rats (2.5 mmole/kg for 5 days or 5 mmole/kg for one day) failed to produce any effect on glutaminase activity. However glutamine synthesis was found to be inhibited when rats were given an acute dose of 5 mmole/kg and sacrificed within 90–150 min, but such an effect was not observed when the dose of lithium chloride was lowered. This indicates that at a therapeutical dose of lithium, as is generally administered, the possibility of inhibition of glutamine synthesis does not arise.

The above results indicate that lithium salts might interfere with the metabolic cycle of glutamic acid–glutamine as a result of which the homeostatic equilibrium of the amino acid transmitter of the glutamate–GABA system may be affected. The inhibition of phosphate activated glutaminase will produce a decreased formation of glutamate from glutamine which eventually is transformed to GABA by GAD [18–21]. However in view of high doses of lithium salts used in the above *in vitro* experiments than is used therapeutically, it appears that the effects observed are unlikely related to the mode of action of lithium *in vivo*.

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REFERENCES

1. M. Schou, *Psychopharmacologia* **1**, 65 (1959).
2. M. Schou, *J. Psychiat. Res.* **6**, 67 (1968).
3. G. Schlagenhauf, J. Tupin and R. B. White, *Am. J. Psychiat.* **123**, 201 (1966).
4. F. V. De Feudis and J. M. R. Delgado, *Nature, Lond.* **225**, 749 (1970).
5. Z. Gottesfeld, B. S. Ebstein and D. Sammuel, *Nature, New Biol.* **234**, 124 (1971).
6. G. Johnson, M. Maccario, S. Gershon and J. Korein, *J. Nerv. Ment. Dis.* **151**, 273 (1970).
7. S. Berl and D. D. Clarke, in *Lithium Research and Therapy* (Ed. F. N. Johnson), p. 425. Academic Press, New York.
8. F. V. De Feudis and R. M. Paolino, *Experientia* **28**, 309 (1972).
9. K. Kuriyama, E. Roberts and J. Vos, *Brain Res.* **9**, 231 (1968).
10. T. M. Brody and J. A. Bain, *J. biol. Chem.* **195**, 685 (1952).
11. M. Errera, *J. biol. Chem.* **178**, 483 (1949).
12. M. L. Horowitz, W. E. Knox and H. P. Morris, *Enzym. Biol. Clin.* **9**, 241 (1968).
13. E. J. Conway and A. Byrne, *Biochem. J.* **27**, 419 (1933).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. M. Dixon, *Biochem. J.* **55**, 170 (1953).
16. W. W. Ackermann and V. R. Potter, *Proc. Soc. exp, biol. Med.* **72**, 1 (1949).
17. C. A. Woolfolk, B. Shapiro and E. R. Stadtman, *Archs Biochem. Biophys.* **116**, 177 (1966).
18. H. F. Bradford, H. K. Ward and A. J. Thomas, *J. Neurochem.* **30**, 1453 (1978).
19. F. A. Henn, M. N. Goldstein and A. Hamberger, *Nature, Lond.* **249**, 663 (1974).
20. A. Martinez-Hernandez, K. P. Bell and M. D. Norenberg, *Science, N.Y.* **195**, 1356 (1977).
21. A. Schousboe, L. Hertz, G. Svenneby and E. Kvamme, *J. Neurochem.* **32**, 943 (1979).

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Effect of naturally occurring coumarins on the activity of drug metabolizing enzymes*

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Hepatic enzymes that metabolize foreign compounds have been shown to be affected by the treatment of the animals with a variety of environmental factors such as drugs, polycyclichydrocarbons, pesticides, industrial products and dietary factors [1–5]. It was demonstrated that these foreign compounds caused biphasic responses on the liver microsomal enzymes [6], eliciting marked changes in the pharmacological and toxicological action of drugs.

In continuing research on medicinal plants affecting drug-metabolizing function in the liver, it was found that *Angelica* spp exhibited potent biphasic effects on barbiturate action [7, 8]. Systematic fractionation of the roots of *Angelica koreana* monitoring by bioassay led to isolation of active principles which were identified as coumarins [9]. This finding and previous reports [10–12] that several synthetic anticoagulant coumarins induced drug-metabolizing enzymes (DME) prompted us to investigate the effect of some structurally related natural coumarins on DME in order to elucidate the structure–activity relationship.

Materials and methods

Animals. For *in vivo* studies, male albino mice weighing 20 ± 3 g were used. The animals were fed lab chows and tap water *ad lib.* Constant-temperature environments were maintained throughout the experiments. For enzyme preparations, male Sprague–Dawley (CD strain) rats weighing 200–250 g were used.

Materials. Coumarins shown in Fig. 1 were isolated from the Umbelliferae. SKF-525A was a gift from Smith, Kline & French (Philadelphia, PA).

Measurement of hexobarbital- (HB) induced sleeping time. The effect on the HB-induced sleeping time was investigated in two phases. During the first phase mice were administered i.p. with each compound 30 min prior to the injection of HB-Na (50 mg/kg i.p.) and the duration of the loss of the righting reflex was estimated. In the

* Part 5 in the series: Studies on crude drugs acting on drug-metabolizing enzymes. For Part 4 see ref. [9].

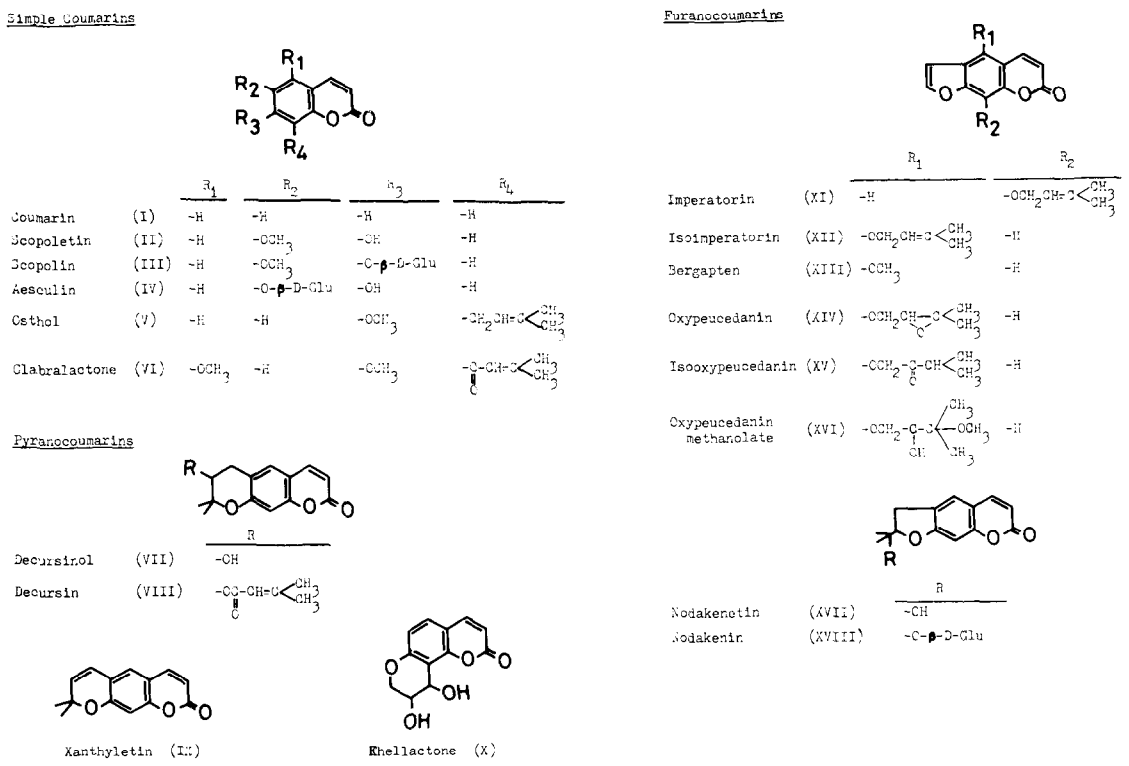

Fig. 1. Structure of coumarins.

second-phase experiment, mice were pretreated with three consecutive daily administrations of each compound. Forty-eight hours after the last treatment, HB-Na (100 mg/kg i.p.) was injected and the duration of sleeping time was estimated.

Measurement of HB level in serum. Each compound was pretreated according to the same schedule (both the first and second phase) as earlier.

Thirty minutes after the injection of 100 mg/kg HB-Na, mice were killed by cutting the carotid artery and blood was carefully collected. Blood from two mice were pooled and the HB concentration in serum was measured according to the procedure described by Cooper and Brodie [13].

Measurement of strychnine mortality. Strychnine nitrate was injected i.p. at a dose of 1 mg/kg to mice 30 min after the administration of the samples. The convulsive death of 20% of the animals was induced at this dose of strychnine nitrate. Mice were observed for 30 min and the mortality was recorded.

In vitro *experiments. Enzyme preparation*: Livers of rats were excised and immediately weighed. They were washed twice in ice-cold isotonic phosphate buffer (1.15% KCl, pH 7.4) and then homogenized in 5 vol. of the buffer. The homogenate was centrifuged for 20 min at 10,000 *g* and the supernatant was used in the enzyme assay. The protein concentration in supernatant was determined by the method of Lowry *et al.* [14], using bovine serum albumin as a standard.

DME assay: The oxidative metabolisms of HB and aminopyrine (AP) were studied. For HB hydroxylation the incubation medium consisted of liver homogenate (1 ml), NADP (0.65 μmoles), glucose 6-phosphate (10 μmoles), nicotinamide (50 μmoles), $MgCl_2$ (25 μmoles), HB-Na (1.94 μmoles) and an inhibitor, in a total volume of 5.1 ml. One hour after incubation at 37°, HB remaining was measured [13]. For AP *N*-demethylation, the medium contained the same cofactors as for HB hydroxylation, semicarbazide (45 μmoles), AP (5 μmoles) and an inhibitor, in a total volume of 6.1 ml. Thirty minutes after incubation at 37°, the formaldehyde generated was measured by the method of Nash [15]. Inhibitors were solubilized in EtOH, the final concentration being 1.96%. Control flasks contained an equivalent concentration of EtOH.

Results and discussion

In Table 1, the results are shown of the effects of 18 naturally occurring coumarins on HB-induced hypnosis and strychnine mortality in mice as well as on HB and AP metabolisms *in vitro*.

All the furanocoumarins tested (XI–XVI) exhibited not only a prolonging effect on HB action in the first phase but also a shortening effect in the second phase. The potency of imperatorin (XI) and isoimperatorin (XII) was as strong as that of SKF-525A. Xanthyletin (IX), which is a pyranocoumarin, was active in the first phase only and much less active than the furanocoumarins.

Dihydrofuranocoumarins, dihydropyranocoumarins and simple coumarins, however, produced no activity at the dosage used.

The furanocoumarins also caused a remarkable increase in strychnine toxicity whereas the pyranocoumarins showed a relatively weak activity. The other coumarins, however, did not affect mortality. The enhancement of strychnine toxicity by these coumarins, therefore, suggested that their HB hypnosis-prolonging action did not result from the simple potentiation of a CNS depressant action of drugs without an alteration in the rate of HB hydroxylation but from the retardation of drug metabolism. This was confirmed by the measurement of the HB level in serum of mice 30 min after the pretreatment of the active coumarins. As shown in Fig. 2, all the furanocoumarins caused a marked elevation of the HB level during the first phase and a significant reduction of its level during the second phase; this phenomenon was quite similar to the action of the coumarins on the hypnotic response. Xanthyletin (IX) showed a weaker but significant elevation of the HB level

Table 1. Effects of coumarins on HB-induced hypnosis, strychnine mortality and DME *in vitro*

Compound	HP hypnosis†		Strychnine mortality‡	DME activity	
	First phase	Second phase		HP hydroxylase§	AP *N*-demethylase‖
Control	24.3 ± 3.9	73.0 ± 5.5	2/10	14.7 ± 0.77	13.7 ± 0.18
SKF-525A	182.2 ± 30.5***	32.5 ± 4.5***	10/10	7.7 ± 1.77*	6.2 ± 0.30***
Phenobarbital	30.9 ± 8.8	22.7 ± 3.4***	2/10	–	–
I	26.3 ± 3.6	73.3 ± 6.6	2/10	14.3 ± 1.07	13.4 ± 0.23
II	24.7 ± 7.3	81.2 ± 8.7	3/10	13.2 ± 0.66	13.1 ± 0.32
III	22.7 ± 2.9	75.5 ± 7.2	2/10	–	–
IV	22.9 ±2.6	73.2 ± 5.7	3/10	15.1 ± 1.01	13.8 ± 0.18
V	30.5 ± 5.7	67.7 ± 4.2	3/10	12.8 ± 1.83	12.7 ± 0.17
VI	22.8 ± 9.5	91.9 ± 12.5	2/10	13.2 ± 0.60	12.1 ± 0.42
VII	29.0 ± 3.2	92.3 ± 8.5	3/10	14.3 ± 2.15	13.3 ± 0.12
VIII	28.2 ± 3.0	90.3 ± 9.0	2/10	15.5 ± 5.16	12.2 ± 0.27
IX	38.4 ± 1.7**	62.9 ± 3.4	4/10	11.0 ± 0.38**	11.6 ± 0.19**
X	21.5 ± 5.8	68.7 ± 3.1	2/10	14.7 ± 1.84	12.0 ± 0.66
XI	246.1 ± 15.6***	48.6 ± 5.7*	9/10	4.7 ± 1.17***	6.8 ± 0.11***
XII	238.6 ± 35.1***	43.5 ± 9.6*	9/10	8.9 ± 1.48***	6.6 ± 0.09***
XIII	218.2 ± 32.5***	48.6 ± 6.5*	7/10	10.1 ± 1.16**	9.8 ± 0.25***
XIV	214.8 ± 33.1***	55.6 ± 2.7*	9/10	8.1 ± 0.21***	7.9 ± 0.20***
XV	111.2 ± 7.1***	55.9 ± 2.4*	8/10	8.9 ± 1.70*	9.1 ± 0.24***
XVI	83.2 ± 8.8***	56.6 ± 4.7*	7/10	10.9 ± 0.97**	11.2 ± 0.29***
XVII	25.5 ± 6.6	67.7 ± 7.3	2/10	14.3 ± 1.39	12.1 ± 0.59
XVIII	19.6 ± 5.0	59.6 ± 7.9	2/10	15.9 ± 3.97	12.1 ± 0.24

All experimental conditions are described in Materials and methods.
† Mean sleeping time (min) ± S.E. of five to six animals given 30 mg/kg i.p. of compound.
‡ No. dead/No. treated with 30 mg/kg i.p. of compound.
§ Mean HB metabolized (μmoles/g protein/hr) ± S.E. of three incubations with 5×10^{-5} M compound.
‖ Mean AP metabolized (μmoles/g protein/hr) ± S.E. of three incubations with 2×10^{-5} M compound.
Significantly different from the control: *P < 0.05, **P < 0.01 and ***P < 0.001.

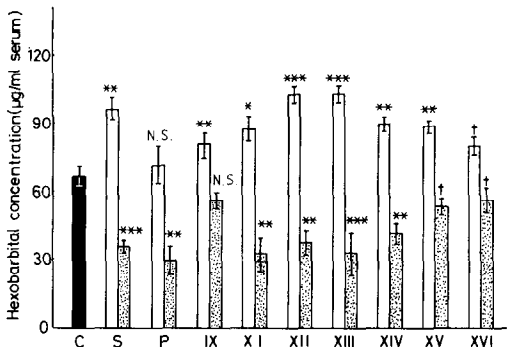

Fig. 2. Effect of coumarins on serum HB level in mice. Experimental conditions are described in Materials and methods. The sample dose was 30 mg/kg i.p. Histobars represent the means ± S.E. of triplicate determinations except in the control, where the number of determinations was seven. C, control; S, SKF-525A; P, phenobarbital; □, single treatment 30 min before test; ■, three consecutive daily treatments 48 hr before test. Significant difference from control: ***P < 0.001; **P < 0.01; *P < 0.02; †P < 0.05; N.S., not significant.

during the first phase only.

Although several coumarins have been previously postulated to be DME inducers [12], Feuer [11] reported that daily treatments of rats for 7 days with coumarin (I), scopoletin (II) and aesculin (IV) (1 mmole/kg) did not affect the liver coumarin hydroxylation activity but imperatorin (XI) was found to be the most effective inducer. This observation is in agreement with our result. In contrast, Emerole *et al.* [16] not only failed to demonstrate the induction of ethylmorphine demethylase activity by pretreatment of animals for 3 days with imperatorin (XI) and oxypeucedanin (XIV) (50 mg/kg) but also found an inhibition of aniline hydroxylase activity without liver lesions. It is well known that a variety of DME inhibitors and inducers produce biphasic alteration in the metabolism of drugs [6]. The differences in action between DME inhibitors and inducers is merely that enzyme inhibitors more strongly inhibited the drug metabolism during the first phase than inducers and enzyme inducers more markedly stimulate the drug metabolism during the second phase than inhibitors. The pattern of alteration of HB concentration in serum and HB action in the animal groups treated with the furanocoumarins in the present study was quite similar to those treated with SKF-525A. These results indicate that the active coumarins may belong to a DME inhibitor rather than an inducer.

Further evidences supporting the liver enzyme inhibitory activity of the coumarins were obtained by the *in vitro* experiments. In coincidence with the results *in vivo*, it was

Table 2. Inhibitory potency of coumarins on HB hydroxylase and AP *N*-demethylase activity

Inhibitor	IC_{50} value ($M \times 10^{-5}$)*	
	HB hydroxylation	AP *N*-demethylation
SKF-525A	5.75	1.11
IX	11.63	10.91
XI	4.57	1.68
XII	5.95	1.45
XIII	9.63	5.00
XIV	8.09	2.04
XV	9.75	3.73
XVI	10.74	9.19

* The concentration that causes 50% inhibition.
For assay conditions, see Materials and Methods.

demonstrated that the furanocoumarins and the pyranocoumarin exhibited significant inhibitory effect on HB hydroxylation and AP *N*-demethylation at concentrations of 5×10^{-5} and 2×10^{-5} M respectively, whereas other coumarins did not affect enzyme activity as expected (Table 1).

The presence of a double bond in a furan or pyran ring attached to the coumarins seems to be essential for the manifestation of the activity, since the furanocoumarins and the pyranocoumarin with a double bond on the heterocyclic rings showed a significant activity while the other coumarins such as dihydrofurano- and dihydropyranocoumarins did not.

As disclosed by the direct comparison of the enzyme inhibitory potency of the coumarins by IC_{50} values (Table 2), imperatorin (XI) and isoimperatorin (XII), which possess an isoprenyl side chain, were found to elicit the strongest inhibitory potency on both HB hydroxylation and AP *N*-demthylation, which was approximately equipotent to that of SKF-525A. The inhibitory potency was decreased in the order of oxypeucedanin (XIV), isooxypeucedanin (XV) and oxypeucedanin methanolate (XVI), indicating that the polarity of the side-chain moiety is associated with a depression of the enzyme inhibitory potency. Xanthyletin (IX), which is devoid of any side chains, showed the least activity.

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REFERENCES

1. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
2. A. H. Conney, M. K. Buening, E. J. Pantuck, C. B. Pantuck, J. G. Fortner, K. E. Anderson and A. Kappas, *Ciba Found. Symp.* **76**, 147 (1980).
3. J. F. Mueller, *Hum. Nutr.* **3B**, 351 (1980).
4. D. M. Goldberg, *Clin. Chem.* **26**, 691 (1980).
5. C. F. Carr, *A. Rev. Pharmac. Toxic.* **22**, 19 (1982).
6. R. Kato, E. Chiesara and P. Vassanelli, *Biochem. Pharmac.* **13**, 69 (1964).
7. W. S. Woo, K. H. Shin, I. C. Kim and C. K. Lee, *Archs Pharm. Res.* **1**, 13 (1978).
8. W. S. Woo, K. H. Shin and K. S. Ryu, *Archs Pharm. Res.* **3**, 79 (1980).
9. W. S. Woo, C. K. Lee and K. H. Shin, *Planta med.* **45**, 234 (1982).
10. G. Feuer and L. Goldberg, *Biochem. J.* **103**, 13p (1967).
11. G. Feuer, *Can. J. physiol. Pharmac.* **48**, 232 (1970).
12. W. A. Ritschel and L. H. Lee, *Drug Dev. ind. Pharm.* **3**, 401 (1977).
13. J. R. Cooper and B. B. Brodie, *J. Pharmac. exp. Ther.* **114**, 409 (1955).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. T. Nash, *Biochem. J.* **55**, 416 (1953).
16. G. Emerole, M. I. Thabrew, V. Anosa and D. A. Okorie, *Toxicology* **20**, 71 (1981).

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Shift in double-sigmoid MAO inhibition patterns in oxygen-saturated reaction mixture

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Stimulation of the reaction velocity of monoamine oxidase (MAO, EC 1.4.3.4) by high oxygen tensions reported long ago [1, 2] has been observed in recent years to be substrate-selective [3–6]. Recently we have demonstrated that oxygen stimulation of rat brain MAO is type-selective [7], type B MAO being much more strongly stimulated than type A MAO. The biphasic inhibition patterns of MAO using common substrates like tyramine by selective inhibitors like clorgyline and deprenyl are often studied to get an approximate idea of the ratio of the two forms of MAO in a particular tissue preparation [8]. In most of these studies, however, little consideration has been given to the concentrations of the substrate amine employed, even though the amine concentrations used in various studies [9–11] can be perceived to have considerable influence on the inhibition patterns obtained. The concentration of the second substrate, i.e. oxygen, has not been given any consideration in these studies even though, in view of the type-selective nature of oxygen stimulation of MAO, oxygen tension in the incubation mixture may be expected to influence the biphasic MAO inhibition patterns considerably.

In the present study the expected shift in the double-sigmoid MAO inhibition curves with clorgyline, deprenyl and harmaline effected by oxgen saturation of the incubation medium was demonstrated. The crude mitochondrial preparations of rat brain and liver were used as the enzyme source. The preparation of crude brain mitochondria and the assay method for MAO activity using tyramine as substrate were as described earlier [12]. The enzyme was allowed 30 min pre-incubation with clorgyline and deprenyl, and 15 min pre-incubation with harmaline in an otherwise complete incubation mixture at 37° prior to the addition of substrate, and the product formed was measured after 15 min incubation. One set of incubation mixtures was equilibrated with air at 37° and was frequently shaken during incubation. The other set was flushed with 100% oxygen through a gas manifold and capillary tubes 5 min prior to the addition of amine substrate. A moderate flow of oxygen was maintained during the incubation period to ensure that the assay mixture remained saturated with oxygen.

Figure 1 shows the dose–response curves of the inhibition of tyramine oxidation by three selective MAO inhibitors, deprenyl, clorgyline and harmaline, in incubation mixtures equilibrated with air and 100% oxygen using the rat brain crude mitochondrial preparation. With each of the three inhibitors there was a noticeable shift in the inhibition curve obtained in the oxygen-saturated assay mixture from the one plotted with air as the gas phase and this shift was most marked in the plateau region. In each case, however, at higher inhibitor concentrations, the two inhibition curves converged again.

Figure 2 shows the results of similar experiments performed with rat liver mitochondrial preparation. The patterns obtained are qualitatively very similar to the patterns