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Haemolytic activity of the alpha and theta toxins of *Clostridium welchii**

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INTRODUCTION

Clostridium welchii type A produces several toxic substances which are lethal, necrotizing, and haemolytic. The lethal and necrotizing properties of this organism have been studied extensively. However, little is known regarding the haemolytic activity of *Cl. welchii*. It is difficult to speculate on the mechanism of haemolysis caused by *Cl. welchii* until the biochemistry and mechanism of the general phenomenon of haemolysis is elucidated.

Of the twelve recognized toxins produced by *Cl. welchii*, only three, alpha, delta and theta toxins, are haemolytic. Since type A strains do not produce the delta toxin, the haemolytic action of type A strains is limited to the activity of alpha and theta toxins. The haemolytic activities of these two toxins are easily recognized; the alpha toxin produces partial haemolysis, whereas the theta toxin produces a complete haemolysis. Non-haemolytic strains of *Cl. welchii* were brought to attention when Hobbs *et al.* (1953) showed that the English 'food poisoning' strains were typically non-haemolytic.

Alpha toxin has been shown by Macfarlane & Knight (1941) to be closely associated, and probably identical, with the enzyme lecithinase found in toxic filtrates of *Cl. welchii*. They suggested that the lecithinase was responsible for the lysis of red cells. The haemolytic activity of the toxin is due to its ability to hydrolyze phospholipid-protein complexes on the surface of susceptible erythrocytes (Macfarlane & Knight, 1941).

Neill (1926) described an oxygen-labile haemolysin present in culture filtrates of *Cl. welchii*. The haemolytic activity of this substance was inactivated by oxidation, and could be restored by the addition of a reducing agent. This oxygen-labile haemolysin was later shown to be identical with theta toxin (Todd, 1941). Although the studies of Todd (1941) and Roth & Pillemer (1955) strongly suggest that this toxin exhibits enzymic properties, no specific substrate has been identified.

Strains of *Cl. welchii* type A have been subdivided into two groups—classical and food-poisoning strains (Hobbs, 1965; Brooks, Sterne & Warrack, 1957). Haemolytic activity is one of the criteria which Hobbs has used to distinguish these two groups. The classical gas-gangrene strains possess heat-sensitive spores, are

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β-haemolytic on sheep blood agar, and produce relatively large amounts of alpha toxin. The food-poisoning strains produce small amounts of alpha toxin but no theta toxin, are heat-resistant and are non- or α-haemolytic on horse-blood agar (Hobbs, 1965). Other workers (Dische & Elek, 1957; Hauschild & Thatcher, 1967) have suggested that food-poisoning strains may be heat-sensitive as well as heat-resistant, and may vary considerably in their haemolytic activity and formation of alpha toxin. Recently Sutton & Hobbs (1968) reported on five outbreaks of food poisoning caused by heat-sensitive strains of *Cl. welchii*. They suggested that perhaps heat-sensitive strains may be implicated in food poisoning outbreaks with unknown aetiology.

Although haemolysis is used as a criterion for the identification of type A strains of *Cl. welchii* the species of red blood cells and the kind of haemolysis observed is often not indicated. The conditions for determining the haemolytic patterns exhibited by *Cl. welchii* have not been standardized. Haemolysis observed on blood agar plates may be influenced by a number of variables: erythrocytes from different animal species, growth conditions of the organism, temperature, and conditions under which the haemolysis is observed. In order to categorize strains of *Cl. welchii* as to haemolytic pattern, it is important to be aware of the range of variations that may be observed. We studied the haemolytic activity of different strains of *Cl. welchii* using erythrocytes from four different animal species in order to determine the relative value of each blood in the detection of haemolysis, as well as to investigate the comparative haemolytic activities of each strain. In addition, the haemolytic activity was compared with the lecithinase activity of *Cl. welchii*.

MATERIALS AND METHODS

Strains of Clostridium welchii

Thirty strains of *Cl. welchii* were employed in this study. All strains were isolated at the University of Montana, Missoula, Montana, from human faeces, soil, and foods. The strains were identified on the basis of morphological and biochemical characteristics.

Lecithinase activity

The lecithinase activity of *Cl. welchii* was assayed by the lecithovitellin method of van Heyningen (1941) as modified by Nakamura & Cross (1968).

Haemolytic activity of Cl. welchii

The haemolytic activity of each strain was assayed by observing haemolysis on 5·0 % blood agar plates. Defibrinated horse, sheep, and rabbit bloods were obtained from Colorado Serum Company, Denver, Colorado; the human blood was drawn in the laboratory and added directly to the medium without the use of an anticoagulant. Actively growing thioglycollate broth cultures (5 hr. at 46° C.) of each strain were centrifuged and the cells washed three times in saline. The cells were suspended in saline and diluted with sterile 0·1 % peptone water in order to obtain approximately 10–50 colonies per plate. After anaerobic incubation at 37° C. for 18 hr., the plates were placed at 5° C. under aerobic conditions for 4–6 hr. to allow

full development of the haemolytic activity of the alpha toxin, a hot–cold lysin. The diameters of ten colonies of each strain on each type of blood were measured using a vernier caliper with an accuracy of 0·1 mm. (General Hardware Mfg., Inc., New York, New York). Haemolytic zones which circumscribed the colony were measured.

Theta toxin produced a zone of complete clearing of blood around the colony. Microscopic examination indicated that all red blood cells were lysed in this area. Alpha toxin produced partial haemolysis which resulted in a zone of discoloration surrounding the colony. In this area of haemolysis only partial lysis of the red cells was observed microscopically.

The effect of temperature and length of incubation on haemolysis by *Cl. welchii* was determined. After an 18 hr. initial incubation period at 37° C. the plates were incubated further aerobically at 37°, 46°, and 52° C. The diameters of each colony and zones of haemolysis were measured periodically for 24 hr.

Haemolytic activity of commercial lecithinase

The haemolytic activity of commercial lecithinase (Nutritional Biochemicals Corporation, Cleveland, Ohio) upon horse, sheep, rabbit and human blood was studied. The lecithinase was diluted in 0·05 M Tris buffer, pH 7·2, in order to obtain concentrations of 20, 40, 80, 100 and 200 μg./ml. An Ouchterlony die (Shandon Scientific Co., London) was used to make wells with a diameter of 3·5 mm. in the blood agar plates. A 1 ml. tuberculin syringe with a 25-gauge needle was used to dispense the lecithinase solutions. One drop of each concentration was added to the wells in the blood agar plates. One drop approximated 0·008 ml. The plates were then incubated at 37°, 46° and 52° C. The diameter of each zone of haemolysis was measured periodically for 24 hr.

The effect of heat on the haemolytic activity of commercial lecithinase was determined. Three tubes containing 100 μg./ml. solution of lecithinase in Tris buffer were placed in water baths preheated to 50°, 60° and 70° C., respectively. Samples were removed after 2, 5, 10, 20, 30, 45 and 60 min. of heating. Unheated samples were used as controls. One drop of each sample was dispensed into wells on sheep blood agar plates. The plates were incubated at 37° C. for 24 hr., and then held for several hours at 5° C. The diameters of haemolysis were measured using a vernier calipers.

RESULTS

Lecithinase activity

The lecithinase activity of thirty strains of *Cl. welchii* is enumerated in Table 1. The mean lecithinase activity of the ten strains isolated from food was $43·5 \pm 22·1$ μg./ml. whereas the mean lecithinase values of the soil and faecal strains were $67·0 \pm 28·3$ and $65·0 \pm 31·5$ μg./ml., respectively.

Haemolytic activity of Cl. welchii

The haemolytic activity of twenty-nine strains of *Cl. welchii* on horse, sheep, rabbit, and human blood is shown in Table 2. Most of the strains produced suf-

ficient theta toxin to produce lysis on all four types of blood. Seventy-nine per cent of the strains produced theta toxin haemolysis on horse blood, 85% on sheep blood, 90% on human blood, and 100% on rabbit blood. The areas of haemolysis produced on rabbit blood were larger than those produced on the other bloods. Therefore, rabbit blood may be considered the ideal blood for the detection of the theta toxin of *Cl. welchii*.

Table 1. *Lecithinase activity of* Clostridium welchii *isolated from human faeces, soil, and food*

Human faeces		Soil		Food	
Strain	μg. toxin/ml. culture filtrate*	Strain	μg. toxin/ml. culture filtrate*	Strain	μg. toxin/ml. culture filtrate*
UM 2	105·0	UM 31	80·0	UM 7	47·5
UM 3	57·5	UM 32	67·5	UM 17	82·5
UM 5	90·0	UM 33	20·0	UM 19	50·0
UM 6	80·0	UM 34	102·5	UM 26	30·0
UM 8	47·4	UM 35	67·5	UM 29	15·0
UM 9	57·5	UM 36	22·5	UM 46	77·5
UM 10	45·0	UM 37	127·5	UM 48	40·0
UM 12	45·0	UM 38	55·0	UM 52	42·5
UM 13	117·5	UM 39	62·5	UM 54	40·0
		UM 43	45·0	UM 55	10·0

* Mean of two determinations.

Only 3 out of 29 (9·6%) strains produced sufficient amounts of alpha toxin to lyse horse blood. These three strains were highly toxigenic (over 100 μg. alpha toxin per ml.) confirming the work of Hall, Angelotti, Lewis & Foter (1963), who reported that a higher concentration of alpha toxin was required to lyse horse erythrocytes. Thirty-eight per cent of the strains produced alpha toxin haemolysis on rabbit blood, 76% on human blood, and 93% on sheep blood. Since sheep erythrocytes are the most susceptible to lysis by alpha toxin, sheep blood should be considered to be the blood of choice for the detection of haemolysis due to alpha toxin. Hall *et al.* (1963) also found that sheep erythrocytes were the most sensitive to lysis by alpha toxin. They suggested that sheep blood may be used to estimate the toxin-producing abilities of *Cl. welchii*.

In our study, horse, rabbit, and human erythrocytes were lysed by some strains which did not haemolyse sheep blood. Thus, it was difficult to determine whether the absence of haemolysis was due to the resistance of the erythrocytes to lysis, or to the differences in the amounts of alpha toxin produced. One strain, UM 37, did not produce alpha-toxin haemolysis on sheep blood, even though it produced high levels of alpha toxin (127·5 μg./ml.) as determined by the lecithovitellin method. Differential susceptibility of erythrocytes of different species to haemolysis may be more significant than the ability of a particular strain to produce alpha toxin.

The strains of *Cl. welchii* isolated from food were less haemolytic than the strains

isolated from faeces or soil. Of the strains isolated from foods, only 43 % produced haemolysis due to alpha toxin and 75 % produced haemolysis due to theta toxin. Sixty per cent of the strains isolated from soil and 61 % of the strains isolated from faeces produced alpha toxin haemolysis on all four bloods. Ninety-three per cent of the strains isolated from soil and 100 % of the strains isolated from faeces produced theta toxin haemolysis.

Table 2. *Haemolytic activity of* Clostridium welchii

Type of blood ...	Haemolysis due to theta toxin: Diameter (mm).*				Haemolysis due to alpha toxin: diameter (mm.)*			
	Horse	Sheep	Rabbit	Human	Horse	Sheep	Rabbit	Human
				Faeces				
UM 2	2·3	4·3	3·6	3·9	7·1	9·5	8·2	11·7
UM 3	2·5	3·2	8·5	1·9	—	9·6	—	—
UM 5	1·9	3·3	3·4	2·3	—	8·0	7·0	5·7
UM 6	2·4	3·4	6·8	2·6	—	8·1	—	7·9
UM 8	2·5	3·1	8·1	2·3	—	9·5	—	9·8
UM 9	1·7	2·6	3·1	2·1	—	7·3	8·7	8·3
UM 10	1·8	2·4	6·8	2·5	—	7·9	—	8·8
UM 12	1·9	2·9	7·6	2·6	—	9·7	—	—
UM 13	2·0	2·0	3·1	1·7	2·3	9·0	6·1	5·5
				Soil				
UM 31	2·4	3·3	8·7	2·9	—	9·8	—	—
UM 32	—	—	5·4	2·2	—	5·5	—	7·1
UM 33	2·7	3·3	9·0	2·7	—	8·3	9·7	9·9
UM 34	1·7	2·7	4·6	2·0	1·9	7·0	9·1	8·3
UM 35	2·5	3·8	4·9	3·2	—	8·5	8·7	7·9
UM 36	2·0	3·1	7·6	2·8	—	8·2	—	9·1
UM 37	—	6·5	5·3	7·2	—	—	5·6	—
UM 38	2·1	3·9	4·7	3·5	—	8·3	8·4	8·5
UM 39	1·9	2·9	8·4	2·4	—	9·2	12·3	10·9
UM 43	2·4	2·5	4·5	2·1	—	4·2	—	5·4
				Food				
UM 7	1·9	2·6	2·2	3·8	—	6·1	7·8	8·6
UM 17	1·5	2·8	6·9	2·3	—	5·5	—	—
UM 19	1·9	2·0	8·8	1·2	—	9·0	—	—
UM 26	2·6	4·0	8·7	3·2	—	9·7	—	10·7
UM 29	1·6	3·2	7·8	2·7	—	7·3	—	—
UM 46	—	4·3	4·0	1·9	—	—	—	5·5
UM 48	—	—	8·2	—	—	9·6	—	9·7
UM 52	—	—	5·7	—	—	7·6	—	8·6
UM 54	3·3	2·3	6·3	2·2	—	5·5	—	8·6
UM 55	—	—	8·1	—	—	9·5	—	8·5

* Mean of ten determinations. —, No haemolysis.

All three strains which produced heat-resistant spores were isolated from food. Two of these strains, UM 48 and UM 55, failed to produce theta toxin on horse, sheep, and human blood. These strains also produced low levels of lecithinase as determined by the lecithovitellin reaction. Therefore these strains fit Hobbs's criteria for food-poisoning strains. However, the majority of strains examined in

this investigation produced theta toxin, and therefore do not fit Hobbs's description of food-poisoning strains of *Cl. welchii*.

The haemolytic activity of the alpha and theta toxins was not strictly dependent on colony size. For example, two strains with identical colonial diameters produced areas of haemolysis varying from 23·8 mm.^{2} to 73·9 mm.^{2} on sheep blood plates.

The lecithinase activity of *Cl. welchii* did not correlate with the haemolytic activity for the majority of the strains. Some strains which produced large amounts of alpha toxin, as measured by the lecithovitellin technique, did not produce large areas of haemolysis. This relationship varied with the type of erythrocytes used to study the haemolytic activity.

Because the area of haemolysis due to theta toxin is considerably smaller than the area due to alpha toxin, it is possible that the theta toxin diffuses more slowly through the blood agar or that it undergoes rapid decomposition. The area of haemolysis due to alpha toxin on all bloods tested was subject to greater variation than the area of haemolysis produced by theta toxin. Of the twenty-three strains which produced both alpha and theta toxin on sheep blood, the standard deviation of the computed areas of theta toxin haemolysis was $\pm 2{\cdot}8$ mm.^{2}, whereas the standard deviation of alpha toxin haemolysis was $\pm 17{\cdot}6$ mm.^{2}. Quantitatively, the production of alpha toxin was much more variable than the amount of theta toxin produced.

Occasionally, the haemolytic patterns on various bloods deviated from the characteristic alpha toxin haemolysis or theta toxin haemolysis usually observed. The haemolytic variability among the strains and difference in the erythrocytes may have been responsible for these atypical zones of haemolysis. It is also possible that these haemolytic peculiarities were due to the unclassified non-alpha-delta-theta type of haemolysis described by Brooks *et al.* (1957) or the hypothetical X and Y haemolysis of van Heyningen (1941). Hall *et al.* (1963) also observed haemolysis produced by type A strains which was not due to alpha, theta, or delta toxins. Conceivably, some strains of *Cl. welchii* produce additional haemolysins which have not been identified.

Effect of temperature and length of incubation

The temperature and length of subsequent incubation similarly influenced both alpha and theta toxin activities. The additional incubation was conducted under aerobic conditions, therefore the additional haemolysis we noted was not due to the growth-stimulating effect of temperature. This treatment probably affected the red blood cells and previously synthesized toxins.

The haemolytic activity due to the theta toxin of *Cl. welchii* UM6 at different temperatures of subsequent incubation is presented in Fig. 1. The diameter of haemolysis was much greater on rabbit blood than on sheep, human or horse blood. On horse blood the haemolytic activity was highest at 52° C., whereas haemolysis was greatest at 37° C. on sheep and human blood. This suggests that the erythrocytes from different species may be reacting differently to the toxin. A number of complex interacting mechanisms may be involved. Higher temperatures may

cause an increased rate of diffusion of the toxin, but in some cases may cause a degradation of the toxin. In addition, higher temperatures may increase the fragility of erythrocytes, thus making them more susceptible to lysis.

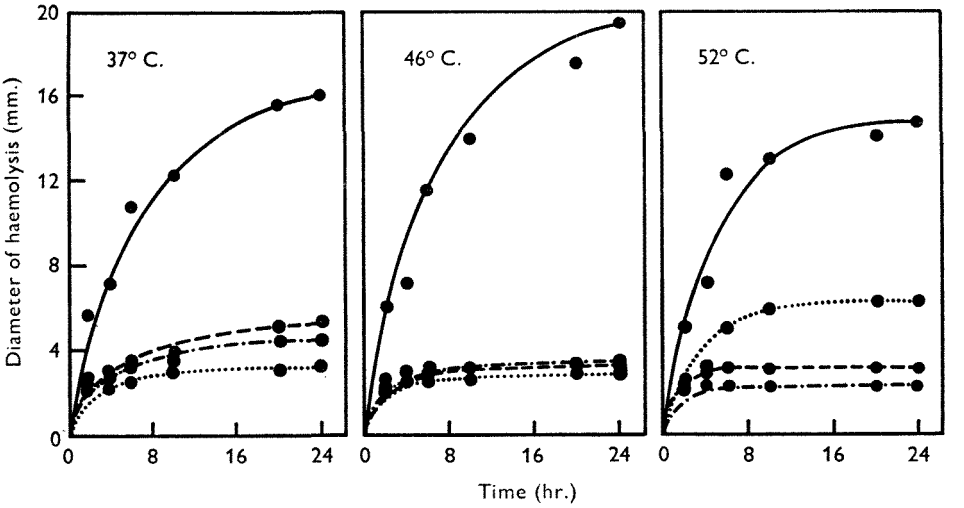

Fig. 1. Haemolytic activity of *Cl. welchii* UM 6 due to theta toxin on erythrocytes of different animal species as a result of additional incubation. —, Rabbit; – – –, sheep; —·—, human; . . . . . , horse.

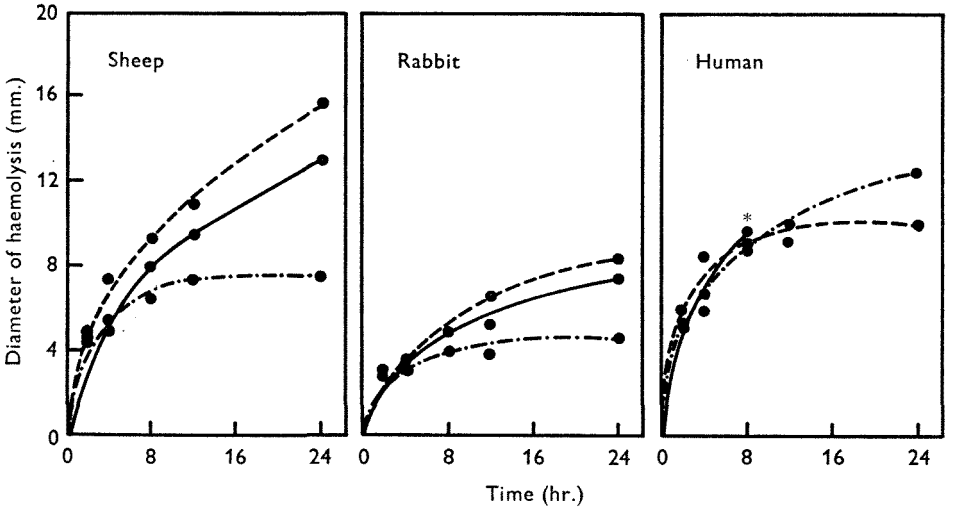

Fig. 2. Effect of time and temperature on the haemolytic activity of commercial lecithinase (80 μg./ml.) —, 37° C.; - – –, 46° C.; — · —, 52° C.;* haemolysis fades and was impossible to measure after 8 hr.

The diameter of haemolysis increased with increasing time of incubation. In studies dealing with the kinetics of haemolysins, Burrows (1951) reported that haemolysis due to alpha toxin of *Cl. welchii* was a function of time, while Bernheimer (1947) found a similar relationship with theta toxin.

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Haemolytic activity of commercial lecithinase

To our knowlege the haemolytic activity of commercial lecithinase has not been related to that of alpha toxin from culture filtrates of *Cl. welchii*. The haemolytic activity of commercial lecithinase was directly proportional to the concentration and length of incubation. Haemolysis was produced by the enzyme on sheep, rabbit and human blood. Neither the commercial lecithinase nor the enzyme produced by the majority of the strains of *Cl. welchii* caused alpha toxin haemolysis on horse blood.

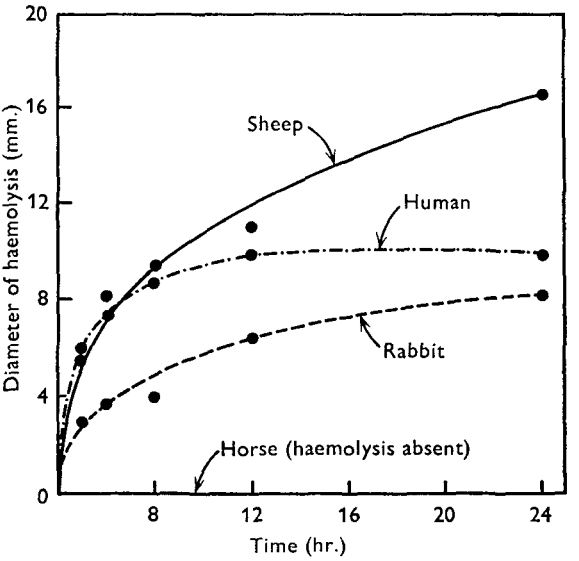

Fig. 3. Haemolytic activity of commercial lecithinase (80 μg./ml.) at 46° C. on erythrocytes of different animal species.

The effect of commercial lecithinase upon erythrocytes from different species is illustrated in Fig. 2. At a concentration of 80 μg./ml. greatest haemolytic activity was observed on sheep blood. Maximum haemolysis occurred when the blood plates containing the commercial lecithinase were incubated at 46° C. Clearly, there was a differential susceptibility of the different bloods to haemolysis by the commercial lecithinase (Fig. 3).

In Fig. 4 the haemolysis due to alpha toxin of *Cl. welchii* UM6 is compared to the haemolytic activity of commercial lecithinase. The concentration of lecithinase tested was 80 μg./ml. and the level of alpha toxin produced by UM6, as measured by the lecithovitellin technique, was also 80 μg./ml.

The close relationship between the haemolytic activity of *Cl. welchii* alpha toxin and commercial lecithinase indicates that these two have similar, if not identical, effects on red blood cells. These data confirm the hypothesis presented by Macfarlane & Knight (1941) that the alpha toxin of *Cl. welchii* is probably identical with lecithinase.

The haemolytic activity of commercial lecithinase was destroyed by exposure to 70° C. for 20 min. (Fig. 5). Partial inhibition of haemolytic activity occurred at

60° C. Little or no inhibition was observed when the enzyme was heated at 50° C. In studies from our laboratory (unpublished) it was found that lecithinase activity, as determined by the lecithovitellin technique, was almost completely destroyed when exposed for 5 min. at 60° and 90° C. Therefore the haemolytic activity of lecithinase appears to be more stable at 60° C. than the lecithovitellin activity of the same preparation. To our knowledge, this is the first report of the effects of heat upon the haemolytic activity of commercial lecithinase.

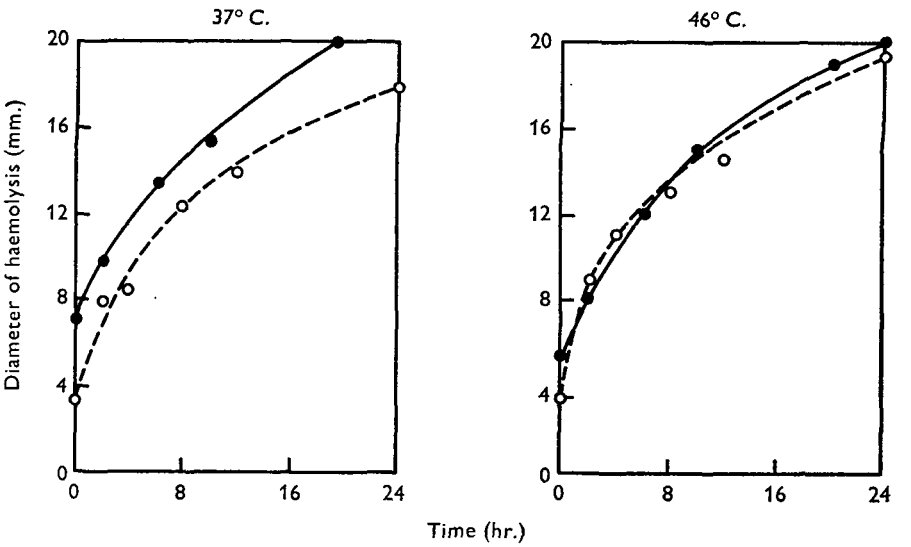

Fig. 4. Comparison of haemolysis due to alpha toxin of *Cl. welchii* UM 6 with haemolysis due to commercial lecithinase. ●—●, UM 6; ○—○, commercial lecithinase (80 μg./ml.).

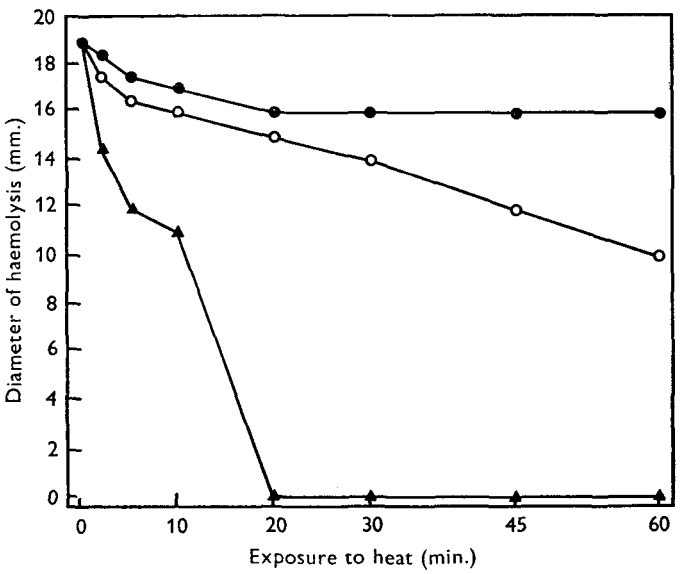

Fig. 5. Effect of heat on the haemolytic activity of commercial lecithinase. ●—●, 50° C.; ○—○, 60° C.; ▲—▲, 70° C.

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DISCUSSION

It is apparent that a number of complex factors are involved in the haemolysis produced by the alpha and theta toxins of *Cl. welchii*. The variation in the differential haemolytic activity of *Cl. welchii* is consistent with other reports (Macfarlane, 1950; Hall & Hauser, 1966). Macfarlane, Oakley & Anderson (1941) concluded that differences in haemolysis by alpha toxin were due primarily to variations in the rate of hydrolysis of phospholipids in the red cells of different species. Obviously, other factors are also involved in differential haemolysis.

Stereochemical configuration is involved in the availability of specific combining sites on the enzyme. This may determine whether the active site of the lecithinase can affect the lecithins of the erythrocyte membrane. The variation in rate of enzyme action with erythrocytes from different species may be due to differences in the rate of absorption of the alpha toxin by the red cells. Variations in the fragility of red blood cells may also affect the degree of haemolysis.

Another factor to be considered is the variation in the dimensions of red cells from different species. The red cells of horse, sheep, rabbit and man are not only of different sizes, but also of different shapes (Ponder, 1948). The size and shape of the erythrocytes may be an important factor in the formation of enzyme-substrate complexes.

Among the various lipid components of red blood cells, the alpha toxin of *Cl. welchii* attacks lecithin, sphingomyelin, and phosphatidylethanolamine. There are only small differences in the amount of total phospholipids in red cells of horse, sheep, rabbit, and man (de Gier & van Deenen, 1961; Ponder, 1948). The differential susceptibility of different erythrocytes to haemolysis cannot be accounted for solely on the basis of the substrate present in the red cell membrane. Haemolytic differences may be due primarily to differences in the relative amounts of specific phospholipids present.

Although the nature of the theta toxin is not known, its mode of action resembles that of an enzyme. Our observations suggest that the mechanism of action of alpha and theta toxin upon blood may be similar. A majority of the strains which produced theta toxin haemolysis also produced alpha toxin haemolysis on the same blood. Temperature and length of subsequent incubation affected similarly the haemolytic activity of both toxins. A clear distinction between the two haemolytic zones was not always possible. When high concentrations of commercial lecithinase were tested, a clear zone of haemolysis within the zone of partial haemolysis was observed.

Gale & van Heyningen (1942) found that theta toxin was detectable in the growth medium a short time before the alpha toxin was detected. On blood plates the zone of alpha-toxin haemolysis surrounds the area of theta-toxin haemolysis. Therefore, it is plausible that the haemolytic activity of theta toxin occurs before that of alpha toxin.

On the basis of these observations, it is possible to hypothesize that one molecular species is responsible for both types of haemolysis. Different active groups present on the toxin moiety may be responsible for both partial and complete

haemolysis. Red blood cells are completely lysed under conditions of maximum enzymic activity. As the enzymic activity decreases, fewer erythrocytes may be acted upon, and partial haemolysis occurs. If only one molecular component is responsible for both types of haemolysis, the alpha-toxin haemolysis may follow theta-toxin haemolysis upon dissociation of the molecule.

SUMMARY

The lecithinase and haemolytic activity of thirty strains of *Cl. welchii* isolated from food, faeces, and soil, was studied. The strains from foods produced smaller amounts of lecithinase and were, in general, less haemolytic than the strains isolated from soil and faeces.

The haemolytic activity of *Cl. welchii* on erythrocytes from different animal species displayed considerable variation. Sheep erythrocytes were the most sensitive to the action of alpha toxin, whereas rabbit blood was most sensitive to haemolysis by theta toxin. The degree of haemolysis was also dependent upon the concentration of the enzyme, and temperature and length of incubation.

The haemolytic activity of commercial lecithinase was observed to be similar to the haemolytic activity of the alpha toxin of *Cl. welchii*. This finding provides further evidence that the haemolytic and lecithinase activities of *Cl. welchii* are due to one substance, the alpha toxin. Exposure of commercial lecithinase to heat resulted in the destruction of its haemolytic properties.

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