A STUDY OF THE CARBOHYDRATE FERMENTATION REACTIONS OF *CLOSTRIDIUM OEDEMATIENS* (*CL. NOVYI*)

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THE currently accepted method of typing *Clostridium oedematiens* (*Cl. novyi*) is based on the detection of different permutations of the soluble antigens in culture products of the organism (Oakley, Warrack and Clarke, 1947). Oakley *et al.* concluded that the results of carbohydrate fermentation tests with the species are rather variable, but standard descriptions of *Cl. oedematiens* still include details of the fermentative properties of the organism (Breed, Murray and Smith, 1957; Willis, 1964). During an intensive investigation of the *Cl. oedematiens* group (Rutter, 1968), strains were isolated from pathological material that gave equivocal results in the serological typing procedure. When I attempted to characterise these strains it became clear that the accepted carbohydrate fermentation test procedures are inadequate for this demanding anaerobe, and it is possible that some of the inconsistencies in previously reported results are attributable to technical difficulties. It was therefore necessary to develop a more reliable testing procedure in order to assess the significance of fermentation reactions in the identification of strains that do not fit readily into the present serological scheme of classification.

MATERIALS AND METHODS

Strains. Twenty-six strains of *Clostridium oedematiens* were used in the present study; their origin has been described previously (Rutter and Collee, 1969). All of the strains were lyophilised as soon as their purity was confirmed; thereafter they were subcultured in cooked-meat broth. Frequent checks of purity were made on aerobic and anaerobic human blood agar plates, and each strain was identified periodically with the immunofluorescent staining procedure, and in haemolytic and lecithovitellin neutralisation tests with appropriate antisera.

Anaerobic jar procedure. Anaerobic jars with a room-temperature catalyst (Baird and Tatlock Ltd, Chadwell Heath, Essex) were employed. Culture media were incubated as a routine at 37°C with 10 per cent. of carbon dioxide added to the anaerobic hydrogen atmosphere.

Culture media. Cooked-meat broth was used as a routine in most of these studies; nutrient broth (Oxoid Ltd, Southwark Bridge Road, London, S.E.1) was used in the preparation of this medium, but it is important to note that the meat particles were prepared according to Cruickshank (1968, p. 757). Peptone-water base and sugar medium 2 were prepared as in Cruickshank (p. 815), and the Brewer medium was as described by Reed and Orr (1941). Carbohydrate solutions were prepared as 10 per cent. solutions and were sterilised by tyndallisation; an appropriate volume of each solution was added to the

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sterile basal medium to give a final concentration of 1 per cent. of the added substrate. The carbohydrates that were tested included dextrin, dulcitol, D-fructose, D-galactose, D-glucose, glycerol, meso-inositol, lactose, maltose, mannitol, D-mannose, salicin, sorbitol, sucrose and D-xylose. All of these carbohydrate preparations were obtained from B.D.H. Ltd, Poole, Dorset.

The fermentation test procedure. In the preliminary tests, 100-ml volumes of cooked-meat broth were used so that sufficient culture was available for several *p*H estimations during prolonged incubation of the tests. Subsequent tests were performed in 14-ml volumes of medium in $6\times\frac{5}{8}$ in. ($15{\cdot}2\times1{\cdot}6$ cm) tubes that contained meat particles to a depth of 1–1¼ in. (2·5–3·2 cm); tube tests gave satisfactory results and are more easily handled. An overnight culture of each strain in cooked-meat broth provided the inoculum; 1 ml of culture was used to seed 100-ml volumes of the fermentation test medium and a 0·1-ml inoculum was used for tube tests. The *p*H values were determined in the following manner: a sample was withdrawn from each culture and the bacterial cells were deposited by centrifugation at 1200*g* in a bench centrifuge (M.S.E. Equipment Co., Manor Royal, Crawley, Sussex); the *p*H of the clear supernatant was then measured with a Pye Dynacap *p*H meter (W. G. Pye & Co. Ltd, Cambridge, England) that had an automatic temperature-compensating device and a Pye Ingold combined glass and reference electrode. The *p*H of the culture-supernatants was determined after incubation of the test medium for 1, 2, 5 and 7 days; it was found that tests read at 2 and 5 days gave the most useful results.

RESULTS

Factors that influence the choice of basal medium for carbohydrate fermentation tests

In tests of carbohydrate fermentation reactions, a basal medium that supports consistent and adequate growth of the organism is necessary. *Cl. oedematiens* did not grow in a simple peptone-water medium, and efforts to provide a suitable physicochemical environment in the medium by adding reduced iron in the form of an iron nail (Hayward and Miles, 1943) were unsuccessful. Attempts to grow the organism in a more nutritious basal medium (sugar medium 2, Cruickshank, p. 815) also gave disappointing results. A semi-solid Brewer medium (Reed and Orr) often supported satisfactory growth of *Cl. oedematiens* provided that the cultures were incubated anaerobically, but frequent failures of growth occurred when fresh isolates of type-B strains were tested. It became clear from these results that a more reliable basal medium was needed. Preliminary experiments had shown that *Cl. oedematiens* grows consistently well in $6\times\frac{5}{8}$ in. ($15{\cdot}2\times1{\cdot}6$ cm) tubes of fluid media that contain meat particles, and a cooked-meat broth medium (CMB) was, therefore, tried as the basal medium in sugar fermentation tests. The results of initial tests confirmed that CMB supports reliable growth of all strains of *Cl. oedematiens*, but certain features of the culture medium necessitated a reappraisal of the fermentation test procedure. For example, gas is produced in ordinary CMB cultures of *Cl. oedematiens*, and the production of gas in cultures with added carbohydrate substrates was not considered significant. In addition, problems associated with the use of indicator dyes (see *Discussion*) suggested that an alternative method of assessing acid production was necessary; accordingly, the *p*H of culture-supernatants was measured with a *p*H meter. The test procedure that was developed is described in another section (see *Methods*).

Carbohydrate fermentation tests with Cl. oedematiens in cooked-meat broth medium

Tests were performed with six type-A strains, ten stock type-B strains and six freshly isolated strains that were provisionally identified as type-B strains

TABLE I

The results of carbohydrate fermentation tests with strains of each type of Cl. oedematiens in a basal medium of cooked-meat broth (see text)

Type (and number of strains tested)	Result of test	Number of strains of *Cl. oedematiens* giving the stated reaction* in relation to number tested in fermentation tests with added						
		dextrin	glucose	glycerol	inositol	fructose	maltose	mannose
A (6)	+	6/6	6/6	5/6	...	...	6/6	2/6
	⊥	...†	...	1/6	...	...	...	2/6
	−	...	...	...	6/6	6/6	...	2/6
B (10 stock)	+	...	10/10	...	10/10	6/10	10/10	10/10
	⊥	...	...	...	...	4/10	...	...
	−	10/10	...	10/10	...	...	...	...
B (6 freshly isolated)	+	...	6/6	1/6	6/6	4/6	6/6	6/6
	⊥	...	...	4/6	...	2/6	...	...
	−	6/6	...	1/6	...	...	...	...
C (1)	+	...	1/1	...	1/1	...	...	1/1
	⊥	...	...	...	...	1/1	...	...
	−	1/1	...	1/1	...	...	1/1	...
D (3)	+	...	3/3	...	3/3	2/3	...	3/3
	⊥	...	...	...	...	1/3	...	...
	−	3/3	...	3/3	...	...	3/3	...

* + = Positive test, *p*H fall >0·6 unit compared with substrate-free control; ⊥ = doubtful positive, *p*H fall 0·3–0·6 unit; − = negative test, *p*H fall <0·3 unit.
† ... = None.

(q.v.), one type-C strain and three type-D strains (*Cl. haemolyticum*) of *Cl. oedematiens*. Carbohydrate substrates representing monosaccharides, disaccharides, polysaccharides, sugar alcohols and glycosides were sterilised separately and added to the basal medium to give a final concentration of 1 per cent. of added fermentable substrate. Initially, 100-ml amounts of fermentation test medium were used for testing each strain with a particular substrate, but in later experiments tubes that contained about 14 ml of medium gave satisfactory results. Control tests in each experiment included sterile culture media, and cultures of *Cl. oedematiens* in CMB without added carbohydrate.

The results of the control tests showed that a fall in *p*H of 0·1–0·4 unit occurs in uninoculated media after anaerobic incubation; this may be related to the absorption of carbon dioxide by the medium. In CMB cultures of

Cl. oedematiens without added carbohydrate, the *p*H fell by 0·1–0·7 unit, and it was clear that fermentable material was present in the basal medium. In tests with added carbohydrates, the *p*H usually fell by a further 1·0 unit when the added substrate was fermented; this frequently occurred within 48 hr, but occasionally it was necessary to incubate the cultures for up to 5 days. A definite positive or negative result was obtained in most of the fermentation tests, but certain strains gave " doubtful positive " results, particularly with glycerol and fructose (see table I).

The results of fermentation tests with strains of each type of *Cl. oedematiens* showed that the organism does not ferment dulcitol, galactose, lactose, mannitol,

TABLE II

*Summary of the carbohydrate fermentation reactions of Cl. oedematiens**

Cl. oedematiens type	Fermentation test result † with						
	dextrin	glucose	glycerol	inositol	fructose	maltose	mannose
A	+	+	+ or ⊥	−	−	+	V
B	−	+	V	+	+ or ⊥	+	+
C	−	+	−	+	⊥	−	+
D	−	+	−	+	+ or ⊥	−	+

* = Negative results were obtained for each type with dulcitol, galactose, lactose, mannitol, salicin, sorbitol, sucrose and xylose (see text).
† + = Positive result; ⊥ = doubtful positive; − = negative result; V = variable result (see table I).

salicin, sorbitol, sucrose or xylose; the fermentation reactions with seven other carbohydrates are summarised in table I. All of the strains fermented glucose. In addition, type-A strains fermented dextrin and maltose; five of the type-A strains fermented glycerol, but the results with mannose were variable. None of the type-A strains fermented inositol or fructose. All of the type-B strains fermented inositol, maltose and mannose; none of these strains fermented dextrin, and " doubtful positive " results were frequently obtained with glycerol and fructose. The type-D strains fermented inositol and mannose; none of these strains fermented dextrin, maltose or glycerol, and two of the strains fermented fructose. One type-C strain was tested and fermented glucose, mannose and inositol. The characteristic fermentation reactions of each type are given in table II.

The characterisation of fresh isolates

The results of fermentation tests with six freshly isolated strains of *Cl. oedematiens* are included in table I; these strains produced a low level of β-antigen in haemolysin tests with human red cells as the substrate (Rutter and Collee) and were provisionally identified as type-B strains. Subsequently,

α-antigen was detected in mouse lethality tests with culture-supernatants of only four of the strains, and the remaining two strains may be regarded either as type-B strains that do not produce lethal amounts of the α-antigen, or as type-D strains that produce a low level of β-antigen. In an attempt to characterise these strains further, their carbohydrate fermentation reactions were investigated. The results of fermentation tests with type-B and type-D strains are similar (see table II), but it is clear that type-B strains consistently ferment maltose; both of the " doubtful " strains described above rapidly fermented maltose and are therefore presumptive type-B strains. Thus it appears that type-B strains exist that do not produce sufficient α-antigen for detection in the mouse lethality test system; the fermentation of maltose may be of value in the identification of these strains.

Discussion

The present study provides a good example of a problem that is frequently encountered in anaerobic bacteriology. Carbohydrate fermentation tests often aid the identification of a fresh isolate, but procedures that ensure the growth of a demanding organism in these tests are usually unspecified. Consequently, the fermentation characteristics of fastidious anaerobic bacteria are regarded as unreliable. The limitations of the currently accepted basal media in fermentation tests with *Clostridium oedematiens* are well demonstrated in the present investigation; " false negative " results are obtained if the organism does not grow in the basal medium, and practical problems associated with *Cl. oedematiens* are well recognised in this respect (Oakley *et al.*, 1947). In order to obtain reliable growth of *Cl. oedematiens* in tubes of fluid media it was necessary to incorporate meat particles in the medium (Rutter, 1968), and the fermentation test medium used in the present study consisted of nutrient broth and freshly prepared meat particles. Commercial preparations of cooked-meat broth are not satisfactory as they do not support reliable growth of *Cl. oedematiens*.

A fermentation test medium that incorporates meat particles was recommended for Gram-negative anaerobes by Khairat (1964), who showed that the constituents of the basal medium that he described are not fermented by *Escherichia coli*. This approach did not seem to be justified in the present study, because considerable differences may exist in the metabolic pathways of *E. coli* and *Cl. oedematiens*; tests in cooked-meat broth (CMB) without added substrates were therefore used as controls. Khairat included an indicator dye and a type of Durham tube in his fermentation tests to detect acid and gas production from added fermentable substrate. Gas production was not considered significant in the present investigation because *Cl. oedematiens* forms gas in the basal CMB medium. Indicator dyes were not incorporated in CMB because many problems are associated with their use; these include (i) the confusing *p*H changes that frequently occur in fermentation media after autoclaving, and during anaerobic incubation of uninoculated media, (ii) the " bleaching " of certain indicators during anaerobic bacterial growth, and (iii) the natural colour of the CMB fermentation test medium. Acid production was therefore detected by measuring the *p*H of culture supernatants with a *p*H meter. The results

obtained with this procedure confirm that the *p*H of uninoculated media falls during anaerobic incubation, and this effect may be partly related to the absorption of carbon dioxide by the medium. It may be argued that the *p*H meter procedure is impracticable as a routine, but it resolved many equivocal results associated with indicator dyes.

Although the present fermentation test procedure employs a basal medium that supports reliable growth of *Cl. oedematiens*, delayed fermentation reactions occasionally occur, and the results obtained after prolonged incubation are not always definitive. Delayed reactions may be related to a diauxie effect in which the strains preferentially utilise substrates in the basal medium before breaking down the added substrate; alternatively, prolonged incubation may allow the development and expression of a mutant population that ferments the added carbohydrate. It is therefore necessary to ensure that fermentation tests are performed in a volume of medium that supports an adequate bacterial population. In some tests, the *p*H of cultures fell by 0·3–0·6 unit, and such results were regarded as doubtful positives. These results are not attributable to inadequate growth in the basal medium; they may be related to a partial breakdown of the added substrate, and utilisation of the intermediate products in reactions with other fermentable materials. In the present study the fermentable material in CMB basal medium appeared to vary with the strain and with different batches of the medium. The use of CMB as the basal medium for fermentation tests should therefore be regarded as a compromise; recent observations on the culture of *Cl. oedematiens* (Moore, 1968) may allow the development of a more simple fluid medium for use in biochemical tests.

There is now good evidence that poorly toxigenic strains of *Cl. oedematiens* exist (Smith, Claus and Matsuoka, 1956; Corbould, 1966), and a system of typing that is based solely on the detection of soluble antigens may founder when strains are encountered that produce inadequate amounts of the key antigens. Two of the freshly isolated strains in the present study provided a good illustration of this problem. Tests that involve other characters would be helpful, and reliable biochemical tests would be useful additions to the identification procedure. The present fermentation technique was used successfully in this laboratory with other demanding anaerobes that do not grow readily in fermentation tests with peptone water as the basal medium; thus, the procedure may have wider applications in anaerobic bacteriology. Within the limits of the present investigation, strains of *Cl. oedematiens* classified on the basis of serological neutralisation tests have characteristic fermentation patterns with dextrin, inositol and maltose that may be of value when problems of taxonomy arise. The type-C strain is not differentiated from type-D strains on this basis, and more information regarding this so-called " non-toxigenic " category is clearly required.

Summary

The carbohydrate fermentation reactions of *Clostridium oedematiens* were critically assessed with particular reference to problems that are encountered in the test procedure. Frequent failures of growth occurred in currently accepted

basal media, and a test procedure was developed with a medium that supports consistent growth of this demanding anaerobe. The medium consists of nutrient broth and freshly prepared meat particles; acid production was measured with a *p*H meter. The results of carbohydrate fermentation tests with 26 strains of *Cl. oedematiens* representing at least one strain of each type suggest that the fermentation patterns may be of value when strains are encountered that do not fit readily into the present serological scheme of classification. The advantages of the fermentation test procedure developed in the present study are discussed in relation to the disadvantages of other fermentation test systems for anaerobic organisms.

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REFERENCES

BREED, R. S., MURRAY, E. G. D., AND SMITH, N. R.	1957.	Bergey's Manual of determinative bacteriology, 7th ed., *London*, p. 651.
CORBOULD, A.	1966.	*Vet. Rec.*, **79**, 861.
CRUICKSHANK, R.	1968.	Medical microbiology, 11th ed., *Edinburgh* and *London*.
HAYWARD, NANCY J., AND MILES, A. A.	1943.	*Lancet*, **1**, 645.
KHAIRAT, O.	1964.	*Canad. J. Microbiol.*, **10**, 513.
MOORE, W. B.	1968.	*J. Gen. Microbiol.*, **53**, 415.
OAKLEY, C. L., WARRACK, G. HARRIET, AND CLARKE, PATRICIA H.	1947.	*Ibid.*, **1**, 91.
REED, G. B., AND ORR, J. H.	1941.	*War Med., Chicago*, **1**, 493.
RUTTER, J. M.	1968.	Ph.D. Thesis, Univ. Edinburgh.
RUTTER, J. M., AND COLLEE, J. G. . .	1969.	*J. Med. Microbiol.*, **2**, 395.
SMITH, L. DS., CLAUS, K. D., AND MATSUOKA, T.	1956.	*J. Bact.*, **72**, 809.
WILLIS, A. T.	1964.	Anaerobic bacteriology in clinical medicine, 2nd ed., *London*, p. 81.