BROTH CULTURES OF BACTERIA THAT ATTRACT FEMALE MOSQUITOES [1, 2]

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For a considerable time, investigators have been reporting that carbon dioxide, moist air, warmth, and human odors, when presented alone or in various combinations, are attractive to female yellow fever mosquitoes, *Aedes aegypti* (L.). Also, most workers agree that the presence of carbon dioxide in quantities above the atmospheric norm will activate mosquitoes to flight (Kahn and Maibach, 1966; Wright *et al.*, 1964; Brouwer, 1960).

Although the role of carbon dioxide as a stimulus in the attraction of mosquitoes has been thus generally accepted, considerable controversy exists about how moisture, warmth, and human odors function in the host-seeking process. Some workers reported that warm, moist convection currents caused the mosquitoes to approach and were not convinced that an odor factor was operative (Wright and Kellogg, 1962). Others concluded that moisture did not play a significant role and that odor was important (Khan *et al.*, 1966). We, too, have observed the response to carbon dioxide countless times and are in agreement that it plays an essential role in the host-seeking behavior at short distances; we do not believe that it, in itself, is an attractant.

Observations during operation of an olfactometer (Schreck *et al.*, 1967) suggested that human hands leave a residue on surfaces that is attractive to female *A. aegypti*. Later, we found that a polyethylene glove worn on the hand for 1 hour attracted 10 to 50 percent of the mosquitoes in the ol-

[1] *Aedes aegypti* Linnaeus (Diptera: Culicidae).

[2] Mention of a proprietary product does not necessarily imply endorsement of this product by the USDA.

factometer when it was tested 5 minutes after removal from the hand and that it lost its attractiveness gradually over 3 hours. The hypothesis developed from these observations made the human residue factor a key, and the line of research that we followed subsequently was the result.

Since a number of organisms and substances are found on human skin (Marples, 1965), an obvious line of inquiry is the investigation of their attractiveness to mosquitoes. Identification of the components of skin secretions such as sweat (Altman and Dittmer, 1961), oils (Haahti *et al.*, 1960), and amino acids (Bauer *et al.*, 1953) has been accomplished, and a number of these materials have been tested with some success as attractants (Roessler, 1963; Brown and Carmichael, 1961; Thompson and Brown, 1955). However, no compound or combination of compounds has yet been demonstrated to equal a hand or an arm as a mosquito attractant.

One consideration that has apparently been overlooked is the presence of microorganisms (of which bacteria are most abundant) on human skin. Scheimann *et al.* (1960) demonstrated the role of bacterial action on skin lipids and the subsequent formation of free fatty acids. Since we had observed in our olfactometer tests that freshly washed hands were less attractive than hands that had not been washed for several hours, the present tests were made to determine whether bacterial action on the skin was responsible for the production of a metabolite that was attractive to mosquitoes.

MATERIALS AND METHODS. Of three colonies of bacteria isolated from smears taken from a human arm, one culture was shown to be much more attractive than the others in preliminary tests. This culture was identified by Dr. Ruth Gordon of the Institute of Microbiology at Rutgers University as *Bacillus cereus* (personal communication). An experiment was therefore made to determine the attractiveness of *Bacillus cereus* to female *A. aegypti*.

The test medium was prepared with 500 ml. of a Bacto nutrient broth in a 2,800-ml. erlenmeyer flask. The flask was sealed with a 2-holed rubber stopper containing two elbows of glass tubing. A short glass tube projected 2 cm. into the flask, and a long tube extended below the surface of the nutrient broth. Short lengths of rubber tubing were attached to the outside ends of the glass tubes. The flask was autoclaved for 15 min. at 15 p.s.i.; after it was removed from the autoclave, the rubber tubes were stoppered with cotton plugs, and the flask was allowed to cool to room temperature.

For each test, 10 ml. of nutrient broth were inoculated with *Bacillus cereus* and incubated for 18 hours. One ml. of the inoculum was then added to the nutrient broth in each of two flasks (A and B); one untreated sterile check flask (C) was also prepared. The three flasks used in each test were incubated at 30° C. for 24, 48, 72, 96, 120, or 144 hours. The entire group of 18 flasks constituted a series, and three series of flasks were incubated and tested.

The tests were conducted in four olfactometers, each containing 125 seven-day-old female *A. aegypti* which had been caged with males but had not received a blood meal. After the allotted incubation time, and 1 hour before testing, flasks A, B, and C were removed from the incubator and allowed to come to room temperature (about 24.5° C.). Flasks A and C were then placed in front of the two test ports of olfactometer No. 1. The short tubes were connected to inlet tubes, each of which connected one of the flasks to one of the ports of the olfactometer (Fig. 1). Gas lines were then attached to the long tube of each flask so that nitrogen bubbled through the medium at 760 ml./min. (measured by a Fisher & Porter variable-area flowmeter). The gas mixture then passed out of the short tubes and into the olfactometer through the ports for 3 min. The test procedure was repeated by using flask B in the second olfactometer; flask C was again used as the sterile check. Flasks A and B were next compared with the check in olfactometers 3 and 4, respectively, by passing nitrogen gas through the flasks as before; in addition, carbon di-

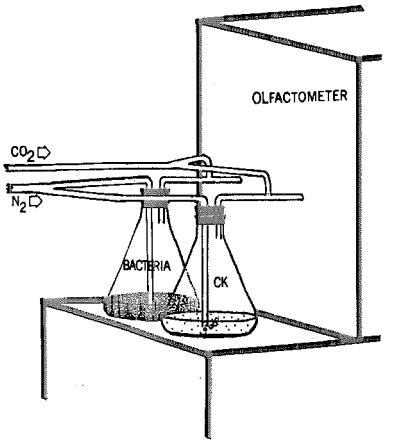

FIG. 1.—Simplified drawing of the apparatus used to test the attractiveness of bacterial cultures. Nitrogen flowed through the flasks and into the olfactometers in all four tests, but carbon dioxide was introduced only into olfactometers 3 and 4.

oxide was supplied at the rate of 10 ml./min. at the exit side of the flasks. (The added carbon dioxide never came in contact with the bacterial cultures.)

The average results obtained in the three series of tests are given in Table 1 and are shown graphically in Fig. 2. Obviously, the bacterial culture contained a substance that attracted the mosquitoes. In the tests with carbon dioxide, the 120-hour culture was more attractive (significant at the 0.01 level of confidence) than cultures at all the other incubation periods. Without carbon dioxide, the 96-hour culture was the most attractive but was not significantly different from the 72- or 120-hour cultures; however, when it was compared with the 144-hour culture, the difference was significant (0.05 level of confidence), and when it was compared with the 24- and 48-hour cultures, the difference was highly significant (0.01 level of confidence). Also, the 24-hour culture was significantly less attractive than the 144-hour culture.

TABLE 1.—Attraction of female *A. aegypti* to emanations from bacterial cultures incubated 24 to 144 hours, with and without the addition of carbon dioxide. (Average of 6 tests).

No. of hr. of incubation	Percentage of mosquitoes trapped (±SD): With CO_2	Without CO_2	Difference (w/ and w/o CO_2)
24	31.5±9.8	12.5±6.7	19.0±7.0
48	40.3±9.5	23.2±8.0	17.2±3.0
72	40.5±5.7	29.5±11.0	11.0±9.7
96	43.3±7.2	42.0±7.8	1.3±8.0
120	65.0±10.0	38.8±5.0	26.2±5.8
144	34.0±5.2	27.0±7.0	7.0±2.2

Records of optical density were made for each of the bacterial cultures with a model DU Beckman quartz spectrophotometer (Fig. 3). Quantitative counts of bacteria were made by the standard method in a Petroff-Hausser counting chamber: a 1:10 dilution in distilled water was made of a sample from each culture after the culture had been tested in the olfactometers; the chamber was charged, and 10 counts were made for each sample; then the data were quantitated and averaged (Fig. 2).

The counts of bacteria showed a general decline in population after 48 hours and for as long as 120 hours; meanwhile, the attraction was increasing gradually from 24 to 120 hours. The readings of optical density agreed with the bacterial counts in showing a peak at 48 hours but changed only slightly after 72 hours. As noted, in all instances when carbon dioxide was used, the attractancy was greater. Although the bacterial population was at its lowest at 120 hours, this culture was the most attractive when carbon dioxide was added.

We do not yet clearly understand what happened in the flask as the nitrogen gas was passed through the broth. Most mosquitoes ceased to respond after 2 minutes to emanations from flasks that had been incubated 24 to 72 hours. When the cultures had been incubated 96 to 144 hours, the attractancy tended to continue throughout the full 3 minutes though it did diminish somewhat the last minute.

In all tests, emanations from the sterile check caused little or no mosquito response: in 48 of 72 check tests, the percentage of trapped mosquitoes was 1 per-

cent or less; 6 percent was the highest recorded.

DISCUSSION AND CONCLUSIONS. The experiments showed that *Bacillus cereus,* when it was cultured in nutrient broth, produced some substance(s) which attracted female *A. aegypti.* Also, we know that bacteria, in general, produce carbon dioxide as a byproduct of metabolism. These tests verify observations by other workers that carbon dioxide has an integral role in mosquito attraction; though it is not itself an attractant, it activates the insects to flight and is probably a basic requisite in the sequence of events which motivate mosquitoes to seek a host.

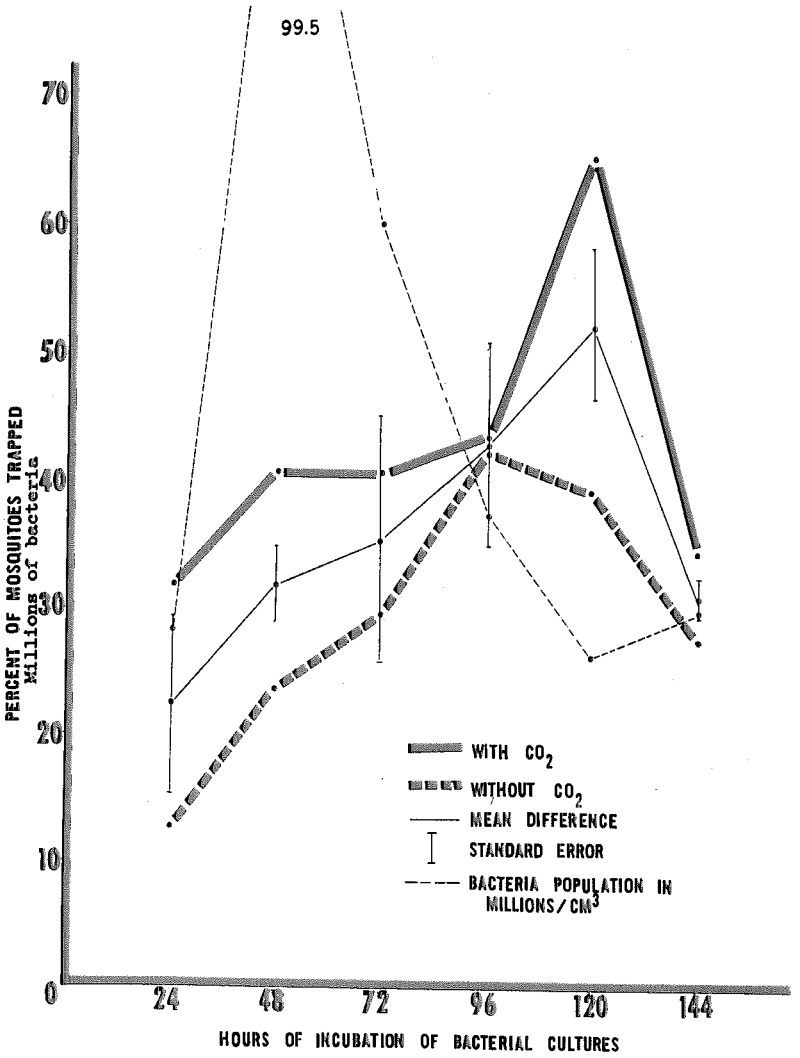

FIG. 2.—Attraction of female *Aedes aegypti* to bacterial cultures and a comparison of the bacterial population and its relationship to length of incubation.

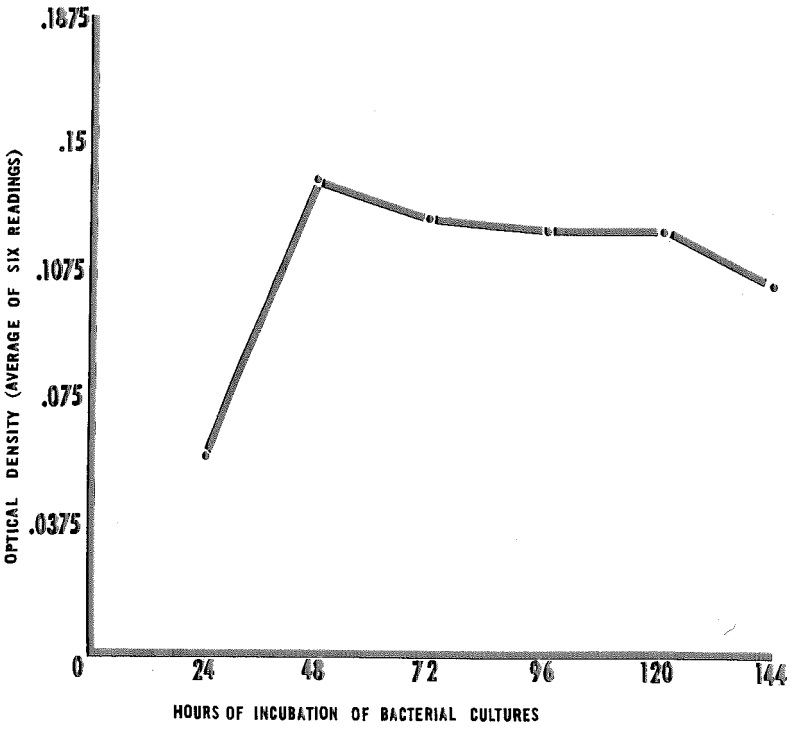

FIG. 3.—Average optical density of samples from each of six bacterial cultures after six periods of incubation.

As previously stated, response to the 24- to 72-hour-old cultures declined rapidly after 2 minutes of testing. However, when these apparently spent flasks were reused in combination with a constant flow of carbon dioxide, the attractancy resumed and was nearly always considerably higher. Perhaps after 2 minutes, the metabolic carbon dioxide in the flasks was diluted sufficiently to cause little or no flight response; thus the mosquito activity ceased. Cultures aged 96 to 144 hours were attractive longer; perhaps because more metabolic carbon dioxide had accumulated during the extended incubation.

The protoplasm of the bacterial cell is similar to all other living protoplasm in that it is composed chiefly of carbon, oxygen, hydrogen, nitrogen, sulfur, and potassium (Frobisher, 1962); however, species may differ in the proportions of the elements present. Also, the dry weight of bacteria may contain from 40 to 90 percent organic matter (Frobisher, 1962), and this organic material (whether in live or dead protoplasm) is a significant member of the skin community. Bacteria contain many complex, nonprotein, nitrogenous substances, including purine bodies, polypeptides and amino acids (Frobisher, 1962). Then an interaction between substrate products of the skin, metabolites of bacterial origin, and the organic matter inherent in bacteria may conceivably be responsible for the attraction of mosquitoes to the human body.

Obviously, in this test, no attempt was made to simulate the conditions on the human skin. The major purpose was to demonstrate a possible direct relationship between microbial activity and mosquito attraction. We still, therefore, need to

find out whether an interaction does exist between microbes (particularly bacteria and yeasts) and the human skin environment and whether production of a mosquito attractant does occur as a result.

SUMMARY. A broth culture of *Bacillus cereus* derived from smears taken from a human arm was attractive to female *Aedes aegypti*. Metabolites and/or decomposition derivatives produced by the action of microorganisms on the human skin may contribute to the attractiveness of the skin to mosquitoes.

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