LI. THE MECHANISM OF THE REVERSAL IN REACTION OF A MEDIUM WHICH TAKES PLACE DURING GROWTH OF *B. DIPHTHERIAE*.

BY CHARLES GEORGE LEWIS WOLF.

*From the John Bonnett Memorial Laboratory, Addenbrooke's Hospital,
Cambridge.*

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IN the preparation of diphtheria toxin for immunising purposes it is known that the initial and final reactions of the medium are factors of prime importance in harvesting a toxin of high potency. The more recent investigations of L. Davis [1918], Bunker [1918, 1919] and Hartley [1922] have placed this beyond a doubt. Bunker, indeed, goes so far as to say that, outside certain well-defined final hydrogen ion concentrations, potent toxins are never harvested, and for this reason has suggested that a determination of the hydrogen ion concentration by modern methods is a criterion for ascertaining when the organism has elaborated the greatest amount of toxin. Hartley questions this and has been able to show that while Bunker's statement is approximately correct, there are cases in which a very high grade antigen is produced outside the limits set by Bunker.

For the purposes of the present paper it is only necessary to refer to the course which the medium takes when reaching this final reaction.

If one starts with a bouillon peptone medium of $p_H = 7.8$, during the earlier period of growth the reaction becomes more acid, often exceeding the neutral point of $p_H = 7.0$. A sudden change in reaction occurs when this acid point is reached and the medium becomes alkaline. The alkalinity may reach the high value of $p_H = 8.6$ or even 8.8. It is during the period of alkalinity that the toxin is formed.

The chemical reactions which occasion the change to the acid side of neutrality and then to the alkaline side with this bacillus have been little studied and not much is known about them. The acid production early received the attention of Theobald Smith [1895, 1899] who believed that it was closely connected with the presence of glucose in the bouillon. Later on he seems to have modified his view and to have attached more importance to the presence of inositol, the so-called muscle sugar, as a producer of acid, and in certain experiments on toxin production he sought to remove all fermentable carbohydrates by the preliminary treatment of the medium with *B. coli* or

yeast. It is noteworthy that he suggests that after this treatment a small amount of glucose should be added to the medium for the more efficient production of toxin. Smith's experiments have had a very considerable influence on the technique of toxin production. Even as late as 1921, Dernby and David [1921] have recommended the preliminary fermentation of media by yeast before proceeding to the inoculation with the diphtheria bacillus.

Smith's statements regarding glucose in the bouillon were based on amounts of gas obtained in a fermentation tube after sowing the bouillon with *B. coli*, and there does not seem to be any real information available as to the amount of glucose in meats as ordinarily purchased. In a subsequent paper I hope to be able to give some data on this point. For the present one may say that, assuming the amount of glucose to be 0.06 %, this would yield, if completely converted into carbon dioxide by fermentation, 0.088 % by weight of this gas. This expressed as gas per 100 g. of meat at room temperature and normal pressure would be about 47 cc. It is improbable that such a reaction occurs. One might also assume that a complete transformation of glucose to lactic acid took place. In this event, the amount of lactic acid produced would be equivalent to 0.66 cc. of normal lactic acid per 100 cc. of nutrient broth. Wolf and Harris (1917, 2) have shown that by adding this amount of lactic acid to broth the reaction shifted to the acid side to about the same extent as is seen when *B. diphtheriae* acts upon peptone bouillon. As the amount of meat corresponding to the bouillon is at least half the quantity reckoned as above, it seems inevitable to conclude that the acids produced are not derived solely from the fermentation of the sugar contained in the medium.

The only statement regarding the amount of inositol to be found in meat is an early one of O. Jacobsen [1871] who estimated that fresh muscle contained 0.035 %. In any case, so far as I am aware, no controls have ever been made to ascertain the effect of inositol added to a medium which has been fermented with yeast or with *B. coli*.

That there are other views on the origin of the acid appears from the discussion which took place between Madsen [1897] and Lubenau [1908]. The former author contended that the reversal of the medium was determined by the initial reaction. Lubenau asserted that with the absence of fermentable carbohydrates there was never any acid reaction, provided that the growth took place in the presence of air. Jacobsen [1911] attempted to reconcile these two points of view and tried to show that peptone also has a capacity for forming acids. Petruschky [1891] also believed that peptone furnishes material for the production of acid substances.

The probability is that the truth lay rather with Madsen. With a high initial alkalinity and enough efficient buffer substances in the medium, despite the formation of acids, the reaction of the medium would never reach an acid point as determined with litmus.

When it came to the question of reversal, the matter was on less certain

ground. The obvious product in fermentation which can lead to an alkaline reaction is ammonia, and to this was attributed the change to an alkaline reaction.

There were certain indications that this could not be the case. For example, Teruuchi and Hida [1912] examined the daily production of ammonia in 2 % bouillon peptone, with and without the addition of small quantities of glucose. While they obtained a small increase of ammonia, commencing on the fourth day, they did not find that the alkalinity bore any relation to the amount of ammonia produced.

The more recent work of Ayers and Rupp [1918] gives a strong clue to the mechanism whereby the medium becomes alkaline. In their paper on the well-known reversion produced by organisms of the colon-aerogenes group it is shown that, with *B. coli*, acid and alkaline fermentations proceed simultaneously. Glucose is converted to organic acids and these, in turn, are transformed to carbonates. They suggest that the reversion of reaction is probably due to bicarbonates. A similar simultaneous acid and alkaline fermentation has been analysed by Harris and the writer [1917, 1] in the case of strongly proteolytic anaerobic organisms. Here the stabilisation of the reaction, in spite of the production of large amounts of organic acids, is due in great part to the production of ammonia. It seemed, therefore, of some importance to ascertain to what the reversal in reaction with *B. diphtheriae* was due.

The first experiment was designed to follow out the changes in hydrogen ion concentration, and volatile acid and ammonia production. For this purpose portions of 100 cc. of a standard bouillon-peptone medium were sterilised in 450 cc. rectangular medicine bottles. These were incubated lying on their sides, so that the surface of medium exposed to the air was about 115 sq. cm. At stated times a bottle was taken from the incubator and the hydrogen ion concentration, volatile acids and ammonia determined. Table I gives the results of the experiment.

Table I.

Hours	p_H	Mgm. ammonia nitrogen per 100 cc.	Volatile acids, cc. N/10 alkali per 100 cc.
Control	7.5	6.13	4.4
24	7.0	7.85	21.2
48	7.1	8.40	24.8
72	7.9	8.98	8.8
96	8.4	8.52	7.2
120	8.5	8.52	6.8
144	8.5	8.42	7.2
168	8.5	8.98	7.8
192	8.4	9.25	8.0
216	8.5	11.20	8.0
240	8.5	10.07	8.0
264	8.5	8.12	6.0

Certain quite clear results emerge from the analyses. Starting with an initial hydrogen ion concentration of $p_H = 7.5$ the reaction follows the usual course, becoming as acid as $p_H = 6.9$. When reversion took place, a final

value of $p_H = 8.5$ was reached which persisted as long as the experiment was continued. The amount of ammonia produced is definite but very small. Starting from an initial value of 6.13 mgm. ammonia N per 100 cc. of medium it does, on one occasion, reach a concentration of 11.2 mgm., but the average is somewhat less than this. This experiment confirms what Teruuchi and Hida found, viz. that the amount of ammonia formed during the metabolism of the bacillus is very small indeed. At the same time a very remarkable change takes place in the volatile acid production. Starting with an amount of acid equivalent to 4.4 cc. of $N/10$ NaOH per 100 cc. of medium, in the first 24 hours the acid had risen to 21.2 cc. with a further rise to 24.8 cc. at the end of 48 hours. With the sudden reversal of reaction from $p_H = 7.1$ to 7.9 a large proportion of the acid disappears and in the fourth sample only 8.8 cc. are found. From this time on the amount of acid rises only slightly with a final alkalinity of the medium of $p_H = 8.5$.

It is thus obvious that a large part of the volatile organic acids formed in the first stages of fermentation are utilised in the sense of Ayers and Rupp to form basic substances.

A second experiment was undertaken to ascertain to what extent the fixed acids took part in the changes above referred to. The results are embodied in Table II.

Table II.

Hours	p_H	CO ₂ , cc. per 100 cc.	Volatile acids N/10 alkali per 100 cc.	Fixed acids N/10 alkali per 100 cc.
Control	7.8	3.0	3.6	27.2
24	7.5	—	4.4	20.5
48	7.3	—	14.8	6.6
72	7.4	—	15.4	11.1
96	7.5	—	15.8	7.4
120	8.1	18.0	7.2	4.5
144	8.2	—	6.8	4.1
192	8.5	27.0	7.2	2.9
240	8.5	27.0	7.4	3.5

The initial alkalinity of the medium was greater than in Exp. 1 and possibly for this reason the medium did not become so acid. The final reaction was, however, the same as in the previous test.

As the amount of medium was not sufficient, no analyses for ammonia were performed. The general trend of the volatile acid curve is the same as in the preceding experiment; one finds a sudden increase in the concentration of these compounds corresponding with a decrease in p_H . The increase in alkalinity is coincident with the sudden lowering of the volatile acids. The course of the fixed acid curve is less easy to explain. From the time of inoculation there is a steady fall, only one value—that obtained at 72 hours—being outside the curve.

The main non-volatile organic acid in bouillon is undoubtedly lactic acid, and this appears to have been metabolised, apparently during the earlier hours of the fermentation, to substances still having an acid reaction and it may well

be that some of these are represented in the increase to be found in the volatile acids. Finally, however, the fixed acids reach a level represented by about 4.0 mgm. of $N/10$ alkali per 100 cc. of medium.

During the course of this experiment I was led to ascertain whether the available carbon dioxide in an old culture was different from that in a medium which had not been inoculated. For this work I employed the van Slyke apparatus, using 1.0 cc. of medium for a determination. It appeared at once that the amount in the former was over five times that of the latter.

Using the present series of cultures it was found that at the 120th hour an amount of carbon dioxide was disengaged by dilute sulphuric acid equivalent to 20.0 cc. of gas per 100 cc. of medium. This was at a time when the reaction of the medium was $p_H = 8.1$. When the fluid reached an alkalinity of $p_H = 8.5$ the amount of carbon dioxide disengaged was 29.0 cc. per 100 cc. of medium. The amount of carbon dioxide disengaged from the control medium was only 3.0 cc., and of this 2.0 cc. were due to gases dissolved in the reagents used for the determination.

It would thus appear that the reversal of reaction was closely connected with the formation of carbonates, confirming what Ayers and Rupp had found with *B. aerogenes*. The matter was therefore studied from the commencement of fermentation.

At the same time it was decided to obtain some information regarding the action of *B. diphtheriae* on certain well-known organic salts. Preliminary experiments performed some time previously made it appear that certain organic acids added to the medium would be available as a source of energy, and a detailed examination of this point was made. Not a great deal of information is available on the latter point.

Table III.

Hours	Control			Sodium succinate			Sodium tartrate		
	Pellicle	p_H	CO_2	Pellicle	p_H	CO_2	Pellicle	p_H	CO_2
0		7.8	1		7.8	1		7.8	1
48	+++	7.6	10	+++	8.0	23	+++	7.3	9
72	+++	8.1	9	+++	8.1	21	+++	8.0	17
120	+++	8.3	23	+++	8.6	57	+++	8.2	19
168		8.3	34		8.6	65		7.9	12
240		7.3?	5?	+++	8.5	71	+++	8.0	11
336		8.3	27		8.6	75		8.2	22
456		8.5	27		8.8	90		8.5	28
624		8.4	21		8.6	56		8.3	13

Hours	Sodium malate			Sodium citrate			Sodium formate		
	Pellicle	p_H	CO_2	Pellicle	p_H	CO_2	Pellicle	p_H	CO_2
0		7.8	1		7.8	1		7.8	1
48	+++	7.7	29	0	7.3	4	+++	7.5	10
72	+++	8.3	50	0	7.2	5	+++	7.5	16
120	+++	8.6	75	0	7.3	7	+++	7.9	20
168		8.6	66		7.5	7		8.3	17
240	+++	8.1?	20?	0	7.5	6	+++	8.3	20
336		8.8	90		8.3	21		8.4	26
456		8.8	92		8.3	23		8.4	26
624		8.6	71		7.4	3		8.3	19

Maassen [1896] many years ago in an elaborate investigation of the action of bacteria on organic salts found in a series of 21 organic acids that malic acid was the only one attacked by *B. diphtheriae* and that very feebly.

Altobelli [1914] in a somewhat recent paper also states that this acid is the only one attacked by *B. diphtheriae*.

In the present experiments the fermentations were carried out in test-tubes, the quantity of medium used being about 10 cc.

The salts were added in a concentration of about 1.0 %. After the addition of the salts the medium was adjusted to $p_H = 7.8$ and sterilised at 120° for 30 minutes. The tubes were all inoculated from the same culture and kept at 37°. The culture used (Park and Williams, No. 8) had been transferred at 24-hour intervals in bouillon peptone and was growing very actively (Table III).

It is clear that various organic acids are utilised in quite a special way by *B. diphtheriae*. The two which are most strongly attacked are malic and succinic acids. Judged by the amount of carbonate formed, succinic acid is nearly as completely used as malic acid and it is difficult to understand how Maassen overlooked its effect on metabolism. Tartaric acid and formic acid do not seem to have been utilised at all, for the amounts of carbonates formed are very close to those given by the control medium. Citric acid appears to have had an inhibiting effect on the growth of the bacillus. Reversal was very much delayed and the alkalinity did not reach the height obtained with other cultures. The carbonate fermentation was below that of the control medium.

A special interest attaches to formic acid, as this compound has been shown to be one of the steps through which the higher acids are finally oxidised to carbon dioxide. In the katabolism of glucose by *B. coli* the intermediary between this compound and carbon dioxide is said to be formic acid [Harden and Penfold, 1912]. This does not appear to be the case with the diphtheria bacillus, for the addition of formic acid to the medium is not associated with an increase in carbon dioxide above that found in the control medium.

SUMMARY.

The reversal of reaction in carbohydrate-free media caused by the growth of *B. diphtheriae* is due, in its acid phase, in part to the production of volatile acids. When the medium reverses to a more alkaline reaction, these acids are converted into carbonates.

Certain organic acids, such as malic and succinic acids, are also utilised to produce carbonates.

The formation of carbonates from the acids examined does not appear to take place with formic acid as an intermediary product.

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