CHANGE OF ACID AGGLUTINATION OPTIMUM AS INDEX OF BACTERIAL MUTATION.

By PAUL H. De KRUIF.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 16, 1921.)

INTRODUCTION.

Two distinct varieties of microbe have been shown to exist in cultures of the bacillus of rabbit septicemia (1). These have been designated as Microbes D and G. Microbe D is the variety isolated from rabbits dead of spontaneous infection with the rabbit septicemia bacillus. It is characterized by diffuse growth in serum and plain broth, forms opaque fluorescing colonies on serum agar, and is highly virulent for rabbits. Microbe G, first discovered accidentally in Microbe D cultures, has been proved to be a true mutant of the parent D form (2). The mutant Microbe G grows in granular fashion in liquid media, forms translucent bluish colonies with no fluorescence, and exhibits extremely low virulence for rabbits. The mutation experiments demonstrating that Microbe D, under controllable conditions, changes into Type G were performed with D strains arising from single individuals isolated by Barber's pipette.

The granular growth of Microbe G in fluid medium is one of its most striking differential characters, and has persisted throughout transplants for more than 1 year. This sedimenting growth of Type G in broth, compared to the evenly suspended, uniformly turbid appearance of broth cultures of Type D led to an examination of the acid agglutination optima of the two types.

Methods.

The method for the determination of the acid agglutination optimum was that of Michaelis (3), later described in full by Beniasch (4). It consisted in mixing carefully prepared suspensions of the organism

to be tested with equal volumes of buffer mixtures of varying C_H^+. Two buffer series were employed, Na lactate-lactic acid and Na acetate-acetic acid. The mixtures were made according to Tables I and II.

TABLE I.

Na Lactate-Lactic Acid Series.

pH	4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Na Lactate N/10.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lactic acid N/10.....	0.06	0.12	0.25	0.5	1.0	—	—	—	—
Lactic acid N.....	—	—	—	—	—	0.2	0.4	0.8	1.6
Distilled water.....	1.54	1.48	1.35	1.1	0.6	1.4	1.2	0.8	—

TABLE II.

Na Acetate-Acetic Acid Series.

pH	5.6	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	
Na Acetate N/10.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Acetic acid N/10.....	0.06	0.12	0.25	0.5	1.0	—	—	—	—	Final volume, 2.1 cc.
Acetic acid N.....	—	—	—	—	—	0.2	0.4	0.8	1.6	
Distilled water.....	1.54	1.48	1.35	1.1	0.6	1.4	1.2	0.8	—	

Preparation of Suspensions.

Microbe G sediments rapidly in fluid media. It fails to remain in even suspension when the sediment from a centrifuged culture is taken up in 0.85 per cent NaCl. If, however, such sediments are repeatedly washed in large volumes of distilled water, very stable suspensions can be obtained. In order to secure perfect comparability, Microbe D was treated in a similar manner, although in this case washing with distilled water is unnecessary and it yields stable suspensions in 0.85 per cent NaCl. The technique of preparation of suspensions was as follows.

5 per cent rabbit serum broth cultures, 24 hours incubation, were centrifuged, the supernatant fluids discarded, and the sediments thoroughly shaken in a volume of distilled water equal to that of the

original culture. The centrifugation and resuspension in distilled water were repeated four times in all. The final suspensions were carefully brought to a uniform turbidity.

EXPERIMENTAL.

All experiments were carried out by adding 1 cc. of distilled water suspension of the microbe in question to an equal volume of each of the buffer mixtures just described. The tubes were carefully shaken, placed in the water bath at 43°C. and readings taken at 1, 2, and 16 hours.

TABLE III.

Acid Agglutination Optimun of Microbes D and G, Strain R 15. Na Lactate-Lactic Acid Buffer Series.

Organism.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH. Final reading.
	pH	4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4	
Microbe D.	1 hour.	0	0	0	0	+	+	0	0	0	3.5–3.3
	2 hours.	0	0	0	0	++	++	+	+	Tr.	
	16 "	0	0	0	0	C	C	+	Tr.	Tr.	
Microbe G.	1 hour.	Tr.+	+	+	++	+	0	0	0	0	4.7–3.8
	2 hours.	Tr.+	++	++	C	++	0	0	0	0	
	16 "	C	C	C	C	+	Tr.+	0	0	0	

In this and the following tables lesser degrees of floculation are recorded as ++, +, and Tr. (trace). C indicates complete flocculation.

The agglutination optimum was considered to be that zone of $C_H{}^+$ where complete flocculation occurred; that is, where the microbes sedimented so perfectly as to leave a water-clear supernatant fluid. Lesser degrees of agglutination are recorded as ++, +, and Tr. (trace). The readings are greatly facilitated by holding the tubes before a powerful beam of light, projected downward in front of a dark background.

Experiment 1. Acid Agglutination Optimum, Microbes D and G, Strain R 15, in Na Lactate-Lactic Acid Buffer Series.—Suspensions of Microbes D and G, isolated from Strain R 15, bacillus of rabbit septicemia, were tested against the Na lactate-lactic acid buffer series. The experiments were carried out as described above. The results are recorded in Table III.

The results recorded in Table III show a definite difference in acid agglutination optimum of the two varieties. The readings, taken at 1,2, and 16 hours, indicate that the reaction does not take place with the speed of that of immune agglutination of the majority of bacteria. It is necessary to allow ample time to elapse before taking the final readings. 16 hours have been found to be sufficient, no material change in readings being noted after this time.

The same suspensions were tested against the Na acetate-acetic acid buffer series, with a similar result recorded in Table IV. Temperature, as before, was 43°C. in the water bath.

TABLE IV.

Acid Agglutination Optimum of Microbes D and G. Na Acetate-Acetic Acid Series.

Organism.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH.
	pH	5.5	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	
Microbe D.	1 hour.	0	0	0	0	0	0	0	+	+	3.5 –3.2
	2 hours.	0	0	0	0	0	0	0	C	C	
	16 "	0	0	0	0	0	0	0	C	C	
Microbe G.	1 hour.	0	0	0	+	++	++	+	0	0	4.75–3.8
	2 hours.	0	0	0	++	C	C	++	0	0	
	16 "	Tr.+	Tr.	++	C	C	C	C	+	Tr.	

The results in the case of the Na acetate-acetic acid series correspond to those of the Na lactate-lactic acid mixtures. The final readings are identical, the only difference lying in a slightly more rapid flocculation in the acetate series.

Variability of Optimum of Microbe G as Compared to that of Microbe D.

A number of strains were now collected, the D and G types isolated and subjected to test with the Na acetate-acetic acid series. The result (Table V) confirms the findings of the previous experiments. Final reading was made after 16 hours with incubation at 43°C. The results are presented in Table V.

It will be noted that the acid agglutination optimum for Microbe D is the same in case of all three of the strains tested. On the other hand, the optimum for Microbe G varies to a considerable extent. This variation is never so great as to prejudice its value as a criterion of differentiation from the parent D form. In all cases complete flocculation of Type G occurs at a distinctly lower $C_H{}^+$ than that of Type D. The difference between the two types in regard to the smallest amount of hydrogen ion in which complete flocculation takes place is never less than 0.6 pH. In short, the organism in the process of mutation gains in sensitivity to flocculation in the presence of H ions.

TABLE V.

Acid Agglutination Optima of D and their Mutant G Forms.
Na Acetate-Acetic Acid Series.

Strain.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH.
	pH	5.6	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	
R 15	D-S 49	0	0	0	0	0	0	0	C	C	3.5 –3.2
	G-S 52	Tr.	Tr.	++	C	C	C	C	+	Tr.	4.75–3.8
	G-S 28*	Tr.	Tr.	C	C	C	C	C	++	++	5.05–3.8
R 11	D-S 43	0	0	0	0	0	0	0	C	C	3.5 –3.2
	G-S 42	0	0	+	+	+	C	C	C	+	4.1 –3.5
R 22	D-S 31	0	0	0	0	0	0	Tr.	C	C	3.5 –3.2
	G-S 32	0	Tr.	+	+	C	C	C	++	Tr.	4.4 –3.8

* G-S 28, a mutant from the same parent D strain as G-S 52.

Sobernheim and Seligmann (5) found a strain of *Bacillus enteritidis* to separate into two races. Beniasch (4) tested the acid agglutination point of this organism and found it to have altered its acid agglutination optimum when tested on two different occasions, a year having elapsed between the two tests. In this work apparently no attempt was made to establish the occurrence of a mutation, or to separate the two varieties.

. Variations in the Agglutination Optimum of Type G.

Table V indicates that the agglutination optima of various strains of Microbe G are not as strictly uniform as those of the parent D type. One of the causes of this variation is passage of the microbe through the animal body. An example of this variation was observed during an attempt to cause reversion of Microbe G to the parent D form.

The Type G strain in question was characteristically of very low virulence. 1.0 cc. of a serum broth culture, injected intrapleurally, was required to produce fatal infection of a 600 gm. rabbit. The organism recovered at necropsy of this animal was cultured and injected into a second animal, and so on. At the third animal passage

TABLE VI.

Effect of Animal Passage on Acid Agglutinability of Type G.

	pH	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2
Microbe R 15 G	Before passage.	Tr.	Tr.	++	C	C	C	C	+	Tr.
	After three animal passages.	C	C	C	C	C	C	C	C	C

the virulence had greatly increased, 10^{-4} cc. of a serum broth culture being fatal. But the organism, far from returning to the uniformly turbid growth character of the Type D form, became more intensely granular in its growth. This characteristic was so marked that difficulty was experienced in preparing the washed suspensions for acid agglutination test.

The acid agglutination reaction of Type G strain after animal passage was compared with the same strain which had been transplanted in parallel in serum broth. The Na acetate-acetic acid buffer series was used. The culture was incubated at 43°C. for 16 hours (Table VI).

It will be seen from Table VI, first, that much less hydrogen ion is required to produce complete flocculation, and second that the optimum is very greatly broadened. It has been widened from

pH 4.7 to 3.8 before animal passage, to pH 5.6 to 3.2 after passage through three rabbits.

Up to the present, the change in acid agglutination optimum that occurs during mutation has been accompanied invariably by a great loss in virulence. For example, all Type D strains tested have been fatal to rabbits in doses of 10^{-5} to 10^{-7} of a serum broth culture. The Type G forms arising from such strains are seldom fatal in 0.5 cc. of undiluted culture. Frequently rabbits are able to resist 1.0 cc.

The experiment just described indicates that the decrease in stability to acid does not necessarily go hand in hand with loss of virulence, and certainly bears no causal relationship to such loss. For, while the stability to the hydrogen ion had greatly *decreased* during animal passage, the virulence had *increased* from 0.5 cc. to 10^{-4} cc.

SUMMARY AND CONCLUSIONS.

A distinct difference in acid agglutination optimum for Type D (bacillus of rabbit septicemia) and its mutant form, Type G, has been observed. The optimum for Type D lies between pH 3.5 and pH 3.0. This changes during mutation, the resulting Type G mutants having in general an optimum lying between pH 4.7 and pH 3.8.

The constancy of the optimum for Type D is very strict, while that for Type G is slightly less so. The variation is never so great as to cause an overlapping of optima and consequent failure of differentiation.

These acid agglutination optima are in the nature of physical constants for the two types and would imply a fundamental difference in the chemical constitution of the organisms.

Animal passage, far from causing a reversion of the mutant Type G to the primordial Type D form, brings about a still greater instability in the presence of H ions.

BIBLIOGRAPHY.

1. De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.
2. De Kruif, P. H., *Proc. Soc. Exp. Biol. and Med.*, 1921, xix, 34.
3. Michaelis, L., *Deutsch. med. Woch.*, 1911, xxxvii, 969.
4. Beniasch, M., *Z. Immunitätsforsch., Orig.*, 1911–12, xii, 268.
5. Sobernheim, G., and Seligmann, E., *Z. Immunitätsforsch., Orig.* 1910, vii, 342.