The results obtained show that the use of the 2 techniques of enzymic hydrolysis and of chromatographic separation (TLC and GLC) can usefully aid in demonstrating the presence of a minor oligosaccharide component in a complex sugar mixture.

‰ *Experimental.* The analyses were carried out on samples the honeydews produced by the following aphids and collected in the years 1978–1981: *C. laricis* on *Larix decidua* Mill., *Tuberolachnus salignus* (Gmelin, 1799) on *Salix* sp., *Chaitophorus tremulae* Koch, 1854 on *Populus tremula* L., *Eucallipterus tiliae* (Linnaeus, 1758) on *Tilia cordata* Mill., *Myzocallis castanicola* Baker, 1917 on *Castanea sativa* Mill., *Aphis craccivora* Koch, 1854 on *Robinia pseudoacacia* L. Reference sugars were obtained from commercial sources.

TLC was performed on standard 20 × 20 cm plates, coated with Kieselgel G (Merck, Darmstadt, FGR), layer thickness 250 μm, with the solvent system chloroform-acetic acid-water (6:7:1), as described in a preceding paper[6]. 15 μl of a 2.5% aqueous solution of freeze-dried material was applied on the plate in 2 cm bands. The spots were detected with the spray reagent diphenylamine aniline phosphate.

GLC was carried out on a Fractovap 2400 V (Carlo Erba, Milano, Italy) dual-column chromatograph, fitted with hydrogen-flame detectors, temperature programming unit, recorder, and electronic digital integrator. Standard twin glass U-columns (3 mm × 2 m) packed with 3% OV 101 on GCP S (100–120 mesh) were used. The separations were generally achieved under the following conditions: initial nitrogen flow-rate, 25 ml/min; air flow-rate, 400 ml/min; linear temperature programming at 3 °C/min (150–300 °C). The trimethylsilyl derivatives were prepared by Ellis's method[7].

Hydrolysis with β-D-fructosidase (Boehringer, Mannheim, FGR) was carried out in the test tube with a 0.01% aqueous solution of the enzyme (30 min at 37 °C), stopping enzyme action by heating for 3 min at 100 °C. Hydrolysates were analyzed by TLC and GLC under the conditions described above.

TLC data for the sugar components of the investigated honeydews and of their invertase hydrolysates. The values in parentheses indicate the number of experimental data used in the determination of the hR_{Glc} values ($hR_{Glc} = 100 \times R_F$ relative to glucose) for each sample

	Standards	Honeydew	Invertase hydrolysate
D-fructose	103–112 (10)	107–113 (12)	110–114 (6)
D-glucose	100	100	100
D-galactose	92–94 (10)	a	a
Sucrose	73–77 (10)	72-76 (12)	–
Maltose	58–62 (10)		
Turanose	56–60 (10)	57–65[b] (12)	56–61[b] (6)
Isomaltose	55–57 (10)	–	–
Trehalose	54–58 (10)	54–58 (5)	c
Melibiose	44–48 (10)	–	44–49 (5)
Raffinose	30–37 (10)	34–37[b] (12)	34–38[b] (6)
Melezitose	32–35 (10)		
Tetrasaccharides[d]	12–15 (10)	15–20 (12)	14–18 (5)

[a] When present in small quantities, galactose is partially masked by glucose. [b] Due to the very close migration rates, the 2 components are not resolved. [c] The corresponding band, too weak, was not sufficiently defined. [d] The standard was stachyose.

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Dinemorphan N-demethylation by mouse liver microsomes

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Summary. Dinemorphan, an antitussive drug, is N-demethylated in vitro by mouse liver microsomes with biphasic kinetics showing two apparent K_m and V_{max}. Moreover, dinemorphan N-demethylation is inhibited by CO, SKF-525A, metyrapone and it is specifically catalyzed by a phenobarbital-inducible form of cytochrome P-450.

Dinemorphan (d-3-methyl-N-methylmorphinan) phosphate (fig. 1) is an antitussive drug, supplied commercially as Astomin®, which has a structure and properties similar to the well known drug, dextromethorphan[2].

In the literature there are no reports on the metabolism of dinemorphan, either in vivo or in vitro. However, when dinemorphan was administered in vivo to several species, N-demethylated metabolites were found in the urine (Yamanouchi Pharm. Co., private communication). The N-demethylation of dextromethorphan has been studied but only in postmitochondrial fractions[3,4]. The present paper is concerned with the N-demethylation of dinemorphan and its kinetic parameters, using liver microsomes from mice treated with various inducers.

Materials and methods. 7–9-week-old male mice (25–35 g) of the Swiss albino CD1 strain (Gentili, Pisa) were housed in clear plastic cages with a bedding of wood shaving and were provided with tap water and food pellets (Altromin®; Rieper, Bolzano, Italy) ad libitum. The mice, when induced, were treated with phenobarbital (PB; 80 mg/kg, i.p.) in saline for 3 days prior to being killed (24 h after final injection), or with a single i.p. injection of 3-methylcholanthrene (3-MC; 80 mg/kg) in corn oil 48 h before sacrifice. Hepatic microsomes were prepared as previously

Apparent V_{max} and K_m values for microsomal dinemorphan N-demethylase activity after different pretreatments

Treatment	Cytochrome P-450 (nmol/mg protein)	V_{max1} (nmol/min/ mg protein)	(nmol/min/ nmol cyto-chrome P-450)	V_{max2} (nmol/min/ mg protein)	(nmol/min/ nmol cyto-chrome P-450)	K_{m1} (mM)	K_{m2} (mM)
Control	0.84 ± 0.11	1.52 ± 0.27	1.81 ± 0.31	3.25 ± 0.64	3.87 ± 0.75	0.19 ± 0.13	1.39 ± 0.41
3-MC	1.31 ± 0.28**	1.34 ± 0.24	1.02 ± 0.18**	2.95 ± 0.87	2.26 ± 0.70*	0.03 ± 0.02	0.38 ± 0.23**
PB	2.15 ± 0.33**	7.98 ± 1.18**	3.70 ± 0.55**	19.15 ± 3.1**	8.9 ± 1.4**	0.03 ± 0.01	0.56 ± 0.24*

Values are reported as an average ± SD for experiments performed at least in duplicate with 3 different preparations of hepatic microsomes for each treatment.
* $p < 0.05$; ** these values differ significantly from those of control microsomes, $p < 0.01$ (Student's t-test).

described[5]. The rate constants of dinemorphan N-demethylase activity were obtained by assaying, after 5 min, the formaldehyde production by the Nash method[6].

Reaction mixtures (3 ml) were incubated at 37 °C under air in a Dubnoff Shaker and, unless otherwise stated, they contained microsomes (1 mg protein/ml, $MgCl_2$ (5 mM), NADP+ (0.5 mM), glucose-6-phosphate (10 mM), EDTA (1 mM), glucose-6-phosphate dehydrogenase (2 units), Tris-HCl (0.1 M, pH 7.4), and the required concentrations of substrate (in Tris-HCl, added by microsyringe). When used, the inhibitors were dissolved in DMSO (20 µl). Microsomal protein concentrations were determined by the method of Lowry et al.[7] using bovine serum albumin as standard.

Cytochrome P-450 was measured as described by Omura and Sato[8] and difference spectra were obtained as previously described[5]. Dinemorphan phosphate was supplied by Gentili (Pisa, Italy).

Results and discussion. Dinemorphan phosphate was N-demethylated by mouse liver microsomes giving rise to formaldehyde. The N-demethylase activity was linear up to 6 min with 1 mg/ml of microsomal proteins from PB-treated mice; with higher concentrations of protein enzyme activity was linear only for shorter times.

The kinetic parameters for the N-demethylation of dinemorphan were determined using microsomes from either untreated, PB-treated, or 3-MC-treated mice. In all cases (treated or untreated mice) the 'double reciprocal' plot showed a break from linearity pointing to different enzymatic components, which appeared to be most widely divergent in PB-microsomes. The Lineweaver-Burk plot for the production of formaldehyde from dinemorphan by microsomes from PB-treated mice is shown in figure 2.

Non-linearity in the 'double reciprocal' plot of monooxygenase activities has been found by other authors for aminopyrine-N-demethylase[9], ethylmorphine N-demethylase[10], ethoxycoumarin O-deethylase[11,12] and styrene monooxygenase[13] activities.

The presence of different cytochrome P-450 species in microsomes[14] may explain the presence of high and low affinity components, although an inhibition by products cannot be excluded as such an effect has been shown with aminopyrine[15].

For control and pretreated mice 2 apparent K_m and V_{max} values could be calculated, and they are presented in the

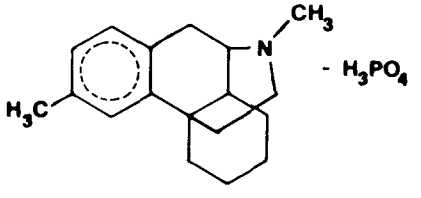

Figure 1.

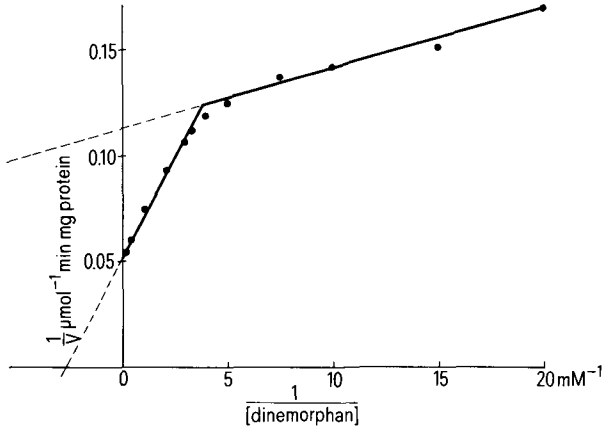

Figure 2. Lineweaver-Burk plot for the production of formaldehyde from dinemorphan by microsomes from PB-treated mice.

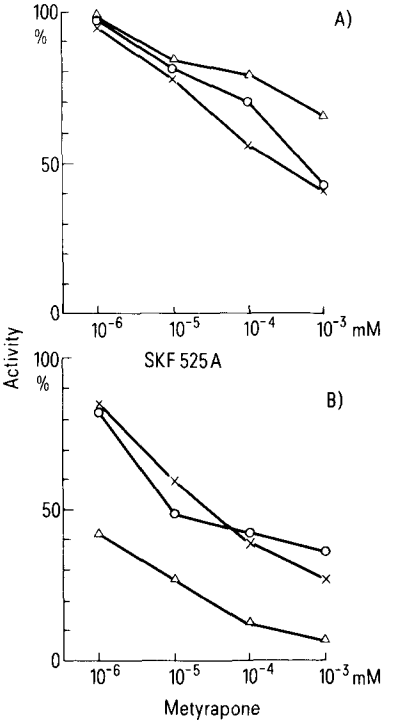

Figure 3. Inhibition of dinemorphan demethylation by SKF 525(A) and by methyrapone(B) in liver microsomes from mice treated with different inducers. Each point is a mean of 2 determinations. The dinemorphan concentration was 5 mM and the control activities for untreated (o), 3MC-treated (△) and PB-treated (×) mice were 3.60, 4.16 and 21.11 nmoles/min/mg protein, respectively. DMSO alone (10 µl) did not inhibit the control activities.

table. Both 3-MC and PB treatment significantly decreased the K_{m2} with respect to the control microsomes.

The highest activity found for the dinemorphan N-demethylase was about one third that of either aminopyrine N-demethylase[16] or ethylmorphine N-demethylase[17] activities determined with microsomes from uninduced mice of the same strain. Phenobarbital treatment increased both the V_{max1} and V_{max2} 5 times per mg of microsomal protein and more than 2 times per nmol of cytochrome P-450, compared to microsomes from control mice.

Thus it appears that PB specifically induced the cytochrome P-450 species which catalyzes the N-demethylation of dinemorphan. Such specificity, in mice of the same strain, was not found for aminopyrine N-demethylase (our unpublished results) or ethylmorphine N-demethylase[17] activities although similar extents of PB-induction, shown by an increase in microsomal protein, were reported. In rats, however, benzphetamine N-demethylase activity was found to be specifically induced by PB treatment[18].

3-MC-treatment did not influence the V_{max} with respect to the control if related to mg of protein, but decreased V_{max1} and V_{max2} if related to nmoles of cytochrome P-450 (table). This shows that 3-MC-inducible cytochrome P-450 species seem to be completely unable to demethylate dinemorphan.

Moreover, dinemorphan was able to bind to the active site of cytochrome P-450, giving a type I binding spectrum. A greater ΔA_{max} was found with PB-microsomes than with 3-MC-microsomes (data not shown).

Dinemorphan demethylation was inhibited by CO and other classical monooxygenase inhibitors such as SKF-525A and metyrapone[19]. SKF-525A strongly inhibited dinemorphan N-demethylase activity in microsomes from PB-induced mice (fig. 3A), while metyrapone, a stronger inhibitor than SKF-525A at all concentrations investigated, is more active towards microsomes from 3-MC-induced mice (fig. 3B).

In conclusion, these results show that dinemorphan, a morphinan-type antitussive drug similar to dextrometorphan, is N-demethylated by liver microsomal monooxygenase and specifically by that from PB-treated mice. Thus, the dinemorphan demethylation can potentially be used as a 'diagnostic' substrate to show if any xenobiotic or treatment, given to mice, is able to induce the PB-like form of cytochrome P-450.

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Migration and dispersal of spermatozoa in spermathecae of queen honeybees (*Apis mellifera* L.)

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Summary. Observations of variability in the phenotypic composition of honeybee colonies through time have led to ad hoc hypotheses concerning the distribution of spermatozoa in multiply mated honeybee queens. These hypotheses are not supported by available data. Serial sections of spermathecae from honeybee queens were examined by light microscopy. No obvious physical evidence was observed of agglomerations, aggregations, clumps, layers, or wads of spermatozoa within spermathecae within 24 h of insemination. A hypothesis is proposed explaining the occurrence of fluctuations of progeny phenotypes.

Taber[1] showed by progeny analysis that sperm utilization by multiply inseminated honeybee queens is not random. He concluded from his study that honeybee spermatozoa are not mixing appreciably within the spermathecae of queens and that spermatozoa are possibly clumping together. However, a look at his data shows that both distinguishable progeny phenotypes were present in significant proportions for all samples of all queens analyzed[2,3].

Kerr et al.[4] proposed high genetic relatedness among honeybee nestmates as a consequence of the physical 'aggregation' and 'agglomeration' of spermatozoa in the spermathecae of queens. Their data, like those of Taber[1],