SPONTANEOUS AGGLUTINATION OF THE CHOLERA VIBRIO IN RELATION TO VARIABILITY

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Spontaneous agglutination is a not uncommon occurrence, especially in the case of organisms of the enteric and diphtheria group, where it causes some difficulty in serological diagnosis. Many investigations have been made to clear up the principles of this phenomenon. Recently Arkwright (1921) has thrown much light on this problem. He was able to isolate from old pure cultures of dysentery, typhoid, paratyphoid and enteritidis (Gaertner) bacilli, two variants which he named the "R" (rough) and "S" (smooth). The "R" variant agglutinates spontaneously in physiological saline and in broth, while the "S" variant forms good emulsions in both. Specific immune sera prepared for these two variants show only slight cross-agglutination. Arkwright regards the two forms as potentially inherent in most of the individuals in the young culture, which may become segregated in different individuals in old cultures. "Rough" varieties have been observed also by Zoeller (1922) in *B. dysenteriae* Shiga and by Schütze (1921) in *B. paratyphosus* B.

Recently De Kruif (1921, 1922*a*, 1922*b*) has reported similar variation in *B. lepisepticum*. From spontaneous pneumonia of rabbits he isolated cultures which he was able to separate into two biotypes. The granular "G" type grows as clumps in broth, yields slightly irregular translucent non-fluorescent colonies on serum-agar, agglutinates easily at 37° C. and 55° C. with immune serum, and is only slightly virulent. The type "D" yields a diffuse turbidity in broth and presents smooth fluorescent colonies on serum-agar. In contact with immune serum it flocculates well at 55° C. but poorly or not at all at 37° C., and is highly virulent. The degree of cross-agglutination between the two types was examined. Serum prepared with the "D" form agglutinated both forms equally (1 in 2000) while that made with the "G" form, had a much lower titre (1 in 50 to 1 in 200) for "D" than for "G" (1 in 1000). The type "G" arose from the parent "D" by mutation.

Mellon (1922) has found in a pleomorphic diphtheroid bacillus two morphological phases of growth: a diplococcoid phase requiring a temperature of 20° C. forming stable emulsions in sodium chloride and Tyrode's solution; and a bacillary phase requiring a temperature of 37° C. and agglutinating spontaneously in both solutions.

A temporary spontaneous agglutinability of cholera-vibrios was observed by Friedberger and Luerssen (1905) in very young freshly isolated cultures; after 18–24 hours' growth at 37° C. this sensitiveness to salt disappeared.

Hamburger observed that cholera-vibrios grown for a long time in immune serum showed the phenomenon of spontaneous agglutination. This character was, however, lost after repeated passages on agar.

Baerthlein (1912) also made a number of observations on variation of the cholera-vibrio, especially on the two well-known variants which are distinguished by forming (1) transparent and (2) yellow and slightly opaque colonies. He found that the serological differences were very slight and he did not describe a spontaneously agglutinable form.

It was with the purpose of searching for similar variants of the cholera-vibrio that the present investigation was undertaken. Two old laboratory strains of vibrio (1) "Konia," a haemolytic strain and (2) "Kolle," non-haemolytic, were inoculated into broth tubes and incubated at 37° C. for 24 hours. The broth tubes were then kept in the dark at room temperature. Subcultured on nutrient agar after 15 days, the "Konia" strain showed two varieties of colony similar to the two types named by Arkwright the "R" and "S." Even after four months, the "Kolle" strain showed no tendency to split off a "rough" variety.

MORPHOLOGY AND BIOLOGY OF THE TWO TYPES OF "KONIA" STRAIN.

The colonies of the "S" type on nutrient agar are circular, with regular, well-defined margins, and, when examined with a low power of the microscope, appear very finely granular. The colonies of the "R" type are larger than the "S" colonies, are flat and thin, and when examined under the low power, they are very granular and have a jagged margin. The "R" form, in ordinary nutrient broth or peptone water, produces a deposit at the bottom of the tube, leaving the supernatant liquid clear. The "S" form produces general turbidity. A pellicle is formed by both types. The precipitation in cultures of the "R" type is no doubt due to the salt content of the medium, since the "R" form produces general turbidity without any deposit if cultured on broth or peptone water diluted with distilled water to one-half or one-quarter of the usual strength; and this is also the case if the medium is prepared with less salt. As regards motility, fermentation of carbohydrates, indol formation and proteolysis, the two types are identical. The haemotoxin-production of the two types was practically the same as is shown by the following experiment:

72 hours' growth of the "R" and "S" types on ordinary broth were filtered through a Berkefeld candle. Different dilutions of the filtrates were prepared with normal saline solution. To 1 c.c. of each dilution, 1 c.c. of a 2 per cent. emulsion of sheep's red blood corpuscles was added. The tubes were kept in the water-bath at 37° C. for two hours and then in the ice chest till next morning.

Result. Haemolysis occurred in practically identical dilutions of the two filtrates, *i.e.* in 1 in 50 but not in 1 in 100.

DEGREE OF AGGLUTINABILITY BY SALTS.

Table 1 shows the agglutinability of the three strains, "Konia," "S" strain and "R" strain, by calcium chloride and sodium chloride.

Table 1.

	NaCl											
	$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$								
"Konia" strain	–	–	–	–								
"S" type	–	–	–	–								
"R" „	+ +	+ +	+	–								
	$CaCl_2$											
	$\frac{M}{2}$	$\frac{M}{4}$	$\frac{M}{8}$	$\frac{M}{16}$	$\frac{M}{32}$	$\frac{M}{64}$	$\frac{M}{128}$	$\frac{M}{256}$	$\frac{M}{512}$	$\frac{M}{1024}$	D.W.	
"Konia" strain	–	–	–	–	–	–	–	–	–	–	–	
"S" type	–	–	–	–	–	–	–	–	–	–	–	
"R" „	+	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	–	–	–	

$\frac{M}{2}$, $\frac{M}{4}$, $\frac{M}{8}$, etc. $= \frac{\text{Molar}}{2}$, $\frac{\text{Molar}}{4}$, $\frac{\text{Molar}}{8}$.

$\frac{M}{2}$ NaCl = 2·9 per cent. $\frac{M}{2}$ $CaCl_2$ = 5·5 per cent. D.W. = distilled water.

Liefmann (1913) described a method which he advocated for differentiating bacterial species by flocculating emulsions with strong solutions of magnesium sulphate. He found that true cholera-vibrios were as a rule completely salted out in 80 per cent. saturation, whereas cholera-like strains almost invariably failed to show precipitation. Greig (1913) and Gildemeister and Günther (1919) repeated and extended these observations. While confirming the general results, they pointed out that exceptions were not infrequent.

Emulsions of the original strain "Konia" and its two variants were tested with strong solutions of $MgSO_4$ and it was found that whereas "Konia" and the "S" form were flocculated in 100 per cent., 90 per cent. and slightly in 80 per cent. of saturated $MgSO_4$, the "R" type was flocculated in all dilutions examined, *i.e.* 100 per cent. to 10 per cent.

Porges and Prantschoff (1906) stated that if an emulsion of spontaneously agglutinating cholera vibrios was heated to 80° C. it was rendered stable, but that its agglutinability by specific serum was not impaired as occurred in the case of an emulsion of *B. typhosus*.

This procedure was tried with the "R" variant, but it was found that the agglutination by salts remained practically the same after heating.

Acid agglutination. Michaelis (1911) found that many species of bacteria were agglutinated by acids and that the agglutination for each species was at its optimum in a particular degree of acidity (*i.e.* of hydrogen-ion concentration). In the coli-typhoid group, he determined the special agglutination optima for *B. typhosus* and *B. paratyphosus* B and recommended this test as a method for the identification of these micro-organisms. Beniasch (1912), working under Michaelis, performed many experiments on the agglutination

of bacteria by acids. He came to the conclusion that the acid agglutination optimum was specific for the bacterial species and that even subgroups of the same species could in some instances be differentiated by this means.

Buffer solutions were prepared and the acid agglutination limits of the "Konia" strain, "S" type and "R" type were tested, by the method of Beniasch.

Result. The optimum agglutination of "S" occurred in a solution of higher acidity than that required by "Konia," while the agglutination of "R" took place over a wider range, with its centre in a lower acidity than the optimum for "Konia."

The agglutination optima for the emulsions were as follows:

"Konia"	[H]	$1{\cdot}1 \times 10^{-3}$
"S"	„	$2{\cdot}2 \times 10^{-3}$
"R"	„	between $2{\cdot}8 \times 10^{-4}$ and $1{\cdot}1 \times 10^{-3}$

According to Beniasch:

V. cholerae	[H]	$0{\cdot}7 \times 10^{-4}$
V. metschnikovi	„	$2{\cdot}8 \times 10^{-4}$

IMMUNITY REACTIONS.

Separate antisera were prepared against the "Konia" strain, the "S" type and the "R" type. Rabbits received intravenous inoculations of heat-killed 24 hour cultures (60° C. for 30 minutes). Three inoculations at 5-day intervals were given, and one week after the last inoculation the animals were bled. The sera were preserved in 0·5 per cent. carbolic acid.

Method of agglutination. The macroscopic method was used. The emulsions were always prepared in distilled water and the serum was diluted with saline solution (0·42 per cent. NaCl). The agglutination was carried out in an incubator at 37° C. for four hours.

Table 2.

Dilution of serum	"Konia" serum tested against			"S" type serum tested against			"R" type serum tested against		
	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type
1/50	+ + +	+ + +	+ +	+ + +	+ + +	+ +	+	+ +	+ + +
1/100	+ + +	+ + +	+ +	+ + +	+ + +	+ +	+	+	+ + +
1/200	+ + +	+ + +	+ +	+ + +	+ + +	+ +	+	−	+ + +
1/400	+ + +	+ + +	+ +	+ + +	+ + +	+	−	−	+ + +
1/800	+ + +	+ + +	+	+ + +	+ + +	+	−	−	+ + +
1/1600	+ +	+ +	(+)	+ + +	+ + +	−	−	−	+ +
1/3200	+ +	+ +	−	+ +	+ +	−	−	−	(+)
1/6400	−	−	−	+ +	+ +	−	−	−	−
1/12800	−	−	−	(+)	+	−	−	−	−
1/25600	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−

+ + + =complete agglutination. + =distinct agglutination.
+ + =good agglutination. (+) =very slight agglutination.

These agglutination tests show that there is only slight cross-agglutination between the "S" and "R" varieties. The "S" type behaved like the parent "Konia" strain.

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Type of agglutination. The two variants showed a marked difference in the type of agglutination. The "S" emulsion was agglutinated in large clumps, settling to a dense deposit at the bottom of the tube, leaving the supernatant fluid nearly clear. Shaking the tube produced a suspension of large clumps. The "R" emulsions after agglutination formed a deposit of fine clumps which made a uniformly turbid emulsion when shaken up.

Absorption tests. These tests were carried out by mixing 1·5 c.c. of serum (diluted to 1/25 in 0·42 per cent. salt solution) with 1·5 c.c. of a strong emulsion of the culture in distilled water. This emulsion was made by adding the growth of one agar slope to 0·5 c.c. of water. After incubation at 37° C. for two hours, the mixture was put in the ice chest till the next day and then centrifuged. The supernatant fluid was examined for its agglutinating power in the way already described.

Table 3.

Dilution of serum	"Konia" serum absorbed with "Konia" and tested against			"Konia" serum absorbed with "S" type and tested against			"Konia" serum absorbed with "R" type and tested against		
	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type
1/100	–	–	+ +	(+)?	–	+	+ + +	+ + +	+
1/200	–	–	+	–	–	+	+ + +	+ + +	+
1/400	–	–	+	–	–	+	+ + +	+ +	+
1/800	–	–	–	–	–	–	+	–	–
1/1600	–	–	–	–	–	–	–	–	–
Control	–	–	–	–	–	–	–	–	–

Table 4.

Dilution of serum	"S" serum absorbed with "Konia" and tested against			"S" serum absorbed with "S" type and tested against			"S" serum absorbed with "R" type and tested against		
	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type
1/100	+	+	+ +	(+)	(+)	+ +	+ + +	+ + +	+
1/200	(+)	(+)	+	–	–	+ +	+ + +	+ + +	(+)
1/400	–	–	+	–	–	+	+ + +	+ + +	–
1/800	–	–	–	–	–	(+)	+ + +	+ + +	–
1/1600	–	–	–	–	–	–	+ + +	+ + +	–
1/3200	–	–	–	–	–	–	+ +	+	–
1/6400	–	–	–	–	–	–	+	(+)	–
1/12800	–	–	–	–	–	–	–	–	–
Control	–	–	–	–	–	–	–	–	–

Table 5.

Dilution of serum	"R" serum absorbed with "Konia" and tested against			"R" serum absorbed with "S" type serum and tested against			"R" serum absorbed with "R" type serum and tested against		
	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type
1/100	–	–	+ + +	–	–	+ + +	(+)?	(+)?	(+)
1/200	–	–	+ + +	–	–	+ + +	–	–	–
1/400	–	–	+ + +	–	–	+ + +	–	–	–
1/800	–	–	+ +	–	–	+ +	–	–	–
1/1600	–	–	(+)	–	–	(+)	–	–	–
1/3200	–	–	–	–	–	–	–	–	–
Control	–	–	–	–	–	–	–	–	–

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The results of the absorption experiments show that "Konia" and "S" sera lost their agglutinins for both these strains when absorbed with the "Konia" and "S" type strains, but not when absorbed with the "R" type strain.

The "R" serum lost its agglutinins when absorbed with the "R" strain but not when absorbed with either "Konia" or "S" strain.

Complement fixation: The serological relations shown to exist between the three varieties were also confirmed by complement fixation experiments.

Technique: The antigen was prepared by emulsifying a 24 hour agar slope culture in 10 c.c. of physiological saline solution (killed by heating at 58° C. for 30 minutes). 0·5 c.c. of the diluted serum + 0·5 c.c. antigen + 0·5 c.c. of fresh guinea-pig serum (diluted 1 : 10) were placed in the water bath for 40 minutes. Then 0·5 c.c. of a 5 per cent. emulsion of sheep's red blood corpuscles + 0·5 c.c. amboceptor (4 m.h.d.) were added. After 30 minutes in the water bath, the tubes were placed in the ice chest and the result read after four hours.

Table 6.

Dilution of serum	"Konia" serum with antigens			"S" type serum with antigens			"R" type serum with antigens		
	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type	"Konia"	"S" type	"R" type
1/50	+	+	+	+	+	+	+	+	+
1/100	+	+	+	+	+	+	+	+	+
1/200	+	+	t.h.	+	+	+	t.h.	t.h.	+
1/400	+	+	t.h.	+	+	−	−	−	+
1/800	+	+	−	+	+	−	−	−	+
1/1600	+	+	−	+	+	−	−	−	t.h.
1/3200	+	+	−	+	+	−	−	−	−
1/6400	t.h.	t.h.	−	+	+	−	−	−	−
1/12800	−	−	−	+	+	−	−	−	−
1/25600	−	−	−	+	+	−	−	−	−
Control serum	−	−	−	−	−	−	−	−	−
Control antigen	−	−	−	−	−	−	−	−	−

+ = complete inhibition. − = no inhibition. t.h. = trace haemolysis.

PATHOGENICITY.

The original "Konia" strain was of a very low virulence, but, in view of de Kruif's results with the two types of *B. lepisepticum*, an experiment was made in order to detect any difference in pathogenicity between the two variants.

Guinea-pigs of about equal weights were inoculated intraperitoneally with 1/20, 1/10, 1/5, 1/2 and 1 of a 24 hours' agar slope culture of both types.

Table 7.

	"S" type	"R" type
1/20 Agar Slope	Survived	Survived
1/10 ,,	Survived	Survived
1/5 ,,	Death	Survived
1/2 ,,	Death	Death
1 ,,	Death	Death

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This experiment was repeated with the same result, from which it would appear that the "S" type is more pathogenic than the "R" type.

Resistance.

It was interesting to see if any difference existed between the two types in their resistance to chemical disinfectants. Two methods of testing this point were used.

Inhibition of growth. To six tubes, each containing 4 c.c. of sterile broth, decreasing quantities of carbolic acid were added. The tubes were then made up with broth to 5 c.c. The inoculation was carried out as follows: 24-hour agar cultures were emulsified each in 2 c.c. physiological saline solution and after being brought to the same opacity, the emulsions were further diluted 50 times. 0·1 c.c. of this dilution was added to the tubes. The tubes were placed in the incubator at 37° C. for 7 days. The results were recorded after 1, 3, 5, and 7 days. Both the "S" and the "R" type multiplied and caused turbidity in 1/1600 carbolic acid but not in 1/800.

Death of organisms. Emulsions were prepared as in the previous experiment. Three drops of the emulsion were added to 5 c.c. of 0·5 per cent. carbolic acid. Directly and after 5, 10, 15, 30 and 60 minutes two loopfuls were inoculated into broth tubes which were incubated and the result recorded as in the preceding experiment.

Result: Both types were viable after 5 but not after 10 minutes.

These experiments show that the two types have practically the same resisting power to the disinfectant used.

The resistance to heat was tested by exposing emulsions to a temperature of 45° C. for varying periods.

Technique. One loopful of a 24 hour agar culture was emulsified in 5 c.c. distilled water. The tubes were put in the water bath at 45° C. and after 5, 10, 15, 20, 30, 40, 50 and 60 minutes one loopful was inoculated into broth.

Result: The "S" type could be subcultured after 40 and the "R" type after 50 minutes but not after longer periods, though the control tubes without carbolic were viable after 60 minutes.

This experiment, which was repeated many times, showed that the "R" variant is to a slight degree more resistant to heat than the "S" variant.

Conclusions.

1. Of two strains of *Vibrio cholerae* tested, one (a haemolytic strain) yielded two variants "S" and "R" resembling those described by Arkwright as occurring in the colon-typhoid group.

2. The "S" variant forms a stable emulsion in physiological saline and causes general turbidity in broth and peptone cultures. The "R" variant agglutinates spontaneously in physiological saline solution. In broth culture it forms a deposit, leaving the supernatant fluid clear. Emulsions in weaker saline solutions are stable, so that agglutination tests can be carried out.

3. The two variants differ in the following characters:
   (*a*) cultural characters on agar,
   (*b*) acid agglutination optima,
   (*c*) agglutination with specific sera, absorption of agglutinins and complement fixation,
   (*d*) pathogenicity,
   (*e*) resistance to heat.
4. They are identical as regards:
   (*a*) motility,
   (*b*) fermentation of carbohydrates,
   (*c*) formation of indol,
   (*d*) proteolysis,
   (*e*) haemotoxin production.

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