

HONEY

A COMPREHENSIVE SURVEY

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CHAPTER 5

COMPOSITION OF HONEY

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5.1 INTRODUCTION

Man is twice indebted to the honeybee for searching earth's fields and forests for their treasured sweetness—for the honey itself, with its variety of appeals to our senses, and for the increased crop yields resulting from inadvertent pollination of the flowers the foraging bee visits. Literally vital to the honeybee colony, honey is to us simply a desirable and delectable variety in our diet. It is a twice-stolen sweet (and stolen sweets are said to be the best)—taken by the bee from the flower (and from the owner of the plot upon which it grows)—and then stolen from the bees by their keepers.

Honey as it is found in the hive is a truly remarkable material. Prepared by the bees from the natural sugar solutions we know as nectar, it is changed from an easily spoiled, thin, sweet liquid to a stable high-density, high-energy food. By inverting the sucrose in the nectar, the bee increases the attainable density of the final product, and thus raises the efficiency of the process in terms of caloric density. At the same time, the resistance of the stored product to spoilage by micro-organisms is greatly increased, because of the higher osmotic pressure attained. A price is paid, however, in terms of the increased tendency of the honey to absorb atmospheric moisture, with consequent liability to fermentation when yeast levels are sufficiently high.

5.2 AVERAGE COMPOSITION OF HONEY*

The composition of a particular honey sample will depend upon two general factors: most important, the composition of the nectar(s) whence

* Discussion in this chapter has been restricted to honey from nectar (floral and extrafloral); honeydew has been excluded because of space limitations, but much useful information on honeydew and honeydew honey is included in Chapter 2.

it originates; of less importance, certain external factors. As detailed in Chapter 2, nectars from different plants vary widely in the identity and concentrations of their constituent sugars; in fact, honey types are ascribed to plant sources by flavour or gross composition alone. Weather or climatic conditions and beekeeper practices in removing and extracting honey may affect composition to a minor extent.

Two considerations make the concept of an 'average composition' of honey somewhat uncertain—the degree to which the analytical methods used to establish the individual values actually reflect the true composition of the sample, and secondly, the considerable variation in composition encountered among honeys because they represent different floral types. This latter factor may be compounded by general differences from one area or country to another in nectar sources available. Hence, the average composition given in Table 5.2/1 of honey from the United States would probably differ from similar values for honeys from regions of differing climate, topography or agricultural pattern.

Table 5.2/1
Average composition of U.S. honey and ranges of values
(White, Riethof, Subers & Kushnir, 1962)*

<i>Component</i>	<i>Average</i>	<i>Standard Deviation</i>	<i>Range</i>
Moisture	17.2	1.5	13.4 - 22.9
Laevulose	38.2	2.1	27.2 - 44.3
Dextrose	31.3	3.0	22.0 - 40.7
Sucrose	1.3	0.9	0.2 - 7.6
'Maltose'†	7.3	2.1	2.7 - 16.0
Higher sugars	1.5	1.0	0.1 - 8.5
Free acid (as gluconic)	0.43	0.16	0.13 - 0.92
Lactone (as gluconolactone)	0.14	0.07	0.0 - 0.37
Total acid (as gluconic)	0.57	0.20	0.17 - 1.17
Ash	0.169	0.15	0.020 - 1.028
Nitrogen	0.041	0.026	0.000 - 0.133
pH	3.91		3.42 - 6.10
Diastase value	20.8	9.8	2.1 - 61.2

* Values as percentage of honey, except the last two entries

† Reducing disaccharides calculated as maltose

Table 5.2/1 shows the average values, with standard deviation and range, obtained in the analysis of 490 samples of U.S. honey representing 3 single floral types and 93 blends of 'known' composition (White *et al.*,

1962). The extreme values found among the samples are shown in the column marked 'range'. A better idea of variability may be obtained by use of the standard deviation (*S*) in the table. With normal distribution of values, about two-thirds of the samples will fall within $\pm 1S$ of the average value shown; 95% will be found in the interval from 2*S* less than the average to the average plus 2*S*. Thus, although in this study the extreme values for laevulose content were 27.2 and 44.3%, 95% of the samples were within the range 34.0 to 42.4% laevulose.

Table 5.2/2 shows average composition of honey samples of various floral types (White *et al.*, 1962). This table will provide an idea of the variability among different kinds of honey. The high dextrose content of fast-granulating cotton honey contrasts with the low values for non-granulating tupelo honey.

The data in Tables 5.2/1 and 5.2/2 were obtained by the most advanced analytical methods, with adequate care that dextrose and laevulose values are accurate and not influenced by other more complex reducing sugars in the sample (White *et al.*, 1962). Many published data on sugar composition of honey (particularly older reports) should be viewed with caution. The nature of the sugar complex of honey is such that results can be misleading unless either a suitable pre-analysis separation of sugar classes is made, or highly specific analytical methods are used. Pourtallier (1962) recognized the inadequacy of the non-specific methods previously used and proposed a modified procedure. It is not clear whether all sources of potential error have been taken into account in his methods. He has examined the application of thin-layer chromatography (1964) and gas-liquid chromatography (1967) to carbohydrate analysis of honey. Echigo (1970) has also reported the use of gas-liquid chromatography. Results on a single sample differed considerably from those by ion-exchange chromatography. Siddiqui (1970) referred to analysis of 95 Canadian samples by a paper-chromatographic procedure but gives only average values and ranges for fructose, glucose, and oligosaccharides, with no information on sample identity or origin. His claim that results by this procedure are 'equally as good as, if not better than, those afforded by other available methods' is not supported by any data and seems highly unlikely.

In Table 5.2/3 are shown representative analytical values for honeys from various areas over the world. The fragmentary nature of the data is apparent. One may generalize that dextrose content is somewhat lower than laevulose content, that moisture content is usually between 15 and 21%, sucrose about 1-3%, and ash between 0.09 and 0.33%. Comparison of data shown in different entries must be made with knowledge of the analytical methods used and of their shortcomings; among the older methods of honey analysis, variability due to methods

can exceed variability due to difference in samples (White, Ricciuti & Maher, 1952). Carbohydrate values obtained by White & Maher's (1954) selective adsorption method are those of Austin (1958), Anderson & Perold (1964), Gryuner & Ariukina (1970), and da Silva Ferreira (1970).

Other factors influencing the composition of honey are the period and conditions of storage. Early data on this subject were reviewed, and storage changes in carbohydrates, acidity, and diastase were quantified, by White *et al.* (1961, 1962). Gonnet (1965) has also examined storage changes in honey, verifying in general the findings of White *et al.*

5.3 CARBOHYDRATES

Table 5.2/1 shows that by far the largest portion of the dry matter in honey consists of sugars. In the main, the sugars are responsible for much of the physical nature of honey, its viscosity, hygroscopicity, granulation properties, energy values, and so on.

5.31 Monosaccharides and disaccharides

In nearly all honey types, laevulose (fructose) predominates; a few honeys—such as rape (*Brassica napus*), dandelion (*Taraxacum officinale*), blue curls (*Trichostema lanceolatum*)—appear to contain more dextrose (glucose) than laevulose. These two sugars together account for 85–95% of honey carbohydrates. More complex sugars (oligosaccharides) made up of two or more molecules of glucose and fructose constitute the remainder, except for a trace of polysaccharide. Recent research in the United States, Japan, and Canada has shown that at least eleven disaccharides are present in honey in addition to sucrose. Most of these sugars are quite rare, and their recovery from honey was the first isolation from natural material. White & Hoban (1959) separated the sugars and, by use of the infra-red spectra of the free sugar and of its acetate, identified maltose, isomaltose, nigerose, turanose, and maltulose. Watanabe & Aso (1960) crystallized the acetates to identify maltose, isomaltose, nigerose, and kojibiose, and tentatively identified leucrose. Further confirmation of the occurrence of all of these sugars (except leucrose) was reported by Siddiqui & Furgala (1967). They attained a more rigorous identification by isolation and characterization of crystalline sugars (sucrose, turanose) or crystalline acetates (isomaltose, kojibiose, maltose, nigerose). In addition, neotrehalose, gentiobiose, and laminaribiose were identified (as acetates). Tentative identification of isomaltulose and maltulose was also reported.

5.32 Tri- and higher saccharides

In their definitive study of honey carbohydrates, Siddiqui & Furgala (1968a) have reported the isolation and identification of eleven oligo-

Table 5.2/3
Composition of honeys from different countries¹

Country	No. samples	Water	Total reducing sugars	Dextrose	Laevulose	Sucrose	Maltose	Dextrin	Ash	Nitrogen	Free acidity ²
EUROPE											
Britain	13	18.9 15.9-23.4	34.6 30.0-36.8	39.8 36.1-44.4							0.68 0.42-1.24
Bulgaria	190	21.3	71.7		1.6				0.15		
Italy (Sicily)			70.9-77.2		1.6-2.1						
Netherlands	41			30.5	41.5		4.6	2.3			
				20.4-39.5	38.1-53.9		1.8-7.5	0.9-4.0			
Portugal	10	18.1		32.2	36.2	0.96	6.68	1.90			0.64
		16.7-19.8		27.2-34.6	31.0-38.2	0.70-1.15	5.13-8.92	1.04-3.56			0.45-0.82
Rumania	257	16.5	75.6	34.0	38.4	3.1		3.8	0.17		
Spain	23	17.3		28.4	36.5	0.9	8.2	1.1	0.18	0.050	0.45
		14.3-21.6		21.2-36.8	31.9-41.7	0.4-2.3	2.4-14.9	0.4-3.5	0.040-0.75	0.014-0.089	0.23-1.20
U.S.S.R. (1970)	10	19.3		32.5	34.9	1.43	4.32	1.26	0.20	0.066	
		17.7-23.6		28.7-36.7	31.5-37.5	0.0-4.75	2.26-6.84	0.10-2.57	0.035-0.34	0.018-0.152	
U.S.S.R. (1963)	217	18.6	73.8	35.9	37.4	2.11			0.16		0.57
		15-23	62.7-84.4	26.4-44.4	21.7-49.7	0-10.3			0.01-0.59		0.09-1.20
Yugoslavia	43	17.7	73.0	35.2	37.3	1.91			0.25		0.38
		13.8-20.8	65.5-79.1	26.1-43.2	30.1-44.9	0.15-4.70			0.07-0.70		0.04-0.85
ASIA											
India (Mahabaleshwar) ³	3 ⁴	17.1		35.1	41.2	2.74		1.5	0.13	0.065	0.23
India ³	12	19.2		35.7	39.3	0.60			0.10		0.45
		16.2-22.1		34.2-39.2	36.8-40.5	0.3-1.0			0.03-0.46		0.25-1.25

Country	No. samples	Water	Total reducing sugars					Ash	Nitrogen	Free acidity ²
			Water	Dextrose	Laeculose	Sucrose	Maltose			
Japan (Aso <i>et al.</i> , 1960)	15	20.5 15.8-26.2	69.2 60.5-76.1	32.6 22.2-38.6	36.0 36.0-48.5	2.83 1.0-5.8				
Japan (Arai <i>et al.</i> , 1960)	30	20.4 13.8-25.4	29.8 20.4-38.7	40.8 30.4-46.0			0.11 0.03-0.46			
Pakistan ³	15	14.3-18.6	39.0-53.8	27.7-34.2	1.9-2.75		0.11-0.32	0.30-0.68		
AFRICA										
Angola	4	19.3 13.4-25.0	33.9 32.1-35.0	36.4 34.2-38.5	0.86 0.15-1.50	6.48 4.56-7.79	0.98 0.52-1.32	0.53 0.22-1.03		
Mozambique	4	18.7 17.4-21.8	32.0 28.6-35.3	36.2 33.6-38.2	1.10 0.65-1.66	6.51 5.10-7.58	1.76 0.82-3.16	0.81 0.32-1.71		
Portuguese Guinea	4	19.4 16.2-20.4	31.2 28.5-34.4	38.3 35.6-40.8	1.06 0.45-2.20	6.36 3.55-8.80	0.80 0.16-1.48	0.98 0.61-1.51		
São Tomé + Príncipe ⁵	4	22.9 19.0-24.6	31.0 28.5-32.4	34.8 33.9-36.2	0.61 0.10-1.00	5.97 5.13-7.34	1.19 0.72-1.36	0.92 0.67-1.40		
South Africa	66	16.2 13.9-18.8	31.5 22.3-39.4	35.5 22.3-40.1	0.54 0.0-6.24	5.4 2.1-10.0	0.50 0.2-3.3	0.33 0.03-0.94	0.043 0.018-0.13	
AMERICA										
Argentina (1945)	58		34.3 29.4-37.2	40.9 37.7-44.9						
Argentina (1930)	16	15.4	74.0		0.72			0.093	0.17	
Canada	40	17.5 ⁶	33.8 30.8-37.4	38.8 35.4-40.7	1.2 0.02-3.4	6.1 4.0-11.1	1.3 0.5-2.8			

Chile	10	16.0	79.2	32.7	44.1 ⁷	2.5			
		14.7-17.9	73.7-80.7	30.1-35.0	39.1-47.0	0.9-4.4			
Uruguay	32	17.3	67.3		4.9		0.15	0.054	
								0.53	
OCEANIA									
Australia	99	15.6	73.5	30.2	43.3 ⁸	2.5	0.17		0.30
New Zealand	21	17.5		36.2	40.0	2.8	0.18	0.040	0.32
		16.2-19.1		32.4-40.2	38.4-42.0	1.5-4.8	0.04-0.39	0.023-0.077	0.13-0.59

Britain—Marshall & Norman (1938)
 Bulgaria—Zoneff (1927)
 Italy (Sicily)—Sorges (1933)
 Netherlands—van Voorst (1941)
 Portugal—da Silva Ferreira (1970)
 Rumania—Pelimon & Baculinschi (1955)
 Spain—Pérez & Rodríguez (1970)
 U.S.S.R.—Gryuner & Arinkina (1970)
 U.S.S.R.—Chugakov (1963)
 Yugoslavia—Černagić *et al.* (1964)
 India (Mahabateshwar)—Phadke (1962)
 India—Giri (1938)
 Japan—Aso, Watanabe & Yamao (1960)
 Japan—Arai *et al.* (1960)

Pakistan—Latif *et al.* (1956)
 Angola—da Silva Ferreira (1970)
 Mozambique—da Silva Ferreira (1970)
 Portuguese Guinea—da Silva Ferreira (1970)
 São Tomé + Príncipe—da Silva Ferreira (1970)
 South Africa—Anderson & Perold (1964)
 Argentina—Ugarte & Karman (1945)
 Argentina—Ceriotti & Delpiro (1930)
 Canada—Austin (1958)
 Chile—Masson & Schmidt-Hobbel (1963)
 Uruguay—Bertullo & Lembo (1943)
 Australia—Chandler *et al.* (1974)
 New Zealand—Thomson (1936)

¹ Single values are averages, others are ranges. All values are given as percentages.
² Calculated as gluconic acid
³ From *Apis cerana indica*; see 17.22
⁴ These three types represent 80% of the area's production
⁵ Islands off West African coast, near Equator
⁶ All Canadian analyses are calculated to 17.5% moisture
⁷ Includes reducing disaccharides, *see text*
⁸ Calculated as difference between total reducing sugars and glucose

saccharides, largely by rigorous procedures. Earlier, Goldschmidt & Burkert (1955*a*) had inferred the presence of melezitose, erlose, kestose, raffinose, and dextrantriose, using paper-chromatographic behaviour and colour reactions for identification. Such procedures alone are not acceptable for absolute identification of sugars: some other physical properties of the sugar or a derivative are needed also. Siddiqui points this out in discussing the alleged presence of raffinose reported by five investigators, which he could not confirm. The carbohydrates reported by Siddiqui & Furgala are: 1-kestose, melezitose, 6^o- α -glucosylsucrose, panose, *iso*-maltotriose, erlose, 3 α -*isomaltosyl*glucose, *isopanose*, maltotriose, *iso*-maltotetraose, *isomaltopentaose* and two not identified. One of the latter was later characterized as α -D-glycopyranosyl-(1 \rightarrow 4)-O-[α -D-glucopyranosyl-(1 \rightarrow 2)-D-glucose] and given the trivial name centose (Siddiqui & Furgala, 1968*b*); it was estimated to constitute 0.0018% of the honey sample. Siddiqui & Furgala (1968*a*) have speculated on the origin of the various oligosaccharides they found in honey; they concluded that the enzymatic production of these materials cannot be explained from our present knowledge of plant, bee, and other insect enzymes.

There are many reports, especially in the earlier literature, of honey 'dextrin'. This has been considered an ill-defined, higher molecular-weight carbohydrate material found in honey, and usually estimated by precipitation with alcohol. This property provided the misleading name, since dextrans (partial hydrolysis products of starch) have similar properties. Rychlik & Fedorowska (1962*a*) have proposed a direct method for dextrin determination, in which sugars are first removed by a preliminary fermentation by baker's yeast. Most of the disaccharides listed above are unfermentable, so they would be measured as 'dextrin' by this procedure—which does not measure 'dextrin', whatever that may be, but rather unfermentable sugars. Many years ago, Barschall (1908) noted that the apparent molecular weight of the dextrin of conifer honey (honeydew honey) corresponded to a trisaccharide; Fellenberg & Ruffly (1933) reported that the dextrans of floral honey were also in this molecular-weight range. With the advent of paper chromatography, it was easily demonstrated that the higher sugars of honey differ from starch dextrans in containing fructose. In fact, the addition of starch syrup to honey can be demonstrated by the presence of a series of glucose saccharides (White, 1959*a*). Helvey (1953), investigating the colloids of honey, reported three components, two of protein nature and one a polysaccharide of molecular weight about 9 000. Siddiqui (1965) isolated a polysaccharide representing 0.002% of honey and showed it to be a highly branched arabogalactomannan (molar proportion 1.0:2.04:4.04) of molecular weight less than 10 000. General structural features were outlined.

5.33 Changes in carbohydrates with time

In modern honey analysis, all the reducing disaccharides are measured together and reported (Tables 5.2/1, 5.2/2) as 'maltose', the best-known member. The amount of such sugars in honey appears to be a function of the period since the honey was ripened, and the storage conditions since that time. Täufel & Müller (1953) suggested that, since they found no minor sugars in pollen, the source of those in honey might be by secondary conversion (enzymes in acid solution) in honey. Later (Täufel & Müller, 1957), however, using both classical methods of quantitative analysis and paper chromatography, they concluded that significant changes in sugar composition of honey do not occur in storage. It has since been shown, using modern analytical methods and statistical treatment (White, Riethof & Kushnir, 1961), that storing honey for two years at 'room temperature' brings about a 69% increase in 'maltose' at the expense of dextrose plus laevulose, which decreased to 86% of its initial value in this period. Earlier, Austin (1958) had proposed that the 'maltose' content of honey would depend to some extent upon apiary management, and on storage temperature and moisture content of honey. The report of Chudakov (1963) that glucose content increase in 8-months' storage (statistically evaluated) must be discounted because of the inadequate analytical procedures used, which would not distinguish between aldose monosaccharides and oligosaccharides. Kalimi & Sohnie (1964*b*) confirmed the increase of higher sugars and decrease of monosaccharides during honey storage at 28–30°C (83–86°F) for 6–12 months.

The increase in the oligosaccharides in honey is caused by two mechanisms: enzyme activity and acid reversion. The sucrose-splitting enzyme present in honey is in reality a transglucosylase (White & Maher, 1953*a*, White & Kushnir, 1967*b*), which synthesizes several of these sugars when it splits sucrose.

Although many of these disaccharides can be split by honey invertase to their constituent monosaccharides under proper conditions, the low water concentration of honey appears to favour a moderate accumulation of the disaccharides, since the free monosaccharides are formed by transfer of the glucosyl residue to water. When solutions of monosaccharides (dextrose, laevulose) remain in concentrated solution in the presence of acids, disaccharides and other carbohydrates are formed (Pigman & Goepf, 1948, page 434). The extent to which these two processes continue is probably limited. An analysis of a honey sample 36 years old (White, Riethof & Kushnir, 1961) showed 16.4% of 'maltose'. The failure of Täufel and Müller to detect these changes was probably due to inadequate analytical procedures.

5.34 Effects of complexity on analysis for sugars

The accurate analysis of honey for individual sugars, even dextrose and laevulose alone, is not simple. The presence of the minor sugars described above introduces errors unless they are removed before analysis, or specific methods of analysis are used. This means that caution must also be exercised when literature reports of the sugar composition of honey, particularly laevulose and dextrose values, are compared. For example, Masson & Schmidt-Hebbel (1963) reported an average dextrose content for 10 samples of Chilean honey as 32.7% (a reasonable value), but the laevulose content was said to be 44.1%. This is higher than the average reported (White *et al.*, 1962) even for tupelo honey, the U.S. honey of highest laevulose content. Although the specific glucose oxidase method was used for dextrose in the Chilean honey (and hence a reasonably good dextrose value obtained), laevulose was estimated simply by subtracting this value from the total amount of reducing sugars, which includes the reducing disaccharides. Such a 'laevulose value' would be considerably higher than the actual laevulose content. The laevulose value should be corrected by subtracting the contribution of the reducing disaccharides, which were not measured; estimating this to be 5-7%, the laevulose value would become 37-39% which appears reasonable.

Another pitfall in honey analysis has been described by White, Ricciuti & Maher (1952): determination of dextrose by a non-specific method for aldose sugar determination, and subsequent estimation of laevulose as the difference between total reducing sugars and the erroneously high 'dextrose' value. The presence of reducing disaccharide aldoses, as listed above, gives too high an apparent dextrose value, and too low an apparent laevulose value. An example of this error is seen in the analysis of 10 Japanese honeys (Watanabe, Motomura & Aso, 1961). Averages of 36.95% dextrose and 36.08% laevulose were reported, which can be compared with other values for Japanese honey in Table 5.2/3.

Further analytical data are needed on the true laevulose and dextrose contents of honey types found elsewhere in the world than in the U.S. and Canada, using suitable analytical methods with adequate controls. Chandler (1974) does not report true laevulose in Australian honeys.

5.4 THE ACIDS OF HONEY

The flavour of honey results from the blending of many 'notes', not the least being a slight tartness or acidity. Not only does this honey characteristic add its note to honey flavour; the level of acidity of honey contributes to its stability towards micro-organisms. In fact, it was early thought that the last act of the bees in ripening honey was to add formic acid to preserve the honey (König, cited by Browne, 1908). As we shall

see later, the bees in effect do increase the acidity of honey during ripening; this perhaps achieves the same end, but not in so direct a manner.

The acidity of honey can be examined in two ways. The kinds of acids present and their relative or total amounts can be considered; or the effect of the acids and other materials which affect acidity, such as minerals, can be expressed in terms of the concentration in the honey of hydrogen ions, which all the acids have in common. A brief review of both aspects is given below.

5.41 Identity of the acids

The complexity of honey extends to the number of acids present. Perhaps largely because newer methods have extended the range of possible investigations, much of our knowledge of the honey acids has been obtained within the past twenty years.

Formic acid, once thought to be 'the' acid of honey, was early recognized (Farnsteiner, 1908, Heiduschka & Kaufmann, 1911, Merl, 1914) to be but a minor component. The following acids have been identified in honey by unequivocal procedures:

acetic	(Stinson <i>et al.</i> , 1960)
butyric	(Stinson <i>et al.</i> , 1960)
citric	(Nelson & Mottern, 1931; Goldschmidt & Burkert, 1955b, Stinson <i>et al.</i> , 1960)
formic	(Vogel, 1882, cited by Farnsteiner, 1908)
gluconic	(Stinson <i>et al.</i> , 1960)
lactic	(Stinson <i>et al.</i> , 1960)
maleic	(Goldschmidt & Burkert, 1955b)
malic	(Hilger, 1904, Nelson & Mottern, 1931, Goldschmidt & Burkert, 1955b, Stinson <i>et al.</i> , 1960)
oxalic	(von Philipsborn, 1952)
pyroglutamic	(Stinson <i>et al.</i> , 1960)
succinic	(Nelson & Mottern, 1931, Stinson <i>et al.</i> , 1960)

The following acids have been identified in honey without rigorous proof of their identity, and it is considered that they are probably present:

glycollic	(Maeda <i>et al.</i> , 1962)
α -ketoglutaric	(Maeda <i>et al.</i> , 1962)
pyruvic	(Maeda <i>et al.</i> , 1962)
tartaric	(Heiduschka & Kaufmann, 1913; Vavruch, 1952)
2- or 3-phosphoglyceric acid	(Subers <i>et al.</i> , 1966)

α - or β -glycerophosphate (Subers *et al.*, 1966)
glucose-6-phosphate (Subers *et al.*, 1966)

Many of the acids listed in the first group have been reported by other investigators besides those cited, but without rigorous proof of identity.

It is now known (Stinson, Subers, Petty & White, 1960; Maeda *et al.*, 1962) that gluconic acid is present in honey in considerable excess over all other acids; it is produced by the action of an enzyme in honey upon the dextrose in it. The various amino acids in honey are dealt with in Section 5.6. Except for gluconic acid, the sources of the various honey acids are not known. Many of the acids are intermediates in the Krebs cycle of biological oxidation and are of widespread occurrence; they may be present already in the nectar.

The identification of gluconic acid in honey provides an explanation of a difficulty long encountered by analysts seeking to measure the total amount of the various acids in honey. This is done by titration with alkali, and an indistinct or fading endpoint is often encountered, which leads to uncertainty or error in the measurement. Gluconic acid exists in solution in equilibrium with its lactone, or internal ester, which does not have an acid function. The proportion present in each form is governed by several factors. The titration of the total acidity of honey can easily be done by a modified procedure (White *et al.*, 1958). Values in Tables 5.2/1 and 5.2/2 for total acidity were determined by this method, as were total acid values reported by da Silva Ferreira (1970) for Portuguese and African honeys.

Since inorganic ions such as phosphate, chloride, and sulphate are known in honey, we may also consider the corresponding acids to be honey constituents.

5.42 Active acidity

All of these acids have in common the dissociation in aqueous solution to provide protons or hydrogen ions. It is to these that much of the 'sourness' and other characteristics of acids are ascribed. A measure of the total concentration of hydrogen ions provides information on the strength of acidity and allows comparisons between materials. This is expressed in the logarithmic *pH* scale, in which *pH* 1 (0.1-molar hydrogen ion concentration) is about the acidity of a dilute solution of an acid like hydrochloric; *pH* 7 represents neutrality. On this scale values for honey fall in the range 3.2 to about 4.5, averaging about 3.9. This value is affected somewhat by the amounts of the various acids present, but mostly by the mineral content—calcium, sodium, potassium, and other ash constituents (Section 5.5). Honeys rich in ash generally show high *pH* values.

Chudakov (1964a, 1964b) has examined the buffer mechanism of the establishment of pH in honey.

5.5 MINERALS

The scientific literature on honey ash falls into three subject categories—amounts of total ash, amounts of the principal constituents, and the identities of minor metallic constituents, which often appear in extremely minute amounts.

Reference to Table 5.2/1 shows that the average ash of U.S. honey is 0.17%, with a range 0.02–1.03%. Table 5.2/3 shows a similar average for other honeys. It is of interest that many years ago in Germany, honeys containing less than 0.1% ash were viewed with suspicion (Schwarz, 1908, Utz, 1908a). A survey of U.S. honeys (White *et al.*, 1962) showed 193 of 492 authentic samples had less than this amount. On the other hand, although the U.S. (advisory) standard permits a maximum of 0.25% ash, 103 of the samples exceeded this.

Tables 5.5/1, 5.5/2, and 5.5/3 summarize the literature on the mineral composition of honey. The data of Table 5.5/1 resulted from the research of Schuette and his colleagues at the University of Wisconsin on United States honeys. Table 5.5/2 gives a summary of data available on honeys from elsewhere. These data were recalculated as far as possible to be comparable to those in Table 5.5/1. Rather than present data on both bases (which is not available for all honeys listed), the results are shown as the percentage of ash for several honeys where the ash content of the honey is not stated in the report, and in parts per million for others, the ash content being given where available. A summary of qualitative (spectrographic) assays of honey minerals appears in Table 5.5/3. Elements listed in Table 5.5/2 were usually reported, but are not included in Table 5.5/3.

5.6 PROTEINS AND AMINO ACIDS

Although it has been known for many years that honey contains protein materials, little is known of their characteristics. The occurrence of protein in honey was used in two ways to demonstrate adulteration of commercial honey. Lund (1909) precipitated proteinaceous material with a tannin solution and attempted to set limits for the volume of the precipitate for genuine honeys. This was later modified (Lund, 1910) using phosphotungstic acid (Voerman & Bakker, 1911) or alcohol (Laxa, 1923). Another means of using honey proteins for this purpose developed from the work of Langer (1903), who demonstrated that serum from an animal immunized with buckwheat honey protein material gave a

Table 5.5/x
Mineral elements of honey*

Mineral element	Honey colour	No. samples	As percentage of ash		As parts per million of honey	
			Range	Average	Range	Average
Potassium (K)	light	13	23.0	35.30	100-588	205
	dark	18	2.0-61.6	33.00	115-4733	1 676
Sodium (Na)	light	13	0.96- 9.26	3.59	6-35	18
	dark	18	0.20-11.20	4.68	9-400	76
Calcium (Ca)	light	14	3.54-13.00	8.77	23-68	49
	dark	21	0.46- 7.30	3.57	5-266	51
Calcium as lime (CaO)	light	14	4.95-18.19	12.27	32-95	69
	dark	21	0.64-10.21	5.00	7-372	71
Magnesium (Mg)	light	14	1.00- 9.24	3.42	11-56	19
	dark	21	0.66-11.47	2.77	7-126	35
Iron (Fe)	light	10	---	---	1.20-4.80	2.40
	dark	6	---	---	0.70-33.50	9.40
Copper (Cu)	light	10	---	---	0.14-0.70	0.29
	dark	6	---	---	0.35-1.04	0.56
Manganese (Mn)	light	10	---	---	0.17-0.44	0.30
	dark	10	---	---	0.46-9.53	4.09
Chlorine (Cl)	light	10	4.52-13.21	10.20	23-75	52
	dark	13	2.26-14.46	9.67	48-201	113
Phosphorus (P)	light	14	1.03- 9.55	6.37	23-50	35
	dark	21	0.84- 6.67	3.67	27-58	47
Sulphur (S)	light	10	5.77-16.24	11.49	36-108	58
	dark	13	2.67-14.36	7.98	56-126	100
Silica (SiO ₂)	light	10	0.58- 2.23	1.60	7-12	9
	dark	10	0.17- 1.79	1.00	5-28	14
Silica, crude	light	14	1.60- 7.07	3.86	14-36	22
	dark	21	1.03- 5.82	2.87	13-72	36

* Schuette *et al.* (1932, 1937, 1938, 1939)

Table 5-5/2
Inorganic constituents of honey

Country honey type	U.S.S.R.		Yugo- slavia ¹ eucalyptus ⁴	Australia eucalyptus ⁴	Sweden ⁵ clinet	Netherlands ⁶	Austria ⁸	Italy ¹²	S. Africa floral	Hungary ¹³ acacia	Australia eucalyptus clover	India ¹⁷
	Germany raspberry- freesia ²	U.S.S.R. buck- wheat ²										
No. samples	—	1	43	1 each of 4 STP	2	3	4	39	17	12	1	1
		Percentage of ash	Parts per million parts honey									
Total ash			2 500 700-7 000	1 830-5 830	890-1 952	4 554-4 902		2 010 370-7 800	270-9 380	790	1 940	1 130
Potassium	25.4-42.2	44.7		578-1 785	318-826	2 090-2 380	502-2 130		141-2 945		1 032	367
Sodium	4.1-7.4	2.3		37-200	36-65	85-121			34-866		17	136
Calcium	1.5-5.7	2.6		107-214	41-56	80-129	128-268		36-164	178	162	76
Magnesium	0.9-1.3	1.5		24-163	7.8-24	32-38	22-93			17	79.5	24
Iron		2.0	8.3 3.5-20.5		1.4-4.9	2.8-4.9		7.7 1.1-20.7	2.65-8.42	2.8	26	6.3
Copper		0.01	8.7 4.5-18.0						0.25-0.83	0.29	0.5	0.6
Manganese		2.1	5.7 3.0-9.2						0.06-0.15	0.30	7.7	0.5
Chlorine		4.1		170-820	180-190	120-210	81-267					

Table 5.5/3
Trace elements in honey*

	<i>Chistov & Silitskaya</i> (1952)	<i>Makarochkin & Yudenich</i> (1960)	<i>Santos Ruiz et al.</i> (1949)	<i>Mladenov</i> (1968)†
Chromium	+			+
Lithium	+		+	
Nickel	+	+	+	+
Lead	+	+	+	
Tin	+	+	+	+
Zinc	+			
Osmium	+			
Beryllium		+		+
Vanadium		+		+
Zirconium		+		+
Silver		+		+
Barium			+	+
Gallium		+		
Bismuth				+
Gold				+
Germanium				+
Strontium				+

* Elements listed in Table 5.5/1 not included

† Quantitative data are in original article

copious precipitate with buckwheat honey. Later development was by Langer (1909), Galli-Valerio & Bornand (1910), and Thöni (1911). Amounts of precipitate, or extent of dilutions showing precipitation, were associated with the degree of admixture of artificial honey. Thöni (1912) used an anti-bee serum for the same purpose. These tests were extensively used in the early part of this century, and Thöni's method was included in the Swiss *Lebensmittelbuch* (Kreis, 1915). By using antiserum techniques, Langer (1915) was able to show the error of Küstenmacher's (1911) contention that the invertase of honey arose from the pollen rather than the bee.

The older work on honey proteins included their assignment to the various classic solubility groups. Moreau (1911a) noted that the common tests for proteins (Millons test, the xanthoproteic reaction, heat coagulation) showed their presence in honey; he reported albumins, globulins, and proteoses. Stitz (1930) found peptones, albumins, and some globulin,

but not protamines, alcohol-soluble albumin, histones, albumoses, and albuminoids. Nucleoprotein was present.

In their work on the colloidal materials of honey, Paine, Gertler & Lothrop (1934) noted that these materials, obtained by ultrafiltration of honey, were more than half protein. The isoelectric point of the colloidal material in the honeys examined was close to 4.3. They also noted that the ultrafiltered honey still retained over half of its original nitrogen content, implying that the common practice of estimating protein in honey by multiplying the nitrogen content by 6.25 is misleading.

Helvey (1953) studied the colloidal materials of buckwheat (*Fagopyrum esculentum*) honey by several physical methods. Ultracentrifugation and moving-boundary electrophoresis both indicated three components, two proteins of approximate molecular weight 146 000 and 73 000, and an (assumed) polysaccharide with a weight of about 9 000.

Crude protein preparations from several types of floral honey and from stores of sugar-fed bees were examined by White & Kushnir (1967*a*), using gel filtration, starch-gel electrophoresis, and ion-exchange chromatography. The number of constituents in the protein fraction varied among the 11 floral types examined from 4 to at least 7; 4 of these components appeared to originate in the bees. Molecular weights of 2 of the bee-imparted proteins were indicated by gel filtration to be about 40 000 and 240 000; other protein materials, of plant origin, showed values of about 98 000 and above 400 000. The portion of nitrogen-containing material passing through the dialysis membrane varied between 35 and 65%.

In the continuing quest for means to distinguish between natural and artificial honeys, minor constituents of honey were frequently examined. In the formol titration, the amino group of an amino acid is blocked by reaction with formaldehyde in neutral solution, which then permits the carboxyl group of the amino acid to be measured. This long-known test was applied to honey by Tillmans & Kiesgen (1927), who proposed that it be used for this purpose. Gottfried (1929), confirming their results, proposed that honeys with a value of 0.3-1.1 (ml 0.1-N NaOH per 20 g honey) be suspected, and assigned values of 0.6-4.0 to genuine honeys. Schuette & Templin (1930) in the United States then applied the test to 15 normal honeys and found an average of 0.41 for their samples (range 0.25-0.76). These low values, together with a lack of reproducibility, convinced Schuette and Templin that the method was of little value. They noted that only 11% of the nitrogen content found by analysis (0.004% N of 0.036%) could be accounted for in the titration. It seems possible that the higher formol titration values of European honeys might reflect a greater content of honeydew honey.

Shortly thereafter, Lothrop & Gertler (1933) proposed a method for

determination of amino acids in honey, and found values for amino nitrogen ranging from 0.0024 to 0.0065% for 10 honeys from which protein had been removed by precipitation. It is interesting that their average value for amino nitrogen determined by a rather cumbersome procedure, 0.0033%, is similar to the average value calculated by Schuette & Templin from formol titration (0.004%). Later, Schuette & Baldwin (1944) studied the free amino acid content of 37 honey samples, harvested in 3 years and representing 20 floral types. Using the Lothrop-Paine procedure, they found an average amino acid content of 0.0034% for light-coloured honeys and 0.0058% for dark honeys (basis not given).

Chistov & Silitskaya (1952) found the nitrogen in honey to be distributed among amines, proteins, amides, amido acids, and small amounts of amino acids.

Later investigations on amino acids in honey gained from the development of paper chromatography. Vavruch (1952) was unable to demonstrate free amino acids by this procedure, and reported several amino acids after tryptic hydrolysis of honey proteins. By concentrating the free amino acids with ion-exchange treatment, Baumgarten & Möckesch (1956) detected a total of 17 amino acids in 15 honey types, the average for each sample being 11. Of the 15 samples, at least 5 contained honeydew honey, which is known to be relatively rich in amino acids (Auclair, 1963, page 468). They proposed that the free amino acids originate from the bee, arguing that the relatively uniform occurrence over a widely varying group of honey types makes it unlikely that they could originate from pollen or nectar. Support for this view is given by the data of Maslowski & Mostowska (1963) who found no qualitative differences between 5 honeys from various plants and one obtained by sugar feeding. Bergner and Körömi (1968) also reported that stores from sugar-feeding of bees contained the same 19 amino acids found in mixed samples of honey. Phadke (1962) noted that tyrosine and tryptophan were present in dark, but not light, *Apis cerana* honeys. A preponderance of proline (45% in Finnish honey and 80% in an imported honey) was found by Komamine (1960), who reported 16 known amino acids and 3 of unknown identity in the honeys, which unfortunately were not characterized as to type. The next most abundant amino acid in the Finnish honey was glutamic acid; in the other sample it was leucine. Komamine noted that, since proline was the principal amino acid found in several pollens (Virtanen & Kari, 1955), at least a part of the honey acids probably originated therefrom. Table 5.6/1 shows the concentration of the various amino acids found by Komamine, recalculated for comparison with other data. The results of Maeda *et al.* (1962) are also shown in the Table; their amino acid analyses for three honey types were determined by the Stein & Moore ion-exchange procedure, which is considered much more reliable

Table 5.6/1
Free amino acids in honey
(mg per 100 g honey)

Honey type	Komamine (1960)		Maeda et al. (1962)			Misusawa & Matsumuro (1968)			Michelotti & Margheri (1969)			Biino (1971)		
	1	2	3a	4	5	6	3b	7	5	8	9	10	11	12
Lysine	0.6	0.4	38.2	8.1	36.7	2.50	2.71	1.85	1.91	1.31	2.07	1.46-2.8		
Histidine			6.7	2.6	10.7	0.94	0.92	0.61	0.93	0.63	0.75	0.56-1.2		
Arginine	0.6	0.0	5.4	5.1	5.8	0.63	0.42	0.33	0.56	+	0.46	0.33-0.53		
Aspartic acid	0.4	0.5	12.3	7.9	17.0	1.81	0.90	0.84	0.86	3.97	0.17	0.06-0.53		
Threonine	0.2	0.2	2.6	0.8	4.5	0.39	0.42	0.35	0.26	0.26	1.10	0.45-1.9		
Serine	0.5	0.5	23.6	3.2	11.8	1.43	0.70	0.65	0.34	0.62	1.19	0.84-1.57		
Glutamic acid	2.5	0.5	19.0	8.3	13.0	1.85	1.91	1.36	1.61	1.34	1.42	1.25-1.80	1.18	1.18
Proline	6.2	19.0	297	134	249	28.71	20.20	22.19	21.06	16.91	14.6	12.5-17.1	53	83.5
Glycine	0.2	0.2	5.9	2.2	3.6	0.31	0.23	0.14	0.12	0.13	0.46	0.33-0.54	0.45	1.80
Alanine	0.6	0.4	10.5	4.6	8.5	0.46	0.53	0.32	0.41	0.31	1.3	0.60-1.65	1.42	2.66
Cystine			6.1	5.5	0.0	-	0.35	0.44	+	+	+	+		
Valine	0.6	0.3	9.7	3.0	7.3	0.52	0.45	0.19	0.46	0.33	0.91	0.71-1.05		
Methionine	0.3	0.0	2.7	1.2	0.8	-	0.05	0.04	0.17	-	+	+	+	0.19

Isoleucine	4.6	2.3	3.6	0.28	0.34	0.12	0.16	0.19	0.77	0.44-1.1	0.52	0.52
Leucine	0.7	0.9	1.4	4.9	0.30	0.34	0.25	0.15	0.58	0.32-0.95	0.52	0.52
Tyrosine	6.9	3.3	6.2	0.49	0.47	0.27	0.26	0.18	2.59	1.3-3.9	0.72	1.45
Phenylalanine	9.6	10.5	11.4	0.93	1.62	0.58	0.54	0.28	16.6	5.0-42.0	2.98	4.28
Tryptophan	0.0	0.0	0.1	+	+	+	-	+	+			

+ indicates traces; - indicates absent; blank indicates not reported.

Honey Types

1. Finnish honey
2. Honey imported into Finland
- 3a. Rape (*Brassica campestris*); 3b gives average of 3 samples
4. Common lime (*Tilia europaea*)
5. Buckeye (*Aesculus turbinata*)
6. Chinese milk vetch (*Astragalus sinicus*), average of 3 samples
7. Mandarin orange (*Citrus ussuriensis*), average of 3 samples
8. Acacia (*Robinia pseudacacia*), average of 2 samples
9. Average for 9 unspecified honey types
10. Range for 9 unspecified types honey (individual values in original)
11. Acacia honey
12. Honeydew honey

than paper chromatography for quantitative work. Twelve Japanese honey samples representing 5 floral types were analysed by this method by Mizusawa & Matsumuro (1968). Averages for each honey type are given in Table 5.6/1, which also shows the average values and ranges found by Michelotti & Margheri (1969) who analysed 9 Italian honey samples of unspecified type by the Stein-Moore procedure. Their values have been recalculated from the 'milligram per kilogram' in the original to permit easy comparison of values. All of the values are reasonably consistent, except that the data of Maeda *et al.* (1962) are uniformly about tenfold higher. Proline is confirmed as the predominant acid; lysine, glutamic, and aspartic acids follow.

Another report of amino acids in honey determined by the automatic analyser is that of Biino (1971). He proposed analysis for proline to indicate the extent of admixture of honeydew with floral sources in honey. His data* (recalculated) for 8 amino acids are included in Table 5.6/1.

It is of interest here to examine the data of Hadorn & Zürcher (1963a), who view the formol number (*see above*) as a useful constant, and who have improved its estimation by combining it with an improved free acid and lactone titration (White, Petty & Hager, 1958). Their average values are in the range 0.3-0.9 meq (milliequivalents) per 100 g; assuming an average equivalent weight of 100 for the amino acids, this corresponds to 30-90 mg%, which does not support the higher values found by Maeda *et al.* (1962). It is evident that further examination of the levels of these compounds in honey would be useful. For comparison, the levels of amino acids in honeydew (as excreted) are of the order of 120 to 1830 mg %, fresh weight; proline is a very minor constituent (Auclair, 1963).

5.7 ENZYMES

The enzymes are among the most interesting materials in honey, possibly have received the greatest amount of research attention over the years, and have supported the greatest burden of nonsense in the lay and even scientific press. The use of enzyme activity in some countries as a test for overheating of honey seems to support by implication the occasional supposition by food faddists that the enzymes of honey have a dietetic or nutritional significance of themselves.

An account of the earlier history of the enzymes of honey is included in published material based on Gothe's dissertation at Leipzig in 1913 (Gothe, 1914a). He noted that honey does not contain lactase, proteases,

* Prof. Biino has confirmed privately that the data in his original article should read 'micromoles', not 'millimols'. Data in Table 5.6/1 have been adjusted accordingly.

or lipases, and possibly not inulase. Catalase was found, and diastase and invertase were present. He reviewed earlier work of Marpmann (1903), who reported proteolytic and alcoholic fermenting enzymes, and whose tests were later shown to be reacting to sugars and acids in the honey. The primary interest in honey enzymes at that time was a possible means for distinguishing between natural and artificial honeys, since the methods of sugar analysis of the time were inadequate. This necessity to detect adulteration was the *raison d'être* for much of the European research on honey and continues even today, with the possible shift of emphasis from detection of adulteration to detection of overheating of honey, which—apart from its obviously detrimental effect on flavour—is supposed to destroy vital, though frequently vague, biological or nutritional qualities of natural honey. Thus, we still see in the literature many articles on the effect of heat on diastase and invertase, and reports of assay results for a given year in a given country, and suggested modifications in assay methods. Relatively little information has appeared on the enzymes themselves—their sources, purification, characteristics, kinetics, and so on. In this review only a sampling of the literature on the enzymes of honey is practicable.

5.71 Honey 'diastase'

The starch-digesting enzyme(s) of honey have been known for many years. As already noted, because of their sensitivity to heat they have been used as indication of honey quality.

The older concepts of amylase activity classified the enzymes into two groups. The α -amylase (amylolytic) group splits the starch chain randomly, producing dextrins, with a slow loss of iodine-produced colour, and relatively little increase in reducing sugars. The β -amylase (saccharogenic) group splits the reducing sugar maltose from the ends of the starch chain, with rapid loss of staining with iodine. In honey analysis, both procedures have been used in diastase assay: loss of colour reaction with iodine, and increase in reducing power. Because the α -glucosidase of honey can further split maltose (produced by β -amylase) and double its effective reducing power, the second procedure can be misleading.

As previously noted, very little work has been done on honey amylases as such. Lampitt, Hughes & Rooke (1930) studied the effect of pH and temperature on both the α - and β -amylase of honey and found them somewhat interrelated. For α -amylase, optimum pH was found in the range 5.0–5.3, the lower value applying at 22–30°C (71–86°F) and the higher value at 45–50°C (113–122°F). The optimum pH for β -amylase was 5.3. Most reports place the optimum pH for honey 'diastase' at 5.3. The lack of adequate control of acidity in the diastase number assay as

outlined by Gothe (1914*b*), and slightly modified by Fiehe & Kordatzki (1928), was shown by Lothrop & Paine (1931*b*) to produce erroneous results in some cases; this was confirmed by Schuette & Pauly (1933). Both studies showed adequate buffering at *p*H 5.3 to be necessary, as did Weishaar's (1933), whose modification was recommended by Kiermeier & Köberlein (1954) and is in general use, though in some laboratories it is being replaced by the more objective method of Schade *et al.* (1958). This method was adapted for a wider variety of instruments and further standardized against the Gothe method (White, 1959*a*) and tested collaboratively (White, 1964*a*). Hadorn (1961) also modified the method of Schade *et al.** Schepartz & Subers (1965) have described an abridged, simplified Schade procedure which would enable a beekeeper or small packer to determine an approximate number without expensive equipment.

No work on these amylases has come to the writer's attention beyond the above study of optimal temperature and *p*H conditions, and many papers relating to the effects of heating honey on its diastatic activity (e.g. Auzinger, 1910*a*, Moreau, 1911*c*, Gothe, 1914*b*, de Boer, 1931, Fiehe, 1931, von Fellenberg & Ruffly, 1933, Bergeret & de Castro, 1943*a*, Kiermeier & Köberlein, 1954, Schade, Marsh & Eckert, 1958, Duisberg & Gebelain, 1958, Hadorn & Kovacs, 1960, Curylo, 1961, Hadorn, Zürcher & Doevelaar, 1962, Hadorn & Zürcher, 1962*b*, White, Kushnir & Subers, 1964, Langridge, 1966, Chudakov, 1966). No definitive reports on the isolation or mode of action of the amylase(s) have yet appeared, though with modern chromatographic methods such research would be facilitated. Schepartz & Subers (1966*a*) have described their preliminary attempt to use such procedures in separating the amylases of honey. A 200-fold purification of α -amylase was attained, but attempts to characterize the enzyme were unsuccessful because of its instability.

Of many reports on heat inactivation of the enzyme in honey that have appeared, those of Schmidt-Nielsen & Engesland (1938), Schmidt-Nielsen & Årtun (1938), and White, Kushnir & Subers (1964) have contained kinetic data. The last-named report points out that the effects of heating and of longer-term storage at lower temperatures are so similar that the half-life of the enzyme can be calculated with a single equation over the temperature range of 10–80°C (50–176°F).

Figure 5.71/1 shows the effect of temperature on the rate of heat inactivation of the contained diastase when full-density honey is heated.

For this inactivation, $\log k = 22.764 - \frac{35\,010}{2.303RT}$. The half-life in days

* Results in the author's laboratory, and informal exchange of samples, indicate that the two modifications produce different results on the same sample (White, Kushnir & Subers, 1964, Kerkaliet & Putten, 1973).

of honey diastase over the temperature range is shown in Figure 5.71/2 and is approximated as follows:

$$\log t_{\frac{1}{2}} = \left(\frac{1}{T} - 0.003000 \right) / 0.000130$$

where T is the temperature in degrees Kelvin between 283 (10°C, 50°F) and 353 (80°C, 176°F).

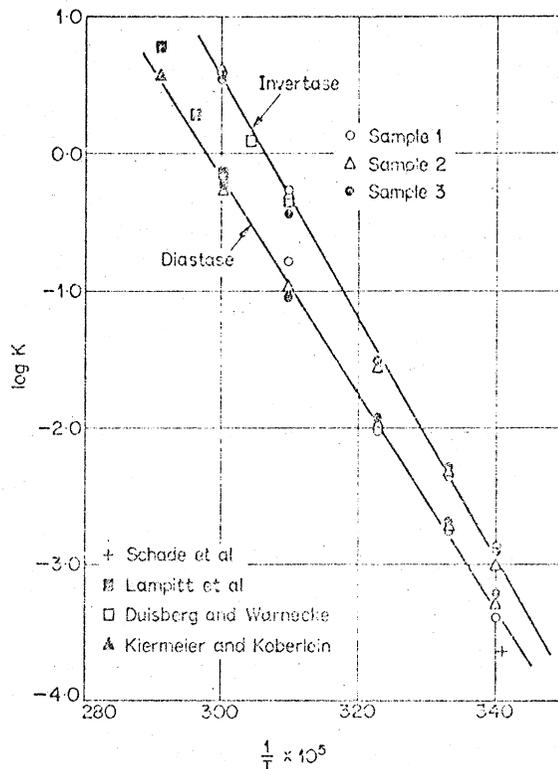


Figure 5.71/1 Effect of temperature on rate of heat inactivation of diastase and invertase in honey. $\left(\frac{1}{T} 0.00280\right)$ corresponds to 84°C, 183°F; 0.00300 to 60°C, 140°F; 0.00320 to 39°C, 102°F; 0.00340 to 21°C, 70°F. (White, Kuslmir & Subers, 1964)

Table 5.71/1 gives calculated half-lives for honey diastase when honey is stored at various constant temperatures. The necessity is obvious for adequate control of heat exposure for honey whose diastase must be preserved.

Gothe (1914b) concluded that the enzyme originated largely from the bees, a very minor portion possibly coming from pollen. Phillips, in his study of the ability of honeybees to survive on various carbohydrates (1927), found that the bees could not utilize raw or cooked starch or dextrans. He found no amylase on the washed lumen of the digestive tract, and though he recognized that the presence of glycogen in certain muscles of the ventriculus required the presence of the appropriate

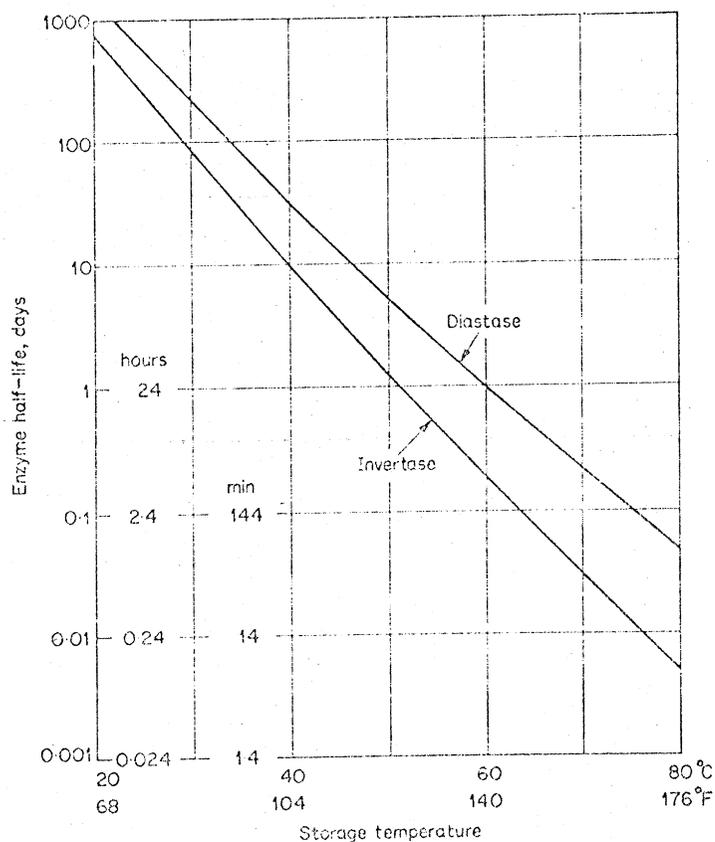


Figure 5.71/2 Approximate time required at a given temperature between 20°C (68°F) and 80°C (176°F) for the diastase and invertase activities of a honey sample to be reduced to one-half of the initial value. (White, 1967a)

enzymes, he held that the origin of diastase was an open question; he appeared to favour pollen grains as the source. Later Vansell & Freeborn (1929) proposed that pollen was the source, on the basis of an apparent correlation between diastatic activity and pollen content, but they were unable to obtain high activities from pollen extracts. Their interest in

the question arose from the low diastase values commonly reported for California honeys in Germany, and the low values found for unheated comb honeys. Lothrop & Paine (1931*b*) noted that the great variation in diastase value among honeys of different floral type supported Vansell's contention.

Table 5.7x/r
Calculated half-lives of honey enzymes
 (White, Kuslmir & Subers, 1964)

<i>Temperature</i>		<i>Half-life</i>	
°C	°F	<i>Diastase</i>	<i>Invertase</i>
10	50	12 600 days	9 600 days
20	68	1 480 "	820 "
25	77	540 "	250 "
30	86	200 "	83 "
32	90	126 "	48 "
35	95	78 "	28 "
40	104	31 "	9.6 "
50	122	5.38 "	1.28 "
60	140	1.05 "	4.7 hr
63	145	16.2 hr	3.0 "
70	158	5.3 "	47 min
71	160	4.5 "	39 "
80	176	1.2 "	8.6 "

Fiehe (1932), in contrast to other workers, considered nectar to be the principal source of diastase, and the bees to be a minor source. Braunsdorf (1932) explained the considerable variability of diastase in 'sugar-fed' honey as the result of several factors—including race and colony strength of the bees, which affect the degree to which they 'work' their stores. He believed that diastase originates both from the nectar and the bees. Later Bartels & Fauth (1933) did not find any relationship between pollen level and diastase content, and suggested the possibility that high ambient temperatures in parts of California caused the low activities. Weishaar (1933) found only 1.5-2.5% of the diastase arising from nectar, 0.25-0.75% from pollen and the remainder from the bees; Gorbach (1942) compared bee and honey amylase(s). He noted an optimal *pH* for the former of 4.8-5.0, with a rapid diminution on both sides (limits 4.0-6.0). The fact that the optimum for honey was slightly different (5.1-5.2) he attributed to plant amylase. He further noted that

bees digest starch with greater difficulty than maltose or sucrose, but more easily than melezitose. This is exactly in opposition to Phillips' results with starch and melezitose. Finally, Ammon (1949) on the basis of similarity between honey diastase and bee diastase—and its long-known presence in honey obtained from sugar-feeding—concluded that the bee is the source. Rinaudo *et al.* (1973), agreed with Ammon.

Lotmar (1935) has studied the apparent discrepancies in reports on the ability of honeybees to utilize starch and dextrans. She fed bees solutions of 8% starch dextrin with 8% sucrose, or with 8% or 16% sucrose solutions alone. The bees survived longer on the dextrin-sucrose solution than on the 8% sucrose. The time by which half of the bees had died (estimated from her chart) was 3.0, 2.5, and 3.5 days respectively. On water alone, half of the bees had died by 1.8 days. Sucrose was fed with dextrin because the latter is tasteless to bees. This procedure when applied to starch, of which only a 5% solution could be made, did not yield appreciable differences in death rates. The starch grains (1.2–5 μ long and 1–2 μ wide) of various pollens were observed to be digested in the bee. The smallest non-pollen starch grains (3.5–5 μ and 6.5–8 μ) were also seen to be partly digested. Large grains, as of pea or wheat starch (about 45 μ long), were not digested.

5.72 Honey invertase

The enzyme responsible for most of the chemical changes that take place when nectar is ripened to honey is the invertase (sucrase, saccharase). It has been known for many years that the bees add invertase to nectar, and it has been long recognized that invertase activity may continue in extracted honey. The subject is dealt with fully in 2.22.

The substrate for invertase is sucrose, which is hydrolysed (eventually) to glucose and fructose. Two general types of invertase are known, fructoinvertase and glucoinvertase. They differ in their mode of action, and earlier workers differentiated them by their behaviour with other sugars and by whether their action was inhibited by glucose or fructose (Neuberg & Roberts, 1946). Bacon and Edelman (1950) and Blanchard & Albon (1950) simultaneously demonstrated that the action of yeast invertase (fructoinvertase) on sucrose is actually a transfer of fructose molecules from sucrose to suitable acceptor molecules (which can be sucrose, water or certain other sugars). The suggestion (Nelson & Sottery, 1924, Papadakis, 1929) that honey invertase is a glucoinvertase was confirmed (White, 1952, White & Maher, 1953*a*) by demonstrating glucose transfer by the enzyme. It was found that separated honey invertase produced several oligosaccharides when acting upon sucrose. All these sugars are eventually hydrolysed to glucose and fructose by prolonged action of the enzyme. The principal of these sugars was identified as a

new trisaccharide, α -maltosyl- β -D-fructoside (White & Maher, 1953b) (also known as fructomaltose, gluco-sucrose, erlose); it has since been found in honeydew of a scale insect (Wolf & Ewart, 1955a), in the digestive systems of many insects, and in blowfly excreta (Gray & Fraenkel, 1953, 1954), as a product of the action of hog intestinal invertase on sucrose (Dahlqvist & Borgström, 1959), and in honey (Siddiqui & Furgala, 1968a). It is likely that honey invertase is actually an α -glucosidase with activity against oligosaccharides having an exposed α -glucosyl group (White & Maher, 1953a).

It is of interest that Bacon & Dickinson (1955, 1957) subsequently established that melezitose, long known as a constituent of manna and honeydew, is also a product of transglucosylation by an aphid enzyme when hydrolysing sucrose. In this case glucose is transferred to the 3-position on the fructose in the sucrose molecule, whereas honey invertase transfers glucose to the 4-position of the glucose moiety of sucrose. The appearance of intermediates in the hydrolysis of sucrose by honey invertase complicates the determination of invertase activity. Erlose, the principal intermediate, is non-reducing and its $[\alpha]_D^{25} = +121.8$ (White & Maher, 1953b), about twice that of sucrose. Appearing during the inversion in amounts up to at least 11% of the starting sucrose (when 25% remains) (White & Maher, 1953a), a considerable error can be introduced in invertase assay in honey if unspecific reducing-sugar methods are used (Moreau, 1911b, Gothe, 1914a, Gontarski, 1957, Rychlik & Fedorowska, 1960), or polarimetric (Duisberg & Gebelein, 1958, Hadorn & Zürcher, 1962a), or iodometric methods for glucose (Kiermeier & Köberlein, 1954). Even such a specific glucose method as glucose oxidase will not provide correct results for the honey invertase assay. Error could be greatly reduced by specifically determining free fructose, but even then a small amount of transfer of glucose to fructose occurs (Dahlqvist & Borgström, 1959). The methods noted above may be of use for routine invertase assay, but should not be expected to give results in agreement with one another.

The research of Nelson and his colleagues (Nelson & Cohn, 1924, Nelson & Sottery, 1924, Papadakis, 1929), mentioned above, is the most detailed study of honey invertase itself. These workers followed the reactions polarimetrically, under the long-accepted assumption that the enzymic hydrolysis of sucrose is a simple splitting of the molecule. Since we now know that the sucrose solution during hydrolysis by honey invertase can contain at least 11% of gluco-sucrose, among other saccharides, and that the specific rotation of the intermediate is nearly twice that of sucrose, about 22% of additional sucrose is simulated. This condition is present to greater or lesser extent during most of the hydrolysis. Rinaudo *et al.* (1973), report that bee-gland and honey

invertases are inhibited by fructose, but not by glucose, and also (in contrast to other workers) that they have no action on maltose.

White & Kushnir (1967*b*) have reported preliminary efforts to characterize the α -glucosidase of honey, the so-called 'honey invertase'. Crude dialysed preparations from several bulk honeys, comb ('single-colony') honeys, and stores from sugar-feeding, were examined. Methods used were chromatography on ion-exchange cellulose, gel filtration with Sephadex G-200, and starch-gel electrophoresis using a high-resolution enzyme assay procedure developed for this work (White & Kushnir, 1966). Ion-exchange chromatography divided the crude α -glucosidase preparation into 3-9 closely spaced components. Material from sugar-feeding (with no plant constituent) was relatively unstable, and showed only one sharp peak. The α -glucosidase elution pattern from honey stored by a single free-flying colony resembled that from a sample of bulk honey derived from many colonies.

All honey α -glucosidase preparations yielded a single peak upon gel filtration with Sephadex G-200, and indicated a molecular weight of about 51 000. Starch gel electrophoresis showed the α -glucosidase complex to have 7-18 components (isozymes). The lowest number was for a sample of comb honey, the highest for a bulk honey clover. In one experiment, the isozymes showed differing degrees of heat sensitivity. Each of the 13 isozymes in one honey sample had the same ratio of activity upon sucrose to that upon maltose; this further confirms the α -glucosidase nature of honey invertase (White & Maher, 1953*a*), which was subsequently questioned by Siddiqui & Furgala (1968*a*).

The α -glucosidase complex from sugar-fed 'honey' was far less stable, and migrated more slowly than that from any of five floral honeys tested, during starch-gel electrophoresis. Taken together with the ion-exchange results, this implies an interaction between the bee and the plant nectar protein constituents.

As with diastase, a number of investigators have examined the effect of storage and heating upon invertase activity of honey (Moreau, 1911*c*, Achert, 1912, Kiermeier & Köberlein, 1954, Duisberg & Gebelein, 1953, Rychlik & Fedorowska, 1962*b*, Curylo, 1961, Gontarski, 1961, Hadorn, Zürcher & Doevelaar, 1962, White, Kushnir & Subers, 1964). Heat inactivation of honey invertase in buffer (pH 5.9 phosphate, 0.01M) proceeds 24 times as rapidly as in full-density honey (White, Kushnir & Subers, 1964).

Figure 5.71/1 shows the effect of temperature on the rate of heat inactivation of the invertase in natural honey. The enzyme is more susceptible to heat damage than is diastase. For the invertase, $\log k =$

$$26.750 - \frac{39\,730}{2.303RT}. \text{ An idea of the half-life of invertase in honey at constant}$$

temperature over the range 283°K (10°C, 50°F) to 353°K (80°C, 176°F) may be obtained from Figure 5.71/2. The equation is

$$\log t_{1/2} = \left(\frac{1}{T} - 0.003083 \right) / 0.000113$$

in the temperature range given above.

Table 5.71/1 shows the half-life of invertase in honey under constant temperature conditions, as calculated by this equation at various temperatures. It can be seen that when invertase activity is to be preserved, attention must be given to proper storage and processing conditions for honey.

It should be noted that assays of either invertase or diastase of authentic unheated honeys give widely varying values. Since these enzymes originate largely in the bee, it would appear at first glance that enzyme levels should be at comparable levels in all genuine honeys. That this is not the case may be related to the degree of manipulation required by the bees to ripen the nectar (*see* 2.22). Schönfeld (1927) showed that as the sucrose content of bees' food increased, the nitrogen content decreased, as did the activity of invertase. Gontarski & Hoffmann (1963) also relate the more intensive working (required for thinner nectar) with higher enzyme activity. The apparent anomaly of high enzyme activity in the stores produced by feeding dry sugar is explained by bees' need to moisten and dilute the food before ingesting it and storing the product (Neprašova & Svoboda, 1956, Krupička, 1959, Simpson, 1964). Sipos (1964), concerned with the normally rather low enzyme content of Hungarian acacia (*Robinia*) honey, states that the enzyme content of stores is influenced by the quantity of daily flow and its sugar content; a rapid flow and high sugar content lead to lower enzyme content. The age of bees is also relevant; Hungarian acacia honey had the highest enzyme content when bees 25-30 days old predominated in the colony. It thus seems generally agreed that a rapid flow of concentrated nectar should result in stores of reduced invertase and diastase content.

While it is not the purpose of this review to examine the enzymes of the bee, mention should be made of the carbohydrases since the α -glucosidase in honey arises from the hypopharyngeal gland of the bee. Gontarski (1954), who reviewed the earlier literature, differentiated between the α -glucosidase of the gut with a *pH*-optimum of sucrose of 4.4-4.8, and the hypopharyngeal α -glucosidase with a corresponding value of 5.9-6.4. He noted that the gland enzyme was soluble, but the enzyme from the gut preparation was particulate; this is not unexpected considering the source, preparation, and function of the enzymes. In his Tables 6 and 7 and Figure 4 he shows the effect of adding glucose and fructose to the reaction of gland enzymes and sucrose. For fructose,

inhibition is directly proportional to fructose concentration. For glucose, the figure shows a considerably greater and less proportional effect, but apparent errors in plotting the data from Table 6 and an apparent error on the blank value makes any conclusions, including his, impossible. It is interesting that he found no inhibition of the gut enzyme with glucose, whereas later, Maurizio (1962c) noted that transglucosylation (which would appear as inhibition) is much greater with the gut enzyme preparation than with gland enzyme preparation. On the basis of its nature as an α -glucosidase, Gontarski proposed that the 'so-called bee diastase' may be identical with the inverting enzyme. Differing stability to heat makes this highly unlikely; in fact this question appears to be settled by the approximate molecular weights reported by White & Kushnir (1967b) of 24 000 for honey amylase and 51 000 for honey α -glucosidase.

Maurizio has for some years studied the actions of the inverting enzymes of the pharyngeal glands and midgut of the bee in order better to understand the digestion and metabolism of carbohydrates by bees and the formation of honey. This work was described in a series of papers (Maurizio, 1957, 1959c, 1961, 1962a, 1962b). Since the subject is discussed in full in Chapter 2, no further comments are included here.

It is generally considered that honey invertase arises from the bee with contributions also from nectar enzymes.* Evidence for the latter is lacking, other than that some nectars may have invertase activity. It has been generalized that plant invertases are of the fructose-transferring type and animal invertases transfer glucose (Bealing, 1953, Gilmour, 1961, page 42). A prominent apparent exception was reported by Zimmermann (1954). In contrast to the demonstration using radioactive carbon that the nectaries of *Euphorbia pulcherima* secreted a transfructosidase with the nectar. (Frey-Wyssling, Zimmermann & Maurizio, 1954), Zimmermann reported that the nectar of *Robinia pseudoacacia* contained an invertase which by transferring glucose instead of fructose 'im Gegensatz zu allen bisher untersuchten Invertasen mit einer saccharosespaltenden Transglukosidase zu tun haben' (Zimmermann, 1954). Such an enzyme had, however, been reported a year earlier in honey (White & Maher, 1953a). In her description of the experimental part, it is noted that over 100 fully unfolded flowers were used for obtaining the nectar. No mention is made of any precaution to exclude bees from the flowers, though Beutler notes (1953) the necessity (and difficulty) of excluding insects from flowers intended for nectar studies. Here again 'enzyme activity' was followed by papergramming nectars stored for at least 60 days. Very small amounts of bee-introduced invertase could provide extensive changes in much less time. Confirmation

* Riaudo *et al.* (1973) conclude it is of bee origin.

of this report of the presence of a glucose-transferring invertase from a plant source with adequate controls would be desirable, since it complicates speculations (Siddiqui & Furgala, 1968a) on the biochemical origin of honey oligosaccharides.

5.73 Glucose oxidase

Early investigations found no evidence for oxidases in honey (Auzinger, 1910a, Moreau, 1911b). A glucose-oxidizing enzyme which formed an acid, probably gluconic, and peroxide, was demonstrated by Gauhe (1941) in the hypopharyngeal glands of the honeybee. She suggested that the organic acid formed acted as a preservative for honey. The presence of the enzyme in honey was not investigated. Gontarski (1948) described an enzyme in the hypopharyngeal glands that oxidizes vitamin C. He based his assumption on the disappearance of ascorbic acid from stores produced by feeding bees with sugar solutions containing ascorbic acid. The enzyme that oxidizes ascorbic acid was later shown to occur in honey.

A few years later Cocker (1951) reported that honey contained an enzyme which produced acid, based on the tendency of honey to become acid on standing after neutralization. White, Petty & Hager (1958) ascribed most of this pH drift after neutralization to lactone hydrolysis, but could not exclude enzymic action. The presence in honey of the glucose oxidase described by Gauhe was shown by White, Subers & Schepartz (1962, 1963), and its action on glucose was shown to produce hydrogen peroxide and gluconolactone. At the same time they showed the cause of the antibacterial effect known as inhibine to be hydrogen peroxide accumulating in the assay plates due to the action of this enzyme system. Adcock (1962) had independently shown inhibine to be destroyed by catalase. A chemical assay for hydrogen peroxide was developed (White & Subers, 1963) to replace the time-consuming biological assay for inhibine, and 90 samples were analysed, of which half were assayed for inhibine by the culture plate assay. The following relation was found between the empirical inhibine number I and peroxide accumulation P (measured in micrograms hydrogen peroxide accumulated per gram of honey in 1 hour under assay conditions): $I=0, P<3.4$; $I=1, P=3.4-8.7$; $I=2, P=8.8-20.5$; $I=3, P=20.6-54.5$; $I=4, P=54.6-174$; $I=5, P>174$. P for cotton honey was found to be especially high (225-360). Conditions affecting the peroxide value were discussed in this paper (White & Subers, 1963). Dustmann (1967a, 1967b) assayed 29 samples of European floral and honeydew honeys for peroxide accumulation and found honey from *Castanea sativa* to be consistently high, values ranging from 120 to 605.

Schepartz & Subers (1964) and Schepartz (1965a, 1965b) have studied the kinetics, inhibition, and specificity of the enzyme. It is unusual in

having a high Michaelis constant ($1.55M$) and optimal substrate concentration ($2.7M$). It appears to be substantially inactive in full-density honey and becomes active upon dilution. Studies have been reported on the effect of heat (White & Subers, 1964a) and light (White & Subers, 1964b) on the accumulation of peroxide in diluted honey. It was found that the glucose oxidase activity in certain honeys was destroyed by visible radiation, the 425–525 nm region being the most effective. The presence in susceptible honeys of a heat- and light-stable non-volatile sensitizer was proposed, which facilitates oxidation of the enzyme, with the greatest effect at pH 3 and becoming negligible at about pH 7 and higher. Honeys were found which lost their peroxide accumulation activity unless weighed for the assay under reduced illumination, but the peroxide accumulation in others was substantially unaffected by direct sunlight. The importance is obvious of immediate adjustment of pH to about 7 before bacteriological assay of inhibine. In any event inhibine is not recommended as a quality factor for honey, because of wide variability in its sensitivity to light and heat and its distribution by floral source. The peroxide accumulation system in honey is at least as sensitive to heat as honey invertase and diastase.

The acid produced by this enzyme is the principal acid of honey (Stinson *et al.*, 1960, Maeda *et al.*, 1962). Since, during storage of full-density honey, acid is produced at a rate of only 0.002–0.012 $\mu\text{g/hr/g}$ honey (White, Subers & Schepartz, 1963)—which is about 1/15 000 of the rate in diluted honey—the 70 mg of gluconic acid/100g honey reported by Maeda *et al.* (1962) would require over eight years' storage of full-density honey for its production. At a reasonable rate for diluted honey (100 $\mu\text{g/hr}^*$) only seven hours would be required for the enzyme system to produce this amount of gluconic acid. Comparison of the above values for gluconic acid with the average lactone content shown in Table 5.2/1 (which represents only a fraction of the total gluconic acid) indicates that actual gluconic acid content of honey may be several-fold higher than the values of Maeda *et al.* Even so, the longer time that would be needed to produce larger amounts during ripening of honey is still in reasonable agreement with the time during which nectar is concentrated to full-density honey in the comb.

If the results of Gontarski (1948) on the 'vitamin C-oxidizing enzyme' are re-examined in the light of the glucose oxidase system, it is evident that his ascorbic acid oxidation could well be caused by the hydrogen peroxide produced by the system, rather than by a direct enzymic action with ascorbic acid as substrate. Unpublished observations in our laboratory with crude enzyme preparations from honey indicate that

* Measured as peroxide accumulation and not as acid production, and hence somewhat low.

ascorbic acid is oxidized by the system only in the presence of glucose, and not in its absence. Gontarski did note that the oxidation was initiated by an extract of hypopharyngeal glands, but he was uncertain if the enzyme was specific, or whether it was Gauhe's glucose oxidase. Schepartz (1966a) examined the effect of ascorbic acid on the glucose oxidase reaction; he found it to be a powerful accelerator, presumably acting by way of product removal and not on the enzyme itself.

5.74 Other enzymes

Honey has been examined for other enzymes from time to time. Marpmann (1903) claimed the presence of inverting, alcohol-forming, proteolytic, and peroxidase enzymes in honey. The first had previously been reported, the second may have been due to fermentation, and the last-named depended upon a colour reaction with peroxide and *p*-phenylenediamine which Auzinger (1910a, 1910b) later showed was due to a chemical rather than an enzymic reaction. Auzinger did agree that a catalase was present, using a 24-hour period for evolution of oxygen from peroxide. No evidence of oxidase or reductase activity was found. Moreau (1911a, 1911b) confirmed Auzinger's results and reported the catalase activity of twenty samples by the Auzinger procedure. Gothe (1914a) could not demonstrate lactase, protease or lipase activity, and questioned the presence of inulase. Gillette (1931) measured evolved oxygen from added peroxide over a two-hour period and suggested that the catalase arose from pollen or yeasts and not from the bee. Occasionally catalase activity is reported for honey samples. Fedotova (1957) reported a range of 0.68 to 9.2 mg peroxide for 33 samples of Uzbekistan honey, alfalfa (*Medicago*) honey showing the highest activity. Działoszyński & Kuik (1963) experienced some doubts about methods; they measured residual peroxide iodometrically, after 10 minutes' incubation. Results for 21 samples averaged 10.8 (range 0-36) mg peroxide per 100 g honey (per 10 minutes?). Gillette's value of 0.7 ml gas would correspond roughly to 7 mg peroxide in 10 minutes.

Schepartz (1966b) reviewed the literature on the occurrence of catalase in honey; he criticized all of the methods used as inappropriate for honey, stating that no unequivocal demonstration of catalase in honey had been accomplished. Using manometric and spectrophotometric procedures, Schepartz provided unequivocal evidence for its occurrence in honey. He determined the reaction with peroxide to be a first-order one, with the *pH* optimum 7-8.5, the substrate concentration optimum 0.018 M, and the Michaelis constant 0.0154 M.

In order to examine a postulate that the peroxide accumulation (inhibine) in honey should be inversely related to catalase content, Schepartz & Subers (1966b) determined catalase in 28 samples which had

previously been assayed for diastase and peroxide accumulation. A direct correlation ($r = +0.76$) was shown between catalase and diastase activities, and an inverse correlation ($r = -0.71$) between catalase and peroxide accumulation; both were significant at the 1% level. Dustmann (1971) reported catalase and 'inhibine' (peroxide accumulation) values for eleven honey samples. The four samples with the highest inhibine values (380-662 μg peroxide per gram honey per hour) were devoid of catalase activity.

Giri (1938) showed that honey contains an acid phosphatase. Using glycerophosphate as substrate, 12 samples of Indian honey were analysed. The enzyme was effective over the pH range 3.5-6.5, showed greatest activity at 35°C (95°F), was activated by Mg^{++} . Działoszyński & Kuik (1963), using Giri's method slightly modified, assayed 25 Polish honeys and obtained an average value of 13.4 mg P/100 g honey/24 hr (range 2.2-52.7). Günther & Burckhart (1967) described a procedure for determining acid phosphatase content of honey using *p*-nitrophenyl phosphate as substrate, with a 3-hour incubation.

Zalewski (1965) examined over 400 samples of honey, pollen, pollen loads, nectar, and bees for acid phosphatase, using phenyl disodium orthophosphate as substrate. Honey averaged 197.2 (range 29.8-2140) micromols per 100 g dry matter. Converted to units used by Działoszyński & Kuik, this corresponds to an average of 5.07, range 0.76-54.8 (assuming a dry-matter content of 82%). A sample of stores from sugar-fed bees was found to contain about one-sixteenth of the average found in honey. High values in nectar and pollen indicate the activity to be largely of plant origin. Heating honey to 60°C or 140°F (10-60 min) reduced activity by about one-third, and to 75°C (167°F) by up to 80%. Storage (at unspecified temperature) destroyed 48-82% of the activity. Acid phosphatase activity was increased over 50% by magnesium ions.

5.8 MINOR CONSTITUENTS

Honey is a complex material; with newer investigations using increasingly acute research tools, the apparent complexity—in terms of numbers and kinds of constituents—appears to increase correspondingly. Identification of these materials is but the beginning; their source or means of entry into honey and their effects on physical, chemical, and biological properties must also be studied, as well as their occurrence among different honey types.

5.81 Honey colour

The substances responsible for the colour of honey are largely unknown. Schuette & Bott (1928) isolated carotene from a buckwheat honey. Later

von Fellenberg & Rusiecki (1938) separated honey colours into two fractions, water-soluble and lipid-soluble. In light-coloured honey, the water-soluble colour was less than the fat-soluble colour, and the opposite was true in dark-coloured honeys. These authors did not think that the carotenoids (fat-soluble) were identical with carotene.

Browne (1908) noted that, of the 92 honeys he analysed, 25 gave a positive test for polyphenolic compounds with ferric chloride, the 5 most intense reactions being from very dark honeys. Oxidation of these compounds can lead to coloured materials. Goodacre (1926) stated that formation of tannic acid in honey is favoured by exposure to air. The development of colour in honey in storage has been ascribed (Milun, 1939) to several factors: combination of tannates and other polyphenols with iron from containers and processing equipment; reaction of reducing sugars with substances containing amino nitrogen (amino acids, polypeptides, proteins); and instability of fructose in acid solution (caramelization). Phadke (1962) noted the absence of tyrosine and tryptophan in light honeys, as contrasted with its presence in dark honeys. Rychlik & Zborowski (1965) were unable to detect either rutin or quercetin in 50 honey samples tested by paper chromatography.

5.82 Aroma and flavour

A discussion of the factors contributing to the honey flavour and aroma will not be attempted here. Maeda *et al.* (1962) ascribed the 'taste' of honey to the sugars, gluconic acid and proline content. But it is evident to anyone who examines a variety of honeys that, although there seems to be a characteristic 'honey flavour', almost an infinite number of aroma and flavour variations can exist. We will here consider only the volatile materials of honey which contribute to the total effect; the field is scarcely developed sufficiently to allow generalizations. It is realized that the various sugars, amino and other acids, tannins and minor non-volatile substances, all contribute to honey flavour. Further, additional contribution to flavour effects of certain honeys may be due to glucoside or alkaloidal compounds specific to the plant source.

The literature on aromatic materials in honey before the introduction of gas-liquid chromatography (GLC) is scanty. Diacetyl (or other diketo-alkane) was identified by Schmalzfuss & Barthmeyer (1929) in very small yield (less than 0.1 ppm) from a German heather honey. Methyl anthranilate was identified in orange honey by Nelson (1930); Lothrop (1932) suggested that its presence provides a specific test for orange honey. More recently, Deshussés & Gabbai (1962) used thin-layer chromatography for this purpose.

The development of gas chromatography has resulted in a great increase of interest in volatile materials from many sources. So far five

reports on the application of this technique to honey analyses are at hand. Dörrscheidt & Friedrich (1962) examined an ether extract of the vacuum distillate from 3 000 kg of honey, but did not get satisfactory results. Best results were obtained by the direct analyses of a sample of vapour phase over honey, using a flame ionization detector; 1.5 cm³ of vapour permitted recording of 31 components, of which only 4 were common to the 6 honeys examined. It was suggested that such chromatograms might serve as 'fingerprints' for identification of honey types. None of the components was positively identified. Merz (1963) used GLC of ether extracts from honey samples as an objective means for assessing honey flavour. He found 5-hydroxymethylfurfuraldehyde (HMF) to be principal peak in organoleptically satisfactory honeys; in honeys of poor (bad, not deficient) flavour, other high-boiling components exceeded HMF in quantity. No other components were identified. Qualitatively, the chromatograms resemble those shown by Dörrscheidt & Friedrich for the ether extract. The volatile carbonyl components of honey were investigated by ten Hoopen (1963). These compounds were isolated as dinitrophenylhydrazones and separated by column and thin-layer chromatography and GLC, and identified by chromatographic behaviour, melting point, and ultraviolet spectrum. Rape and clover honeys yielded greater amounts of material than did thyme honey. Compounds identified were formaldehyde, acetaldehyde, acetone, isobutyraldehyde, and diacetyl.

Cremer & Riedmann (1964) have extended the work of Dörrscheidt & Friedrich, using the same instrument but with a capillary column instead of the packed column. They reported 50 components in the analysis of 10 honeys; 22 were identified, of which only 3 (formaldehyde, propionaldehyde, and acetone) were common to all samples. Their results are shown in Table 5.82/1. Aliphatic alcohols comprised over half of the material examined.

In a later report these investigators (Cremer & Riedmann, 1965) using a Golay column 100 m × 1 mm, have brought the total components separated to 120, more than half being identified. However, only phenylethyl alcohol, n-pentanol, benzyl alcohol and 3-methyl-1-butanol were added to the list of the preceding paper. They noted that a honey sample stored for a year showed an increase in n-pentanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and n-propanol, and suggest that these compounds may originate by fermentation from the corresponding amino acids: norleucine, isoleucine, leucine, and α -aminobutyric acid. They reported that 15 of 22 honeys contained phenylethyl alcohol, and 14 of these also had benzyl alcohol; the former they related to phenylalanine as a precursor. The 6 honeys not containing the last two alcohols were also low in the four alcohols noted above, and were not organoleptically

recognizable as honey. Oxidation of phenylethyl alcohol produces phenylacetic acid. It is of interest that most synthetic honey flavours contain large proportions of methyl, ethyl or propyl phenylacetate and also phenylethyl salicylate or phenylacetate (among many other components); according to Jacobs nearly all phenylacetic esters are characterized by a honey taste and odour (Jacobs, 1955).

Table 5.82/r
Aroma constituents of honey¹

<i>Carbonyls</i>	<i>Alcohols</i>	<i>Esters</i>
Formaldehyde ²	<i>Isopropanol</i>	Methyl formate
Acetaldehyde ²	Ethanol	Ethyl formate
Propionaldehyde	2-Butanol	
<i>Isobutyraldehyde</i> ²	n-Propanol	
Butyraldehyde	3-Pentanol n-Pentanol ³	<i>Other</i> Diethyl ether
<i>Isovaleraldehyde</i>	<i>Isobutanol</i>	
Methacrolein	3-Methyl-2-butanol 3-Methyl-1-butanol ³	
Acetone ²	n-Butanol	
Methyl ethyl ketone	β -Methallyl alcohol 2-Methyl-1-butanol Phenylethyl alcohol ³ Benzyl alcohol ³	

¹ Cremer & Riedmann (1964)

² Also identified by ten Hoopen (1963)

³ Cremer & Riedmann (1965)

It is also of passing interest that a patent for preparing a synthetic honey flavour specifies heating a solution of a monosaccharide with phenylalanine and some derivatives (Morton & Sharples, 1959); thus perhaps a chemical reaction in storage is involved.

The fantastic sensitivity of the newer methods of analysis provides an embarrassment of riches—or what may appear to be riches—in such investigations as these. It is probable that only a few of the compounds that are or will be identified in honey in this manner will actually contribute toward the unravelling of the nature of honey aroma, though the technique may be of value for comparison and 'identification' of floral types of honey on a more or less empirical basis. Merz (1963), using the nose as a detector in GLC, reported only 4 of 20 distinct odour fractions as honey-like.

Methyl anthranilate (MA) is a minor constituent of citrus honey and contributes to its distinctive aroma. Lothrop (1932) described a colour test for this compound in honey which required 1 kg of honey. White (1966) adapted a procedure requiring 10 g of honey and analysed 21 citrus honeys (average 2.87 $\mu\text{g/g}$, range 0.84–4.37) and 12 non-citrus honeys (average 0.07, range 0.00–0.28). He proposed that content of MA might be a useful quality measure for citrus honey if further studies were confirmatory. Knapp (1967), after examining 59 Florida citrus honeys (average 3.29, range 1.6–4.9) and 14 other samples, supported the proposal, but noted that additional work with samples of known source was needed.

Hadorn (1964) noted that orange and lavender honeys, both containing MA, were occasionally deficient in diastase and invertase. He determined that inhibition of enzyme activity by endogenous MA was not responsible for the deficiency.

5.83 Vitamins

As the importance of vitamins in nutrition became known in the 1920s and 1930s, the occurrence of vitamins in honey was studied. Apart from the report of Dutcher (1918), who found negligible amounts of 'water-soluble vitamine' in honey, succeeding workers (Faber, 1920, Hawk, Smith & Bergeim, 1921, Scheunert, Schieblich & Schwanebeck, 1923, Taylor & Nelson, 1929, Hoyle, 1929, Kifer & Munsell, 1929, Trautmann & Kirchhof, 1932) uniformly found no evidence of vitamins A, B₁, B₂, C, D, and E in honey. Markuze (1935) reported that two samples of Polish honey showed presence of vitamin B₂, but no others. All of these investigators used biological assay methods.

By the early 1940s, microbiological and microchemical tests for the vitamins had been improved so much that the very low levels of the various vitamins in honey could be measured. Haydak *et al.* (1942)* and Kitzes, Schuette & Elvehjem (1943) assayed considerable numbers of honeys for thiamine (B₁), riboflavin (B₂), ascorbic acid (C), pyridoxine (B₆), niacin, and pantothenic acid, and a few for biotin and folic acid.

* Values in their Table 2, last column, should be multiplied by 0.01

Table 5.83/1 summarizes the results of these investigations. Both groups of investigators remarked upon the wide variability, both among different samples of similar honeys and among different honey types. The lack of agreement between the two groups for pyridoxine and niacin values was ascribed (Kitzes *et al.*, 1943, Haydak, 1955) to the use

Table 5.83/1
Vitamin content of honeys

Samples	Panto-		Niacin	Thia- mine	Pyri- doxine	Ascorbic acid
	Ribo- flavin	thenic acid				
	(micrograms/100 g honey)					
29 Minnesota ¹	61	105	360 ³	5.5	299	2 400
38 U.S. and Foreign ¹	63	96	320 ³	6.0	320	2 200
21 U.S. 3-7 yr. old ²	22	20	124	3.5	7.6	—
19 U.S. 1-2 yr. old ²	26	54	108	4.4	10.0	—
4 India ⁴	12-54	—	442-978	8-22	—	2 000-3 400

¹ Haydak, Palmer, Tanquary & Vivino (1942)

² Kitzes, Schuette & Elvehjem (1943)

³ Corrected from original data in publication as later shown (Haydak *et al.*, 1943)

⁴ Kalimi & Sohoni (1965)

of chemical methods by Haydak *et al.* and of microbiological methods by the Wisconsin group. Later Haydak *et al.* (1943) found that commercial filtration of honey reduced the vitamin content (except vitamin K) by amounts from 8 to 45%. This was ascribed to the more complete removal of pollen (which is known to contain all the vitamins reported in honey) (Kitzes *et al.*, 1943). The Wisconsin group also found (Kitzes *et al.*, 1943) for a few samples of honey, 0.066 and 3.0 μg per 100 g of biotin and folic acid respectively. Using a chick assay, Vivino *et al.* (1943) found a vitamin K activity for honey, equivalent to about 25 μg of menadione per 100 g honey.

These levels of the various vitamins are so low that they have no real nutritional significance, particularly in view of the amount of honey normally eaten.*

All of the nine early investigators cited at the beginning of this section used bioassays in examining honey for the various vitamins. Seven of them tested for ascorbic acid (vitamin C) and found none (Faber, 1920, Hawk, Smith & Bergeim, 1921, Scheunert, Schieblich & Schwanebeck,

* It can be calculated that honey supplies about 3% of the thiamine and 6% of the niacin required for the metabolism of the contained sugars.

1923, Hoyle, 1929, Trautmann & Kirchhof, 1932, Markuze, 1935). As noted in Table 5.6/2, Haydak *et al.* (1942) reported an average value for 67 honeys of 2.3 mg per 100 g honey. They used a chemical method, and reviewed chemical determinations of ascorbic acid in honey prior to their paper: Griebel (1938) reported 160-280 mg/100 g for mint honey and 7-22 for others. The mint honey was bioassayed, and protected guinea pigs at 1 g per day, which corresponds to about 100-200 mg ascorbic acid/100 g (Rosenberg, 1942, page 337). Kask (1938) found an average of 4.9 mg/100 g (range 0-20) for Estonian honey, noting that the vitamin was relatively stable in honey. Werder & Antener (1938) found a range of 1.1 to 14.6 mg/100 g for 19 samples; Becker & Kardos (1939) found 31-89 mg/100 g for honey from *Castanea sativa* by chemical means, but bioassay did not demonstrate any activity. Kardos stated later (1941) that the indophenol-reducing substances of acacia, fruit-bloom, and *Stachys* honeys were not ascorbic acid, but protein decomposition products. Nevertheless, Griebel & Hess (1939) isolated the strong reducing substance from thyme and mint honeys as a derivative whose melting point agreed with that from the ascorbic acid derivative. Their chemical analyses for buckwheat, mint, and thyme honeys gave 19, 103, and 391 mg/100 g respectively. An analysis of mint and thyme nectars (Griebel & Hess, 1940) showed 288 and 222 mg/100 g, giving calculated values 39.4 and 88.3 for the corresponding honeys if no loss were sustained in ripening. Hansson (1949) reviewed the status at that time. As noted in 5.73, Gontarski (1948) described an ascorbic acid oxidizing enzyme in honey; the oxidation however is probably by hydrogen peroxide produced by glucose oxidase. This enzyme system operates well in diluted honey and only very slowly in full-density honey. Since the amount in honey is quite variable (White & Subers, 1963), rate of destruction of natural or added ascorbic acid would vary widely. Lüttge (1962c), using the Roe method, found 162 mg/100 g (dry wt) for a mint honey, present only as ascorbic acid. None of the other honeys tested contained any. The nectar of *Mentha aqualica* was found to have 10 mg ascorbic acid per gram of sugar. Kalimi & Schonie (1965), using a microchemical method, found 2-3.4 mg/100 g in four Indian honeys, with one-fifth lost after storage at 28-30°C (83-86°F) for one year.

Since ascorbic acid is important only because of its biochemical significance, and honey is a very complex mixture, it would seem reasonable to defer to biological assay values for the determination of this vitamin in honey.

The bioassay value deduced from Griebel's (1938) statement (1 g honey/day protecting guinea pigs from scurvy) agrees reasonably with their chemical value for mint honey. Rahmanian *et al.* (1970) have found high ascorbic acid values (118-240 mg/100 g) for three samples of honey

of unknown source from the mountainous Damavand area in Iran. The analysis was done by several procedures, including a specific chemical method (thin-layer chromatography of the dinitrophenyl-osazone); the results were about 90% as high as those found by the dichlorophenol-indophenol titration. The sample containing 118 mg/100 g was assayed with guinea pigs. Weight gains of animals fed daily 5 mg ascorbic acid or 4 g honey did not differ significantly. Rahmanian *et al.* reported an ascorbic acid requirement of 3-6 mg/day for the animals they used, and concluded that the honey actually contained 75-150 mg/100 g of vitamin C. They suggested the possibility of encouraging the use of honey from this region as a means of helping to relieve the marginal vitamin C deficiency often found in Iran.

Schepartz (1966a), reporting that ascorbic acid is a powerful activator of the glucose oxidase system by way of product removal, stated that because ascorbic acid is oxidized by peroxide from this system, it is not likely that ascorbic acid in the reduced form would be found in a honey containing significant glucose oxidase activity.

When considering the possible interactions between ascorbic acid, glucose oxidase and catalase in honey, it must be remembered that conditions in full-density honey depart so greatly from optimal assay conditions that little or no interaction is likely to occur. During ripening of the nectar to honey, conditions are much more favourable to enzyme action.

5.84 Hydroxymethylfurfuraldehyde (HMF)

This compound, which may be formed by the decomposition of fructose in the presence of acid, was originally thought not to be a constituent of honey in the hive. Modern sensitive quantitative measurements have shown, however, that even fresh honey contains small amounts of HMF (0.06-0.2 mg/100g; White, Kushnir & Subers, 1964, Hadorn & Kovacs, 1960, Winkler, 1955). HMF in honey has received considerable attention over the years: at least a hundred papers devoted to the subject are available. In the early days its presence in honey, as revealed by such qualitative tests as those of Fiehe (1908b) and Feder (1911), was taken to be evidence of the addition of invert sugar; but as soon as the Fiehe test was described it was severely criticized (Bremer & Spornagel, 1909, von Raumer, 1908, Drawe, 1908, Hertkorn, 1909, Klassert, 1909, von Raumer, 1909), because heated, though authentic, honey gave a result interpreted as positive. Many modifications are recorded, but the use of a qualitative test for the purpose carries a number of disadvantages. Use of quantitative procedures would be preferable; numerical values for permitted levels could then be used (Hadorn & Kovacs, 1960). Several studies of quantitative methods have been made. For specific information the reader is referred to the original papers. Optical methods based on

ultraviolet absorption by HMF are described (Schou & Abildgaard, 1931, Lampitt & Billham, 1936, Winkler, 1955, Franzke & Iwainsky, 1956, Gautier, Renault & Julia-Alvarez, 1961c, Romann & Staub, 1961); paper chromatography (Franzke & Iwainsky, 1956, Gautier, Renault & Julia-Alvarez, 1961b); a 'quantitated' Fiehe reaction (Schade, Marsh & Eckert, 1958, Gautier, Renault & Julia-Alvarez, 1961a, Gonnet, 1963); and, probably the best, the chemical procedure of Winkler (1955) which does not require extraction.

When quantitative methods are used, it can be seen that the early confusion about the validity of the Fiehe test was caused by the differing amounts of HMF present from various causes (heating, storage, added with invert sugar) and the sensitivity of the tests in various hands. Hadorn & Zürcher (1962b) examined the effect of processing-heating on HMF. Gonnet (1963) has pointed out that room-temperature storage led to an increase in HMF, whereas cool storage retarded it. A review of the effect of storage and heating of honey on HMF content is in the paper of White, Kushnir & Subers (1964). The effect of storage and heating of three honeys is shown in Figure 5.84/1, where it can be seen that the logarithm of 'time needed to reach a given HMF level' is linearly related to temperature over a range of (at least) 20-75°C (68-167°F). The considerable differences in rate even among the few honeys examined precludes a mathematical expression of the relationship.

Duisberg & Hadorn (1966) used the Winkler method to examine over 1 600 honeys for HMF; they reported most honeys to contain less than 10 ppm. On the other hand Hallermayer (1969), in discussing quality evaluation of honey, noted a mean value of 33 ppm in 1 500 samples of commercial honey.

5.95 Toxic substances

Since tremendous numbers of organic compounds are synthesized by various plants, many with marked pharmacological activity, it is not surprising that occasionally such materials are found in honey. Possibly many more compounds are present in nectar, but are not detected in the honey because the physiological response (taste, toxicity) is not sufficiently apparent, or in some cases because their action on the bees prevents storage of the nectar. The compounds mentioned in this category are not honey constituents in the sense that they are common to all or most honeys; they have been reviewed by White (1973).

Instances of toxic reactions from ingestion of certain kinds of honey have been reported since antiquity. By far the largest number concern honey from the Ericaceae (*Rhododendron*, *Azalea*, *Andromeda*, *Kalmia* spp.). Various articles (Kebler, 1896, Fühner, 1926, Howes, 1949, Palmer-Jones, 1947a, Gruch, 1957, Carey *et al.*, 1959) review poisoning by honey

from these sources. Recent instances of human poisoning by these honeys are described by Ungan (1940), Jones (1947), Povchenko (1950) and Scott *et al.* (1971). A toxic compound was isolated by Plugge from honey (Carey *et al.*, 1959) in 1883, and by others since that time. Pulewka (1949)

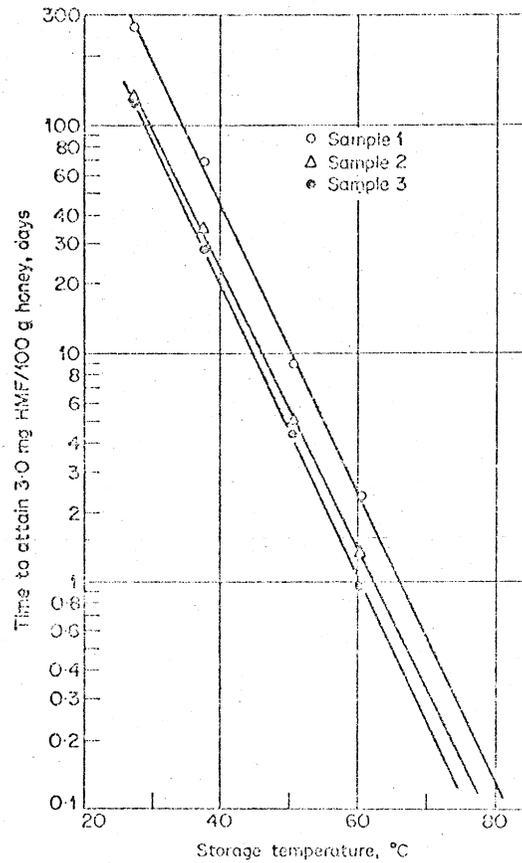


Figure 5.84/1 Effect of storage (processing) temperature on the accumulation of HMF in three honey samples.

(White, Kushnir & Subers, 1964)

and Popova *et al.* (1960) described biological tests for the toxic material in honey. White & Riethof (1959) described a chemical test for this toxic principle in honey, acetylandromedol. Scott *et al.* (1971), investigating a toxic British Columbia honey, isolated three toxic substances in the sample, which was of unknown origin. No acetylandromedol was detected, but three other related compounds were detected by thin-layer chromatography: andromedol, anhydroandromedol, and desacetyl pieristoxin B.

Hazslinsky (1956) described a toxic effect of a Hungarian honey which he ascribed to belladonna (*Atropa*) alkaloids from the nightshade, but Örosi-Pál (1956) claimed that the source was Egyptian henbane (*Datura metel*) and that the poisoning was by scopolamine, not atropine. Lehrner (1955) and Sviderskaya (1959) have described honey poisoning ascribed to atropine; in the latter article the source was said to be *Datura stramonium* and *Hyoscyamus niger*.

Wiley (1892, page 750) described an occurrence of poisoning by honey from the yellow jessamine (*Gelsemium sempervirens*) in which three persons died, of twenty affected. Analysis of the honey showed a large amount of gelsemine present.

Juritz (1925) discussed so-called 'Noors' honey from several species of *Euphorbia* in South Africa which produces a strong burning sensation in the throat. Sanna (1931) reported that a Sardinian honey with a sour, bitter taste was found to contain the glucoside arbutin, derived from *Arbutus unedo*.

Bitter honeys are not uncommon; Joachim & Kandiah (1940) attributed bitterness in a Ceylon honey to alkaloids from pollen of the Ceará rubber plant. Very little chemical work has been done with bitter honeys; likewise, little information is available on the materials responsible for the strong, sometimes nauseating odours and flavours of certain honeys, such as that from *Melaleuca* (Taylor, 1956), *Agave* (Pellett, 1947, page 97) and privet (*Ligustrum*).

Palmer-Jones (1947b) described an outbreak of poisoning in New Zealand, ascribed to eating honey. A new compound, mellitoxin, was isolated from the honey (Sutherland & Palmer-Jones, 1947a). It was shown to be related to tutin, a picrotoxin previously isolated from *Coriaria* species in New Zealand. In a search for the source of contamination in the field, it was noted that honey gathered during the blooming period of the suspected plant, *Coriaria arborea*, was not toxic. The source was then found to be honeydew on the leaves of the plant (Paterson, 1947). Subsequently Hodges & White (1966) isolated tutin and hyenanchin (mellitoxin) from a toxic honey, and Turner & Clinch (1968) tested 150 New Zealand honeys for toxin by both a biological assay and thin-layer chromatography. Samples found toxic by the mouse test (Clinch, 1966) showed spots corresponding to hyenanchin alone, or hyenanchin and tutin. Some indication of other similar substances was obtained.

g.36 Lipids

A qualitative investigation of the ether-extractable lipids in a cotton honey was described by M. R. Smith (1963, Smith & McCaughey, 1966). He found glycerides, sterols, and possibly phospholipids. Using thin-layer and gas chromatography, he identified the acids as palmitic acid 27%,

oleic acid 60%, with small amounts of lauric, myristoleic, stearic, and linoleic acids. Twelve compounds were separated by thin-layer chromatography; ten were unsaturated, one was acid, with three gave a positive reaction to the antimony trichloride test for carotenoids.

Traces of beeswax may be microdispersed in extracted honey; they are probably introduced from the uncapping procedure. This subject is not considered here; such wax should have the general composition of beeswax (Callow, 1963).

5.87 Miscellaneous materials with biological activity

It is probable that materials additional to those described are present, and will be reported, in honey as isolative and analytical procedures improve. For example, (+)-2-hydroxy-3-phenylpropionic acid was isolated from a toxic honey sample (Hodges & White, 1966). Two materials not yet discussed are choline and acetyl choline. Neumann & Habermann (1950-51) showed that honey contained a material that caused contraction of isolated muscle preparations, and reported that the activity was equivalent to a content in honey (and in stored syrup) of 0.2-2.5 μg acetyl choline per gram of honey. By pharmacological methods Marquardt & Vogg (1952) showed that this cholinergic* factor of honey is probably acetyl choline. About thirty times as much choline was also probably present. Assay of 156 honeys showed acetyl choline levels of 0.06-5 mg/100 g (Marquardt, Aring & Vogg, 1953). Goldschmidt *et al.* (1952) also believed that choline was the cholinergic substance, and reported honey to contain 6 mg/100 g of choline. Later (Goldschmidt & Burkert, 1955*b*) they presented further evidence that the factor was acetyl and not propionyl or formyl choline. Schuler (1957) agreed. Watanabe (1955*a*) found no acetyl choline in a floral nectar (Japanese camellia) or in six pollens. The pollen of *Alnus sieboldiana* did contain acetyl choline, and all pollens contained choline. Confined bees were fed (Watanabe, 1955*b*) invert sugar both without and with (10 $\mu\text{g}/\text{ml}$) acetyl choline. The corresponding stored 'honeys' were found to have a biological activity corresponding to about 0.2 $\mu\text{g}/\text{ml}$ of acetyl choline, stores from the food containing acetyl choline showing slightly more. Watanabe concluded that acetyl choline in honey is not derived from pollen or nectar, but is formed by biosynthesis and secreted into the honey. A review on the subject is in the literature (Marquardt & Spitznagel, 1956).

Smith *et al.* (1969) assayed eight honey samples of different floral sources from various regions for several previously alleged biological activities. Yeast growth stimulation greater than that from 1 μg of biotin was found; no real effect was seen on wrist-joint stiffness in guinea pigs.

* A material acting as a chemical transmitter of nerve impulses from parasympathetic nerve ends to the effector organ.

Some samples increased the numbers of roots on plant cuttings, but no oestrogenic activity for rats was found. Rats gained more weight on diets in which honey replaced sucrose or cornstarch.

We have seen that at least 181 substances are known to be present in honey. This total will increase with time, but our increasing knowledge of its complexity must not diminish the pleasure of eating honey, the only sweetener that need not be processed before we enjoy it.