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THE SOMATIC ANTIGENS OF THE *CL. WELCHII* GROUP OF ORGANISMS

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INTRODUCTION

THE object of the work described in the present paper has been to examine the affinities of the somatic antigens of a group of organisms closely related in morphological and biological characteristics to the classical *Bacillus aerogenes capsulatus* (*Cl. welchii*) of Welch & Nuttall (1892). The summary, which is given below, of the information leading to the present taxonomy of these organisms shows the position in which the various types stand in relation to one another.

Qualitative differences in the toxic metabolites of the *Cl. welchii* group have been demonstrated by Dalling and his colleagues (1931, 1938), Wilsdon (1931), Glenny and his colleagues (1933), Mason (1935) and others. At the present time at least four types of these organisms (Wilsdon's A, B, C, D) are recognized according to the quality and number of the toxins they have been shown to elaborate *in vitro*. Each type had already been raised to the status of a separate species characterized by the power to produce one or more toxins (Glenny's α, β, δ, ϵ, etc.); each toxin exhibiting a high degree of antigenic specificity. The species at present recognized are as follows:

Species	Type (Wilsdon)	Toxins produced (Glenny)
Cl. welchii (Classical; Welch & Nuttall)	A	α
Bacillus agni (L.D. *bacillus*; Dalling)	B	α, β, ϵ (traces γ, δ)*
Bacillus paludis (McEwen)	C	α, β, δ (traces γ)
Bacillus ovitoxicus (Bennetts)	D	α, ϵ

* The γ toxin is apparently a minor component.

Raising the status of these types to the rank of species may be helpful at the present time, but it is questionable whether the physiological property of toxin production will prove a sufficiently constant feature to be of value in the taxonomy of the group as a whole. The inherent practical difficulties involved in the production of toxin may be alone sufficient to make typing on this basis unreliable. Further, there is already evidence to suggest that variation of strains within each type is a frequent occurrence. Thus certain strains may become apparently completely atoxic or they may lose the power to produce one or other of the toxins (Dalling, 1931; Mason, 1935) whereas others within type A probably produce several specific toxic substances (Prigge, 1936, 1937; Stewart & Clampit, 1938; Weinberg, 1938; Ipsen & Davoli, 1939).[1]

Attempts to classify members of this group of organisms on a basis of the specific affinities of their bacterial antigens have met with little or no success. It must be emphasized, however, that most of the attempts have probably been carried out with classical type A strains. The early work in this field is briefly reviewed in the Medical Research Council Report (1919) and the conclusion is drawn that the agglutination test is of no value in the diagnosis of *Cl. welchii*. Howard (1928) examined the cross-agglutination reactions of sixty strains of *Cl. welchii* with fifteen immune sera prepared in the rabbit. She found only one

[1] Glenny's (1933) terminology for toxins has been used throughout the text. It should be clearly understood, therefore, that the α toxin here described may in many instances include several specific toxic components.

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strain that failed to be agglutinated by any of the sera and four others that were agglutinated only by the homologous serum; the remainder showed a widely variable degree of cross-agglutination. Wilsdon (1931) tested twenty-three strains representative of his toxigenic types A, B, C, D in cross-agglutination with eleven sera prepared in the rabbit by immunizing with selected strains. He found that strains which proved to be closely related in their toxin-antitoxin reactions showed little in common as regards their agglutination reaction and his conclusion was that there is little likelihood of forming a satisfactory classification of the group on the basis of the agglutination reaction. Henriksen (1937) examined the agglutination and complement-fixation reaction of twenty-five strains of *Cl. welchii* isolated from human faeces. Six of the strains were used to prepare immune sera in the rabbit and the immune body in each instance appeared to be strictly strain specific. An interesting feature of the work was the apparent diversity of serological types that might be obtained from the same sample of faeces. Meisel (1938) states that strains of *Cl. welchii* which show slight morphological and biochemical differences fall into different serological groups. He obtained the same grouping in complement-fixation tests using as antigen a polysaccharide fraction obtained from the organisms by an alkaline extraction process. Kreuzer (1939) examined strains representative of Wilsdon's types A, B, C, D and found that each type possessed a specific thermostable "O" antigen. The number of strains of each type examined is not given and heterogeneity of the bacterial antigens within the type is not considered.

Cl. welchii frequently exhibits wide variation in the morphology of the colony and the cell. The work on this subject which has been reviewed by Weinberg (1936) and Henriksen (1937) shows that little attention has been given to the serology of the bacterial antigens occurring in the numerous stable variants that have been described. McGaughey (1933) and Henriksen (1937), however, have examined in some detail the serological behaviour of so-called smooth types and rough variants obtained therefrom and in certain respects their results are contradictory. Thus McGaughey found that the agglutinogens of two stable rough variants which appeared as spontaneous dissociants from the smooth form were serologically specific. Henriksen, on the other hand, failed to observe a serological change in spontaneously-occurring rough variants but by cultivating the smooth forms in homologous immune serum he obtained variants with a different antigenic structure.

The experiments described in the following pages are concerned mainly with an antigenic analysis of Wilson's types B, C, D of *Cl. welchii* but for comparative purposes certain strains of the classical type have also been examined. Special attention is given to the possibility of more than one somatic antigen conferring specificity on a particular type or strain. A heat-stable O antigen similar to that first described by Felix & Robertson (1928) as occurring in certain species of anaerobes is present in all of the strains now examined. In addition to this antigen a heat-labile somatic component has been found in certain strains. Throughout the text this labile antigen and the corresponding immune body are designated by the letter "L". The research has been carried on intermittently over a period of five years during which time the influence of bacterial variation on the serological behaviour of particular strains has also been recorded.

Methods and materials

The strains examined. Wilsdon's terminology as recorded above is used throughout the text. Strain histories are given under the appropriate headings. Each strain complied with the accepted cultural and biochemical activities of the group and the purity of the culture was regularly checked by recourse to plating on 1% Difco proteose peptone meat infusion

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agar containing 0·5% glucose. Except where otherwise stated the colonial forms selected in each plating as typical *Cl. welchii* complied with the text-book description as "low convex, amorphous, with smooth surface and entire edge". There was, however, considerable variation in the density of the colony which in some instances was completely opaque and in others was translucent and openly granular. So-called "mucoid" variants were not infrequent and the rough forms which appeared in certain strains were isolated for further study.

Antigens for in vitro *tests.* The organisms were grown under atmospheric conditions at 37° C. for 18 hr. in a deep flask containing tryptic digest broth with 1% glucose. Growth deposits, obtained by centrifuging, were washed twice in a large volume of distilled water. The organisms so obtained were resuspended in distilled water to opacity 10 × 5 on Brown's scale and then differently treated according to requirements. For *in vitro* tests the organisms were used directly as "L" suspensions or alternatively after steaming at 100° C. for 2½ hr. as "O" suspensions.

Preparation of immune rabbit sera. Two types of immune serum were prepared. One (O serum) by injecting intravenously the O suspension described above, the other (L serum) by injecting similarly the "L" suspension to which 0·4% of formalin solution had been added and then stood for 48 hr. at room temperature before use. No difficulty was experienced in getting a satisfactory agglutinin response after six to eight injections of either type of antigen given at intervals of 2–4 days. The animals were bled from the jugular vein before immunization and again 7 days after the last injection of antigen. Occasionally a second and third course was given.

The agglutination test. The method of test is described here in some detail because it is believed that much of the discordance between the reports of earlier workers may be attributed to the widely variable methods they employed. The test proper is carried out in tubes approximately 1·25 cm. in diameter and 5 cm. in length (narrow tubes of the Dreyer pattern make the reading of an "end-point" extremely difficult). The serum in each tube is adjusted to a total volume of 1·0 c.c. with 0·42 or 0·85% salt solution and the antigen is of such a density that the addition of one drop gives the necessary opacity. In all tests normal serum and NaCl controls are included. The tubes are incubated at 43–45° C. for 4 hr. when a preliminary reading is taken; the final reading is made after 24 hr. at room temperature. With a few exceptions the stability of the L and O suspensions is completely satisfactory although the sensitivity to immune-body of L suspensions prepared from the same strain appears to vary from time to time. In satisfactory tests, after standing 24 hr., normal serum and NaCl control tubes have a clearly defined "ring" deposit with an entire margin and on gentle shaking this deposit is readily and evenly dispersed; suspensions that are less stable either show a deposit with a "fluffy" margin or there is no evidence of the "ring". Positive agglutination with O suspensions in low dilutions of serum is unmistakable and typical of the "granular" type; frequently in higher dilutions a negative result will be recorded unless the tubes are shaken gently and the deposit disturbed when, in fact, a strongly positive reading may be obtained. Agglutination with the L suspension is also of the granular type but the clumps are much less compact and are readily dispersed. Frequently also L suspension in the presence of pure O serum gives a lower reading of O agglutinin content than is obtained with an O suspension.

Results of experiments

Special attention has been given to the complex *Cl. welchii* types B, C and D of Wilson and representative strains which had been fully examined for toxin production were kindly supplied by Prof. Dalling and Miss H. E. Ross. The type A strains were obtained from the collection of my colleague Dr

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Muriel Robertson. Their capacity for toxin production varied considerably but all of them elaborated a measurable amount of α toxin.

In the analysis of the antigens of these strains the production of agglutinins in rabbits, direct and cross agglutination and absorption of agglutinins were considered. The stability in salt solution of suspensions prepared from certain strains varied from time to time. A few were regularly unsatisfactory and their serological behaviour was examined by testing with heterologous strains the antibody they produced in rabbits as well as by their ability to absorb agglutinins from heterologous sera.

Examination of type B strains (L.D. *bacillus*). Fourteen strains were selected, all isolated from the intestines of lambs suffering from dysentery-like diseases. Four were from Scotland, five from England, four from Wales and one from South Africa (this latter strain was a culture from a single cell prepared by Dr J. H. Mason, Onderstepoort). Thirteen of these strains produced Glenny's α, β and ϵ toxins. The other, which had appeared as a spontaneous variant of one of the strains isolated in Scotland, had apparently lost the power of elaborating ϵ toxin. L and O sera were prepared as described above against five representative strains including the aberrant type just mentioned and the South African strain.

Complete identity of the heat-stable O antigen was established for thirteen of the fourteen strains. The South African culture appeared to possess a strictly specific O antigen. It failed to absorb O antibody from any of the heterologous sera and the O antibody to this strain failed to give more than a trace of agglutination with heterologous strains. These findings are recorded in Tables 1 and 2, which show respectively the results of a cross O agglutination test and a cross O absorption test with representative strains and sera.

Table 1. *Type B. Cross O agglutination test*

Rabbit O immune serum prepared from strains	Titre of O agglutination with strains: Scotland		England		Wales		South Africa
	1	2	3	4	5	6	7
1	1000	500	1000	1000	1000	500	0
2	500	500	1000	500	500	500	0
3	2000	1000	2000	2000	1000	2000	0
5	1000	1000	500	1000	1000	1000	0
7	0	0	0	0	0	0	1000
Normal rabbit serum	0	0	0	0	0	0	0
0·42% NaCl	0	0	0	0	0	0	0

Titre 0 = a negative result in a dilution 1 : 50.

An examination of the L sera prepared by immunizing with the formolized L suspension revealed the presence in most of the strains of a relatively labile somatic antigen which was serologically distinct from the O antigen. In the first place it was observed that the agglutinin titre of an L serum in the presence of L antigen was generally 10–20 times greater than that obtained with heated O antigen. If the O antibody was removed from such a serum by

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Table 2. *Type B. Cross-absorption of O agglutinins*

Agglutination with strain		O serum (Scotland 1) absorbed in dilution 1 : 50 with strains								Unabsorbed serum (Scotland 1)
		Scotland			England		Wales		South Africa	
		1		2	3	4	5	6	7	
Scotland I	Absorbing opacity	10	10 × 5	10 × 5	10 × 5	10 × 5	10 × 5	10 × 5	10 × 5	—
	Titre of agglutination	100	0	0	0	0	0	50	500	1000

Agglutination with strain		O serum (South Africa) absorbed in dilution 1 : 50 with strains								Unabsorbed serum (South Africa 7)
		South Africa		Scotland		England		Wales		
		7		1	2	3	4	5	6	
South Africa 7	Absorbing opacity	10	10 × 5	10 × 5	10 × 5	10 × 5	10 × 5	10 × 5	10 × 5	—
	Titre of agglutination	50	0	1000	500	1000	1000	1000	500	1000

Titre 0 = a negative result in a dilution 1 : 50.

Suspensions for absorption twice washed in distilled water; heated for 2½ hr. in distilled water (opacity 10 × 10), diluted as required and then centrifuged. The serum dilution in 0·42 % NaCl added to the centrifuged deposits using 1 c.c. serum dilution per 1 c.c. original suspension; incubated 2 hr. 37° C. and centrifuged.

absorption with O suspension, then the agglutinin titre with L antigen remained unaltered. On the other hand, L antigen removed the O antibody and reduced the L agglutinin titre virtually to zero. It was also observed that sera prepared by immunizing with O antigen failed to develop any trace of this L agglutinin. Table 3 shows the results of an absorption test designed to demonstrate these points. Portions of one O and one L serum have been absorbed respectively with O and L antigen and subsequently tested for the presence of the two types of agglutinin.

Table 3. *Type B. Agglutinin absorption test illustrating the presence of L antigen*

Serum: Type	Serum: Absorbed in dilution 1 : 50 with homologous suspension	Agglutination with homologous suspension	Serum dilution: 50	100	200	500	1000	2000	5000	10,000	20,000
"L"	O	O	−	−	−	−	−	−	−	−	−
		L	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	±
	L	O	−	−	−	−	−	−	−	−	−
		L	+ +	+	−	−	−	−	−	−	−
	Unabsorbed	O	+ + +	+ + +	+ + +	+	±	−	−	−	−
		L	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	±
"O"	O	O	−	−	−	.					
		L	−	−	−	.					
	L	O	−	−	−	.					
		L	−	−	−	.					
	Unabsorbed	O	+ + +	+ + +	+ + +	+ ±	±	−	−	−	−
		L	+ + +	+ + +	+ +	+	(±)	−	−	−	−

Opacity of L suspension for absorption 10 × 15 and of O suspension 10 × 5.

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This labile agglutinogen (L) was found to be present in all but two of the strains. The aberrant type which was known to have lost its power to elaborate ϵ toxin and the South African strain failed completely to stimulate the production of L antibody and they gave no agglutination reactions indicative of the presence of a labile component. These points are illustrated in the cross-agglutination test with three sera and representative strains recorded in Table 4.

Table 4. *Type B. Cross L agglutination test*

Rabbit L immune serum (O absorbed) prepared from strains	Titre of L agglutination with strains						
	Scotland		England		Wales		South Africa
	1	2	3	4	5	6	7
Scotland 1	20,000	0	20,000	20,000	500	20,000	0
Wales 5	100	0	200	100	5000	100	0
South Africa 7	0	0	0	0	0	0	0

Titre O = a negative result in a dilution 1 : 50.
O antibody removed from each serum by absorption with homologous O suspensions.
Strains: Scotland 1 is typical of 11 strains.
Scotland 2 and South Africa 7 are apparently devoid of L antigen.
Wales 5 has a specific L antigen.

Examination of type C strains (B. paludis). Ten strains were selected all of which were isolated from sheep diagnosed to be suffering from "Struck". Six were isolated by Dr McEwen from sheep in Kent and four by Dr Montgomerie in north Wales. Each strain produced α, β and δ toxins. L and O immune sera were prepared against five of them. Cross-absorption tests showed that all strains possessed an identical O antigen. On the other hand sera prepared against the formolized suspensions contained no trace of L antibody similar to that produced by B strains.

Examination of type D strains (B. ovitoxicus). Thirteen strains were examined. They were all isolated from sheep suffering from one form or another of so-called infectious entero-toxaemia and each one was known to produce α and ϵ toxins. The countries of their origin were, England (3 strains), Scotland (3 strains), Wales (3 strains), Australia (2 strains), America (2 strains). Seven representative strains were used in the preparation of L and O sera.

In contrast with types B and C the O antigens of type D strains exhibited a widely diverse specificity. The thirteen strains fell into at least seven strictly specific O groups, six of which are comprised as follows:

O group	Number of strains	Country of origin
1	1	America
2	2	Australia
3	2	Scotland
4	1	Wales
5	2	1 England; 1 Scotland
6	2	1 Wales; 1 America

The O antigen of three strains, one from Wales and two from England, failed to come within any of the above groups and O-immune serum for each

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was not prepared. The apparent indiscriminate distribution of specific O antigens among strains from different sources makes it improbable that these three strains would all come within a seventh group. In fact these thirteen strains might be divided into not less than nine groups. The strains within any group, however, show a complete identity of their O antigen and the degree overlap of one group with another, is either *nil* or negligible.

The seven L sera were examined by methods similar to those used in testing type B strains. L antibody was found to be present in five and apparently absent from two of these sera. By employing those containing L antibody in cross-agglutination and agglutinin absorption tests L antigen was detected in nine of the thirteen strains and appeared to be absent in four. The positive strains fell into three groups according to the specificity of the L component. One of these L antigens was shared by the two Australian strains, another by strains isolated in Great Britain and the third by one of the American strains. It will be observed that the grouping made according to the specificity of the L antigen coincides with the grouping according to the country of origin of the strains. This may readily be due to chance selection.

L group	Number of strains	Country of origin
1	2	Australia
2	1	America
3	6	1 England; 3 Scotland; 2 Wales
L antigen apparently absent	4	1 America; 1 Wales; 2 England

The antigenic composition of this group is obviously very complex and these preliminary observations may require modification when a more extensive series of strains have been examined.

Examination of type A strains (*classical* Cl. welchii). The wide diversity in antigenic specificity among type A organisms which has been demonstrated by many workers made it improbable that an examination of any small series of strains would offer a clue to the general classification of this type of *Cl. welchii*. The ten strains examined, therefore, were selected principally for purposes of comparison with types B, C and D. Nine were isolated from gangrenous conditions in the human subject, three in England, four in France and two in Germany. The tenth was isolated from the intestinal contents of a healthy sheep. Their capacity for toxin production varied considerably but all elaborated a measurable amount of α toxin.

L and O sera were prepared against five representative strains. The O antibody in each serum reacted strongly with the homologous strain but there was no cross reaction with any of the others. Further, no evidence was obtained for the presence in any of the strains of a labile antigen similar to that in types B and D.

Cross agglutination reactions of the various types. Tests have shown that each type or race within the type possesses a strictly specific O antigen. Faint traces of agglutination of type B strains with types C and D antisera (and

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vice versa) were detected but absorption of the sera with heterologous types failed to lower the agglutinin value for the homologous strain. Type A strains failed to react with any heterologous sera.

The L antigen complex of types B and D had certain elements that were common to both types. Thus the L antigen present in all but three of type B strains was closely related to the labile antigenic substance of Australian type D strains. Cross-absorption tests, however, showed that these two antigens were not identical but had a dominant common factor. L antigens present in the other type D strains had some minor factor common with type B strains.

The L antigen and ϵ toxin. The labile somatic antigen was found only in those strains that produce ϵ toxin, i.e. types B and D. In the early stages of the work it was not known that certain strains of these types apparently devoid of L antigen could produce this toxin. Indeed the absence of the relatively labile somatic component from the variant of the type B strain which no longer produced ϵ toxin suggested that there might be a close parallelism between the bacterial antigen and this toxin. The evidence was further supported by experience with one type D strain which, when it was received, was known to produce ϵ toxin but after some short subculture failed completely to stimulate the production of L antibody. Prof. Dalling then retested this culture and found that it had lost the capacity to produce ϵ toxin.

In view of these findings it seemed desirable to examine more closely the relationship of the L antigen to this toxin. Now apart from the strict antigenic specificity of ϵ toxin, two factors clearly distinguish it from either the α or β toxins. Thus Wilsdon (1931) showed that it was relatively resistant to the action of heat, and Bosworth & Glover (1934) have recorded the remarkable property of enhancement of its toxicity brought about by the action of trypsin. The influence of heat and of trypsin on the L antigen was, therefore, investigated. Table 5 records the results of an experiment to determine the heat stability of this substance when used as antigen in agglutination tests. The suspension was prepared as described above and portions of it were heated for

Table 5. *Agglutination test illustrating the heat lability of the L antigen*

Treatment of L suspension (type D strain)	Agglutination with homologous L serum (O absorbed) dilution									Normal serum dilution		NaCl control
	50	100	200	500	1000	2000	5000	10,000	20,000	50	100	0·42 %
Untreated	+ + +	+ + +	+ + +	+ + +	+ + +	+ + ±	+ +	+	(±)	(±)	–	–
Heated 30 min. at 80° C.	+ + +	+ + +	+ + +	+ + +	+ + +	+ ±	+ +	+	(±)	–	–	–
Heated 1 hr. at 80° C.	+ + +	+ + +	+ + +	+ + +	+ +	+	±	–	–	–	–	–
Heated 30 min. at 100° C.	+ + +	+ +	+	±	(±)	–	–	–	–	–	–	–
Heated 1 hr. at 100° C.	±	–	–	–	–	–	–	–	–	–	–	–
Heated 2 hr at 100° C.	(±)	–	–	–	–	–	–	–	–	–	–	–

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varying periods at 80 and 100° C. respectively. It is seen from the table that the antigen is almost completely destroyed after heating for 1 hr. at 100° C. Suspensions thus treated were also found to be devoid of agglutinogenic activity and failed also to absorb L agglutinin. This finding is in close agreement with Wilsdon's observation concerning the critical temperature and time of exposure necessary for the destruction of ϵ toxin.

Experiments were designed to observe the action of trypsin (Liquor trypsinae Co.) on the L antigen of the intact cell. It was considered possible that if this antigen had a chemical structure not dissimilar to that of ϵ toxin then digestion with trypsin might either remove the L antigen from the cell or liberate reasonable amounts of the toxin from it. Entirely negative results, however, were obtained under all conditions of experiment. Small amounts of toxin were in fact detected in the supernatant fluid from trypsinized suspensions which, prior to the action of trypsin, were apparently devoid of toxin but this may well have been due to the action of trypsin on residual amounts of ϵ toxin. The evidence obtained, therefore, suggests that L antigen and ϵ toxin are unrelated but it cannot be accepted as a proof in this direction because as Morgan & Partridge (1940) have indicated, the digestive action of trypsin on certain bacterial antigens depends to a large extent on whether the antigen is attached to the cell or removed therefrom before digestion is commenced.

The most suggestive evidence that L antigen and ϵ toxin are not related is the fact that certain strains known to produce this toxin apparently fail to stimulate in rabbits the production of L antibody and also that sera containing a high titre of L antibody may be practically devoid of ϵ antitoxin.

Most of the strains (types A, B, C, D) were tested in the presence of serum by the Indian ink method for the presence of capsules. By this negative method of testing all strains were shown to have a clear zone of varying diameter around the bacillus. If this phenomenon be accepted as evidence for the presence of a capsule then all strains were encapsulated. L antigen, however, was not detected in any type A or C strains and it is improbable, therefore, that capsular material *per se*, is the labile antigenic substance.

COLONIAL VARIANTS ASSOCIATED WITH SEROLOGICAL CHANGE

It has already been mentioned that colonial forms of the strains examined in the foregoing experiments conformed to the text-book descriptions of typical *Cl. welchii*. A 1% Difco proteose peptone meat infusion agar containing 0·5% glucose was used for all plating and on this medium a considerable number of strains produced colonial variants. These dissociant forms varied greatly in appearance from one plating to another, but certain strains threw off variant colonies with apparently constant characteristics. Certain of these latter forms which were strongly reminiscent of Arkwright's (1921) rough forms among Salmonella were selected for serological study. They

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were obtained from two strains of type B (one typical and the other no longer produced ϵ toxin), one of type C and one of type D. Their appearance on agar plates resembled closely McGaughey's (1933) variant I of a *Cl. welchii* type A strain. They were all openly granular, somewhat translucent and had an irregular margin. After the first few subcultures and platings all but one of these variants bred true and have continued to do so for about 3 years. The variant from the type D strain, however, has continued to yield a small proportion of typical *Cl. welchii* colonies. Apart from their colonial development the cultural and biochemical characteristics of these variants remained typical of the type from which they were derived and morphologically the cells were indistinguishable from classical *Cl. welchii*.

A serological examination of these variants showed that the B and C types had entirely lost the power to absorb the O agglutinin specific for the parent strain. The type D variant could still absorb O antibody, but it was found that if estimated on a quantitative basis this variant was much less efficient than the original strain. Table 6 records the results of an experiment in which portions of an O serum specific for the type were absorbed respectively with the typical form of the organism and with the rough variant. The absence of O antigen is clearly demonstrated. Table 6 also records the influence of absorption of an L serum (from which O antibody had previously been removed) with the normal and with the variant form of one of the type B strains. It is seen from the table that whereas the rough change denoted a loss of specific O substance the variant still possessed a full complement of the labile somatic component.

Table 6. *Illustrating the antigenic composition of a type B rough variant*

Serum	Agglutination with homologous suspension	Serum absorbed in dilution 1 : 50 with suspension	Serum dilution 50	100	200	500	1000	2000	5000	10,000	20,000
O	O	R variant	+ + +	+ + +	+ + +	+ +	+	±	–	–	–
		S form (normal)	–	–	–	–	–	–	–	–	–
		Unabsorbed	+ + +	+ + +	+ + +	+ +	+	±	–	–	–
L (O absorbed)	L	R variant	+ +	±	–	–	–	–	–	–	–
		S form (normal)	+ +	+	–	–	–	–	–	–	–
		Unabsorbed	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ ±	+

The L serum was one from which the O antibody had already been absorbed.

Formolized suspensions of the R and S forms were used for absorption according to the method described under Table 2. The opacity of the suspensions for absorption was 10 × 15.

These findings have been substantiated by an examination of the antibody response in rabbits to immunization with variants. One of the type B variants as well as that of type C failed to stimulate the production of O antibody specific for the parent strain. The other type B variant was apparently not entirely devoid of specific O substance because it was found that after three courses of immunization one of the three rabbits produced a serum with a low titre of antibody. The type D variant contained sufficient specific O substance

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to immunize rabbits effectively. One of the type B variants and the type D also produced L antibody in rabbits. As noted above, the parent strain of the other type B variant was itself a variant in respect of the fact that it contained no labile somatic component and did not produce ϵ toxin.

Suspensions prepared from these variants were so highly sensitive to the precipitating action of electrolytes that they could not be reliably employed in sensitive agglutination tests. From certain rough tests, however, it appeared that an antibody common to all was produced in response to immunization with any one of them. It also seemed probable that sera prepared by immunizing with heterologous smooth organisms contained an antibody associated with these rough forms. If this finding is substantiated it might form a useful method of establishing the group relationship of these otherwise diverse serological types.

In view of the serological and cultural characteristics of these variants it seemed to be important to establish whether concurrently with the loss of the specific O antigen their power to elaborate one or other of the multiple toxins had disappeared. Through the kind co-operation of Miss H. E. Ross of the Wellcome Physiological Research Laboratories, the toxin-producing capacity of the variants and their parent cultures were examined. No evidence of change in this direction was found. The cultures were sent for examination as specimens to be identified, and from an examination of the toxins they produced, Miss Ross succeeded in placing each parent culture and the corresponding variant in their proper order.

Summary and conclusions

The complex group of organisms with cultural and biochemical properties closely similar to the classical *Cl. welchii* are at present differentiated into four types according to the quality and number of the toxins they elaborate *in vitro*.

The present study is concerned with the specific affinities of the bacterial antigens of representative strains from these four types. The experiments confirm earlier reports concerning a wide diversity in the specificity of the somatic antigens of the classical type A strains. The bacterial antigens of the types B, C and D, however, have been shown to possess interesting similarities and affinities:

(1) The O antigen of type A (classical *Cl. welchii*) strains is strictly strain specific.

(2) The type B (L.D. bacillus) strains isolated in Great Britain share an identical O antigen but a strain from South Africa had a strictly specific O component.

(3) The O antigen of the type C (*B. paludis*) strains isolated in Great Britain is identical.

(4) The type D (*B. ovitoxicus*) strains exhibit wide diversity in the specificity of their O component. Thirteen strains were placed in not less than seven groups.

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(5) Cross O reactions between various types is negligible or entirely absent.

(6) In addition to the heat-stable O antigen certain strains possess a heat-labile somatic component. This antigen occurs only among those types (B and D) that produce ϵ toxin but it is probably not present in all strains.

(7) Colony variants reminiscent of the typical rough forms of Gram-negative organisms are frequently encountered among strains of each type. Certain of these forms appear as stable variants and they are characterized serologically by the loss of O antigen specific for the strain.

(8) The rough forms still produce toxins characteristic of the type to which the parent culture belongs.

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