

SUBJECT: Progress Report--Evaluation of Honey for Selected Biological Properties. Contract No. 12-111-100-2596(73).
Period covered: May 12, 1958 to November 12, 1958.

OBJECTIVES OF THE PERIOD

1. During this first period work was started on the bactericidal effect of the various honeys.
2. A study was made of the bactericidal effect of the pollen suspended in raw honey.
3. An estimation of the relative buffer capacities of the honeys was carried out.
4. The influence of the pH of honey upon inhibition of bacterial growth was studied.

EXPERIMENTAL METHODS

The bacterial inhibiting effect in honey was studied by means of the Pad-Plate method of Kohler and Broquist (1). The procedure was modified by use of a different assay medium for Bacillus subtilus and Micrococcus flavus. In addition to these organisms Bacillus cereus and Sarcina lutea were employed. Stock cultures were maintained ^{on} Penassay seed agar¹, and transferred every two weeks. The broth medium consisted of 3% B.B.L. Trypticase Soy Broth. The cultures of B. cereus and B. subtilus were incubated at 37° C, while S. lutea and M. flavus were incubated at 27° C. Difco Brain Heart Infusion was used as the assay medium for B. cereus and B. subtilus. Penassay seed agar was used as the assay medium for S. lutea

¹Difco Laboratories, Detroit, Michigan.

and M. flavus². Recently, it was found that Penassay seed agar can be used as the assay medium for all four bacteria.

All honeys were tested in the raw state at concentrations of 1, 10, 20, 40, and 100%. The control sugar solution contained 32% glucose, 39% fructose, 8% maltose, 2% raffinose, 2% sucrose, and 17% water.

To determine the inhibiting effect contributed by the suspended pollen, different honeys were treated as follows: cotton honey was diluted to 40% with water and centrifuged at 17,000 rpm for 30 min.; 40% cotton honey was filtered through a Seitz filter; and undiluted sweet clover honey was filtered through a sintered glass funnel. Also, 25 ml. of 100% cotton honey was dialyzed in a cellulose membrane against an equal volume of water. Each of the resulting solutions was compared with a similar dilution of untreated honey by the Pad-Plate method.

It was of interest to determine the relative buffer capacity of honey, since a solution strongly buffered to an acid pH above the optimum pH of the organism would inhibit its growth. The capacity of the buffer system in honey was determined in 8 ml. of a 1:1 dilution of honey, buffered control sugar solution and non-buffered control sugar solution by titration with 0.04 N NaOH. The results are expressed as the ml. of 0.04 N NaOH required to bring the solution to pH 7, divided by the total change in pH. The buffered control sugar solution, pH 3.6, was composed of 13.65 ml. of 0.1 M citric acid, 6.44 ml. of 0.1 M Na₂HPO₄, and the sugars in the percentages stated above.

²Tentative Methods for the Determination of Antibiotics in Animal Feeds, August, 1953. Department of Health, Education and Welfare; Food and Drug Administration, Division of Antibiotics, Washington, D. C.

The contribution of pH to the inhibiting effect of a given honey was determined by comparison of the inhibition caused by control sugar at pH 6.3 and 4.2, buffered control sugar at pH 7.0 and 3.6, and honey at pH 4.1, (pH of the honey in natural state) and pH 7.3.

RESULTS ACCOMPLISHED

Antibacterial Effect of Honey

The inhibiting effect of the various honeys as determined by the Pad-Plate procedure is shown in Table I.

Table I. Inhibiting Effect of Honey

| Honey, % | <u>B. cereus</u> | | | | | <u>B. subtilis</u> | | | | | <u>M. flavus</u> | | | | | <u>S. lutea</u> | | | | |
|-----------------------------------|------------------|----|----|----|---|--------------------|----|----|----|---|------------------|----|----|----|---|-----------------|----|----|----|---|
| | 100 | 40 | 20 | 10 | 1 | 100 | 40 | 20 | 10 | 1 | 100 | 40 | 20 | 10 | 1 | 100 | 40 | 20 | 10 | 1 |
| Sweet Clover, Alfalfa (Budge) | S | - | - | - | - | S | - | - | - | - | + | + | + | + | - | + | + | + | S | - |
| Tulip | + | + | - | - | - | + | - | - | - | - | + | + | + | - | - | + | + | - | - | - |
| Xtra white orange | + | - | - | - | - | S | - | - | - | - | + | + | - | - | - | + | + | - | - | - |
| Tupelo | + | - | - | - | - | S | - | - | - | - | + | + | + | - | - | + | - | - | - | - |
| Buckwheat | + | S | - | - | - | + | - | - | - | - | + | - | - | - | - | + | - | - | - | - |
| Sweet Clover, Alfalfa (Powers) | + | S | - | - | - | - | - | - | - | - | + | + | S | - | - | + | + | + | + | - |
| Fall Flower | + | - | - | - | - | + | - | - | - | - | + | - | - | - | - | + | - | - | - | - |
| Cotton | + | - | - | - | - | + | - | - | - | - | + | + | + | S | - | + | + | + | + | - |

NOTE: + definite inhibition
 - no inhibition
 S slight inhibition

This chart indicates that all the honeys at the 100% level and some at the 10% level caused inhibition of M. flavus and S. lutea. Sweet clover

and cotton honey showed the greatest inhibiting effect. The control sugar solution showed very slight inhibition of all four bacteria. The results show that some honeys do contain an inhibiting factor. All tests were run in triplicate.

Inhibiting Effect of Pollen

In order to study the contribution of suspended pollen to the inhibiting effect of the various honeys, a considerable portion of the pollen was removed by centrifuging or filtering. The inhibiting effect of the clarified honey was compared with that of the untreated honey by the Pad-Plate method. In one experiment cotton honey was dialyzed for 24 hours and the inhibiting effect of the dialysate was compared with that of the dialysis bag contents and with the original honey prior to dialysis. The results are shown in Table II. S. lutea was used as the test organism.

Table II. Comparative Bactericidal Effects of Untreated Honey and Clarified or Dialyzed Honey

| | Diameter of Inhibiting Ring | | | | Average |
|--|-----------------------------|------|------|-----|----------------|
| | Replicates | | | | |
| | mm. | mm. | mm. | mm. | mm. |
| Cotton honey, 40% | 9.1 | 8.8 | 8.9 | 8.6 | 8.8 |
| Cotton honey, centrifuged, 40% | 8.4 | 8.1 | 8.0 | 8.1 | 8.2 |
| Cotton honey, 40% | 8.6 | 8.8 | 8.9 | | 8.8 |
| Cotton honey, filtered ¹ , 40% | 0 | 0 | 0 | | 0 ³ |
| Sweet Clover honey, 100% | 11.6 | 16.0 | 18.1 | | 15.2 |
| Sweet Clover honey, filtered ² , 100% | 10.6 | 12.6 | 13.2 | | 12.1 |
| Cotton honey, dialyzed | 8.8 | 8.1 | 8.1 | | 8.3 |
| Dialysate of Cotton honey | 8.0 | 7.6 | 7.9 | | 7.8 |
| Cotton honey, 100% | 10.9 | 16.1 | 14.0 | | 13.7 |

¹Seitz filter

²Sintered glass funnel, medium porosity

³Some slight inhibition

When cotton honey was diluted to 40% with water and centrifuged to remove the suspended pollen, the inhibition of S. lutea was decreased as shown with four replicates. No pollen was found in the centrifuged solution when checked under a microscope.

Filtering a 40% solution of cotton honey through a Seitz filter reduced the inhibition almost to zero. Also, the inhibition of S. lutea by sweet clover honey was lowered by filtering through a sintered glass funnel.

The dialyzing experiment shown in Table II indicates that the inhibiting factor is dialysable. This confirms the results reported by Prica (2), Dold (3), and Plachy (4). Since the filtering experiments indicate that the inhibiting factor is associated with the pollen, it may be concluded that the inhibiting factor is very loosely linked to the pollen.

The Capacity of the Buffer System in Honey

The capacity of the buffer system was determined as previously described. The results were as follows.

| | <u>pH</u> | <u>Capacity</u> ml./pH unit |
|------------------------|-----------|--------------------------------|
| Control sugar | 4.5 | 0.10 |
| Buffered control sugar | 3.6 | 1.57 |
| Cotton honey | 3.8 | 1.27 |
| Sweet Clover honey | 3.9 | .47 |

The buffer capacities of the two honeys tested showed considerable variation. However, according to the results in Table III the buffered sugar control had a capacity sufficiently great so that its pH was maintained even in contact with the well-buffered medium.

Effect of pH on Inhibition

With S. lutea as the test organism for the Pad-Plate test, buffered and non-buffered control sugar solutions were compared to sweet clover honey to determine the contribution of pH to the inhibiting effect.

Table III. Effect of pH on Bacterial Inhibition

| | pH | Diameter of Inhibition Rings | | | Average |
|------------------------|-----|------------------------------|------|------|---------|
| | | mm. | mm. | mm. | |
| Control sugar | 6.3 | 0 | 0 | 0 | 0 |
| Control sugar | 4.2 | 0 | 0 | 0 | 0 |
| Buffered control sugar | 7.0 | 0 | 0 | 0 | 0 |
| Buffered control sugar | 3.6 | 1.56 | 1.45 | 1.68 | 1.56 |
| Sweet Clover, 80% | 7.3 | 1.02 | 1.29 | 1.01 | 1.11 |
| Sweet Clover, 80% | 4.1 | 1.01 | 0.98 | 0.89 | 0.96 |
| Sweet Clover, 100% | 4.7 | 1.42 | 1.32 | 1.48 | 1.41 |

The only control sugar solution which caused inhibition was the one buffered to pH 3.6. Therefore, in this case the acid pH caused definite inhibition. The 100% sweet clover honey had almost the same inhibiting power as the control sugar buffered to pH 3.6, but the buffered control had a stronger buffering system than the honey. Also, the 80% sweet clover honey at pH 7.3 had almost the same inhibiting effect as it did at pH 4.1, indicating that the acidity of honey was not responsible for the inhibition of bacterial growth.

FUTURE EXPERIMENTS

1. The antibacterial experiment will be repeated with heat treated honey to determine whether the inhibiting effect is heat-labile.
2. Work will be started on the yeast growth effect.

LITERATURE CITED

1. Kohler, A. Richard and Broquist, Harry P.
Determination of Aureo^Mmycin in Tissues by a Modified Pad-Plate Assay Method.
Lederle Laboratories Division, American Cyanamid Company, Pearl River,
New York.
2. Prica, M. Z. Hyg. Infektionskrankh. 120, 437 (1938).
3. Dold, H. D.; Du, H. and Dziao, S. T. Ibid 120, 155 (1938).
4. Plachy, E. Zentr. Bakt. Parasitenk., 11 Abt. 106, 401 (1944).