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On the inducing activity of eucalyptol

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Eucalyptol increases the microsomal activity of rat liver after a single dose given subcutaneously. Further doses do not enhance the effect. Eucalyptol does not affect the concentration of cytochrome P_{450} in liver microsomes. In this it differs from phenobarbitone. Both drugs increase the biliary flow but only phenobarbitone enhances the excretion of conjugated bilirubin after an intravenous load of bilirubin. The results suggest that the induction of liver microsomal enzymes elicited by eucalyptol differs from that elicited by phenobarbitone.

The administration of eucalyptol to rats (Jori, Bianchetti & Prestini, 1969; Jori, Bianchetti & others, 1970), guinea-pigs and mice (unpublished results) produces an increased activity of drug-metabolizing enzymes in liver. Furthermore, animals pretreated with eucalyptol show a reduced sensitivity to pentobarbitone and lower plasma and brain concentrations of the barbiturate (Jori & others, 1969; Jori & others, 1970). We have compared eucalyptol with a classic inducer, phenobarbitone.

MATERIALS AND METHODS

Eucalyptol (gas chromatographically pure) was given subcutaneously diluted with arachis oil, or undiluted by inhalation to female Sprague-Dawley rats (160 ± 10 g). Two rats at a time were placed in a round cage (4 dm^3) with solid sides, for the period of inhalation and eucalyptol was nebulized by means of pressurized air at a rate of approximately 50 mg/min during 15 or 30 min. Phenobarbitone was given intraperitoneally in saline. The treatments were repeated for several days and the *in vitro* and *in vivo* determinations were made at various intervals from the last administration.

In vitro *experiments.* The animals were killed, the livers were immediately frozen on dry ice and stored at −20°. Tissues were homogenized with a glass potter in KCl 1·15%, centrifuged at 4° at 9000 *g*. The supernatant was used directly for enzymatic assays in amounts corresponding to 640 mg of fresh tissue.

The incubation mixtures contained besides the liver homogenate (9000 *g*) $MgCl_2$ (25 μmol), nicotinamide (50 μmol), glucose-6-phosphate (50 μmol), NADP (1·5 μmol), and one of the following substrates: aminopyrine (5 μmol), *p*-nitroanisol (1·5 μmol) or aniline (5 μmol). The corresponding metabolites, 4-amino-antypirine, *p*-nitrophenol, and *p*-aminophenol were determined as reported by Gilbert & Goldberg (1965). The measurement of microsomal cytochrome P_{450} concentrations using CO as a ligand, was made on the microsomal fraction according to Omura & Sato (1964). Microsomal proteins were determined according to Lowry, Rosebrough & others (1951) with a semi-automatic device.

In vivo *experiments.* Biliary flow was measured on rats anaesthetized with ethyl urethane (1·25 g/kg) at various times after the last administration of the inducing

agent or the solvents. The bile duct was surgically exposed by a midline abdominal incision and cannulated. The biliary excretion of injected bilirubin (12·5 mg/kg, i.v.) was measured in similar experimental conditions. The bilirubin determination in the bile was according to Jendrassuk & Cleghorn (1936).

RESULTS

The increase in microsomal enzymatic activity induced by eucalyptol was evident 18 h after the last administration but not after 48 h (Table 1). An initial high dose of eucalyptol produced an increase in microsomal activity which was not further increased by successive doses. Phenobarbitone produces a similar increase to eucalyptol after a single treatment at the dose used, and repeated administrations produced a cumulative effect (Table 2).

Eucalyptol induces an increase in the *O*-demethylation of *p*-nitroanisol and the hydroxylation of aniline which is due to an increase of V_{max} without any change on the K_m (Fig. 1).

Eucalyptol did not modify liver weight, or the concentration of the cytochrome P_{450}. Phenobarbitone, on the other hand, causes a dose-dependent increase of both

Table 1. *Effect of eucalyptol and phenobarbitone on liver microsomal enzymes.*

Treatment	Interval between the last treatment and death (h)	Enzymatic activity (m μmol/g liver h) ± s.e.	
		p-Nitrophenol	*p*-Aminophenol
Arachis oil	—	344 ± 22	578 ± 68
Eucalyptol	18	480 ± 50*	712 ± 90†
	48	374 ± 41	586 ± 60
Saline	—	372 ± 49	587 ± 63
Phenobarbitone	24	1087 ± 118*	911 ± 112*
	48	1438 ± 55*	1495 ± 180*

Eucalyptol (500 mg/kg, s.c.) and phenobarbitone (50 mg/kg, i.p.) were given daily for 3 days Determinations were done on the 9000 *g* supernatant fraction of liver homogenates. The figures indicate the metabolites formed from *p*-nitroanisol and aniline respectively, incubated with the enzymatic preparation.

* $P < 0{\cdot}01$ versus the corresponding control group.

† $P < 0{\cdot}05$ versus the corresponding control group.

Table 2. *Effect of single or repeated treatments of eucalyptol or phenobarbitone on microsomal enzyme activity.*

Treatment (no of rats)	mg/kg	Days of treatment	*p*-Nitrophenol	*p*-Aminophenol	4-Aminoantipyrine
Controls (5) ..	—	—	408 ± 38	344 ± 28	141 ± 18
Eucalyptol (5) ..	500 s.c.	1	752 ± 101†	521 ± 81†	323 ± 67†
	500 s.c.	3	841 ± 53*	592 ± 59*	318 ± 44*
Phenobarbitone (5)..	40 i.p.	1	671 ± 40*	415 ± 16	223 ± 23†
	40 i.p.	3	1336 ± 91*	793 ± 78*	740 ± 127*

The animals were killed 18 h and 24 h after the last administration of eucalyptol and phenobarbitone respectively. Enzymatic activity was measured on the 9000 *g* supernatant fraction of liver homogenates, corresponding to 640 mg of fresh tissue. The figures indicate the amount (nmol/g h) formed from the incubated substrates *p*-nitroanisol, aniline and aminopyrine respectively.

† $P < 0{\cdot}05$ * $P < 0{\cdot}01$

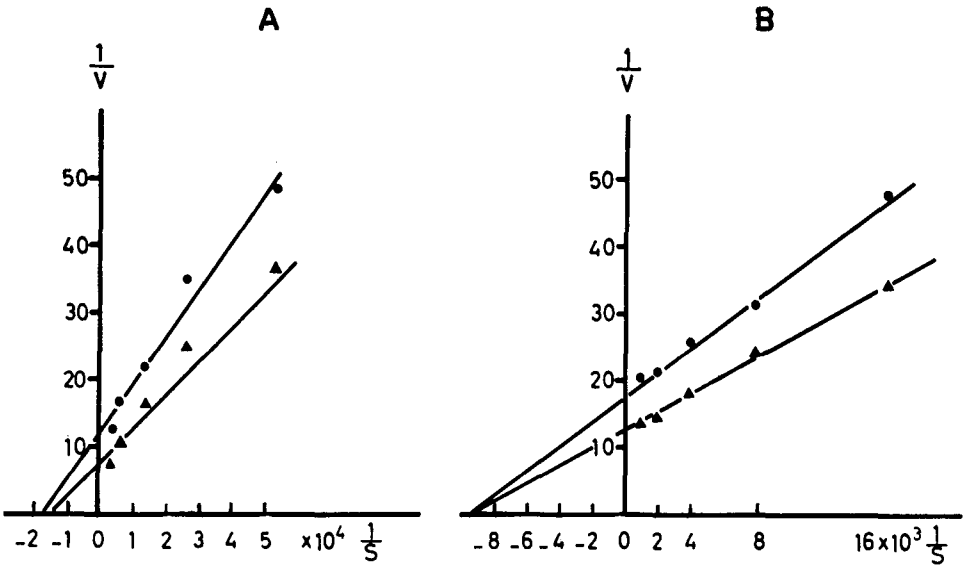

FIG. 1. Effect of eucalyptol pretreatment on the kinetic constants for *p*-nitroanisol *o*-demethylation and aniline *p*-hydroxylation. Eucalyptol (500 mg/kg s.c.) was given daily for 3 days. Animals were killed 24 h after the last treatment. Determinations were made on the supernatant of the 9000 *g* fraction of 3 pooled liver homogenates for each curve. An amount corresponding to 640 μg of fresh tissues was incubated with cofactors (see material and methods). 1/S is the reciprocal of the molar concentration of *p*-nitroanisol (A) and aniline (B), while 1/V expresses the reciprocal of the formation of *p*-nitrophenol (A) and *p*-aminophenol (B) in nmol/g h from the controls (●—●) and eucalyptol-treated rats (▲—▲).

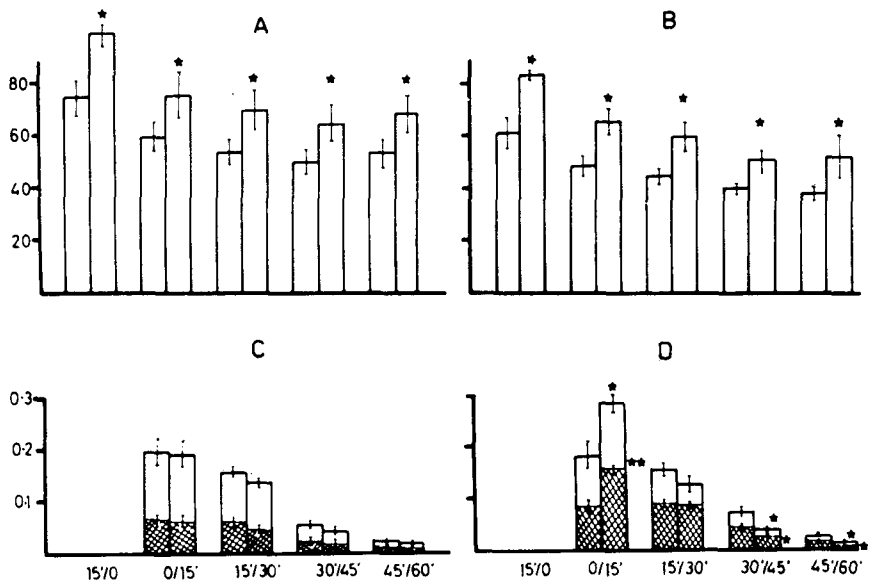

FIG. 2. Effect of eucalyptol and phenobarbitone on the biliary flow and the bilirubin biliary excretion after a bilirubin load. Eucalyptol (500 mg/kg s.c. daily for 3 days) (A–C) and phenobarbitone (80, 80 and 50 mg/kg i.p. in 3 days) (B–D) treated rats were cannulated 24 h after the last administration. The left of each pair of columns represents the biliary flow (A–B) or the biliary excretion (C–D) of the control rats during periods of 15 min and the right represents that of the treated animals. In C and D the shaded and the unshaded portions of each column represent respectively the excreted amount of conjugated and free bilirubin. Vertical bars represent the s.e. (n = 5 each point).

* $P < 0{\cdot}05$ and ** $P < 0{\cdot}01$ versus the control group.

Table 3. *Effect of eucalyptol and phenobarbitone on liver weight, cytochrome P_{450} and microsomal enyzme activity.*

Treatment (no of rats)	mg/kg	Liver weight g ± s.e.	Microsomal enzyme activity: *p*-Nitrophenol	*p*-Aminophenol	4-Aminoantipyrine	P_{450} Cytochrome nmol/mg proteins
Controls (10) ..		4·64 ± 0·14	467 ± 50	510 ± 56	172 ± 20	0·73 ± 0·04
Eucalyptol (8) ..	500 s.c.	4·49 ± 0·16	764 ± 40*	769 ± 84*	342 ± 17*	0·70 ± 0·07
Phenobarbitone (8)	10 i.p.	4·78 ± 0·07	834 ± 64*	807 ± 96*	379 ± 40*	0·94 ± 0·03*
Phenobarbitone (6)	25 i.p.	5·04 ± 0·14*	1245 ± 61*	1079 ± 54*	584 ± 45*	1·27 ± 0·12*
Phenobarbitone (6)	50 i.p.	5·96 ± 0·13*	1645 ± 75*	1355 ± 108*	876 ± 111*	1·23 ± 0·04*

Rats were given eucalyptol and phenobarbitone at the doses indicated daily for 3 days and they were killed 24 h after the last administration. Microsomal enzyme activity was measured on the 9000 *g* supernatant fraction of liver homogenastes as the amount of metabolites formed (nmol/g h) from the corresponding substrates *p*-nitroanisol, aniline and aminopyrine respectively. Cytochrome P_{450} was measured on the microsomal fraction obtained after centrifugation at 105 000 *g*.

† $P < 0{\cdot}01$ versus control group. * $P < 0{\cdot}05$ versus control group.

Table 4. *Effect of eucalyptol on bile flow.*

Treatment (no. of rats)	No. of days	Interval between the last administration and bile collection (h)	Bile excretion (ml ± s.e.) 0–1 h	0–2 h
Controls (7)	—	—	0·75 ± 0·04	1·40 ± 0·07
Eucalyptol I (6) ..	4	18	1·16 ± 0·11*	1·84 ± 0·13*
Eucalyptol s.c. (6) ..	4	18	1·02 ± 0·09*	1·78 ± 0·19*
Eucalyptol s.c. (4) ..	1	18	1·06 ± 0·06*	1·79 ± 0·14*
Controls (6)	—	—	0·67 ± 0·06	1·16 ± 0·09
Eucalyptol s.c. (6) ..	3	48	0·65 ± 0·02	1·18 ± 0·03

Rats were given eucalyptol by aerosol inhalation (I) daily for 4 days for 15, 15, 30, 30 min respectively or subcutaneously (s.c.) at the doses of 500 mg/kg for 1,3 or 4 days. The bile flow was measured in anaesthetized rats (ethyl urethane 1·25 g/kg, i.p.) immediately after surgical cannulation of the choledocus.

* $P < 0{\cdot}01$ versus the corresponding control group.

parameters, increasing the P_{450} even at doses eliciting an increase of the microsomal enzyme activity similar to that induced by eucalyptol (Table 3). Table 4 shows the biliary flow during 2 h in controls and in rats treated with eucalyptol according to different schedules. The treatment produced a significant increase in bile flow; no differences were observed between 1 or 3 days of treatment. The effect disappeared 48 h after the last dose of eucalyptol.

Fig. 2 shows the biliary excretion of bilirubin, relative to the biliary flow after an exogenous load of bilirubin (12·5 mg/kg, i.v.), in control, phenobarbitone- and eucalyptol-treated rats. Eucalyptol did not modify the excretion of bilirubin, while phenobarbitone enhanced the excretion of conjugated bilirubin during the first collection period.

DISCUSSION

Jori & others (1969, 1970) presented evidence that eucalyptol increases drug metabolism of different substrates, suggesting a phenobarbitone-like activity on liver

microsomal enzymes. The data here reported show instead that eucalyptol stimulation differs in some aspects from phenobarbitone induction.

The enhanced microsomal metabolizing activity evoked by phenobarbitone is long-lasting and returns to control levels only 5 days after the last administration (Orrenius & Ericsson, 1966). However, the effect of eucalyptol at a dose eliciting maximum stimulant properties disappears in a shorter time. We have confirmed that eucalyptol decreases pentobarbitone sleeping time in rats (Jori, Bianchetti & Prestini, 1969) and have shown that the liver microsomal enzyme activity is normal 48 h after the last treatment of eucalyptol.

The cumulative effect of phenobarbitone on induction (Ernster & Orrenius, 1965; Argyris, 1968), was not seen with eucalyptol with which we observed a maximum increase after the first injection. This might be related to the short half-life of eucalyptol or to a possible "autoinduction" so that the second dose is metabolized faster than the first. Eucalyptol does not increase the cytochrome P_{450} in liver microsomes when the enzymatic activity was stimulated. On the contrary, phenobarbitone enhances both microsomal enzymatic activity and the P_{450} level. This agrees with previous findings (Orrenius, 1965; Remmer & Merker, 1965). Other compounds such as caffeine and dimethylsulphoxide (DMSO) reported to act as inducer agents on the microsomal enzyme activity (Mitoma, Lombrozo & others, 1969; Stock, Hansen & Fouts, 1970) do not increase the P_{450} content of microsomes (Stock & others, 1970; Lombrozo & Mitoma, 1970). That increase in drug metabolism after these inducing agents may be correlated with increased rate of reduction of cytochrome P_{450} must be investigated. Davies, Gigon & Gillette (1969), have demonstrated that cytochrome P_{450} reduction is the limiting step for the rate of drug metabolism by microsomal enzymes.

The bile flow is increased to a similar extent by eucalyptol and by phenobarbitone and this effect follows the time course of the variation of microsomal enzyme activity by eucalyptol, this is in agreement with results for phenobarbitone (Klaassen, 1969). However, according to Klaassen (1970) the rate of the total and the conjugated bilirubin excretion into the bile is increased by phenobarbitone but not by eucalyptol.

We have found (unpublished) that glucuronyltransferase was not stimulated when *p*-nitrophenol and bilirubin were used as substrates for liver preparations from animals pretreated with eucalyptol. Since variations in substrate specificities for such enzymes have been found (Isselbacher, Chrabas & Quinn, 1962; Dutton & Lawes, 1966), these data do not exclude the possibility that eucalyptol may enhance the glucuronation of other substrates (Hohenwallner & Klima, 1971).

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