SUBJECT: Progress Report---Evaluation of Honey for Selected Biological Properties.

Contract No. 12-14-100-2596(73).

Period Covered: November 12, 1958 to May 12, 1959.

OBJECTIVES OF THE PERIOD

1. During this period tests were conducted to determine the heat lability of the inhibiting factor in honey.
2. An attempt was made to distill the inhibiting factor from raw honey.
3. Chromatography of the inhibiting factor was attempted on paper with Sarcina lutea as the test organism.
4. Conditions were determined for studying yeast growth effect in honey and a calibration curve was established.

EXPERIMENTAL METHODS

During the period covered by this report tests were conducted to determine the heat lability of the inhibiting factor in honey. The assay method was the Pad-Plate method of Kohler and Broquist, as previously reported. When honey was diluted with water to 83% or less by weight, the variation in inhibiting effect among the replicates was small. However, when undiluted honey was assayed, accurate pipetting was next to impossible because of the high viscosity. Thus, the method employed was to dip the pads in the honey and then allow them to drain uniformly before they were placed on the agar plate. This is the method used by Kohler and Broquist in testing antibiotic substances with their Pad-Plate method. When undiluted honey was so tested, considerable variation occurred among replicate pads. In some cases it was possible to reduce this large variance by dividing the diameter of the outer ring of inhibition by an inner ring which was presumably caused by the high concentration of sugars. This was a valid treatment since the diameter of the inner ring is proportional to the amount of honey on the pad, and this calculation resulted in a much smaller variation. Difficulty was encountered most of the time, however, in distinguishing this inner ring, and the large variation nullified any significant differences which may have been present.

In all of the studies in which honey was heated, timing was started when the temperature of the honey reached 71°C. The temperature was maintained at $71^\circ \pm 1^\circ$ during the period stated.

A study was carried out to determine whether the inhibiting factor was heat-labile or merely lost by volatilization. Honey was heated for $12\frac{1}{2}$ hours in sealed or in unsealed containers and then diluted to 83% by weight with water and assayed for inhibition.

Schuler and Vogel (1) reported the isolation of volatile components from honey which inhibited the growth of _Escherichia coli_, but the honey type was not reported. Based on this work attempts were made in this laboratory to distill the inhibition factor from cotton honey. The first distillation was carried out under a vacuum of 4-6 mm. Hg. One fraction distilled at 43°C, while a second distilled over the range 50-60°C. In this experiment ice water was circulated through the condenser system. Two dry ice traps were utilized in a second distillation at which time three fractions were collected. These distilled at 25°/16 mm, 50-60°/7 mm, and 60-95°/7 mm, respectively, over a period of six hours.

Paper chromatography has been used to separate antibiotics such as penicillin fractions and streptomycin fractions (2). Some preliminary studies were carried out in the work reported here to determine whether the inhibiting factor in honey could be separated by the same method. Cotton and sweet clover honey were diluted to 50% with water and spotted on Whatman No. 1 paper. These were developed for 16 hours in a 4:1:5 butanol-acetic acid-water system, air-dried, and placed on an agar plate seeded with S. lutea. The time of contact of the paper with the agar plate seemed fairly critical; a contact period of 15 minutes yielded the best results in this preliminary work. The agar plates were then incubated at 30°C for 15 hours, after which time they were examined for zones of inhibition, which would correspond to the position of the inhibiting fractions on the chromatogram.

While the work described above was in progress, attempts were being made to grow the yeast Saccharomyces cerviseae in a biotin-free medium with the addition of increasing increments of biotin to derive a standard growth curve. The procedure of Hertz (3) was followed in which the organism maintained on a complete medium was transferred with several washings to a biotin-free medium.

RESULTS ACCOMPLISHED

While studying the heat-lability of the bacterial inhibitor in honey, relatively small standard deviations were achieved in a few cases in which a smaller ring inside the ring of inhibition could be observed. The values in Table I were obtained by dividing the diameter of the outer ring by the diameter of the inner ring. By this method significantly lower values of inhibition resulted for the heated samples than for the unheated controls.

Table I. Inhibition[1] of *M. flavus* by undiluted Fall Flower Honey after heating at 71°C

Replicates	Unheated Control	Period heated, hours			
		½	2	4	26
1	18.2[1]	14.0	13.2	13.5	12.4
2	17.8	13.0	12.2	11.5	11.5
3	17.4	12.5	11.6	11.5	11.2
4	16.5	12.6	11.8	13.2	11.0
5	18.1	13.1	12.6	11.5	11.7
6	17.0	12.2	-	13.6	11.8
Mean ± S.D.[2]	17.5 ± .66	12.9 ± .63*	12.3 ± .64*	12.5 ± 1.05*	11.6 ± .49*

[1]expressed as (diameter of inhibition ring, mm./diameter of inner ring) x 10

[2]mean ± standard deviation of the mean

*significantly lower than control at 5% level of probability

When undiluted cotton honey was tested for inhibition, no inner ring could be detected. The data in Table II for cotton honey heated for various times show a considerably larger standard deviation than did fall flower honey as mentioned above. Despite this large variance cotton honey heated for 4 hours showed significantly less inhibition than the unheated control.

When cotton honey was heated for 4 hours and then diluted to 67% with water, a lowering of the inhibition effect is observed which is just barely significant when compared with the control ($t=2.14$ at $P=.05$ for 16 d.f.). Even though smaller standard deviations were obtained at this dilution (Table III) than with the undiluted honey (Tables I and II), the inhibition factor was apparently diluted to the point where mechanical error in measurement exceeded any noticeable loss in activity.

Table II. Inhibition of M. flavus by undiluted Cotton Honey after heating at 71°C

Replicates	Unheated Control mm.	Period heated, hours			
		½ mm.	2 mm.	4 mm.	26 mm.
1	21.4	17.2	16.4	16.1	15.0
2	21.3	21.0	19.5	18.6	16.1
3	18.5	18.3	17.9	18.9	17.1
4	20.6	19.0	21.8	19.1	17.2
5	20.4	20.1	19.1	17.5	19.0
6	20.0	18.8	20.8	17.0	15.6
Mean ± S.D.	20.4 ± 1.06	19.0 ± 1.33	19.2 ± 1.95	17.8 ± 1.16	16.7 ± 1.42

Table III. Inhibition of S. lutea by Cotton Honey heated at 71°C and diluted to 67%

Replicates	Plate 1		Plate 2	
	Unheated Control mm.	Heated ½ hour mm.	Unheated Control mm.	Heated 4 hours mm.
1	8.5	8.1	9.3	8.9
2	8.2	8.0	9.8	9.1
3	8.6	8.3	9.3	8.9
4	8.1	8.2	8.9	8.9
5	8.0	8.0	9.2	8.2
6	8.1	7.9	8.7	8.8
7	8.0	8.0	8.9	8.5
8	7.9	8.1	9.0	8.8
9	8.1	8.1	9.1	9.0
Mean ± S.D.	8.2 ± .23	8.1 ± .12	9.1 ± .32	8.8 ± .28*

*significantly lower than control at 5% level of probability

The results described in Table III indicate that the inhibiting factor in fall flower and cotton honey is heat-labile (or lost through volatilization.) However, this loss of activity would appear to be negligible in the first 30 minutes of heating since honey heated for 26 hours at 71°C still retained some of its inhibition effect.

In order to determine whether the inhibition factor in honey was heat-labile or merely lost by volatilization, cotton honey and fall flower honey were heated for 12½ hours at 71°C either in open beakers or in sealed flasks. After rapid cooling, the honey samples were diluted to 83% with water, and unheated and heated samples of each honey were tested in replicates of 9 for inhibition of the two microorganisms as notes. The mean values obtained are listed in Table IV.

Table IV. Inhibition of S. lutea and M. flavus by 83% Fall Flower Honey and 83% Cotton Honey Heated at 71°C in Sealed or Open Containers

Microorganism	COTTON HONEY			
	Sealed		Open	
	Control mm.	Heated mm.	Control mm.	Heated mm.
S. lutea	9.8 ± .20	9.6 ± .54	11.5 ± .69	9.7 ± .30[a]
M. flavus	8.8 ± .29	9.2 ± .37[b]	10.6 ± .90	10.1 ± .85

	FALL FLOWER HONEY			
	Sealed		Open	
	Control mm.	Heated mm.	Control mm.	Heated mm.
S. lutea	10.0 ± .22	8.7 ± .49[a]	9.9 ± .62	9.6 ± .37
M. flavus	9.0 ± .43	8.0 ± .30[a]	10.0 ± .86	9.8 ± .79

[a]significantly lower than corresponding control at % level of probability

[b]significantly greater than corresponding control at % level of probability

In general the honey samples heated in sealed flasks showed a decrease in inhibition as compared to the unheated controls. This appears to be evidence of heat-lability of the inhibition factor. Heating samples in open beakers resulted in only one case where activity was decreased.

Several attempts have been made to chromatograph the honey inhibition factor. The results so far, however, have not been very consistent. Inhibition was observed on one plate in a position corresponding to a spot with an Rf of 0.25 on the paper. When the same honey sample was chromatographed under identical conditions and treated with reagent to reveal the position of the sugars, the Rf corresponded with that of the inhibition factor. This work could be extended to other solvent systems in order to separate the factor from the sugars. Of course, it is possible that the factor is intimately related to the sugars and difficult to separate by ordinary methods.

Two fractions were collected during the first vacuum distillation of cotton honey; that which distilled at 43°C showed a very slight inhibition, and the second fraction (50-60°C) showed no inhibition. The three fractions collected during the second trial yielded the following results: 25°C, no inhibition; 50-60°C and 60-95°C, very slight inhibition. It would appear that the inhibiting factor in cotton honey is not as volatile as the factor reported by Schuler and Vogel.

FUTURE EXPERIMENTS

1. The yeast growth factor in the various raw and heat-treated honeys will be investigated.
2. A study of the rooting effect in the various honeys will be carried out.

LITERATURE CITED

1. Schuler, R. and Vogel, R. Wirkstoffe des Bienenhonigs, Arzneimittel Forsch. 6, 661-663 (1956).

2. Block, Richard J. "Paper Chromatography" Academic Press, Inc.; New York, 1952. Pp. 144-151.

3. Hertz. P. "Modification of the yeast-growth assay method for biotin" Proc. Soc. Exp. Biol. Med. 52, 15 (1943).