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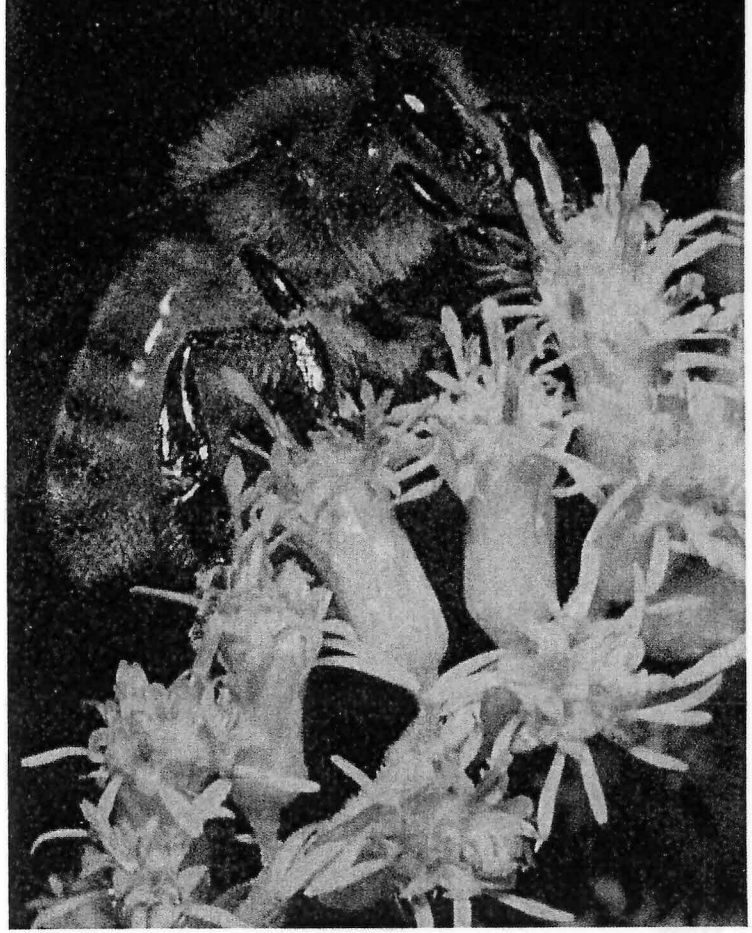
THE ANTIBACTERIAL ACTIVITY OF HONEY

by

Peter Molan



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THE ANTIBACTERIAL ACTIVITY OF HONEY
1. The nature of the antibacterial activity

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Introduction

Honey has been used as a medicine since ancient times in many cultures[63, 86](fig. 1), and is still used in 'folk medicine'[55]. The use of honey as a therapeutic substance has been rediscovered by the medical profession in more recent times, and it is gaining acceptance as an antibacterial agent for the treatment of ulcers and bed sores, and other surface infections resulting from burns and wounds[4, 135]. In many of the cases in the cited reports, honey was used on infections not responding to standard antibiotic and antiseptic therapy. It was found in almost all of the cases to be very effective in rapidly clearing up infection and promoting healing. Honey has also been found to be effective in treating bacterial gastroenteritis in infants[47].

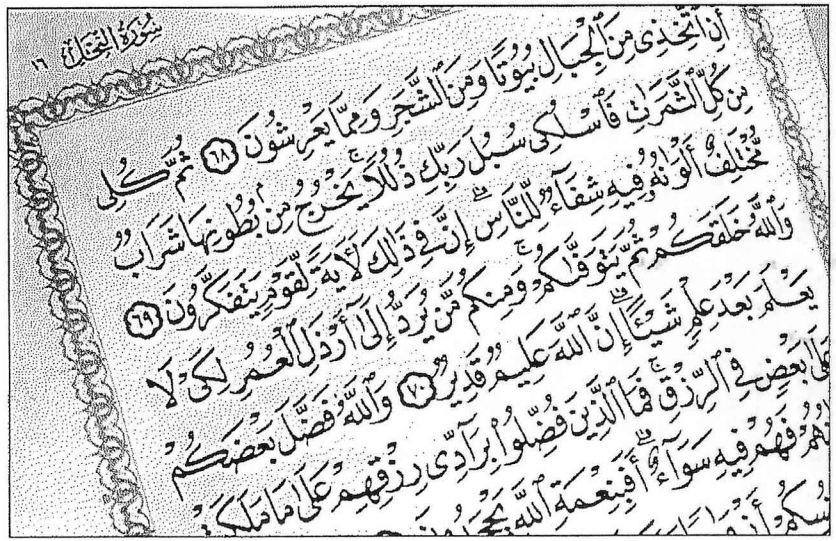

FIG. 1. The Koran, *circa* 590 AD.

Translation:
68. And thy Lord taught the Bee
To build it cells in the hills,
On trees, and in (men's) habitations;
69. Then to eat all ...../.....: there issues
From within their [the bees] bodies
A drink of varying colours,
Wherein is healing for men.

Some infections caused by some of the species of bacteria that have been found to be sensitive to the antibacterial activity of honey[78]

Pathogen	Infection caused
Bacillus anthracis	anthrax
Corynebacterium diphtheriae	diphtheria
Escherichia coli	diarrhoea, septicaemia, urinary infections, wound infections
Haemophilus influenzae	ear infections, meningitis, respiratory infections, sinusitis
Klebsiella pneumoniae	pneumonia
Listeria monocytogenes	meningitis
Mycobacterium tuberculosis	tuberculosis
Pasteurella multocida	infected animal bites
Proteus species	septicaemia, urinary infections, wound infections
Pseudomonas aeruginosa	urinary infections, wound infections
Salmonella species	diarrhoea
Salmonella cholerae-suis	septicaemia
Salmonella typhi	typhoid
Salmonella typhimurium	wound infections
Serratia marcescens	septicaemia, wound infections
Shigella species	dysentery
Staphylococcus aureus	abscesses, boils, carbuncles, impetigo, wound infections
Streptococcus faecalis	urinary infections
Streptococcus mutans	dental caries
Streptococcus pneumoniae	ear infections, meningitis, pneumonia, sinusitis
Streptococcus pyogenes	ear infections, impetigo, puerperal fever, rheumatic fever, scarlet fever, sore throat, wound infections
Vibrio cholerae	cholera

In the ancient use of honey as a medicine there was no knowledge of it having antibacterial properties — it was just known to work. In more recent times, now that it is known that festering wounds are the result of infection by micro-organisms, honey is used on the basis of it being an antibacterial substance, but the nature and extent of its antibacterial activity is not widely known. A large amount of research work has been done on the antibacterial activity of honey, but the results of this remain unknown to most users of honey because the work is so widely spread over time, and is published in different journals and in different languages. Because it is important to be aware of the research findings to realize the full potential of honey as a therapeutic substance, this review has been prepared to bring together what is known about the antibacterial activity of honey.

Reports of antimicrobial activity of honey

Experimental approach

The antibacterial activity of honey appears to have been reported first by van Ketel in 1892 (cited by Dustmann[35]). The next report was by Sackett in 1919[95]. He also reported that the antibacterial potency was increased by limited dilution of honey, an observation that was hard to explain. More intensive study did not commence until the work of Dold *et al.* in 1937[27]. They introduced the term 'inhibine' for the antibacterial activity of honey, a term which has been widely used since in the literature on honey.

Since then there have been many reports. Some have been of simple testing that

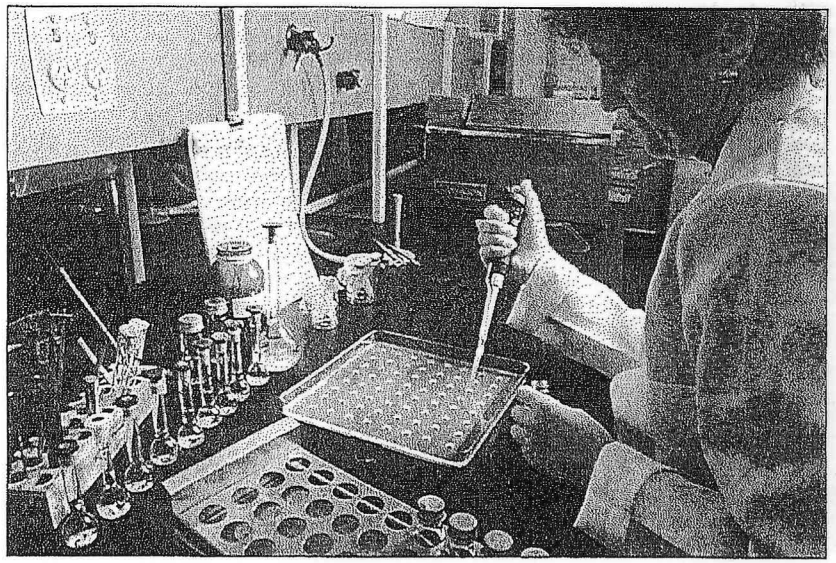

FIG. 2. Honey solutions being pipetted into wells in an agar plate (the agar is impregnated with *Staphylococcus aureus*).

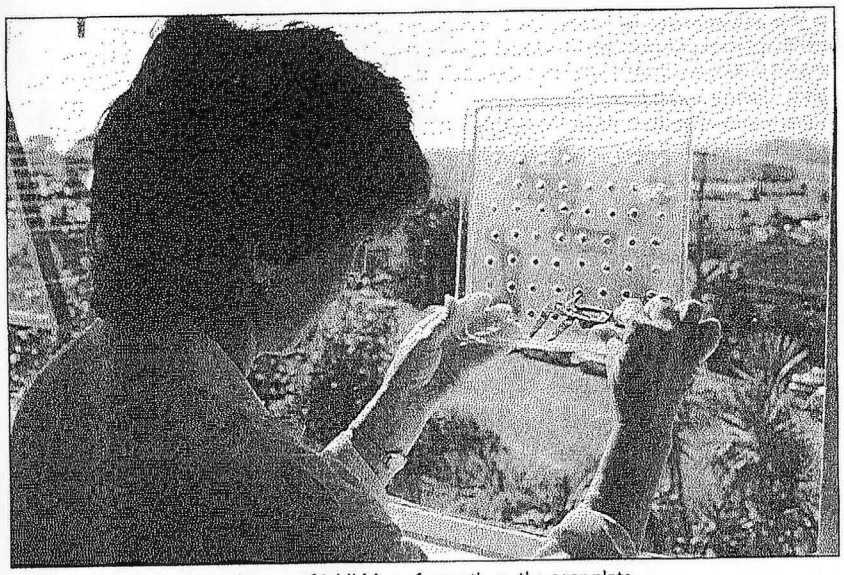

FIG. 3. Measuring the size of zones of inhibition of growth on the agar plate.

has shown honey to have antibacterial activity: these have often been done without recognition of the prior discovery of this by others. Most, however, have involved investigation of the activity spectrum of honey (i.e. determining which species of micro-organism are sensitive to the action of honey), or comparison of different types of honey for the potency of their action against one or more species of bacteria. Also there have been many investigations of the nature of the antibacterial substances present.

In studies where the potency of the antibacterial activity of honeys has been measured, this has involved the use of one form or another of two standard microbiological techniques. In the agar diffusion assay technique, a small quantity of honey, or a solution of honey, is applied to a nutrient agar plate inoculated with a microbial culture (fig. 2). While the plate is incubating, the honey diffuses out into the agar from its point of application. Where the concentration of honey in the agar is high enough to inhibit growth of the culture no colonies develop, and a clear zone is seen around the point of application of the honey. The size of the clear zone is a measure of the potency of the honey (fig. 3). However, because the honey is diluted as it diffuses into the agar, the effective antibacterial concentration of the honey in this type of assay is always lower than the concentration of the solution applied. In the other type of assay, honey is incorporated in the nutrient agar or in the nutrient broth in which the culture is grown. By using a series of different concentrations of honey it is possible to find the minimum inhibitory concentration for each honey. Whether diluted by extensive diffusion in the first method, or as a further step in a dilution series in the second, the more potent the antibacterial activity of a honey, the more it can be diluted and still retain its inhibitory action.

None of the methods mentioned can show whether the action of honey is bactericidal (i.e. lethal to the bacteria). If no colony development occurs in the period of incubation, it can only be taken as a bacteriostatic action (i.e. inhibition of growth of the bacteria). Demonstration of bactericidal activity requires subsequent culturing in fresh nutrient medium to see if the test micro-organisms survived exposure to the honey.

Species found to be susceptible

The microbial species that have been found to be sensitive to the antimicrobial activity of honey are listed in table 1. Many of the reports, especially the older ones, use names no longer in common use for many of the bacterial species: the currently used names for these species are listed in table 1, as identified from past and present editions of *Bergey's manual of determinative bacteriology*[12,13].

Table 1 also shows the lowest concentration of honey reported to show an antibacterial effect against each species in each study. In many of the studies this concentration is not necessarily the minimum inhibitory concentration. In some cases the testing for susceptibility was done with a single concentration of honey. In others, where a dilution series was used, activity was found at the lowest concentration in the series. It is possible that activity could have been detected at lower concentrations in all of these instances, if lower concentrations had been used in the testing.

In some of the reports, results are given of the testing of susceptibility to more than one type of honey. In these instances the results presented in table 1 are those obtained with the most active honey used. The decision to do this was based on the finding in many other studies that honeys vary very widely in their antibacterial potency, many having no detectable antibacterial activity (see later). As one of the aims of this review is to show the potential of honey for use as an antibacterial agent, the results are therefore presented of what can be achieved with honeys of high activity, rather than what is achieved if unselected honeys are used.

The concentrations of honey used in the assays of antibacterial activity are given in most of the reports as percentages, but in many of the reports there is no notation of whether it is grams of honey per 100 g of solution (% wt/wt), grams of honey per 100 ml of solution (% wt/vol), or millilitres of honey per 100 ml of solution (% vol/vol). As honey is a liquid of high density, the way the percentage is calculated makes a substantial difference to the value given. Where it cannot be deduced from the description given of the way the solutions were prepared, it is assumed that the values given are % vol/vol. If in any instance the assumption is incorrect, the actual concentration of honey that caused the observed antibacterial effect would have been lower than the value given in table 1. To facilitate comparison between the reports, all values for the concentration of honey used are given in this review as % vol/vol, these being calculated on the basis of honey having a density of 1.4 g/ml[125].

Antifungal activity

Although an earlier brief review[49] of the biological effects of honey expressed the

TABLE 1. A summary of the reports of the antimicrobial activity of honey, showing the species affected and the concentration (% by volume) of honey used in the testing. Results for the most active honey are shown where more than one honey was used. The lowest concentration with activity is shown if more than one concentration was used.

§ signifies an agar diffusion assay — the active concentration is lower than the value given, the honey being diluted by diffusion into the agar.
Values are in brackets where completeness of inhibition was not stated (the results were reported as "sensitive to honey").
? signifies that the actual concentration used was not given in the report.

Species inhibited	Conc. of honey (%) for complete microbicidal action	Conc. of honey (%) for complete inhibition of growth	Conc.of honey (%) for partial inhibition of growth
BACTERIA			
Alcaligenes sp.		10§[54]; 100§[82]	
Alcaligenes faecalis	7.4[95]		
Bacillus sp.	50[15]		
Bacillus alvei		extract[42, 118]	
Bacillus anthracis	2.5[20, 57]	1.3[20, 57]; 5§[3]; 17[27]; 20[40]; 100§[43]	
Bacillus cereus		17[27]; 42§[90]; ≤100§[106]; 100§[82]	
Bacillus cereus var. mycoides		17[27, 83]; 100§[82]	8[27]
Bacillus larvae			extract[42]
Bacillus megaterium		extract[118]	
Bacillus pumilus	1.3[20, 57]	1.3[20, 57]; 13[27]; 17[83]; 25§[67]; ?§[88]	8[27]; 25[61]
Bacillus stearothermophilus		42§[90]	
Bacillus subtilis	[50 partial][69]	10§[54]; 10[42]; 13[27]; 17[83]; 20[17]; 42§[90]; ≤100§[106]; 100§[69, 103, 119]; (?)[70]; distillate[59, 79]; extract[8, 42, 118]	5[17]; 8[27]; 25[61]

TABLE 1. (continued)

Species inhibited	Conc. of honey (%) for complete microbicidal action	Conc. of honey (%) for complete inhibition of growth	Conc.of honey (%) for partial inhibition of growth
Citrobacter freundii		3.6[50]; 10§[92]	
Corynebacterium diphtheriae	5[20, 57]; 17[84]	2.5[20, 57]; 10§[54]; 25§[67]; 25[28]	
Edwardsiella tarda	99[115]		
Escherichia coli	7.4[95]; 9[84]; 20[40]; 30[15]; 99[115]; [25 partial][69]	0.25§[3]; 2[25]; 3.1[30]; 3.6[50]; 4.5[131]; 5[21]; 5–6[52]; 6.25[94]; 7.6[81]; 10§[54, 92]; 10[51]; 12.5[44]; 20[17, 20, 40, 57]; ≤25[77]; 25§[67, 85, 92]; 25[61]; 40[56]; 100§[37, 43, 69, 82, 103, 112, 119]; (10)[53]; (?)[70]; ?[133]; ?§[88]; distillate[79, 113]	0.7[131]; 1.4[50]; 5[17]; 10[54]; 17[27, 83]; extract[42]; distillate[101]
Haemophilus influenzae		10[56]	
Klebsiella sp.		10§[54]; (10)[53]	
Klebsiella pneumoniae	15[95]; 20[20, 57]	10[20, 57]; 25§[67]; 25[61]; 50[56]; 100§[119]; distillate[79, 113]	40[56]
Listeria monocytogenes		≤25[77]; 30[56]	
Micrococcus sp.		10§[54]	
Micrococcus luteus		10§[106]; 42§[90]	
Mycobacterium tuberculosis	100[105]	4.5[83]	1.2[83]
Neisseria sp.		10§[54]	
Pasteurella multocida		(?)[70]	
Proteus sp.		10§[54]; 20[17]; (10)[53]; extract[42]	5[17]
Proteus mirabilis	30[15]	3.6[131]; 6.4[50]; 20[17]; 40[56]; 100§[37]; distillate[79]	1.4[131]; 5[17]

TABLE 1. (continued)

Species inhibited	Conc. of honey (%) for complete microbicidal action	Conc. of honey (%) for complete inhibition of growth	Conc.of honey (%) for partial inhibition of growth
Proteus morganii		0.25§[3]	
Proteus vulgaris	23[95]; 99[115]	0.6[25]; 5–6[52]; 10§[92]; 20[20, 57]; ≤36[35]; extract[42]	10[54]
Pseudomonas sp.		(10)[53]; 100§[82]	
Pseudomonas aeruginosa	10[20, 57]; 20[52]; 99[115]	3[25]; 3.1–6[44]; 5[20, 21, 57]; 5-6[52]; 6.4[50]; 10§[54]; 13[27]; ≤25[77]; 25[61]; 30[56]; ≤36[35]; 100§[119]; distillate[79, 113]; extract[118]	0.7[131]; 8[27]; 17[83]; 100§[37]; extract[42]
Pseudomonas fluorescens		8.3[81]; 25[61]	
Salmonella sp.		10§[54]; 25§[85]; 40[56]; 100§[43]	30[56]; extract[42]
Salmonella cholerae-suis	7.4[95]		extract[42]
Salmonella dublin			
Salmonella enteritidis	7.4[95]; 99[115]		5[17]; extract [42]
Salmonella gallinarum		10[17]; ≤36[35]	
Salmonella paratyphi-A	7.4[95]	17[83]; extract[118]	
Salmonella pullorum		≤36[35]	extract[42]
Salmonella schottmuelleri	7.4[95]	25§[67]	17[27, 83]
Salmonella typhi	99[115]	0.25§[3]; 10[54]; 30[55]; extract[118]	20[56]
Salmonella typhimurium	99[115]	5[21, 131]; 10§[92]; ≤25[77]; 100§[82]; extract[118]	
Salmonella typhosa	7.4[95]; 20[40]	17[27, 83]; 20[40]; 25§[67]	
Sarcina lutea		10§[106]; 25[61]; ≤36[35]; 50§[92]; ≤100§[87]; 100§[82]	
Sarcina orangea		25§[67]	
Serratia marcescens	99[115]	5[131]; 10§[54]; 13[27]; 25§[67, 92]; 25[61]; 50[55]; distillate[79]	0.7[131]; 8[27]; 17[83]; 40[56]
Shigella sp.		10§[54]	

TABLE 1. (continued)

Species inhibited	Conc. of honey (%) for complete microbicidal action	Conc. of honey (%) for complete inhibition of growth	Conc.of honey (%) for partial inhibition of growth
Shigella boydii		40[56]	
Shigella dysenteriae	7.4[95]	6.9[65]; 8.3[108]; 17[27]; 20[40]	17[27]
Shigella flexneri	5[20, 57]	0.5§[3]; 1[25]; 2.5[20, 57]; 5[52]; 10§[92]; 10[51]	17[27, 83]
Shigella sonnei		0.8[25]; 5–6[52]; 17[83]	10[54]
Staphylococcus sp.	20[57]; 30[15]	3[25]; 5–6[52]; 9[51]	8[51]
Staphylococcus aureus (albus)		10[17]; 25[61]; (10)[53]; extract[118]	5[17]; 17[27, 83]
Staphylococcus aureus	1.3[20, 57]; 1.5[14]; 9[84]; 20[40]; 50[1, 69]	0.3[35]; 0.5§[3]; 0.6[20, 57]; 1[72]; 1.56[94]; 2.9[50]; 3[96, 127, 128, 130]; 3.1[30]; 3.6§[4]; 3.6[1]; 4[132]; 4.5[122]; 5[21, 31, 121]; 3.1–6[44]; 6[129]; 6.3[73]; 9[81]; 10[29]; 10§[54, 92]; (10)[53]; 20[40]; ≤25[77]; 25§[67]; 25[61]; 50§[8, 16, 17]; 50[56]; 100§[37, 43, 82, 103, 112, 119]; (?)[70]; ?§[88]; distillate[79, 113]; extract[118]	0.4[131]; 1.4[50]; 17[27, 83]; 20[8]
Streptococcus sp.	2.5[20, 57]; 30[15]; 33[84]	2.5[20, 57]; 5[21]; 5.4[81]; 30[56]; ≤36[35]; (10)[53]	20[56]
Streptococcus faecalis		6.9[66]; 7.1[50]; 8.3[108]; 10§[54]; 20[17]; 25§[92]; 40[56]; 100§[82]; distillate[79]	5[17]; 30[56]
Streptococcus mitis		10§[54]	
Streptococcus mutans		100§[36]	
Streptococcus pneumoniae		10§[54]	

TABLE 1. (continued)

Species inhibited	Conc. of honey (%) for complete microbicidal action	Conc. of honey (%) for complete inhibition of growth	Conc.of honey (%) for partial inhibition of growth
Streptococcus pyogenes	0.6[20, 57]	0.6[20, 57]; 2.9[131]; 10§[54]; 20[56]; 100§[37, 103]; 25§[67]	0.7[131]; 10[56]
Streptococcus salivarius			25[61]
Streptomyces sp.		17[27]; 20[56]	
Vibrio cholerae		17[27]	
Vibrio cholerae biotype *Proteus*			
FUNGI			
Aspergillus flavus		60[123]; 75[85]	25[85]
Aspergillus fumigatus		3.1[30]	
Aspergillus niger		75[85]; distillate[79]	25[85]
Aspergillus parasiticus		60[123]	
Candida albicans	100[15]; distillate[79]	1.6[30], ≤100§[87]; distillate[80, 113]	
Candida pseudotropicalis	10[15]		
Candida reukaufii	50[15]		
Candida stellatoidea	50[15]		
Candida tropicalis	50[15]		
Candida utilis		(?)[70]	
Penicillium sp.		3.1[30]; distillate[79]; ?§[88]	
Penicillium chrysogenum		75[85]	25[85]
Saccharomyces sp.	[50 partial][69]		

opinion that honey had no effect on fungi beyond its osmotic action, the data in table 1 show that some honeys, at least, must have antifungal factors present, as some fungi are inhibited under conditions where the sugar content of the honey is clearly not responsible.

Non-specific reports

Two studies have been carried out on the antimicrobial activity of honey against unidentified micro-organisms in soil, water and air. Growth of colonies from 70–90% of the bacteria and 30–60% of the fungi from sewage, soil, air and tap water was found to be prevented by 25% honey[85]. Growth of colonies from airborne contaminants was found to be prevented completely by 20% honey and partially by 2% honey, the survivors being mainly fungi[54].

Differences in susceptibility between species

The relative sensitivity of various species of micro-organisms to honey is of great interest, as more resistant species may be able to overcome the inhibitory effects of the honey in areas of an infection where the honey is at lower concentrations. However, the nature of the studies carried out so far limit the accuracy of quantitative comparisons between species in their sensitivity to the antibacterial effect of honey. Because of this, and because the values given are not necessarily the minimum inhibitory concentrations, comparison of the sensitivity of various species is not possible by reference to the values given in table 1.

The major differences in findings on the sensitivity of each species are more likely, however, to be due to differences in the honeys used. Many workers have demonstrated that not all honey samples have the same degree of antibacterial activity (see later), therefore the sensitivity of species cannot be compared using the results from different studies, as the honeys used in the studies may have had widely differing antibacterial activity. The sensitivity of species relative to each other can be validly determined within a single study in which the same honey and same test conditions are used. Even so, the relative sensitivity of species could be found to be different within another study because species could respond differently to the different types of antibacterial factor that may be present in a different honey. This difference in ranking of sensitivity has been demonstrated by Willix[131] in a specific study of this point using two honeys known to have different types of antibacterial factors present. It was also observed by Popeskovik *et al.*[82], and further evidence of it can be seen in the data of others who worked with larger numbers of honeys[3, 52, 94].

Where the effect of a honey, or a group of honeys, on a number of species has been assayed under the same conditions within one study, sensitivities can be compared and the relative sensitivity of the species tested ranked. *Staphylococcus aureus*, a species included in most of these comparative studies, can be seen to be one of the species most sensitive to honey[17, 20, 35, 40, 50, 52, 54, 57, 61, 69, 79, 82, 84, 92, 103, 118, 119, 131]. (This is of medical significance because this species, as a result of its wide resistance to antibiotics, has become the major cause of wound infections and septicaemia in hospitals[62]). The relative sensitivity of other species is not so discernible because of

the marked variation from study to study. This almost certainly reflects the differences in the antibacterial factors in the honeys used in the various studies.

Explanation of the antibacterial activity of honey

Osmotic effect

Honey is a saturated or super-saturated solution of sugars, the water content usually being only 15–21% by weight[124]. Of the solids in honey, 84% is a mixture of the monosaccharides fructose and glucose[125]. The strong interaction of these sugar molecules with water molecules leaves very few of the water molecules available for micro-organisms. This 'free' water is what is measured as the *water activity* (a_w): mean values for honey have been reported as 0.562 and 0.589[91], 0.572 and 0.607[10], and 0.62[117]. Although some yeasts can live in honeys that have a high water content, causing spoilage of the honey, the a_w of ripened honey is too low to support the growth of any species, no fermentation occurring if the water content is below 17.1%[5].

Many species of bacteria have their growth completely inhibited by the a_w being in the range 0.94–0.99[60, 102]. These values correspond to solutions of a typical honey (a_w of 0.6) of concentrations from 12% down to 2%, calculated on the basis of the concentration being proportional to –log a_w[102]. On the other hand, some species have their maximum rate of growth when the a_w is 0.99[102], so inhibition by the osmotic (water-withdrawing) effect of dilute solutions of honey obviously depends on the species of bacteria.

Fungi are generally much more tolerant of low a_w than bacteria are[60], so the reports of antifungal activity with diluted honey indicate that there is more involved than just the sugar content of the honey. Likewise, *Staphylococcus aureus* has an exceptionally high tolerance of low a_w, yet is one of the species most sensitive to the antibacterial activity of honey. For complete inhibition of growth of *S. aureus* the a_w has to be lowered below 0.86[18, 19, 60], which would be a typical honey at 29%. There have been many reports of complete inhibition of *S. aureus* by honeys much more dilute than that.

The results of some experiments have demonstrated quite clearly that there is much more than an osmotic effect involved. In one study with *S. aureus*, honeys were dialysed to remove the sugar, yet complete inhibition was observed with some at dilutions down to 1.5% honey[35]. In another study[4], honeys were tested at a concentration of 18% in an agar diffusion assay, where the activity of many honeys was below the level of detection: the activity of others was up to 20 times higher than the minimum detectable. In a similar study[72] a honey of low antibacterial activity showed no activity against *S. aureus* when tested at a concentration of 50% in an agar diffusion assay that allowed activity to be detected in an active honey diluted to a concentration of 1%. The range of a_w found in honey (0.47–0.70[91]) could account for only a two-fold difference in activity due to osmotic effects.

Further indication that the antibacterial activity of honey is due to a lot more than just the removal of water from bacteria is seen in the results of the many studies in which the antibacterial activity of honey has been compared with that of 'artificial honey' (a solution of sugars of the same proportions as typically in honey).

In one study, 13 species[83], and in another study, 15 species[27], were found to be substantially or completely inhibited by honey at 17% in the nutrient agar, but were not inhibited by artificial honey in its place at the same concentration. A bacteriostatic action against five species seen with 20% honey was not seen with 20% artificial honey[40]. Bacteriostatic and bactericidal activity against 12 species was seen with honey diluted to concentrations of 20% down to 0.6%, but with artificial honey only bacteriostatic activity was seen, only with dilutions down to 20%, and only against certain Gram-positive species[20]. Honey diluted 1 in 10 was found to inhibit *S. aureus*, *Shigella flexneri* and *Escherichia coli*, but a 76% solution of glucose used as an artificial honey was not inhibitory when diluted 1 in 5[51]. *Streptococcus faecalis* and *Shigella dysenteriae* were found to be completely inhibited by 8.3–21.6% honey but not by 25% artificial honey[108]. In another study these species were found to be completely inhibited by 10–25% honey but not by 25% artificial honey[66]. No inhibition of *Corynebacterium diphtheriae* was seen with 25% artificial honey, but strong inhibition was seen with 25% natural honey[28]. In tests involving *S. aureus*, *Pseudomonas aeruginosa* and a strain of *Streptococcus*, a marked lack of antibacterial activity was observed in artificial honey compared with that in various types of natural honey[81].

In other studies inhibition was observed with artificial honey, but greater inhibition was seen with natural honey. A very low degree of inhibition of *E. coli* and *S. aureus* was seen with artificial honey compared with that from natural honeys[61]. With five species of bacteria only partial inhibition of growth was seen with artificial honey at 20% compared with complete inhibition with natural honeys at concentrations down to 5%[21]. There was 60% inhibition of growth of *E. coli* with artificial honey at 20% compared with complete inhibition with natural honey at 6–12%[81]. Larger zones of inhibition were seen in an agar diffusion assay against *E. coli* and a strain of *Salmonella* with natural honey than with artificial honey[85]. A similar finding was made in another study with *E. coli*, *Bacillus pumilus*, *S. aureus* and a strain of *Penicillium*[88]. Complete inhibition of growth of *Aspergillus niger*, *A. flavus* and *Penicillium chrysogenum* was seen with 75% natural honey, but only partial inhibition with 75% artificial honey[85]. To achieve 50% inhibition of growth of *Proteus mirabilis*, 3.6% natural honey was required but artificial honey had to be at a level of 14%[131]. Recombining the components of honey in proportions equivalent to their original levels in honey, complete inhibition of *S. aureus* was seen at a concentration equivalent to 7.7% honey; no inhibition was seen to result from the sugars alone at a concentration equivalent to 12.9% honey[127]. High levels of activity against *S. aureus* were found in an agar diffusion assay with 50% solutions of honey, but there was no inhibition when the honeys were replaced with an artificial honey[8]. However, using a different assay method, in which the honeys were not diluted by diffusion, at a concentration of 20% the artificial honey gave approximately 20% inhibition of growth.

Thus it can be concluded that both the osmolarity and additional factors are involved in the antibacterial activity of honey, their relative importance depending on the sensitivity of the species and the level of the additional factors in any honey. Some species of bacteria, with little tolerance of low a_w, are likely to be inhibited by quite low concentrations of honeys that have nothing more than their sugar content at work. Other species of bacteria, and fungi, tolerant of lower a_w, can still be inhibited by very low concentrations of some honeys if these contain high levels of other antibacterial factors.

Acidity

Some of the early thinking on the explanation of the antibacterial activity of honey considered the acidity of honey to be important[84, 95]. Honey is characteristically quite acidic, its pH being between 3.2 and 4.5[124]. This acidity is due primarily to the content of gluconolactone/gluconic acid present as the result of enzymic action in the ripening nectar, average values of 0.23–0.98% being reported in honey[124]. However, studies in which acidity was taken into account found no correlation between antibacterial activity and the pH of the honeys studied[10, 24, 61, 81, 94, 108]. Because there may be different degrees of buffering in different honeys, the pH is not necessarily an indication of the titratable acidity which is what would determine the final pH when honey is diluted by a neutralizing medium. Even so, in a study in which a buffered gluconolactone/gluconic acid solution was made up to match the composition of the most acidic honey sample, this solution at the equivalent concentration of 25% honey showed no detectable activity in an agar diffusion assay in which the honey gave a clear zone of 23 mm diameter at 12.5%[72]. The concentration of gluconolactone/gluconic acid in this experiment with *S. aureus* was 0.2%. In different work with this species[23] no inhibition was seen with gluconic acid added to nutrient broth at levels up to 0.25%. In other studies on honey, marked antibacterial activity was still found when the honeys were neutralized before assay, ruling out any contribution from the acidity to the antibacterial activity observed[20, 42, 51, 77, 83, 84, 85, 88, 132].

Although these observations point to the acidity of honey being unimportant, they do not mean that acidity does not contribute to the antibacterial activity of honey. Pothmann[83] measured the pH of the nutrient broth containing the minimum inhibitory concentration of honey (4.5%) for *Corynebacterium diphtheriae* and found it to be 6.2. With this species the lowering of the pH of the growth medium was of consequence, as the minimum inhibitory concentration of neutralized honey was found to be 10%. The low pH of honey was found to be of effect in the inhibition of *Bacillus cereus* also: inhibition by 50% honey in an agar diffusion assay was lost if phosphate buffer was added to bring the pH to 6.1–6.5[90].

The low pH of honey would be inhibitory to many animal pathogens, with their optimum pH for growth normally falling between 7.2 and 7.4, and with minimum pH values for growth of some common wound infecting species being: *E. coli*, 4.3; *Salmonella* species, 4.0; *Pseudomonas aeruginosa*, 4.4; *Streptococcus pyogenes*, 4.5[111]. Under experimental conditions, especially with heavily diluted honeys, the growth medium used tends to neutralize the acidity of the honey so that it does not cause inhibition, but when honey is used as a dressing on a wound or ulcer, bacteria may be in contact with honey that is much less diluted, and the acidity could well be of importance. The fairly strong buffering capacity of body fluids would most likely neutralize the acidity of honey in other situations where there is greater dilution of honey.

Hydrogen peroxide

The possibility that hydrogen peroxide could be the substance responsible for the antibacterial activity of honey was investigated by Adcock because both hydrogen peroxide and the antibacterial activity of honey are destroyed by exposure to light. He reported in 1962 that the antibacterial activity of honey could be removed by the addition of catalase, and measured the presence of hydrogen peroxide in honey[1]. The topic was also studied by White *et al.* who had found that the major acid in honey is gluconic acid[107]. They reported in 1963 that it was produced by the action of glucose oxidase which produced hydrogen peroxide in the reaction, and they showed a direct relationship between the hydrogen peroxide produced and the 'inhibine number' of various honeys[127].

That antibacterial activity could result from such enzyme activity was not a surprise as it had been found well before in a different system. When following up Fleming's work on the antibacterial properties of *Penicillium notatum*, Coulthard *et al.*[23] obtained erratic results which were traced to the potent activity of a second factor, notatin, present in addition to penicillin. They found notatin to be a combination of the enzyme glucose oxidase with glucose, and showed the activity of notatin to be due to the production of hydrogen peroxide. Others working on the antibacterial property of honey have since demonstrated antibacterial activity to result from a combination of glucose oxidase and glucose[8, 35, 36, 126].

It was reported by Gauhe in 1941 that glucose oxidase is present in the hypopharyngeal glands of the honey bee, and that the contents of the honey sac become acidic on standing[41]. The glucose oxidase in honey was found to strongly resemble the enzyme in the hypopharyngeal glands of the bee[99], and is assumed to be secreted along with other enzymes from the hypopharyngeal glands into the nectar to assist in the formation of honey[64]. Gauhe suggested that this would be of advantage in preservation of the honey. This function of glucose oxidase may account for its unusual production by an animal species[99]. The hydrogen peroxide produced at the same time would be of effect only during the ripening of honey however, as full-strength honey has a negligible level of hydrogen peroxide (undetectable[85, 128], or < 10 mmol/kg[127]).

White *et al.*[127] found that the enzyme is practically inactive in full-strength honey, it giving rise to hydrogen peroxide only when the honey is diluted. On dilution the activity increases by a factor of 2500–50 000[126]. This explains the paradoxical finding of Sackett[95] that the deleterious effect of honey on the survival of bacteria put in it was increased by dilution of the honey. It also brings into question the conclusion reached by some that hydrogen peroxide is not responsible for the antibacterial activity of honey[85, 90] when their conclusion was based on finding a low level of hydrogen peroxide in honey assayed undiluted.

In most of the studies on the antibacterial activity of honey, solutions of honey diluted to 50% or below have been used, so the enzyme would have been active. Thus a good relationship has been observed between the antibacterial activity of diluted honey samples and the level of hydrogen peroxide that accumulated in them on incubation[8, 35, 127, 128, 129]. The involvement of hydrogen peroxide in the antibacterial activity of diluted honey is also supported by the finding that all or a substantial part of the detected activity can be removed by the addition of enzymes that destroy hydrogen peroxide (catalase, or peroxidase plus a hydrogen donor)[1, 4, 8, 50, 72, 92, 127, 131].

The antibacterial activity arising from enzymatic production of hydrogen peroxide accounts for many of the discrepancies in earlier observations on the molecular weight of the antibacterial factor in honey. It has subsequently been demonstrated that if honey is dialysed, removing the sugars, the enzyme is retained and will give

rise to hydrogen peroxide if glucose is added back to it[35, 127]. Prior to this, some thought that the antibacterial factor was of high molecular weight and some of low. Their conclusions can be explained by looking at their experimental conditions: hydrogen peroxide would have been produced by the the enzyme in the dialysis retentate with glucose added[35, 127], but not without it added[81]; when the diffusate was recovered, concentrated and tested, it would have contained hydrogen peroxide produced in the diluted honey during dialysis[81]. Adsorption of the enzyme on to asbestos would account for removal of activity by Seitz filtration[20, 27, 61, 81, 108]: proteins are known to be adsorbed[127]. It has also been found that activity is removed by a Berkefeld filter (diatomaceous earth) and by adsorption on to clay soil, bolus alba and kaolin[27]. The activity found to pass through a Seitz filter when 50% honey was used[84] could have been hydrogen peroxide produced in the diluted honey: however, it may have been that the EK-coated Seitz filter used did not adsorb the enzyme.

One question that has not been addressed in the literature on the subject is why, when the enzyme and its substrate, glucose, are together in honey, glucose oxidase is inactive until the honey is diluted. The most likely explanation is that its activity is suppressed by the unfavourable pH in ripened honey. The enzyme has an optimum pH of 6.1, with a good activity from pH 5.5 to pH 8, but the activity drops off sharply below pH 5.5 to near zero at pH 4[99]. The pH measured in the dilution series of agar plates in an assay of the inhibine number of a honey of pH 3.9 was found to be from 5.5 to 6.4[130]. White *et al.*[127] observed that with some honeys, diluted without buffering, the maximum rate of production of hydrogen peroxide is found at the intermediate inhibine number dilutions and not at the lowest dilutions as expected. This phenomenon was not observed if dialysed honey was used with glucose added back, but was observed when dried honey was added instead as the source of glucose. These findings could easily be explained by the acidity of some honeys keeping the pH too low for the enzyme unless well diluted.

Although most of the acidity in honey is due to the gluconic acid that arises from the activity of glucose oxidase[107], the suppression of the enzyme's activity appears to be due to the resultant pH rather than to the reaction product *per se*: in a buffered system no inhibition at all was seen with 10 mmol/litre gluconic acid or gluconolactone[99]. Nor, it is reported[98], does the other reaction product, hydrogen peroxide, cause inhibition at the levels that are produced. The latter finding is brought into question, however, by data presented from studies with honey[127] and with the isolated enzyme[98] which show the rate of reaction to be falling off over a short period of time, a period in which denaturation of the enzyme at the temperature of incubation would not be noticeable[129]. Removal of the hydrogen peroxide produced, by the addition of ascorbic acid, gave a five-fold increase in the rate of reaction[98]. Even so, the level of hydrogen peroxide is so low in full-strength honey that product inhibition of the enzyme can be ruled out as an explanation of why the enzyme is not active before dilution.

The possibility of substrate inhibition can also be ruled out on consideration of the finding that glucose concentrations beyond those occurring in honey do not suppress the rate of reaction[99]. In fact, the optimum substrate concentration for the glucose oxidase in honey is exceptionally high (1.5 mol/litre[99]), this being well suited

to the enzyme's functioning in ripening honey. (The concentration of glucose in ripened honey is around 2 mol/litre.)

Not so well suited is the enzyme's requirement for a minimum of 100 mmol/litre of sodium for maximum activity[99]. The levels of sodium in honey range from 0.3–41 mmol/litre, but would typically be 2–3 mmol/litre[124]. If honey were diluted by body fluid, the requirement for sodium would easily be met. In laboratory assays of its antibacterial activity the situation could be different, depending on the composition of the medium used to dilute the honey.

Consideration needs also to be given to the effect of dilution on the concentration of substrate, with the enzyme requiring such a high level of glucose for maximum activity. The rate of production of hydrogen peroxide decreases acutely when the level of glucose is lowered, as would happen when honey is diluted a lot. This causes a complication in interpreting the inhibine number (see later) as a measure of antibacterial activity. Normally an assay of minimum inhibitory concentration would be expected to give a linear measurement of the concentration of antibacterial substance present. Samples under test are each diluted to the level at which the response is the same. Usually this means that if one sample has twice the antibacterial activity of another it would have to be diluted twice as much to be at this level. The complication in determining the inhibine number is that the bacteria are responding to a secondary substance (hydrogen peroxide), not to the substance being diluted. It has been clearly demonstrated[127] that a constant response to a constant level of hydrogen peroxide is occurring in the assay at the minimum inhibitory concentration of honey. However, the degree of dilution necessary to achieve this level of production of hydrogen peroxide is not linearly related to the level of glucose oxidase in the nutrient agar because the reduction in substrate concentration gives a sharp decline in the rate of production of hydrogen peroxide.

This is well demonstrated in the data from a study in which hydrogen peroxide was assayed in the plates of a dilution series for determination of the inhibine number[127]. In this study dialysed glucose oxidase from honey was used , with glucose added back at the same levels as would be present in the usual dilutions of honey in the assay. The amount of hydrogen peroxide measured at the greatest dilutions was disproportionally low, but was found to be much more in proportion to the concentration of glucose oxidase if glucose was added at the same level as in the least dilution. It is also shown in a study of 45 honey samples in which it was found that the inhibine number (i.e. the stepwise dilution) correlated with the logarithm of the level of accumulation of hydrogen peroxide in the samples assayed with them all diluted to the same degree (20%)[128]. A completely different result was seen when an agar diffusion assay was used, in which the honey samples were all assayed at the same degree of dilution (50%): there was found to be a significant ($P = 0.001$) *linear* correlation between the antibacterial activity and the level of accumulation of hydrogen peroxide in the 37 samples studied[8].

The non-linearity of the inhibine number as a measure of antibacterial activity was recognized by Duisberg and Warnecke in 1959[31]. They devised a formula to obtain a linear measure:

$$\text{concentration of inhibine} = \frac{100}{(30 - 5) \times \text{inhibine number}}$$

This non-linearity would apply to the results of most of the studies of antibacterial activity in honey in which dilution methods have been used: only one study[35] kept the level of glucose constant. Thus the results from these studies will underestimate the true potential of honey as an antibacterial agent. The actual antibacterial activity at high dilution may be considered to be the more appropriate measure in the context of the action of honey diluted to low levels by body fluids. However, it is the full potential to produce hydrogen peroxide that should be compared when considering the effectiveness of a honey in the treatment of an infection, and a linear measure is better for this.

The amount of hydrogen peroxide produced in diluted honey is clearly high enough to give a substantial antibacterial activity. When the levels of hydrogen peroxide accumulating in the agar plates of an inhibine-number assay were monitored, it was found that the minimum inhibitory concentration of the honeys corresponded with an accumulation of 0.05 mmol/litre in 1 h, 0.07 mmol/litre in 2 h, and 0.12 mmol/litre in 4 h[127]. A study of the accumulation of hydrogen peroxide in 90 samples of honey diluted to 14% and incubated for 1 h found values ranging from 0 to 2.12 mmol/litre (mean 0.47, s.d. 0.55)[128]. A similar assay of 31 samples by another researcher found values ranging from 0 to 0.95 mmol/litre (mean 0.32, s.d. 0.27)[8]. Another study, carried out with 36% honey, found in the 25 samples assayed the level of hydrogen peroxide accumulated ranged from 0.11 to 0.58 mmol/litre (mean 0.22, s.d. 0.13)[1]. Two other studies, in which the dilution of the honey was not stated, gave results for the production of hydrogen peroxide per hour per gram of honey. Expressed as the rate for a 14% solution of honey, these would translate to 0.02 to 3.89 mmol/litre (mean 1.48, s.d. 1.50, n = 11)[33] and 0.14 to 3.66 mmol/litre (mean 1.24, s.d. 1.18, n = 9)[35].

There have been several reports on the levels of hydrogen peroxide required for antibacterial activity. In work with *Bacillus cereus*[90] it was found that to obtain clear zones in an agar diffusion assay with hydrogen peroxide applied to the paper disks used, a minimum of 5.9 mmol/litre was required. (There would, however, have been a substantial dilution of the applied solution as it diffused from the small paper disk into the mass of agar in this work, so the effective level of the hydrogen peroxide would have been much lower.) In the early work on notatin[23] it was found that *S. aureus* failed to grow in 24 h in nutrient broth containing hydrogen peroxide at 0.29 mmol/litre but grew at 0.15 mmol/litre. This was confirmed by others working with *S. aureus*[127] who found only one colony grew on a nutrient agar plate containing 0.29 mmol/litre hydrogen peroxide, and none at the next level tested, 0.5 mmol/litre. In another study with *S. aureus*[8] it was found that 20% inhibition over an incubation period of 16 h corresponded with an accumulation of 0.12 mmol/litre hydrogen peroxide from the glucose oxidase-glucose system used to generate it.

It is possible that hydrogen peroxide has an even greater potential for inhibiting bacteria when in honey than when it is tested on its own. It appears that hydrogen peroxide is itself not antibacterial, the antibacterial action being due to damagingly reactive hydroxyl free radicals generated by the catalytic action of traces of metal ions from the bacterial cells[114]. The bactericidal action of hydrogen peroxide can be potentiated by ascorbic acid (vitamin C), especially in the presence of certain metal ions[89]. With ascorbic acid at 0.1 mmol/litre and hydrogen peroxide at 1–10 mmol/litre a powerful bactericidal effect was observed[68]. The sporicidal action of hydrogen peroxide has been found to be markedly increased by copper at 10

mmol/litre[120]. It has also been found that the antibacterial potency of hydrogen peroxide is increased ten-fold by 0.83 mmol/litre iron, copper, chromium, cobalt or manganese, but these destabilize hydrogen peroxide solutions so cannot be added to an antiseptic preparation[65]. However, when honey is used as an antiseptic the hydrogen peroxide is generated *in situ* so its stability is unimportant. It has been observed that the addition of 9.7 mmol/litre ascorbic acid to honey glucose oxidase in fact stimulates a five-fold increase in turn-over of the enzyme as its product (hydrogen peroxide) is removed[99]. As bactericidal free radicals would be generated in the removal of hydrogen peroxide, high levels of hydrogen peroxide do not have to be reached. The levels of ascorbic acid found in honey have been up to 22 mmol/litre, although more typically the level would be 0.2–0.3 mmol/litre[124]. The levels of iron, copper, manganese and cobalt in honey have been found to be 0.01–0.60, < 0.01–0.28, < 0.01–0.80, and 0.01–0.03 mmol/litre respectively[124]. These are not inhibitory to glucose oxidase[99]. Thus in some honeys at least, there is the potential for the generation of free radicals, catalysed by ascorbic acid and metal ions, from the decomposition of the hydrogen peroxide produced on dilution.

It is suggested that this decomposition reaction may be the reason why hydrogen peroxide went out of favour as an antiseptic, unfavourable results being obtained with the unstabilized preparations in use at that time[114]. An upsurge of interest in more recent times, with good germicidal activity being reported, has been pointed out now that stable preparations are in use[114]. Hydrogen peroxide was widely used at one time, but went out of favour also on the theoretical grounds that some species of bacteria possess the enzyme catalase which decomposes hydrogen peroxide[26]. Note should be taken, however, of the finding that the catalase activity of strains of *S. aureus* does not correlate with their sensitivity to hydrogen peroxide[7].

Catalase is also present in plasma, at a mean level of 6.9 units/ml, (i.e. 6.9 mmol/litre of hydrogen peroxide removed per minute). That present in exuding plasma in a wound could be augmented by catalase released from dead leucocytes. Although this catalase would be considered to reduce the antibacterial activity of honey by removal of the hydrogen peroxide generated, it could in the process be itself generating antibacterial activity in the form of free radicals[58]. This, and the possible augmentation of the leucocytes' own production of hydrogen peroxide for the killing of ingested bacteria, could account for the clinical observation that honey is a more effective bactericide *in vivo* than *in vitro*[37].

If a solution of hydrogen peroxide is used as an antiseptic it is likely to be far less effective than a 'slow release preparation' in the form of honey. Catalase is active with high concentrations of hydrogen peroxide but is of low activity with physiological levels[22]. Unexpectedly high levels of catalase were found to be necessary to destroy the antibacterial activity of honey[1, 127]. A further consideration is that myeloperoxidase, the enzyme that generates the active free radicals from hydrogen peroxide in the leucocytes, is inactivated by excess hydrogen peroxide[58], being denatured by levels above 2 mmol/litre[2].

Other factors

Since the work of White *et al.* established that hydrogen peroxide is responsible

for antibacterial activity in honey, the term inhibine has in many cases been used interchangeably with hydrogen peroxide in the literature on honey, the authors doing so obviously not considering other factors beyond acidity and osmolarity to be involved. However, there is much evidence of there being other antibacterial factors, some of significant activity.

There has been much disagreement about the existence of non-peroxide antibacterial substances in honey, some authors being of the opinion that they account for little if any of the activity[35, 75, 128] and others that they account for all of the activity[42, 71, 85] beyond that due to the acidity and high osmolarity of honey. Mostly it is accepted that both types of activity occur, to different degrees in different honeys. The evidence for the existence of non-peroxide factors is mainly in the form of the peroxide-generating system failing to account for all of the observed non-osmotic antibacterial activity, but there have also been some reports of isolation of antibacterial substances from honey that are not hydrogen peroxide.

The level of hydrogen peroxide accumulating in honey can vary according to the floral source because of negative influences from various other components (see later), but should be at its maximum in honey produced by bees fed on sugar syrup instead of nectar. In this case the negative influences from various plants would not be present to counteract the production of hydrogen peroxide by the enzyme secreted into the honey by the bees. Yet it was found that the bacteriostatic activity against *E. coli* and *S. aureus*[61], and against these and three other species[21], was low in honey from sugar-fed bees . Also it was found that whereas complete bactericidal action against *Mycobacterium tuberculosis* took one day in sainfoin-lavender (*Onobrychis viciifolia - Lavandula* sp.) honey, and two days in honeydew honey, it took four days in honey from sugar-fed bees[105].

The existence of non-peroxide antibacterial factors is indicated also by findings that the antibacterial activity does not correlate completely with the rate of accumulation of hydrogen peroxide in honey samples[1, 8, 35, 127, 128]. In one study it was found that honeys producing hydrogen peroxide when diluted were not antibacterial, and the ones that were antibacterial did not produce any significant amount of hydrogen peroxide[90]. However, this extreme case may have been the result of *Bacillus cereus* being used in this study instead of the usual *S. aureus*. The use of test species possibly more resistant than *S. aureus* to hydrogen peroxide could also explain the finding[85] that *Bacillus subtilis* and *Saccharomyces cerevisiae* were no more sensitive to honey than they were to a sugar solution of the same osmolarity, yet *E. coli* and a strain of *Salmonella* were sensitive.

It could also be the explanation for Gonnet and Lavie[42] concluding that hydrogen peroxide is not involved in the antibacterial activity of honey. Their conclusion was based on the finding that heating honey for 1 h at 75–80°C did not destroy its activity against *B. subtilis*. If this species were less sensitive to hydrogen peroxide and more sensitive to the non-peroxide factor present, then denaturation of glucose oxidase by heating (see later) would have made little difference. Others have found that heating honey causes loss of activity against some species whilst it is retained against others[31, 61, 85].

The finding of antibacterial activity in honey that is stable to heating has been an indication in several other studies of the existence of non-peroxide antibacterial factors. Although the stability of glucose oxidase can vary according to the presence of different plant-derived components in honey (see later), there have been reports of honeys with stability well in excess of this variation.

In a study of some Jamaican honeys, the activity of the two most active honeys was not reduced by steam-sterilizing. In three less active ones it was reduced by boiling, and in the least active honey it was destroyed by boiling[54]. Activity with a very high stability to heating has also been found in New Zealand manuka (*Leptospermum scoparium*) honey[72] and other honeys of unspecified floral source[59, 90]. A study of some Romanian honeys found that conifer honeydew honey, which had exceptionally high activity, contained a heat-stable as well as a heat-sensitive antibacterial factor[24]. Heat-stable activity has been reported in other honeys also[8, 17, 24, 52].

More direct evidence for the existence of non-peroxide antibacterial factors in honey is seen in the reports of activity persisting in honeys treated with catalase to remove the hydrogen peroxide activity[1, 4, 8, 50, 72, 90, 92, 131]. In the first study in which catalase was added to remove the hydrogen peroxide, substantial antibacterial activity remained in many of the honeys yet direct assay of the level of hydrogen peroxide present showed that the catalase had been completely effective[1]. It was reported that the residual activity could be removed by the addition of higher levels of catalase, greatly exceeding those required to destroy the amount of hydrogen peroxide present. It was suggested that the catalase in this case could be having an effect on components other than hydrogen peroxide. This would be feasible if the catalase generated reactive free radicals as discussed above.

High levels of non-peroxide activity were found in some New Zealand honeys with sufficient catalase added to remove hydrogen peroxide at a level one hundred times higher than that with activity equivalent to the most active honey in the study[72]. Manuka honey was found to have a particularly high level of this type of activity[72]. In a later study[4] finding similar results, it could be seen that the catalase was effective in use, in that it removed all detectable activity from honeys with very high levels of activity. In this study of 345 samples, non-peroxide activity was found to be associated only with honey from vipers bugloss (*Echium vulgare*) and manuka. In the former, of relatively low activity, it accounted for 75% of the total activity; in the latter, of relatively high activity, it accounted for 90% of the total activity. The possibility was investigated that the activity remaining in manuka honey after the addition of catalase was the result of a component of this honey inhibiting the enzyme, but it was shown that inhibition did not occur[4].

In another study on honey[8] it was found that whereas catalase removed the antibacterial activity detectable by an agar diffusion assay, it had no effect on the inhibition of bacterial growth in nutrient broth assessed after 16 h incubation. The hydrogen peroxide content at the end of 16 h in the latter assay was far too low to account for the inhibition when catalase was not added, suggesting that the bacteria had removed it. Further investigation of the residual inhibitory activity led to the extraction and identification of pinocembrin as an antibacterial component of honey[8].

Further investigation[9] of this non-peroxide activity indicated that propolis was the most likely source of the pinocembrin. This compound is the major flavonoid in propolis, and the flavonoid composition of honey and propolis have a similar pattern. However, flavonoids dissolve only a little into honey: the level of pinocembrin was found to be only 1–2% of what would be required to account for the observed

non-peroxide activity. The occurrence of a considerable level of this heat-stable activity in honey from sugar-fed bees suggested that it is produced by the bee rather than coming from a plant source. The possibility that the heat-stable non-peroxide antibacterial activity derived from the bee is the bacteriolytic enzyme lysozyme was excluded by the finding that the honey used in the study had no detectable activity in a standard test for lysozyme.

Lysozyme has been identified in honey[71], occurring at a level of 5–10 mg/ml usually, occasionally at 35–100 mg/ml (expressed as concentration of egg-white lysozyme of equivalent activity), if the honey is freshly extracted from the comb. The level was found to be much lower in older samples. It is questionable, however, whether lysozyme activity is of any significance in the non-peroxide antibacterial activity in honey that has been reported by others, because much of the work has been done with samples that had not been recently extracted. Also, in the study identifying lysozyme[71] the test species used was *Micrococcus lysodeikticus*, a bacterial species traditionally used for this purpose because of its high sensitivity to lysozyme. Species used in other studies would probably be less susceptible to it.

Investigation of an ether extract of manuka honey by preparative thin-layer chromatography led to the identification of some components with antibacterial activity: 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid), methyl 3,5-dimethoxy-4-hydroxybenzoate (methyl syringate), and 3,4,5-trimethoxybenzoic acid[93]. Another phenolic acid with antibacterial activity, 2-hydroxy-3-phenylpropionic acid, was identified as the major component of the ether extract of manuka honey observed by gas chromatography-mass spectrometry[110]. The same study found 1,4-dihydroxybenzene as the major component of the ether extract of vipers bugloss honey. Subsequent quantitative work[74] showed that the non-peroxide antibacterial activity of viper's bugloss honey could be accounted for entirely by its content of 1,4-dihydroxybenzene, but in manuka honey only 1.6–3.2% was due to 2-hydroxy-3-phenylpropionic acid, and 0.2–0.35% to 3,5-dimethoxy-4-hydroxybenzoic acid. The other antibacterial components identified were found to make an insignificant contribution to the antibacterial activity. Additionally, 2-hydroxybenzoic acid was found to contribute 0.2–0.3%.

A similar conclusion, that the major antibacterial component remains to be identified, has to be reached on considering the findings of Tóth *et al.* in their gaschromatographic analysis of the steam-distilled oil obtained from honey[113]. Although the terpenes and benzyl alcohol identified may have known antibacterial properties, the quantities present were far too low to be of any consequence.

Others have also found volatile antibacterial substances in honey. Some Bulgarian honeys were found to have a bactericidal component which gave zones of inhibition extending up to 15 mm from glass cups in which the honey was placed on agar plates[20, 57]. A similar effect may have been the explanation for the observation made in other work, that when more than six honey-soaked paper disks were placed on each plate in an agar diffusion assay of honey, the size of the clear zone around each disk was larger[90]. Loss of volatile antibacterial substances could explain the finding that the antibacterial activity was reduced by bubbling air through honey, an experiment performed in an attempt to explain the loss of activity only in honeys that had been opened frequently during storage[66]. The study

with the Bulgarian honeys found that the volatile activity was lost if honeys were left open for 24 h at 37°C.

Some researchers have been able to distil antibacterial activity from honey. Fractional distillation of honey under vacuum (18 mm Hg) gave rise to a potently antibacterial distillate boiling at 25–26°C[101]. This distillate was collected at a rate of 0.4–70 mg/kg of honey, depending on the source of the honey. None could be obtained from the honey produced by bees fed on syrup. Another study using fractional distillation found that antibacterial activity could be collected in the fraction boiling at 95°C[59]. This activity was light-sensitive but heat-stable. Other workers distilled a 'yellowish-brown oil' from honey in the boiling range 123–126°C[79]. This distillate was easily dissolved in water.

The differences found in the boiling points of the distillates by various workers make it clear that more than one compound is involved in the non-peroxide antibacterial activity of honey. Roth *et al.*[90] also concluded that more than one substance exists because not all of the honeys they studied could have their non-peroxide antibacterial activity extracted into ether.

Roth *et al.*[90] found that the non-peroxide antibacterial activity was extracted almost completely by ether, but only slightly or not at all by petroleum ether, ethyl acetate, and methylene chloride. Schuler and Vogel[101] were able to extract activity into ether, a little into chloroform, and none into propanol. They were also able to detect activity in the urine of people fed 50 g of honey, the maximum activity being present 3 h after eating the honey. (No activity was detected in control urine.) Gonnet and Lavie[42] found that the antibacterial activity (against *Bacillus subtilis*) in honey could be partly extracted with acetone, and extracted totally with alcohol. Lavie[59] reported subsequently that hot alcohol was twice as effective as cold alcohol in extracting the activity, and cold alcohol was twice as effective as cold acetone. The alcohol extract was water-soluble, and the activity was increased three times by extracting this solution into ether. Vergé[118] also found that activity could be extracted into alcohol, acetone and ether, but the antibacterial activity in the honey he used was extracted best into acetone. This activity was decreased by exposure to heat and light. Dustmann[35] found that activity could be extracted into acetone, but it was only a small fraction (often less than 2%) of the activity due to hydrogen peroxide. Chambonnaud[16, 17] similarly found that 2.5–5% of the total activity in honey could be extracted into acetone. Lindner[61] also found that most activity remains in honey extracted with solvents.

There is clearly much variation in the findings of non-peroxide antibacterial factors in honey, and in the quantitative importance of these factors in the antibacterial activity of honey. A problem in considering quantitative aspects is that in many of the studies, extracted antibacterial factors have been concentrated to a level above that at which they occur in the honey. The variation seen beyond that introduced by different degrees of concentrating almost certainly reflects differences in the degree of contribution of antibacterial phytochemicals made by the source plants through the nectar or honeydew collected by the bees (although it is possible that the phytochemicals themselves are without antibacterial activity until acted upon by enzymes from the bee). There have been some attempts, with success, to enhance the process by feeding bees on extracts of various herbs to increase the antibacterial activity of the honey produced[30, 69, 134].

The likely significance of non-peroxide factors in a clinical situation was investigated by Willix[131] who compared the susceptibility of common wound-infecting species of bacteria to a honey with high activity due mostly to hydrogen peroxide, and to manuka honey with activity due mostly to non-peroxide factors. The species tested were *E. coli, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhimurium, Serratia marcescens, Staphylococcus aureus* and *Streptococcus pyogenes*. It was found that both honeys were very active against the range of species tested, but the order of sensitivity for the species tested was quite different for the two types of honey. The concentrations of honey needed to achieve 50% inhibition of growth of each species over 8 h were 3.9, 2.6, 5.4, 1.3, 2.4, 2.7 and 1.4% respectively for the honey with activity due to hydrogen peroxide, and 0.8, 4.7, 5.4, 1.3, 3.4, 0.9 and 2.2% respectively for the honey with non-peroxide activity.

Conclusion

Honey has been shown convincingly to have a potent antibacterial activity, effective against a very broad spectrum of species, and to have antifungal properties as well. The activity seen with dilute solutions of honey clearly indicates that there is much more than the high sugar content of honey involved in its antibacterial action. This additional antibacterial activity is due to hydrogen peroxide produced by enzymatic activity in the honey, and in some honeys to plant-derived antibacterial substances as well.

Part 2 of this review (*Bee World* 73 (2) 1992) will cover the very large variation that has been found in the antibacterial potency of different honeys, and the loss of activity that results from inappropriate handling and storage of honey.

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References

A full list of references cited in this part 1 of a 2 part series will be printed at the end of the final part of the article.

[Both parts of this article will be reprinted as 'M' series reprint number M124 – Ed.]

THE ANTIBACTERIAL ACTIVITY OF HONEY
2. Variation in the potency of the antibacterial activity

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Introduction

Honey is gaining acceptance by the medical profession for use as an antibacterial agent for the treatment of ulcers and bed sores, and other surface infections resulting from burns and wounds[4, 135]. In many cases it is being used with success on infections not responding to standard antibiotic and antiseptic therapy. Its effectiveness in rapidly clearing up infection and promoting healing is not surprising in light of the large number of research findings on its antibacterial activity, covered in Part 1 of this review (*Bee World* 73(1): 5–28, 1992).

None of the reports in the medical literature, however, mention any selection of the honey used for the treatment of infections. Although it is recognized that honey has antibacterial activity, it is not generally realized that there is a very large variation in the antibacterial potency of different honeys, and that the antibacterial properties can be easily lost by inappropriate handling and storage of honey. Part 2 of this review covers the research that has been done on these aspects: giving regard to these findings should result in a more rational usage of honey in medicine and allow its full potential as an antibacterial agent be achieved.

Variation in antibacterial activity

A common feature of all of the reports in the medical literature on the use of honey as an antibacterial agent is that no consideration is given to the selection of type of honey for therapeutic use. Aristotle, *c* 350 BC[6], and Dioscorides, *c* AD 50 [46], recommended that honey collected in specific regions and seasons (and therefore presumably from different floral sources) be used for the treatment of different ailments. Such considerations have continued into present-day folk medicine: the strawberry-tree (*Arbutus unedo*) honey of Sardinia is valued for its therapeutic properties[38]; in India lotus (*Nelumbium speciosum*) honey is said to be a panacea for eye diseases[39]. In modern clinical practice, however, these views have gone unnoticed, as have the laboratory findings of large differences in the antibacterial potency of honey from different floral sources.

Degree of variance observed

In almost all studies in which more than one type of honey has been used, differences in the antibacterial activity of the honeys have been observed. The degree of difference observed has in some cases been very large, and in many others where it has been smaller this possibly is the result of a more limited range of testing rather than of less variance in the activity of the honeys. In many studies the antibacterial activity of different honeys has been compared by way of the inhibine

number determined by the method devised by Dold and Witzenhausen for such comparisons[29].

Dold and Witzenhausen coined the term 'inhibine number' in 1955[29] to describe the degree of dilution to which a honey will retain its antibacterial activity. This is a term that has been widely used since as a measure of the antibacterial activity of honey. The inhibine number involves a scale of 1 to 5 representing sequential dilutions of honey in 5% steps, from 25% to 5%. There have since been various minor modifications to this method so that the actual concentration corresponding to the inhibine number reported may vary. One modification has been to estimate fractional inhibine numbers by visual assessment of partial inhibition on the agar plate with the concentration of honey that just allows growth[1, 31, 108]. Another modification[96] has been the use of double-strength nutrient in the dilution mixture to keep the concentration of nutrient constant throughout the series: in the original method of Dold and Witzenhausen[29] this varied considerably. The effect of differences between methods on the comparability of the inhibine numbers from different studies has been discussed by White *et al.*[127].

In most of the studies measuring the inhibine number of honeys, activity has been found to range over the five-fold difference in concentration in the dilution series[1, 52, 61, 94, 96, 121, 122, 127, 128, 129, 130, 132]. In three other studies[21, 73, 108] activity was found to range over a four-fold difference in concentration in the dilution series. With some honeys not active at the highest concentration tested in some of the studies, and others still active at the greatest dilutions, it is possible that if greater and lesser degrees of dilution had been included in the testing then a wider range of activities would have been detected. One study using a wider range of dilutions (honey from 50–0.25%) found the minimum inhibitory concentrations of the honeys tested to range from 25% to 0.25%[3]. Another, testing from 50% to 0.4% found the minimum inhibitory concentrations to range from greater than 50% (i.e. not active at 50%) to 1.5%[35]. Other studies with wide ranges tested also found some honeys without activity at the highest concentration tested, and other honey with activity at the lowest concentration tested: the ranges were from 20–0.6%[14] and 50–1.5%[20, 57].

When the data are examined, activities are seen to be fairly well spread over these ranges. Duisberg and Warnecke[31] plotted the distribution of the activity of 131 samples of honey tested, and found that it deviated from a normal Gaussian distribution because of the large number of samples with low activity. (In 7% of the samples the activity was below the level of detection.) They attributed this to destruction of activity by exposure to heat and light, and estimated that 50% of the samples had lost more than half of their original activity, and 22% had lost more than three-quarters. Another study of 345 samples of honeys[4] also found a large number with low activity (36% of the samples had activity near or below the level of detection), the rest having almost a Gaussian distribution over a twenty-fold range of activity.

Association with floral source

Although some have concluded that honey from certain plants has better antibacterial activity than that from others, there is not enough evidence for such definite conclusions to be justified. Some of these conclusions are based on data from very

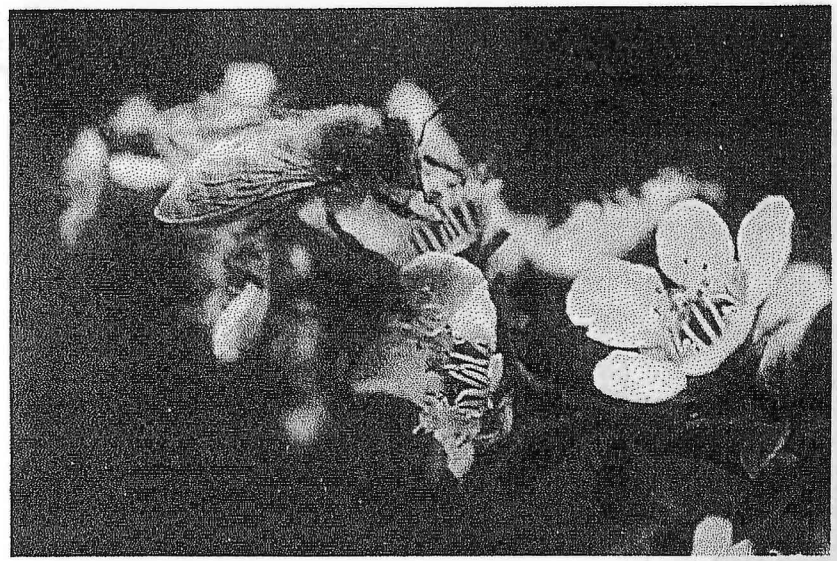

FIG. 4. Honey bee foraging on manuka flowers in New Zealand.

small numbers of samples. Other studies, though, have shown that there can be a large variation in the activity of different samples from the same plant source. Because of this, and because of the likelihood of misidentification of the source, the impossibility of getting a truly unifloral honey, and of variation associated with instability of the activity (discussed later), small numbers of samples cannot be taken as being representative of a particular source of honey. Even large-scale studies produce data of limited usefulness in this respect: because there are so many different plant species from which honey is produced, not many can be looked at from each. However, honeys from some sources have been been studied in large enough numbers or have been included in enough different studies for some trends to be noted.

There have been several studies in which dark honey from the conifer forests of the mountainous regions of central Europe have been found to have particularly high activity[14, 16, 17, 24, 25, 35, 52, 61, 81, 94, 103]. This honey is not from a nectar source, but from honeydew, produced by aphids sucking the sap from the leaves of the trees. Honey from sweet chestnut (*Castanea sativa*), a nectar source, has also been reported to have high activity[14, 35], but it is dark in colour and thus is considered to be partly derived from honeydew[14]. Another dark coloured honey, from manuka (*Leptospermum scoparium*)in New Zealand (fig. 4), has also been found to have a high level of activity[4, 73]. Roth *et al.*[90] commented on the association of high activity with dark coloured honeys in their study of Canadian honeys.

Heather (*Erica* spp.) honey, which has a fairly dark colour, has been found to have a high level of antibacterial activity in one study[4], but a fairly low[14, 94] or low[35, 73] level of activity in others. Rape (*Brassica napus*) honey has also been found to have a

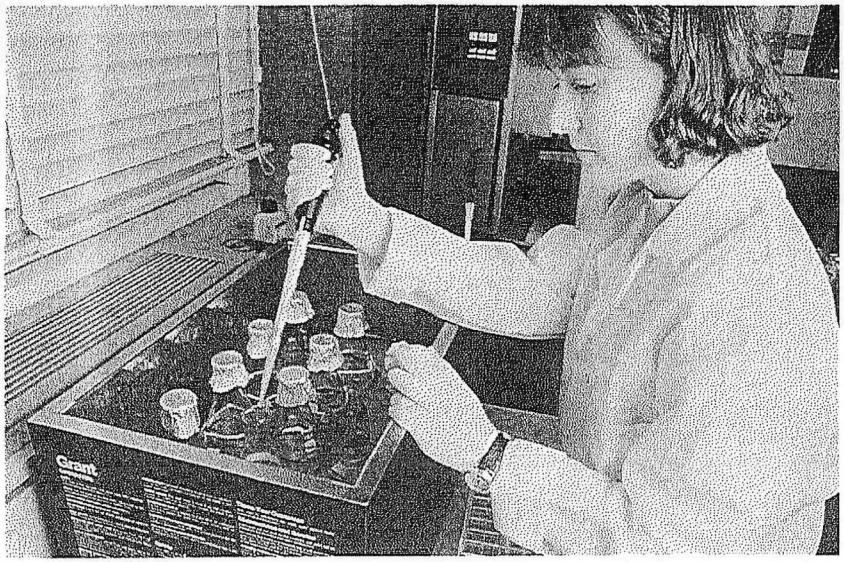

FIG. 5. Sampling broth cultures to measure the rate of bacterial growth with different concentrations of honey added.

high level of activity in one study[4], but a fairly low[54] or low[35, 61, 77] level of activity in others. In several studies linden (*Tilia cordata*) honey has been found to have a fairly high level of activity[14, 61, 77, 94], but a fairly low level of activity in others[35, 52]. Clover (*Trifolium* spp.) honey has been consistently found to have low activity[4, 14, 73], and cotton (*Gossypium hirsutum*) honey high activity[103, 106, 128].

Reasons for variance

The water activity of honey varies relatively little, and is not of much importance in the antibacterial effect of the dilute solutions of honey used to study the antibacterial activity of honey. Although the acidity of honey varies considerably, this too is likely to be of little consequence when the honey is in dilute solution in nutrient broth for testing its effect on bacterial cultures, as the broth buffers the acidity (fig. 5). The major variations seen in overall antibacterial activity are due to variation in the level of hydrogen peroxide achieved, and in some cases to the level of non-peroxide factors. The latter was found to be responsible for much of the activity in honeys with high levels of antibacterial activity in a study of 64 samples[72]. The content of non-peroxide factors is obviously related to the floral source, and sometimes it can account for the major part of the antibacterial activity in a honey, as is found with manuka honey[4]. The level of hydrogen peroxide achieved can also be related to the floral source, as components from some floral sources can affect both the production and the destruction of hydrogen peroxide (discussed below). There is a dynamic equilibrium: the level of hydrogen peroxide depends upon the balance between the rate of its production and the rate of its destruction[128].

Hydrogen peroxide obviously must be degraded, or else full-strength honey would contain substantial amounts of it, and any dilution of honey would eventually achieve inhibitory levels.

From the first work demonstrating that hydrogen peroxide is responsible for antibacterial activity in honey, it was realized that hydrogen peroxide is destroyed by components of honey. When testing *Staphylococcus aureus* for its susceptibility to added hydrogen peroxide, it was found that higher levels had to be added to achieve an inhibitory effect if honey was present[127]. Hydrogen peroxide was found to rapidly disappear when added to dilute honey, and, except in samples accumulating very high levels, the level of hydrogen peroxide accumulated from enzymatic action was seen to decline with time[128].

Of the factors possibly involved in the destruction of hydrogen peroxide, an obvious component to consider was catalase. This enzyme had long been thought to be present in honey[97], and was unequivocally shown to be present by Schepartz[97] in 1966. Catalase comes from the pollen and nectar of certain plants; more coming from the nectar[33]. Honeys from some floral sources have been found to have very high levels of catalase, and these honeys accumulate low levels of hydrogen peroxide: the ones accumulating high levels of hydrogen peroxide had low levels of catalase[33, 35]. There was some deviation from the inverse correlation seen in these studies, but this could well have been the result of non-peroxide antibacterial factors giving higher levels of activity, or prior denaturation of glucose oxidase giving lower levels. The latter would probably have been the explanation for the group of honeys with low antibacterial activity and low catalase activity found in another study of 28 samples[100]. Excluding this group, in this study a highly significant inverse correlation was found between catalase activity and accumulation of hydrogen peroxide.

Not all the variation, however, in the destruction of hydrogen peroxide associated with floral sources is due to the plants contributing catalase to the honeys. It has been found that the disappearance of hydrogen peroxide added to honey occurs even if honey is boiled beforehand to inactivate the catalase, indicating that a chemical degradation is involved as well as the enzymatic destruction[127]. This could well be the metal-catalysed reaction with ascorbic acid discussed earlier

The floral source can influence the production as well as the destruction of hydrogen peroxide, thus affecting the balance between these and lowering the level of accumulation. Very large differences have been found between honeys from different floral sources in the thermal stability of their glucose oxidase content[129]. A similar finding has been made in respect of the sensitivity of glucose oxidase to denaturation by light, a photosensitizing component responsible for the photo-oxidation of the enzyme being partially characterized in this study[130]. Of course, the influence of these factors on the antibacterial activity depends on the degree of exposure of honey samples to heat and light before they are assayed, but it is likely that much of the variation seen in the antibacterial activity of honeys reflects the history of those honeys. The level of antibacterial activity in a honey has for a long time been taken as an indication of whether or not a honey has been subjected to heating in its processing, although with the realization that it depends on other factors as well, this measure is no longer recommended[129].

Bactericidal or bacteriostatic action?

Duration of bacteriostasis

Most of the reports on the antibacterial activity of honey do not allow a distinction to be made between whether a honey is killing the bacteria or whether it is just stopping the bacteria from growing. Although no growth may have been seen over the period of observation, sometimes up to four days, in the absence of other evidence this only can be taken to be a *bacteriostatic* action, even if termed a *bactericidal* action by some authors[14, 67, 81]. A bactericidal action only can be concluded to have been observed in those studies where subculturing in a honey-free medium after initial exposure to honey shows no subsequent growth, which is what is recorded as bactericidal action in table 1. There may have been a bactericidal action in additional instances, but this cannot be known without the additional experimentation needed to demonstrate it.

Limited experimentation in these studies may also have left instances of bacteriostasis unobserved. In most cases a bacteriostatic action was demonstrated by lack of visible growth at the end of a period of incubation, a single observation being made at the end. It is likely, especially in the many studies with a long period of incubation, that growth would have ceased well before the observation was made: in batch culture, exhaustion of nutrient or build-up of toxic end-products can limit growth in quite a short time. In these cases a partially inhibited culture could 'catch up' an uninhibited control at this point of limited growth before the growth was observed. It is also possible that complete inhibition of growth could have gone unobserved in these studies: in other studies involving monitoring throughout incubation there is evidence of bacteria overcoming the antibacterial activity of honey after a period of inhibition[25, 52, 101]. However, the period was found to be longer with higher concentrations of honey, and seven major wound-infecting species of bacteria were found to be kept in a state of complete inhibition for 8 h if the concentration of honey was increased to between 3% and 10%[131].

Complete inhibition of growth maintained over a long period is obviously an important feature in controlling infections. Also of relevance is that if bacteria are kept in a state of bacteriostasis for a long period, their capacity to recover is lost[109]. In most of the cases of complete inhibition listed in table 1, the period of study over which this was maintained was 18–24 h.

Bactericidal action of honey

Whether or not honey has a bactericidal action appears to be very much a matter of the time of exposure of the cells to the honey. A gradual decline over 24 h was seen in the number of viable cells of several species of bacteria killed by 10% honey[25]. In another study[84] a bactericidal action was seen against *Escherichia coli* with 17% honey after 24 h, but 48 h was required for bactericidal action with 9% honey: with *S. aureus* 24 h was required for bactericidal action with 33% honey, 48 h was required for bactericidal action with 25% honey, and 96 h for bactericidal action with 9% honey. Another factor which contributes to this variation is differences in the susceptibility of the species being used for testing. The action of 20% honey was found to be bactericidal on only two out of six species of bacteria tested[52]. Observation of the bactericidal action of 50% honey on 12 species[20] revealed that Gram-positive species generally were the first to be killed, starting to die after 1 h of exposure, with complete killing after 3–24 h. Gram-negative species generally began to die after 4–6 h, complete killing taking up to 48 h. A comparison of ten species of bacteria exposed to eight different honeys at 50% concentration[57] found that the time required for a complete bactericidal action ranged from 3 to 48 h, there being four-fold differences between the honeys, and larger differences between the species. Another study[69] also showed that the time required for bactericidal action depends on the species of bacteria, and on the concentration of honey: *E. coli* with 50% honey grew for 2 h then began to decline in the number of viable cells; *S. aureus* showed a decline by 1 h, with complete killing after 4 h with 50% honey, but only partial killing by 5 h with 25% honey.

An incomplete bactericidal action in the time allowed would have been taken as no bactericidal action by some investigators who simply looked for growth after exposure: a small number of surviving cells would give this. Thus it was concluded that there was no bactericidal action on 6 species by 5–10% honey over a period of 5 h[50]. Likewise it was concluded that there was no bactericidal action on *S. aureus* by 15% honey over a period of 5 h[127], and by 29% honey over a period of 36 h[131]. Also with 11 species exposed to 10% honey for 8 h it was concluded that the action was only bacteriostatic[54], but it was noted that on subsequent examination there was evidence of damage to the bacterial cells.

It is known that vegetative cells of bacteria will die off slowly at sub-optimal levels of water activity (a_w)[76]. Even so, in full strength honey this can take up to 34 days for *Salmonella* at 18–20°C[115], and up to 2 years at 10°C[116]. However, in another study[95] much shorter times were found to be needed (up to 3 days at room temperature). Differences in the composition of the honeys used could well account for these differences in findings: different times were found to be required for different honeys to kill a particular species within the same experiment in two studies[95, 105]. Differences in the composition of the honeys used could also account for differences in conclusions on whether the antibacterial action of honey is bacteriostatic or bactericidal. In several studies[14, 20, 40, 112, 131] only some of the honeys tested had a bactericidal activity at the concentrations used.

It may just be a matter of longer times or higher concentrations being required for bactericidal activity to be seen: many bacteriostatic substances are bactericidal at higher concentrations[109]. Low a_w may greatly influence the microbicidal effect of other factors[76], thus these factors may be of more consequence in honeys at high concentrations.

Although hydrogen peroxide gives bacteriostasis with *S. aureus* at 0.29 mmol/l or lower[8, 23, 127], it has been found that 29 mmol/l hydrogen peroxide is required to kill *E. coli* and *S. aureus* in 1 h[65], and 8.8 mmol/l to achieve a kill rate of 80% in 1 h with seven strains of bacteria[114]. The quantities of hydrogen peroxide that are produced in honey (discussed earlier) are unlikely to accumulate to such levels, but could be high enough in some honeys to be bactericidal over a longer period of exposure, especially with the influence of a low a_w and with potentiation by metal ions and ascorbic acid. The presence of plant-derived bactericidal factors in some honeys, helped by the low a_w may also account for some honeys being bactericidal.

Whether or not honey is bactericidal is of little practical significance however. Some of the antibiotics in common use in medical practice have only a bacteriostatic

action[104]. Complete bacteriostasis, maintained by regular application of honey, would be sufficient to allow the healing process to work successfully. The fairly rapid clearing up of infections that is found to occur under a dressing of honey[11, 15, 32, 48] may be a result of bactericidal action from prolonged exposure, or possibly be a result of the natural defence system being more successful with multiplication of bacterial cells held in check.

Stability of antibacterial activity

The instability of honey inhibine was first recognized in 1937 by Dold *et al.*[27], who found that it was destroyed by heating and by exposure to light. These observations have since been confirmed by numerous other researchers, but there have been differences in the degree of instability reported.

Sensitivity to heat

The initial report (Dold *et al.*[27]) on the loss of antibacterial activity on exposure of honey to heat was of complete loss of inhibition by 17% honey after exposure of 50% honey to 100°C for 5 min, 80°C for 10 min, or 56°C for 30 min. However, this did not mean that antibacterial activity was lost completely: if the unheated honey had been of just high enough activity to inhibit growth when at 17%, not much activity would have to be lost on heating for inhibition no longer to be seen. This also applies to the similar finding of Pothmann[83] that exposure of honey to 100°C for 5 min or 56°C for 1 h caused complete loss of inhibition by 17% honey.

In later reports the researchers used a dilution series for the assay of activity. Although complete loss of inhibition in their studies still did not mean that antibacterial activity was lost completely, its reduction to a level below detectability would generally represent a loss of 80% or more, if not a complete loss. In these reports 'complete loss' was found to result from exposure of honey to: 100°C for 30 min[51]; 100°C for 15 min[96]; 90°C for 8 h[45]; 100°C for 5 min, 90°C for 15 min, 70–80°C for 20–30 min, and 56°C for 60 min[40]; 80°C for 15 min[108]; 80°C for 30 min[52]; 60°C for 15 min[132]; and from use of 'heated honey' (no details given)[36]. An almost complete loss was found on heating honey for 100°C for 10 min[17].

In another report[20] the activity was not lost completely after exposure of honey to 100°C for 15 min, but was reduced to the same level as that of artificial honey, indicating that all activity other than that due to osmolarity had been destroyed. A similar finding was made with honey boiled for 10 min[81]. Others also have found that only part of the antibacterial activity is destroyed by heating honey. Exposure of honey to 100°C for 10 min caused complete loss of activity against seven species of bacteria, but only partial loss of activity against *Bacillus pumilus* and a strain of *Streptomyces*, and no loss of activity against *Bacillus subtilis* and *Sarcina lutea*[61]. In another report[42] about half of the activity against *B. subtilis* was found to be heat-stable. Heating honey at 56°C for 30 min caused a loss of activity that was greater against some species than against others[3]. The presence of both heat-stable and heat-sensitive factors has been reported by others also[8, 16, 17, 24, 52].

The retention of part of the activity reported in instances where honey has been subjected to lesser degrees of heating probably results from there being only partial destruction of the heat-sensitive factor, rather than a heat-stable factor being responsible. The minimum inhibitory concentration of honey was found to increase from 4% to 8% after exposure of honey to 46°C for 8 h, to 12% after exposure to 52°C for 8 h, and to 16% after exposure of honey to 55°C for 8 h[132]. Also reported[108] was complete loss of activity after exposure to more than 65°C for less than 4 h, a heavy but not complete loss after exposure to 56°C for 24 h, but no loss after exposure to 40°C for 96 h. In another report[51] there was complete loss of activity after exposure to 100°C for 30 min, but no loss after exposure to 56°C for 30 min. Also reported[84] was complete loss of activity after exposure to 100°C for 5 min, but only partial loss after exposure to 60°C for 1 h.

The stability of the antibacterial activity in heated honey has been found to depend on the pH, activity being more rapidly lost at low pH[45].

There are some large differences in the findings on the stability of the antibacterial activity of honey at lower temperatures, but generally the conclusion has been that it is stable below 40°C. No decrease in antibacterial activity was seen in 20 honeys held at 40°C for 96 h[31], as in the case mentioned above[108], nor in honey held at 37°C for 24 h[20]. This is to be expected when it is borne in mind that the temperature in the beehive where honey can spend quite a long time is around 34°C. It may not be as stable at this temperature when diluted: the rate of production of hydrogen peroxide drops off with time[127], and the amount of hydrogen peroxide present after 16 h was found to be much lower than that present after the first hour[8]. Others have also reported that honey is less stable when diluted[3, 16, 17, 27, 28, 42]. This could be a consequence of the build-up of gluconic acid, or of damage to the glucose oxidase from free radicals generated from hydrogen peroxide as discussed earlier. The latter suggestion is supported by the finding with the isolated enzyme that addition of a high level of hydrogen peroxide inactivated it after about 30 min[98]. However, it has been reported that 50% honey held at room temperature for 100 h does not lose its antibacterial activity[42, 127].

There are several indications of the antibacterial activity of honey being very stable at room temperature. In one study[121] of a large number of honeys it was noted that 43 of the 85 honeys with high antibacterial activity were 9 months to 1 year old, and some were 2 years old. It was stated that the antibacterial activity could be retained on long storage in the laboratory if the honey was kept away from light and high temperatures. Another study[4] of a large number of honeys also found high activity in old samples of honey, some up to 5 years old, there being no correlation between the activity and the age of the honey. A study of 18 honeys found some of the most active to be 2–3 years old[16, 17]. Storage of honey without deterioration of its antibacterial activity for several months at 20°C[42], also for 2 years at 25–30°C[122], has been reported. Little or no loss of activity was found in honeys stored for 1 year at room temperature in closed containers, but loss of activity was noted in samples that had been opened frequently[66]. On the other hand almost complete loss of activity was found on storage of honeys for 18 months at 4°C in the dark[30, 94]. Also reported[8] has been a loss of 15–16% in 3 months and 24–27% in 6 months storage at 20–25°C.

These differences in findings could be the result of there being differences in the stability of various honeys. Marked differences in the proportions of activity lost

at each temperature tested have been reported by several authors[8, 31, 42, 54, 61]. A 70-fold difference was found between honeys in the half-life of the peroxide-accumulating system of various honeys[129]. This appeared to be related to the floral source of the honey, prompting the suggestion that plant-derived substances influenced the stability of the glucose oxidase. As the half-life of the enzyme isolated from honey is approximately 5 min at 50°C[99], and the half-life of the peroxide-accumulating system was found to range from 2.8 to 6.1 h at 55°C[129], it appears that this influence is a stabilizing one.

The half-life of the peroxide-accumulating system was determined for higher temperatures as well. In addition to their own measurements, White and Subers[129] estimated the half-life of the antibacterial activity in honey from the data published by others on its decline on heating. They found that at 65°C it ranged from 36 s to 4.5 min, and at 70°C, with a larger number of samples, it ranged from a few seconds to 1 hour. Their estimates from others' data were 4.5 h at 62.8°C, and 10 h at 57°C.

Sensitivity to light

It has been known since some of the earliest work on the antibacterial properties of honey that the activity is unstable in light. Dold *et al.* in 1937[27] reported that honey lost its ability to inhibit bacterial growth (tested in a 17% solution) after exposing a thin film of it to sunlight. Others have since confirmed this observation. Exposure of honey in a layer 1–2 mm thick to sunlight for 15 min was found to result in complete loss of non-osmotic activity[130]. When not spread out in a thin layer it has not been found to be so sensitive: almost complete loss of activity after 18 days in direct sunlight[61], gradual disappearance of activity when exposed to direct sunlight but not with diffuse daylight[40], and a significant reduction in activity in honey samples stored for 3–6 months on open shelves (more than twice that lost in the same samples stored in a dark cupboard)[8] have been reported. No loss of activity was found, however, when a thin film of honey was exposed for 1 h to an ultraviolet (UV) lamp (254 nm)[17].

A large loss of activity was found in honey left for 8 months on a window-sill on the sunny side of the building if stored in 1 or 2.5 litre jars made from clear polystyrene, but not if stored in jars made of white or ivory polyethylene with low transmission of light of wavelength below 400 nm[122]. Glass jars coated with a film to absorb UV light were only partially successful in this study in preventing the loss of activity, indicating the necessity to protect from light of wavelengths up to 400 nm. Similar findings were made in another study: honey stored for 5–7 months by the window in the sun lost about half of its activity if kept in UV-absorbing glass jars, but kept all its activity if kept in jars made of dark glass[31]. This protection by absorption of light can occur within the honey itself, as is seen with the greater stability of bulk quantities compared with thin films. Dark-coloured honey was found to be more light-stable than light-coloured honey[8], presumably because it lets less light into the bulk of the honey. However, the sensitivity to light has been observed to depend on the floral source of the honey[35]: in a 500 g jar kept in sunlight, some floral types of honey were found to lose their activity completely in only 48 h[35], and a reduction of up to 67% in the production of hydrogen peroxide

The components responsible for the antibacterial activity of honey

Acidity

The pH of honey is low enough to slow down or prevent the growth of many species of bacteria, but this acidity may be neutralized if honey is diluted with buffering solutions such as body fluids.

Osmolarity

The high sugar content of honey makes the water unavailable for micro-organisms: no bacteria or fungi can grow in fully ripened honey, but the more diluted honey becomes, the more species can grow in it.

Hydrogen peroxide

The glucose oxidase enzyme activated by dilutions of honey generates hydrogen peroxide which generally is the major antibacterial factor in honey. This enzyme is inactivated by heating honey, and by exposure to light in some honeys which contain a sensitizing factor. Some honeys also contain substances which destroy the hydrogen peroxide generated by the enzyme.

Other components

Honeys from some floral sources contain various antibacterial substances, presumably produced by certain species of plants, which in some case can account for a large part of the antibacterial activity of honey.

was found 4–5 cm in from the glass after only 6 h[34]. Difference in floral source could account for the finding in an early study that honey was nearly insensitive to diffuse daylight, it standing for 20 months in the laboratory[84].

Although hydrogen peroxide is degraded by exposure to light, this cannot account for the sensitivity of the antibacterial activity of honey to light as there is so little hydrogen peroxide present in full-strength honey. It has been found that in fact it is the glucose oxidase, that generates the hydrogen peroxide, that is sensitive to light[130]. The marked disagreement seen in observations on the stability of the antibacterial activity of honey to light can be explained by the finding that a photosensitizer is necessary for the photo-oxidation of the enzyme, and that it occurs in different amounts in different honeys[130]. Another variable could be the dependence of the light-sensitivity on the pH, which can vary between 3.2 and 4.5[124] in different honeys. The sensitivity of the glucose oxidase activity to light was found to be minimal at pH 8 but to increase sharply from pH 5 downwards[130]. It is not known whether it is the photosensitizer or the enzyme that is influenced by the pH.

The hydrogen peroxide accumulating system in honey was found to be strongly influenced by diffuse daylight and the light from fluorescent tubes[128], which is more detrimental than the light from incandescent bulbs[34]. Nearly half of the enzyme activity was found to be lost in a 10 g sample of honey left for 2 h on the laboratory bench in a 50 ml beaker[130]. The sensitivity of the photosensitizer/enzyme was found, using various lamps and filters, to be greatest to light in the wavelength band 425–525 nm[130].

There have also been reports of non-peroxide antibacterial factors being light-sensitive[59, 118].

Conclusion

It has been shown that the potency of the antibacterial activity can vary very markedly. The number of variable factors involved makes it impossible to predict with any certainty that a particular honey will have a high antibacterial activity. Because of this, honeys purveyed for therapeutic use should be assayed for their antibacterial activity as a form of quality assurance.

Consideration should also be given to the way that honey is processed if it is intended for sale as an antibacterial product (fig. 6). Honey is often pasteurized, at a temperature of 70–75°C, to destroy yeasts that can spoil a honey with a high water content, or to dissolve sugar crystals that could initiate granulation in a liquid honey. In view of the short half-life of the antibacterial activity at pasteurization temperatures, it is clear that pasteurization of honey is undesirable if the honey is to be used as an antiseptic. It would also be advisable to keep any other warming of the honey during processing to a minimum, and to store it at cool temperatures.

Another consideration regarding processing for marketing liquid honey should be the likely effect on antibacterial activity when honey is filtered to remove pollen and other particles which can initiate granulation, since it has been found that glucose oxidase is adsorbed on to the asbestos filter pads in Seitz filtration. It remains to be determined whether the enzyme is removed by absorption on to filtration aids used in the clarifying of liquid honeys, but some of the filtration aids used are very effective in removing proteins from other products.

As a further precaution against possible loss of antibacterial activity, honeys with high activity should not be blended with honey of low activity: a honey with low activity could well have components present that destroy antibacterial activity.

Loss of antibacterial activity on exposure to light is another important consideration. Because there is little certainty about which floral sources give honeys that are sensitive to light, and because some can be very sensitive, it is important that honey intended for therapeutic use be protected from light to prevent possible reduction of its antibacterial activity. For retail sale it could well be packaged in brown glass containers like other medical products.

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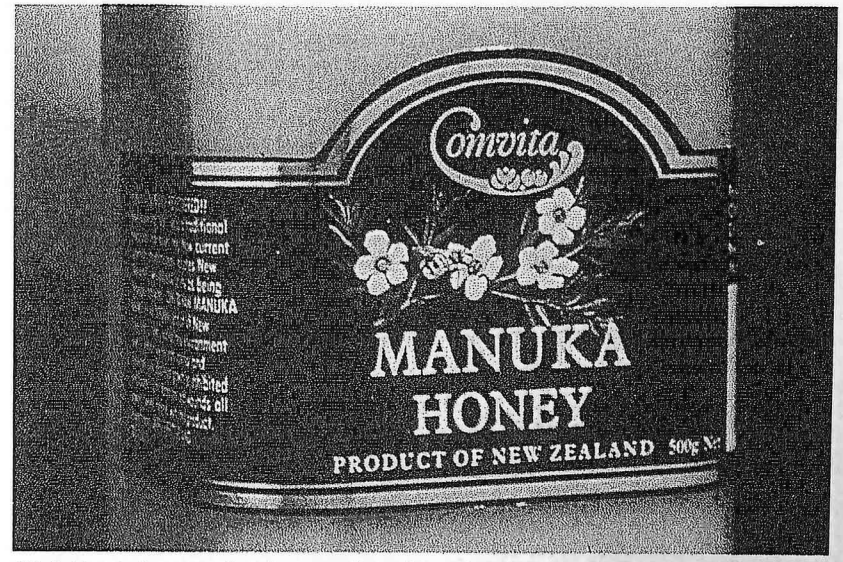

FIG. 6. Manuka honey marketed as an antibacterial product.

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