SOME ECOLOGICAL RELATIONS OF PSEUDOMONAS AERUGINOSA TO CLOSTRIDIUM BOTULINUM TYPE C

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Considerable work has been done in recent years by various workers on the causes of, and remedies for, Western Duck Sickness (see references). Comparatively little attention has been given, however, to the symbiotic or antagonistic properties of other microorganisms.

Recently such studies have been undertaken at the Bear River Wildlife Disease Research Station some of which are presented in the following:

In April 1942 an outbreak of botulism occurred at the Weber Bay Refuge near Ogden, Utah. During routine bacteriological examinations it was found that *Pseudomonas sp.* occurred with surprising regularity in the intestinal tract of the various ducks as shown in table 1.

As our organisms showed certain discrepancies with cultural characteristics as given in Bergey's Manual two cultures were sent to Dr. C. P. Hegarty, of the Georgetown University Medical School, who was kind enough to identify the organisms as *Pseudomonas aeruginosa.* These two cultures were used in the following experiments.

In the course of studying these organisms culturally their great oxygen-consuming properties and ability to render the media highly alkaline were noted. These characteristics suggested symbiotic possibilities in relation to *Clostridium botulinum* type C, so a study was undertaken.

The changes in hydrogen ion concentration and dissolved oxygen occurring after inoculation of strain 604 into nutrient broth, as determined by the use of a potentiometer and Winkler's method for determining dissolved oxygen, showed that all traces of detectable oxygen disappeared between 4½ and 5 hours and the pH began to rise after about 10 hours incubation, reaching a high of 9.0 and attaining optimum conditions for growth of *C. botulinum* in 24 to 48 hours.

Following these determinations attempts were made to grow *C. botulinum* type C and *P. aeruginosa* together in nutrient broth without artificial production of anaerobiosis. Growth of *C. botulinum* was evidenced by daily microscopic examinations and toxicity determined by mouse inoculation tests checked against antitoxin. The differing gram-staining properties and size of the two organisms makes microscopic differentiation simple. Toxicity and growth of the botulism organisms developed faster when inoculated into a two-day-old culture of *Pseudomonas* than when the two organisms were inoculated simultaneously. *C. botulinum* did not grow or develop toxin when inoculated into nutrient broth without *Pseudomonas.*

Previous experiments (Quortrup and Holt) had indicated great detoxifying properties of various bacteria, so this character of *Pseudomonas* was determined.

Bacteria-free botulinus toxin was mixed with nutrient broth and inoculated with *Pseudomonas*, and toxicity tests made from time to time. Repeated tests showed that the botulinus toxin was not destroyed after being incubated at 37°C. under the mentioned conditions for 192 hours, using inoculations of 1/1000 ml. containing 100 MLD's of botulinus toxin.

As detoxifying properties of *Escherichia coli* had been demonstrated in earlier work, the following experiments were conducted.

Tests were made on *E. coli* and *P. aeruginosa* separately in order to determine their respective properties which are shown in table 2. It is readily seen that oxygen is absorbed rapidly by both organisms but that the pH reactions go in opposite directions.

Attempts to grow *C. botulinum* in various media in the presence of *E. coli* were unsuccessful. When *C. botulinum*, *P. aeruginosa* and *E. coli* were incubated together in nutrient both botulinus toxin could be demonstrated by mouse inoculations. It was apparent that *P. aeruginosa* was able to outgrow *E. coli* and this explains the loss of the detoxifying properties of this organism.

TABLE 1

SPECIES EXAMINED	PSEUDOMONAS PRESENT	PSEUDOMONAS NOT PRESENT
Gadwall	4	1
Shoveler	4	0
Redhead	2	0
Pintail	6	0
Baldpate	2	0
Scaup	2	1
Greenwinged Teal	0	9

As *Salicornia rubra* had previously been found to be an excellent medium for *C. botulinum*, some of these plants were collected and the foliage placed in Erlenmeyer flasks. A sufficient amount of distilled water was added to cover the *Salicornia*. No sterilization or artificial production of anaerobiosis was attempted. One of these flasks was inoculated with *C. botulinum*; another with *C. botulinum* and *P. aeruginosa*; and a third with *C. botulinum* and *E. coli*. Botulinus toxin developed only in the flask containing the *Pseudomonas* and was detected after 72 hours incubation when inoculated in 1/100 ml. amounts into mice, or 2 ml. amounts into ducks; 10 ml. of the supernatant fluid produced typical symptoms of botulism in a greenwinged teal following oral administration and resulted in death in 36 hours.

This experiment was followed by another in which *Salicornia* and a mixture of *Potamogeton* and *Cladophora* were used. These media were placed in open pans of about 6 liters capacity and covered with water, after which a loopful of *C. botulinum* and 100 ml. of *Pseudomonas* broth were added. The material used in this experiment closely simulates patches of decaying vegetation found in the duck marshes during the sickness period. The mixture was placed without

previous incubation in cages containing four ducks. A typical reaction observed in one of the experiments is shown in table 3, indicating a high toxicity and such flooded material should be considered a potential toxin-producing medium under actual field conditions.

TABLE 2

E. COLI 586—IN LACTOSE BROTH			P. AERUGINOSA 604		
Incubated	Oxygen	pH	Incubated	Oxygen	pH
hours			*hours*		
0	3.8	6.8	0	3.8	6.8
4	3.8	6.8	4	2.4	6.8
5	3.2	6.5	5	0	6.9
6	0	6.5	6	0	6.9
7	0	6.1	7	0	7.0
18	0	5.6	18	0	7.2
24	0	5.2	24	0	7.3
42	0	4.8	42	0	6.8
48	0	4.9	48	0	6.3
72	0	5.0	72	0	5.9
120	0	5.4	120	0	5.9
144	0	5.1	144	0	6.6
192	0	5.1	192	0	8.0
216	0	5.1	216	0	8.6
240	0	5.1	240	0	8.8
288	0	5.1	288	0	8.9

TABLE 3

DATE	G. W. TEAL NO. 1	G. W. TEAL NO. 2	PINTAIL NO. 1	PINTAIL NO. 2
September 19	—	—	—	—
20	—	—	x	—
21	xx	—	xx	—
22	xx	x	xx	x
23	xx	x	xx	xx
24	xxx	x	xx	xx
25		x	xx	xx
26		x	xxx	xx
27		—		x
28		—		—

—, negative; x, symptoms; xx, severely afflicted; xxx, death.

The same material was tested under identical conditions with inoculation of *C. botulinum* alone; *C. botulinum* together with *P. aeruginosa*; and *C. botulinum* with *E. coli*. Only when *P. aeruginosa* was present was toxin produced.

Another series of experiments in which water samples containing concentrations of plankton were tested showed that seven such field-collected samples were non-toxic when collected. Two of the seven samples became toxic when inoculated with *C. botulinum*, but five out of seven became toxic when inoculated with *P. aeruginosa* also.

During the 1942 season a survey was conducted showing that *P. aeruginosa* could be isolated with great regularity from water samples collected in duck marshes at many points in the Salt Lake valley in Utah as well as at Des Lacs Lake in North Dakota.

SUMMARY

Great oxygen-consuming and alkali-producing capacities of *Pseudomonas aeruginosa* were observed. These two properties indicate symbiotic relations of *Clostridium botulinum type C*, which were confirmed by various tests. *P. aeruginosa* was isolated from the intestinal tract of six species of ducks.

It was found that it was possible to produce botulinus toxin using common marsh vegetation as a medium merely by inoculating with *P. aeruginosa* and *C. botulinum*. Botulinus toxin was not produced in the presence of *Escherichia coli* alone but when *P. aeruginosa* was added this organism would outgrow *E. coli*, and botulinus toxin could be produced.

Cultures of *P. aeruginosa* did not destroy bacteria-free botulinus toxin after 192 hours incubation.

The presence of organisms such as *P. aeruginosa* readily explains the occurrence of botulism in areas where it is normally not expected, for instance, in water having a depth in excess of 12 inches but containing masses of decomposing vegetation. Fecal deposits from duck concentrations and bird colonies undoubtedly play an important rôle.

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