

SUMMARY

A reproducible procedure is presented for the determination of hydrogen peroxide accumulation in diluted honey. Results are given for 90 honey samples, for 45 of which the inhibine value was also determined. The inhibine number of honey is shown to be linearly related to the logarithm of the hydrogen peroxide accumulation.

INTRODUCTION

Inhibine, an antibiotic principle reported in honey by Dold, Du, and Dziao (1937) and confirmed by Prica (1938) and Plachy (1944), has been assayed by its effect on the growth of *Staphylococcus aureus* on nutrient agar plates containing graded amounts of honey (Dold & Witzhausen, 1955). This assay has been used in studies of the occurrence and stability of inhibine.

We have recently (White, Subers & Schepartz, 1962, 1963) demonstrated that the antibacterial effects shown by honey in the inhibine assay result from accumulation of hydrogen peroxide produced by a natural glucose oxidase system in honey. Addition of *o*-dianisidine and peroxidase to the agar medium in the bacteriological assay permitted an estimation of peroxide (i.e. hydrogen peroxide) production during the assay. Although the procedure gave reasonably reproducible values, it is not accurate enough for research use and somewhat cumbersome for routine determination of peroxide production. Furthermore, it is probable that the antibacterial effects displayed are more directly related to the rate of peroxide accumulation in the assay medium, representing the excess of production over any peroxide destruction by honey constituents, than to peroxide production alone.

Adcock (1962) has independently suggested a possible connection between inhibine and peroxide values, having found that inhibine can be destroyed by catalase. He found little correlation between inhibine values and peroxide values, however, and was unable to explain this.

We have developed an assay for the accumulation of hydrogen peroxide in diluted honey, and compared the results of the assay with standard bacteriological inhibine assays, in 45 honey samples. A logarithmic relation between inhibine number and peroxide accumulation is shown. Full-density honey has been found (White, Subers & Schepartz, 1962) to contain 0.3 parts per million of hydrogen peroxide or less. All the peroxide measured in the assay is thus produced during the incubation.

METHODS

Production of colour by *o*-dianisidine and peroxidase in the presence of hydrogen peroxide is the basis of the procedure finally adopted. In preliminary work involving the measurement of peroxide present in diluted buffered honey incubated under various

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conditions, a considerable lack of reproducibility was noted. This was traced to the relation of surface area exposed to depth of solution, and led to the adoption of a rolling type of agitation to provide optimal and reproducible surface exposure.

Assay for peroxide accumulation in honey

Reagents

Buffer: 0.4 M phosphate, pH 6.5.

Peroxide reagent: dilute 5 ml. buffer, and 10 mg. o-dianisidine (3,3'-dimethoxybenzidine, Practical, Eastman Organic Chemicals, Rochester, N.Y.*) in 2 ml. 95% ethyl alcohol to 200 ml. with water. Make fresh daily.

Peroxidase: 2 mg. peroxidase (Sigma Chemical Company, St. Louis, Mo., type 1, horseradish) is dissolved in 50 ml. 0.01 M phosphate buffer, pH 6.5.

Apparatus

A Tissue Culture Rollordrum (Model TC 5 with drum to hold 16 250-ml. centrifuge bottles, New Brunswick Scientific Company, New Brunswick, N.J.) operating at 20 r.p.m. in an incubator at 37°C. is used.

Bottles: narrow-mouth, screw-neck, 8-oz. [225 ml.] round flint glass bottles are used, with moulded plastic screw caps with conical polyethylene liners (Poly-seal, A. H. Thomas Company, Philadelphia, Pa.). Such caps outlast all other types used.

Procedure

Weigh under minimal illumination 10 g. honey to the nearest 0.01 g., add 5 ml. buffer, transfer to a 25-ml. volumetric flask, and make to volume with water. After the buffer is added the enzyme is no longer light-sensitive, and ordinary laboratory illumination may be used. To the 8-oz. bottle is added 10 ml. honey solution and 10 ml. water. The bottle is capped and warmed to 37° in a bath, without agitation. It is placed in the roller drum at 20 r.p.m., 37°, for 1 hour.

Three test-tubes are meanwhile prepared for each sample: two containing 6.00 ml. reagent and sufficient water to make 2.0 ml. when added to the sample volume, and the third (blank) tube containing water in place of the reagent. After the 1 hour's incubation, the bottle is removed, and an appropriate volume (0.1 to 2.0 ml. depending on peroxide content) of the incubated solution is added to each tube within 5 minutes. After mixing, 1 drop of peroxidase solution is added to each tube, the contents mixed, and the absorbance at 450 m μ is determined between 5 and 10 minutes after the final mixing.†

The reagent is calibrated with aqueous dilutions of hydrogen peroxide, the stock solution of which is standardized by Kingzett's iodide method (Scott, 1939).

The result of the assay is expressed as micrograms hydrogen peroxide accumulated per hour per gram of honey.

Inhibine assay

The procedure of Dold and Witzenhausen, as described by Schade *et al.* (1958), was used.

* Mention of a company or its products does not constitute endorsement by the Department over others not named.

† Blanks containing reagent and no peroxidase give very slightly lower values for the assay, as some bleaching of honey colour takes place in the incubated solution in the presence of peroxidase. Determination of peroxide content of unincubated solutions showed none to be present within the limit of the method.

RESULTS AND DISCUSSION

The honey concentration and the pH during the assay are those of the first plate in the bacteriological assay as used in our previous work (White, Subers & Schepartz, 1963). During the development of the procedure it was noted that the peroxide accumulation values obtained were strongly influenced by exposure of the undiluted honey to light, either diffuse daylight or fluorescent-tube illumination in the laboratory. This is avoided by weighing the sample in low-level illumination. Further studies of the effect of ultraviolet and visible radiation on inhibine and peroxide accumulation have been made and will be reported later.

Table 1 shows the results of analysis of 90 samples of honey by the method described here. Of these samples, 45 were also assayed by the bacteriological procedure for inhibine, to clarify the relationship between peroxide accumulation and inhibine number. In the inhibine assays, no effort was made to assign fractional values between whole numbers as other workers have done (Stomfay-Stitz & Kominos, 1960; Adcock, 1962; Duisberg & Warnecke, 1959).

TABLE 1. Peroxide accumulation, inhibine, and diastase values for honey

No. ^a	Honey type	Diastase ^b	Inhibine	Peroxide accumulation ^c
2	Alfalfa <i>Medicago sativa</i>	14.2	0	4.3
5	Alfalfa	18.2	0	3.0
11	Alfalfa	22.2	—	104
14	Alfalfa	21.9	—	8.5
19	Alfalfa	—	3	23
34	Alfalfa—sweet clover <i>M. sativa</i> — <i>Melilotus</i>	12.6	—	30
74	Basswood—Clover <i>Tilia americana</i> — <i>Trifolium</i>	30.9	1	9.4
79	Lima Bean <i>Phaseolus lunatus</i>	16.0	0	0
85	Blackberry <i>Rubus</i> spp.	14.0	4	107
89	Spring blend	42.3	—	150
103	Summer blend	20.0	—	97
104	Summer blend	10.8	0	1.0
126	Fall blend	19.4	3	3.5
151	Boneset <i>Eupatorium</i> spp.	25.6	1	0.9
152	Buckwheat <i>Fagopyrum esculentum</i>	46.2	—	4.5
153	Buckwheat	31.6	4	72
158	Wild buckwheat <i>Erigonium fasciculatum</i>	33.0	—	102
168	Chinquapin <i>Castanea pumila</i>	31.6	—	111
174	Alsike and Sweet clovers <i>Trifolium hybridum</i> — <i>Melilotus</i>	18.2	2	23
178	Crimson clover <i>Trifolium incarnatum</i>	31.9	—	92
193	Sweet clover	22.6	0	6.6
200	Sweet clover	10.2	0	5.0
234	White clover <i>Trifolium repens</i>	61.2	—	16.5
236	White clover	27.3	—	0.7
249	Clover—Spring blend	29.4	—	260
293	Clover—Cotton	29.3	—	145
308	Cotton <i>Gossypium hirsutum</i>	12.2	4	132
309	Cotton	21.8	—	80
311	Cotton	23.6	4	162
313	Cotton	—	5	325
321	Cranberry <i>Vaccinium macrocarpon</i>	26.7	—	130
324	Eucalyptus	31.6	4	173
326	Fireweed <i>Epilobium angustifolium</i>	17.6	—	115
331	Gallberry <i>Ilex glabra</i>	21.4	3	38
332	Gallberry	18.0	—	2.0

No. ^a	Honey type	Diastase ^b	Inhibine	Peroxide accumulation ^c
336	Goldenrod <i>Solidago</i>	24.0	0	0.7
338	Goldenrod	50.8	—	0.3
340	Goldenrod—Aster	46.2	—	102
349	Heartsease <i>Polygonum</i> spp.	35.3	2	15
355	Horsemint <i>Monarda punctata</i>	21.7	5	252
359	Lespedeza <i>Lespedeza sericea</i>	43.5	—	89
361	Black locust <i>Robinia pseudoacacia</i>	14.6	—	86
365	Manzanita <i>Arctostaphylos</i>	7.8	—	2.4
369	Mesquite <i>Prosopis glandulosa</i>	8.3	0	9.0
372	Mint <i>Mentha</i>	15.0	0	0.0
374	Mustard <i>Brassica campestris</i>	18.8	4	90
378	Orange <i>Citrus sinensis</i>	15.8	0	1.7
383	Orange—Grapefruit <i>Citrus sinensis</i> — <i>C. paradisi</i>	8.7	3	35
384	Orange—Grapefruit	11.5	4	50
387	Orange—Grapefruit	7.5	—	51
389	Orange—Grapefruit	9.1	—	23
391	Orange	16.9	—	135
394	Palmetto <i>Sabal</i> spp.	11.8	2	7.0
397	Saw palmetto <i>Serenoa serrulata</i>	21.1	—	150
401	Peppermint <i>Mentha piperita</i>	17.1	—	115
403	Peppervine <i>Ampelopsis</i>	6.7	—	3.1
409	Purple loosestrife <i>Lythrum salicaria</i>	30.6	—	4.7
410	Raspberry <i>Rubus occidentalis</i>	14.0	2	15
415	Rosinweed <i>Grindelia squarrosa</i>	55.6	2	9.0
424	Sourwood <i>Oxydendrum arboreum</i>	15.6	2	18.5
425	Sourwood	8.6	—	3.4
430	Spanish needle—Heartsease <i>Bidens</i> — <i>Polygonum</i>	32.6	—	52
431	Spanish needle—Heartsease	43.5	4	175
442	Star thistle <i>Centaurea solstitialis</i>	36.4	—	81
449	Trefoil—Vetch <i>Lotus corniculatus</i> — <i>Vicia</i> spp.	10.3	—	1.4
453	Tulip tree <i>Liriodendron tulipifera</i>	18.5	3	33
454	Tulip tree	13.2	—	0
460	Tulip tree—Blend	17.6	—	124
464	Tupelo <i>Nyssa ogeche</i>	17.1	4	42
469	Vetch <i>Vicia</i>	16.9	—	19
470	Vetch	21.7	0	68
471	Vetch	21.1	4	170
472	Vetch	5.9	—	3.4
481	Hairy vetch <i>V. villosa</i>	11.3	—	5.7
499	Oak honeydew <i>Quercus</i>	41.4	5	182
503	Honeydew	48.4	—	63
HS33	Fall blend	30.6	4	120
HS35	Fall blend	45.8	4	100
HS36	Clover	15.1	2	13.3
HS37	Fall blend	46.2	4	83
HS38	Cotton <i>Gossypium hirsutum</i>	—	5	310
M261	Tupelo	16.1	3	16.8
M262	White clover <i>Trifolium repens</i>	—	0	0.0
M266	Cotton	—	—	360
M267	Cotton	—	—	320
M268	Cotton	—	5	332
M269	Cotton	—	—	245
M270	Cotton	—	—	325
M271	Cotton	—	—	225
M512	Tupelo	17.6	—	0.9

^a Sample numbers without letters are those of honeys whose complete description and analyses appear in Technical Bulletin 1261 (White *et al.*, 1962). All samples were unheated except M262.

^b Grams starch hydrolysed per 100 g. honey per hour at 40°.

^c Micrograms hydrogen peroxide accumulated per g. honey in 1 hour under assay conditions.

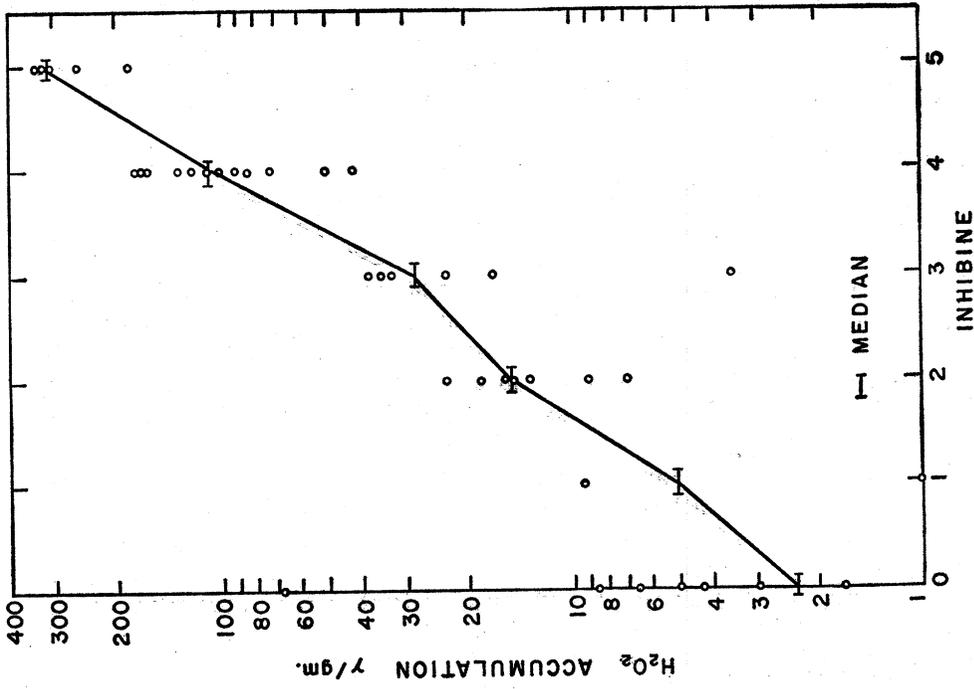


Fig. 1. Relation between hydrogen peroxide accumulation by honey solutions and inhibine number. Five values with inhibine numbers of 0 and peroxide accumulation of less than 1 γ/g. are not shown.

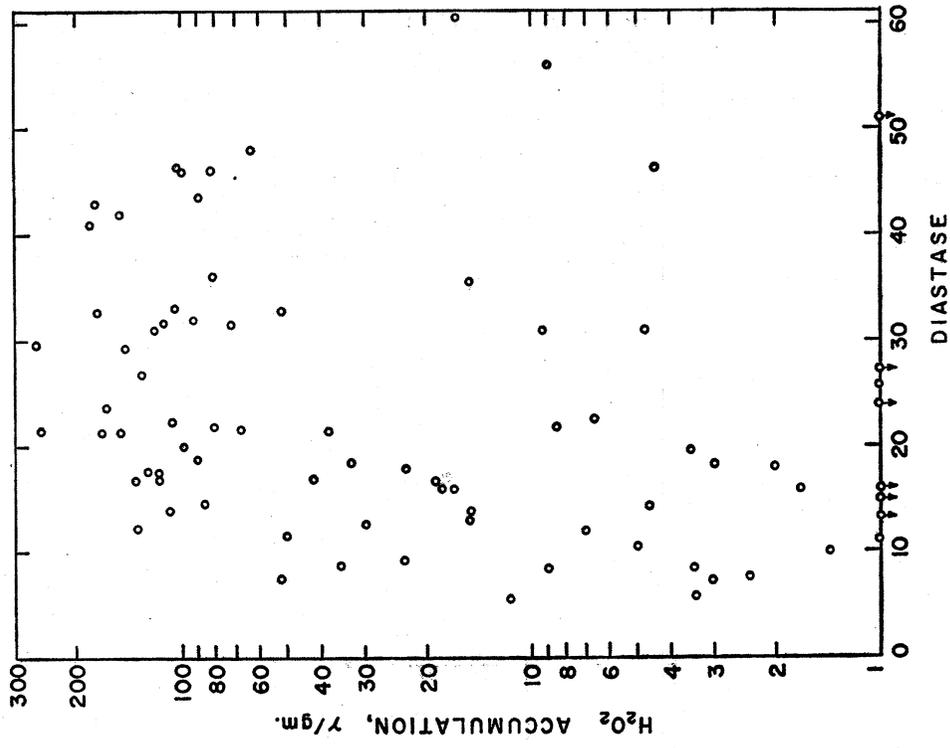


Fig. 2. Relation between hydrogen peroxide accumulation and diastase values of honey

Fig. 1 shows the relation obtained between inhibine number and peroxide accumulation. A certain degree of 'overlap' between adjacent inhibine numbers was found (and is to be expected), because of the relative insensitivity and inaccuracy of the inhibine assay.

Several values may be noted that fall far from the medians. These have been checked, especially the sample with an inhibine number 0 and peroxide value 68. Insufficient material was available to determine the cause of the apparent anomaly; it is possible that a growth factor was present in the sample. Growth was present, but considerably reduced, in plates corresponding to inhibine numbers 1 and 2, and somewhat reduced in the third plate.

TABLE 2. Relation between inhibine number and peroxide accumulation

<i>Inhibine No.</i>	<i>Peroxide accumulation^a</i>
0	<3.4
1	3.4- 8.7
2	8.8- 20.5
3	20.6- 54.5
4	54.6-174
5	>174

^a Micrograms hydrogen peroxide accumulated per g. honey in 1 hour under assay conditions.

In Fig. 1 the median values of peroxide accumulation for each inhibine number are indicated. An approximate classification into inhibine classes from peroxide values can be made, as shown in Table 2.

Adcock (1962) determined the peroxide content of diluted honey incubated 1 hour at 37°, and obtained values varying from 2.08 to 11.52 ml. 0.01 N thiosulphate per 100 g. honey (equivalent to 35-196 γ H₂O₂/g. by our notation). Although he was able to destroy all inhibine values with catalase, his data show no quantitative relation between the peroxide value of honey as determined by his procedure and the inhibine value.

In examining the data at hand, it became evident that certain honey types show interesting trends. The high values obtained for cotton honey (308, 313, 311) led us to examine several additional samples of cotton honey. The last six values (M-266 - M-271) shown in Table 1 are of samples of cotton honey selected at random.* All show very high peroxide accumulation.

Since both diastase and glucose oxidase probably originate within the bee, it might be expected that samples which are rich in diastase are also high in glucose oxidase. Fig. 2 shows how the peroxide accumulation values for the samples in Table 1 compare with the diastase values. The relationship is far from striking. The peroxide accumulation value as assayed is not a direct measure of glucose oxidase in honey, but rather of the

* We are indebted to R. L. Church, and the Superior Honey Company of Phoenix, Arizona, for providing these samples.

difference between peroxide production by the enzyme and its destruction by various constituents of the honey. Hydrogen peroxide disappears rapidly when added to dilute honey; after incubation the peroxide value of the solution declines, except with samples showing very high values. Minor components arising from the nectar or pollen, and oxidizable by peroxide, may vary in amount and identity from honey to honey and may be absent from some. The consistently high values found for cotton honey may reflect the absence from this honey of some factor which destroys peroxide—or enzymes—as well as a high enzyme content. Thus the peroxide accumulation value for a honey is affected by the glucose oxidase content (which appears to be introduced during ripening by the bees) and also by various minor components in the honey (from sources such as nectar, pollen, yeasts) which interact with the enzyme or with the hydrogen peroxide produced. It may also be affected to a greater or lesser extent by the handling, storage, and processing of the honey after it is removed from the hives. All these factors can therefore affect the inhibine value of the honey, as defined by Dold *et al.* (1937).

The effects of heat and light on the peroxide accumulation value of honey have been studied and will be reported in detail; anomalous results of some investigators on heat and light sensitivities of honey inhibine will also be discussed.

It appears from the data we have at hand that peroxide accumulation by diluted honey accounts for a major part of the non-osmotic antibacterial effect of honey, but there appear to be minor levels of antibacterial action due to other materials in honey. Lavie has demonstrated that acetone or alcohol will extract a heat-stable material from undiluted honey which has antibacterial activity. Until recently, differences in heat sensitivity between substances he examined and inhibine described by the German workers led Lavie to reject the latter's concept of inhibine (Gonnet & Lavie, 1960). But he now agrees with us (Lavie, 1963) that Dold's inhibine is enzyme-produced hydrogen peroxide and heat-sensitive, and states that the relatively heat-stable antibacterial materials from honey with which he and his colleagues have been concerned form a different category of antibacterial factors in honey.

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