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Studies on Honey Inhibine. 4. Destruction of the Peroxide Accumulation System by Light

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SUMMARY

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Conflicting reports on the sensitivity of honey inhibine (accumulated hydrogen peroxide) to light are resolved by demonstration of great variability in the effect of light on the peroxide accumulation value of different honeys. Destruction of the glucose oxidase in honey by photo-oxidation has been observed. This enzyme, responsible for peroxide production in diluted honey, is destroyed in either full-density or diluted honey by visible radiation, with the 425-525 m μ region most effective. General laboratory illumination, particularly fluorescent-tube lighting, is highly destructive to inhibine in a sensitive full-density honey. The liability of the inhibine of a honey sample to photo-destruction depends on the presence of a heat- and light-stable, non-volatile sensitizing material; photo-oxidation is greatest at pH 3 and negligible at pH 6-7. Immediate adjustment of the pH of honey samples prepared for bacteriological inhibine assay is therefore essential. Inhibine content of honey is not recommended as a quality factor, because of wide variation in light and heat susceptibility and in distribution according to floral source.

INTRODUCTION

In the initial report on inhibine, an antibacterial substance in honey, Dold *et al.* (1937) stated that inhibine content of honey was reduced by sunlight. A honey which originally prevented bacterial growth on agar at the 17% level was found after 1 hr of exposure in a thin layer to sunlight to allow such growth. Prica (1938) generally confirmed the existence of an antibacterial principle, but noted that it was nearly insensitive to diffuse daylight, resisting 20 months standing in the laboratory. Duisberg and Warnecke (1959) examined in some detail the effect of light on inhibine. Storage of samples of honey in clear glass jars near a window for 5-7 months destroyed 55% of the inhibine and 36% of saccharase. In another set, 11 samples lost an average of 34% of their inhibine and 31% of their saccharase in clear glass; in dark ultraviolet-absorbing glass, the corresponding losses were 0 and 15%. Although Prica had rejected the possibility of inhibine being an enzyme, because of its light sensitivity, it is noteworthy that Duisberg and Warnecke's data show the extent of light de-

struction under their conditions to be similar for inhibine and saccharase.

We have recently shown (White *et al.*, 1963) that "inhibine" is the result of accumulated hydrogen peroxide on bacteria in inhibine assay plates; the peroxide originates from the action of the natural glucose oxidase in honey on the glucose therein. Natural full-density honey contains no hydrogen peroxide (<0.3 ppm); the enzyme system is virtually inoperative in full-density honey but becomes active, producing hydrogen peroxide and gluconic acid (gluconolactone), when the honey is diluted. As measured by increase in acidity during long-time storage, the system produces in full-density honey about 0.002-0.012 μ g H₂O₂/g honey /hr. Other components of the honey probably destroy the peroxide as it is produced at this low rate.

We recently described a chemical assay for hydrogen peroxide accumulation in diluted honey (White and Subers, 1963) which we have shown to be logarithmically related to the inhibine number obtained by microbiological assay. The much greater convenience and sensitivity of the peroxide

volume within the sac being 30 ml. Portions of each solution (10 ml) were assayed for peroxide accumulation after the addition of 1.9 g glucose (corresponding to the equivalent amount of honey). The unexposed control showed $71.6 \mu\text{g H}_2\text{O}_2/\text{g}$ original honey, and the irradiated sample $1.7 \mu\text{g/g}$. The loss of activity on irradiation is thus not due to the production of low-molecular-weight peroxide-destroying substances or other materials interfering with the assay, but rather to a destruction of the enzyme. Samples of honey (#HS 37) were exposed to sunlight for times between 5 sec and 3 min. Fig. 1 shows the effect on the

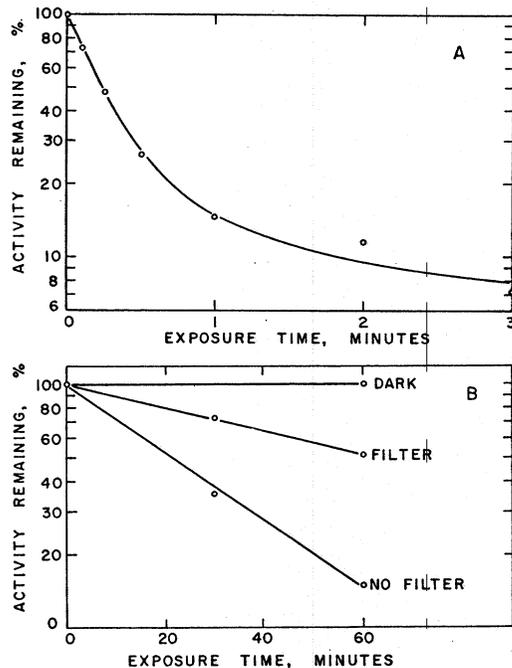


Fig. 1. Destruction of peroxide accumulation value of honey by radiation. A) Honey (10 g) in thin layer in 8.5-cm flat dish exposed to sunlight through 3-mm window glass with air mass about 1.5. B) Honey (10 g) as in A exposed on laboratory bench to overhead fluorescent tube illumination at 1.7-m distance (no filter); in light-tight dish covered by Noviol B (1.5 mm) filter; and held in darkness for indicated times.

peroxide accumulation value. Between 0 and 75% destruction the inactivation appears to follow first-order kinetics.

Effect of radiation source. Samples of honey (#HS 37) were exposed to unfiltered (the "Blacklite" tube had a dark visible-absorbing envelope and the sun exposure

was through window glass as previously noted) radiation from several sources for appropriate times. Results are shown in Table 1. The intensities of radiation shown therein were calculated for the sample distances. Comparison of the effects of the "Blacklite" and the fluorescent laboratory lights indicates the visible light to be considerably more destructive than ultraviolet.

To eliminate the ultraviolet, samples were exposed through 1.5-mm thickness of Noviol B filter (Corning Glass Co., Corning, N. Y.), which transmits only radiation longer than $440 \text{ m}\mu$. Table 2 shows the effect of such radiation on the enzyme system. The less destructive sources (sun, incandescent, mercury arc) are similar in producing a considerable portion of their radiation in the infrared when filtered by Noviol. The more effective fluorescent tube lighting, when filtered by Noviol B, has an output between 440 and $604 \text{ m}\mu$, with a maximum at $484 \text{ m}\mu$. Visible radiation in the 440–600 $\text{m}\mu$ region is thus quite destructive to the peroxide accumulation system in honey.

To locate more closely the spectral region responsible for the inactivation, samples (#HS 37) were exposed to several sources through Wratten filters. Results of such irradiation with the high-pressure Hg arc are shown in Table 3. An attempt was made to calculate the relative effectiveness of the various spectral regions, using the manufacturers' values for arc output. It is assumed in the calculations that the difference in degree of inactivation of a sample exposed through different filters is due to the radiation passed by one filter but not passed by the second. Since the filters do not transmit 100% of the unblocked radiation, but average about 87%, the loss values for the honey peroxide accumulation system were corrected for this as follows: In Table 3 it is seen that unfiltered radiation caused a 72% loss of activity, while interposition of a Wratten 1 filter reduced the loss to 51%. This filter absorbed strongly from the far ultraviolet to $356 \text{ m}\mu$ but transmitted only 87% of the remaining visible radiation. The reduction of the activity loss of the sample from 72 to 51% is thus due not only to removal of the

assay over the biological assay of Dold and Witzhausen (1955) has permitted us to examine further the effect of light on peroxide accumulation (inhibine) in honey.

We found that honeys fall into two general categories with respect to the effect of light on peroxide accumulation. Some are so sensitive that they may be handled and weighed only under greatly reduced illumination, while others resist direct sunlight. The effect of wave length of light has been examined; sensitivity is greater to visible light (425–525 $m\mu$) than to ultraviolet radiation. The resistance to illumination of the inhibine of some honeys seems to be due to the absence therefrom of a sensitizing substance, responding to blue light, which inactivates the enzyme by oxidation. Adjustment of the pH to about 6.5 virtually eliminates the photo-oxidation of inhibine.

EXPERIMENTAL

Peroxide accumulation and inhibine assays. These were carried out as previously described (White *et al.*, 1963; White and Subers, 1963). No peroxide (by "peroxide," hydrogen peroxide is always meant herein) is present in undiluted honey before or after irradiation; peroxide is produced only after the honey sample is diluted. All peroxide values given in this study resulted from the action of the enzyme system during incubation, after irradiation or other treatment. When tested before 1-hr incubation, the peroxide content of solutions was negligible. The peroxide accumulation activity is reported as μg of hydrogen peroxide accumulated per g of honey per hr.

Honey samples. Much of the work was done on samples from a 60-lb lot of northeastern (U. S.) fall-flower (Aster-goldenrod) honey (# HS 37), unheated and naturally granulated. An Arizona cotton honey (# HS 38) was also received in a 60-lb lot. Other samples were received in smaller quantity from producers. These were stored at -20°C . The 60-lb lots were kept at $4-6^{\circ}\text{C}$. All were protected from light at all times.

Light sources. *Fluorescent "black light" tube.* A Sylvania (no endorsement implied) 15-W "Blacklite" tube whose envelope absorbed most visible radiation was used. It emitted about 2.1 W of radiation, continuous between 310 and 394 $m\mu$ (max. 350 $m\mu$) with Hg lines at 313, 368, 404 overlaid.

High-pressure quartz Hg arc. This was a Hanovia Utility model lamp with 100-W high-pressure quartz Hg vapor arc tube # 616A-13, with re-

flector, with energy distribution, according to manufacturer, as shown in Table 4.

Incandescent tungsten. A General Electric standard 100-W inside-frosted incandescent light bulb was employed, emitting 0.12 W between 310 and 400 $m\mu$ and 9.3 W in the 400–700 $m\mu$ region (Weitz, 1956).

Laboratory illumination. General laboratory illumination was by multiple General Electric cool white 40-W fluorescent tubes in diffusing fixtures, with approximate output of each lamp 0.27 W 320–400 $m\mu$ and 8.0 W 400–760 $m\mu$ (Weitz, 1956). The level of illumination at the point of sample exposure (bench) was about $110 \mu\text{W}/\text{cm}^2$, continuous from 370–605 $m\mu$ (max. 440, 488, 564 $m\mu$), with Hg lines between 313 and 570 $m\mu$ overlaid.

Sunlight. Bright sunlight through 3-mm window glass which transmits only above 310 $m\mu$; air mass about 1.5, exposures between 11 A.M. and 1 P.M. (local time).

Filters. Wratten dyed gelatin light filters in "B" glass (Eastman Kodak Co., Rochester, N. Y.) were used. The filters used transmitted an average of 87% of radiation of longer wave length than a given cut-off value and were stated to transmit freely in the infrared. The locations of the absorption edges of the filters and the emission characteristics of the light sources were verified with a Bausch and Lomb Spectronic 505 recording spectrophotometer.

RESULTS AND DISCUSSION

As noted during development of the assay for peroxide accumulation in honey (White and Subers, 1963), the values were influenced by the illumination received by the honey. A 10-g sample held undiluted in a 50-ml beaker on the laboratory bench for 2 hr before analysis gave a value of 49 μg $\text{H}_2\text{O}_2/\text{g}$ honey; one analyzed immediately after weighing showed 88. Another held 2 hr in the dark gave a value of 80. The difference between 80 and 88 was later found to be due to exposure during weighing. Each 10-g sample of honey was spread in an 85-mm-diameter crystallizing dish in a 1–2-mm layer for subsequent light exposures and was weighed under minimal light.

When samples were exposed for varying times (1–15 min) to direct sunlight, activity loss increased with exposure time, with 15 min causing complete destruction. A 10-g sample exposed to sunlight for 15 min and an unexposed sample were dialyzed overnight against running tap water, the final

Table 4. Effect of spectral region on photoinactivation of peroxide accumulation system in honey.^a

Spectral region		Source output (W)	Exposure ^b (J/cm ²)	Activity loss (%)	Exposure for 50% inactivation (J/cm ²)
Limits (m μ)					
<356		4.4	0.96	13	4.8
356-425		2.3	0.51	10	3.3
425-464		0.82	0.18	11	1.1
464-525		0.68 ^d	0.15	20	0.5
525-572		1.5	0.33	4	5.5
572-610		1.5	0.33	0	∞
610-670		0.0	0.00	0	∞
>670		2.46	0.55	6	6.1

^a Analysis of data from Table 3; high-pressure quartz Hg arc radiation.

^b At sample.

^c Corrected for filter transmission of 87% in "transparent" region of filter.

^d Measured value; manufacturer's literature showed no value.

system in honey appears to be more sensitive to visible than to ultraviolet radiation. This was confirmed in another series of illuminations by a source of continuous visible light, a 100-W incandescent tungsten lamp. Uncertainties due to the low output of Hg radiation in the 464-525-m μ region were thus eliminated. Table 5 shows the results

Table 5. Effect of filters on inactivation of peroxide accumulation system in honey by incandescent lamp radiation.^a

Filter	Spectral region (m μ)	Peroxide accumulation (μ g/g)	Activity loss (%)
None	310-IR ^b	27	77
1	356-IR	45	63
2A	425-IR	46	62
4	464-IR	58	52
16	525-IR	93	23
23	572-IR	116	4.1
29	610-IR	116	4.1
89	670-IR	116	4.1
....	Dark	121	0

^a Honey (10 g) in thin layer, exposed at 13.5 cm for 20 min in light-tight box with opening for 5 \times 5-cm Wratten filters as shown; filters cooled by air stream. Source, 100-W incandescent bulb.

^b According to manufacturer, dyed gelatin filters transmit freely in the infrared.

of a 20-min illumination by this radiation, which is nearly all in the visible and infrared regions. Table 6 shows the effect of spectral region on the photoinactivation. The apparently high effectiveness of the 310-356-m μ region is believed not to be real but to be due to uncertainties in the filter effect and particularly in the very low relative lamp

output at the extreme ultraviolet end of its range. When considerably more radiation is available in the ultraviolet (Table 4), the relative effectiveness is much lower. In both Tables 4 and 6, the 425-525-m μ regions appear to be most effective.

The work described above was carried out with one lot of honey. Table 7 shows the effect of radiation from various sources on the peroxide accumulation system of several other honey samples. These include dark- and light-colored honeys; no relation between gross composition and light susceptibility was noted. Honey color did not seem to be a factor. All of the samples appear less sensitive to light than the one fortuitously used for the work described above; two other samples of similar nature are also quite sensitive to light.

Effect of dilution and pH. It had been noted that honey solutions prepared for assay of peroxide accumulation (pH about 6.2) were not sensitive, even to 5 min of exposure to sunlight, in contrast to the original-density honey. Samples of the same honey were accordingly diluted to various moisture contents between 25 and 60%; upon exposure to light they uniformly lost their activity. Samples of the same honey (#HS 37) were then adjusted to various pH values with alkali, exposed, and assayed. Results appear in Table 8. Adjustment to pH 6 completely protected the peroxide accumulation system from photodestruction, with an intermediate degree of protection found

Table 1. Effect of radiation from various sources on peroxide accumulation value of honey.^a

Source	Radiation						Activity loss (%)	Effective-ness ^c
	Intensity ^b (mW/cm ²)		Time (min)	Exposure (J/cm ²)				
	Ultra-violet	Visible		Ultra-violet	Visible	Total		
Incandescent	0.055	4.37	20	0.07	5.25	5.31	77	2.5
Hg Arc	3.5	2.4	10	2.10	1.45	3.55	72	1.9
"Blacklite"	1.56	0.04	30	2.80	0.07	2.87	25	6.9
Lab. lights	0.004	0.106	30	0.007	0.19	0.20	64	0.14
Sun	2.8	40	1	0.17	2.4	2.6	87	0.88

^a Honey (10 g) in thin layer exposed to unfiltered radiation 13.5 cm from artificial sources (except about 1.7 m from laboratory lights) for indicated times. Intensity of laboratory lights estimated by photometric measurement at sample level. Controls held equivalent times in dark, assayed with exposed samples. Sunlight intensity approximated from data of Forsythe and Christison (1930).

^b At sample.

^c Exposure (Joules/cm²) required to reduce activity to 50%.

Table 2. Effect of visible radiation from various sources on peroxide accumulation value of honey.^a

Source	Radiation			Activity loss (%)	Effective-ness ^b
	Intensity (mW/cm ²)	Time (min)	Exposure (J/cm ²)		
Incandescent	2.65	20	3.16	52	3.0
Hg arc	1.65	10	0.99	24	2.5
Lab. lights	.065	30	0.12	27	0.26
Sun	25	1	1.50	40	2.0

^a Honey (10 g) exposed as in Table 1 to specified radiation through 8 × 8-cm filter of Noviol B, 1.5 mm. Other conditions as in Table 1. Intensities calculated for 445-700-m μ radiation.

^b Exposure (J/cm²) required to reduce activity to 50%.

Table 3. Effect of filters on inactivation of peroxide accumulation system in honey by mercury arc radiation.^a

No.	Filter		
	Spectral region (m μ)	Activity (μ g/g)	Activity loss (%)
None	222-1367	24	72
1	356-1367	42.5	51
2A	425-1367	51	41
4	464-1367	61	30
16	525-1367	78	10
23	572-1367	82	6
29	610-1367	80	8
89	670-1367	80	8
Dark	none	87	0

^a Samples (#HS 37, 10 g) exposed in thin layer for 10 min at 13.5 cm in light-tight box with opening for 5 × 5-cm Wratten filters as shown; filters cooled by air stream. High-pressure Hg arc.

ultraviolet <356 m μ but also to reduction of intensity of the transmitted radiation to 87% of the unfiltered value. Algebraic calculation shows that the 21% difference is distributed as follows: 13.5% due to destruc-

tion by ultraviolet <356 m μ , and 7.6% due to absorption of damaging visible radiation by the filter. Similarly, the 14% difference between no filter and the No. 1 filter for tungsten radiation is divided: 4.7% due to ultraviolet below 356 m μ , and 9.3% due to reduction of intensity of visible radiation by the filter. This correction is applied only to the first filter in the series.

Table 4 shows the relative effectiveness of the various spectral regions for inactivation of the peroxide accumulation system of honey. The values in the last column give the calculated exposure required for destruction of half of the activity, assuming first-order reaction. The 464-525-m μ region appears to be most effective. The manufacturer's data sheets for the arc show no lines emitted in this region, but examination by the spectrophotometer indicated the 491.6 and 496.0 m μ Hg lines to be present.

Contrary to photoinactivation in simple enzyme systems, the peroxide accumulation

Table 8. Effect of pH on photosensitivity of peroxide accumulation system.^a

pH	Activity (μg/g)
4.4	7.0
4.7	20
5.1	26
5.4	58
6.0	126

^a Honey (10 g #HS 37) and 5 ml H₂O adjusted with 0.1N NaOH to pH shown, made to 25 ml; 10-ml portions exposed for 5 min in 8.5-cm flat dishes to sunlight through 3-mm window glass. Solutions transferred to assay bottle, 2 ml 0.4M sodium phosphate, pH 6.5, and 8 ml H₂O added before incubation; pH of all samples after incubation, 6.25.

 Table 9. Effect of pH on stability of peroxide accumulation system.^a

pH	Activity			
	Dark (μg/g)	Loss ^b (%)	Irradiated (μg/g)	Loss ^c (%)
3.0	72	33.3	0	100
4.0	104	3.7	8.5	91.8
5.0	108	0	75	30.5
6.0	107	0.9	87	18.7
7.0	98	9.2	84	14.2
8.0	89	17.5	83	6.7
9.0	78	27.8	71	9.0

^a Honey (10 g #HS 37) and 5 ml H₂O adjusted with 0.1N NaOH to indicated pH, made to 25 ml, 10 ml exposed in 8.5 cm flat dish to Hg arc at 16 cm through 2 cm H₂O for 10 min. Another 10 ml held similarly in dark during time of irradiation of first. Solutions assayed within 20 min of initial dilution.

^b Based on pH 5 dark value.

^c Based on corresponding dark value.

ing markedly with the acidity of solution. The unexposed honey solutions were also unstable, with a maximum stability at pH 5-6, decreasing in both directions. Measurement of H₂O₂ content of the exposed and unexposed solutions in the pH 5 set before incubation showed 2.0 and 2.1 μg/ml. No corrections were made for these negligible amounts.

Presence of a sensitizer. Equal weights of a relatively resistant honey (#HS 38, cotton honey) and of the sensitive #HS 37 were thoroughly mixed without exposure to light. The mixture and the individual components were exposed to 5 min of sunlight and assayed as usual. The results (Table 10) indicate that the less stable honey contains a sensitizing material that acts to in-

 Table 10. Effect of light-sensitive honey on stability to light of peroxide accumulation system of light-stable honey.^a

Sample		Activity		
No.	Type	Dark (μg/g)	Irradiated (μg/g)	Loss (%)
HS 37	Fall blend	115	0.75	99.5
HS 38	Cotton	318	143	55.2
	Mixture, 1:1	186	5.2	97.5

^a Ten g indicated honeys exposed at original density in thin layer for 5 min to sunlight through 3 mm window glass. Mixture made by grinding samples in mortar in darkness.

crease the sensitivity of the "resistant" system in the mixture upon irradiation. The effect is not due to pH; that of the sensitive honey was 3.9, the other 4.0. The greater effectiveness of visible light over ultraviolet in the inactivation also implies the presence of an intermediate energy transfer or sensitizing agent. A similar experiment is described in part 1 of Table 11, except that the honey was in solution rather than at full density (17.5% moisture). Results were similar to those described in Table 10. It is possible that the sharp increase in light susceptibility of the system between pH 4.0 and 5.0 (Table 9) is due to an effect on the sensitizer as well as on the enzyme.

Stability of the sensitizer. Using the irradiation system described in the pH study, three separate samples of sensitive honey (#HS 37) were respectively irradiated, boiled 1 min, and separated by dialysis into high- and low-molecular weight portions. Each was mixed with an equal weight of the less sensitive honey, irradiated, and assayed. As seen in Table 11, part 2, the sensitizer appears to be stable to radiation and heat, and to pass through the dialysis membrane. The latter fraction shows reduced sensitizing activity; the aqueous solution outside the membrane (6 changes of liquid totaling 2.2 L) had been concentrated to honey density by vacuum evaporation at about 50°C. To determine whether the agent was in fact somewhat volatile, 10 g of sensitive honey was steam-distilled in a one-piece micro-Kjeldahl apparatus (Steyermark *et al.*, 1951), collecting 20 ml. Ten g of insensitive honey was made to 25 ml with the distillate. This solution showed a 15% loss in activity on irradiation. This may be compared with

Table 6. Effect of spectral region on photoinactivation of peroxide accumulation system in honey.^a

Spectral limits (m μ)	Source output (W)	Exposure ^b (J/cm ²)	Activity ^c loss (%)	Exposure for 50% inactivation (J/cm ²)
310-356	0.02	0.012	4.7	0.18
356-425	.14	.096	1.0	5.76
425-464	.28	.16	10	1.02
464-525	.69	.38	29	0.78
525-572	1.01	.59	19	1.92
572-610	1.11	.66	0	∞
610-670	2.16	1.22	0	∞
>670	76.5	4.30	4.1	94

^a Analysis of data in Table 5, radiation from 100-W incandescent tungsten lamp.

^b At sample.

^c Corrected for filter transmission of 87% in "transparent" region of filter.

Table 7. Effect of light on peroxide accumulation values of several honey samples.^a

No.	Sample Type	Activity		
		Dark (μ g/g)	Irradiated (μ g/g)	Loss (%)
Fluorescent laboratory lights, 1 hr				
168	Chinquapin	131	132	0
460	Tulip tree-clover	124	139
11	Alfalfa	106	114
M 268	Cotton	332	318	1.2
HS 35	Fall blend	93	8.2	91
HS 33	Fall blend	102	14	86
HS 37	Fall blend	97	11	89
Mercury arc, 10 min				
85	Blackberry	110	87	21
361	Locust	85	65	23
324	Eucalyptus	155	105	32
442	Star thistle	81	71	12
499	Oak honeydew	175	168	4
HS 37	Fall blend	87	24	72
Sunlight, 15 min				
168	Chinquapin	131	107	20
361	Locust	85	58	32
460	Tulip tree-clover	124	110	11
11	Alfalfa	106	81	22
442	Star thistle	81	66	18
499	Oak honeydew	175	170	3
HS 37	Fall blend	72	1.7	97.5

^a Samples (10 g) exposed in thin layer as indicated (intensities in Table 1). Control samples spread in thin layer in darkness for equal time.

between the natural pH (3.9) and this value. To examine other effects of pH on the system, the experiment was repeated over a wider range, with controls held in darkness. Working under a Wratten No. 2 photographic safelight, honey solutions were adjusted in pH and aliquots were exposed to the mercury arc or held in darkness. A 2-cm

layer of water in a dish between arc and sample protected the latter from heat of the arc. Each was immediately incubated after pH adjustment to 6.25, as described in Table 9. About 20 min elapsed between the original pH adjustment and the incubation. Table 9 shows the system to be most stable to radiation at pH 8, with sensitivity increas-

visible light, oxygen, and a sensitizing material. That a photosensitizer is not removed by the vacuum treatment is shown in the second line of Table 12, where the solutions were first evacuated, then air was admitted, before exposure. The oxidation would appear to differ from other photo-oxidations in visible light, such as that of proteins and enzymes by methylene blue (Weil *et al.*, 1952; Weil and Buchert, 1952; Weil and Seibles, 1955), and of α -amylase by riboflavin (Galston and Baker, 1949). The former showed a pH maximum around 9, whereas the latter was most rapid at pH 5.9–6.6; the present oxidation is most rapid at or below pH 3.

Inhibine values of irradiated honeys. Inhibine assays were made on several irradiated honey samples by the modification by Schade *et al.* (1958) of the Dold and Witzenhäusen (1955) bacteriological procedure using *Staphylococcus aureus*. The results (Table 13) were as expected from our previous correlation (White and Subers, 1963) of inhibine and peroxide accumulation values. Neither the original procedure nor that of Schade *et al.* provides for any control of the pH of the inhibine plates. The pH of the nutrient agar medium is 6.7. The addition of unbuffered honey solution of pH 3.9 produces a pH range of 5.5–6.4 in the plates containing the greatest and least amounts of honey. The effect of radiation on peroxide accumulation is definitely greater at pH 5.5 than at pH 6.4, but the 1:1 honey solution used in preparing the plates is at its natural pH of 3.5–4.5 and may be highly sensitive to radiation before it is mixed with the nu-

trient medium. This introduces a variable into the assay, even though the samples be weighed, as previously suggested (White and Subers, 1963), in minimal light. *For the inhibine assay, therefore, the honey should immediately be diluted with a buffer of pH 6.4, as is done in the peroxide accumulation assay.* Variable inhibine results obtained in this laboratory may be traced to this factor. In the second paper in this series (White and Subers, 1963), Table 1, which relates inhibine values and peroxide accumulation for 45 honey samples, has one sample (#470) with an inhibine number of 0 (permitted growth on all plates) and a peroxide value of 68. Normally, an inhibine number of 0 is shown only by samples with peroxide accumulation values of less than 3.4 $\mu\text{g/g}$. As noted therein, insufficient sample was available for more than cursory examination, but it has since been found that immediate adjustment of the pH of this honey solution to 6.25 did produce a plate upon which growth was not permitted, implying an inhibine number greater than zero. The peroxide accumulation system of this sample may have been unusually light-sensitive.

It is now clear that the inhibine value of honey is influenced by so many factors that its absence from a honey sample may not be interpreted as evidence of lower quality or overheating of the honey. However, the presence of an appreciable amount of inhibine in a honey otherwise in question because of low diastase or invertase content might justify placing such a honey into an acceptable category. Were it affected only by heat and uniformly present in all honeys

Table 13. Inhibine values of irradiated honeys.^a

Sensitive honey		Resistant honey	
Control, unexposed	4	Control, unexposed	5
3 min, sun, original density	3	5 min, sun, original density	5
5 min, sun, original density	2	5 min, sun, pH 6.5, diluted	5
3 min, sun + Noviol B, original density	4		
1 hr lab. lights, original density	2	Mixture	
10 min, Hg arc, original density	3	Control, unexposed	4
5 min, sun, pH 3.9, diluted	2	5 min, sun, original density	1
5 min, sun, pH 6.5, diluted	5	5 min, sun, pH 6.5, diluted	5

^a Ten g honey (# HS 37, sensitive; # HS 38, resistant) exposed as shown at original density in thin layer at distances shown in previous tables. Diluted honey: 10 ml of 40% (w/v) solution exposed. Each assayed for inhibine by Schade *et al.* (1958) modification of the Dold and Witzenhäusen (1955) method.

Table 11. Effect of pretreatment of photosensitive honey on subsequent sensitizing action.^a

Material	Activity		
	Irradiated ($\mu\text{g/g}$)	Control ($\mu\text{g/g}$)	Loss (%)
Part 1. Resistant honey, 10 g	242	268	9.7
Resistant honey, 5 g	82.5	88	9.1
Sensitive honey, 10 g	6.7	82	91.8
Sensitive honey, 5 g	6.1	19.5	68.8
Sensitive, 5 g, + resistant, 5 g	6.1	177	66.0
Part 2. Pretreated sensitive honey mixed with resistant honey			
(a) Irradiated 10 min	34	92.5	64
(b) Boiled 1 min	39	93	58
(c) Dialyzed; inside sac	47.5	53.5	11.2
(d) Dialyzed; passing sac	89	130	31.5
(e) (d), with 5 g sugar *	26.5	26.8	1.1
(f) (c) plus (d) (equiv. to 5 g honey)	3.7	11.2	67

^a Part 1. Indicated amounts of honey made to 25 ml, 10-ml portions used in 8.5-cm-diam. flat dishes for irradiation (10 min, Hg arc at 16 cm through 2 cm H₂O), and dark control. Part 2. Sensitive honey (#HS 47) in amounts equivalent to 5 g honey treated as shown, mixed with 5 g resistant honey (#HS 38), made to 25 ml, and 10 ml used for irradiation and for controls as in Part 1.

^b 2.5 g each of glucose and fructose.

the 9.7% loss shown by the insensitive honey using water as solvent. A repetition of the experiment using 5 g of resistant honey and the distillate of 10 g sensitive honey gave a loss of 5% on irradiation.

Ten grams of sensitive honey were diluted to 25 ml and extracted with three 25-ml portions of diethyl ether. The evaporated extract and the extracted residue, freed of ether, were tested for sensitizing action. The resistant honey mixed with the extract showed a 10.5% loss of activity on irradiation, while the ether-extracted honey when mixed with the resistant honey (10 g) caused a 45.5% loss of activity on irradiation. The sensitizing material is thus non-steam-volatile and not extracted from honey by ether.

The optimum honey concentration in the peroxide accumulation assay is that provided by a 10-g sample (10 g of honey per 25 ml solution, further diluted 1 to 1). The effect of halving this may be seen in part 1 of Table 11. At 10 g, glucose is about 0.4M during the incubation. The peroxide accumulation value decreases markedly above and below this value. A substrate concentration of 1.5M was found to be optimum for the separated enzyme (Schepartz and Subers, 1964) in the absence of inhibitors and peroxide-destroying materials found in honey.

Differences in degree of destruction of the peroxide accumulation system in Tables 10 and 11 are due to the greater radiation intensity used in Table 10.

Nature of photoinactivation of glucose oxidase in honey. Aliquots of a solution of photosensitive honey were irradiated under reduced pressure as detailed in Table 12. Removal of most of the air, by reduction of the pressure to 7 mm Hg, reduced photo-destruction of the enzyme to a very low level; hence the reaction appears to be a photo-oxidation of the enzyme requiring

Table 12. Effect of absence of air on photosensitivity of honey solutions.^a

Condition during exposure	Activity		
	Dark ($\mu\text{g/g}$)	Irradiated ($\mu\text{g/g}$)	Loss (%)
(a) In vacuum	82	81	1
(b) Evacuated, air admitted	87	10.5	88
(c) In air	82	11	86

^a Sensitive honey (#HS 37, 40% solution w/v in water); 10 ml in 500-ml filter flask treated as follows:

- Evacuated to 7 mm Hg, sealed; one exposed to Hg arc at 16 cm through 2 cm H₂O for 10 min, one held 10 min in dark. Air admitted and assayed.
- Evacuated as above, held 10 min *in vacuo*. air admitted; one exposed as above, one held in dark, both assayed.
- Sample in open flask, one exposed as above, one held in dark, both assayed.

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it might serve to indicate heating history of honey. We have shown, however, in an earlier paper (White and Subers, 1964) that a wide variation (70-fold or more) exists among honeys in heat sensitivity of the peroxide accumulation system, and that the floral type of honey also has an influence. The variable sensitivity of the system to illumination is a further complication; commercial blending of various lots of honey may well increase the light sensitivity of the bulk by introducing the natural sensitizing material. Whatever the natural role of inhibine may be, honey is not exposed to light in nature and is not subject to this deterioration. Extracted honeys which are quite high in inhibine are probably low in or lack the sensitizer and are relatively insensitive to radiation. Where honey is handled in bulk, self-absorption of the harmful radiation may effectively reduce light damage to inhibine. Insufficient information is available to make conclusions on floral types of honey showing high photosensitivity, but it does appear that the inhibine of fall-flower honey (aster-goldenrod) is highly sensitive to light.

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