In conclusion and in addition to former results [3, 5] on the stimulatory effect of Silibinin on RNA and protein synthesis we show here that the drug increases DNA replication by about 23–35% in hepatectomized rat livers. This again gives proof for the liver cell regenerating capacity of the flavonolignane derivative supporting the clinical reports [2]. Our experiments with some malign cell lines cells should be seen as a first attempt to investigate any influence of Silibinin on malignant tissues and to ascertain that there are no stimulatory effects which should be a precondition for a clinical use.

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Effect of oxygen concentration on the metabolic pathway of anisole in rat liver microsomes

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The microsomal mixed-function oxidase system containing cytochrome P-450, which utilizes molecular oxygen, catalyzes the metabolism of a wide variety of endogenous and xenobiotic compounds. Recently, several groups [1–4] have reported the effects of oxygen concentration on the metabolism of several compounds. Jones [5] mentioned that many drug-metabolizing reactions require molecular oxygen either directly as a substrate or indirectly because of their dependence upon cellular energetics. His group also studied oxidation and conjugation reactions of drugs at low oxygen concentration [6–8]. However, no studies have been done on the effect of oxygen concentration on the different types of reactions in the metabolism of a substrate which are catalyzed by cytochrome P-450. In this study, we chose anisole as a substrate, because it was shown by Daly *et al.* [9, 10] that the mixed-function oxidase system metabolizes anisole by two principal oxidation pathways, O-dealkylation and aromatic hydroxylation, in spite of its simple structure, and we investigated the effect of oxygen concentration on the metabolic pathway of anisole.

Methods

Liver microsomes were prepared from male Wistar rats (*ca.* 150 g) that had been pretreated with sodium phenobarbital (60 mg/kg in saline, i.p., 3 days).

Standard gases (1.0% and 10% O_2 in N_2) were purchased from the Takachiho Chemical Industry, and we obtained gas mixtures of other desired oxygen concentrations (2.0%, 4.0% and 6.0% O_2 in N_2) with a gas divider apparatus (SGD-XC 0.5 l, STEC Inc.). From the calibration by Clark oxygen electrode measurements, the oxygen concentrations in the assay solutions equilibrated by the gases (1.0, 2.0, 4.0, 6.0, 10% O_2 in N_2 and air) were determined to be 24, 34, 54, 74, 113 and 223 μM respectively.

The formation rates of metabolites of anisole at various oxygen concentrations were determined according to the methods of Holtzman *et al.* [11], with slight modifications. The system consisted of gassing towers with rubber seals at the top, connected with stainless steel tubes. The gas was passed through the first gassing tower containing 0.1 M phosphate buffer (pH 7.4). Then the humidified gas was

passed successively through the towers containing the components (microsomes, glucose-6-phosphate dehydrogenase, NADP and glucose-6-phosphate) in the same buffer. To avoid denaturing the microsomes and glucose-6-phosphate dehydrogenase by gas bubbling, the gas was simply passed over them for 2 hr. The final reaction vessel was a 50-ml Erlenmeyer flask. The mixture of microsomes and glucose-6-phosphate dehydrogenase in the buffer was transferred to the reaction vessel with a gas-tight syringe, and then anisole in 20 μl of acetone was added. The reaction was initiated by addition of the mixture of NADP and glucose-6-phosphate, followed by preincubation for 3 min. The final volume of the mixture was 2.52 ml, and it contained 4 mg of microsomal protein, 0.4 mM NADP, 4 mM glucose-6-phosphate, 100 mM KCl, 10 units of glucose-6-phosphate dehydrogenase, and 2.0 mM anisole. The reaction was carried out at 37° with continuous gassing (100–200 ml/min) and shaking (100 strokes/min). The reaction was terminated by the addition through the seal of 15% $ZnSO_4$ solution (1 ml). The seal was removed, and sat. $Ba(OH)_2$ (1 ml) was added. The mixture was centrifuged, and the metabolites were extracted from the supernatant fraction (3 ml) with CH_2Cl_2 (7 ml × 3). The combined organic phase was carefully concentrated under reduced pressure to prevent loss of the metabolites. Then *N,O*-bis(trimethylsilyl)trifluoroacetamide and internal standard (2,4,6-trimethylphenol) were added, and the mixture was heated at 70° for 1 hr. Samples were analyzed by gas chromatography.

Results and discussion

The metabolites of anisole produced by phenobarbital-induced rat liver microsomes were the O-demethylated product (phenol) and aromatic hydroxylated products (*p*-hydroxyanisole and *o*-hydroxyanisole), though no *m*-hydroxyanisole was formed. To examine possible subsequent metabolic reaction steps, the total demethylation activity was assayed by the method of Nash [12]. The total demethylation activity (3.00 nmoles/mg protein/min) was nearly equivalent to the formation of phenol (2.95 nmoles/mg protein/min). Hence, it was considered that the determination of phenol, *p*-hydroxyanisole and *o*-hydroxyanisole would account for the whole metabolism of anisole.

Figure 1 shows the time courses of the formation of phenol, *p*-hydroxyanisole and *o*-hydroxyanisole from anisole by phenobarbital-induced rat liver microsomes at 223 μM O_2 (air) and 24 μM O_2 (1% O_2 in N_2). The formation of all metabolites at 24 μM O_2 was significantly reduced as compared with that at 223 μM O_2. It is clear that the formation rates of metabolites of anisole were affected by oxygen concentration.

When the relationship between the rates of metabolite formation of anisole and oxygen concentration was examined (Fig. 2), the formation rate of *o*-hydroxyanisole was saturated at about 35 μM O_2, while the formation rates of phenol and *p*-hydroxyanisole were not saturated even at 223 μM O_2 (air).

Figure 3 shows the relative ratios of phenol and *p*-hydroxyanisole formed with respect to *o*-hydroxyanisole formation at each oxygen concentration. The relative ratios gradually decreased with decreasing oxygen concentration, and phenol formation fell below *o*-hydroxyanisole formation (ratio less than unity) at about 60 μM O_2. It is of interest that the composition of metabolites formed from a substrate is greatly dependent on oxygen concentration.

In the report of Erickson *et al.* [1], it was presumed that the oxygen pressure in the liver was 26 torr (35 μM). As can be seen from Fig. 3, the compositions of metabolites at 35 μM O_2 and 223 μM O_2 (air) were significantly different. For this reason, it is necessary to carry out detailed *in vitro* studies in order to permit extrapolation to the *in vivo* situation. For example, Jones [5] predicted that the metabolism of many drugs that are substrates for cytochrome P-450 would be decreased during even mild hypoxia. This study suggests that in hypoxia, not only may the metabolism of drugs be decreased, but also the metabolic pattern or pathway may be changed.

In summary, the ratio of metabolites of anisole formed by rat liver microsomes varied as a function of oxygen concentration. Thus, the oxygen concentration is very important in studies of drug metabolism *in vitro*, and we may predict that the *in vivo* metabolic pathways of drugs may be changed in hypoxia.

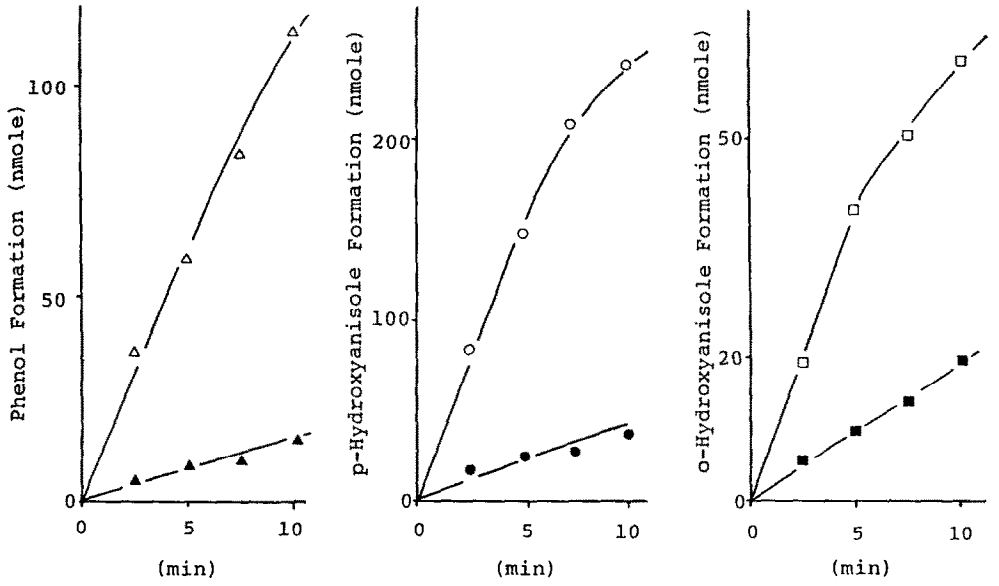

Fig. 1. Time courses of metabolite formation from anisole by phenobarbital-induced rat liver microsomes at 24 and 223 μM O_2. The reaction mixture (2.52 ml) contained the microsomes (4 mg protein), NADP (0.4 mM), glucose-6-phosphate (4 mM), glucose-6-phosphate dehydrogenase (10 units), KCl (100 mM) and anisole (2.0 mM, added in 20 μl of acetone) in 0.1 M, pH 7.4, phosphate buffer. The incubations were carried out at 37°, at 24 μM O_2 (▲,●,■) and 223 μM O_2 (△,○,□).

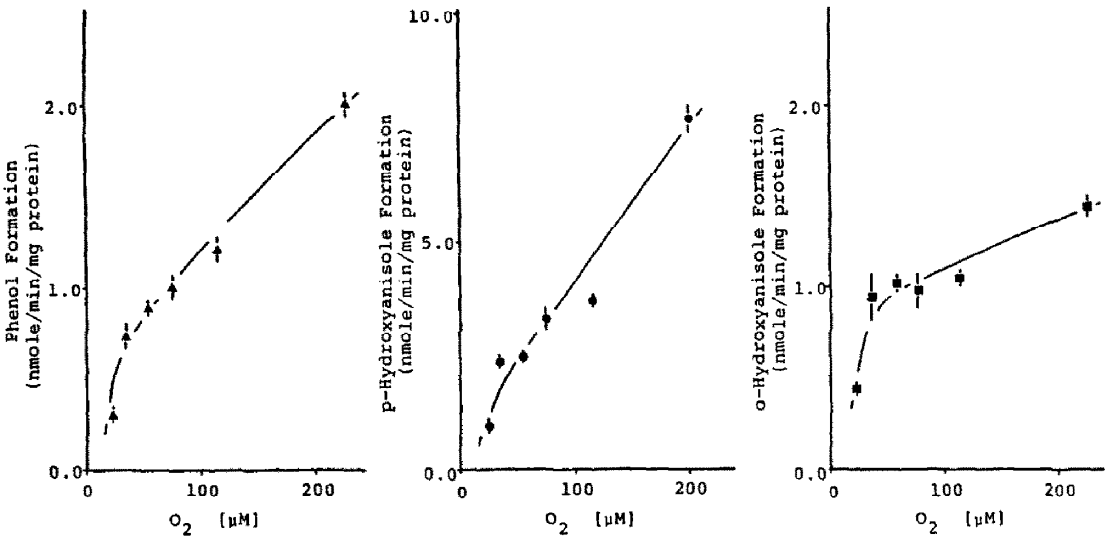

Fig. 2. Rates of formation of anisole metabolites by phenobarbital-induced rat liver microsomes as a function of $[O_2]$. Incubations were carried out as described in the legend of Fig. 1. The rates of metabolite formation were calculated from the amount of metabolites at 5 min. Each value is the mean ± S.E. of three or four experiments.

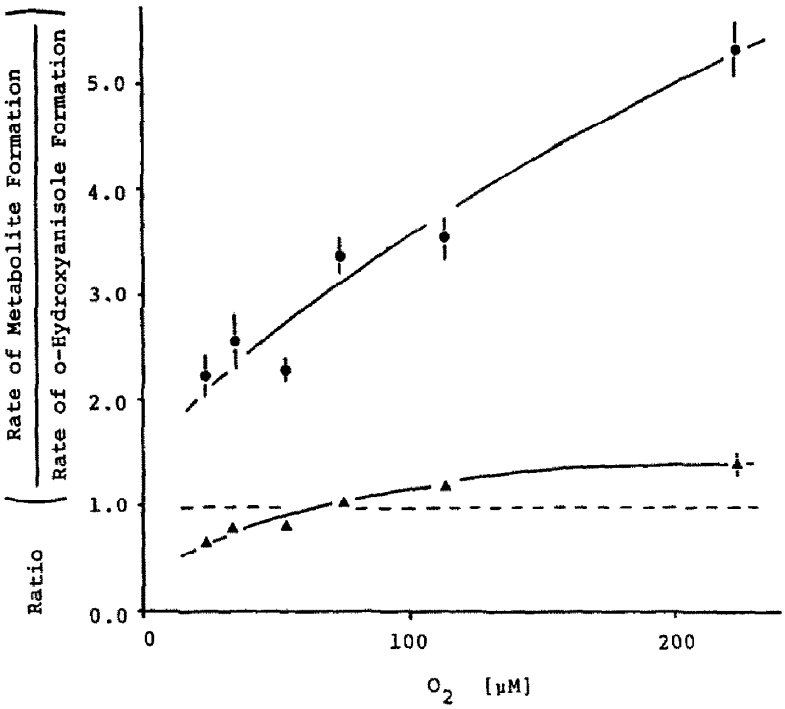

Fig. 3. Relative ratios of formation of anisole metabolites by phenobarbital-induced rat liver microsomes as a function of $[O_2]$. The relative ratios of phenol and *p*-hydroxyanisole formed were calculated based on *o*-hydroxyanisole formation at each oxygen concentration from the data in Fig. 2. Key: phenol (—▲—); *p*-hydroxyanisole (—●—); and *o*-hydroxyanisole (– – – –). Each value is the mean ± S.E. of three or four experiments.

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Availability of plasma sulfate for conjugation of salicylamide in dogs*

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A failure of sulfoconjugation of phenolic substrates to increase in proportion to dose, observed in several species [1], could be due to a limitation in the rate of any one, or more, of several steps of sulfoconjugation [2] or to depletion of body stores of cosubstrate precursors [3–5]. In contrast to humans, sulfoconjugation of salicylamide in dogs shows this kinetic behavior even when plasma sulfate concentrations are normal [6] or increased [2]. Thus, one might hypothesize that transport into or activation of sulfate at the sites of sulfoconjugation could be responsible for limiting the rate of metabolism after increasing doses of the drug in the dog.

We report here studies designed to determine the source of sulfate used for the sulfoconjugation reaction and the rate of incorporation of plasma inorganic sulfate into salicylamide given intravenously to dogs. The plasma inorganic sulfate pool was labeled with [^{35}S]sulfate. Then the incorporation of radiolabeled sulfate into salicylamide-sulfate was determined by comparing the specific activities of inorganic sulfate in plasma and salicylamide-sulfate in urine.

Materials and methods

Chemicals. [^{14}C]Salicylamide [2] was synthesized by ICN (Irvine, CA). [^{35}S]Sulfate (1070 Ci/mmole, primarily as the ammonium salt) was purchased from Amersham (Arlington Heights, IL).

Animals. Three conditioned male mongrel dogs weighing 20–26 kg were studied using the experimental techniques described previously [2].

Rate of appearance of salicylamide-sulfate in the plasma. [^{14}C]Salicylamide, 7.3 μmoles/kg (sp. act. = 275 μCi/mmole), in 10 ml normal saline, was administered intravenously to a dog over 1 min. Blood samples were collected at 1, 2, 3, 4, 5, 6, 8, 10, 13, 16, and 22 min. Plasma concentrations of [^{14}C]salicylamide-sulfate were measured.

Fate of administered salicylamide-[^{35}S]sulfate. To prepare salicylamide-[^{35}S]sulfate, 40 μCi of inorganic [^{35}S]sulfate and 32 μmoles/kg of salicylamide were administered in the same solution (45 ml 0.9% NaCl) intravenously to a dog over 1 min. Salicylamide-[^{35}S]sulfate was extracted from the 24-hr urine collection and counterextracted as described previously [7].

The isolated salicylamide-[^{35}S]sulfate (containing 40 μmoles of salicylamide-sulfate) was given intravenously over 17.5 min to a different dog. Urine was collected for 6 hr. The specific activity of salicylamide-[^{35}S]sulfate, extracted from the urine, was compared with that of the administered compound.

Rate of incorporation of [^{35}S]sulfate into salicylamide-sulfate. Normal saline containing unlabeled salicylamide, 7.3 μmoles/kg, and inorganic [^{35}S]sulfate, 10 μCi, was given intravenously (10 ml) to three dogs over 30 sec. Blood samples were obtained at 0, 1, 2, 3, 4, 5, 6, 9, 11, 14, 18, 22, 26, and 30 min and urine was collected over 6 hr. The specific activity of salicylamide-[^{35}S]sulfate in the urine was compared with that of inorganic sulfate in the plasma.

Rate of incorporation of [^{35}S]sulfate into salicylamide-sulfate after a bolus of sodium sulfate. To prelabel the metabolically-active sulfate pool, inorganic [^{35}S]sulfate, 10 μCi, was given intravenously over 30 sec to three dogs. Fifteen minutes later, an aqueous solution containing sodium sulfate, 1.13 mmoles/kg, and unlabeled salicylamide, 7.3 μmoles/kg, was infused simultaneously into two separate forelimb veins over 1.2 min. Blood and urine samples were collected and data were analyzed as described in the preceding section.

Assays. Concentrations of unlabeled salicylamide in the plasma were measured by high performance liquid chromatography (HPLC) [7]. Total [^{14}C] and [^{14}C]salicylamide concentrations in the plasma were measured as described previously [6]. The concentration of [^{14}C]salicylamide-sulfate was calculated by subtracting [^{14}C]salicylamide concentration from the total [^{14}C] concentration. Previous studies [6] indicated that at salicylamide doses of 36 μmoles/kg, or less, the sulfate conjugate constituted greater than 97% of the radioactivity appearing in the urine. Therefore, other [^{14}C]radiolabeled metabolites were unlikely to be present in the plasma.

Concentrations of inorganic [^{35}S]sulfate in the plasma were determined as follows. Plasma, 100 μl, was added to Aquasol (New England Nuclear, Boston, MA), 15 ml, for counting of total [^{35}S]-activity. Because molar concentrations of salicylamide-sulfate in the plasma after a 7.3 μmole/kg salicylamide dose (Fig. 1A) were less than 1% of molar concentrations of inorganic sulfate in the plasma (about 1 mM), the contribution of salicylamide-[^{35}S]sulfate to total plasma radioactivity was assumed to be less than 1% and, therefore, unimportant. [^{14}C]Salicylamide and [^{35}S]sulfate were not administered in the same studies.

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