SOME CULTURAL REACTIONS OF *CLOSTRIDIUM TETANI*

A. T. WILLIS AND KATHLEEN WILLIAMS

Public Health Laboratory, Lewsey Road, Luton

PLATES XXV-XXIX

THERE are conflicting views concerning the proteolytic and gelatinase activities of *Clostridium tetani*.

Some contemporary workers consider that the organism is non-proteolytic (Reed and Orr, 1941; Willis, 1964). Smith (1955), and Smith and Holdeman (1968), on the other hand, stated that "Coagulated serum or egg is softened and digested", and Breed, Murray and Smith (1957) recorded in Bergey's *Manual* that coagulated albumin is slowly liquefied. Wilson and Miles (1964) and Cruickshank (1965) also regarded the organism as proteolytic. The absence of any demonstrable proteolytic effect on milk medium was noted by Adamson (1919-20), Hall (1922), Reddish and Rettger (1924), Fildes (1925), Spray (1936) and Reed and Orr, and, though old cultures of *Cl. tetani* in cooked meat broth may show slight blackening, there is no clear evidence that this is due to digestion (Adamson and Cutler, 1917; Robertson, 1917; Adamson; Hall; Reddish and Rettger).

Reports in the literature suggest that any gelatinase activity of *Cl. tetani* is slow, late and variable (Adamson; Hall; Kendall, Day and Walker, 1922; Reddish and Rettger; Fildes; Walbum and Reymann, 1934; and Spray). Although Adamson stated that gelatin was completely liquefied in 3-4 days, Kendall *et al.* reported that it was not softened after 3 weeks' incubation. Hall reported that whilst some strains of *Cl. tetani* liquefied gelatin, others merely lowered its solidification point. Similarly, Fildes noted that "Although the tetanus bacillus leads to a liquefaction of gelatin . . . nevertheless this does not take place with equal facility with all strains. Whereas some will cause liquefaction in 3 or 4 days at 37°C, others will require 3 or 4 weeks or must be tested more than once".

In the present study of 71 strains of *Cl. tetani*, we have re-examined the proteolytic and gelatinase activities of the organism using a variety of complex protein and gelatin media. In the course of these investigations, a new and almost species-specific reaction of *Cl. tetani* in cultures on milk agar was noted, and incidental observations were made on the inhibitory effect of horse tetanus antitoxic sera on the swarming growth of the organism in surface culture. The 71 strains were also examined for deoxyribonuclease and phosphatase activity.

MATERIALS AND METHODS

Organisms

A total of 71 strains of *Cl. tetani* was examined, together with strains of *Cl. histolyticum* (NCTC7123), *Cl. sporogenes* (NCTC 275, 276, 532, 533), *Cl. welchii* type A (NCTC 8799, 9851, 10239, 10240), *Cl. septicum* (NCTC 281, 284, 547), *Cl. chauvoei* (NCTC8596), *Cl. oedematiens* type A (NCTC538), *Cl. oedematiens* type B (NCTC9691), *Cl. bifermentans* (NCTC8780), *Cl. botulinum* type A (NCTC7272), *Cl. botulinum* type B (NCTC7273),

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Cl. botulinum type C (NCTC 3732, 8264) and *Cl. tetanomorphum* (NCTC543). Organisms other than *Cl. tetani* were used for control and comparison purposes.

Of the 71 strains of *Cl. tetani*, 26 were isolates from soil samples collected in different parts of England and Wales, and 30 were strains isolated from new disposable bed-pans; these were kindly provided by Drs W. J. Ryan and J. H. Payne. The remaining 15 strains, kindly provided by Dr S. P. Lapage, were National Collection strains as follows: NCTC 2933, 2934, 2935, 2936, 2937, 2938, 5405, 5409, 9568, 9569, 9570, 9571, 9573, 9574 and 9575. Twenty-three of these 71 strains were non-toxicogenic.

Culture media and reagents

The nutrient base used in all the following media was heart infusion broth prepared from fat-free ox hearts. Unless otherwise stated, agar media contained 3.0 per cent. of Davis New Zealand agar. Sterilisation was at 121°C for 15 min. For satisfactory surface growth of exacting species such as *Cl. oedematiens* type B, cysteine hydrochloride (0.05 per cent.) was added to the medium (Moore, 1968).

Robertson's cooked meat medium, Loeffler's inspissated serum, fresh horse blood agar and heated blood agar were prepared as described by Collins (1967).

Glucose gelatin and gelatin agar (Frazier, 1926) were as described by Willis and Hobbs (1959).

Heated serum agar was as described by Willis and Hobbs, except that sucrose and neutral red were omitted from the medium, and horse serum was used in place of human serum.

Heated egg albumin agar was prepared in the same way as heated serum agar, egg albumin being substituted for horse serum.

Milk agar was as described by Reed and Orr.

Fibrin agar was prepared from human thrombin and fibrinogen (Lister Institute) as described by Jacobs, Willis and Goodburn (1964); fibrin agar with trypsin inhibitor was prepared in the same way, except that soy bean trypsin inhibitor (Worthington Biochemical Corporation) was added (Lack and Wailling, 1954).

Gelatin disks used were Charcoal Gelatin Discs (Oxoid).

Phenolphthalein phosphate agar was as described by Bray and King (1943).

Deoxyribonucleic acid (DNA) agar was as described by Jacobs *et al.*

Inoculation of media

The agar media were spot- or streak-inoculated in the usual way with a charged loop. For spot inocula, a 9-cm petri dish conveniently accommodated five cultures. The tubed media were inoculated with a pasteur pipette after dissolved oxygen had been driven off by steaming and cooling. Care was taken to inoculate the glucose gelatin while it was still fluid. All inoculated media were incubated at 37°C in the anaerobic jar. In all cases inocula came from fresh 18–24-hr cooked-meat broth cultures of the clostridia listed above, and, where appropriate, positive and negative controls were included with each batch of organisms examined. In some cases, "half-antitoxin" plates were employed, treated with commercial horse tetanus antitoxic serum* (Wellcome).

Reading of results

Glucose gelatin. Duplicate cultures were read after 2 and 4 days' incubation. Liquefaction was recorded if the medium remained fluid after half an hour in the cold room at 4°C.

Gelatin agar. Cultures were tested for gelatinase activity after 48 hours' incubation by flooding the plates with acid mercuric chloride solution (15 per cent. mercuric chloride in 20 per cent. hydrochloric acid). Gelatinase-producing cultures were surrounded by a zone of clearing in the medium.

* This rather clumsy phrase has to be used as it is extremely improbable that the effects of the serum recorded in this paper are due to tetanus antitoxin.

Charcoal gelatin disks. A charcoal gelatin disk was incubated in a cooked-meat broth culture of the organism under test, and was examined after 2 and 4 days' incubation. Gelatinase activity was indicated by release of charcoal particles into the medium.

Cooked-meat broth. Cultures were read at weekly intervals for 4 wk. Proteolysis was indicated by breakdown and discoloration of the meat particles.

Loeffler's inspissated serum. Heavily inoculated cultures in quadruplicate were read after 1, 2, 3 and 4 weeks' incubation. Proteolysis was indicated by the development of surface erosion or by disintegration or liquefaction of the medium.

Milk agar, heated serum agar, heated egg albumin agar and heated blood agar. Duplicate cultures were read after 2 and 4 days' incubation. Proteolysis was indicated by the development of zones of clearing or partial clearing about areas of growth.

Fibrin agar and fibrin agar with trypsin inhibitor. Duplicate cultures on each medium were read after 2 and 4 days' incubation. Proteolysis was indicated by the development of

TABLE I
Gelatinase activity of 71 strains of Cl. tetani determined by three different methods

Incubation period (hr)	Number (and percentage) of strains giving a positive result with		
	glucose gelatin	gelatin agar	charcoal gelatin disks
48	16 (22.5)	71 (100)	17 (23.9)
96	28 (39.4)	...	70 (98.5)

... = Not tested.

zones of clearing about areas of growth on both media; kinase activity was indicated by clearing on the plain fibrin agar plate only.

Phenolphthalein phosphate agar. Duplicate cultures on this medium were exposed to ammonia vapour and read for phosphatase activity after 2 and 4 days' incubation.

DNA agar. Cultures on plates were flooded with 1.5N-HCl after 48 hours' incubation; positive colonies showed an area of clearing around them.

RESULTS

Gelatinase and proteinase activity

The findings for the gelatinase and proteinase activity of the 71 strains of *Cl. tetani* examined are summarised in tables I and II.

Only 16 of the strains of *Cl. tetani* showed gelatinase activity in the conventional nutrient glucose gelatin medium after 48 hours' incubation, and only 17 showed activity in the charcoal gelatin disk test, at a time when all 71 strains gave clear positive reactions in the gelatin agar test of Frazier. After 4 days' incubation, when the experiment was stopped, only 28 strains were gelatinolytic as judged by the glucose gelatin test, but 70 were positive in the charcoal disk test. The single negative strain in the disk test was also negative in the conventional glucose gelatin. Although it seems likely that the number of positive strains in nutrient glucose gelatin would have increased with more extended incubation, 4 days seemed an adequate period if the test was to be regarded as

useful. In any event, the high sensitivity of the Frazier test (100 per cent. of strains positive after 48 hr), and of the charcoal gelatin disk test (99 per cent. positive after 96 hr), indicates that the nutrient glucose gelatin method should be abandoned.

Cultures of the 71 strains of *Cl. tetani* on the seven different complex protein media were uniformly non-proteolytic. A proportion of the strains produced sharp zones of clearing on fibrin agar, but because this activity was abolished by soy bean trypsin inhibitor, this fibrinolysis was considered to be due to a kinase and not to a true proteinase.

TABLE II
Proteolytic activity of 71 strains of Cl. tetani on seven different media

Incubation period (days)	Number of strains producing a positive result with							
	cooked-meat broth	inspissated serum	heated serum agar	heated egg albumin agar	heated blood agar	milk agar	fibrin agar	fibrin agar plus trypsin inhibitor
2	0	0	0	0	0	0*	25	0
4	0	0	0	0	0	0*	49	0
7	0	0	...	...	...	...	...	...
14	0	0	...	...	...	...	...	...
21	0	0	...	...	...	...	...	...
28	0	0	...	...	...	...	...	...

... = Not tested.

* = Intense diffuse opacity produced by all strains.

A rennin-like activity of Cl. tetani

In the course of investigating the proteolytic activity of *Cl. tetani* in cultures on milk agar, it was noted that all strains produced an intense diffuse opacity in the medium similar in appearance to that caused by lecithinase C-producing organisms on egg yolk agar. This reaction was evident only in cultures on firm (3 per cent. agar) milk plates, in which restriction of bacterial growth, and consequently of the opacity, permitted the detection of this change in the medium. From previous experience it was known that lactose-fermenting organisms, such as *Cl. welchii*, may produce this sort of appearance on milk agar by acid precipitation of the caseinogen (Willis, unpublished). It seemed unlikely, however, that this was the mechanism of opacity production by *Cl. tetani*, because this organism is in our hands entirely non-saccharolytic. The only other explanation that might account for the effect is the production by *Cl. tetani* of a rennin-like enzyme which would cause enzymatic clotting or precipitation of the milk as opposed to acid curdling (see Hall).

Effect of rennin and Cl. tetani culture supernates on milk agar. A 30 per cent. solution of rennin (Griffin and George) in sterile distilled water was spotted on duplicate plates of firm milk agar, and wells were punched in the medium with a cork borer and filled with the enzyme solution. Similar plates were prepared

using phenolised tetanus culture-supernatants instead of rennin solution. All plates were incubated aerobically and anaerobically at 37°C. After 24 hr the enzyme- and culture-supernatant-treated areas of both the aerobic and anaerobic plates showed zones of diffuse opacity identical in appearance to that produced by the growing culture of *Cl. tetani* on anaerobic milk agar (figs. 1 and 3).

Rennin-like activity in milk broth. Some strains of *Cl. tetani* cause simple clotting or precipitation in cultures in whole milk, a reaction that is doubtless due to their rennin-like activity. However, this reaction is inconstant from strain to strain, and varies even with the same strain when tested on different occasions. Moreover, the reaction, when it does occur, is always late to develop, and may require as long as 2–3 weeks' incubation (Hall; Reddish and Rettger; Spray; Reed, 1948). Among our own 71 strains tested in whole-milk medium, none produced any detectable change after 48 hours' incubation. After 7 days' incubation, three cultures showed simple coagulation of the milk, and after 14 days 52 cultures showed either a simple clot or partial precipitation of the casein; the remaining 19 cultures were negative.

In view of the constancy of the reaction of *Cl. tetani* and its speed of development in 10 per cent. milk agar, experiments were conducted with a nutrient broth medium containing concentrations of skimmed milk varying from 5 to 20 per cent. Fresh young cultures of *Cl. tetani* were inoculated into these media and incubated anaerobically for 18 hr at 37°C. Uninoculated control tubes were included, together with tubes of the medium inoculated with *Cl. sporogenes* (proteolytic), and with a few drops of 10 per cent. rennin solution.

All 71 strains of *Cl. tetani*, and tubes inoculated with rennin showed an identical result, which was best developed in 10 per cent. milk broth. There was complete precipitation of the milk, with a coarse white floccular deposit in a virtually clear supernatant fluid. The opalescent, semi-opaque appearance of the uninoculated medium was unaltered, whilst tubes containing *Cl. sporogenes* showed almost complete clearing of the medium with a little fine white bottom deposit consisting mainly of bacterial cells (fig. 2).

A variety of clostridia was then tested on milk agar, and in 10 per cent. milk broth for comparison with the reactions of *Cl. tetani*. These results are summarised in table III.

As might be expected, proteolytic species (*Cl. sporogenes*, *Cl. bifermentans*, *Cl. histolyticum* and *Cl. botulinum* types A and B) produced an early precipitate or opacity in the medium, followed by rapid digestion and clearing. Lactose-fermenting organisms, notably *Cl. welchii* and *Cl. septicum*, produced an early precipitate or opacity due to acid formation; in the fluid medium this was distinguished from the rennin reaction of *Cl. tetani* by the addition of a little phenol red indicator at the end of the incubation period. Because the strains of *Cl. oedematiens* types A and B produced reactions identical with those of *Cl. tetani*, it is concluded that these organisms also produce a rennin-like enzyme. As might be expected, the precipitate of caseinogen produced by lactose-fermenting organisms redissolved when the cultures were neutralised, in contrast with that due to rennin and to cultures of *Cl. tetani*, the development of which was favoured by an alkaline reaction and was optimal at pH 9.

Inhibition of the rennin-like activity of Cl. tetani by Cl. tetani antitoxic sera. The qualitative effect of tetanus antitoxic sera on the rennin-like activity of *Cl. tetani* was examined in two ways. (1) "Half-antitoxin" plate cultures were prepared on firm milk agar, two strains of *Cl. tetani* being accommodated on each plate. Duplicate plates were prepared in each case, commercial horse tetanus antitoxic serum (Wellcome) being used for one plate and horse *Cl. welchii* typing control serum (Wellcome) for the other. Plates were incubated anaerobically at 37°C for 24 hr. (2) Phenolised culture-supernatants prepared from 24-hr cultures of *Cl. tetani* in cooked-meat broth were pipetted into wells punched into plates of milk agar. Horse tetanus antitoxic serum and

TABLE III

Reactions of some clostridia in milk broth and milk agar during 48 hours' incubation

Organism (and number of strains)	Reaction during 48 hours' incubation in			
	milk agar		milk broth	
	Opacity—→Clearing		Precipitate—→Digestion	
<i>Cl. welchii</i> type A (4)	+	—	+	—
<i>Cl. chauvoei</i> (1)	—	—	—	—
<i>Cl. septicum</i> (3)	+	—	+	—
<i>Cl. botulinum</i> type A (1)	+	+	+	+
<i>Cl. botulinum</i> type B (1)	+	+	+	+
<i>Cl. botulinum</i> type C (2)	—	—	—	—
<i>Cl. oedematiens</i> type A (1)	+	—	+	—
<i>Cl. oedematiens</i> type B (1)	+	—	+	—
<i>Cl. bifermentans</i> (1)	+	+	+	+
<i>Cl. sporogenes</i> (4)	+	+	+	+
<i>Cl. histolyticum</i> (1)	+	+	+	+
<i>Cl. tetanomorphum</i> (1)	—	—	—	—
<i>Cl. tetani</i> (71)	+	—	+	—

Cl. welchii typing control serum were allowed to diffuse against the supernatants from adjacent wells, duplicate plates being incubated at 37°C under aerobic and anaerobic conditions.

In all the half-antitoxin milk agar plate cultures, the opalescence reaction of *Cl. tetani* was inhibited by tetanus antitoxic serum, but not by the *Cl. welchii* control serum. In both the aerobic and anaerobic punch-hole diffusion plates, the culture-supernatants of *Cl. tetani* produced an intense opalescence that was inhibited only by the tetanus antitoxic serum (figs. 3 and 4).

Effect of tetanus antitoxin on the growth of Cl. tetani in plate cultures

In the course of studying the inhibitory effect of *Cl. tetani* antitoxic serum on the rennin-like activity of *Cl. tetani*, the non-motile variant, Tulloch type VI (NCTC 9569), was cultured on half-antitoxin milk agar plates that contained only 1.5 per cent. of agar. It was thought that the lower concentration of agar

CULTURAL REACTIONS OF *CL. TETANI*

FIG. 1.—Acrobic milk agar punch plate, treated with *Cl. tetani* culture-supernatant (top hole) and rennin solution (bottom hole). $\times \frac{3}{4}$.

FIG. 2.—Cultures of *Cl. sporogenes* (centre) and *Cl. tetani* (right) in 10 per cent. milk broth. Un-inoculated control (left). $\times \frac{3}{4}$.

CULTURAL REACTIONS OF *CL. TETANI*

FIG. 3.—Culture of *Cl. tetani* on firm milk agar. Tetanus antitoxin serum on the left-hand half of the plate inhibits the diffuse rennin opacity. $\times \frac{3}{4}$.

FIG. 4.—Aerobic milk agar punch plate, treated with *Cl. tetani* culture-supernatants (top holes) and tetanus antitoxic serum (bottom holes). $\times \frac{3}{4}$.

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FIG. 5.—Edge of confluent growth of *Cl. tetani* type VI (non-motile) on the antitoxic serum-free half of a half-antitoxic serum (tetanus) fresh blood agar plate. Note the short fine rhizoidal outgrowths. $\times 18$.

FIG. 6.—Edge of confluent growth of *Cl. tetani* type VI (non-motile) on the antitoxic serum-treated half of a half-antitoxic serum (tetanus) fresh blood agar plate. Rhizoidal outgrowths are absent. $\times 22.5$.

CULTURAL REACTIONS OF *CL. TETANI*

FIG. 7.—Motile strain of *Cl. tetani* on half-antitoxic serum (tetanus) fresh blood agar. The fine spreading growth is inhibited by tetanus antitoxic serum on the left-hand half of the plate. Natural size.

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FIG. 8.—Discrete colony of a motile strain of *Cl. tetani* on fresh blood agar containing tetanus antitoxic serum to a concentration of 40 units tetanus antitoxin per ml. $\times 42.5$.

FIG. 9.—Motile daughter colonies of a motile strain of *Cl. tetani* on fresh blood agar containing tetanus antitoxic serum to a concentration of 40 units tetanus antitoxin per ml. $\times 42.5$.

might allow the development of wider and more photographable zones of opacity.

The most striking feature of these cultures, however, was complete abolition on the antitoxic serum half of the plates of the small rhizoidal outgrowths that are characteristic of surface colonies of these organisms (figs. 5 and 6). Because commercial tetanus antitoxic sera are prepared by the injection of filtrates of autolysates of *Cl. tetani* into the horse, the most likely explanation for this was that antibody to the vegetative organism, probably somatic antibody, was causing "agglutination" of the growing surface cultures. If this was so, then one would expect the horse tetanus antitoxic serum to have a similar restricting influence on the surface growth of motile strains of the organism.

Accordingly, our motile strains of *Cl. tetani* were cultured on "half-antitoxin" fresh horse blood agar plates containing 1.5 per cent. of agar. In every case there was complete inhibition of swarming growth on the "antitoxin" half of the plate. When examined under the plate microscope, colonies and areas of confluent growth of *Cl. tetani* on antitoxin-containing media showed a highly irregular edge, but the finely filamentous outgrowths that are characteristic of spreading cultures were absent. These were replaced by short coarse rhizoids of truncated appearance, which not infrequently gave the impression that they had been turned back upon themselves. In addition, some strains produced small motile daughter colonies, similar in appearance to those described by Turner and Eales (1941) (figs. 7-9).

This principle was applied to the separation of other anaerobes from mixtures with *Cl. tetani* in plate cultures. Mixtures of either *Cl. welchii* type A, *Cl. bifermentans*, *Cl. oedematiens* type A, *Cl. sporogenes*, *Cl. histolyticum* or *Cl. septicum* with *Cl. tetani* were tested on "half-antitoxin" plates of horse blood agar. In most cases it was a simple matter to pick off discrete colonies of the required organism from the "antitoxin" half of the plate; the untreated half of each plate showed a confluent growth of *Cl. tetani* overlying colonies of the other species. In other cases, however, there was not complete inhibition of swarming, probably due to uneven application of antibody. Incorporation of tetanus antitoxic serum in the plating medium at a concentration of 40-60 units of antitoxin per ml, however, has been uniformly successful in inhibiting the spreading growth of *Cl. tetani*, and the method is recommended, especially when *Cl. tetani* is a troublesome contaminant in mixtures with other exacting clostridia. Although we have not investigated the matter, media containing antitoxic serum may provide a useful alternative to firm agar when viable counts of *Cl. tetani* are required.

Phosphatase and DNase activities of Cl. tetani

None of the 71 strains of *Cl. tetani* showed evidence of phosphatase activity when tested by the method of Bray and King.

In the plate test for DNase activity, 40 of the 71 strains (56 per cent.) gave clear-cut positive results.

DISCUSSION

Apart from the neurotoxin (tetanospasmin) and the oxygen-labile haemolysin (tetanolysin), little close attention has been paid to the production by *Cl. tetani* of other enzymic substances. The relevant earlier reports on gelatinase and proteinase activities have been mentioned already. The organism is well known as non-lipolytic and as a non-lecithinase C-producer. Hall (1922) suggested that the late coagulation produced by some strains in whole-milk medium might be due to a rennin-like enzyme. In an investigation of fibrinolysin production by gas gangrene anaerobes, Reed, Orr and Brown (1943) failed to detect fibrinolytic activity amongst any of five toxigenic strains of *Cl. tetani*, whilst Bray and King (1943) reported that the two strains they tested on their phenolphthalein phosphate medium were phosphatase-negative. Several clostridia are known to produce deoxyribonucleases (Oakley and Warrack, 1951; Warrack, Bidwell and Oakley, 1951), but this enzyme has not been sought in cultures of *Cl. tetani*.

The method used for testing a growing organism for any particular property clearly may have a significant effect on the result of the test, and therefore on the property assigned to the organism. In general terms it may be said that the most practically useful methods are those that are simple to perform and produce an unequivocal end-point after a reasonably short incubation period. The advantages to be gained by the careful choice of method are well illustrated by our findings in the gelatinase tests, amongst which the gelatin agar plate method of Frazier was superior in every respect to the other methods examined, giving 100 per cent. clear-cut positive results in 48 hr.

For detection of bacterial proteinases in cultures in cooked-meat broth and inspissated serum, fairly overt proteolytic activity is required. On the other protein media used, however, and especially on heated serum agar, milk agar and fibrin agar with trypsin inhibitor, in which comparatively low concentrations of substrate are used, even minor degrees of proteinase activity produce rapid and conspicuous changes that rarely need subjective interpretation. Our failure to demonstrate proteinase production by any of the strains of *Cl. tetani* on any of the media employed leads to the conclusion that the organism is rightly regarded as non-proteolytic.

In contrast to the earlier findings of Reed *et al.*, more than half of our strains were fibrinolytic, an activity that is almost certainly due to a kinase rather than a proteinase, since it is inhibited by soy bean trypsin inhibitor. Because Reed and his colleagues examined only five strains of the organism, it is possible that they studied a random sample of genuinely non-fibrinolytic organisms. On the other hand, their method for the detection of fibrinolysis, in which the indicating effect is dissolution of a fibrin clot (Tillett and Garner, 1933), is less sensitive than the fibrin agar method of Jacobs *et al.* (1964).

Oakley and Warrack, and Warrack *et al.*, who were concerned with the quantitative determination of deoxyribonuclease activity of *Cl. welchii* and *Cl. septicum*, used a number of indicating effects; these included the change in staining properties of fixed films of rabbit leucocytes, viscometric measurements on solutions of sodium deoxyribonucleate, and the "ACRA" test. None of

these tests is particularly convenient for the all-or-none qualitative determination of deoxyribonuclease activity, although the rabbit leucocyte test was recently employed by Arseculeratne, Panabokké and Wijesundera (1969) as the indicator for DNase activity of *Cl. sordellii*. Much less troublesome and time-consuming is the DNA agar medium originally described by Jeffries, Holtman and Guse (1957). By this method, more than half of our strains gave clear-cut positive results. There was no apparent relation between the abilities of strains to produce tetanospasmin, DNase and fibrinolysin.

The earlier suggestion put forward by Hall that the coagulation of milk by some strains of *Cl. tetani* is due to a rennin-like enzyme has been confirmed. This part of the present investigation developed following the incidental observation that all of our strains of *Cl. tetani* produce zones of diffuse opacity in cultures on firm milk agar. Unless the strains are non-motile, the reaction cannot be observed on 1.5 per cent. agar because of confluent growth. This rennin-like activity of *Cl. tetani*, which was shown to be present in culture-supernatants, to be active under both aerobic and anaerobic conditions, and to be inhibited by tetanus antitoxic sera, was also easily detectable in 10 per cent. milk broth cultures, but much less conveniently and certainly in whole-milk cultures. Reactions indistinguishable from those of *Cl. tetani* are produced in both milk broth and milk agar by *Cl. oedematiens* types A and B, and are probably also due to a rennin-like enzyme. Similar reactions produced by lactose-fermenting organisms, and especially *Cl. welchii*, are due to acid, rather than to enzymatic, precipitation. This is easily demonstrated by adding a little indicator (phenol red) to a culture in milk broth after incubation to show the production of acid, or by neutralising the culture, when the precipitate redissolves.

Although whole-milk medium is widely used for the identification and classification of clostridia, its chief interest today is a historical one. All the information that can be obtained (often with difficulty and uncertainty) from cultures of clostridia in whole milk is more easily, more quickly and much more certainly obtained by other methods. Whole-milk cultures should be abandoned; definitive information about proteolysis, enzyme clotting and lactose fermentation is obtained by the use of 10 per cent. milk agar or milk broth, and a conventional lactose fermentation test.

The inhibitory effect of commercial horse tetanus antitoxic sera on the swarming growth of *Cl. tetani* is doubtless due to the presence of antibody to the vegetative cell. Although we have not followed the matter up experimentally, it seems probable that somatic antibody is the factor involved, since strains of *Cl. tetani* are known to possess a common O-antigen, but may have type-specific H-antigens. In any event, tetanus antitoxic sera incorporated into solid media at a concentration of 40–60 units of antitoxin per ml had no adverse effect on the growth of other clostridia tested, but prevented the swarming of *Cl. tetani* and allowed the development of discrete colonies. By this means we have been able to isolate other clostridia from mixtures with *Cl. tetani* without difficulty, both by selection of discrete colonies, and by allowing spreading species such as *Cl. septicum* to swarm from the restricted growth of *Cl. tetani*.

SUMMARY

Probably because *Cl. tetani* is a well-defined species whose recognition is rarely difficult, no recent attempts have been made to clarify the present uncertainties about its gelatinolytic and proteolytic activity. In the present study of 71 strains of the organism, all of them were shown to produce gelatinase, and none was proteolytic.

A proportion of the strains (49 of 71) produced a fibrinolytic enzyme, which behaved like a kinase.

A proportion of the strains (40 of 71) produced a deoxyribonuclease.

None of the strains produced phosphatase.

All 71 strains produced an intense diffuse opacity on milk agar, and complete precipitation of casein in milk broth due to the production of a rennin-like enzyme. This enzyme was present in culture-supernatants, was active under both aerobic and anaerobic conditions, and was specifically inhibited by tetanus antitoxic sera. With the exception of *Cl. oedematiens* types A and B, the reactions of all other clostridia tested on milk agar and in milk broth were dissimilar to those of *Cl. tetani*.

Commercial horse tetanus antitoxic serum inhibits the swarming growth of *Cl. tetani* in plate cultures. Advantage may be taken of this in the separation of other anaerobic organisms from mixtures with *Cl. tetani*.

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