The Production by Certain Species of *Clostridium* of Enzymes Disintegrating Hide Powder

BY D. G. EVANS

National Institute for Medical Research, Hampstead, London

SUMMARY: The production by certain species of *Clostridium* of enzymes disintegrating hide powder was investigated by measuring the lytic action of broth cultures and toxic filtrates on finely divided hide powder suspended in an agar gel.

Cl. histolyticum was the most active producer of enzyme, *Cl. welchii* A was less active and *Cl. sporogenes* and *Cl. bifermentans* only moderately active. *Cl. tetani*, *Cl. oedematiens* and *Cl. septicum* produced no such enzyme. The lytic enzyme of *Cl. histolyticum* is not the lethal toxin.

Among strains of *Cl. welchii* type A, enzyme production, α -toxin production and ability to cause fatal infection in guinea-pigs are associated.

There was some evidence that the different enzymes affecting hide powder are antigenically related, but no definite conclusion is possible, since the antisera employed may have contained antibodies to the lytic enzymes of a number of different organisms.

Maschmann (1938) claimed that *Cl. welchii* elaborated an enzyme which splits collagen. This he named collagenase but recorded no experiments demonstrating its specific action. Later Jennison (1945) recorded the disintegration of collagen fibres by enzymes produced in actively growing cultures of aerobic and anaerobic organisms. Macfarlane & MacLennan (1945), confining their study to *Cl. welchii* Type A, found that culture filtrates were able to break down muscle by their action on the collagen of the reticulin scaffolding. They regarded the active substance as a collagenase and suggested that it was responsible for the muscle destruction observed in cases of gas gangrene caused by *Cl. welchii* A and thus played an important part in the pathogenesis of the disease. The collagenase of *Cl. welchii* A was shown by Oakley, Warrack & van Heyningen (1946) to be immunologically distinct from the other known antigens present in culture filtrates, and *Cl. welchii* A antisera contained a distinct anticollagenase. Evans (1945*b*, 1947) showed that the anticollagenase alone had no protective action in experimental *Cl. welchii* A infections, and concluded that although collagenase may be responsible for the muscle destruction observed in *Cl. welchii* A infections, there was no evidence to suggest that it had any significant role in determining whether fatal infection would follow the injection of a given inoculum of *Cl. welchii* A.

The strains of *Cl. welchii* A used in these experimental infections all produced collagenase *in vitro*, though in variable amounts. Oakley *et al.* (1946), in estimating the anticollagenase potency of *Cl. welchii* antisera, obtained identical results using as substrate either collagen prepared from horse tendon or commercial hide powder. Hide powder, although not a pure substrate, is a rich and convenient source of collagen and was employed in the tests described below. The disintegration of hide powder, however, is not a specific

indication of collagenase action but provides reasonable presumptive evidence that a collagenase is present. A study has been made of the *in vitro* production by a number of strains of *Cl. welchii* A and by other species of *Clostridium*, of enzymes with ability to disintegrate hide powder.

METHODS

The production of lytic enzymes by *Cl. welchii* A was first demonstrated by incorporating the hide powder in the culture medium. Hide powder was passed through a sieve, 60 meshes to the inch, dried over P_2O_5 *in vacuo* for 2 days and sterilized by dry heat at 100° for 1 hr. To 100 ml. of saline 4 g. of the hide-powder preparation were added and a smooth suspension obtained by vigorous agitation. This was added to melted Fildes's agar medium, 12 % by volume, and pour-plates made in which the hide-powder particles were distributed densely and uniformly. Sterilization of the hide powder was necessary to eliminate contaminants, but it is improbable that the dry heating caused significant degradation of the collagen, for similar results were obtained with unsterilized hide powder.

After 24 hr. anaerobic growth on this medium strains of *Cl. welchii* A gave colonies surrounded by a zone in which complete dissolution of the hide powder had occurred and which increased in size after a further 24 hr. incubation. With different strains the size of the zone of clearing varied from a faint narrow zone to one three times the diameter of the colony.

The method was, however, unsuitable for comparing the lytic enzyme production of different strains of *Cl. welchii* A, or for detecting lysis by species of *Clostridium* that grow in widely spreading colonies. To obviate these difficulties, the amount of enzyme produced in broth cultures was titrated in cups cut in non-nutrient hide powder-agar plates. These plates were made by adding 2 ml. of the saline hide-powder suspension to 15 ml. of melted 4 % agar in saline and pouring the mixture into a Petri dish. Cups of 7 mm. diameter were cut with a cork-borer, and the amount of enzyme in a culture was then titrated by pipetting into each cup approximately 0.1 ml. of falling dilutions of an 18 hr. culture in rabbit-liver broth and incubating aerobically at 37°. After 24 hr., actively lytic strains gave around the cup a distinct concentric zone completely free from hide-powder particles; the zone increased in size after a further 24 hr. incubation, when the final readings were made. There was no disadvantage in filling the cups with whole-broth culture, since there was no possibility of the growth of anaerobes occurring on this medium. Moreover, tests made with the clear supernatant fluid from centrifuged cultures gave results identical with those using whole culture.

RESULTS

Lysis of hide powder by Clostridium welchii A

All the thirty strains of *Cl. welchii* A which were used in a previous investigation (Evans, 1945*a*), and which had been preserved in the dry state in the meantime, were found to produce enzymes disintegrating hide powder. The

zone of clearing was 25 mm. in diameter with the most active strains and less than 10 mm. in diameter with the least active. The strains were classified into three groups according to the diameter of the zone and arranged in order of α -toxin production (Table 1). Strongly lytic cultures were active when diluted 1:100, but those which gave small zones were inactive when diluted 1:4 and in some cases 1:2. When enzyme production by the thirty strains of *Cl. welchii* A is compared (Table 1) with α -toxin production and the ability to cause fatal infection in guinea-pigs (Evans, 1945*a*), it is evident that there is a general association between these three properties.

Table 1. *Comparison of hide-powder enzyme production with other properties of thirty strains of Clostridium welchii A*

Enzyme production: + = <10 mm., ++ = 10–15 mm., and +++ = >15 mm. zone of hide-powder clearing.

α -toxin production: figures = number of units of α -antitoxin required to neutralize the α -toxin in 1 ml. of culture.

Virulence for guinea-pigs: + = virulent; – = avirulent.

Strain	<i>In vitro</i> production of		Virulence for guinea-pigs
	Hide-powder enzyme	α -toxin	
S107	+++	1.60	+
SR12	+++	0.95	+
A119	+++	0.85	+
A117	+++	0.60	+
A118	+++	0.60	+
SR9	+++	0.35	+
Rosher	+++	0.25	+
G5g	+++	0.25	+
3893	+++	0.20	+
S1a	++	0.20	+
BB	+++	0.20	+
BS1	+++	0.20	+
3895	++	0.15	–
26	+++	0.15	+
A102	+	0.15	–
274	++	0.10	+
Mills	++	0.10	+
7731	++	0.10	+
A19	++	0.10	±
PL	+	0.05	+
5053	++	0.05	+
529	++	0.03	–
P5706	+	0.03	–
Corcoran	++	0.03	–
D5	++	0.03	–
4226	+	0.02	–
A78b	+	0.02	–
A38b	+	0.02	–
D3a	+	0.02	–
A13b	+	0.02	–

The zones of hide powder clearing were evidently produced by a true collagenase, since the activity of the cultures was neutralized by three of Dr Oakley's *Cl. welchii* A antisera in proportion to their anticollagenase

content. (1) Serum R5434 contained 50 units (Oakley, *et al.* 1946) of anti-collagenase and 0.2 unit of α -antitoxin per ml., (2) serum R6423 contained 75 units of α -antitoxin per ml. but no detectable anticollagenase, and (3) serum Ex1055 contained 2500 units of anticollagenase and 500 units of α -antitoxin per ml. With each of the thirty strains of *Cl. welchii* A, mixtures of equal parts of an 18 hr. culture and each serum were held for 1 hr. at room temperature and then pipetted into the hide powder-agar-cup plates, which were incubated at 37° for 48 hr. Serum R6423 had no effect on the zones of clearing produced by each of the thirty strains; the zones were similar in size to those produced by mixtures of culture and normal horse serum. On the other hand, serum R5434 completely inhibited the clearing produced by twenty-one of the strains; the remaining nine strains, all of which produced large zones, were partly neutralized, 20 mm. zones being reduced to less than 12 mm. in diameter. The more potent anticollagenase serum Ex1055 completely neutralized the clearing by all thirty strains.

Lysis of hide powder by other species of Clostridium

The following strains were tested for enzyme production: *Cl. sporogenes*: M1f, M41a, M3g, M5f; *Cl. oedematiens*: H1, Jolly, M131o, M4t; *Cl. bifermentans*: M74, M16, M15h, M58e, M1e; *Cl. septicum*: VS189, VS54, M16m, M40f; *Cl. tetani*: T67, T279; *Cl. histolyticum*: CN1693, CN950, CN919, CN920, CN949; and a non-pathogenic *Clostridium* with morphological and biochemical properties almost identical with those of *Cl. histolyticum* but which did not produce *histolyticum* toxin: strains M37e, M55, M17l.

The M strains were isolated from war wounds by Mrs E. M. Miles, and the *Cl. histolyticum* strains were supplied by Miss Helen E. Ross of the Wellcome Physiological Research Laboratories. The strains had been preserved either in alkaline-egg medium or in the dry state, and were subcultured a number of times to bring them into an actively growing condition. For the titration of enzyme each strain was grown in rabbit-liver broth for 18–20 hr.

Cultures of all the *Cl. histolyticum* strains produced rapid and complete dissolution of hide powder; even after 3 hr. a distinct rim of clearing was visible around the cups and at 24 hr. the diameter of the zone was 20 mm., increasing to an average of 30 mm. after a further 24 hr. incubation. The titres of these cultures were as high as 1:250. Each of the five strains of *Cl. histolyticum* produced, in guinea-pigs, an infection showing extensive muscle destruction which, with the infecting doses employed, was not always accompanied by the death of the animal.

Cultures of *Cl. bifermentans*, *Cl. sporogenes* and the three *Cl. histolyticum*-like strains also contained enzymes disintegrating hide powder. Their behaviour, however, differed from that of *Cl. welchii* A and *Cl. histolyticum* cultures. Although the zones of clearing at 24 hr. were distinct and 15–20 mm. in diameter, the clearing was only partial. Large numbers of unchanged hide-powder particles were visible within the zones, while other particles appeared to be only slightly affected. After a further 24 hr. incubation, the zones became completely clear, although they were not well defined. The concentration of

enzyme in these cultures was not high, for dilutions of 1:32 had little or no activity. The low concentration may have been responsible for the slow dissolution of the hide powder, for a concentrated filtrate of *Cl. bifermentans* culture produced in 24 hr. a zone completely free from hide-powder particles.

No enzyme attacking hide powder was demonstrable in the cultures of *Cl. oedematiens*, *Cl. septicum* and *Cl. tetani*. Two strains of each species proved to be highly pathogenic for guinea-pigs, so that with these organisms at least, virulence and enzyme production are not associated.

Some of Jennison's (1945) results have been confirmed in this investigation. He found that cultures of *Cl. histolyticum*, *Cl. sporogenes* and *Cl. bifermentans* disintegrated collagen fibres and that *Cl. histolyticum* was the most active. He reported, however, that *Cl. welchii* did not affect the collagen substrate, a result which may well have been due to poor collagenase production by the strains of *Cl. welchii* he used.

Lysis of hide powder by toxic filtrates

A number of dry preparations of ammonium sulphate precipitates from toxic filtrates of various organisms of the gas gangrene group, were tested by the cup-plate method for enzyme activity. Forty mg. of each toxin were dissolved in 1 ml. of saline, giving solutions containing many mouse lethal doses/ml. The toxic precipitates from *Cl. tetani*, *Cl. oedematiens* and *Cl. septicum* contained no enzyme which disintegrated hide powder, whereas those of *Cl. histolyticum* and *Cl. welchii* A produced clear wide zones in less than 24 hr. By titration it was found that the smallest concentration of toxin showing enzyme activity (M.E.D.) was 0.5 mg./ml. with the most potent *Cl. welchii* A preparation and 0.004 mg./ml. with the most potent *Cl. histolyticum* toxin.

Five different dry ammonium sulphate precipitates, prepared from *Cl. histolyticum* (I-V) in different institutes throughout the world, were titrated in parallel for enzyme activity and lethal power on intravenous injection in mice (Table 2). It is evident from the inconsistent ratios of enzyme activity to toxicity that the factors responsible for these two effects are not the same.

Table 2. *Hide-powder enzyme activity and toxicity of Clostridium histolyticum toxins*

<i>Histolyticum</i> toxin sample	Minimal enzyme concentration (mg./ml.)	Minimal lethal dose for mice (mg.)	Ratio M.E.D. : M.L.D.
I	1/256	1/32	8:1
II	1/16	1/32	1:2
III	1/128	1/8	16:1
IV	1/64	1/16	4:1
V	1/128	1/8	16:1

Neutralization of enzyme by antisera

The cup-plate method was also used in an attempt to investigate the antigenic relationships of the enzymes attacking hide powder produced by the different organisms. Three antisera were employed: (1) *Cl. welchii* A horse anti-

toxin Ex1055, (2) the International Standard *Cl. histolyticum* antitoxin reconstituted so that 280 units of antitoxin were contained in 1 ml.; this antiserum was also from a horse, and (3) *Cl. bifermentans* rabbit antiserum 2718 (Miles & Miles, 1947). Each serum was titrated for neutralization property with a number of homologous and heterologous enzyme preparations. Twofold dilutions of antiserum were made, and to each dilution an equal volume of the enzyme preparation was added. In most tests the mixtures were so constituted that the concentration of enzyme in each cup was eight times the smallest concentration which gave a reaction (8 M.E.D.). It was, however, necessary in some cases, where neutralization was only slight, to diminish the concentration of enzyme in the cup in order to obtain an end-point in the titration.

Table 3. *Neutralization of hide-powder enzyme by various antisera*

The figures indicate the reciprocal of the serum dilution, and the figures in brackets the number of minimal enzyme doses (M.E.D.) used in the titration.

Enzyme preparation		Neutralization titres of antisera		
		<i>welchii</i> (Ex 1055)	<i>histolyticum</i> (Int. St.)	<i>bifermentans</i> (2718)
<i>Cl. welchii</i> :	Toxin Welchpool	2048 (8)	32 (8)	< 2 (2)
	Culture Rosher	2048 (8)	32 (8)	< 2 (2)
<i>Cl. histolyticum</i> :	Toxin I	2 (4)	512 (8)	< 2 (2)
	Toxin II	2 (4)	1024 (8)	< 2 (2)
	Culture CN 920	2 (4)	512 (8)	< 2 (2)
	Culture CN 1693	2 (4)	512 (8)	< 2 (2)
<i>Cl. bifermentans</i> :	Culture M58e	64 (8)	32 (8)	128 (8)
	Culture M15h	32 (8)	16 (8)	64 (8)
<i>Cl. sporogenes</i> :	Culture M1f	32 (8)	8 (8)	2 (8)
	Culture M5f	32 (8)	8 (8)	4 (8)

Some evidence was obtained (Table 3) of an antigenic relationship between the enzymes of the different organisms. There were, however, inconsistencies in the results, such as the low titres of *welchii* antiserum with *histolyticum* antigens compared with the relatively higher titres of *histolyticum* antiserum with *welchii* antigens, and also the inability of the *bifermentans* antiserum to show cross-reactions with either *welchii* or *histolyticum* antigens, although both *welchii* and *histolyticum* antisera reacted with *bifermentans* enzyme. It was, of course, possible that the horse antisera contained antibodies to the lytic enzymes of a number of different organisms, naturally produced in the animals from which the sera were obtained. Nothing was known of the anti-enzymic properties of the sera of the animals before they were used for preparing the antisera, and there was evidence that the *histolyticum* serum came from a horse with some experience of *Cl. welchii* A antigens. More was known of the *bifermentans* antiserum since it was prepared in a rabbit which had received only *Cl. bifermentans* culture, and its greater specificity may have been a result of this, although, on the other hand, it may have been due to its low homologous titre. It is clear that if the antigenic relationship of the enzymes pro-

duced by the different organisms were to be investigated, it would be necessary to prepare antisera in selected animals whose sera before immunization were entirely free from antibodies to enzymes disintegrating hide powder.

DISCUSSION

From the results of this investigation it may be stated that the ability of various species of *Clostridium* to elaborate enzymes affecting hide powder is not related to the pathogenicity of the species, for both pathogenic and non-pathogenic organisms produce enzymes which disintegrate hide powder and, furthermore, a number of pathogenic species of *Clostridium* show no such enzyme activity. It is noteworthy, however, that of the pathogenic organisms examined, *Cl. welchii* A and *Cl. histolyticum*, both of which elaborate the lytic enzyme, produce lesions with extensive muscle destruction, whereas muscle disintegration does not occur in lesions caused by *Cl. tetani*, *Cl. oedematiens* and *Cl. septicum* which do not produce the enzyme.

There is clearly an association between enzyme production and virulence of strains of *Cl. welchii* A, but so far there is no evidence to suggest that the ability to produce enzymes disintegrating hide powder in any way determines virulence, which appears to depend mainly on the production of the lethal α -toxin (Evans, 1945*b*, 1947).

The part played by the antigens of *Cl. histolyticum* in infections produced by this organism has not been so fully investigated, but *Cl. histolyticum* produced in guinea-pigs a non-fatal infection with extensive muscle destruction. It is reasonable, therefore, to suppose that the enzyme disintegrating hide powder is associated with muscle destruction, and that when fatal infection occurs it is probably due to the lethal toxin of *Cl. histolyticum*.

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REFERENCES

- EVANS, D. G. (1945*a*). The *in vitro* production of α -toxin, θ haemolysin and hyaluronidase by strains of *Cl. welchii* type A, and the relationship of *in vitro* properties to virulence for guinea-pigs. *J. Path. Bact.* **57**, 75.
- EVANS, D. G. (1945*b*). Gas gangrene. *Lancet*, ii, 478.
- EVANS, D. G. (1947). Anticollagenase in immunity to *Cl. welchii* type A infection. *Brit. J. exp. Path.* **28**, 24.
- JENNISON, M. W. (1945). Bacterial collagenase. *J. Bact.* **50**, 369.
- MACFARLANE, R. G. & MACLENNAN, J. D. (1945). The toxæmia of gas gangrene. *Lancet*, **2**, 328.
- MASCHMANN, E. (1938). Die Anaerobiase der Gasbranderreger. *Biochem. Z.* **297**, 284.
- MILES, E. M. & MILES, A. A. (1947). The lecithinase of *Clostridium bifermentans* and its relation to the alpha-toxin of *Clostridium welchii*. *J. gen. Microbiol.* **1**, 385.
- OAKLEY, C. L., WARRACK, G. H. & VAN HEYNINGEN, W. E. (1946). The collagenase (κ toxin) of *Cl. welchii* type A. *J. Path. Bact.* **58**, 229.

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