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BIOSYNTHESIS OF WAX IN THE HONEYBEE, *APIS MELLIFERA* L.

GARY J. BLOMQUIST, ARTHUR J. CHU and STEPHEN REMALEY
Department of Biochemistry, University of Nevada, Reno, NV 89557, U.S.A.

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Abstract—The composition of the cuticular wax of the honeybee is quantitatively different from that of the comb wax. The major component of the cuticular lipids is hydrocarbon, which comprises 58% of this wax. In contrast, hydrocarbon comprises only 13–17% of the comb wax, and monoester is the largest component (TULLOCH 1971). The major fractions of the cuticular wax were analyzed by gas–liquid chromatography, and were shown to be qualitatively similar to those of the comb wax. The incorporation of injected radio-labelled acetate into hexane extractable wax by worker honeybees not actively producing comb wax resulted in the recovery of much of the radioactivity in the hydrocarbon fraction. In insects actively producing comb wax, a higher percentage of radioactivity was recovered in the monoester fraction. A dramatic effect of age on the distribution of radioactivity from acetate into the various wax fractions from honeybees studied in the summer months was observed. In insects from eleven to eighteen days following emergence to adults, the major wax component synthesized was monoester, whereas in younger and older insects, hydrocarbon was the major wax component formed. Both *in vivo* and *in vitro* experiments using insects actively producing comb wax showed that the abdomen produced significant amounts of monoester, hydrocarbon, and other esters, whereas the thorax synthesized mostly hydrocarbon. These data show that the epidermal cells and wax glands each produce a wax with a distinct composition, and that the age and seasonal differences observed in wax synthesis are due to the presence or absence of active wax glands.

Key Word Index: honeybee, *Apis mellifera*, beeswax, hydrocarbons, wax esters.

INTRODUCTION

BEESWAX consists of complex mixtures of hydrocarbons, esters, and other aliphatic compounds. It is one of the oldest commerical products in man's history, and its chemistry has been extensively studied (TULLOCH 1970, 1971; TULLOCH and HOFFMAN 1972; HOLLOWAY 1969). However, despite a great deal of interest in the chemistry of beeswax, there have been few studies on its biosynthesis. *In vivo* work by YOUNG (1963) and PIEK (1964) showed that radio-labelled acetate was incorporated into beeswax, and recently BLOMQUIST and RIES (1979) and LAMBREMONT and WYKLE (1979) have described a microsomal system which synthesizes the cuticular and comb wax esters.

Considering the different functions of the comb wax and the cuticular wax in the honeybee, and the fact that comb wax is produced by abdominal glands while the cuticular wax is most probably produced by epidermal cells, it was thought that the cuticular wax might have a different chemical composition from that of the comb wax. To explore this possibility, the cuticular wax of the honeybee was analyzed and the results presented in this paper show that its composition is quantitatively different from that of comb wax.

The development of wax glands in the honeybee has been shown to be an age-related phenomenon (GARY 1975). Since considerable differences were observed between the composition of the cuticular and comb waxes of the honeybee, the effect of age on the incorporation of labelled acetate into the various wax fractions was compared. These data, along with studies demonstrating differences in wax production between wax gland enriched and epidermal cell enriched preparations suggest that the age-related and seasonal differences in the composition and production of wax by the honeybee are due to the presence or absence of active wax glands.

MATERIALS AND METHODS

Insects

Colonies of the honeybee *Apis mellifera* L. were obtained locally and maintained on the University farm. Insects were used throughout the year as specified.

Radioactive substrates

Sodium [2-^{3}H]-acetate (6 Ci/mmole) and sodium [1-^{14}C]-palmitate (52 mCi/mmole) were purchased from ICN Pharmaceuticals, Irvine, California. Sodium [1-^{14}C]-acetate (57 mCi/mmole), sodium [1^{14}C]-stearate (50 mCi/mmole) and sodium [1-^{14}C]-oleate (52 mCi/mmole) were purchased from Research Products International, Elkgrove, Illinois. [1-^{14}C]-glucose (40 mCi/mmole) was purchased from New England Nuclear, Boston, Massachusetts. [R-^{3}H]-tetracosanol was prepared as described earlier (BLOMQUIST and RIES 1979).

Extractions and separations

Lipids were extracted by immersion of the insects in hexane for 10 min followed by rinsing with another aliquot of hexane. The lipid fractions were separated on Adsorbosil-2 thin layer chromatography (TLC) plates developed first in hexane and then twice in hexane:diethyl ether (95:5, v/v). Samples were visualized with Rhodamine 6G under u.v. light, and compared to hydrocarbon, monoester, diester, triester

and fatty acid standards. Each fraction was scraped into a test tube, and lipid was isolated by extraction with diethyl ether (hydrocarbon, esters) or diethyl ether with 0.1% formic acid (free fatty acids and more polar lipids). Quantitation was achieved by weighing each fraction after removal of the solvent under a stream of nitrogen. Known amounts of ^{14}C labelled hydrocarbon and fatty acids were taken through this procedure to determine recovery efficiencies. Between 98 and 100% of the radioactivity from hydrocarbon samples and 93–100% from fatty acid samples was recovered.

Hydrocarbons were separated into saturated and unsaturated fractions by TLC on Absorbosil-2 TLC plates impregnated with 10% (w/w) silver nitrate developed in hexane. Alternatively, samples were separated on 5 cm × 0.5 cm i.d. columns of BioSil A impregnated with 20% (w/w) silver nitrate. Alkanes were eluted with 8 ml hexane and alkenes with 8 ml of hexane-diethyl ether (50:50, v/v) (MAJOR and BLOMQUIST 1978). Gas–liquid chromatography (GLC) was performed on a 1.8 m × 0.3 cm i.d. 3% (w/w) SE-30 on Supelcoport column, temperature-programmed from 200 to 320°C at 8°per min.

Wax monoesters were analyzed on the same column described above, temperature-programmed from 240 to 340°C at 4° per min with a final hold time of 10 min. Esters were saponified by refluxing in 10% (w/w) potassium hydroxide in methanol for 1 hr. The fatty acids were methylated by the addition of 14% BF_3 in methanol, kept at 60°C for 1 hr, water added, and the solution extracted with hexane. The methyl esters and primary alcohols were separated on Absorbosil-2 TLC plates developed in hexane:diethyl ether:formic acid (40:10:2 by vol.). The alcohols were acetylated with acetic anhydride in pyridine. The fatty acid methyl esters were analyzed in a 1.8 m × 0.3 cm i.d. 15% (w/v) EGS-Y on Chromosorb W column, temperature-programmed from 190 to 220°C at 2° per min. The acetylated alcohols were analyzed under the same conditions described for hydrocarbon analysis.

The free fatty acids were methylated with 14% BF_3 in methanol as described above. The methyl esters were analyzed on a 1.8 m × 0.3 cm i.d. 3% (w/w) SE-30 on Supelcoport column, temperature-programmed from 180° to 310°C at 8° per min. The methylated free fatty acids and acetylated alcohols from the monoesters were checked for unsaturation by silver nitrate TLC developed in hexane:diethyl ether (45:5, v/v).

In vivo *studies*

Radio-labelled substrates in 2–5 μl of water (acetate, glucose) or a Tween-80 emulsion (palmitate, stearate, oleate, and tetracosanol) were injected into each insect between the sixth and seventh sternites with a needle inserted parallel to the abdomen to about the third sternite before expulsion of the syringe contents. In one experiment, substrates were layered on the surface of the insect. After 2 hr, the insects were killed and extracted by immersion in hexane as described above. In one study, the thorax and abdomen were excised and extracted separately.

Age studies

Frames with large numbers of pupa were removed from a strong hive and kept at 30° to 34°C. Each day the frames were examined, and newly emerged adults were collected. The insects were marked with an acrylic paint on the dorsal thorax, and returned to the hive. Insects of known age (±12 hr) were removed from the hive as needed.

In vitro *studies*

Adult workers were placed on ice, and kept at 0–4°C during subsequent manipulation. Abdomens were excised, separated into ventral and dorsal halves after an incision on each side of the abdomen, and fat body tissue removed. Thoraxes were excised and used intact. The tissue was placed into incubation vials with labelled substrates as indicated in the text and 0.5 ml of a saline solution containing: NaCl (12g/l), KCl (0.5 g/l), $CaCl_2$ (1,5 g/l), $MgCl_2$ (0.53 g/l) and $NaHCO_3$ (0.2 g/l) added. The vials were incubated for 2 hr at 37°C, and then extracted by the method of BLIGH and DYER (1959).

Determination of radioactivity

After separation of the lipid components by TLC as described earlier, each fraction was scraped into a scintillation vial. Ten millilitres of a counting fluor containing 0.4% 2,5-diphenyloxazole and 4% (w/v) fumed silica (as a thixotropic gel) in toluene was added. Samples were assayed for radioactivity for 10 min using a Beckman liquid scintillation counter. Counting efficiency was determined with an external standard and ranged from 85 to 90% for ^{14}C and 46 to 50% for ^{3}H.

For some samples, radioactivity on TLC plates was detected with a Packard radiochromatogram scanner using helium-isobutane (99.5:0.5) as a quench gas. The monoesters from some samples were isolated and separated into the acyl and alcohol moieties as described earlier, and the radioactivity in each fraction determined by liquid scintillation counting.

The incorporation of radiolabelled precursors into individual hydrocarbon components was determined by radio-gas–liquid chromatography (radio-GLC) using a Hewlett Packard GLC interfaced to a Packard combustion flow-through proportional counter. Hydrocarbons were separated on a 1.8 m × 0.3 cm i.d. 3% (w/w) Dexil-400 on Chromosorb W column temperature programmed from 200 to 300°C at either 2° or 4° per min. Counting efficiency for ^{14}C was about 40%.

RESULTS AND DISCUSSION

Composition of the comb and cuticular wax

A number of studies have established that the largest component of the comb wax of honeybees is monoester (TULLOCH 1971; TULLOCH and HOFFMAN 1972; HOLLOWAY 1969), with hydrocarbon comprising a lesser percent of the comb wax (Table 1). In contrast, an examination of the cuticular lipids of the honeybee (Table 1) shows that in extracts obtained from insects during the winter months, hydrocarbon was the major fraction, followed by monoester and lower amounts of diester, triester, free fatty acids and

Table 1. A comparison of the composition of the cuticular and comb wax of the honeybee *A. mellifera* L.

Class	Percent by weight Cuticular wax*	Comb wax†
Hydrocarbon	58‡	13–17
Monoester	23	31–35
Diester	9	10–14
Triester	2	3
Free fatty acids and more polar material	9	34

*Cuticular wax was obtained from insects not actively producing comb wax during March 1978. Material was extracted with hexane, separated by TLC, and weights determined as described in Methods. Three groups of about 300 insects per group were used.

†Data taken from TULLOCH (1970, 1971), TULLOCH and HOFFMAN (1972), STRANSKY *et al.* (1971), and STRANSKY *et al.* (1972).

‡The range for each value is less than 20% of the mean.

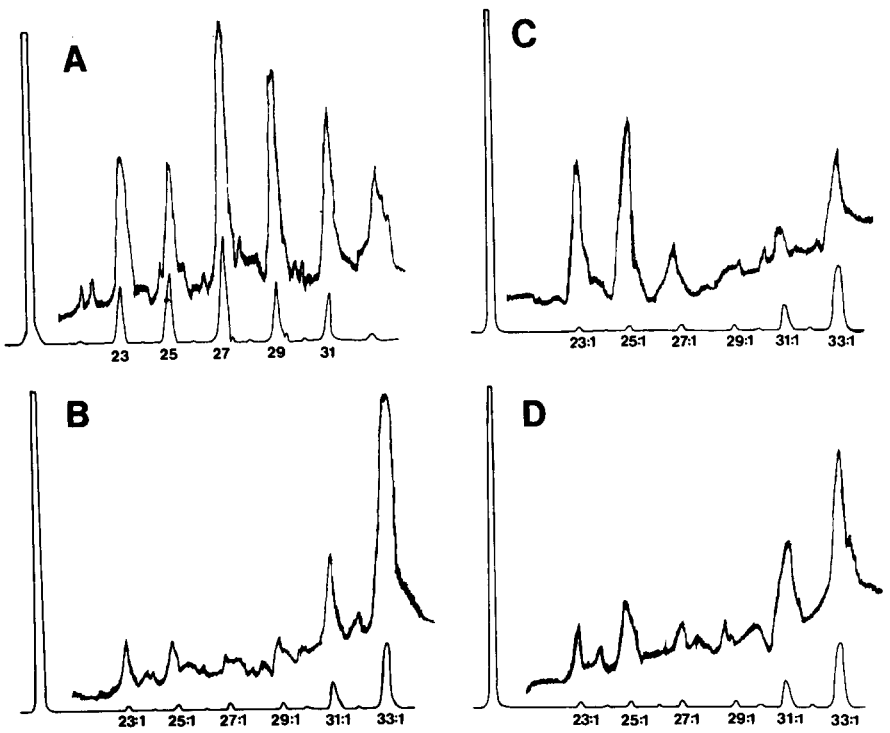

Fig. 1. Radio-GLC of the hydrocarbons from the honeybee, *A. mellifera* L. after the incorporation of labelled substrates. Substrates were injected into the abdomen of the insect, and after 2 hr the insects were killed, extracted, hydrocarbon isolated, separated into alkane and alkene fractions, and analyzed by radio-GLC as described in Methods. The lower line is the mass trace and the upper line is the radioactivity trace. The numbers are the carbon numbers, with the first number representing the carbon number and the second the number of double bonds. (A) Distribution of [1-^{14}C]-acetate into saturated hydrocarbons. (B) Distribution of [1-^{14}C]-acetate into the unsaturated hydrocarbons. (C) Distribution of [1-^{14}C]-oleate into unsaturated hydrocarbons. (D) Distribution of [1-^{14}C]-stearate into the unsaturated hydrocarbons.

more polar material. In extracts of insects obtained during the summer months, when some of the insects were actively producing comb wax, a lower percentage of hydrocarbon and a higher percentage of monoester were observed (data not shown), suggesting that these extracts contained mixtures of comb and cuticular wax. Considering the different functions of the comb and cuticular waxes, and the high percentage of hydrocarbon in the cuticular waxes from a number of other insects (BLOMQUIST and JACKSON 1979; NELSON 1978), such differences between comb and cuticular wax were not surprising.

It has been shown that saturated hydrocarbons comprise 66–78% of the comb wax hydrocarbons (STREIBL *et al.*, 1966), whereas alkanes comprise 48% of the cuticular hydrocarbons (BLOMQUIST *et al.*, 1980), with 52% of the hydrocarbon comprised of unsaturated components. In both the comb and cuticular wax, the shorter chain C_{23}, C_{25}, C_{27} and C_{29} alkenes were primarily (Z)-9 components, the C_{31} alkene about equally (Z)-8 and (Z)-10, and the C_{31} alkene primarily (Z)-10 (STREIBL *et al.*, 1966; BLOMQUIST *et al.*, 1980). Since the compositions of the hydrocarbons from the comb and cuticular wax of the honeybee differed somewhat quantitatively, and honeybees have been shown to generally produce comb wax from days twelve to eighteen after adult emergence (GARY 1975), and examination of the composition of the hydrocarbons from insects of various ages was undertaken. Dramatic differences were observed in the hydrocarbon compositions between insects of different ages. There was an increase in the saturated component from 29.0% at day 7 to 76.2% at day 26 (Table 2). Similarly, there was an increase in the number of shorter chain length components with age. At day 7, components with chain lengths from C_{23} to C_{29} comprised 25.2% of the hydrocarbons, whereas by day 26 they comprised 87.0% of the hydrocarbon fraction. The composition of the hydrocarbons of the insects 7, 9, 16, 18, 22, and 26 days after adult emergence are listed in Table 2, along with the percentage of saturated and unsaturated components and shorter chain and longer chain components. The changes in the composition of the hydrocarbons appear to be an age related phenomenon, and do not show clear differences between times when insects were producing comb wax (wax scales were observed on most insects from day 11 to day 18) and when they were presumably producing only cuticular wax (older and younger insects).

The monoesters of the cuticular wax are similar to those of the comb wax (TULLOCH, 1971). The cuticular wax monoesters range in carbon chain length from C_{36} to C_{50} (Table 3), and are comprised mainly of fatty acids of 16 and 18 carbons, and of saturated primary alcohols of C_{20} to C_{32}. The major primary alcohol is tetracosanol, which comprises 42% of this fraction (Table 3).

Table 2. Changes in the composition of the major hydrocarbons of the honeybee with age

Hydrocarbon component	Percentage composition 7 days	9 days	16 days	18 days	22 days	26 days
nC_{23}	2.8†	2.5	2.3	4.1	3.8	25.8
$nC_{23:1}$	0.7	0.4	0.3	0.2	1.1	4.6
nC_{25}	3.0	3.0	2.5	5.7	5.7	27.2
$nC_{25:1}$	1.3	0.9	1.1	1.3	2.3	7.6
nC_{27}	7.8	9.4	7.8	11.9	14.5	12.4
$nC_{27:1}$	0.7	0.3	0.2	3.2	1.1	1.8
nC_{29}	7.1	10.4	9.9	10.0	9.0	6.8
$nC_{29:1}$	1.8	1.6	2.2	1.1	2.6	0.8
nC_{31}	6.9	8.5	8.3	7.5	5.7	3.6
$nC_{31:1}$	19.4	17.7	22.8	19.6	17.2	3.2
nC_{33}	1.4	1.5	1.4	1.0	1.0	0.4
$nC_{33:1}$	43.6	41.2	40.1	32.4	35.2	5.6
$nC_{35:1}$	3.5	2.6	1.1	0.7	0.8	0.2
% Saturated	29.0	35.3	32.2	40.2	39.7	76.2
% Unsaturated	71.0	64.7	67.8	59.8	60.3	23.8
% C_{23}–C_{29} Components	25.2	28.5	26.3	37.5	40.1	87.0
% C_{31}–C_{35} Components	74.8	71.5	73.7	62.5	59.9	13.0

* Insects were used during June 1979. Material was extracted, separated, and analyzed by GLC as described in Methods. Identification of each component has been previously reported (BLOMQUIST *et al.*, 1980).

† Values are the mean of two groups of fifteen insects per group. The range for each value was less than 15% of the mean.

The major free fatty acid in the cuticular lipids of the honeybee is tetracosonoic acid (Table 4). Lesser amounts of free fatty acids in the range C_{16}–C_{36} are present, and all the free fatty acids are saturated. The composition of the free fatty acids present in the cuticular waxes is similar to that reported for the fatty acids of comb wax (TULLOCH 1971). Because of the small amounts of di- and triesters in the cuticular wax, further characterization of these fractions was not undertaken.

Table 3. Composition of the monoesters and their component fatty acids and alcohols from the cuticular wax of the honeybee *A. mellifera* L.*

Carbon number	Percent composition
wax esters	
36	Tr†
38	4‡
40	26
42	14
44	9
46	14
48	24
50	8
Resulting fatty acid	
16:0	54
18:0	6
18:1	36
20:0	Tr
22:0	Tr
Unidentified	3
Resulting primary alcohols	
20	4
24	42
26	15
28	9
30	14
32	16

* Insects were used in March 1978. Material was extracted, isolated by TLC, and analyzed by GLC as described in methods.

† Trace is less than 0.5 and greater than 0.1%.

‡ Values are the mean of three determinations. The range for each value is less than 10% of the mean.

Biosynthesis of beeswax: hydrocarbons

Labelled acetate, oleate and stearate were readily incorporated into the hexane-extractable wax of the honeybee. The movement of radio-label into the various fractions from acetate was shown to be a seasonal and age-related phenomenon (Table 5 and Figs. 2 and 3). Using mixed-age insects in July 1979, $0.23 \pm 0.07\%$ of [1-^{14}C]-acetate, $0.02 \pm 0.01\%$ of [1-^{14}C]-stearate, and $0.03 \pm 0.01\%$ of [1-^{14}C]-oleate was incorporated into hydrocarbon. Of the labelled

Table 4. Free fatty acids of the cuticular wax of the honey bee *A. mellifera* L.*

Fatty acid	Percentage composition
16:0	7†
18:0	7
22:0	4
24:0	29
26:0	12
28:0	11
30:0	9
32:0	9
34:0	9
36:0	3

* Cuticular wax was obtained from insects not actively producing comb wax in March 1978. Material was extracted, separated by TLC, methylated, and analyzed by GLC as described in Methods.

† Values are the mean of three determinations. The range for each value is less than 20% of the mean.

Table 5. Incorporation of [1-^{14}C]-acetate into the wax of the honeybee *A. mellifera*

Fraction	Percent distribution: Insects not producing comb wax*	Insects actively producing comb wax†
Hydrocarbon	47±3‡	32±6
Monoester	18±2	35±7
Diester	7±1	9±2
Triester	12±1	3±1
Free fatty acid and more polar lipid	16±3	21±4

* Insects used in April 1978 when they were not actively producing comb wax.

† Insects were actively producing comb wax as evidenced by wax scales on the ventral abdomen. Insects were used in June 1978.

‡ Values are the mean ± S.D. Five groups of five insects per group were used in each experiment.

acetate incorporated into hydrocarbon, 63.6±8.2% was recovered in the saturated components and 36.4±8.2% in the alkenes. A similar distribution was observed for stearate, with 65.4±3.1% recovered in the alkane fraction and 34.6±3.1% in the unsaturated hydrocarbons. In contrast, [1-^{14}C]-oleate preferentially labelled the alkene fraction, with 62.4±4.6% of the radio-label incorporated into hydrocarbon recovered in the unsaturated hydrocarbons. All values ± standard deviation.

An examination of the incorporation of these substrates into each hydrocarbon component separated by radio-GLC showed that [1-^{14}C]-acetate labelled all components [Fig. 1(A), (B)]. The major alkane labelled was *n*-heptacosane, with lesser amounts of radioactivity incorporated into the other components. The major alkene labelled from [1-^{14}C]-acetate was the C_{33} component, with significant amounts of radioactivity also incorporated into the C_{31} alkene [Fig. 1(B)]. The incorporation of [1-^{14}C]-oleate into the saturated alkanes was similar to that of [1-^{14}C]-acetate (data not shown), suggesting that it labelled the alkanes after β-oxidation to acetyl-CoA. An examination by radio-GLC of the incorporation of [1-^{14}C]-oleate into the alkene fraction [Fig. 1(C)], showed that [1-^{14}C]-oleate preferentially labelled the shorter chain (Z)-9 C_{23} and C_{25} components [Fig. 1(C)]. This suggests that oleate was directly elongated and decarboxylated (MAJOR and BLOMQUIST 1978; CHU and BLOMQUIST, unpublished observation) to form the (Z)-9 alkenes, and was probably β-oxidized to acetyl-CoA which then labelled the (Z)-10 C_{33} alkene at lower levels. [1-^{14}C]-stearate was incorporated into each of the saturated alkanes, and was preferentially incorporated into the C_{33} and C_{31} alkenes [Fig. 1(D)]. Lesser amounts of radioactivity from this substrate were also incorporated in the C_{25} and C_{23} alkenes. These data show that the hydrocarbons are readily formed from acetate, and suggest that the alkenes are formed from fatty acids desaturated at the (Z)-8, (Z)-9, and (Z)-10 positions. The (Z)-9 components are apparently elongated and decarboxylated to form the shorter chain C_{23}, C_{25}, C_{27}, and C_{29} alkenes, whereas the (Z)-8 and (Z)-10 intermediates are used to form the longer chain C_{31} and C_{33} alkenes. An examination of the fatty acids of the honeybee showed that the 18:1 fatty acid was exclusively ω-9 (BLOMQUIST, unpublished).

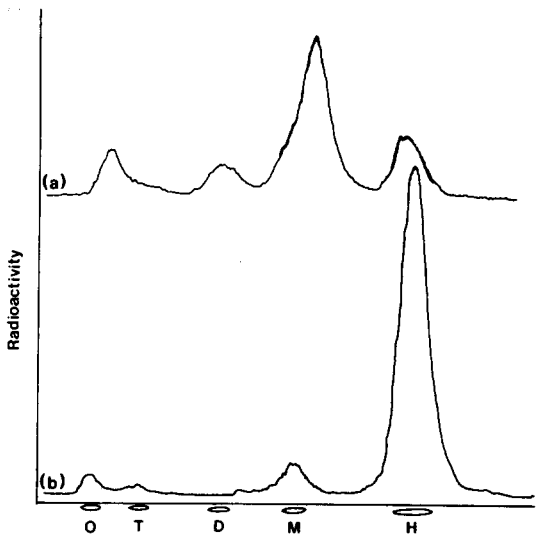

Fig. 2. Radio-TLC trace of hexane-extractable lipids formed from labelled acetate by worker honeybees 14 (a) and 26 (b) days following adult emergence. Insects were injected with [1-^{14}C]-acetate, sacrificed after 2 hr, extracted and the lipid fractions analyzed as described in Methods. H = hydrocarbon, M = monoester, D = diester, T = triester and O = origin.

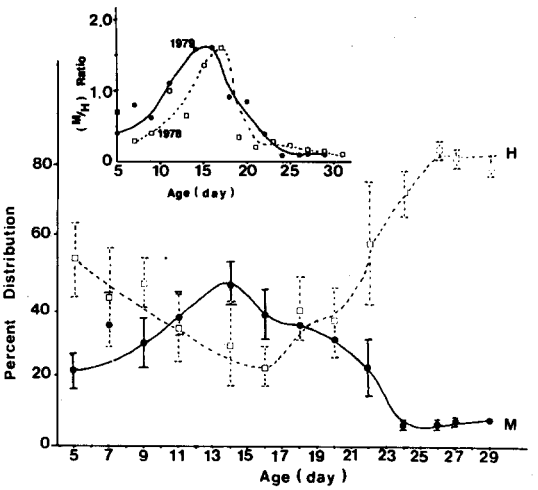

Fig. 3. Distribution of [1-^{14}C]-acetate into the hydrocarbon and monoester fractions in worker honeybees from 5 to 29 days after emergence to adult. Insects were used in June 1979. Insects of appropriate age were injected with [1-^{14}C]-acetate, killed after 2 hr, and extracted and analyzed as described in Methods. H = hydrocarbon, M = monoester. The inset shows the monoester–hydrocarbon ratio for the same experiment performed in 1978 and 1979. In the experiment in 1978, [^{3}H]-acetate was used rather than [1-^{14}C]-acetate. The error bars represent the standard deviation of the data from five groups of five insects per group.

Monoester biosynthesis

The results of examining the distribution of radio-labelled [1-^{14}C]-acetate and [1-^{14}]-glucose between the alcohol and acyl groups of the monoesters showed

Table 6. Incorporation of labelled acetate, palmitate, tetracosanol, and glucose into the alcohol and acid portions of monoesters in the honeybee *A. mellifera* *

Substrate	Radioactivity incorporated into monoesters %	
	Acyl group	Alcohol
[1-^{14}C]-Acetate	40±8†	60±8
[1-^{14}C]-Glucose	41±7	59±7
[1-^{14}C]-Palmitate	69±5	31±5
[R-^{3}H]-Tetracosanol	0±1	100±1

* Labelled substrates were injected just beneath the cuticle of the ventral abdomen. After 2 hr the insects were killed, extracted, and the monoesters isolated by TLC. The monoesters were saponified, methylated, and the alcohols and methyl esters were separated by TLC and assayed for radioactivity by liquid scintillation counting as described in Methods.

† Values are the mean ± S.D.

that these labelled substrates were present in both fractions. Two-fifths of the label from acetate and glucose were incorporated into the acyl portion of the monoester and three-fifths into the alcohol portion (Table 6), which mirrors the mass distribution of each component. The acyl portion contains C_{16} and C_{18} components, whereas the alcohol portion consists of C_{20}–C_{32} components. Labelled palmitate was preferentially incorporated into the acyl portion of the monoester (Table 6), suggesting that some of this substrate was directly esterified as the acyl-CoA derivative (BLOMQUIST and RIES 1979). [R-^{3}H]-tetracosanol was incorporated exclusively into the alcohol portion of the wax ester.

The incorporation of [R-^{3}H]-tetracosanol and [1-^{14}C]-palmitate varied considerably depending upon the method of application. Labelled tetracosanol was incorporated into monoester when the substrate was injected into the insect to the extent of 0.96±0.3% of administered radio-label, compared to 7.2±2.4% when it was applied to the surface of the insect (Table 7). In contrast, labelled palmitate was more efficiently incorporated into monoester when it was injected into the insect (Table 7). Experiments with isolated cuticle showed that both substrates were incorporated into monoester more efficiently when the substrate was applied to the inner surface of the cuticle prior to incubation. These data show that the site of esterification is closely associated with the epidermal cells.

Incorporation of [1-^{14}C]*-acetate into the major lipid fractions*

Since the main difference between the comb and cuticular waxes of the honeybee was the variation among the lipid classes, it was of interest to determine if the same differences would be apparent in the incorporation of radioactive substrates into each fraction in insects not producing comb wax and in insects with active wax glands. Insects used during April 1978 readily incorporated labelled acetate into hexane-extractable waxes, with much of the label incorporated into hydrocarbon (Table 5), and lower percentages into the esters and other lipids. This distribution of label is similar to that of the cuticular wax (Table 1). In honeybees used in June and with active wax glands (evidenced by the production of wax scales on the ventral abdomen) lower amounts of hydrocarbon and higher amounts of monoester were produced (Table 5). Since this distribution of label was intermediate between those of the comb and cuticular waxes (Table 1), it probably reflects the biosynthesis of both types of wax.

Age studies

It is generally accepted that the activities in which bees engage (i.e. cell cleaning, feeding larvae, building activities, gathering nectar and pollen, etc.) are age-related phenomena, although there is great flexibility in the age–activity relationship. The comb wax of bees is secreted by wax glands located on the ventral abdomen, and the wax glands are best developed and most productive in bees twelve to eighteen days old (GARY 1975). Since the composition of cuticular and

Table 7. Incorporation of [1-^{14}C]-palmitate and [R-^{3}H]-tetracosanol into the monoesters of honeybees *in vivo* and by cuticle preparations

Experiment *in vivo**	Percent of substrate incorporated into monoesters	
	[R-^{3}H] tetracosanol	[1-^{14}C] palmitate
Injected	0.96±0.3†	0.58±0.1
Layered on surface	7.2±2.4	0.1± <0.01
Cuticle Tissue‡		
Applied to inner surface	8.8±3.0	3.0±0.7
Applied to outer surface	4.9±1.3	0.7±0.1

* Labelled substrates in a Tween-80 emulsion were either injected just beneath the abdominal cuticle or layered on the surface of the insect. After 2 hr, the animals were killed, extracted, lipids separated by TLC, and the monoester fraction assayed for radioactivity by liquid scintillation counting as described in Methods.

† Values are the mean ± S.D.

‡ Epidermal tissue was obtained, and labelled substrates layered on the surface. The tissue was then incubated for two hours in 1 ml of insect saline at 37°C. Lipids were extracted with chloroform, separated by TLC and assayed for radioactivity by liquid scintillation counting as described in Methods.

comb waxes of honeybees differs considerably and the distribution of labelled acetate into the various wax fractions is different between bees producing comb wax and those not actively producing comb wax, a study was undertaken to monitor the incorporation of labelled acetate into the various wax fractions from adult emergence until the insects were one month old (during June, 1979).

Between days 5 and 22, 0.6–0.9% of the radioactivity from the labelled acetate administered was incorporated into hexane-extractable wax by worker honeybees. In older insects (days 24–29), a higher percentage of the labelled acetate (1.2–1.5%) was incorporated into wax.

Considerable differences were observed in the distribution of label into the various wax fractions during different age periods. An example of the type of data obtained is presented in Fig. 2, which shows the relative amount of label incorporated into each lipid fraction in 14- and 26-day-old insects. The major wax fraction labelled from [1-^{14}C]-acetate by 14-day-old insects was monoester, with lesser amounts of radioactivity incorporated into hydrocarbon and the other esters. In contrast, 26-day-old insects incorporated labelled acetate primarily into the hydrocarbon fraction.

Most of the radioactivity throughout the twenty-nine day time period examined was incorporated into hydrocarbon and monoester, and the percent distribution of these components is plotted in Fig. 3. Hydrocarbon was the major fraction labelled between days 5 and 10, and days 19 and 29, whereas monoester was the major component labelled between days 11 and 18. Almost identical data were obtained in a similar experiment in June 1978 using ^{3}H-acetate. The inset (Fig. 3) shows the monoester–hydrocarbon ratio for both the 1978 and 1979 experiments. In addition, 5–9% of the labelled acetate incorporated into wax was recovered in the diester fraction up to day 20, and 1–3% between days 22 and 29. The incorporation of label into triester was also quite low throughout the time period, ranging between 3 and 9% of the label incorporated into wax. Wax scales were usually observed on the ventral abdomen from 11 to 18 days after adult emergence, indicating that comb wax synthesis was taking place at this time. The production of comb wax correlates closely with the increase in monoester synthesis and the decrease in hydrocarbon synthesis from day 11 to day 18. These data are consistent with the composition of the comb and cuticular wax, and the distribution of label into the various wax fractions by insects actively producing comb wax and those which do not have active wax glands.

Many other factors in addition to age can effect wax production in the honeybee, such as season, the presence of a queen, the need for comb wax production, the size of the colony, etc. One of the most easily controlled factors is the time of year in which the experiments were performed. The results in this paper show that the season can play an important role in comb wax production. When an age–wax production study was performed in September 1978, hydrocarbon was the major component synthesized throughout the time period (Fig. 4). Thus, the increase in monoester synthesis observed in the June experiments appears to be an indicator of comb wax production, and was not observed in the September experiments. Likewise, wax scales were not observed in honeybees used in September.

It should be pointed out that whatever physiological factors are responsible for inducing and turning off wax production in a strong 'normal' hive, the factors only effect insects of certain ages. Older and younger insects subject to the same behaviour cues do not produce comb wax. However, under special conditions, bees of various ages can apparently be induced to produce comb wax (GARY 1975). Further studies are needed to determine the control mechanism(s) for the production of comb wax in the honeybee.

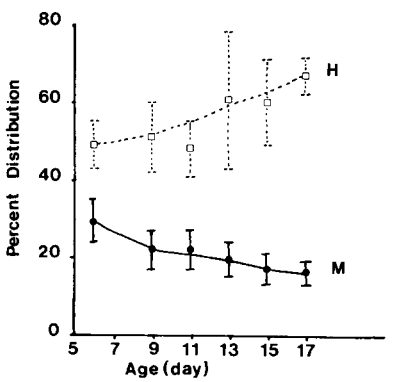

Fig. 4. Distribution of [1-^{14}C]-acetate into the hydrocarbon and monoester fractions in worker honeybees from 5 to 17 days following emergence to adults. Insects were used in September 1978. Insects of appropriate age were injected with [1-^{14}C]-acetate, sacrificed 2 hr later, and extracted and analyzed as described in Methods. H = hydrocarbon, M = monoester. The error bars represent the standard deviation of data from five groups of three insects per group.

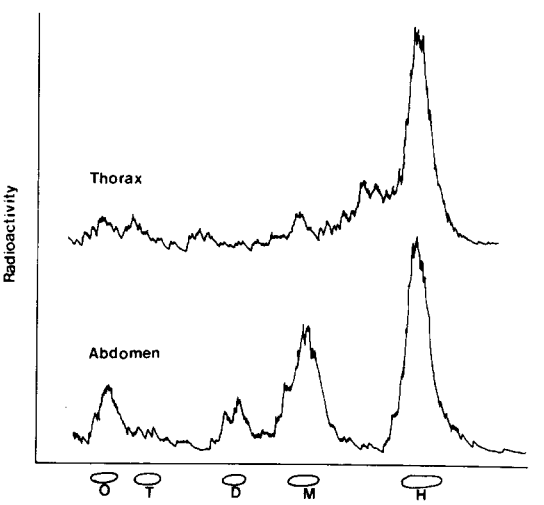

Fig. 5. Radio-TLC trace showing the distribution of [1-^{14}C]-acetate into wax fractions in the thorax and abdomen of worker honeybees actively producing comb wax. Insects were injected, killed after 2 hr, and the lipid extracted and analyzed as described in Methods. H = hydrocarbon, M = monoester, D = diester, T = triester, and O = origin.

Location of wax synthesis

Since it appeared that the wax produced by the wax glands differed appreciably from the cuticular wax, the distribution of labelled acetate into the various wax fractions was compared in the abdomen (which contained the wax glands) and thorax. After injecting the labelled substrate into the abdomen and allowing a 2 hr incubation period, the thorax and abdomen were excised and analyzed separately. The data (Fig. 5) showed that the abdomen had appreciable amounts of esters, whereas the thorax produced hydrocarbon almost exclusively.

The incorporation of [1-^{14}C]-acetate into hydrocarbon and monoester by fat body, thorax, and both dorsal and ventral cuticle tissue from the abdomens of bees actively producing comb wax was determined. Fat body tissue did not efficiently incorporate labelled acetate into either hydrocarbon or monoester. The ventral abdominal cuticle, which contained the wax glands, incorporated labelled acetate preferentially into monoester, producing a wax with a monoester–hydrocarbon ratio of 2.82 ± 1.60. The dorsal abdominal cuticle tissue incorporated labelled acetate roughly equally into monoester and hydrocarbon fractions (monoester–hydrocarbon ratio of 1.15 ± 0.2), whereas the thorax tissue preferentially incorporated labelled acetate into hydrocarbon (monoester–hydrocarbon ratio of 0.28 ± 0.09). These data demonstrate that the site of cuticular wax synthesis in the honeybee, as in other insects (ARMOLD and REGNIER 1975; BLOMQUIST and KEARNEY 1976; CONRAD and JACKSON 1971; NELSON 1969; DIEHL 1973, 1975), is closely associated with the cuticle. It further demonstrates that the production of large amounts of monoester is dependent upon the wax gland.

In one of the first reports on the biosynthesis of beeswax, PIEK (1964) found that when worker honeybees were fed with deuterated water, [1-^{14}C]-acetate or [U-^{14}C]-glucose, the label was incorporated into the hydrocarbons and free acids but not into the wax esters. He postulated that the oenocyte cells of the wax gland synthesize the wax acid and hydrocarbon components of beeswax from acetate, but that the fat cells in the gland do not take up acetate and therefore the wax esters are not labelled. LAMBREMONT and WYKLE (1979) suggested that Piek's failure to see incorporation of labelled precursors into the ester fractions might have been due to the very low specific activities of the labelled compounds fed to the bees. Another possibility, based on the result of this paper, is that Piek might have observed primarily cuticular wax synthesis during the 10–20 days in which labelled precursors were fed to the young bees (PIEK 1964). If this were the case, one would expect to observe the hydrocarbon labelled much more readily than the wax esters. Whatever the explanation for Piek's data, the results of this paper emphasize the importance of controlling the age and physiological state of the insect when studying wax production in the honeybee.

The biosynthesis of hydrocarbons in several cockroaches and termites has been shown to occur by the decarboxylation of very long chain fatty acids (MAJOR and BLOMQUIST 1978; CHU and BLOMQUIST, unpublished), whereas monoester synthesis probably occurs by the reduction of very long chain fatty acids to primary alcohols (LAMBREMONT 1972) followed by esterification with fatty acyl-CoAs (BLOMQUIST and RIES 1979; LAMBREMONT and WYKLE 1979). It is tempting to speculate that the production of hydrocarbons by epidermal tissue and the production of predominantly wax esters by wax gland tissue may reflect differences in the amount of very long chain fatty acid decarboxylase and reductase activity in these tissues. Further work is required to verify this hypothesis.

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