	0·8	1·1	0·6	0·0
Tartaric	1·0	1·2	1·0	1·2	0·9	0·8	1·0	0·5	0·6	1·2	0·4	0·2
Citric	1·0	1·2	1·0	1·1	1·2	0·8	1·0	1·4	1·0	1·2	1·0	0·4
Pellicle	+	++	+	−	+	++	+	+	++	−	++	−

Table VI. *Average alkalinity (pH alkaline change) produced in seven days' growth with pH adjustments in varying acids.*

*p*H 6·8. Formol titration = 40. Buffer index = 1·8. Reserve alkalinity = 1·05. Average *p*H alkaline change in two non-toxic and ten toxic strains.

	Casein digest		Casein digest + peptone		Peptone 1 %		Peptone 3 %		Unfermented veal broth + 1 % peptone		Total	
Acids	N.T.	T.	N.T.	T.	N.T.	T.	N.T.	T.	N.T.	T.	N.T.	T.
HNO_3	1·6	0·6	1·6	1·2	1·4	0·5	0·7	0·6	1·2	0·5	6·5	3·4
HCl	1·8	0·6	1·4	0·9	1·2	0·3	0·4	0·3	1·2	0·5	6·0	2·6
H_2SO_4	1·5	0·2	1·2	0·8	1·1	0·5	0·7	0·6	1·4	0·3	5·9	2·4
Phosphoric	1·8	0·3	1·2	0·6	1·2	0·4	0·5	0·5	1·0	0·2	5·7	2·2
Formic	1·3	0·3	1·6	1·0	0·9	0·4	0·1	0·2	1·2	0·3	5·1	2·2
Acetic	1·6	0·4	1·6	1·3	1·5	0·8	0·4	0·7	1·3	0·4	6·4	3·6
Propionic	1·4	0·2	1·5	0·7	1·3	0·6	0·3	0·2	1·3	0·4	5·8	2·1
Butyric	1·5	0·3	0·9	0·9	1·0	0·2	0·4	0·5	1·1	0·4	4·9	2·3
Oxalic	1·5	0·2	1·4	0·9	0·8	0·3	0·0	0·9	1·4	0·4	5·1	2·7
Lactic	1·8	0·5	1·6	1·0	0·8	0·6	0·4	0·7	1·1	0·4	5·9	3·2
Tartaric	1·5	0·2	1·1	0·7	0·9	0·6	1·5	0·6	1·3	0·3	6·3	2·4
Citric	1·8	0·5	1·2	1·0	1·1	0·1	0·7	0·6	1·2	0·3	6·0	2·5
	19·1	4·3	16·3	11·0	13·2	5·3	6·1	6·4	14·7	4·4		

N.T. = non-toxic. T. = toxic.

https://doi.org/10.1017/S0022172400031399 Published online by Cambridge University Press

It indicates therefore the average *p*H change produced by non-toxic and toxic strains in all the several acidified media, and also the average effect of each type of acid on the bacillary metabolism.

With regard to the media, it will be noted that the non-toxic strains grow and function equally well in all except the 3 per cent. peptone, which inhibits their proliferation and action.

The toxic types appear to require more precise nutrients; their alkalinity production is enhanced in casein digest plus peptone, but retarded in simple casein digest, peptones or unfermented veal broth plus peptone. From the proliferation standpoint, however, although it is not evident from this table, good growth is yielded by all the media, but 3 per cent. peptone requires a longer time for the attainment of heavy growths.

The differential action of the acids is more pronounced with the toxic strains of bacilli. Acetic, nitric and lactic are associated with a larger amount of alkalinity. The non-toxic strains produce greater total alkaline change, and are less affected by the individual acids, although in both types the monobasic fatty acids, butyric and formic, produce a delaying effect.

THE TIME FACTOR IN REACTION CHANGES.

If it is conceded that the attainment of a favourable alkaline zone is a necessary prelude to toxin formation, and its precise limitation a safeguard against toxin destruction, then a knowledge of the average time required for the several stages is of importance.

The whole of the strains employed in this investigation were therefore used for experiments intended to yield such information. Nitric acid was chosen as the adjuvant, and casein digest, veal broths and peptones as culture media.

Standard loopsful of 18 hours' broth cultures of each strain were sown into equal quantities of the media. After incubation at 37° C. with adequate oxygenation, readings of the opacity and *p*H changes were made every four hours up to 72 hours, and thereafter at intervals of 24 hours. The abbreviated results are included in Table VII.

The figures show that when glucose is added to the medium, none of the toxic or non-toxic strains induces an alkaline change. The toxic varieties make the media much more acid than the others.

In unfermented veal broth, the non-toxic strains form much alkali, the toxic strains do not produce any. This difference is of practical value as a preliminary indication in routine virulence work.

In the other types of media, the non-toxic bacilli reach the favoured zone within 40 hours, but exceed it in four days.

The toxic organisms develop the alkaline change more gradually, and show a time relation to the amount of acidity previously developed. In simple casein digests, 10–14 days are required for the bacilli to form sufficient alkali to reach the favoured zone: in casein digest with peptone it is attained in $2\frac{1}{2}$–3 days. The same time is necessary for fermented veal broth plus peptone,

but in simple peptone solutions the period is extended to six days. In unfermented veal broth without peptone, the danger period of excessive alkalinity commences on the fourth day.

Table VII. *Time periods of reaction changes.*

Variations in *p*H changes in different media. HNO_3 adjustment. Standard loopsful sown from 18 hours' cultures. Formol titration = 40. Incubated flat at 37° C. Average figures from ten toxic and two non-toxic strains.

		Non-toxic strains							Toxic strains						
		Acid, hours	Alkaline, days						Acid, hours	Alkaline, days					
Media *p*H 7·4	Buffer index	30	2	3	4	6	10	14	30	2	3	4	6	10	14
Casein digest	1·8	7·28	7·4	7·7	7·7	8·6	8·65	9·1	7·12	0	7·54	7·56	7·56	7·75	8·4
Casein digest + 1 % glucose	2·1	7·12	0	0	0	0	0	0	6·0	0	0	0	0	0	0
Unfermented veal broth	1·8	7·2	7·5	7·7	7·7	8·5	9·0	10·0	6·8	0	0	0	0	0	0
Fermented veal broth	2·0	7·3	8·0	8·6	8·7	9·0	9·1	9·3	6·93	7·8	8·2	8·4	8·4	8·5	8·5
Peptone 2 %	1·8	7·4	8·2	8·3	8·4	9·0	9·2	9·9	7·35	7·6	7·7	7·78	7·9	8·02	8·4
Peptone 3·3 %	2·5	7·4	7·7	7·9	8·0	8·3	8·4	9·0	7·4	7·7	7·8	7·8	7·9	8·0	8·1
Casein digest + 1 % peptone	2·1	7·3	8·0	8·4	9·0	9·0	9·2	9·3	6·9	7·45	8·0	8·1	8·2	8·3	8·4
Fermented veal broth + 1 % peptone	2·2	7·3	7·7	7·9	9·0	9·0	9·0	9·5	7·18	7·8	8·1	8·2	8·2	8·3	8·3

The average time required for the first manifestation of alkaline change is recorded in Table VIII.

It will be noted that in broth with initial *p*H reaction of *p*H 6·8, the alkaline turn is not delayed to any extent as compared with growth at 7·4. In the former less acid is produced: in curves constructed from the changes

Table VIII. *Effect of initial* pH *on the time periods of alkalinity.*

Two non-toxic, four slightly toxic and six highly toxic strains. HNO_3 adjustment. Various broths containing phenol red as indicator.

		Alkaline change, hours			Attain *p*H 8·0, days		
Media *p*H 6·8	Buffer index	N.T.	S.T.	T.	N.T.	S.T.	T.
Casein digest	1·8	30	30–60	30–60	4–7	—	—
Fermented veal broth	2·1	16	30–50	20–48	3–4	3–6	3–5
Unfermented veal broth	2·6	80	—	—	7	—	—
*p*H 7·4							
Casein digest	1·8	25	40	40	2	8–10	8–10
Fermented veal broth	2·0	22	22	23–30	2½	2¼	2–2½
Unfermented veal broth	2·4	50	—	—	6	—	—
Peptone 2 %	1·8	30	24	24–30	2	3½–7	2–5
Peptone 3·3 %	2·5	38	19–24	22–24	3¼	5–8	5–8
Casein digest + 1 % peptone	2·3	28	28–40	28–40	2	2–3½	2–2½
Fermented veal broth + 1·5 % peptone	2·4	24	28–40	24–34	3	2–3	2¼–3

N.T. = non-toxic. S.T. = slightly toxic. T. = toxic.

in each of the cultures and media, the acid production is shown to cease at approximately the same *p*H level.

As a further guide in the use of the presumptive test in unfermented veal broth, the non-toxic strains produce alkalinity at 50 and 80 hours respectively, while the toxic strains fail to exhibit an alkaline phase.

The period necessary for the attainment of the favourable alkaline zone appears, therefore, also to vary with the initial reaction and the type of medium. Fermented veal broth plus peptone or casein digest plus peptone of *p*H 7·4 yields the earliest change, the average for all the non-toxic strains being 2–3 days.

THE USE OF ACIDIFIED MEDIA IN VIRULENCE TESTS FOR DIPHTHERIA BACILLI.

The results so far obtained tend to show that the inclusion of dilute nitric or acetic acids in certain culture media accelerates the growth of diphtheria bacilli and ensures an early attainment of the alkaline zone favourable for toxin formation. Whether or not this addition will constitute an improvement in the technics usually employed for virulence testing may now be considered.

For the determination of the toxicity of strains of diphtheria bacilli isolated from carriers or convalescents it is deemed necessary to obtain a copious growth of organisms as well as a sufficiency of toxin. "The latter is but one element in the disease producing power of the bacillus and toxin production in bouillon may not be a true index of the toxin production in mucous membranes." (Kolmer, 1920.)

The test tube findings must therefore be submitted to animal experiments. For this purpose diphtheria bacilli isolated from swabs taken for virulence tests are grown in veal or casein digest broths containing sterile normal acetic or nitric acids in quantities sufficient to make the fluid equal to a *n*/200 of the acid. 1 c.c. of 1/1000 phenol red is added for each 50 c.c. of medium. The flasks are incubated on the flat at 37° C. Daily readings are carried out. Directly the reaction reaches *p*H 8·0 the culture is well shaken and varying amounts are injected into 250 grm. guinea-pigs with the usual antitoxin controls.

Table IX contains a record of a typical experiment. Omitting the dilutions of acids which act unfavourably, it shows that with the addition of acetic or

Table IX. *Comparison of toxic potency of diphtheria bacilli grown in ordinary and acidified culture media.*

*p*H = 7·4. Buffer index = 3·1. Subcutaneous inoculation into 250 grm. guinea-pigs with antitoxin controls.

	Strain D 1 (toxic)			Strain D 2 (non-toxic)		
	Time to reach *p*H 8·0	M.L.D.	Death	Time to reach *p*H 8·0	M.L.D.	Death
	hours	c.c.	hours	hours	c.c.	hours
Fermented veal broth + 2 % peptone	45	0·35	40	35	0	—
Casein digest + 2 % peptone	50	0·4	44	40	0	—
Fermented veal broth + 2 % peptone + 1/200 *n*/acetic acid	50	0·1	44	40	0	—
Fermented veal broth + 2 % peptone + 1/200 *n*/nitric acid	50	0·25	60	45	0	—
Casein digest + 2 % peptone + 1/200 *n*/acetic acid	50	0·15	45	45	0	—
Casein digest + 2 % peptone + 1/200 *n*/nitric acid	50	0·3	55	45	0	—

nitric acids, the favoured zone is more rapidly attained, and the bacillary growth and toxin potency are increased when compared with the results in non-acidified media. It also excludes any alteration of potency when non-toxic strains are subjected to similar conditions.

Summary.

1. The growth of diphtheria bacilli in sugar-free pure casein digest broth may be accelerated by the addition of dilute nitric, acetic and lactic acids, and delayed by certain monobasic fatty acids.

2. The limiting acid zone for toxic and non-toxic varieties is unaffected by initial dilute acidifications by several acids, but increased by the addition of citric acid.

3. The time periods and intensity of the reaction changes produced by diphtheria bacilli growing in a sugar-free casein digest medium are altered by the addition of certain acids. Toxic strains produce earlier and more progressive alkalinity when dilute acetic or nitric acid is included in the medium: and later and lessened alkalinity in the presence of butyric, formic, and propionic acids. Certain other typical acids do not exert any obvious action. Non-toxic strains are less affected by the selected acids, although butyric acid generally retards reaction changes.

4. The time required for virulence testing is decreased, and the potency of the bacillus under examination is more evident when 1/200 *n* acetic or nitric acid is added to the culture medium. Acidified casein digest plus peptone broth yields practically the same results as acidified fermented veal peptone infusion. The acidification does not affect the metabolism of non-toxic strains.

5. The disadvantages of unfermented meat infusions and the ascribed harmful effects of the fermentation products in fermented meat broths, may perhaps be avoided by the substitution of casein digest peptone medium. The advantages of the starting action of the glucose may be retained by adding acetic or nitric acid to the casein digest.

6. Individual strains of diphtheria bacilli differ considerably in their capacity of altering the reaction of culture media, and in their toxic potency. It does not seem permissible to apply to newly isolated organisms all the published results obtained from cultures that have been repeatedly sub-cultured and established as toxin producers in nutrient bouillon. As a presumptive test in the virulence testing of bacilli just transferred from human tissues to artificial culture media the tendency of non-toxic strains to produce early formation of alkali in adequately buffered and adjusted unfermented veal broth may be thought worthy of extended trial.

REFERENCES.

BUNKER (1919). Studies of the Diphtheria Bacillus in Culture. *Journ. of Bacteriol.* IV. 379.

DAVIS (1920). Bacteriologic Peptone in Relation to the Production of Diphtheria Toxin and Antitoxin. *Ibid.* V. 477.

DAVIS and PERRY (1919). The Role of the Amino-acids in the Metabolism of Bacterium Diphtheriae. *Ibid.* IV. 237.

DERNBY and DAVID (1921). A Study of the Preparation of Diphtheria Toxin. *Journ. of Pathology*, XXIV. 157.

HALL, WALKER (1913). The Purification of Silk Peptones for Bacteriological Purposes. *Ibid.* XIX. 286.

—— (1918). Amino-acid Content of Nutrient Media. *Brit. Med. Journ.* II. 398.

—— (1922). Indicators for Culture Media containing Varying Acids and Buffers. *Brit. Journ. Exper. Pathology*, III. 182.

—— (1922). Action of Dilute Acids in Blood Cultures. *Journ. of Pathology*, XXV. 297.

HALL, WALKER and FRASER (1922). Action of Dilute Acids in Bacterial Growth in Optimum *p*H Concentration. *Ibid.* XXV. 19.

KOLMER (1920). *Infection, Immunity and Specific Therapy*, p. 115.