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 pres-

ence 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.

## Acknowledgements

I wish to thank the reference and interloan librarians at the University of Waikato and the librarians at IBRA in Cardiff for their help in obtaining the articles reviewed in this paper. Also, Lorenzo Alibardi, Niaz Al Somai, Roman Antoszewski, David Foreman, Karin Klages, Anna Kurman, Ken McNeil and Anika Schroeder for their help in translating articles written in languages foreign to me. I also wish to thank my wife for assistance with the references, critical reading of the manuscript, and her forbearance whilst I have been working on this undertaking. Photographs used in figures 2 and 3 were taken by the Photographic Unit of the University of Waikato.

## 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.]