THE ANTIGENIC STRUCTURE AND SPECIFICITY OF LUMINOUS BACTERIA

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Although the immunological reactions of the luminous bacteria have received little study, they justify investigation from three points of view: first, the nature of their antigenic structure; second, the extent of species specificity in the reactions concerned; and, finally, the significance of antigenic components in the luminescent system.

This study has included agglutination, agglutinin absorption, and precipitation tests in relation to whole cells as well as the filtrates obtained from cytolytic products. These procedures were applied to all the well-authenticated species available. Previous studies in which immunological techniques were employed have dealt almost exclusively with whole cells and with only very restricted groups of species or strains (Ballner, 1907; Ninomya, 1924; Meissner, 1926; Majima, 1931). Varying degrees of specificity and the occurrence of group reactions have been noted in these limited studies. Johnson (1941) cytolyzed the cells of two marine species in distilled water, and found a specific agglutination of the "ghosts" as well as the normal cells, and a specific precipitation of Berkefeld filtrates of the cytolyzates.

The phenomena associated with the cytolysis of these organisms have been extensively investigated from the point of view of physiological activity (Harvey, 1915; Hill, 1929; Korr, 1935a, 1935b; Johnson and Harvey, 1937, 1938) as well as the fine structure of the cells (Johnson, Zworykin, and Warren, 1943). In the report by Harvey and Deitrich (1930), however, the production of antibodies against the oxidative enzyme (luciferase) concerned in the luminescence of extracts of the invertebrate animal *Cypridina* is described.

Although a decrease in light intensity of luminous bacteria has been observed in the presence of immune serum (Ninomya, 1924), the results thus far reported have evidently been due entirely to the agglutination of the cells. A loss of a completely adequate gaseous respiratory exchange occurs concomitantly with agglutination. Furthermore, as observed in the present study, easy confirmation of this explanation may be obtained by observing the reappearance of the original light intensity upon vigorously shaking the agglutinated cells.

MATERIALS AND METHODS

The species used in these studies are *Vibrio albensis* (Lehmann and Neumann, 1901)[2]; *Vibrio phosphorescens*[3]; *Achromobacter harveyi* (Johnson and Shunk,

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[2] Cultures of these species, under the generic name *Photobacterium*, were obtained in 1939 from the Delft Collection, through the kindness of Professor A. J. Kluyver.

[3] Original culture was kindly supplied by Professor M. H. Soule.

1936); *Achromobacter fischeri* (Beij.) Bergey *et al.* (Bergey, 1934; Johnson and Shunk, 1936); *Photobacterium phosphoreum* (Cohn) Beij. (1912, 1916)[2]; *Bacillus pierantonii* (Zirpolo, 1918)[2]; *Bacillus sepiae* (Zirpolo, 1917)[2]; and *Photobacterium splendidum* (Beijerinck, 1916)[2]. Of the eight species, six are marine forms and were cultivated on 3 per cent NaCl beef infusion agar. The remaining two, *Vibrio albensis* and *Vibrio phosphorescens*, are "fresh water" species and were cultivated on the same medium as the marine species, except that the medium contained only 0.9 per cent NaCl. The medium was adjusted to pH 7.3 and sterilized at 15 pounds' pressure for 15 minutes. *P. phosphoreum*, a psychrophilic type, was cultivated at 15 C., but the others were incubated at 25 C.

These organisms are as authentic as could be obtained. Their possible identity with organisms described in the early literature (Beijerinck, 1889, 1890, 1916; Dahlgren, 1915; Gorham, 1903; Lehmann and Neumann, 1901; Mangold, 1910; Migula, 1897, 1900; Molisch, 1912) cannot be fully established in all cases. The literature concerning the luminous species, especially in regard to nomenclature, is unusually confusing.[4] The problem is perhaps somewhat further complicated by the occurrence of variants (cf. Beijerinck, 1912; Doudoroff, 1938; Giese, 1943). The characteristics of the species used in this study have, therefore, been reinvestigated and are summarized in tables 1, 2, and 3.

The organisms that have been previously studied are *Vibrio rumpel* (Ballner, 1907); *Vibrio pierantonii, Coccobacillus pierantonii, Bacillus sepiae* (Meissner, 1926); and *Vibrio pierantonii, Coccobacillus pierantonii, Vibrio euprima, Micrococcus sepiola, Coccobacillus tolega, Vibrio yasakii* (Majima, 1931).

EXPERIMENTAL

Agglutination Reactions

Luminous cultures were suspended in sterile blanks of the appropriate salt concentration. Rabbits were then injected in duplicate with each organism

[4] For example, Fischer, in 1888, discovered what he termed an "*Einheimischer Leuchtbacillus*" in the water of the Kiel harbor. In 1889 Beijerinck included all luminous bacteria in the special genus *Photobacterium* and renamed Fischer's bacterium, *Photobacterium fischeri*. Katz described an organism which he designated as *Bacillus argenteo-phosphorescens I.* Migula (1897, 1900) maintained that this organism was a variety of *Photobacterium fischeri* (Beij.) and classified it as such in his *System der Bakterien* (1900). However, Katz had previously isolated *Bacillus argenteo-phosphorescens* which Migula (1900) assumed to be a synonym of *Bacillus argenteo-phosphorescens I.* In 1897 Migula rejected Beijerinck's *Photobacterium* genus for this organism and included it in the genus *Bacillus*, i.e., *Bacillus fischeri* (Beij., Mig.). Flügge (1896) reclassified this organism under *Bacillus phosphorescens-indigenus* Kruse. Further synonyms have been given: *Vibrio fischeri* (Beij.) (Lehmann and Neumann, 1901), *Achromobacter fischeri* (Beij.) Bergey *et al.* (1930).

A further illustration of the susceptibility of these organisms to possible errors in classification appears in Bergey's *Manual of Determinative Bacteriology*, 5th edition, 1939. Under the description of *Pseudomonas phosphorescens* (Fischer) Bergey, the following organisms are cited as synonyms: *Photobacterium phosphorescens* (Beijerinck, 1890); *Bacillus phosphorescens* (Fischer, 1888); and *Bacterium phosphorescens* (Migula, 1900). However, when the literature is consulted, it is apparent that *Bacillus phosphorescens* is evidently not synonymous with any of the other organisms mentioned above. *Photobacterium phosphorescens*, as Bergey indicates, is synonymous with *Bacterium phosphorescens*, whereas *Bacillus phosphorescens* is a distinct species (Migula, 1897).

TABLE 1

Major characteristics of eight species of luminous bacteria

ORGANISM	MORPHOLOGY	MOTILITY	FLAGELLA (ELECTRON MICROSCOPE)	CAPSULE*	OPTIMUM NaCl CONCENTRATION	GELATIN (LIQUEFACTION)	REDUCTION OF NITRATE TO NITRITE	INDOLE†	BLOOD AGAR			STARCH DIGESTION	H_2S
									Growth	Luminescence	Hemolysis‡		
Fresh-water species—0.9% NaCl included in media													
					%								
V. albensis....	Vibrio single and pairs 1.2–2.1 μ gram–	+	Mono-tri-chate	–	0.9	++	+	+	++	+	Beta	+	–
V. phosphorescens.....	Vibrio single and pairs 0.8–2.5 μ gram–	+	Mono-tri-chate	–	0.9	++++	+	+	++	+	Alpha	+	–
Marine species—3% NaCl													
A. harveyi.....	Rods—str., curved single and pairs. Ends: pointed, rounded, 1.2–2.3 μ gram–	+	Peri- or lopho-tri-chate	–	3	+++	+	+	++	+	–	+	+
A. fischeri.....	Rods—str., curved single and pairs. Ends: pointed, rounded, 0.9–1.8 μ gram–	+	Peri- or lopho-tri-cate	–	3	+	+	–	++	+	–	–	+
P. phosphoreum	Oval cocci single and pairs 1.2–2.2 μ gram±	–	–	+	3	–	+	–	+	+	–	–	–
B. pierantonii.	Rods—str., curved rounded ends 0.9–2.2 μ gram –	+	?	–	3	+	+	–	++	+	Alpha	–	–
B. sepiae.....	Rods—str., single, pairs, chains, 1.2–2.6 μ gram–	+	Peri- or lopho-tri-cate	–	3	+++	+	+	++	+	Alpha	+	+
P. splendidum	Rods—str. single and pairs 1.0–2.8 μ gram –	+	Peri- or lopho tri-cate	–	3	+++	+	+	++	+	Alpha	+	–

* Welch's method.

† Gore's method.

‡ The hemolysis results are unaccountably variable.

TABLE 2

Characteristics of luminous bacteria (con.)

ORGANISM	GLUCOSE		LACTOSE		SUCROSE		KOSER'S CITRATE MEDIUM		GROWTH IN PEPTONE BROTH (72 hours)				
	Acid	Gas	Acid	Gas	Acid	Gas	Growth	Luminescence	8–10 C	15 C	20 C	25 C	37 C
Fresh-water species—0.9% NaCl													
V. albensis	+	−	−	−	+	−	+	+	−	+	++	+++	++++
V. phosphorescens	+	−	−	−	+	−	+	++	−	++	++++	++++	++++
Marine species—3% NaCl													
A. harveyi	+	−	−	−	+	−	++++	++++	−	+++	++++	++++	++++
A. fischeri	+	−	−	−	−	−	−	−	−	+++	+++	+++	−
P. phosphoreum	+	+	−	−	+	−	+	++	++	++++	++	+	−
B. pierantonii	+	−	−	−	+	−	++	±	−	+++	+++	+++	++++
B. sepiae	+	−	−	−	+	−	+	−	−	+++	++++	++++	++++
P. splendidum	+	−	−	−	−	−	++	−	−	++++	++++	++++	++++

TABLE 3

Characteristics of luminous bacteria (cont.)

ORGANISM	LUMINESCENCE (72 HOURS)					PERSISTENCE OF LUMINESCENCE (DAYS)					PER CENT NaCl TOLERANCE (PEPTONE BROTH)			
											Growth		Luminescence	
	8–10 C	15 C	20 C	25 C	37 C	8–10 C	15 C	20 C	25 C	37 C	Min	Max	Min	Max
Fresh-water species—0.9% NaCl														
V. albensis	−	++	++	+++	+++	−	11	19	16	11	0	4.1	0	1.2
V. phosphorescens	−	−	++++	++++	+++	−	15	25	26	6	0	4.5	0	2.1
Marine species—3% NaCl														
A. harveyi	−	±	++	++	−*	−	33	32	34	2	0.6	8.7	0.6	6.9
A. fischeri	−	++	++++	++++	−	−	48	46	43	−	0.6	6.9	1.5	6.0
P. phosphoreum	++	++++	++++	++	−	71	59	43	23	−	0.9	5.4	0.9	5.1
B. pierantonii	−	+	+	++	++	−	7	17	12	−	0.3	6.9	2.4	4.1
B. sepiae	−	−	++	++++	−	−	17	26	19	−	0.6	8.7	6.0	7.8
P. splendidum	−	++	+++	+++	−*	−	32	18	13	2	0.3	9.0	0.6	6.9

* Luminescence appears at an earlier incubation period.

over a period of 5 weeks. The animals received 1-ml suspensions of freshly prepared luminous 18- to 24-hour cultures at 48-hour intervals over a 3-week period. A rest period of 7 days followed, after which they received a 2-ml intra-

TABLE 4

Agglutination and cross agglutination of eight species of luminous bacteria with rabbit antisera prepared from the following luminous cell antigens: V. albensis, A. fischeri, A. harveyi, V. phosphorescens, P. phosphoreum, B. pierantonii, B. sepiae, and P. splendidum

(The table includes all cross agglutinations that were found)

ANTIGEN	DILUTION OF ANTISERUM (FINAL DILUTION)												CONTROL
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	1:20480	1:40960	
A. Anti-albensis													
V. albensis	4	4	4	4	4	4	3	2	2	1	1	0	0
V. phosphorescens	2	2	2	2	2	1	1	1	0	0	0	0	0
B. Anti-phosphorescens													
V. phosphorescens	4	4	4	4	4	4	4	2	1	1	0	0	0
V. albensis	4	4	4	4	4	4	3	2	1	0	0	0	0
C. Anti-harveyi													
A. harveyi	4	4	4	4	3	2	2	1	0	0	0	0	0
P. splendidum	2	2	1	1	1	0	0	0	0	0	0	0	0
D. Anti-splendidum													
P. splendidum	4	4	4	4	3	2	2	2	1	1	0	0	0
B. sepiae	3	1	1	1	±	0	0	0	0	0	0	0	0
E. Anti-sepiae													
B. sepiae	4	4	4	4	3	2	1	1	0	0	0	0	0
F. Anti-fischeri													
A. fischeri	4	4	4	4	4	4	3	2	1	0	0	0	0
G. Anti-phosphoreum													
P. phosphoreum	4	4	4	4	3	3	2	1	1	0	0	0	0
H. Anti-pierantonii													
B. pierantonii	4	4	4	4	4	4	4	3	2	2	1	0	0

0 indicates no agglutination; 1, trace; 2, moderate; 3, almost complete; and 4, complete agglutination.

venous injection. After a second rest period of similar length, the rabbits were bled by heart puncture. The serum of each rabbit was tested at definite intervals during the period of injections for agglutination titer.

Antisera were set up in dilutions ranging from 1:20 to 1:40,960, using the appropriate salt concentration for the dilution of the antisera. To each tube

and the control, 0.5 ml of the homologous antigens were added. At the same time, cross agglutination reactions were carried out with each of the 8 species. All tubes were incubated in a 25 C water bath for 4 hours, after which they were stored overnight in an icebox and read the following morning.

The study of the agglutination reactions was complicated by the autoagglutination of several species. This was especially true of *V. albensis* and *A. fischeri*. However, this was remedied by centrifuging and washing the cells thoroughly.

An examination of the data reveals that *A. fischeri*, *P. phosphoreum*, *B. pierantonii*, and *B. sepiae* are antigenically specific. Cross agglutination occurs between *V. albensis* and *V. phosphorescens*, between *A. harveyi* and *P. splendidum*, and between *P. splendidum* and *B. sepiae*.

The cross agglutination of *A. harveyi* and *P. splendidum* has been confirmed by Johnson (unpublished). However, since only *A. harveyi* antiserum will react with *P. splendidum* and not vice versa, the relationship is obviously not of a reciprocal nature. This is also true in the case of *P. splendidum* antiserum and *B. sepiae* antigen. On the other hand, the *V. albensis*-*V. phosphorescens* cross reaction occurs reciprocally with these two organisms. It is interesting to note that although anti-*V. albensis* serum cross-agglutinates *V. phosphorescens*, the homologous strain is agglutinated to a higher titer. When anti-*V. phosphorescens* serum is used, an equal titer occurs with both antigens.

Agglutinin Absorption

For agglutinin absorption tests, heavy suspensions of cells were harvested in the appropriate sodium chloride solution, centrifugalized, and washed once. Absorptions were carried out in a final 1 to 20 dilution of serum. The tubes were well shaken and then incubated at 25 C for 3 hours. This was followed by strong centrifuging, i.e., until the supernatant was practically clear. This procedure was repeated with the supernatant serum and a fresh suspension of cells. Following the second incubation period, the tubes were left in the icebox overnight before centrifuging.

The results of the agglutinin absorption tests, which are shown in table 5, confirm the results of the straight agglutination reactions. It is evident that *P. splendidum* almost completely absorbs its corresponding antibody from anti-*A. harveyi* serum without appreciably changing the agglutination titer of the serum with respect to *A. harveyi*. Similarly, *B. sepiae* absorbs its antibody from anti-*P. splendidum* serum without altering the titer of the serum for *P. splendidum*.

Cross absorption experiments were set up for both *V. albensis* and *V. phosphorescens*. Although it was not possible[5] for *V. albensis* to absorb the entire *V. phosphorescens* agglutinin from the anti-*V. phosphorescens* serum, a good fraction of the heterologous agglutinin was absorbed. On the other hand, the ab-

[5] In view of the close antigenic relationship between *V. albensis* and *V. phosphorescens*, cross absorption experiments were repeated several times. Modifying the technique, i.e., dilution of sera, incubation period, and temperature, did not alter the absorption results.

sorption of anti-*V. albensis* serum by *V. phosphorescens* resulted in an almost complete absorption of antibodies for both antigenic factors. In addition, *V. phosphorescens* almost completely absorbs from its homologous antiserum both *V. albensis* and *V. phosphorescens* agglutinins. These conclusions are reached from the agglutination reactions with the absorbed antisera (table 5).

Practically a complete absorption of anti-*V. albensis* serum occurs in the presence of *V. phosphorescens* antigen. A significant fundamental agglutinogenic similarity exists. Anti-*V. phosphorescens* serum will not only agglutinate both *V. phosphorescens* and *V. albensis* to full titer, but agglutinins of either species are almost completely absorbed by that organism.

TABLE 5

The effect of absorbing cross-agglutinative immune sera with the heterologous antigens

DILUTION OF ABSORBED SERUM													
Antiserum-antigen	Agglutinating organism	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	1:20480	1:40960	CONTROL
Anti-splendidum absorbed by *B. sepiae*	*B. sepiae*	±	±	0	0	0	0	0	0	0	0	0	0
	P. splendidum	4	4	4	4	2	2	2	1	1	1	0	0
Anti-harveyi absorbed by *P. splendidum*	*P. splendidum*	1	±	0	0	0	0	0	0	0	0	0	0
	A. harveyi	4	4	4	4	3	1	1	1	0	0	0	0
Anti-albensis absorbed by *V. phosphorescens*	*V. phosphorescens*	1	1	1	0	0	0	0	0	0	0	0	0
	V. albensis	1	±	0	0	0	0	0	0	0	0	0	0
Anti-phosphorescens absorbed by *V. albensis*	*V. albensis*	1	1	0	0	0	0	0	0	0	0	0	0
	V. phosphorescens	2	2	2	1	1	1	0	0	0	0	0	0
Anti-phosphorescens absorbed by *V. phosphorescens*	*V. albensis*	1	1	±	0	0	0	0	0	0	0	0	0
	V. phosphorescens	1	0	0	0	0	0	0	0	0	0	0	0
Anti-albensis absorbed by *V. albensis*	*V. albensis*	1	1	0	0	0	0	0	0	0	0	0	0
	V. phosphorescens	2	2	1	1	0	0	0	0	0	0	0	0

Antigenicity of Filtrates After Cytolysis

The antigenic properties of filtrates are of particular interest because the internal components of the cell rather than the entire cell are largely involved. Electron micrographs (Johnson, Zworykin, and Warren, 1943) have conclusively shown that distilled water cytolysis of luminous cells is rapid but is not accompanied by a complete disintegration of the cell wall. Furthermore, the end product of cytolysis is a cell structure which is for the most part devoid of internal components. The question arose as to whether the filtrates so prepared were capable of producing immune sera which would not only precipitate the filtrates themselves but would also agglutinate intact luminous cells.

Filtrates were prepared and standardized by the following procedure: 5 per cent by moist weight of luminous bacteria were cytolyzed in distilled water. The disappearance of luminescence was indicative of this cytolysis. The prepara-

TABLE 6

Agglutination and cross agglutination of luminous bacteria with rabbit antisera prepared from cytolyzed filtrate antigens. Wherever single antigens are listed, no cross agglutination occurred

ANTIGEN	1:2	1:4	1:8	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	CONTROL
A. Anti-albensis														
V. albensis	4	4	4	4	4	4	4	4	3	3	2	1	0	0
V. phosphorescens	4	4	4	4	4	4	4	4	4	2	2	1	0	0
B. Anti-phosphorescens														
V. phosphorescens	4	4	4	4	4	4	4	4	3	2	2	0	0	0
V. albensis	4	4	4	4	4	4	4	4	2	2	2	±	0	0
C. Anti-harveyi														
A. harveyi	4	4	4	4	2	1	1	0	0	0	0	0	0	0
P. splendidum	2	2	1	0	0	0	0	0	0	0	0	0	0	0
D. Anti-splendidum														
P. splendidum	4	4	4	3	2	2	2	1	0	0	0	0	0	0
B. sepiae	3	2	1	0	0	0	0	0	0	0	0	0	0	0
E. Anti-sepiae														
B. sepiae	4	4	4	4	3	1	1	1	0	0	0	0	0	0
F. Anti-fischeri														
A. fischeri	4	4	4	3	3	2	1	±	0	0	0	0	0	0
G. Anti-phosphoreum														
P. phosphoreum	2	2	2	1	1	1	0	0	0	0	0	0	0	0
H. Anti-pierantonii														
B. pierantonii	4	4	4	3	3	2	1	1	0	0	0	0	0	0

tion of filtrates of the fresh-water forms were more difficult. However, *V. albensis*, the more resistant to cytolysis of the two, served as the distilled water "standard" for *V. phosphorescens*. In this way, some degree of standardization of the filtrate potencies is maintained. Clear filtrates were obtained with Berkefeld filters.

For the production of antisera, rabbits received intravenous injections of filtrates. Five successive injections were administered at 2-day intervals. Be-

ginning with 1 ml, the dosage was doubled with each injection. Following a rest period of 7 days, two 20-ml injections of filtrate were made at 3-day intervals. After a second rest period of 7 days, the animals were bled aseptically from the heart and the serum collected. Cross agglutination experiments were set up in the usual manner.

The results are summarized in table 6. With the exception of a general decrease in the agglutinating titers[6] and an antigenic difference in the *V. albensis-V. phosphorescens* cross agglutination, the data are in complete agreement with the straight agglutination reactions. A further interesting point was the almost identical cross agglutination results with either *V. albensis* or *V. phosphorescens*. This relationship, as shown in table 6, is discussed later.

Precipitation Reactions

The antigen was prepared and standardized by the same method as employed in the antifiltrate agglutination study. Precipitin ring tests were performed with antisera prepared by injections of rabbits with both luminous cell antigen and filtrate antigen, respectively. For each test 0.2 ml of a 1:3 dilution of antiserum was carefully overlaid by 0.2 ml of antigen. Dilutions of antigen ranged from 1:1 to 1:160. Three controls detected any possibility of autoprecipitation.

The results of the cross precipitation reactions with luminous cell antibodies are found in table 7. It is apparent that the precipitin reactions reveal a mosaic of antigens within the bacterial cell in addition to a number of antigenic components which are common to several species.

Both *V. albensis* and *V. phosphorescens*, in addition to having mutual antigenic groups, still retain a similar titer relationship. Curiously enough, anti-*A. fischeri* serum has all the components necessary for the precipitation of any of the remaining species. In addition, a minor *A. fischeri* factor is present in the antisera of the other species. It is then valid to conclude that a common *A. fischeri* component is present in all species. The *A. harveyi-P. splendidum* cross precipitation is of a reciprocal nature. Furthermore, anti-*A. harveyi* serum will precipitate *P. phosphoreum*, *B. pierantonii*, and *B. sepiae* antigens. The *P. splendidum-B. sepiae* kinship is also of a reciprocal nature. It is to be noted that *B. pierantonii* serum possesses only one nonspecific component (*A. fischeri*).

The results of the cross precipitation tests with filtrate immune sera are shown in table 8. There is obviously much more cross precipitation than with the luminous cell immune sera. The *V. albensis-V. phosphorescens* reciprocal relationship is enhanced by the appearance of *A. harveyi* antibody in both sera. The antigenic makeup of *A. fischeri* and *B. sepiae* remain unchanged. Furthermore, anti-*A. harveyi* serum will duplicate the results of the anti-*A. fischeri* serum. In addition, *B. sepiae* and *P. splendidum* now possess a similar mosaic of antigenic components. The only marked change in antigenic structure is shown by *B. pierantonii*. With only one nonspecific precipitation factor when

[6] Although several additional injections of filtrate antigens were made, no appreciable increase in the agglutinating titers of the antisera resulted.

TABLE 7

Precipitation and cross precipitation results of luminous bacteria. I. Luminous cell immune sera

ANTISERA (1:3)	P. PHOSPHOREUM									B. PIERANTONII									P. SPLENDIDUM								
	Antigen Dilutions																										
	Undiluted	1:10	1:20	1:40	1:80	1:160	Control	Normal serum	Antigen + salt	Undiluted	1:10	1:20	1:40	1:80	1:160	Control	Normal serum	Antigen + salt	Undiluted	1:10	1:20	1:40	1:80	1:160	Control	Normal serum	Antigen + salt
V. albensis	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
V. phosphorescens	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
A. harveyi	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−
P. splendidum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
B. sepiae	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−
A. fischeri	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−
P. phosphoreum	+	+	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
B. pierantonii	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	V. albensis									*A. fischeri*									*A. harveyi*								
V. albensis	+	+	+	+	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
V. phosphorescens	+	+	+	+	+	−	−	−	−	+	+	±	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−
A. harveyi	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	+	+	+	±	−	−	−	−	−
P. splendidum	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
B. sepiae	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
A. fischeri	+	+	+	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	+	+	−	−	−	−	−	−	−
P. phosphoreum	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. pierantonii	±	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE 8

Precipitation and cross precipitation results of luminous bacteria. II. Filtrate immune sera

ANTISERA (1:3)	P. PHOSPHOREUM									B. PIERANTONII										B. SEPIAE										P. SPLENDIDUM									
	Antigen dilutions																																						
	Undiluted	1:5	1:10	1:20	1:40	1:80	Control	Normal serum	Antigen + salt	Undiluted	1:5	1:10	1:20	1:40	1:80	Control	Normal serum	Antigen + salt	Undiluted	1:5	1:10	1:20	1:40	1:80	Control	Normal serum	Antigen + salt	Undiluted	1:5	1:10	1:20	1:40	1:80	Control	Normal serum	Antigen + salt
V. albensis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−
V. phosphorescens	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	+	+	+	−	−	−	−	−	−
A. harveyi	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	±	+	+	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−
P. splendidum	+	+	+	−	−	−	−	−	−	−	±	+	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	−
B. sepiae	+	−	−	−	−	−	−	−	−	−	+	+	±	−	−	−	−	−	+	+	+	+	+	+	−	−	−	+	+	+	+	−	−	−	−	−
A. fischeri	+	+	+	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
P. phosphoreum	+	+	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−	−	−	−	−	−
B. pierantonii	+	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	+	+	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−
	V. albensis									*A. fischeri*									*A. harveyi*									*V. phosphorescens*								
V. albensis	+	+	+	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−
V. phosphorescens	+	+	+	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−
A. harveyi	+	+	−	−	−	−	−	−	−	+	+	+	±	−	−	−	−	−	+	+	+	+	+	−	−	−	−	+	+	+	−	−	−	−	−	−
P. splendidum	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. sepiae	±	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	+	+	+	±	±	−	−	−	−	−	−	−	−	−	−	−	−	−
A. fischeri	+	+	+	±	−	−	−	−	−	+	+	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−	−	+	−	+	±	−	−	−	−	−
P. phosphoreum	−	±	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−
B. pierantonii	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−

combined with luminous cell immune serum, the filtrate immune serum of this organism precipitates six additional filtrates. *P. phosphoreum* occupies an exactly similar kinship with both *B. sepiae* and *P. splendidum*.

The precipitin tests appear to be highly sensitive in seeking out the component antigens in luminous bacteria filtrates. Prevalent nonspecific reactions in addition to weak precipitinogenic components are significant in the general antigenic system. It is evident from the enumeration of components present that the system is complex. Tests with luminous bacteria immune sera show a more specific antigen-antibody relationship than similar reactions with filtrate immune sera.

DISCUSSION

The results seem to indicate rather clearly that the six marine species of luminous bacteria studied, apart from minor agglutinins in two species (*A. harveyi* and *P. splendidum*), are fairly specific in their agglutinin reactions.

The fresh-water forms, *V. albensis* and *V. phosphorescens*, present a much closer cross agglutination relationship. They are also much more agglutinogenic than the marine forms. Immune sera will give a higher titer in a shorter immunization period. Whether this is due to the character of the antigenic reacting groups is not known.

With one exception, the results indicate that the specificity of the agglutinations with filtrate immune sera agrees with the results of luminous cell immune sera. This suggests that the agglutinin factors derived from luminous cells are all present in their filtrates and points to the classification of the latter as complete antigens.

It is apparent that the cultural as well as immunological characteristics of *V. albensis* and *V. phosphorescens* are for the most part similar. However, *V. phosphorescens* possesses a more intense luminescence, is more stable in suspension, and is more complete antigenically. These facts suggest the possibility that *V. albensis* is a "rough" variant of *V. phosphorescens*, although differences in colony form are not apparent.

The precipitation studies of luminous bacteria exhibit much less specificity than the agglutination reactions and, in addition, a marked nonspecific reaction at low titer. Nevertheless, the differentiation of the various species is facilitated by the higher titers of the homologous antisera.

Any consideration of the specific antigens involved in the precipitation reactions of luminous bacteria is more or less speculative. The marked group phenomena which characterize the results are of special interest since not merely different strains, or even species, but actually different genera are represented. This study is not comprehensive enough to justify any recommendations as to the most desirable place, in the classification of bacteria, for these organisms; nor for renaming or systematizing the group itself.

It is conceivable that these serological reactions are linked with similarity in the chemical structure of particular cellular components in which the luminescent system is of prime importance, since luminescence is perhaps the most outstand-

ing common property of the different organisms. A possible role played by the components of a common luminescent system of the organisms, in influencing antigenicity of the intact luminous cell, has not been established. Although no experiments were devised or conducted for the purpose of making a positive contribution to the problem of the mechanism of the luminescent system, comments on the subject can be presented on the basis of observations made during the course of this investigation.

Ballner (1907) reported no inhibition of luminescence with immune sera. Although Ninomya (1924) found an inhibition with immune sera, he showed that a correlation existed between the degree of agglutination and the inhibition of luminescence. It is highly probable, as suggested by Ninomya, that these inhibitions are due merely to an intense clumping of cells during agglutination reactions which interferes with an adequate gaseous exchange. The possibility of a specific antigen-antibody reaction which influences this inhibition was considered as a secondary factor by that author.

Harvey and Deitrich (1930) investigated the possible antigenic properties of the *Cypridina* luminescent system, i.e., luciferase and luciferin. They reported the production of an antiluciferase serum which combined with active luciferase. When this luciferase plus antibody was now added to luciferin, the luminescent reaction did not occur. It is apparent that the enzyme which is involved in the luminescent reaction of *Cypridina*, and which is assumed to be responsible for the same reaction in luminous bacteria, is antigenic.

The observation of numerous cases of both normal and immune sera in contact with luminous bacteria has revealed no evidence of a corresponding immunological interference with the light-emitting reactions in the living cells. In no instance was there an inhibition of luminescence, except by agglutination. The amount, and therefore potency, of such an enzyme antigen, of course, must also be considered in explaining an apparent absence of a specific luminescent antibody. Furthermore, luminescence is actually increased in the presence of both normal and immune sera. This is undoubtedly due to the addition of a nutrient substrate (Ninomya, 1924; Johnson, 1936; van Schouwenburg, 1938).

Thus, it is clear that an antiluciferase antibody is either not present in the immune serum or else, if it is present, it does not have access to the light-emitting enzyme in the intact bacterial cells. This point is of particular interest for its possible significance in connection with the problems of the site of oxidative systems in living bacteria. If it may be assumed that bacterial luciferase is antigenic, the results would indicate that the luminescent enzyme is not directly exposed to immunological combination at the cell surface.

SUMMARY

The antigenic properties of eight species of luminous bacteria are reported. Evidence was sought from the results of agglutination, agglutinin absorption, and precipitation.

Cross agglutination results indicate that specific agglutinogenic properties are present in *Achromobacter fischeri*, *Bacillus sepiae*, *Bacillus pierantonii*, and

Photobacterium phosphoreum. Cross reactions occur between *Achromobacter harveyi* and *Photobacterium splendidum*, between *Photobacterium splendidum* and *Bacillus sepiae*, and between *Vibrio albensis* and *Vibrio phosphorescens*.

The nature of the *V. albensis-V. phosphorescens* kinship is different from the other cross reactions. At least two major antigens are present in *V. phosphorescens*, whereas *V. albensis* contains a major and minor antigen.

Agglutinin absorption tests confirm the existence of major and minor agglutinins in the case of *A. harveyi-P. splendidum*, and *P. splendidum-B. sepiae*. Cross agglutinin absorptions with *V. albensis-V. phosphorescens* indicate a significant agglutinogenic similarity.

Filtrate antigens of luminous bacteria will give rise to agglutinins which exhibit specificities characteristic of luminous cell agglutinins.

A mosaic of common antigens is revealed by cross precipitation with both immune filtrate sera and immune luminous cell sera. An intensified group reaction is found with the immune filtrate sera. These results are discussed in relation to the chemical composition of cellular structures and a luminescence factor of possible antigenic significance.

The luminescence of living cells was not directly affected by any of the immunological reactions studied.

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REFERENCES

BALLNER, F. 1907 Über das Verhalten von Leuchtbakterien bei der Einwirkung von Agglutinations-serum und anasthesierenden chemischen Agenzien, nebst Bemerkungen über Pfanzennarkose. Zentr. Bakt. Parasitenk., II, **19**, 572–576.

BEIJERINCK, M. W. 1889 Le *Photobacterium luminosum*, bactérie lumineuse de la Mer du Nord. Arch. Neérland. Sci., **23**, 401–415. Les bactéries lumineuses dans leur rapports avec l'oxygène. *Ibid.*, 416–427.

BEIJERINCK, M. W. 1890 Over Lichtvoedseelnen plastisch Voedsel van Lichtbakterien. Mededeelingen Akademie Wetenschappen, Afdeeling Natuurkunde, 2 Reeks, Deel VII, Amsterdam, p. 239.

BEIJERINCK, M. W. 1912 Mutation bei Mikroben. Folia Microbiologica, Delft, **1**, 4–97.

BEIJERINCK, M. W. 1916 Die Leuchtbakterien der Nordsee im August und September. Folia Microbiologica, Delft, **4**, 15–40.

BERGEY, D. H., *et al.* 1930, 1934, 1939 Manual of determinative bacteriology. 3d, 4th, and 5th ed. Williams & Wilkins Co., Baltimore.

DAHLGREN, U. 1915 The production of light by animals. J. Franklin Inst., **180**, 513–537, and subsequent articles.

DOUDOROFF, M. 1938 Lactoflavin and bacterial luminescence. Enzymologia, **5**, 239–243.

FISCHER, B. 1888 Über einen neuen lichtentwickelnden Spaltpilz. Zentr. Bakt. Parasitenk., **3**, 105, 137.

FLÜGGE, C. 1896 Die Mikroorganismen. 3 Aufl. C. Flügge, Leipzig. Bd. I, 166.

GIESE, A. C. 1943 Studies on the nutrition of dim and bright variants of a species of luminous bacteria. J. Bact., **46,** 323–331.

GORHAM, F. G. 1903 The photogenic bacteria. Unpublished MSS., Biology Department, Princeton University.

HARVEY, E. N. 1915 The effect of certain organic and inorganic substances upon light production by luminous bacteria. Biol. Bull., **29,** 308–311.

HARVEY, E. N., AND DEITRICH, J. E. 1930 The production of antibodies for *Cypridina* luciferase and luciferin in the body of a rabbit. J. Immunol., **18,** 65–71.

HILL, S. E. 1929 The penetration of luminous bacteria by the ammonium salts of the lower fatty acids. J. Gen. Physiol., **12,** 863–872.

JOHNSON, F. H. 1936 The aerobic oxidation of carbohydrates by luminous bacteria and the inhibition of oxidation by certain sugars. J. Cellular Comp. Physiol., **8,** 439–463.

JOHNSON, F. H. 1941 Immunological reactions of marine luminous bacteria. J. Bact., **41,** 67.

JOHNSON, F. H., AND HARVEY, E. N. 1937 The osmotic and surface properties of marine luminous bacteria. J. Cellular Comp. Physiol., **8,** 167–178.

JOHNSON, F. H., AND HARVEY, E. N. 1938 Bacterial luminescence, respiration and viability in relation to osmotic pressure and specific salts of sea water. J. Cellular Comp. Physiol., **11,** 213–232.

JOHNSON, F. H., AND SHUNK, I. V. 1936 An interesting new species of luminous bacteria. J. Bact., **31,** 585–592.

JOHNSON, F. H., ZWORYKIN, N., AND WARREN, G. 1943 A study of luminous bacterial cells and cytolysates with the electron microscope. J. Bact., **46,** 167–187.

KORR, I. M. 1935a The relation between cell integrity and bacterial luminescence. Biol. Bull., **58,** 347–354.

KORR, I. M. 1935b An electrometric study of the reducing intensity of luminous bacteria in the presence of agents affecting oxidations. J. Cellular Comp. Physiol., **6,** 181–216.

LEHMANN, K. B., AND NEUMANN, R. O. 1901 Atlas and principles of bacteriology. W. B. Saunders and Co., Philadelphia.

MAJIMA, R. 1931 Studies on luminous bacteria. III. On the fermentation of carbohydrates and the immune agglutination by various luminous micro-organisms. Sei-i Kwai Med. J., **50,** 41–67.

MANGOLD, E. 1910 Die Produktion von Licht. Winterstein's Handbuch der vergleichenden Physiologie, **3** (2nd half), 225–392. G. Fischer, Jena.

MEISSNER, G. 1926 Bakteriologische Untersuchungen über die symbiontischen Leuchtbakterien von Sepien aus dem Golf von Neapel. Zentr. Bakt. Parasitenk., II, **67,** 194–238.

MIGULA, W. 1897, 1900 System der Bakterien. G. Fischer, Jena.

MOLISCH, H. 1912 Leuchtende Pflanzen. G. Fischer, Jena.

NINOMYA, R. 1924 Der Einfluss von Antikörper und Komplement auf biologische Functionen von Bakterien. I. Der Einfluss specifischer Amboceptoren mit and ohne Komplementzusatz auf das Leuchtvermögen von Leuchtbakterien. Z. Immunitäts., **39,** 498–512.

SCHOUWENBURG, I. L. VAN. 1938 On respiration and light emission in luminous bacteria. Thesis. Delft.

ZIRPOLO, G. 1917 Richerche su di un bacillo fosforescente che si svilluppa sulla *Sepia officinalis* (*Bacillus sepiae*, n. sp.). Boll. soc. natural. Napoli, **30,** 47.

ZIRPOLO, G. 1918 I. Batterii fotogeni degli organi luminousi die *Sepiola intermedia* Naef (*Bacillus pierantonii* n. sp.). Boll. soc. natural. Napoli, **30** (Ser. 210) Atti, 206–220.