Pharmaceutical Research, Vol. 8, No. 7, 1991

Research Article

Structure–Metabolism Relationships in the Hydrolysis of Nicotinate Esters by Rat Liver and Brain Subcellular Fractions

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Received October 30, 1990; accepted January 12, 1991

Rat liver and brain subcellular esterase activities toward nicotinic acid esters were studied, under varying conditions, such as pH, organic solvents, protein concentration, duration of incubation, and substrate concentration. Esterases in each subcellular fraction displayed activities that obey Michaelis–Menten kinetics, although subcellular fractions are heterogeneous. The K_m values were of the same magnitude, and the V_{max} values were lower in microsomes than in cytosol of the liver. Brain activities normalized to protein concentration, were much lower than liver activities, aromatic nicotinates being the best substrates in both tissues. Myelin and brain mitochondria of nerve-ending and neuroglial origin display esterase activity toward phenyl nicotinate. In contrast to brain esterases, liver esterases appear homogeneous, and esterase activities in both tissues react differently to changes in pH. Qualitative and quantitative structure–metabolism relationships are not suggestive of tissue-specific ester hydrolysis.

KEY WORDS: nicotinic acid esters; carboxylesterases; subcellular fractions; structure–metabolism relationships.

INTRODUCTION

Nicotinic acid plays an essential physiological role as precursor of cofactors to many vital enzymes, and it is a therapeutical agent capable of reducing high blood levels of triglycerides, cholesterol, and lipoproteins associated with atherosclerosis. Vasodilating and fibrinolytic properties have also been described (1). Because of its rapid elimination and short half-life, the required high doses of nicotinic acid cause several unpleasant side effects limiting patient compliance (2). Several prodrugs of nicotinic acid have been developed, among them nicotinate esters, which may have pharmacokinetic advantages as compared to the parent acid. Ester prodrugs must undergo activation in the body through chemical and/or enzymic hydrolysis. The latter is catalyzed by esterases, which are widely distributed in most if not all mammalian tissues.

Most of the liver esterase activity is attributable to an homogeneous population of enzymes, i.e., carboxylesterases localized in hepatocytes (3). Hydrolytic activities toward several ester drugs such as aspirin have been assessed in liver subcellular fractions (lysosomes, mitochondria, microsomes, or cytosol) of several animal species.

In contrast to liver esterases, literature reports indicate that brain esterases form a heterogeneous mixture of enzymes with broad overlapping substrate selectivities and inhibitor sensitivities, and human brain esterases markedly differ from hydrolytic enzymes in other tissues (4).

The tissue or organ specificity of prodrug activation may be desirable in some cases; the target organ for the hypolipemic action of nicotinic acid is mainly the liver (1), while in the case of cerebral insufficiency related to circulatory problems, nicotinic acid should exert its vasodilating effect specifically upon brain capillaries (5) to avoid the unpleasant flush associated with systemic treatment.

Several authors have studied the influence of chemical structure on the hydrolysis of a variety of esters, for example, esters of *n*-fatty acids (6), salicylic acid (7), steroidal hormones (8), benzodiazepines (9), a metabolite of 5-fluorouracil (10); γ-aminobutyric acid (GABA) (11), benzoic acid (12), or the anticonvulsant nipecotic acid (13). In many cases, however, a limited set of compounds was considered, whose structural variations were often restricted to aliphatic or phenylic esters. Frequently, the biological parameters determined were rate constants such as log k or $t_{1/2}$, but we have chosen to determine the kinetic constants K_m and V_{max}, whose ratio is related to $t_{1/2}$ and which describe the substrate binding step and the catalytic step, respectively.

In other experiments, the kinetic constants of a series of nicotinate esters were measured for their hydrolysis catalyzed by a purified hog liver carboxylesterase and human plasma (in preparation). Here, we investigated their enzymatic hydrolysis in biological media such as rat liver and brain subcellular fractions. Among the full set of available nicotinate esters (14), 16 compounds were selected, which feature various alkyl, arylalkyl, and aryl substituents showing the largest possible variations in physicochemical properties. It has already been demonstrated that the half-life of chemical hydrolysis of most nicotinate esters falls in the range 100–1000 hr at pH 7.4 and 37°C (15). The influence

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0724-8741/91/0700-0832$06.50/0

of pH on rat brain and liver esterase activities toward two nicotinates was also determined. Our purpose was to compare a series of substrates under "standard" conditions, as this may provide some insight into the capacity of the two organs to activate potential prodrugs of nicotinic acid.

MATERIALS AND METHODS

Chemicals

The esters of nicotinic acid were synthesized in our laboratory according to known methods (14) with the exception of four compounds which were obtained from commercial sources or kindly donated (see Table I).

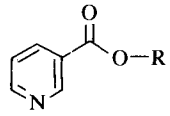
Solvents and chemicals were of analytical or HPLC grade. The Tris buffer [tris(hydroxymethyl)aminomethane] was purchased from Merck (Darmstadt, Germany), and the MES (morpholinoethanesulfonic acid and Na^+ salt) and TES buffers (2-{[tris(hydroxymethyl)methyl]amino}-ethanesulfonic acid and Na^+ salt) from Calbiochem (La Jolla, CA). Ficoll 400 was obtained from Pharmacia (Uppsala, Sweden).

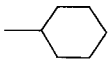
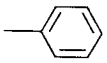
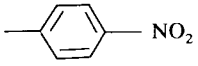
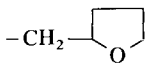
Preparation of Liver Subcellular Fractions

Male Sprague–Dawley rats of body weight 180–200 g (Madörin Kleintierfarm, Füllinsdorf, Switzerland) were used throughout. The animals were fasted overnight, then anesthetized with an ip injection of pentobarbital (80 μg/kg), and the livers of four animals were perfused 15 min with 15 ml/min 10 m*M* Tris/HCl, pH 7.4, containing 0.9 g/L NaCl to remove blood, since albumin and hemoglobin are known to display esterase activity at least in humans. The remaining hemoglobin (<5%) in liver subcellular fractions was quantified as the CO complex (λ_{max}, 419 nm) using a Perkin Elmer UV PE 557 spectrophotometer (Norwalk, CT).

Four grams of liver were homogenized in 12 ml of a 0.01 *M* Tris/HCl solution (pH 7.4) containing 0.32 *M* sucrose ("isolation medium") in a Potter S Braun homogenizer consisting of a glass body and a Teflon pestle and maintained on

Table I. Structure of Investigated Substrates



Nicotinate	R	Nicotinate	R
Methyl[a]	$-CH_3$	Cyclohexyl	
Ethyl[b]	$-CH_2CH_3$		
Isopropyl	$-CH(CH_3)_2$	Phenyl	
n-Butyl	$-(CH_2)_3CH_3$		
t-Butyl	$-C(CH_3)_3$	Benzyl[a]	$-CH_2$ 
n-Hexyl[c]	$-(CH_2)_5CH_3$		
n-Octyl	$-(CH_2)_7CH_3$	*p*-Nitrophenyl	
3-Hydroxypropyl	$-(CH_2)_3OH$		
Methoxyethyl	$-CH_2\ CH_2OCH_3$	Phenoxyethyl	$-CH_2-CH_2-O$ 
1-Carbamoylethyl	$-CH(CH_3)CONH_2$		
2-Tetrahydrofurfuryl[d]			

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[c] Ega-Chemie, Steinheim/Albuch, Germany.
[d] Ciba-Geigy AG, Basel, Switzerland.

ice. Homogenates were diluted to 28 ml prior to centrifugation.

Liver subcellular fractions were prepared according to Ref. 16. The homogenates were centrifuged 5 min at 1700*g* (Centrikon T 2070 Kontron, Zürich, Switzerland). The crude nuclear pellet was suspended in the isolation medium and recentrifuged 5 min at 1700*g*. Both 1700*g* supernatants were then mixed and centrifuged 20 min at 11,000*g*. The 11,000*g* pellet yielded a crude mitochondrial fraction. Microsomes and cytosol were obtained from the 11,000*g* supernatant at the end of a 60-min 105,000*g* centrifugation. Crude mitochondrial and microsomal fractions were resuspended in the isolation medium and recentrifuged to yield "washed" fractions.

Preparation of Brain Subcellular Fractions

Eight rats were decapitated, and their brains removed and dissected on ice to remove blood vessels and pial membranes. Four grams of brain were homogenized in 15 ml of Tris/sucrose solution in a manual Dounce (Bellco, Vineland, NJ) glass tissue grinder kept on ice. Homogenates were diluted to 28 ml prior to centrifugation, and the procedure to isolate the crude mitochondrial and microsomal fractions and the cytosol was the same as before, with the only difference that the 105,000*g* centrifugation to produce microsomes lasted 90 min instead of 60 (17) to increase the yield in brain microsomal proteins.

The crude mitochondrial pellet (11,000*g* pellet) was resuspended to a final volume of 9 ml in a 3% Ficoll 400 solution layered on 19 ml of a 6% Ficoll 400 solution to yield mitochondria of neuroglial origin. Another subpopulation of mitochondria could be isolated from the crude mitochondrial fraction, using a three-phase density gradient prepared with Ficoll solutions of 13% (11 ml) and 7.5% (11 ml); the third phase consisted of a 7-ml suspension of crude mitochondria in isolation medium. This procedure yields myelin and synaptosomes, i.e., vesicules containing mitochondria of nerve-ending origin. The whole procedure for the isolation of rat brain subcellular fractions was reproduced from Walther *et al.* (17,18), who checked the preservation of mitochondrial integrity and the lack of microsomal contamination by mitochondrial fragments with marker enzymes.

Protein Determination

Protein content in liver and brain subcellular fractions was determined according to the procedure of Lowry *et al.* (19) with bovine serum albumin as a standard.

Assay of Nicotinate Ester Hydrolase Activities

Samples of liver and brain subcellular fractions were incubated with nicotinate ester in closed glass tubes standing in a rotating (260-rpm) water bath (Infors WTR-1, Zürich, Switzerland) at 37°C. The pH of the incubation medium is specified for each experiment. The protein samples were preincubated for 3 min for temperature equilibration (37°C), after which 50 μl of a solution of ester of varying concentrations were added; addition of the substrate defined $t = 0$.

The product of enzymic hydrolysis (nicotinic acid) was extracted and quantified by a method previously described (20). Esterase activities are expressed as nanomoles of nicotinic acid formed per minute of incubation and per milligram of total proteins (nmol/min/mg).

Influence of Organic Solvent on Esterase Activities

Most of the nicotinate esters were not sufficiently soluble to allow investigation over a wide enough range of concentrations (0.1–5 K_m). It was therefore necessary to select an organic cosolvent that would exert the least possible influence on esterase activities.

Triplicate samples containing 300 μg of microsomal proteins suspended in 950 μl of a pH 7.4 Tris/HCl buffer were incubated with a 50-μl solution of ethyl nicotinate in buffer, acetonitrile, acetone, or DMSO to give a final substrate concentration of 5.6×10^{-3} *M*. Cosolvent concentration in the incubation medium was thus limited to 5% (v/v). Activities were measured after 5 min at 37°C.

Influence of Protein Concentration and Duration of Incubation

Physiological conditions of pH (7.4) and temperature (37°C) were used in these experiments. Triplicate samples of liver mitochondrial proteins, containing 14–1380 μg of total proteins/ml, were incubated with 5.9×10^{-4} *M* ethyl nicotinate added as a DMSO solution. Chemical hydrolysis was negligible (15).

Brain microsomes (160–2010 μg proteins/ml) were incubated 30 min with 5×10^{-4} *M* phenyl nicotinate, as preliminary assays had shown that rat brain esterases are quite active toward this substrate. Here it was necessary to take chemical hydrolysis into account by incubating blanks, devoid of proteins, under identical conditions and subtracting the results from the observed biological hydrolysis.

The influence of time was assessed by incubating liver mitochondria (290 μg proteins/ml) with the same concentration of ethyl nicotinate as before for 1, 2, 5, 10, 20, and 40 min, and brain microsomes (500 μg proteins/ml) with phenyl nicotinate for 10, 30, 60, and 120 min.

Subcellular Distribution of Liver Esterase Activity

Specific activities were measured after 5 min of incubation of 2.9×10^{-3} *M* ethyl nicotinate dissolved in DMSO (final concentration, 5%) at a pH of 7.4 and a temperature of 37°C, in the presence of liver homogenate or subcellular fractions (nuclear, mitochondrial and microsomal fractions, and cytosol).

Influence of pH on Rat Liver and Brain Subcellular Hydrolytic Activities

Liver microsomes (70 μg proteins/ml), brain microsomes (850 μg proteins/ml), or cytosol (850 μg proteins/ml) were incubated for 5, 30, and 60 min, respectively, at 37°C, with 0.6 m*M* phenyl nicotinate or 18 m*M* ethyl nicotinate, at different pH values between 3.5 and 10. The incubation media were buffered with TES or MES (pH 3.5 to 7) and Tris/HCl (pH 7.4 to 10). For each pH, a blank without proteins was incubated, and nicotinic acid released by chemical hydrolysis quantified and subsequently subtracted from the total amount formed in the presence of proteins.

Determination of K_m and V_{max} Values by Iterative Nonlinear Optimization

Triplicates of six solutions of increasing concentration of nicotinate esters (containing 5%, v/v, DMSO) were incubated at 37°C and pH 7.4 with samples of brain or liver subcellular fractions. Equations of a rectangular hyperbola corresponding to Michaelis–Menten kinetics were fitted to the untransformed experimental data by iterative nonlinear optimization using an IBM AT personal computer.

Quantitative Structure–Metabolism Analysis of the Data

The search for the best combination of physicochemical properties quantifying nicotinate esters (independent or explanatory variables) (15) in correlation with log V_{max}, pK_m, or log V_{max}/K_m (dependent variables or target properties) was done with the software SAS (SAS Institute Inc., Cary, NC) running on a Vax-8550. The selection criterion was the squared correlation coefficient (r^2). The equations were calculated and cross-validated (cross-validated squared correlation coefficient r^2_{c-v} (21) with SYBYL (Tripos Associates) running on a SUN SPARC 1 workstation.

RESULTS AND DISCUSSION

Influence of Organic Solvents on Esterase Activities

Among the three solvents studied, dimethylsulfoxide (DMSO) had little effect on microsomal activity toward ethyl nicotinate, while acetone and acetonitrile were inhibitory (Table II). The influence of organic solvents depends on their concentration and might not be identical toward all isoenzymes present in subcellular fractions. Further, we compared V_{max} and K_m for ethyl nicotinate in buffer and in 5% DMSO. The values (Table III) confirm that the influence on esterase activity toward this ester (as described by V_{max}) is negligible, while K_m is increased.

Even a 5% concentration of solvent was not enough to prepare a proper concentration range for the determination of V_{max} for 4-nitrophenyl nicotinate in the liver microsomal fraction. In contrast, this was possible for the *n*-hexyl and *n*-octyl nicotinates due to their low K_m values (hence a lower concentration range) and despite their poor solubility.

Table II. Influence of Organic Solvents on the Activity of Rat Liver Microsomes Toward Ethyl Nicotinate

Cosolvent (5%, v/v)	Activity ± SD[a]
Control[b]	100%
Acetonitrile	42 ± 3%
Acetone	59 ± 2%
Dimethylsulfoxide	88 ± 5%

[a] Activity expressed as a percentage of control activity (n = 3).
[b] Conditions: pH 7.4 (Tris buffer); temperature, 37°C; no cosolvent.

Table III. Influence of Dimethylsulfoxide (5%, v/v) on Ethyl Nicotinate Hydrolysis in Rat Liver Microsomes

Michaelis–Menten constant	Tris buffer[a]	DMSO[b]
K_m ± SD (10^{-4} *M*)	15 ± 3	62 ± 20
V_{max} ± SD (nmol/min/mg prot.)	1990 ± 190	1880 ± 120

[a] Tris/HCl buffer, pH 7.4; 37°C.
[b] Tris/HCl, pH 7.4, +5% (v/v) DMSO; 37°C.

Esterase Activities as a Function of Protein Concentration

The amount of nicotinic acid formed by enzymic hydrolysis of ethyl nicotinate increases linearly with the content in liver mitochondrial proteins up to 700 μg/ml (r^2 = 0.995; data not shown), above which a curvature occurs, possibly because of product inhibition by nicotinic acid or because initial reaction rates are no longer observed.

The brain microsomal activity toward phenyl nicotinate increases linearly with protein concentration up to 1000 μg/ml (r^2 = 0.991; data not shown). The amount of nicotinic acid formed by chemical hydrolysis during the 30-min incubation is readily detectable (0.35 nmol/min).

These two experiments, and the two following ones, served to test whether our experimental conditions allowed measurements of initial rates. Subsequent experiments with brain subcellular fraction incubation media contained 850 μg/ml proteins, and with the liver fractions, between 70 and 350 μg/ml.

Esterase Activities as a Function of Incubation Time

Within experimental error, rat liver mitochondrial hydrolytic activity toward ethyl nicotinate was proportional to the duration of incubation for 10 min, after which a curvature is observed (data not shown). Subsequent incubations of liver samples therefore lasted 5 or 10 min.

Rat brain microsomal activity toward phenyl nicotinate was proportional to the duration of incubation up to 60 min (r^2 = 0.991), with a modest fall-off during the next hour (data not shown). All subsequent incubations of brain samples lasted 30 or 60 min.

Subcellular Localization of Rat Liver Hydrolytic Activity Toward Ethyl Nicotinate

Many authors have demonstrated the high esterolytic activity of microsomes, although other authors consider this microsomal activity to be at least partly artifactual (24). In this study, 46% of total rat liver esterase activity was found in the microsomal fraction. These results are similar to those obtained with acetylsalicylic acid (25). Further, 17% of rat liver hydrolytic activity toward ethyl nicotinate is located in the mitochondrial fraction, which includes lysosomes and peroxisomes, two types of organelles containing hydrolytic enzymes, when prepared according to the present protocol. Mitochondrial esterases are reported to be similar to microsomal enzymes (3).

The presence of specific esterases in nuclear and cytosolic fractions is still under debate. In this study, 14 and 5% of total rat liver activity toward ethyl nicotinate, respec-

tively, were found in those two fractions. However, many authors consider nuclear esterase activity to be due to contamination by large fragments of the endoplasmic reticulum, although von Deimling and Boecking (26) have demonstrated a specific esterase activity in nuclear envelopes by means of a histochemical study on whole cells. We have measured only weak activity in the cytosol, which is possibly due to the solubilization of labile microsomal esterases (25).

Subcellular Localization of Rat Brain Hydrolytic Activity Toward Phenyl Nicotinate

Phenyl nicotinate was chosen following preliminary assays for its reactivity toward brain esterases. The kinetic constants V_{max} and K_m obtained in several cerebral subcellular fractions are reported in Table IV, showing that the highest activities are located in myelin and synaptosomes. Eto and Suzuki (27) found a myelin-specific cholesterol ester hydrolase in rat brain, which was different from myelin-associated nonspecific esterase (28). We have also found ethyl and *n*-hexyl nicotinates to be hydrolyzed by myelin esterases, although at a much slower rate (results not shown). It has often been suggested that most, if not all, esterase activity associated with isolated myelin arises from artifactual redistribution from other sites in the cell; however, a study by Rumsby *et al.* (29) of nonspecific hydrolytic activities of bovine myelin toward naphthyl esters showed that the myelin enzymes are intimately associated with the membrane structure and do not result from contamination.

Influence of pH on Rat Liver and Brain Esterase Activities

An aliphatic and an aromatic ester were chosen for this study, previous experiments having shown that aromatic esters were hydrolyzed faster than aliphatic esters in brain but not in liver subcellular fractions, suggesting different populations of enzymes.

The substrate concentrations (18 m*M* ethyl and 0.6 m*M* phenyl nicotinate, respectively) were nonsaturating. As shown in Fig. 1, the influence of pH on liver microsomal esterolytic activity toward phenyl nicotinate is modest, a 25% variation being observed between pH 4.3 and pH 9.1. A first region of optimal pH, although barely significant, can be observed at pH 6.4 (90% of maximal activity); an optimal value for ethyl nicotinate is also found at pH 6.4 (60% of maximal activity). The maximal activity (100%) toward phenyl nicotinate is displayed in a broad pH domain between 7.4

Table IV. Hydrolysis of Phenyl Nicotinate in Rat Brain Subcellular Fractions[a]

Fraction	K_m (SD)	V_{max} (SD)
Myelin	6.41 (0.90)	48.0 (3.9)
Synaptosomes	3.01 (0.43)	27.4 (1.8)
Microsomes	23.9 (2.0)	0.89 (0.22)
Mitochondria[b]	35.1 (1.1)	3.98 (0.24)
Cytosol	7.3 (0.2)	1.60 (0.12)

[a] K_m as m*M*. V_{max} as nmol nicotinic acid/min/mg protein. Conditions: pH 7.4 in Tris/HCl buffer, 5% (v/v) DMSO; temperature, 37°C.

[b] Mitochondria of neuroglial origin.

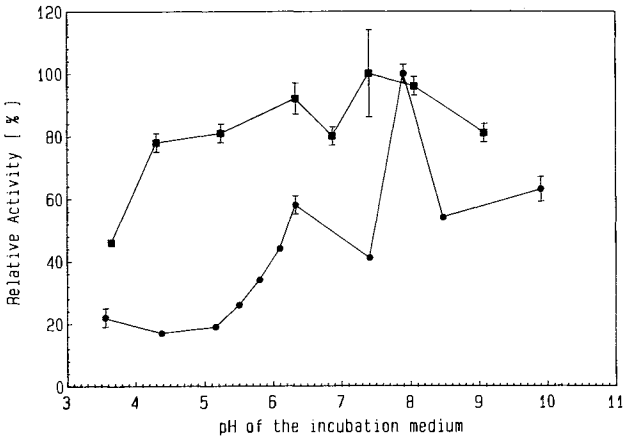

Fig. 1. Influence of pH on rat liver microsomal esterase activity. (■) Phenyl nicotinate; (●) ethyl nicotinate.

and 8. In contrast, a well-defined optimal pH can be seen for ethyl nicotinate at pH 7.9. The most important difference between the two esters is their rate of hydrolysis at physiological pH (7.4), which is optimal toward phenyl nicotinate but weak (41% of maximal activity) toward ethyl nicotinate. This result suggests the existence of an esterase with preference for aryl esters. Many reports confirm that liver carboxylesterases optimally hydrolyze a variety of substrates at pH values between 7 and 9 (3,25).

The relative brain microsomal activity toward ethyl nicotinate (Fig. 2) in the acidic range is only slightly lower than the maximal rate measured at pH 6.9. This is not true for phenyl nicotinate, whose hydrolysis is slow below pH 6. This activity is absent from brain cytosol (Fig. 3), where the optimal pH is 7.4; with ethyl nicotinate a cytosolic activity was detectable only at pH 6.9 (75% of main activity) and 7.4 (main activity). At other pH values enzymatic hydrolysis was not significantly greater than chemical hydrolysis. This result is in agreement with published data (4,27) showing brain esterases to be an heterogeneous population of enzymes with different and/or diffuse pH optima.

Influence of Substrate Concentration on Rat Liver and Brain Subcellular Hydrolytic Activities Toward Nicotinate Esters

Nicotinate esters were incubated over 50- to 100-fold

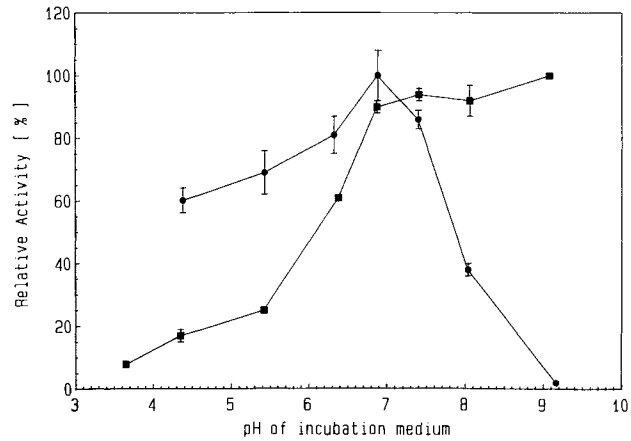

Fig. 2. Influence of pH on rat brain microsomal esterase activity. (■) Phenyl nicotinate; (●) ethyl nicotinate.

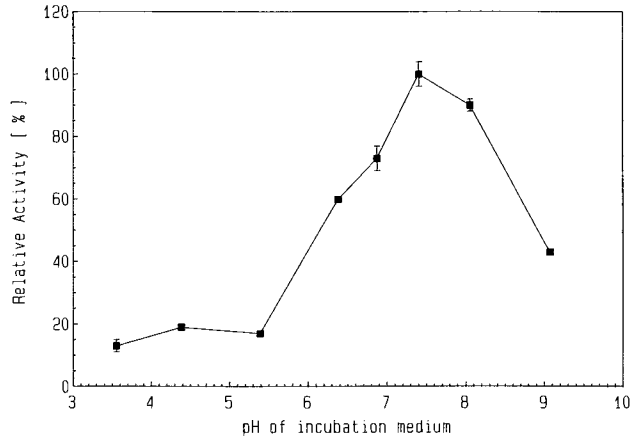

Fig. 3. Influence of pH on rat cytosolic esterase activity toward phenyl nicotinate.

concentration ranges to ensure sufficient accuracy in the determination of K_m. The results for hepatic subcellular fractions are presented in Table V. In general, V_{max} values are highest in microsomes and lowest in cytosol, while K_m values are comparable in the three fractions. Thus, the highest V_{max} values are displayed by the phenyl ester, i.e., 5770, 1860, and 335 nmol/min/mg proteins in microsomes, mitochondria, and cytosol, respectively. Nicotinates with the highest affinity (e.g., the *n*-hexyl and *n*-octyl esters) are among the poorest substrates.

For the study of brain subcellular fractions, five substrates were selected from preliminary experiments and for maximal physicochemical and structural diversity. The results (Table VI) show for each substrate comparable V_{max} values in the three fractions, in contrast to the liver. The K_m values also are comparable. Here again, the phenyl ester is the best substrate, while the *n*-hexyl ester has a high affinity but is slowly hydrolyzed.

Liver and brain fractions show similar K_m values, indicating similarities in the enzymes involved. However, V_{max} values are 2–3 orders of magnitude larger in hepatic than in cerebral microsomes and about 2 orders of magnitude larger in the mitochondrial and cytosolic fractions of liver than brain.

Quantitative Structure–Metabolism Relationships

The biological data in Tables V and VI were analyzed for quantitative relationships with physicochemical parameters, including the previously reported lipophilicity of compounds (14) (expressed as log k^0_w determined by RP-HPLC), steric parameters such as molar refractivity MR and dimensions of the substituents [Verloop's parameters L (length), B_1 and B_5 (breadth)], and electronic parameters such as $\sigma_{Hammett}$ (30) and previously reported ^{13}C-NMR chemical shifts of the nicotinates (15). The correlation analyses were performed by multiple linear regression (MLR) using the cross-validation procedure, which tests the predictive utility of the QSARs. In each cycle, one of the data points is left out, the model rederived, the omitted target property predicted, and its residual calculated to yield ultimately a cross-validated correlation coefficient r^2_{c-v}; value of r^2_{c-v} greater than 0.5 are considered to be reasonable. This approach provides strong assurance that the QSAR is not a chance correlation. The following best questions were thus obtained:

$$pK_{m\ \text{liver microsomes}} = -0.23(\pm0.05)(\log k^0_w)^2 + 1.8(\pm0.3)\log k^0_w + 0.36\ (\pm0.38) \quad (1)$$

$$n = 10,\quad r^2 = 0.913,\quad r^2_{c-v} = 0.871,\quad S = 0.202,\quad F = 36.8,\quad \log k^0_{w\ opt.} = 3.8$$

where n is the number of data points, r^2 is the squared correlation coefficient, r^2_{c-v} is the squared cross-validated correlation coefficient, S is the standard deviation of regression,

Table V. Hydrolysis of Nicotinate Esters in Rat Liver Subcellular Fractions[a]

Nicotinate	Microsomes		Mitochondria		Cytosol	
	K_m (SD)	V_{max} (SD)	K_m (SD)	V_{max} (SD)	K_m (SD)	V_{max} (SD)
Methyl	12.0 (2.8)	1740 (140)	6.21 (1.90)	396 (34)	3.60 (0.61)	65.4 (3.0)
Ethyl	7.90 (3.58)	2280 (380)	2.89 (0.42)	393 (19)	2.83 (0.68)	104 (8)
Isopropyl	1.25 (0.13)	245 (9)	0.931 (0.151)	66 (4)	0.912 (0.161)	12.0 (0.6)
n-Butyl	0.40 (0.06)	977 (41)	0.332 (0.081)	306 (28)	0.403 (0.131)	65.4 (6.1)
t-Butyl	ND	ND	1.56 (0.21)	7.6 (0.3)	14.6 (3.5)	62.1 (8.7)
n-Hexyl	0.125 (0.026)	244 (8)	0.217 (0.019)	102 (2)	0.169 (0.018)	14.7 (0.4)
n-Octyl	0.267 (0.082)	197 (19)	0.218 (0.053)	79 (7)	0.063 (0.010)	18.0 (0.8)
3-Hydroxypropyl	ND	ND	ND	ND	NS	NS
Methoxyethyl	ND	ND	ND	ND	2.60 (0.37)	284 (11)
1-Carbamoylethyl	0.217 (0.082)	10.1 (1.3)	ND	ND	—[b]	—
Cyclohexyl	ND	ND	ND	ND	0.185 (0.103)	6.80 (0.81)
Tetrahydrofurfuryl	3.91 (0.18)	3380 (60)	7.59 (0.78)	1440 (60)	14.0 (3.0)	441 (49)
Phenyl	1.27 (0.13)	5770 (230)	0.957 (0.136)	1860 (90)	0.678 (0.077)	335 (12)
Benzyl	0.428 (0.113)	1270 (100)	0.313 (0.031)	363 (11)	0.410 (0.036)	75.1 (2.1)
Phenoxyethyl	1.10 (0.08)	1440 (50)	0.741 (0.112)	490 (31)	0.344 (0.072)	73.8 (5.1)
p-Nitrophenyl	NS	NS	0.470 (0.021)	671 (16)	NS	NS

[a] K_m as m*M*. V_{max} as nmol nicotinic acid/min/mg protein. NS, saturation not reached; ND, not determined. Conditions: pH 7.4 in Tris/HCl, 5% (v/v) DMSO; temperature, 37°C.

[b] Insufficient activity to measure K_m and V_{max}.

Table VI. Hydrolysis of Five Nicotinate Esters in Rat Brain Subcellular Fractions[a]

Nicotinate	Microsomes		Mitochondria[b]		Cytosol	
	K_m (SD)	V_{max} (SD)	K_m (SD)	V_{max} (SD)	K_m (SD)	V_{max} (SD)
Ethyl	3.92 (0.55)	1.34 (0.06)	18.0 (1.5)	0.68 (0.03)	15.6 (1.8)	1.02 (0.05)
n-Hexyl	0.322 (0.052)	0.56 (0.04)	0.264 (0.081)	0.87 (0.07)	0.776 (0.021)	0.23 (0.02)
Cyclohexyl	0.640 (0.149)	0.12 (0.01)	1.27 (0.25)	0.13 (0.01)	0.598 (0.246)	0.03 (0.003)
Phenyl	0.886 (0.217)	23.9 (2.0)	3.98 (0.24)	35.1 (1.1)	1.60 (0.12)	7.30 (0.20)
Benzyl	1.16 (0.14)	1.44 (0.07)	8.20 (0.89)	3.60 (0.13)	1.61 (0.32)	1.42 (0.12)

[a] K_m as m*M*. V_{max} as nmol nicotinic acid/min mg protein. Conditions: pH 7.4 in Tris/HCl buffer, 5% (v/v) DMSO; temperature, 37°C.
[b] Mitochondria of neuroglial origin.

and F is the Fischer test of significance. The value of log $k^0_{w\ optimal}$ is calculated with the indices given by the equation. The standard deviations are in parentheses.

One data point (carbamoylethyl nicotinate) was an obvious outlier (residual greater than 2 S) and was excluded from the above equation. This ester is the most hydrophilic one among the whole set (log k^0_w = 0.46), but although it is very poorly hydrolyzed, its pK_m is very high, which might indicate a nonproductive binding to the enzyme.

A very similar quadratic equation was derived from pK_m values measured in liver mitochondria:

$$pK_{m\ \text{liver mitochondria}} = -0.20\ (\pm 0.06)\ (\log k^0_w)^2 + 1.5\ (\pm 0.4)\ \log k^0_w + 0.75\ (\pm 0.47) \quad (2)$$
$$n = 12, \quad r^2 = 0.816, \quad r^2_{c-v} = 0.729, \quad S = 0.252, \quad F = 20.0, \quad \log k^0_{w\ opt.}\ 3.9$$

The contribution of the quadratic term to the pK_m measured in liver cytosol is smaller than in microsomes and mitochondria, as shown by the following equation:

$$pK_{m\ \text{liver cytosol}} = -0.094\ (\pm 0.030)\ (\log k^0_w)^2 + 1.0\ (\pm 0.2)\ \log k^0_w + 1.6\ (\pm 0.2) \quad (3)$$
$$n = 11, \quad r^2 = 0.957, \quad r^2_{c-v} = 0.935, \quad S = 0.130, \quad F = 88.3$$

The t-butyl and tetrahydrofurfuryl esters displayed unexpectedly low affinities and were not included in Eq. (3).

Equations (1)–(3) indicate that lipophilicity plays a major role in the binding of nicotinate esters to membranal and cytosolic esterases. However, the three equations, and hence the interactions they describe, are not similar enough for a global equation to be derived.

Parabolic relationships allow the calculation of optimal values of log k^0_w, which were found to be 3.8 for microsomal and 3.9 for mitochondrial esterases, respectively. The optimal value given for cytosolic enzymes falls outside the explored range and cannot be considered.

Due to the limited set of nicotinates studied with rat brain subcellular fractions, only one independent variable is permissible, and the following linear relationships were derived:

$$pK_{m\ \text{brain microsomes}} = 0.54\ (\pm 0.12)\ \log k^0_w + 1.8\ (\pm 0.3) \quad (4)$$
$$n = 5, \quad r^2 = 0.860, \quad r^2_{c-v} = 0.691, \quad S = 0.172, \quad F = 18.5$$

$$pK_{m\ \text{brain mitochondria}} = 0.93\ (\pm 0.26)\ \log k^0_w + 0.38\ (\pm 0.63) \quad (5)$$
$$n = 5, \quad r^2 = 0.808, \quad r^2_{c-v} = 0.699, \quad S = 0.364, \quad F = 12.6$$

$$pK_{m\ \text{brain mitochondria}} = 0.43\ (\pm 0.07)\ L + 0.031\ (\pm 0.434) \quad (6)$$
$$n = 5, \quad r^2 = 0.922, \quad r^2_{c-v} = 0.889, \quad S = 0.231, \quad F = 35.5$$

For this set of five nicotinate esters log k^0_w and Verloop's L parameter contain overlapping information (intercorrelation r^2 = 0.64). Lipophilic interactions thus play an important role for the binding of nicotinate esters to rat brain membrane-bound esterases, as is the case for the liver.

No satisfactory equations were found between the other physicochemical parameters and log V_{max} or log V_{max}/K_m, the latter expressing the selectivity of substrates for the enzymes. The fact that V_{max} was not amenable to QSAR analysis is, in itself, worth noting. The K_m parameters being an expression of affinity/binding result from intermolecular interactions describable by physicochemical parameters such as lipophilicity. In contrast, V_{max} essentially expresses rates of biochemical reactions involving cleavage and formation of covalent bonds. In selected situations, electronic/steric parameters such as Taft's E_s or Hammett's σ could be correlated with reaction rates, but this was not the case here.

CONCLUSION

This study shows the predominantly microsomal location of hydrolytic activity in rat liver, while the relatively high activity found in myelin and synaptosomes of rat brain is noteworthy. While K_m values for a given substrate are comparable between fractions and in the two tissues, V_{max} values are 2 orders of magnitude larger in liver than in brain. Marked substrate selectivities are seen, which, however, do not appear to differ significantly between the two organs.

Lipophilic interactions seem to play a major role in the binding of nicotinate esters to rat liver and brain subcellular fractions. This finding indicates that, at least for esters of this type, tissue-selective hydrolysis cannot be achieved.

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