THE RELATIONSHIP BETWEEN MORPHOLOGY, COLONIAL APPEARANCE, AGGLUTINABILITY, AND VIRULENCE TO MICE OF CERTAIN VARIANTS OF *BACTERIUM AERTRYCKE*.

By G. S. WILSON.

(*From the London School of Hygiene and Tropical Medicine.*)

(With Plates I–III.)

Evidence has been adduced (Wilson, 1928) to show the existence in cultures of *B. aertrycke* of discontinuous variations in virulence to mice. Side by side, in the same culture of a given strain, it proved possible to demonstrate the presence of both virulent and avirulent organisms. It was further noticed that the fall in virulence of a smooth strain, consequent on daily sub-culture under certain environmental conditions, was accompanied by the appearance of one or more variants that were either completely avirulent or were only weakly virulent. The tentative conclusion drawn from these observations was that the fall in virulence of a given strain is due not to a simultaneous fall in the virulence of each of its constituent organisms, but to a replacement of the highly virulent organisms by organisms of a lower degree of virulence.

The present paper is concerned with a closer study of the normal smooth and the variant strains of *B. aertrycke* which were encountered during the course of these and subsequent experiments. It appeared desirable to find out whether, by paying adequate attention to the morphological, colonial, and serological properties of a given strain, it was possible to distinguish virulent organisms, on the one hand, from avirulent, or weakly virulent organisms, on the other. Accordingly, during a long series of virulence experiments made on cultures grown under different atmospheric conditions, these properties were carefully studied and the results correlated with those obtained by inoculation of mice.

Technique.

(1) *Virulence.* In the majority of experiments a 24-hour broth culture of the strain to be tested was diluted with Ringer's solution to such an extent that 0·5 c.c. of the last dilution contained approximately 100 viable organisms. This quantity was inoculated intraperitoneally into 20 parti-coloured mice, weighing between 17 and 23 grm., and a count of the viable organisms was made on the actual inoculum used. In certain experiments the growth on a 24-hour agar slope culture was suspended in Ringer's solution and diluted till 0·5 c.c. of the last dilution contained approximately 1000 viable organisms;

https://doi.org/10.1017/S0022172400010275 Published online by Cambridge University Press

this quantity was inoculated into 20 mice, and counts were made as before. The mice were kept in single cages, and each animal that died was examined for the presence of *B. aertrycke*. No mouse was regarded as having died from a specific infection, unless this organism was recovered from the tissues. The survivors at the end of a fortnight were killed; the spleen was seeded into broth and incubated; this culture was plated out on to MacConkey 1–5 days later, and an attempt made to recover the specific organism.

(2) *Colonial appearance.* From the 1/1000 dilution in Ringer of the broth culture used for inoculating mice, five agar plates were streaked by means of a wire loop; on each 4-inch plate eight streaks were made at approximately 1 cm. intervals, followed by a second series of eight streaks at right angles to the first. By this method it was generally possible to obtain a relatively large number of well-isolated colonies. After 24 hours' incubation at 37° C. the colonies were examined macroscopically, with a magnifying glass, and with a binocular microscope (Leitz plate binocular model, × 10) using both transmitted and reflected light. The colonies were described, particular attention being paid to the shape, size, elevation, structure, surface and edge. If more than one type was present on the five plates examined the different types of colonies were counted, so as to obtain an approximate estimate of the proportion in which they were present in the whole culture. Photographs of the colonies were then taken, using oblique transmitted light and a magnification of eight diameters.

(3) *Morphology.* A film was made from each different type of colony appearing on the plates described under (2). The organisms were rubbed up in a small drop of formol on a new and specially cleaned slide. After the film had dried, it was fixed in absolute alcohol for 10 minutes, and stood in the air till the alcohol had completely evaporated. It was then stained for 1 hour in a 1/20 dilution of Giemsa in distilled water of *p*H 7·4; differentiation was carried out with one change of 33 per cent. alcohol and two changes of distilled water, the changes being made at $\frac{1}{2}$-minute intervals. The slide was air-dried, and examined with a 2 mm. objective of N.A. 1·3. The organisms were described, special notes being taken of their shape, size, curvature, arrangement and variability. A certain number in each film were carefully measured by means of an eyepiece micrometer. Photographs of the different types were then taken. *Note.* In order to facilitate comparison between the different types, it is important that all films should be made of approximately the same degree of thickness; a film of moderate thickness is to be preferred, since this renders evident the intra-colonial arrangement of the organisms.

(4) *Motility.* A 24-hour broth culture at 37° C. of the strain used for inoculating mice was examined for motility by the hanging-drop method. An approximate estimate was made of the proportion of motile organisms present.

(5) *Growth in broth.* A 24-hour casein broth culture at 37° C. was examined, and notes taken of the degree and nature of the turbidity, the presence of a surface pellicle or ring growth, and the degree and type of deposit. Using

the opacity method, an estimate was then made of the degree of turbidity after shaking.

(6) *Saline agglutination.* A 24-hour agar slope culture at 37° C. was washed off with about 5 c.c. of distilled water. 0·2 c.c. of this suspension was then added to four Dreyer's agglutination tubes containing respectively 0·8 c.c. distilled water, 0·8 c.c. of 0·25 per cent. NaCl solution, 0·8 c.c. of 1 per cent. NaCl solution, and 0·8 c.c. of 5 per cent. NaCl solution. The tubes were incubated for 24 hours in a water-bath at 55° C., and the results read against a brightly illuminated dark background.

(7) *Agglutination by a smooth and rough O-serum.* A 24-hour agar slope culture at 37° C. was washed off with distilled water, standardised by opacity to about 1000 million organisms per c.c. and steamed for 1 hour to destroy the heat-labile agglutinogens. This suspension was tested against a smooth O-serum prepared against *B. aertrycke* (Glasgow strain), and a rough O-serum prepared against *B. enteritidis*. The serum dilutions were made in 0·25 per cent. saline. The results were read after 18 hours' incubation at 55° C.

(8) *Acid agglutination.* A 24-hour agar slope culture at 37° C. was washed off with distilled water, and standardised by opacity to about 1000 million organisms per c.c. This suspension was then tested against Beniasch's acid series (Beniasch, 1912; Arkwright, 1928); the tubes were incubated in a water-bath at 55° C., and readings taken up to 3 hours.

The study of the serum and acid agglutinability of the organisms was not commenced till towards the end of the present series of experiments, so that results with these tests are available for only a comparatively small number of strains. Apart from these, a permanent record has been preserved of the properties of each individual strain, the results being entered upon a large card, which likewise bears photographs of the colonial and morphological appearances. Altogether 135 strains have been studied.

The four different types of *B. aertrycke*.

Before recording the data obtained during the examination of these strains, it will make for simplicity if the conclusions reached from this study are stated immediately. Briefly, it may be said that four main types of this organism have been recognised. These types have been distinguished on the basis of colonial and morphological appearances, of virulence, and to a less extent of agglutinability. In any one type the first three properties are closely correlated, so that by a study of the colonial and morphological appearances it is possible to estimate the approximate virulence of the organism.

Type A. The normal smooth virulent form.

Agar colonies. Circular, about 3 mm. in diameter, low convex in elevation with a slightly bevelled margin; viewed by transmitted light the structure is slightly irregular, having an appearance not unlike that of beaten glass; the surface, when viewed with a binocular microscope (× 10), has a finely granular,

glistening, beaten-copper appearance, and gives a fairly good mirror effect, *i.e.* the image of the window bars, for example, seen when the colony is observed by reflected light, is not greatly distorted; the edge is entire when viewed macroscopically, but under the binocular microscope it appears very slightly crenated and shows shallow radial striations confined to the extreme periphery of the colony (Pl. I, fig. 1). Though this is the normal appearance of the A type, a certain amount of variation may occur. Thus, the colony may be slightly smaller, of almost amorphous structure, and have a smooth very finely granular surface, with an almost perfect mirror effect (Pl. I, fig. 2). Or it may be slightly larger, and have a rougher surface of a moderate degree of granularity, with a rather poor mirror effect (Pl. I, fig. 3). Or, more rarely, it may be still larger, with a diameter of 4–5 mm., and present an amoeboid appearance; the surface of this type has a fine to moderate degree of granularity, with a variable mirror effect (Pl. I, fig. 4).

Morphology. The organisms appear as straight rods and are generally 1–2·5 μ long and 0·5–0·6 μ wide. They are arranged in bundles, which tend to have a slipping appearance, so that the end of each organism is placed more or less opposite the middle of the organism next to it. Within the bundle the individual organisms are set closely together, there being as a rule no clear space between them. The bundles themselves in a thin film may be more or less isolated (Pl. II, fig. 11), but in a film of moderate thickness they are arranged in a polygonal manner, which is very characteristic (Pl. II, fig. 12). Staining is fairly uniform, but larger, more deeply stained organisms are not uncommon.

Motility. Nearly all strains of this type are motile; only occasional exceptions have been encountered. In a 24-hour broth culture about 10–40 per cent. of organisms exhibit motility.

Growth in broth. The usual picture is that of a moderate uniform turbidity, with a moderate powdery deposit disintegrating completely on shaking. A true surface pellicle is uncommon, but a surface ring growth is generally present.

Saline agglutination. With a few exceptions, the organisms are not agglutinated by 4 per cent. saline.

O-serum agglutination. As a rule agglutination occurs to titre with an *aertrycke* smooth O-serum, but fails completely with an *enteritidis* rough O-serum.

Acid agglutination. Either no agglutination at all is seen within 3 hours, or a trace appears in tubes 4 to 8.

Virulence. Inoculated intraperitoneally in a dose of about 100 viable organisms, strains of this type kill on an average about 14 out of 20 mice. Fewer than 9, or more than 18 deaths, are uncommon. With a dose of 1000 organisms the number of deaths is about 16. Whether the variability of the results, which occurs even with pure line strains tested repeatedly, is due to a change in the virulence of the organisms, or to an alteration in the suscepti-

bility of the animals, it is impossible to say, but evidence has been accumulating to suggest that the latter factor may be of considerable importance.

Type B. The smooth weakly virulent form.

Agar colonies. Circular, 1–2 mm. in diameter, convex or umbonate in elevation, with a slight bevelling of the margin; structure is amorphous; the surface, viewed under the binocular microscope, has a very finely granular appearance, is smooth, glistening, and gives a perfect mirror effect; the edge is entire (Pl. I, fig. 5). Quite frequently the colonies are rather larger, and have a slightly raised conical projection in the centre (Pl. I, fig. 6). Sometimes an even larger colony may be formed, 2–3 mm. in diameter, which is very definitely umbonate, and has a beaten-copper surface (Pl. I, fig. 7); except for the umbonation, these colonies are not easy to distinguish from those of type A.

Morphology. The organisms appear as short fat coccoid forms, staining fairly uniformly. There is often considerable variation in size, from very small coccoid forms, on the one hand, to definite rods, on the other. The coccoid forms are arranged in pairs, the bacillary forms are more often single. The average size is about $0{\cdot}7$–$2{\cdot}0\,\mu \times 0{\cdot}6$–$0{\cdot}8\,\mu$ (Pl. II, fig. 13). In a film of moderate thickness, though the majority of the organisms are arranged singly or in pairs, occasionally they are collected into curious groups, having a superficial resemblance to a diagram illustrating the distribution of isobars in a cyclonic formation (Pl. II, fig. 14). Within these groups the members are arranged in more or less concentric circles, and at approximately equidistant intervals from each other, a clear space being visible between adjacent organisms. There is none of the typical bundle formation nor polygonal distribution of type A.

Motility. The majority of the strains are motile, but a considerable proportion show no evidence of motility. In a 24-hour broth culture it is uncommon to find more than 5–10 per cent. of motile organisms.

Growth in broth. There is usually a moderate to dense uniform turbidity, with a fairly heavy powdery deposit, disintegrating completely on shaking. A surface ring growth is always present, and in about half the strains an actual pellicle is formed.

Saline agglutination. There is no agglutination with 4·0 per cent. saline.

O-serum agglutination. The three strains that have been tested have agglutinated to titre with an *aertrycke* smooth O-serum, and have failed to react, or have reacted only in a low dilution, with an *enteritidis* rough O-serum.

Acid agglutination. There is generally agglutination of a finely granular type in tubes 4–8 within 3 hours.

Virulence. Injected intraperitoneally in a dose of about 100 viable organisms, strains of this type kill about one to two mice out of 20; with a dose of 1000, the usual number of deaths is three to four.

Type C. The rough, ichthyotic, weakly virulent form.

Agar colonies. Circular, 4–5 mm. in diameter, of low convex or slightly umbonate elevation, with little or no bevelling of the margin. Structure resembles frosted glass. The surface under the binocular microscope is moderately to coarsely granular, and has an irregular, rather honeycombed, appearance; it is dull and has practically no mirror effect; it is smoother around the centre than at the periphery. The edge is slightly undulate and finely dentate (Pl. I, fig. 8). The most striking feature of this type of colony is the appearance, under the binocular microscope, of definite imbrications near the edge; the colony, in fact, looks as if it was made up of fish scales; hence the term ichthyotic. Provided the depth of agar in the plate is not too great, the whole colony, viewed under the microscope by transmitted light, has a silvery appearance, which contrasts with the yellowish brown colour of the other types.

Morphology. The organisms at first sight give the impression of being long, curved, rather delicate rods, but on closer examination their length is found to be subject to great variation. In a single film the majority of the organisms are 1–5 μ long and 0·5–0·6 μ broad; but besides these there are almost invariably a number of long, curved filamentous forms, which are characteristic of this type (Pl. II, fig. 15). The filaments are broader than the rods, but the exact breadth of any individual filament varies from one portion to another. The rods stain more or less uniformly; the filaments usually show sections of uneven staining. The general arrangement of the organisms is in narrow bundles, which exhibit a very marked slipping appearance (Pl. II, fig. 16). Often the individual members in a bundle are so strung out as to assume an almost end-to-end appearance. One of the most striking features is the irregularity in length of the different members in a given bundle; short, long, and even filamentous forms are found side by side in a single bundle. It is common for one or more rods to be arranged at distant intervals along one or other side of a filament (Pl. II, fig. 15 and Pl. III, fig. 17).

Motility. Strains of this type are actively motile, but the motility is confined chiefly to the shorter rods. Generally about 10 per cent. of the organisms appear to be motile.

Growth in broth. There is a moderate, uniform turbidity, and a moderate powdery deposit disintegrating on shaking. A surface ring growth is always seen, but a true pellicle is uncommon.

Saline agglutination. With occasional exceptions, the organisms are not agglutinated by 4 per cent. saline.

O-serum agglutination. Two strains have been examined. Agglutination occurs to about one titre with an *aertrycke* smooth O-serum, and to a low titre with an *enteritidis* rough O-serum.

Acid agglutination. Three strains have been examined. Usually a finely granular type of agglutination is visible in tubes 4–8 of the series.

Virulence. Injected intraperitoneally in a dose of about 1000 viable organisms, strains of this type kill on an average five mice out of 20. Too few strains have been tested in a dose of 100 organisms to obtain comparable figures.

Type D. The true rough avirulent type.

Agar colonies. Irregularly circular, 4–6 mm. in diameter, of raised elevation with a marked concave bevelling of the margin. Structure resembles coarsely frosted glass. The surface is coarsely granular, and is very irregular, bearing a resemblance to a relief-map; it is dull, and has no mirror effect; frequently there is a small umbilicated depression at the centre. The edge is irregularly undulate with small secondary crenations (Pl. I, fig. 9). When fully developed this type is usually unmistakable; but if, for one reason or another, it has not reached its full size, it is apt to be confused with type C. The chief points of difference are: (1) type C is low convex in elevation and shows practically no bevelling of the margin, whereas type D is raised, and shows a marked concave bevelling of the margin; (2) the periphery of type C is imbricated, and the whole colony looks, under the binocular microscope, as if composed of silvery scales; type D shows no imbrication, and is of a yellowish brown colour under the microscope; (3) the central portion of type C colonies tends to be smoother than the periphery, and the granularity of the surface extends down to the very edge; with type D, the rough appearance extends over the whole colony with the exception of the bevelled margin, which is comparatively smooth. Imperfectly developed colonies may be mistaken for the rougher forms of type A (Pl. I, fig. 10); as a rule the D colonies are larger and less circular in shape and have a definitely concave bevelled edge; umbilication, if present, is a further point suggestive of the D type.

Morphology. The organisms appear as straight rods, from 1–3 μ long by 0·6–0·7 μ broad, staining fairly uniformly. They are arranged singly, in Chinese-letter form, or in dense clumps of varying size (Pl. III, figs. 18, 19). As a rule, in any given preparation, all three types of distribution are seen. The arrangement of type A organisms in slipping bundles, on the one hand, and of type D organisms in Chinese-letter formation, on the other, is undoubtedly due to the peculiar mode of division within the growing colony. This difference has already been pointed out by Nutt (1927), who made observations on smooth and rough strains of *B. aertrycke* in agar-film preparations under dark-ground illumination. She drew attention, likewise, to the fact that in the rough type the bacilli are more adherent than in the smooth; this clearly accounts for the tendency of rough organisms to form dense clumps, in which the individual members can be made out only with difficulty.

Motility. The majority of strains are motile, but as a rule only quite a low proportion, 1–5 per cent., of organisms in a 24-hour broth culture show motility.

Growth in broth. Considerable variation is seen with different strains. Perhaps the most typical appearance is a slight flocculo-granular turbidity with a heavy flocculo-granular deposit, which does not disintegrate com-

pletely on shaking. A surface ring is always present, and, not infrequently, a true surface pellicle. Other strains give a uniform turbidity with a moderate to heavy powdery or viscous deposit.

Saline agglutination. Many strains are agglutinated by 0·8 per cent. saline, and nearly all by 4 per cent. The few instances in which a rough strain, as judged by colonial and morphological appearances, remained stable in 4 per cent. saline were those of cultures which had recently gone rough, and in which presumably the transition was not quite complete.

O-serum agglutination. Agglutination occurs to a very low titre, or not at all, with an *aertrycke* smooth O-serum, and to a moderate titre with an *enteritidis* rough O-serum.

Acid agglutination. Strains of this type are agglutinated over a wide range, and exhibit a specially heavy degree of flocculation in tubes 3–6. The flocculi are coarse and of a curdy appearance—very different from the finely granular flocculation of the other types.

Virulence. Injected intraperitoneally in a dose of 100 or 1000 organisms, strains of this type rarely kill more than one mouse out of 20; usually no deaths occur at all.

SEROLOGICAL ANALYSIS OF A, B, C AND D TYPES.

Agglutinating sera were prepared by the intravenous injection of rabbits with suspensions made by washing off 24-hour agar cultures at 37° C. with absolute alcohol, heating in a water-bath at 55° C. for 2 hours, leaving in the ice-chest overnight, centrifuging, decanting the alcohol, and suspending the deposit in distilled water. The resultant sera, which contained O-agglutinins only, were then used for a series of cross-absorption experiments. Absorption was carried out by the fractional method, the mixtures being kept for 4 hours in the incubator, and overnight in the ice-chest. The suspensions used for absorption were prepared by washing off 24-hour agar cultures at 37° C. with distilled water, steaming for 1 hour, centrifuging, and re-suspending in a small quantity of distilled water. The same suspensions, diluted to contain 1000 million organisms, were used for agglutination. The results are given in Tables I–IV.

Tables I, II and III show that A, B and C remove all agglutinins from each serum, but that D is unable to remove any. From Table IV it is seen that A, B and C can partly absorb the agglutinins from D-serum. This last experi-

Table I. *A-serum. Absorption carried out with serum dilution of* 1/5.

		A-serum absorbed with			
Antigen	Unabsorbed	A	B	C	D
A	80 tr. 160	—	—	—	80 tr. 160
B	640	—	—	—	—
C	1280	—	—	—	—
D	—	—	—	—	—

tr. = trace.

https://doi.org/10.1017/S0022172400010275 Published online by Cambridge University Press

Table II. *B-serum. Absorption carried out with serum dilution of* 1/5.

		B-serum absorbed with			
Antigen	Unabsorbed	A	B	C	D
A	320	tr. 20	—	—	—
B	640	tr. 20	tr. 20	tr. 20	640 tr. 1280
C	2560	—	—	20 tr. 40	—
D	—	—	—	—	—

tr. = trace

Table III. *C-serum. Absorption carried out with serum dilution of* 1/20.

		C-serum absorbed with			
Antigen	Unabsorbed	A	B	C	D
A	640	—	—	—	—
B	320	—	—	—	—
C	2560	—	—	—	2560
D	—	—	—	—	—

Table IV. *D-serum. Absorption carried out with serum dilution of* 1/5.

		D-serum absorbed with			
Antigen	Unabsorbed	A	B	C	D
A	—	—	—	—	—
B	320	—	20	—	—
C	80	—	—	—	—
D	1280	320	160	80	40

ment was repeated on two or three occasions, using different suspensions; the results agreed in showing that B and C always absorbed a greater proportion of agglutinins than A, and in one experiment A failed to absorb any at all.

It appears from these absorption tests that A, B and C all contain a smooth O-antigen, while D contains a pure rough O-antigen. In addition to the smooth O-antigen, B and C likewise contain a considerable proportion of rough O-antigen, which in A is present only in minimal amount.

Constancy of the A, B, C and D types.

To ascertain whether the differential characters of the four types enumerated above remained constant over a reasonable time and under reasonable conditions of sub-culture, four colonies were chosen, conforming respectively to the A, B, C and D types. Each was seeded into broth, incubated for 24 hours at 37° C., and plated out on agar. The following day a single colony was picked off into broth, which was again incubated for 24 hours and plated on agar. This broth-agar sequence was repeated three times weekly; over the week-end the agar plates were incubated for 48 hours instead of for 24. At the outset the virulence of each type was tested in the usual way on a batch of 20 mice; the test was repeated on the 65th and the 88th broth sub-cultures after 22 and 29 weeks, respectively, from the commencement of the experiment. During the whole of this time the four types retained their differential characteristics unaltered. The results of the virulence experiments are given in Table V.

It will be noticed that the virulence of the A, B and C types is rather lower than usual, but there is no indication of any change in virulence during the process of repeated sub-culture.

The result of this experiment leaves no doubt that the four types of *B. aertrycke* described represent fixed variants, whose properties remain unaltered over considerable periods of time.

Table V. *Constancy of virulence of four particular strains of types A, B, C and D.*

Date	Exp. no.	No. of mice injected	Dose	Died	Specific deaths	Killed	Spleens infected	Total infected	Mean expectation of life in days
7. xi. 28	47 A 1	20	1464	13	13	7	7	20	10·85
1. iv. 29	47 A 66	20	554	9	9	11	11	20	11·45
4. vi. 29	47 A 88	20	641	12	12	8	8	20	9·65
7. xi. 28	47 B 1	20	1300	0	0	20	17	17	14·0
1. iv. 29	47 B 66	20	810	3	3	17	16	19	13·6
4. vi. 29	47 B 88	20	684	0	0	20	17	17	14·0
7. xi. 28	47 C 1	20	802	3	1	17	17	18	12·95
1. iv. 29	47 C 66	20	541	1	1	19	15	16	13·8
4. vi. 29	47 C 88	20	651	2	1	18	11	12	13·15
7. xi. 28	47 D 1	20	498	1	1	19	13	14	13·95
1. iv. 29	47 D 66	20	1144	0	0	20	13	13	14·0
4. vi. 29	47 D 88	20	920	1	1	19	3	4	13·65

THE COMPOSITION OF DIFFERENT STRAINS OF *B. AERTRYCKE*.

Having defined the four different types of *B. aertrycke*, we may now consider the results of examining the composition of a number of different strains of this organism. Altogether 135 strains have been studied; of these, 36 were single-colony cultures, 54 were whole cultures containing only one type of colony, and 45 were whole cultures containing more than one type of colony.

Single-colony cultures.

The strains were allocated to their different types on the basis of morphological and colonial appearance. The results of this grouping, together with the virulence results, are shown in Table VI.

Table VI.

Type	No. of strains	Average no. of specific deaths following intraperitoneal inoculation of 20 mice with about 1000 viable organisms
A	5	14·8 ± 4·45
B	13	3·4 ± 3·76
C	13	4·9 ± 3·74
D	5	0·4 ± 0·49
	36	

Whole cultures with only one type of colony.

The results are given in Table VII. One strain proved impossible to type, the colonial appearances resembling those of A, the morphological appearances those of D.

Table VII.

Type	No. of strains	Average no. of specific deaths following intra peritoneal inoculation of 20 mice with about 100 viable organisms	Average no. of specific deaths following intra peritoneal inoculation of 20 mice with about 1000 viable organisms
A	24	14·63 ± 3·08	—
A	9	—	16·56 ± 3·54
B	7	1·86 ± 3·23	—
C*	1	—	—
D	9	0·11 ± 0·31	—
D	3	—	1·33 ± 1·89
Untyped	1	—	—
	54		

* This strain was the only C type in the series; on the one occasion on which it was tested it killed eight mice in a dose of about 100—an unusually large number of deaths for this type.

Whole cultures with more than one type of colony.

In all, 45 strains fell into this category, but of these it proved possible to allocate only 23 to their respective types. In the remaining 22 strains one or other of the constituent colonial types was atypical, or disagreed with the classification based on morphology. With only three exceptions these strains

Table VIII. *Dose of about* 100 *organisms.*

Exp. no.	Type	Organisms injected	Type	Organisms injected	Type	Organisms injected	Specific deaths
10 *b*	A	62·0	B	129·0	—	—	20
7	A	14·0	C	34·0	—	—	9
16 *a*	A	87·5	C	0·5	—	—	16
16 *b*	A	310·0	C	2·0	—	—	12
17	A	106·0	C	11·0	—	—	11
19	A	146·0	C	35·0	—	—	13
13	A	35·7	D	0·3	—	—	9
22	A	86·0	D	34·0	—	—	5
27	A	16·0	D	131·0	—	—	14
31	A	113·0	D	23·0	—	—	8
32	A	73·0	D	17·0	—	—	9
33	A	68·0	D	24·0	—	—	15
34 *a*	A	58·8	D	1·2	—	—	14
19 *c*	A	147·0	B	11·0	D	19	19
10 *c*	B	32·0	C	60·0	—	—	0
11	B	35·0	C	82·0	—	—	6
12	B	73·0	C	41·0	—	—	7
13	B	31·0	C	17·0	—	—	2
24	B	9·0	C	64·0	—	—	0
14	B	10·0	D	31·0	—	—	1
15	B	47·0	C	71·0	D	2	0
21	B	23·0	C	53·0	D	28	0

Dose of about 1000 *organisms.*

8	A	13 approx.	B	987 approx.	—	—	13
7	A	260·0	C	618·0	—	—	13
10 *c*	B	405·0	C	764·0	—	—	8
11	B	375·0	C	869·0	—	—	10
12	B	729·0	C	410·0	—	—	12
13	B	718·0	C	396·0	—	—	13
14	B	188·0	D	600·0	—	—	3
15	B	492·0	C	745·0	D	25	1

https://doi.org/10.1017/S0022172400010275 Published online by Cambridge University Press

were being submitted to daily sub-cultivation in atmospheres of different constitution—a process which is liable to lead to the development of variant colonies. It would appear probable that these strains were in a transitional phase, which rendered their exact typing impossible.

The composition of the 23 strains that were successfully typed is given in Table VIII.

An analysis of the instances in which type A colonies were mixed with those of other types, taking the lower doses only, is given in Table IX.

Table IX.

	Dose of about 100 organisms	
Types	No. of strains	Average no. of specific deaths
A+B	1	20·0
A+C	5	12·2
A+D	7	10·6
A+B+D	1	19·0
	14	12·43 ± 4·12

Considering that in some of these experiments the dose of type A organisms was very low, it is surprising that the average number of specific deaths was as high as 12·43; this figure is not greatly below that of 14·63, which was obtained with the whole cultures consisting purely of type A bacilli. It would appear as if admixture of avirulent with virulent bacilli has little or no effect in diminishing the virulence of the latter organisms. There is a suggestion that admixture with the completely avirulent type D bacilli may afford a partial exception to this rule, but the figures are clearly insufficient to prove this. When, in fact, a given strain comprises organisms showing discontinuous variations in virulence, the virulence of the whole culture is similar to that of the most virulent variant. The same conclusion was reached in a previous paper (Wilson, 1928) as the result of study along slightly different lines. An analysis of the comparatively avirulent types is given in Table X.

Table X.

	Dose of about 100 organisms		Dose of about 1000 organisms	
Types	No. of strains	Average no. of specific deaths	No. of strains	Average no. of specific deaths
B+C	5	3·0	4	10·75
B+D	1	1·0	1	3·0
B+C+D	2	0·0	1	1·0
	8	2·0 ± 2·67	6	7·83 ± 4·45

It will be noticed that when the comparatively avirulent types B, C and D, were inoculated in one combination or another in an aggregate dose of about 100 organisms, the average number of specific deaths was only 2·0; but when the dose was increased to 1000, the average number of specific deaths rose to 7·83. The reason for this increase is not clear. If reference is made to Table VI, it will be seen that when pure type B, C and D strains, derived from single colony cultures, were inoculated in a dose of about 1000 organisms, the average number

of specific deaths was 3·4, 4·9 and 0·4 respectively. On the other hand, when they were present in combination in a mixed culture (Table X), the average number of specific deaths was 7·83. The suggestion is either (1) that in the whole cultures the types were not completely pure; that is, they had assumed the colonial and morphological appearances of the pure types, while still retaining some of the virulence of the organisms from which they were originally derived; or (2) that there were present in the whole cultures a few virulent type A bacilli, which, though practically or completely absent in the lower doses, might yet have been present in sufficient numbers in the larger doses to cause an appreciable increase in the mortality. Which of these explanations is correct it is impossible from the available data to decide with certainty. In a previous paper in which a similar phenomenon was noticed, we favoured the latter explanation, but more detailed study has led us to doubt whether this is sufficient to account for all cases. Some light is thrown on the problem by the examination of the 22 strains which it proved impossible to type completely. Of these 22 strains, 18 were tested in a dose of about 100 organisms; the average number of specific deaths caused was $10{\cdot}77 \pm 4{\cdot}94$. The difficulty in typing these strains arose chiefly from the presence in them of colonies that appeared to be of a modified A type; whether these were type A colonies, or whether they belonged to other types bearing a superficial resemblance to A, it was impossible to decide. From the relatively high average mortality, however, it would appear probable that they were really type A colonies, which had taken on some of the characters of the B, C or D types, and which had lost some of the virulence of the original A type. Reverting to the observation that, in whole cultures consisting of one or more of the B, C or D types, an increase in the dosage from 100 to 1000 organisms was often accompanied by a marked rise in the number of specific deaths, we consider the most probable explanation to be that such strains were comprised of organisms which were in a transitional phase, and which, though partly resembling one of the avirulent types in morphological or colonial appearance, still retained a certain degree of virulence. In other words we suggest that during the transition from a highly virulent to an avirulent type, there may be an intermediate stage in which the organisms do not conform definitely to any of the four types described.

Discussion.

The results recorded in this paper seem adequate to justify the conclusion that there are at least four well-defined types of *B. aertrycke*, which may generally be recognised by an examination of their morphological and colonial appearances; that, of these types, A is highly virulent to mice, B and C have a very low degree of virulence, while D is almost or completely avirulent. Analysis of a large number of strains that were being submitted to daily sub-cultivation in atmospheres of different constitution showed (1) that a marked fall in virulence was always accompanied by the appearance of one or more of

the relatively or completely avirulent types of variant; (2) that so long as type A bacilli were present in the strain in adequate numbers, even though they formed only a small proportion of the total organisms present, the virulence remained comparatively high, but that when all type A bacilli were replaced by type B, C or D bacilli, so far at least as could be ascertained by examination of the colonies on five plates, the virulence fell considerably. On no occasion have we encountered a strain containing type A bacilli which was avirulent, when tested in a dose of 100; nor have we met with a virulent strain, as determined by this dosage, which failed to contain bacilli of this type.

The mechanism responsible for the genesis of avirulent variants from an initially virulent culture is by no means clear. Whether their appearance is comparatively sudden, or whether they result from a gradual change in the A form, has not been ascertained with certainty; the method employed during the series of experiments recorded in this paper was not suited to determine this point. The most that can be said is that if a strain, which is being submitted to daily sub-cultivation under conditions known to lead to a diminution in virulence, is examined at intervals of a week or more, it will be found that at some period avirulent variants of the B, C or D types make their appearance, and henceforward gradually increase in proportion to the original A type, till ultimately they replace this type completely. In some cultures transitional forms appear, which do not conform definitely to any of the four types. A close study of these transitional forms has not been made.

SUMMARY AND CONCLUSIONS.

1. Analysis of a series of 135 strains of *B. aertrycke* indicates that at least four well-defined types may be recognised on the basis of morphology, colonial formation, virulence to mice, and to a less extent agglutination by salt, serum, and acids. These types comprise: (i) type A—the normal smooth virulent form; (ii) type B—a smooth form of low virulence; (iii) type C—a roughish, so-called ichthyotic, form of low virulence; (iv) type D—the really rough avirulent form. Between these well-defined types certain transitional forms may sometimes be recognised, the virulence of which is frequently of an order intermediate between that of the A type on the one hand, and the B, C, or D types on the other.

2. Evidence is brought to show that if a virulent strain is sub-cultured daily, under conditions known to lead to a fall in virulence of the whole culture, a gradual replacement occurs of the virulent type A bacilli by the avirulent B, C or D types. So long as an adequate proportion, which is very small, of type A bacilli persists in the culture the virulence of the whole culture remains fairly high; but when all type A bacilli have been replaced by bacilli of the B, C or D types the virulence of the whole culture falls considerably.

We would express our thanks to the Government Grant Committee of the Royal Society for financial aid in part of this investigation.

EXPLANATION OF PLATES.

Types of *B. aertrycke.*

PLATE I.

Fig. 1. A type. Smooth virulent form. Normal type of colony. (× 8)

Fig. 2. A type. Smooth virulent form. Smoother type of colony. (× 8)

Fig. 3. A type. Smooth virulent form. Rougher type of colony. (× 8)

Fig. 4. A type. Smooth virulent form. Amoeboid type of colony. (× 8)

Fig. 5. B type. Smooth weakly virulent form. Normal type of colony. (× 8)

Fig. 6. B type. Smooth weakly virulent form. Slightly larger, umbonate colony. (× 8)

Fig. 7. B. type. Smooth weakly virulent form. Larger, umbonate colony, with beaten-copper surface. (× 8)

Fig. 8. C type. Rough ichthyotic weakly virulent form. Normal type of colony. (× 8)

Fig. 9. D type. True rough avirulent form. Normal type of colony. (× 8)

Fig. 10. D type. True rough avirulent form. Smoother type of colony. (× 8)

PLATE II.

Fig. 11. A type. Smooth virulent form. Isolated bundle formation. (× 1000)

Fig. 12. A type. Smooth virulent form. Bundles arranged polygonally. (× 1000)

Fig. 13. B type. Smooth weakly virulent form. Isolated coccoid and bacillary forms. (× 1000)

Fig. 14. B type. Smooth weakly virulent form. Isolated organisms and a large "cyclonic" bundle (× 1000)

Fig. 15. C type. Rough, ichthyotic weakly virulent form. Typical bacillary and filamentous forms. (× 1000)

Fig. 16. C type. Rough, ichthyotic weakly virulent form. Slipping bundle arrangement. (× 1000)

PLATE III.

Fig. 17. C type. Rough, ichthyotic weakly virulent form. Showing short rods arranged at intervals on either side of the filaments. (× 1000)

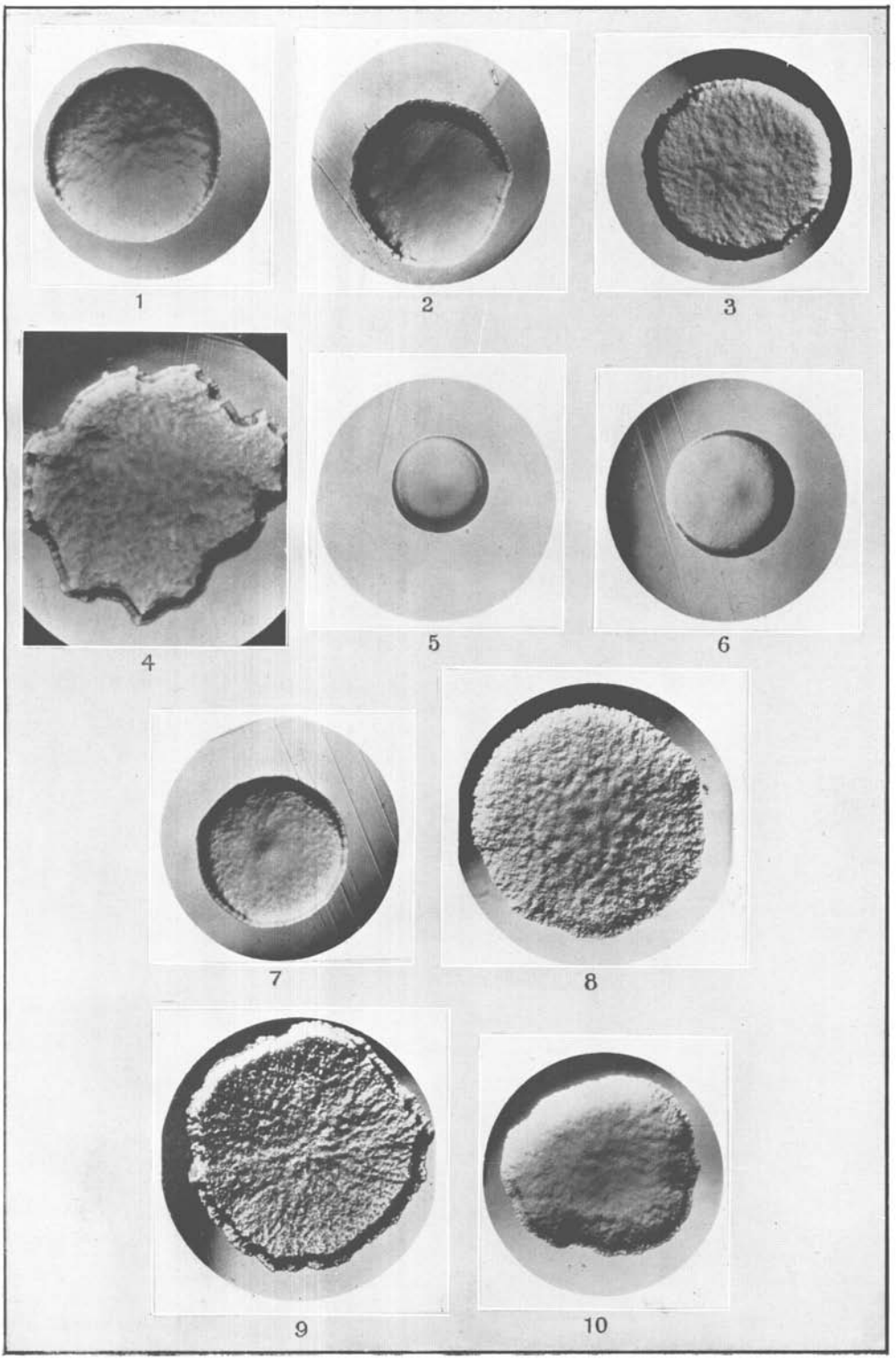
Fig. 18. D type. True rough avirulent form. Chinese-letter formation and a dense clump. (× 1000)

Fig. 19. D type. True rough avirulent form. Chinese-letter formation and small dense clumps. (× 1000)

REFERENCES.

ARKWRIGHT, J. A. (1928). *J. Path. and Bact.* **31**, 665.
BENIASCH, M. (1912). *Zeitschr. f. Immunitätsf.* **12**, 268.
NUTT, M. M. (1927). *J. Hygiene*, **26**, 44.
WILSON, G. S. (1928). *Ibid.* **28**, 295.

(*MS. received for publication* 16. XI. 1929.—Ed.)



https://doi.org/10.1017/S0022172400010275 Published online by Cambridge University Press

17

18

19

https://doi.org/10.1017/S0022172400010275 Published online by Cambridge University Press