71. The Effect of the *p*H and the Presence of Glucose during Growth on the Production of α and θ Toxins and Hyaluronidase by *Clostridium welchii*

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When grown in a suitable medium *Cl. welchii*, type A, excretes α and θ toxins, hyaluronidase and proteolytic enzymes. α toxin has been shown by Macfarlane & Knight [1941] to be closely associated, and probably identical, with an enzyme, lecithinase; θ toxin acts as a haemolysin when in a reduced state [Todd, 1941], but the mechanism of its action has not yet been elucidated. The excretion and adaptive nature of hyaluronidase have been studied by McClean & Hale [1941]. When grown in an acid medium or in the presence of a high concentration (2%) of glucose, the organism develops histidine decarboxylase, thus giving rise to histamine in the medium [Gale, 1941]. This enzyme is only formed when growth occurs at *p*H $< 5{\cdot}5$ and the object of this communication is to establish (1) the optimum medium *p*H conditions for the production of α and θ toxins and hyaluronidase, and (2) the part played by glucose in the medium in the production of these substances. The amino-acid metabolism of *Cl. welchii* has been studied by Woods & Trim [1942].

Methods

The organism used for the whole of this work was *Cl. welchii*, type A, strain S 107. The medium used for growth of the organism was that described by Macfarlane & Knight [1941] with and without 0·45% glucose, with the exception that 1·5% 'Allenbury's Beef Extract' was substituted for the sodium sulphate protein-free extract of horse-meat. Preliminary experiments indicated that it was not possible to control the *p*H of the medium by the addition of buffer solutions, since the presence of phthalate or borate inhibits the growth of the organism. It was therefore necessary to control the *p*H during growth by the addition of sterile acid or alkali as required and the following apparatus was devised. 500 ml. medium were sterilized by filtration through a Ford G.S. filter and transferred with sterile precautions to a three-necked flask. A hydrogen electrode, sterilized in alcohol, was introduced, together with one arm of a sterile agar-bridge, through one neck of the flask. A delivery tube reaching to the bottom of the flask and bent over outside to form a siphon acted as a sampling device and passed through the second neck. The third neck was fitted with a wide glass tube reaching to the surface of the medium and plugged with a sterile bung: sterile *N* NaOH or HCl was added through this tube as required by means of a Pasteur pipette and the medium stirred after each addition by a stream of N_2 passed to the bottom of the flask through a third tube in this neck. The *p*H of the medium was measured by means of the hydrogen electrode connected to a valve-potentiometer. The flask was incubated in a bath maintained at 37° and inoculated from a 12-hr. meat-tube culture of the organism. At suitable intervals after inoculation the medium was stirred and approximately 10 ml. samples withdrawn by the siphon. The dry weight of organism/ml. medium was estimated by the use of a photo-electric turbidimeter previously calibrated for the organism concerned [Clifton, Mueller & Rogers, 1935]. The logarithm of the dry weight was plotted against the age of the culture and the generation time, representing the time taken for the dry weight of organism to become doubled during the logarithmic phase of growth, calculated from the linear portion of the curve.

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The sample was then clarified by adjusting to neutrality, adding a knife-point of kieselguhr and centrifuging down the kieselguhr and the adsorbed organism. The following estimations were carried out on the clear supernatant fluid.

1. α toxin was estimated by the method of van Heyningen [1941*a*]; activity expressed as E.U./ml. medium or mg. dry weight of organism.

2. θ toxin was estimated by measuring the time in which an aliquot of a given sample brings about complete haemolysis of a red blood cell suspension under standard conditions. When necessary the samples were treated with thiolacetic acid to reduce the toxin. The suspensions of red cells and the dilutions of toxin are made in isotonic 0·0665 *M* phosphate buffer *p*H 6·5 so that the α toxin is completely inhibited [van Heyningen, 1941*b*]. The sheep red cell suspension used is approximately 1·5%, and is made up daily from a stock standard approximately 25% suspension, the preparation of which has already been described [van Heyningen, 1941*b*]. A flat (tintometer) cell, 4 mm. wide, is suspended in a water bath at 38° against the inside of the front glass wall of the thermostat tank. Immediately behind the cell, and attached to it by means of a rubber band, is a microscope slide with a fine vertical line scratched on it. Behind this device is an electric light bulb. 1 ml. of an appropriate dilution of the toxin is pipetted into the cell, and when it has reached 38° (1 min.) 1 ml. of the 1·5% red blood cell suspension, previously warmed to 38°, is added. The time in which the *fine detail* of the scratch on the microscope slide becomes visible through the haemolysing red cell suspension is taken as the time for complete haemolysis. This end-point can be observed fairly easily with practice; when the haemolysis time is 100 sec. the end-point can be determined within ± 3 sec., and the percentage error appears to be the same over a range of 50–300 sec. From the time taken for complete haemolysis the corresponding number of units of θ toxin in the aliquot used can be determined by reference to a previously determined standard activity curve. One θ unit is arbitrarily defined as the amount of θ toxin which will bring about complete haemolysis of a standard, approximately 0·75%, suspension of sheep red cells at 38° in 100 sec. The error of this method appears to be within ± 5%.

3. Toluene was added to clear samples which were sent to Dr McClean for estimations of the hyaluronidase titre. This enzyme was titrated by the mucin clot prevention (M.C.P.) test which depends upon the determination of the highest dilution of the sample which, when incubated for a standard time with a substrate mixture composed of a hyaluronic acid-protein complex, will destroy the power of this mixture to form a typical 'mucin clot' on the addition of acetic acid. A complete description of this test together with a study of its correlation with other methods of estimating hyaluronidase and diffusing activity is being prepared for publication. Most of the 24-hr. samples, which contained the highest titre of enzyme, were also titrated by the viscosimetric method [McClean & Hale, 1941]; there was good agreement in the relative values obtained by these two methods. Hyaluronidase activity is expressed as M.C.P. titre/ml. of medium or per mg. dry weight of organism.

All estimations other than hyaluronidase determinations were made on samples taken from the first appearance of turbidity and at half-hour intervals from then until growth ceased. Hyaluronidase estimations were made on samples taken (1) during the middle of the growth phase, (2) at the end of the period of active growth and (3) 24 hr. after inoculation. Activities have been calculated in all cases per ml. medium and per mg. dry weight of organism; experience shows that the latter is the most consistent way of expressing activities in cultures in which the total crop may vary slightly from culture to culture.

EXPERIMENTAL

Fig. 1*a* shows the variation of the generation time with the *p*H of the medium in which growth occurs. *Cl. welchii* will grow in the medium described at any *p*H between 5 and 8·5, although the generation time is too long below *p*H 5·5, and the total crop of organisms

then obtained too small, for useful experimental work. Glucose slightly decreases the generation time in most cases, its greatest effect being in the alkaline growth range. Fig. 1 *b* shows the variation of the total crop (i.e. maximum dry weight observed) obtained at various growth *p*H values; glucose markedly stimulates the production of organism.

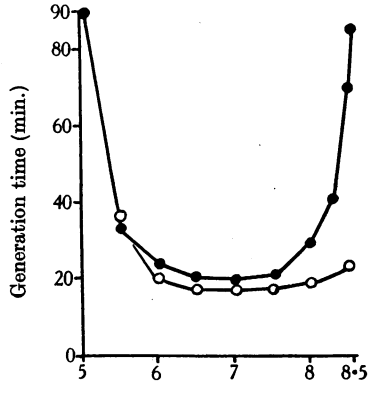

Fig. 1 *a*.

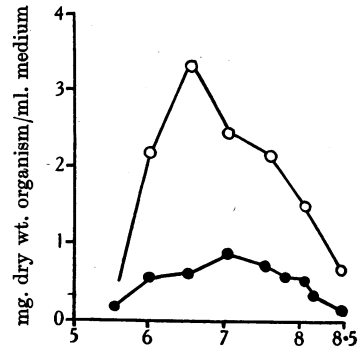

Fig. 1 *b*.

Fig. 1. (*a*) Variation of generation time with growth *p*H (*Cl. welchii*). (*b*) Variation of total crop with growth *p*H. •—• No glucose. o—o 0·45% glucose.

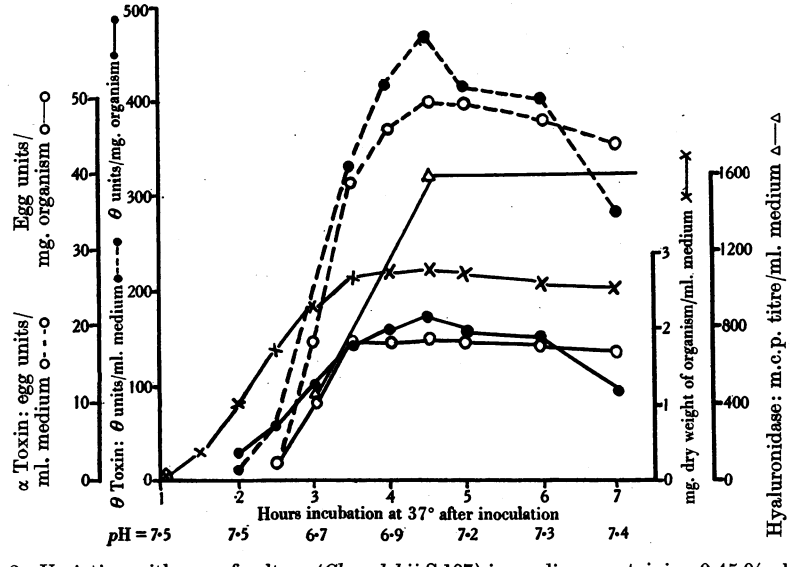

Fig. 2. Variation with age of culture (*Cl. welchii* S 107) in medium containing 0·45 % glucose at an initial *p*H of 7·5. Growth temp. = 37°.

Fig. 2 shows the variation of toxin and hyaluronidase production with the age of the culture. It can be seen that θ toxin appears in the medium a short time before the α toxin and that both toxin and hyaluronidase activities of young cultures are small; they increase as growth proceeds and reach a maximum at about the time that active cell division ceases. After this stage the activities of both α and θ toxins decrease in the medium but hyaluronidase activity remains constant or, in some cases, even increases.

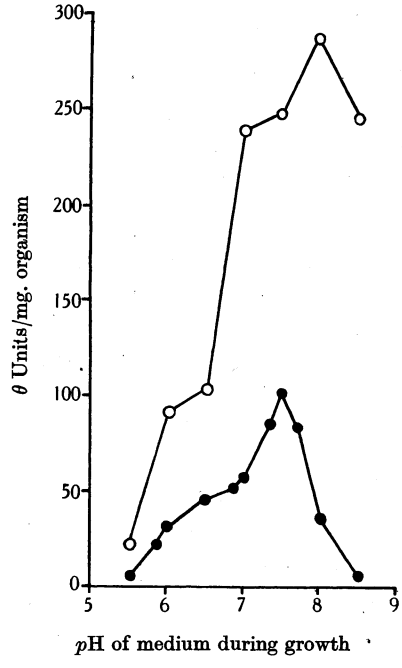

Fig. 3. Variation of θ toxin production per mg. organism with growth *p*H.
●—● No glucose. o—o 0·45 % glucose present.

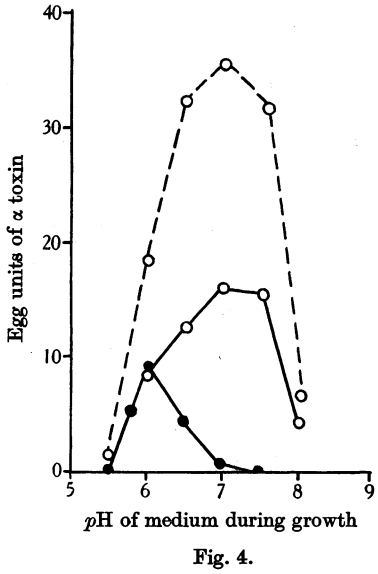

Fig. 4.

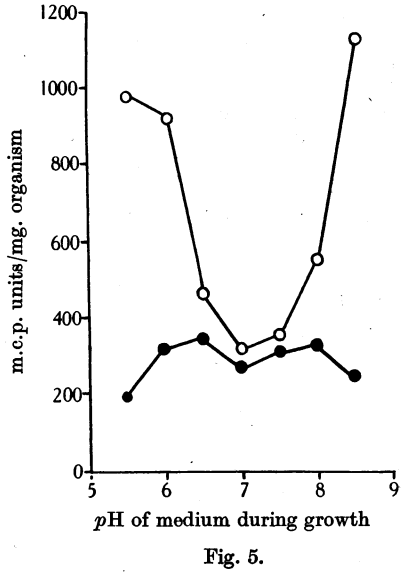

Fig. 5.

Fig. 4. Variation of α toxin production with growth *p*H. ●—● Egg units/mg. organism: no glucose. o—o Egg units/mg. organism: 0·45 % glucose. o- - -o Egg units/ml. medium: 0·45 % glucose.

Fig. 5. Variation of hyaluronidase production per mg. organism with growth *p*H. ●—● No glucose. o—o 0·45 % glucose.

The case quoted represents the conditions holding in a medium containing 0·45% glucose with the *p*H initially adjusted to 7·5, no attempt being made to control the *p*H during growth. This case represents the usual conditions of growth used for toxin production, but the general shape of the curves for toxin, hyaluronidase and organism production against time remains the same for all conditions of growth studied. The figures quoted below for toxin content of media are calculated for the time of maximum toxin production in each case, while the hyaluronidase activities are calculated from the activity of the 24-hr. sample divided by the maximum dry weight of organism recorded during growth.

Two series of experiments along these lines were carried out: first the formation of toxin, etc., was studied in glucose-free media adjusted to *p*H values from 5·5 to 8·5 with intervals of 0·5 *p*H unit; secondly, the series was repeated with the addition in each case of 0·45% glucose to the medium. The *p*H was maintained at a steady value in each experiment; in the absence of glucose this is not difficult as the drift of *p*H towards neutrality due to the metabolic activities of the growing organism is easily countered, but when glucose is present in the medium it is necessary to add about 1 ml. of *N* NaOH every 5 or 10 min. during the period of rapid growth in order to neutralize the acid produced by fermentation. Fig. 3 shows the production of θ toxin per mg. dry weight of organism at the various medium *p*H values tested: in the absence of glucose there is a marked production optimum at a growth *p*H of 7·5; the presence of glucose markedly increases the yield of toxin at all growth *p*H values and results in an apparent shift of the optimum production *p*H to 8·0. Fig. 4 shows the corresponding variations of α toxin; in the absence of glucose α toxin production is negligible except for growth *p*H values between 5·5 and 6·5; the presence of glucose results in an entirely different curve, with a greatly increased toxin production over the range 6–8, and with an optimum at 7·0–7·5. The growth *p*H for optimum production of α toxin thus shifts from 6·0 in the absence of glucose to 7 or 7·5 in its presence. The yield of α toxin/ml. medium shows an optimum when the medium *p*H is stabilized at 7·0.

Fig. 5 shows the variation of hyaluronidase production/mg. dry weight of organism with medium *p*H. In the presence of glucose there is a minimum production of hyaluronidase at a growth *p*H of 7 and the production increases rapidly as the growth *p*H value becomes either acid or alkaline, so that the highest yields per mg. dry weight of organism are obtained at the extremes of the growth range. In the absence of glucose, the much smaller growth makes the hyaluronidase activity too small to be estimated with accuracy but it appears probable that the shape of the production-*p*H curve is similar to that obtained in the presence of glucose, especially around the centre part of the growth range. The activities estimated for a growth *p*H of 7 with and without glucose indicate that glucose does not significantly stimulate the production of hyaluronidase per mg. dry weight of organism.

Finally, the estimations for media containing glucose and adjusted to initial *p*H values of 8·5, 7·5, 6·5 and 5·5 were repeated, but in these cases the *p*H was allowed to alter without control. The results are set out in Table 1 and indicate that an initial *p*H of 7·5 gives the highest production of both α and θ toxins estimated per ml. medium. It appears that the yield of α toxin when the *p*H is uncontrolled is slightly higher than when the *p*H is stabilized, but insufficient experiments have been carried out to be certain on this point.

pH stability of α and θ toxins. α and θ toxins, in the crude bacterial filtrate, appear to be quite stable in the *p*H range used in these growth experiments. 10 ml. aliquots of a toxic filtrate were adjusted to the *p*H values, determined with the glass electrode, shown in Table 2, and left at 22° for 5 hr. After that time, the aliquots were adjusted to neutrality, made up to 20 ml. and their α and θ activities determined.

Table 1. *Production of α and θ toxins and hyaluronidase by* Cl. welchii *growing in the presence of glucose with varying initial* p*H*

Initial *p*H	Age of culture hr.	*p*H	mg. organism/ml. of medium	α toxin E.U./ml.	α toxin E.U./mg.	θ toxin θ U./ml.	θ toxin θ U./mg.	Hyaluronidase Titre/ml.	Hyaluronidase Titre/mg.
8·5	6½	8·08	0·650	4·2	6·5	57	79	80	120
	8½	7·40	1·400	33	23·5	468	334	—	—
	24	—	—	—	—	—	—	1280	910
7·5	3	6·75	1·700	18	10·6	170	100	400	235
	4½	7·11	2·700	50	18·5	475	176	1600	592
	24	—	—	—	—	—	—	1600	592
6·5	2½	6·39	0·575	1·6	2·8	16	28	80	140
	5	6·36	3·37	40	12	220	66	640	190
	24	—	—	—	—	—	—	1280	380
5·5	4½	5·25	0·102	1·1	11	1·8	17	10	98
	5½	5·42	0·05	3·3	?	2·2	?	10	?
	24	—	—	—	—	—	—	100	?

In each case readings are given for (1) middle of growth phase, (2) end of period of active growth, and (3) 24 hr. after inoculation.

Table 2. p*H stability of α and θ toxins*

*p*H	Percentage of maximum activity after 5 hr. at 22° α	θ	*p*H	Percentage of maximum activity after 5 hr. at 22° α	θ
2·5	33	28	7·3	100	100
3·3	38	42	8·5	100	78
4·1	72	85	9·1	81	61
5·3	96	100	9·8	60	49
6·3	96	98			

Discussion

Figs. 3 and 4 indicate that glucose is toxigenic for α and θ toxins; this action of glucose cannot be ascribed to changes in medium *p*H produced by fermentation acid, since the *p*H of the medium has been controlled in all the experiments involved and, furthermore, toxin production is not favoured by an acid growth medium. Since α toxin is not produced at growth *p*H values >7 in the absence of glucose it is possible to produce a culture filtrate containing θ toxin free of α toxin by growing the organism at *p*H 7·5 in the absence of glucose.

It is interesting to note that the maximum production of α and θ toxins takes place when the growth *p*H is in the neighbourhood of 7. This *p*H is also the optimum for the growth of the organism. Hyaluronidase production, on the other hand, is lowest in this range.

Summary

Cl. welchii, type A, strain S 107 has been grown in a peptone-salt-beef extract medium with and without glucose; the *p*H has been stabilized in each case at various values within the growth range 5·5–8·5. Under these conditions the production of α and θ toxins and hyaluronidase per mg. dry weight of organism varies with the age of the culture, being small in young cultures and increasing to a maximum at the time when active cell division ceases. In the absence of glucose, θ toxin production per mg. dry weight of organism is maximum for a growth *p*H 7·5; glucose increases the yield of toxin at all *p*H values and shifts the growth *p*H for maximum production to 8·0. In the absence of glucose α toxin is produced only when the medium *p*H lies between 5·5 and 7·0 with a maximum production at *p*H 6·0; in the presence of glucose the maximum production takes place at medium *p*H values 7·0–7·5. The formation of hyaluronidase per mg. dry weight of organism is least when the medium *p*H is 7·0 and increases as the medium *p*H becomes more acid or alkaline.

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