

2107

STUDIES ON HONEY INHIBINE. 3. EFFECT OF HEAT

JONATHAN W. WHITE, JR., & MARY H. SUBERS

SUMMARY

The effect of heating honey upon its peroxide accumulation value (inhibine) has been examined. Wide variation in sensitivity to heat was noted among 29 samples, which reduces the utility of the inhibine value as a measure of the heat exposure of honey. Between 55 and 70°C., the half-life of the peroxide accumulation system is a logarithmic function of temperature, with a slope approximately that for honey diastase and invertase. In the samples examined, inhibine shows a sensitivity to heat as great as, or greater than, these two enzymes.

INTRODUCTION

The literature shows a marked lack of agreement on the effect of heat on inhibine, the antibacterial principle of honey reported by Dold, Du and Dziao (1937) and defined by the assay procedure of Dold and Witzenhausen (1955). Now that inhibine is known to be hydrogen peroxide accumulation, brought about by the action of honey glucose oxidase during the assay (White, Subers & Schepartz, 1963), the question of the heat lability of inhibine may profitably be re-examined.

The classification of inhibine as heat-labile was originally based on the report (Dold *et al.*, 1937) that it is destroyed by heating a 50% honey solution for 5 minutes at 100°C., 10 minutes at 80°, or 30 minutes at 56°.

Schade, Marsh and Eckert (1958), on the basis of two experiments, stated that inhibine was more heat-labile than diastase, with an approximate half-life of 4·5 hours at 62·5°C. They noted, as did Warnecke and Duisberg (1958), honey samples with low enzyme and high inhibine contents, and vice versa. We have also reported this (White & Subers, 1963).

Duisberg and Warnecke (1959), using the assay of Dold and Witzenhausen, disagreed with Dold *et al.* regarding sensitivity at 56°C., but found some reduction in activity. They noted that the 0-5 inhibine scale proposed by Dold and Witzenhausen is not suitable for measuring and reporting loss of inhibine. They reported that (for 20 samples) the sensitivity of inhibine at 50-56° was very similar to that of invertase. Their data show the average half-life for inhibine to be 10 hours at about 57°C; a half-life for invertase of 10 hours at about 58-59°C. can also be calculated from their results.

Stomfay-Stitz and Kominos (1960) assayed 16 United States honeys by the Dold technique, but using different organisms. They found that 24 hours at 56°C. reduced the inhibine values of five samples from 7-11 to 3 (their notation), equivalent to a reduction from 3-5 to about 1·5 on the Dold scale. At 80°, 15 minutes reduced the values to zero.

Gonnet and Lavie (1960) have vigorously questioned the heat-sensitivity of the antibiotic factor; they found it to be fairly heat-stable, 30 minutes at 80° destroying about half the activity (Dold scale). They were unable to reproduce the German results, though using the same methods (but not the recommended organism). They noted that

honeys vary widely in their response to heat, some being very sensitive to short moderate heating, though they lose only part of their activity, and others being much more resistant. Much of Gonnet and Lavie's work was concerned with solvent extracts of honey; antibacterial activity obtained in this way resisted 15 minutes' autoclaving at 120°C. Lavie (1963) has recently agreed with our view that inhibine is hydrogen peroxide formed during the assay, and believes that honey contains another group of antibacterial factors; substances which are volatile, photolabile, extractable by solvents, and more heat-stable than inhibine.

Examination of the paper by Dold, Du and Dziao shows that the method of assay they originally used for heat lability did not detect *destruction* of inhibine, but demonstrated only that the inhibine was reduced sufficiently to allow bacterial growth on a plate containing 17% honey; in most cultures growth did not occur at this honey concentration before heating. This corresponds to an inhibine number (as later defined by Dold and Witzenhausen) of about 2·5. As we now measure inhibine, 'destruction' means reduction of the inhibine number to zero. Thus, re-interpreting the data of Dold and his colleagues, it is not known whether the conditions they recorded *destroyed* the inhibine; they showed only that inhibine is somewhat heat-sensitive.

In summary, different honeys differ widely in the response of their antibacterial activity to heat; the conclusions of Dold *et al.* (1937) regarding heat destruction of inhibine are not acceptable; several investigators (Duisberg & Warnecke, Schade *et al.*, Stomfay-Stitz & Kominos) report inhibine to be heat-sensitive; finally Gonnet and Lavie find varying response—but never complete destruction—of the antibacterial activity by heat, with considerably more resistance to heat than has been found by other workers.

Since we are convinced that nearly all the non-osmotic antibacterial activity of the honey samples we have examined is enzymic in origin, and that the agent primarily responsible (H_2O_2) is essentially absent from undiluted honey, we have considered the problem as one of enzyme inactivation by heat. As previously noted (White & Subers, 1963), the inhibine value of a sample of honey depends not only upon the activity of glucose oxidase in producing peroxide, but also on the possible destruction of the peroxide by other honey constituents. The relation between H_2O_2 content of an incubated honey solution and the inhibine number (Dold) of the honey is logarithmic. Hence the use of inhibine numbers in evaluating the effect of a treatment on the actual concentration of the antibiotic material is misleading. A reduction in inhibine from 4 to 2·25 (such as that reported by Gonnet and Lavie by heating at 75–80°C. for 1 hour) may appear minor, but in terms of peroxide production it represents a drop from 60–175 to about 15–30 γ/g .

We have determined the effect of heating four undiluted honeys on their peroxide accumulation values, examined a number of other samples in less detail, and also carried out several Dold inhibine assays of heated honeys.

METHODS

Honey samples

All samples of honey were unheated, either by the producers or in the laboratory. Samples in the HS series were from 60-lb. [27-kg.] lots stored at all times at about 4°C. Other samples, of the 1956 and 1957 crops, were originally obtained from their producers

for an analytical survey of United States honey (White, Riethof, Subers & Kushnir, 1962), and had been stored at -20°C . since their arrival, shortly after extraction.

Weighing and handling of samples

Because of the great sensitivity of the peroxide accumulation system to light, all samples were weighed in a semi-darkened room, and heating was done under heavily shaded diffused daylight. After dilution with buffer, samples are no longer light-sensitive.

Hydrogen peroxide accumulation

This was determined as previously described (White & Subers, 1963).

Inhibine assay

The Dold and Witzenhausen (1955) method as described by Schade *et al.* (1958) was used.

Heating of samples

For short-time heating, 4-g. samples of honey in 18×150 -mm. test-tubes were warmed in a bath 5°C . higher than the intended temperature, while being stirred with a thermometer. When the desired temperature was reached, they were transferred to a bath at that temperature; at the end of the time they were cooled rapidly. Samples heated for longer times (55°) were contained in small, closed jars.

RESULTS AND DISCUSSION

Four honey samples were heated at 55° , 60° , 65° and 70°C . for appropriate times. Straight lines over the entire range were rarely obtained by plotting the logarithm of remaining activity against time; deviation was greatest at 60° . In general, however, the portion of the curve from 100% to about 40% activity was linear; deviation was at lower activities (longer heating times). In those tests where data extended over a sufficient time span, the experimental points fit a curve calculated for a mixture of two components showing straight lines through the origin (100% remaining), their slopes indicating relative heat-resistance and heat-sensitivity (for HS33, half-lives of 4 and 0.4 hours). These four samples (HS33, HS35, HS36, HS37) showed such a great sensitivity to heat that reliable values for the course of the destruction at 70° could not be obtained; less than 15% activity remained after 5 minutes; in the one attempt with shorter times, 15% remained after one minute at 70° . The graphical analysis described above for HS33 indicated a half-life for this sample of 0–2 min. at 70° .

To summarize the results, Fig. 1 shows the time required for loss of half of the activity (peroxide accumulation) at various temperatures; the values were obtained from the plotted lines for the individual samples. Half-lives at 65°C . for the peroxide accumulation system ranged from 0.01 to 0.075 hours (36 seconds–4.5 minutes); at 55° , from 2.8 to 6.1 hours.

Several of the heated samples represented in Fig. 1 were also subjected to the bacteriological assay for inhibine (Table 1). The table also includes inhibine numbers predicted from the peroxide values, by the relation given by White and Subers (1963). The inhibine number predicted agrees with the number found, except for the third assay, which gave a value of 3, whereas the 20.2 peroxide value is slightly below the lower limit for 3 (20.6); it is therefore listed as 2.

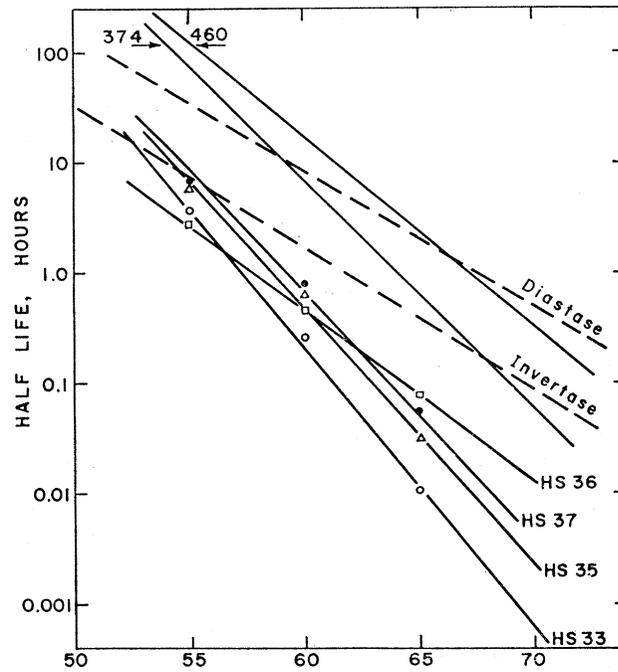


Fig. 1. Effect of temperature on the half-life of diastase, invertase, and the peroxide accumulation system in honey

TABLE 1. Inhibine values of heated honey^a

Treatment	Peroxide value	Inhibine	
		predicted ^b	found
none	107	4	4
6 hr., 55°C.	36	3	3
16 hr., 55°	20.2	2	3
2 hr., 60°	29.5	3	3
6 hr., 60°	12.3	2	2
5 min., 70°	24	3	3
15 min., 70°	8.2	1	1
5 min., 75°	2.0	0	0
15 min., 75°	3.0	0	0

^aSample HS37

^bPredicted from the peroxide accumulation value by the relation shown in Table 2 of White & Subers 1963)

For an estimate of the variability in heat resistance among different honeys, 29 samples were heated at 70°C. for 10 minutes (Table 2). About two-thirds of the samples lost more than 80% of their activity, five lost 60–80%, two lost 40–60%, and two were more heat-resistant. Portions of the last two honeys (374, 460) were also heated at 60° and 65°; their half-lives are shown in Fig. 1.

TABLE 2. Effect of heat^a on peroxide accumulation value of honey

No. and type ^b	Peroxide accumulation ^c			Inhibine equivalent ^d	
	initial	heated	loss %	initial	heated
11 Alfalfa	104	23.5	77	4	3
89 Alfalfa - Sweet clover	150	3.4	98	4	1
103 Summer blend	97.5	9.7	90	4	2
168 Chinquapin	131	123	6	4	4
178 Crimson clover	92.5	12.5	86	4	2
200 Sweet clover - alfalfa	6.2	0	100	1	0
249 Clover blend	260	75	71	5	4
293 Clover - cotton	115	18.5	84	4	2
311 Cotton	162	11.2	93	4	2
321 Cranberry	130	31.5	76	4	3
340 Goldenrod - aster	102	5.7	94	4	1
355 Horsemint	252	23.0	91	5	3
359 Lespedeza	95	0.7	99	4	0
361 Black locust	85	21.8	74	4	3
374 Mustard	90	38.6	57	4	3
387 Orange - grapefruit	51	4.7	91	3	1
389 Orange - grapefruit	23.3	4.2	82	3	1
391 Orange - grapefruit	135	13.7	90	4	2
397 Palmetto	150	22.2	85	4	3
401 Peppermint	115	3.6	97	4	1
431 Spanish needle	175	21.2	88	4	3
442 Star thistle	81	17.2	79	4	2
460 Tulip tree - blend	124	78	37	4	4
471 Vetch	170	3.1	98	4	0
499 Oak honeydew	175	81	54	5	4
HS33 Fall blend	118	8.5	93	4	1
HS35 Fall blend	96	9.0	91	4	2
HS37 Fall blend	116	10.0	91	4	2
HS38 Cotton	310	11.3	96	5	2

^aSamples heated 10 minutes at 70°C.

^bComplete information on samples appears elsewhere (White *et al.* 1962)

^cMicrograms peroxide per gram of honey under assay conditions

^dCalculated

For comparison, the half-lives of diastase and invertase in honey, as reported earlier (White, Kushnir & Subers, 1963), are also given in Fig. 1. The heat resistance of the peroxide accumulation system (inhibine) of the two more stable honeys is comparable with that of these two enzymes; that of most of the samples is considerably less.

Though the number of honeys examined was limited, the heat resistance of their peroxide accumulation systems varied 70-fold or more. Table 2 shows inhibine numbers for the samples, derived from the peroxide values. Because of the logarithmic nature of the relationship, a loss in peroxide accumulation of 82% is equivalent to a 47% loss in inhibine number.

The wide range of sensitivity of inhibine to heat found in the few samples examined here casts further doubt on the usefulness of the inhibine value as a means of assessing the previous exposure of honey to heat, as proposed by other workers. A strong influence of floral type upon peroxide accumulation was indicated earlier (White & Subers, 1963) and should be further investigated before this property of honey is used as a criterion of quality.

ACKNOWLEDGEMENT

The assistance of A. I. Schepartz with the inhibine assays is appreciated.

REFERENCES

- DOLD, H., DU, D. H. & DZIAO, S. T. (1937) Nachweis antibakterieller, hitze- und lichtempfindlicher Hemmungsstoffe (Inhibine) im Naturhonig (Blütenhonig). *Z. Hyg. InfektKr.* 120 : 155-167
- DOLD, H. & WITZENHAUSEN, R. (1955) Ein Verfahren zur Beurteilung der örtlichen inhibitorischen (keimvermehrungshemmenden) Wirkung von Honigsorten verschiedener Herkunft. *Z. Hyg. InfektKr.* 141 : 333-337
- DUISBERG, H. & WARNECKE, B. (1959) Erhitzungs- und Lichteinfluss auf Fermente und Inhibine des Honigs. *Z. LebensmittUntersuch.* 111 : 111-119
- GONNET, M. & LAVIE, P. (1960) Influence du chauffage sur le facteur antibiotique présent dans les miels. *Ann. Abeille* 3 : 349-364
- LAVIE, P. (1963) Sur l'identification des substances antibactériennes présentes dans le miel. *C.R. Acad. Sci., Paris* 256 : 1858-1860
- SCHADE, J. E., MARSH, G. L. & ECKERT, J. E. (1958) Diastase activity and hydroxy-methyl-furfural in honey and their usefulness in detecting heat alteration. *Food Res.* 23 : 446-463
- STOMFAY-STITZ, J. & KOMINOS, S. D. (1960) Ueber bakteriostatische Wirkung des Honigs. *Z. Lebensmitt-Untersuch.* 113 : 304-309
- WARNECKE, B. & DUISBERG, H. (1958) Die bakteriostatische (inhibitorische) Wirkung des Honigs. *Z. LebensmittUntersuch.* 107 : 340-344
- WHITE, J. W., JR., KUSHNIR, I., & SUBERS, M. H. (1963) Effect of storage and processing temperatures on honey quality. *Food Tech.* 18(4) : 153-166
- WHITE, J. W., JR., RIETHOF, M. L., SUBERS, M. H. & KUSHNIR, I. (1962) Composition of American honeys. *Tech. Bull. U.S. Dep. Agric. No.* 1261 : 124 pages
- WHITE, J. W., JR. & SUBERS, M. H. (1963) Studies on honey inhibine. 2. A chemical assay. *J. apic. Res.* 2(2) : 93-100
- WHITE, J. W., JR., SUBERS, M. H. & SCHEPARTZ, A. I. (1963) The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. *Biochim. biophys. Acta* 73 : 57-70