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DRUG-INDUCED PORPHYRIN BIOSYNTHESIS—XIV
ROLE OF LIPOPHILICITY IN DETERMINING PORPHYRIN-INDUCING ACTIVITY OF ESTERS AND AMIDES AFTER BLOCKADE OF THEIR HYDROLYSIS BY BIS-[*p*-NITROPHENYL]PHOSPHATE*

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Abstract—The porphyrin-inducing activity of a series of aromatic esters and amides was measured in chick embryo liver cells. Sterically unhindered aromatic esters which are inactive in the absence of a carboxylesterase inhibitor, bis-[*p*-nitrophenyl]phosphate (BNPP), are markedly active in the presence of BNPP. On the other hand, the potency of sterically hindered esters is similar in the presence and absence of BNPP. In contrast to the aromatic esters, both sterically hindered and unhindered aromatic amides are active in the absence of BNPP. A high correlation was shown between porphyrin-inducing activity and lipophilicity of aromatic amides and esters after inhibition of aromatic ester hydrolysis by BNPP. It was concluded that porphyrin-inducing activity of aromatic esters and amides depends upon lipophilicity and resistance to rapid metabolism to compounds of lower lipophilicity.

Studies of the relationship between chemical structure and porphyrin-inducing activity have shown that a series of aliphatic and aromatic esters and amides are potent as porphyrin-inducing agents, while the corresponding acids are devoid of activity [1-3]. Recent evidence suggests that, in order for a chemical to cause porphyrin accumulation, it must remain in the liver for a period of at least several hr in order to induce and maintain high levels of δ-aminolevulinic acid (ALA)-synthetase [4–7]. Consequently, it follows that a porphyrin-inducing drug should possess, in addition to other features, appropriate chemical properties that prevent it from being rapidly metabolized and inactivated by the liver. In earlier studies [2, 3], we attempted to demonstrate that porphyrin-inducing activity was confined to aromatic and aliphatic esters and amides which were sterically hindered from hydrolysis to the corresponding acids. This hypothesis could not account for the relative porphyrin-inducing activity of the compounds studied and has been modified to take into account the importance of lipophilicity [8]. In recent studies of aliphatic amides [9], we have shown that porphyrin-inducing activity in chick embryo liver cells could be correlated with two properties of the compounds, viz. lipophilicity and resistance to rapid hydrolysis to compounds of lower lipophilicity. Bis-[*p*-nitrophenyl]phosphate (BNPP) [10, 11] is a specific relatively non-toxic inhibitor of liver carboxylesterase (carboxylic-ester hydrolase, EC 3.1.1.1.). The liver carboxylesterase also possesses amidase activity [12], and the hydrolysis of several amides can be inhibited by BNPP. The objective of this study was to determine whether the porphyrin-inducing activity of aromatic esters and amides could be correlated with lipophilicity after blockade of hydrolysis to compounds of lower lipophilicity with BNPP.

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EXPERIMENTAL

Fertilized eggs of a white Leghorn strain were obtained from Archers Poultry Farm, Brighton, Ontario. The eggs were stored in the refrigerator at 4° for no longer than 7 days prior to incubation at 38° at a relative humidity of 68 per cent. The age of the embryo was taken as the number of days from the onset of incubation.

Source of compounds and reagents. BNPP was prepared by the condensation of *p*-nitrophenol with phosphorous oxychloride [13]. The following compounds were purchased from Aldrich Chemical Co.: 3,5-dimethylbenzoic acid, 2,4,6-trimethylbenzoic acid and 2-(2,4,6-trimethylphenyl)-ethanoic acid (mesityl acetic acid). Ethyl benzoate and benzamide were obtained from Eastman Organic Chemicals and 2-(phenyl)-ethanoic acid and 2,4,6-trimethylbenzoic acid were purchased from Canadian Laboratory Supplies. The remaining esters and amides were synthesized by methods described previously [1, 2].

Trypsin (2.5% in saline) and powdered basal medium (Eagle) containing Earle's salts and glutamine were purchased from Grand Island Biological Co. Pooled bovine serum (lot 35) was purchased from Pentax, Inc., Winley–Morris Co., Ltd.

Cell culture techniques. Chick embryo liver cell cultures were prepared using the procedure of Granick [14] with the following modifications [15]: 17-day-old chick embryo livers were removed and a cell suspension was prepared in a mixture of 10 ml of 2.5% trypsin in saline and 10 ml magnesium- and calcium-free Earle's solution. The cell suspension was centrifuged at 250 *g* for 5 min, the supernatant was discarded and the cells were resuspended in warm growth medium (3 ml/liver); 0.2 ml of this suspension was added to each Petri dish, containing 5 ml of warm growth medium and placed in a Napco incubator at 37° and a constant air flow of 8.4 litres/min. The air flow was adjusted to contain 5% CO_2. After

24 hr of incubation, the media were removed from the cells and replaced with fresh media.

BNPP (50 μg) in 95% redistilled ethanol (5λ) or ethanol alone (5λ) was added to the cell cultures which were then returned to the incubator for 1 hr. Drugs dissolved in ethanol (10 μl) were then added to the cell cultures and the cultures were re-incubated. The porphyrin and protein content of the cells and medium was measured 24 hr later [14].

RESULTS AND DISCUSSION

The porphyrin-inducing activity of a series of analogues of ethyl benzoate has been shown previously to correlate with the degree of steric hindrance to hydrolysis of the ester group provided by adjacent *ortho*-methyl groups [1, 2]. Moreover, sterically unhindered esters, such as ethyl benzoate, which exhibited no activity in the absence of BNPP, were shown to be markedly active in the presence of BNPP [16]. On the other hand, the potency of sterically hindered esters such as ethyl 2,4,6-trimethylbenzoate was found to be similar in the presence and absence of BNPP [16]. The porphyrin-inducing activity of a series of analogues of benzamide is shown in Table 1. The results obtained in the present study using a quantitative procedure for porphyrin analysis are in general agreement with results previously obtained using a qualitative procedure for porphyrin analysis [1, 2]. In contrast to the aromatic esters [16], steric hindrance to the hydrolysis of the amides provided by the presence of *ortho*-methyl groups plays no role in the activity of these compounds, and sterically unhindered compounds such as benzamide, 3,5-dimethylbenzamide, 3-phenylpropanamide, 2-(2,4,6-trimethylphenyl) ethanamide and 3-(2,4,6-trimethylphenyl)propanamide display activity. Since the free acid analogues of the above aromatic amides are inactive, it follows that hydrolysis must be a slow process. In view of the above considerations, one would not expect BNPP pretreatment of liver cells to result in markedly increased activity of sterically unhindered aromatic amides as was the case with sterically unhindered aromatic esters. To see if this idea was correct, the activity of the sterically unhindered aromatic amides, 3-phenylpropanamide and 2-(2,4,6-trimethylphenyl) ethanamide, was measured before and after BNPP pretreatment of liver cells. No increase in porphyrin-inducing activity was observed (Table 1).

The relative biological activity of many different series of drugs can be correlated either linearly or parabolically with their lipophilicity [17–19]. Therefore, we have attempted to utilize the procedures developed by Hansch and co-workers to assess the importance of lipophilicity in determining porphyrin-inducing activity of the aromatic amides. As a measure of lipophilicity, Hansch and co-workers used log P, where P is the octanol-water partition coefficient of a drug. The log P values of benzamide (0.64), of 3-phenylpropanamide (0.91) and of 2-phenylethana-

Table 1. Porphyrin accumulation in response to benzamide analogues in a primary culture of chick embryo liver cells

Compound*	Concn (μg/ml)	Porphyrin accumulation† (ng/mg protein) In absence of BNPP	In presence of BNPP (10 μg/ml)
3-Phenylpropanamide	30	12.0 ± 2.5 (4)	19.5 ± 4.7 (4)
	150	188.0 ± 48.2 (5)	142.3 ± 54.6 (5)
	300	422.3 ± 86.2 (5)	251.1 ± 31.5 (5)
2-(2,4,6-Trimethyl-phenyl) ethanamide	10	245.5 ± 33.2 (4)	244.8 ± 12.0 (3)
	30	399.6 ± 58.7 (4)	415.9 ± 18.5 (4)
	100	406.6 ± 14.6 (5)	393.0 ± 30.0 (4)
3-(2,4,6-Trimethyl-phenyl) propanamide	1	15.2 ± 1.6 (4)	
	3	73.9 ± 14.7 (4)	
	10	410.6 ± 18.2 (4)	
	40	674.8 ± 46.2 (4)	
Benzamide	500	63.1 ± 23.5 (4)	
	1000	625.6 ± 142.2 (4)	
	1500	1127.1 ± 132.6 (3)	
3,5-Dimethyl-benzamide	150	808.0 ± 25.6 (4)	
	250	697.0 ± 25.1 (4)	
	400	743.1 ± 59.2 (4)	
2,4,6-Trimethyl-benzamide	30	546.6 ± 30.6 (4)	
	100	645.6 ± 33.9 (4)	
	200	754.0 ± 17.7 (4)	

* Control experiments were carried out as follows: 95% ethanol (5 μl) was added to three dishes and BNPP (50 μg) in 95% ethanol (5 μl) to three dishes. After 1 hr of incubation, 95% ethanol (10 μl) was added to the dishes which were reincubated for 24 hr. The porphyrin content of the dishes was then determined. The 95% ethanol control had 10.7 ± 0.9 ng/mg of protein and the BNPP 95% ethanol control had 8.8 ± 0.2 ng/mg of protein.

† The values shown represent the mean of the number of determinations shown in parentheses ± standard error.

Table 2. Observed and calculated concentrations of aromatic amides and esters which give the same porphyrin-inducing activity as AIA (10 μg/ml)

Compound	Log *P*	Log 1/*C*		Δ Log 1/*C*
		Obs'd	Calc'd	
Linear case*				
Ethyl benzoate	2.64	3.36	3.59	0.23
Ethyl 2-(phenyl) ethanoate	2.30	3.65	3.44	0.21
Ethyl 3,5-dimethylbenzoate	3.76	3.97	4.11	0.14
Ethyl 2,4,6-trimethylbenzoate	4.32	4.04	4.36	0.32
Ethyl 2-(2,4,6-trimethyl) ethanoate	3.98	4.45	4.21	0.24
Ethyl 3-(2,4,6-trimethyl-phenyl) propanoate	4.50	4.48	4.45	0.03
Benzamide	0.64	2.08	2.68	0.60
3,5-Dimethylbenzamide	1.76	3.19	3.19	0.00
2,4,6-Trimethylbenzamide	2.32	3.43	3.45	0.02
2-(2,4,6-Trimethylphenyl) ethanamide	2.13	3.55	3.36	0.19
3-(2,4,6-Trimethylphenyl) propanamide	2.58	3.80	3.57	0.23
3-Phenylpropanamide	0.91	3.21	2.80	0.41
Parabolic case†				
Ethyl benzoate	2.64	3.36	3.69	0.33
Ethyl 2-(phenyl) ethanoate	2.30	3.65	3.53	0.12
Ethyl 3,5-dimethylbenzoate	3.76	3.97	4.12	0.15
Ethyl 2,4,6-trimethyl-benzoate	4.32	4.04	4.28	0.24
Ethyl 2-(2,4,6-trimethyl-phenyl) ethanoate	3.98	4.45	4.19	0.26
Ethyl 3-(2,4,6-trimethyl-phenyl) propanoate	4.50	4.48	4.32	0.16
Benzamide	0.64	2.08	2.50	0.42
3,5-Dimethylbenzamide	1.76	3.19	3.23	0.04
2,4,6-Trimethylbenzamide	2.32	3.43	3.54	0.11
2-(2,4,6-Trimethylphenyl) ethanamide	2.13	3.55	3.44	0.11
3-(2,4,6-Trimethylphenyl) propanamide	2.59	3.80	3.67	0.13
3-Phenylpropanamide	0.91	3.21	2.70	0.51

* Linear case: log $1/C = 0.458 \log P + 2.384$; n = 12; $r = 0.897$; S.E.M. of estimate 0.299 (3).

† Parabolic case: log $1/C = -0.067 (\log P)^2 + 0.812 \log P + 2.011$; n = 12; $r = 0.910$; S.E.M. of estimate 0.296 (4).

mide (0.45) were available [20, 21], and the log *P* values of the other amides (Table 2) were calculated by adding a value of 0.56 for each —CH_3— group added to the benzene ring. Hansch and co-workers have defined the relative biological activity of a drug in terms of log 1/*C*, where *C* is the molar concentration of a drug producing a standard biological response. For our studies, we have defined the porphyrin-inducing activity observed 24 hr after the addition of AIA (10 μg/ml) to chick embryo liver cells as the standard biological response. Dose-response relationships were determined for each amide and the molar concentration (*C*) of each drug, which gave the same response as AIA (10 μg/ml), in the same experiment, was determined. Observed log 1/*C* values (Table 2) were derived from this information.

The degree of correlation between log 1/*C* and log *P* was determined for the aromatic amides. For this purpose regression analysis by the method of least squares was used to determine equations defining the 'best' fit straight line (equation 1) and the 'best' fit parabola (equation 2) through the data.

The equations derived were:

$$\begin{aligned} \log 1/C &= 0.645 \log P + 2.098; \\ n &= 6; \\ r &= 0.850; \\ \text{S.E.M. of estimate} &= 0.352. \end{aligned} \quad (1)$$

$$\begin{aligned} \log 1/C &= -0.266 (\log P)^2 \\ &\quad + 1.487 \log P + 1.576; \\ n &= 6; \\ r &= 0.865; \\ \text{S.E.M. of estimate} &= 0.387. \end{aligned} \quad (2)$$

where n is the number of data points, *r* is the correlation coefficient, and S.E. is the standard error [17, 19]. Our analysis indicated that $F_{1,4}$ for the linear case was 10.394 ($F_{1,4}\ \alpha_{0.05}$ is 7.71) and $F_{2,3}$ for the parabolic case was 4.469 ($F_{2,3}\ \alpha_{0.05}$ is 9.55). It was concluded that a linear relationship existed between lipophilicity and the porphyrin-inducing activity of aromatic amides.

Since the aromatic esters previously studied [16] and the aromatic amides are congeners of benzoic

acid, the possibility was considered that a single equation might describe a relationship between lipophilicity and the porphyrin-inducing activity of both aromatic esters and amides. The log P value of the aromatic esters (Table 2) was calculated as follows. The log P values of ethyl benzoate (2.64), ethyl 2-(phenyl) ethanoate (2.30) and methyl 3-(phenyl) propanoate (2.32) were available [21]*, and the log P values of the other esters (Table 2) were obtained by adding a value of 0.56 for each methyl group added to the benzene ring and of 0.5 when an ethoxycarbonyl group was substituted for a methoxycarbonyl group [22]. Dose response curves were constructed for each aromatic ester, in the presence of BNPP, in a single chick embryo liver cell culture experiment. This procedure was adopted in order to eliminate experiment-to-experiment variation. From each dose-response curve, the molar concentration (C) of each ester was determined, which gave the same porphyrin-inducing activity as a dose of AIA (10 μg/ml) in the same experiment. From this information observed log $1/C$ values were obtained (Table 2). The log P values and the log $1/C$ values of the aromatic esters, in the presence of BNPP, and the corresponding values determined for the aromatic amides, in the absence of BNPP, were combined (Table 2) and regression analysis of the data gave equations 3 and 4:

$$\begin{aligned} \log 1/C &= 0.458 \log P + 2.384; \\ n &= 12; \\ r &= 0.897; \\ \text{S.E.M. of estimate} &= 0.299; \end{aligned} \tag{3}$$

$$\begin{aligned} \log 1/C &= -0.067 (\log P)^2 \\ &\quad + 0.812 \log P + 2.011; \\ n &= 12; \\ r &= 0.910; \\ \text{S.E.M. of estimate} &= 0.296. \end{aligned} \tag{4}$$

For each compound, the log P value, the experimentally observed log $1/C$ value and the log $1/C$ value calculated to fit equations 3 and 4 are given in Table 2. The experimentally observed values of log $1/C$ for each drug are plotted against the log P values in Fig. 1 and the 'best' fit straight line (defined by equation 3) was drawn through them. Our analysis indicated that $F_{1,10}$ for the linear case was 41.2 ($F_{1,10}$ α $_{0.001}$ is 21.04) and $F_{2,9}$ for the parabolic case was 21.74 ($F_{2,9}$ α $_{0.001}$ is 16.39). Thus, a linear equation suffices to correlate lipophilicity with the porphyrin-inducing activity of aromatic esters and amides. Since the above correlation cannot be achieved if the activity of aromatic esters is measured in the absence of BNPP pretreatment, it is clear that neither steric factors nor lipophilicity alone can explain the porphyrin-inducing activity of these compounds but that consideration of both factors is required.

*C. Hansch, private communication (1975).

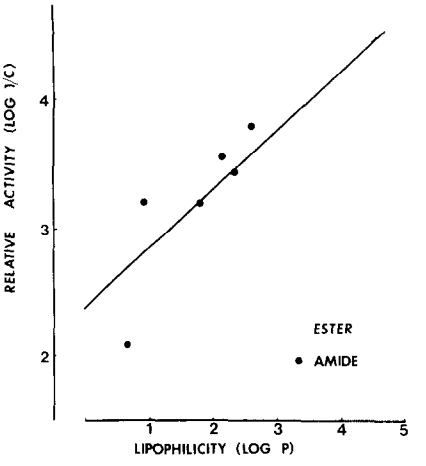

Fig. 1. Relationship between lipophilicity and porphyrin-inducing activity of aromatic esters in the presence of BNPP and of aromatic amides alone.

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