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IDENTIFICATION AND CHARACTERIZATION OF HEPATIC CARBOXYLESTERASES HYDROLYZING HYDROCORTISONE ESTERS

BASHEER ALI, SWARAJ KAUR, EDWIN C. JAMES and SURENDRA S. PARMAR*

Departments of Physiology and Surgery, University of North Dakota, School of Medicine, Grand Forks, ND 58202, U.S.A.; and Jawaharlal Nehru Laboratories of Molecular Biology, Department of Pharmacology and Therapeutics, King George's Medical College, Lucknow 226003, India

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Abstract—The present study has provided evidence for the existence of three distinct carboxylesterases involved in the hydrolysis of steroid esters, where two enzymes are possibly responsible for the metabolism of hydrocortisone hemisuccinate (HCHS) at pH 5.5 and 8.0, and a third enzyme for the metabolism of hydrocortisone acetate (HCAC) at pH 8.0, in isolated rat liver microsomes. The activity of all three enzymes in rat liver was induced significantly by the administration of phenobarbital while no such function in enzyme activity was observed in animals receiving 3-methylcholanthrene or benzo[*a*] pyrene under similar experimental conditions. The increase in the activity of HCHS esterase I (HCHS-E_1) active at pH 5.5, HCHS esterase II (HCHS-E_2) active at pH 8.0, and HCAC esterase (HCAC-E) was approximately 7 to 8, 3- and 3-fold respectively. On the other hand, the degree of induction of nonspecific microsomal carboxylesterase acting on *p*-nitrophenylacetate (PNPA) was significantly less. The K_m values for the hydrolysis of HCHS at pH 5.5 and 8.0 and HCAC by rat liver microsomes obtained from control rats were 2.45, 2.02 and 1.6 mM, respectively, and these K_m values were not changed significantly in preparations obtained from rats treated with phenobarbital. The distinct *in vitro* responses displayed by hepatic microsomal steroid esterases to various inhibitors were able to distinguish three different enzymes which also differed from nonspecific carboxylesterases. The activity of HCAC-E was inhibited by $NaAsO_2$ and $AgNO_3$ while that of HCHS-E_1 and HCHS-E_2 remained unaffected. Selective inhibition of HCHS-E_1 by NaF, $HgCl_2$ and *p*-chloromercuribenzoate and that of HCHS-E_2 by $NiSO_4$ indicated the possible existence of different enzymes or isozymes of a carboxylesterase catalyzing HCHS hydrolysis. The effects elicited by the inhibitors on the activity of PNPA esterase were different from those observed with steroid esterases. Furthermore, the present study has also indicated species variations in the distribution of steroid esterases in the livers of rat, mouse, dog and cat.

Steroid esters are widely used in therapy due to their prolonged physiological and pharmacological actions. Steroid esters have been demonstrated to undergo hydrolytic metabolism in liver and other tissues [1–3] and recently in blood [4, 5], and the degree of hydrolysis affects their pharmacological activity. Earlier studies have indicated that a purified microsomal nonspecific carboxylesterase from pig liver possesses high affinity for uncharged steroid hormone esters when enzyme activity was determined at pH 8.0 but the esterase was unable to hydrolyze the charged esters, namely hemisuccinate conjugates of prednisolone and hydrocortisone. Recent investigations undertaken to characterize rat liver steroid esterases have indicated the presence of two microsomal carboxylesterases of B-type (EC 3.1.1.1) specific for the hydrolysis of charged and uncharged esters at pH 5.5 and 8.0 respectively. The activities of these two enzymes were inhibited differentially by serine and sulfhydryl function inhibitors. These results, however, failed to offer substantial evidence to distinguish steroid esterases from nonspecific microsomal carboxylesterases because the hydrolysis of acetylsalicylic acid, a substrate used for assessing nonspecific carboxylesterases, was also inhibited by these inhibitors. The hydrolysis of acetylsalicylic acid in rat liver has been shown recently to be catalyzed by two microsomal B-esterases that are substrate specific, active at pH 5.5 and 7.4, and can be distinguished from nonspecific carboxylesterases responsible for the hydrolysis of *p*-nitrophenylacetate (PNPA), and cholinesterase [6]. At present, the characteristics of steroid esterases are not fully understood as compared to other hepatic microsomal carboxylesterases/amidases which are responsible for the metabolism of drugs [7, 8], carcinogens [9] and other xenobiotics [10]. It is thus difficult to provide identification and differentiation of steroid esterases from other hydrolases which are known to exist in multiple forms in rat liver [10] and are induced by phenobarbital [11–13] but not by 3-methylcholanthrene or benzo[a]pyrene [13].

The present study has been undertaken to characterize rat liver microsomal esterases responsible for the hydrolytic metabolism of hydrocortisone hemisuccinate (HCHS) and hydrocortisone acetate (HCAC), which represent charged and uncharged steroid hormone esters respectively. Identification of different enzymes involved in the hydrolysis of steroid esters has been accomplished by investigating their pH optima, kinetic parameters, species vari-

* Address all correspondence to: Dr. S. S. Parmar, Department of Physiology, The University of North Dakota, School of Medicine, Grand Forks, ND 58202.

ations and susceptibility to various inhibitors. The properties of steroid esterases were also compared with those of nonspecific microsomal carboxylesterase, using PNPA as the substrate. In addition, attempts have been made to characterize the enzyme-inducing effects of phenobarbital, 3-methylcholanthrene and benzo[*a*]pyrene on these hydrolases in order to study their enzyme inducibility and genetic control.

MATERIALS AND METHODS

Materials. Bovine serum albumin, 3-methylcholanthrene, 3,4-benzo[a]pyrene and *p*-chloromercuribenzoate were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Crystalline hydrocortisone hemisuccinate, trimethamine (Tris) and ethylenediaminetetracetate (EDTA) were purchased from Glaxo Laboratories (India) Ltd. *p*-Nitrophenylacetate was obtained from Sisco Research Laboratories Pvt. Ltd., India. Eserine sulfate (physostigmine sulfate) was supplied by Boeringer, Ingelheim, Germany. Metal salts and other chemicals were of analytical grade and obtained from commercial sources. All reagents were prepared in deionized water.

Experimental. Female albino rats (120–180 g) having free access to food and water were used throughout the study. Earlier studies designed to investigate the dose-dependent kinetics of the induction of rat liver microsomal carboxylesterase during hydrolytic metabolism of α-naphthylacetate by repeated phenobarbital and dichloro diphenyltrichloroethane (DDT) administration showed that a critical high dosage of the inducer must be administered to achieve maximum induction of the enzyme [14]. Hence, phenobarbital, 3-methylcholanthrene and benzo[a]pyrene were administered intraperitoneally to rats at a dose of 80 mg/kg in peanut oil for 7 consecutive days as reported earlier [13]. An equal amount of peanut oil administered to control rats was found to have no effect on the enzymes investigated in this study. All rats, unless otherwise mentioned, were killed 18–20 hr after administration of the last dose. The rats were killed by decapitation, and livers were immediately removed, cut into pieces, washed thoroughly with ice-cold 1.15% KCl (w/v) to remove blood, and homogenized in 1.15% KCl with a Potter–Elvehjem homogenizer. Liver microsomes were prepared by differential centrifugation of the homogenate by the procedure described earlier [6] and were found to possess the bulk of the steroid esterase activity (55–65%), indicating microsomal origin of the three enzymes. Hence, isolated liver microsomes were used as the source of the hydrolases in the present investigation. The enzyme activity was determined in tissue homogenates of different species and isolated rat liver microsomal preparations.

Assay of steroid esterases. The activity of esterases hydrolyzing HCHS and HCAC was assayed by following the disappearance of the substrates spectrophotometrically. The rate of the hydrolysis of HCHS, unless otherwise specified, was determined at pH 5.5 and 8.0 in order to assess the activity of HCHS-E_1 and HCHS-E_2 respectively. The reaction mixtures (1.5 ml) containing 0.1 M acetate buffer, pH 5.5, or 0.1 M Tris–HCl buffer, pH 8.0, 0.005 M HCHS and liver microsomes equivalent to 4.8 and 0.8 mg protein for HCHS-E_1 assay or 4 and 1.7 mg protein for HCHS-E_2 assay from control and phenobarbital-treated rats, respectively, were incubated at 37° for 30 min. For HCAC-E assay, the reaction mixtures (1 ml) containing 0.1 M Tris–HCl buffer, pH 8.0, 0.004 HCAC in dimethyl sulfoxide (DMSO), and liver microsomes equivalent to 1.6 and 0.54 mg protein from control and phenobarbital-treated rats, respectively, were incubated at 37° for 5 min. The reaction was terminated by the addition of alkaline hydroxylamine hydrochloride, and unutilized steroid esters were estimated spectrophotometrically [15]. Under the experimental conditions used in the present study, the rate of reaction was linear with respect to time and enzyme concentration.

Assay of p-*nitrophenylacetate esterase*. The activity of nonspecific carboxylesterase was determined spectrophotometrically by following the hydrolysis of PNPA to *p*-nitrophenol [16]. The reaction mixture (1 ml) containing 0.1 M Tris–HCl buffer, pH 7.4, 0.003 M PNPA and liver microsomes equivalent to 0.1 mg protein was incubated at 25° for 10 min. The reaction was terminated by the addition of trichloroacetic acid (TCA) (10% w/v), and a suitable aliquot of the supernatant fraction, obtained after centrifugation, was made alkaline with Tris–HCl buffer (1 M, pH 7.4) to obtain a yellow color which was read at 415 nm.

Protein determination. Protein was determined by the method of Lowry *et al.* [17] using bovine serum albumin as the reference standard.

Treatment with modifiers. The *in vitro* effects of modifiers including various anions and cations on steroid esterases and PNPA esterase were investigated by preincubation with rat liver microsomes before the addition of the substrate. Suitable controls containing modifiers were also run under similar experimental conditions. Since the carbamoylation of HCHS-E_1 did not occur at acidic pH, eserine preincubation [6] with the microsomes was carried out at pH 7.4 with Tris–HCl buffer (0.01 M) for 10 min at 37° to ensure complete carbamoylation; then the pH of the reaction mixture was brought to 5.5 with acetate buffer (0.1 M) to assay the enzyme activity. Various concentrations of the modifiers ranging between 1×10^{-5} and 5×10^{-3} M were employed to assess their inhibitory effectiveness toward different hydrolases. The concentration of the inhibitor giving 50% inhibition (I_{50}) of phenobarbital-induced steroid esterases and PNPA esterase was estimated from log dosage–percent enzyme activity plot.

RESULTS

The optimum pH for the hydrolysis of HCHS by liver microsomes from control rats was found to range between pH 5.5 and 8.0 (Fig. 1A). The pH–activity curve of the esterase hydrolyzing HCAC showed a single peak between pH 8.0 and 8.5 (Fig. 1B). As is evident from Table 1, the rate of hydrolysis of steroid esters in rat liver was several times less as

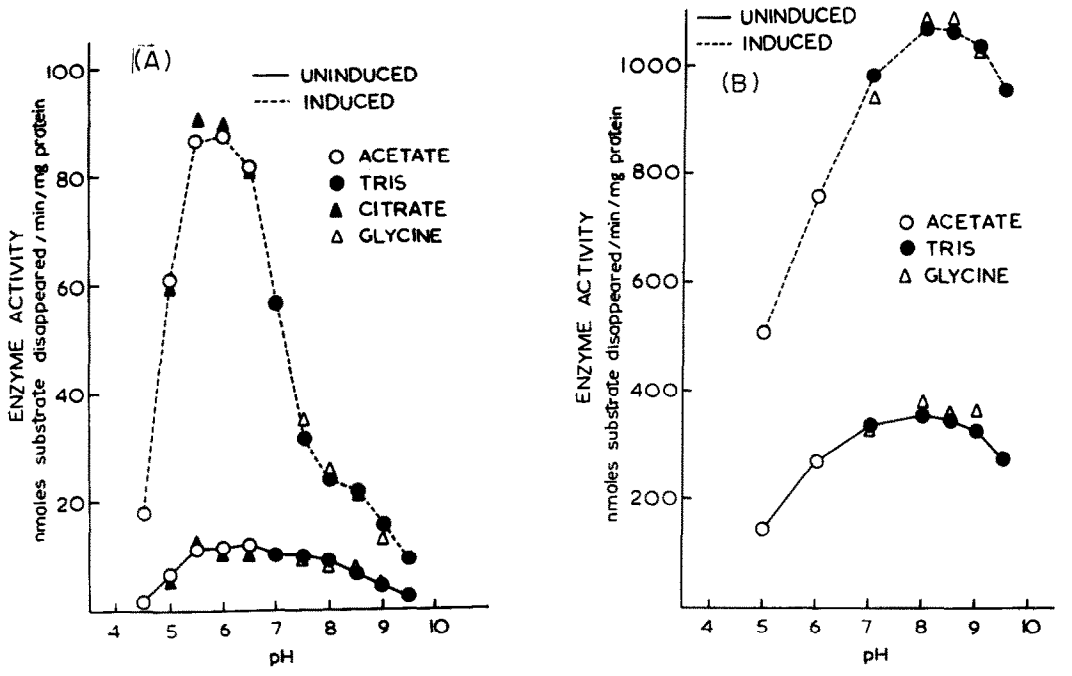

Fig. 1. pH-activity profiles of uninduced and phenobarbital-induced rat liver microsomal esterases hydrolyzing HCHS (A) and HCAC (B). The following buffer systems were used at a final concentration of 0.1 M. For acidic pH range (4.5 to 6.5), acetic acid–sodium acetate and citric acid–Na_2HPO_4 buffers were used. For alkaline pH range (7.0 to 9.5), Tris–HCl and glycine–NaOH buffers were used. Each point is the average of determinations with three to four animals.

compared to the hydrolysis of PNPA by nonspecific carboxylesterase.

Comparative effects of intraperitoneal administration of 80 mg/kg of phenobarbital, 3-methylcholanthrene and benzo[a]pyrene to rats daily for 7 days on the hydrolytic metabolism of hydrocortisone esters and PNPA by liver microsomes are presented in Table 1. Phenobarbital caused a marked increase in the rate of hydrolysis of HCHS, HCAC and PNPA, while 3-methylcholanthrene and benzo[*a*]-pyrene were devoid of any effect under similar experimental conditions. The activity of steroid esterases was found to remain unaltered in animals given a single intraperitoneal dose of phenobarbital (80 mg/kg) and killed after 2 hr or by the addition of phenobarbital (1 mM) to *in vitro* incubation mixtures. The pH–activity curves for HCHS hydrolysis by hepatic microsomes from control and phenobarbital-treated rats showed that phenobarbital induced the hydrolysis of HCHS at pH 5.5 by approximately 7-fold, whereas at pH 8.0 the increase in activity was only approximately 3-fold (Fig. 1A and Table 1). The carboxylesterase, responsible for the hydrolysis of HCAC, referred to as HCAC-E, was induced approximately 3-fold (Fig. 1B and Table 1). The activity of phenobarbital-induced steroid esterases, like uninduced enzymes, was not changed significantly with different buffer systems (Fig. 1, A and B). The induction of microsomal PNPA esterase elicited by phenobarbital under these conditions was only 1.6-fold.

Kinetic constants for the hydrolysis of hydro-

Table 1. Effects evoked by phenobarbital, 3-methylcholanthrene and benzo[*a*]pyrene administration on steroid esterases and PNPA esterase in rat liver microsomes*

	Enzyme activity [nmoles substrate disappeared or product appeared·min^{-1}·(mg protein)$^{-1}$]			
	HCHS-E_1	HCHS-E_2	HCAC-E	PNPA esterase
Control	12.1 ± 0.68	9.5 ± 0.62	331 ± 18	1560 ± 92
Phenobarbital	83.7 ± 9.2†	25.1 ± 2.0†	937 ± 66†	2500 ± 141†
3-Methylcholanthrene	13.5 ± 1.1	7.7 ± 0.59	370 ± 25	1776 ± 105
Benzo[*a*]pyrene	11.7 ± 0.75	9.3 ± 1.2	286 ± 35	1444 ± 66

* Values are the mean ± S.E. of six to eight rats.
† $P < 0.001$ compared to control.

Table 2. Kinetic parameters for hydrolysis of hydrocortisone esters by hepatic microsomal esterases of rat*

Enzyme	K_m (mM)		V_{max} [nmoles substrate disappeared·min^{-1}·(mg protein)$^{-1}$]	
	Uninduced	Induced	Uninduced	Induced
HCHS-E_1	2.45 ± 0.18	2.22 ± 0.15	15.8 ± 1.2	129 ± 14
HCHS-E_2	2.02 ± 0.17	2.31 ± 0.21	10.6 ± 0.8	29.6 ± 14
HCAC-E	1.60 ± 0.11	1.38 ± 0.06	365 ± 28	1100 ± 98

* Values are expressed as mean ± S.E of four determinations. The microsomes from phenobarbital-treated rats were used as the source of induced esterases.

cortisone esters by normal and phenobarbital-induced rat liver microsomal esterases were determined by following the Lineweaver–Burk plots [18] and are recorded in Table 2. The K_m values of the steroid esterases induced by phenobarbital were not changed significantly as compared to the values obtained for enzymes from normal animals, while the V_{max} values were remarkably higher for enzymes obtained from animals exposed to phenobarbital.

Further characterization of HCHS-E_1, HCHS-E_2 and HCAC-E, and their differentiation from PNPA esterase are based on the *in vitro* responses displayed by phenobarbital-induced microsomal esterases to various inhibitors. The effects of the modifiers and the I_{50} values of the inhibitory modifiers are recorded in Tables 3 and 4 respectively. The hydrolysis of HCAC was selectively inhibited by 2×10^{-3} M of $NaAsO_2$ and $AgNO_3$, whereas that of HCHS remained unaffected. The activities of HCHS-E_1 and HCAC-E were, however, inhibited 46.6 and 100% respectively, when the concentration of $AgNO_3$ was increased to 5×10^{-3} M. NaF, $HgCl_2$ and *p*-chloromercuribenzoate were found to be selective inhibitors of HCHS-E_1 (Table 3). A small decrease in the rate of HCHS hydrolysis at pH 8.0 may be attributed to an overlapping of HCHS-E_1 activity in the alkaline pH range. The effect of NaF on HCHS hydrolysis at different pH with two buffer systems, shown in Fig. 2, further illustrates selective susceptibility of hydrolysis occurring at acidic pH of NaF inhibition.

As is evident from Table 3, the response of PNPA esterase to the inhibitors differed from those of steroid esterases. The activities of hepatic microsomal steroid esterases and PNPA esterase were not affected by Ca^{2+}, Mg^{2+}, and EDTA. There was 53.3, 42,8, 69.0 and 48.2% inhibition of HCHS-E_1, HCHS-E_2, HCAC-E and PNPA esterase, respectively, by 5×10^{-4} M eserine.

The hydrolytic activities of liver microsomes obtained from normal rats towards HCHS at pH 5.5 and 8.0, HCAC and PNPA were inhibited 80, 5.6, 27.2 and 54.6%, respectively, by 5×10^{-3} M NaF and 68.7, 2.8, 86.3 and 9.4%, respectively, by 5×10^{-4} M $HgCl_2$. The inhibition of HCHS-E_1, HCHS-E_2, HCAC-E and PNPA esterase from control animals by 1×10^{-3} M *p*-chloromercuribenzoate was 50.5, 0, 40.7 and 2.8% respectively.

The distribution of steroid esterases and PNPA esterase in the liver homogenates of rat, mouse, dog and cat is illustrated in Fig. 3. Livers of other species, like that of rat, also hydrolyzed HCHS at two optimum pH, i.e. 5.5 and 8.0. Mouse in comparison to other animals, showed the highest activity for HCHS at both pH levels. The activity of PNPA esterase was not significantly different in these animals. NaF (2×10^{-3} M) inhibited HCHS hydrolysis at pH 5.5 and 8.0 in mouse liver by 82.3 and 15.2% and in cat liver by 88.2 and 48.2% respectively. There was 50.2 and 50.6% inhibition of HCAC-E by NaF in liver homogenates of mouse and cat respectively.

Table 3. Differential responses of phenobarbital-induced rat liver microsomal steroid esterases and PNPA esterase to inhibitors*

Inhibitor	Concn. (mM)	Enzyme activity [nmoles substrate disappeared or product appeared·min^{-1}·(mg protein)$^{-1}$]			
		HCHS-E_1	HCHS-E_2	HCAC-E	PNPA esterase
None		90.2 ± 4.5	28.5 ± 1.9	1011 ± 65	2370 ± 102
$NaAsO_2$	2	96.2 ± 3.8	29.3 ± 0.91	576 ± 25	1303 ± 35
$AgNO_3$	2	83.2 ± 6.1	27.3 ± 0.81	353 ± 15	2559 ± 169
NaF	5	3.96 ± 0.1	20.2 ± 0.53	535 ± 25	1279 ± 35
$HgCl_2$	0.5	7.21 ± 0.19	23.9 ± 0.68	647 ± 15	2085 ± 100
p-Chloromercuribenzoate	1	3.51 ± 0.12	23.3 ± 0.82	505 ± 36	2488 ± 81
$NiSO_4$	2	82.9 ± 3.9	5.13 ± 0.20	717 ± 21	2227 ± 74
$ZnSO_4$	0.5	57.7 ± 1.8	7.98 ± 0.31	0	2109 ± 141

* Values are the mean ± S.E. of four to five experiments.

Table 4. Inhibitory effectiveness of modifiers for phenobarbital-induced hepatic steroid esterases and PNPA esterase*

Inhibitor	I_{50} (M)			
	HCHS-E_1	HCHS-E_2	HCAC-E	PNPA esterase
$NaAsO_2$	NI†	NI	2.3×10^{-3}	2.6×10^{-3}
$AgNO_3$	NI	NI	5.8×10^{-4}	NI
NaF	1.4×10^{-3}	$>5 \times 10^{-3}$	$>5 \times 10^{-3}$	$>5 \times 10^{-3}$
$HgCl_2$	1.5×10^{-4}	1.2×10^{-3}	6.5×10^{-4}	$>5 \times 10^{-3}$
p-Chloromercuribenzoate	3.9×10^{-4}	$>5 \times 10^{-3}$	1×10^{-3}	NI
$NiSO_4$	NI	7.8×10^{-4}	2.8×10^{-3}	NI
$ZnSO_4$	7.1×10^{-4}	3×10^{-4}	1.4×10^{-4}	$>5 \times 10^{-3}$

* The I_{50} value is the concentration of the inhibitor causing 50% inhibition of the enzyme activity. Various concentrations of inhibitor ranging between 1×10^{-5} and 5×10^{-3} M were used. Each experiment was done in duplicate, and the I_{50} values are the mean of two separate experiments.
† NI = no inhibition.

DISCUSSION

Recent studies have shown that uncharged steroid hormone esters (acetate esters of methylprednisolone, hydrocortisone and cortisone) are hydrolyzed by hepatic microsomal carboxylesterases in rat and other mammalian species at a pH between 8.0 and 8.5, whereas charged steroid hormone esters (hemisuccinate esters of methylprednisolone and hydrocortisone) undergo hydrolytic metabolism at pH 5.5 [19]. It has also been noted that rat liver, compared to liver from other species, had considerably lower hydrolytic activity towards these steroid esters. However, these results have not been able to provide a convincing explanation for the hydrolysis of oxazepam hemisuccinate observed in alkaline conditions by others [20]. The results of our present study have suggested that the hydrolysis of HCHS by rat liver microsomes occurs at two optimum pH, i.e. 5.5 and 8.0, and possibly two enzymes or isozymes, namely HCHS-E_1 and HCHS-E_2 which are active in acidic and alkaline medium, respectively, are responsible for the hydrolytic metabolism of HCHS. The optimum pH for HCAC hydrolysis by microsomal HCAC-E in alkaline medium was in agreement with that reported previously for uncharged steroid esters [19].

The possibility of the presence of at least three steroid hormone esterases in rat liver was provided further support by these results revealing heterogeneity in the responses of these enzymes to phenobarbital treatment. The activities of steroid esterases and PNPA esterase were found to be increased by repeated administration of phenobarbital by nonspecific induction of hepatic microsomal carboxylesterases. The degree of induction of steroid esterases was found to be remarkably higher not only than that of PNPA esterase but also that observed with other hepatic microsomal carboxylesterases/amidases or B-esterases (EC 3.1.1.1) hydrolyzing procaine [21], PNPA [12], acetylsalicylic acid, acetanilide and 2-acetylaminofluorene, a carcinogen [13],

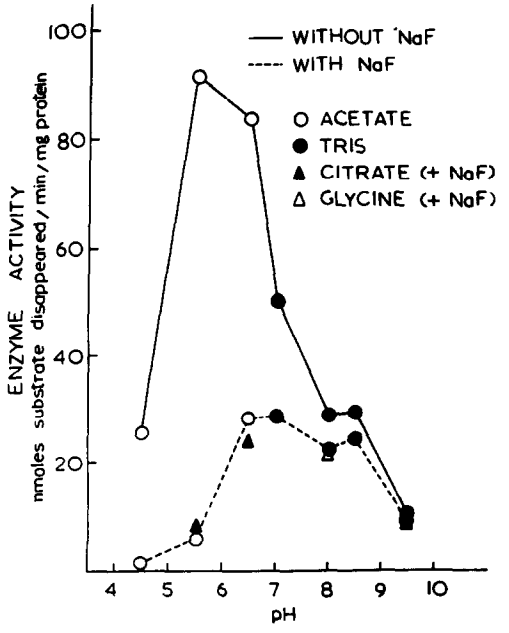

Fig. 2. Effect of NaF (5×10^{-3} M) on HCHS hydrolysis by phenobarbital-induced rat liver microsomes. The buffer systems used were as described in the legend of Fig. 1. Each point is the average of experiments with three animals.

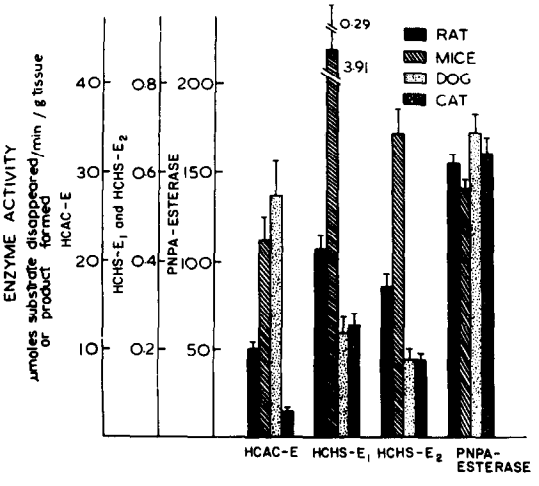

Fig. 3. Distribution of steroid esterases and PNPA esterase in livers of various species. Values are average mean of six to eight rats, eight mice (Albino), three dogs and four cats.

where the degree of induction observed was approximately 2-fold in rats pretreated with phenobarbital. The increase in the activity of HCHS-E_1 elicited by phenobarbital was of particular interest since the observed induction was more than double that obtained for HCHS-E_2. These results have provided support to the view that the hydrolysis of HCHS and probably other charged steroid esters is catalyzed by at least two enzymes or isozymes that have independent genetic regulation. Since the rate of hydrolysis of HCHS at pH 5.5 and 8.0 by uninduced and induced microsomes with different buffers was the same, the possibility of a single enzyme exhibiting different degrees of hydrolysis in acidic and alkaline medium due to different buffer ionic composition gets little support. Interestingly, 3-methylcholanthrene and benzo[*a*]pyrene did not influence the activity of hydrolases which were examined in the present study, and these results have thus suggested selectivity in their responses to these inducers. Likewise, recent studies have reported that microsomal carboxylesterease and amidases of B-type, responsible for the hydrolytic metabolism of acetylsalicylic acid, procaine, PNPA, acetanilide and 2-acetylaminofluorene in rat liver, are not affected by 3-methylcholanthrene and benzo[*a*]pyrene treatment [13].

The results of the kinetic studies on steroid esterases in rat liver microsomes from control and phenobarbital-treated rats showed no statistically significant changes in the K_m values of these enzymes, whereas V_{max} values were increased markedly. These observations have thus provided further support to the view that the enhanced hydrolytic metabolism of the steroid esters is presumably due to an increased *de novo* synthesis of these esterases.

Differential responses displayed by hepatic steroid esterases and PNPA esterase to various inhibitors have helped in distinguishing specific steroid esterases as well as in their differentiation from nonspecific carboxylesterases. HCHS-E_1 could be dissociated from HCHS-E_2 by selective inhibition of the activity of HCHS-E_1, by NaF and -SH function inhibitors, $HgCl_2$, and *p*-chloromercuribenzoate, and the activity of HCHS-E_2 by $NiSO_4$. The inhibition of carboxylesterases, responsible for the hydrolysis of charged and uncharged steroid hormone esters by organophosphates reported earlier has led to the conclusion that the active site of these enzymes, like B-esterases, comprises serine as an essential group for activity [19]. The results of the present study have shown that the catalytic activity of these enzymes was independent of Ca^{2+} and Mg^{2+}, and the enzyme activity was inhibited only by a high concentration of eserine. On the basis of these observations, the steroid esterases can thus be classified as B-esterases in accordance with the scheme introduced by Aldridge [22]. Further studies dealing with the purification and electrophoretic separation of these steroid esters hydrolyzing carboxylesterases should be able to provide additional information regarding the molecular weights,substrate specificity and other molecular properties of their isolated isozymic forms.

Species variations observed in the rate of hydrolysis of HCHS at pH 5.5 and 8.0, and HCAC in the present study have provided additional support to the possible involvement of at least three esterases in other mammalian livers also, of which two enzymes are responsible for the hydrolytic metabolism of HCHS while another enzyme acts on HCAC.

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REFERENCES

1. W. Dirscherl and U. Dardenne, *Biochem. Z.* 325, 195 (1953).
2. C. Schöttler and K. Krisch, *Biochem. Pharmac.* **23**, 2867 (1974).
3. T. Oshima, E. Najajima and K. Hattori, *Pharmac. Res. Commun.* **12**, 139 (1980).
4. D. C. Garg, P. Ng, D. J. Weidler, E. Sakmar and J. G. Wagner, *Res. Commun. Chem. Path. Pharmac.* **22**, 37 (1978).
5. C. Myers, O. Lockridge and B. N. LaDu, *Drug Metab. Dispos.* **10**, 279 (1982).
6. B. Ali and S. Kaur, *J. Pharmac. exp. Ther.* **226**, 589 (1983).
7. K. Moroi and T. Satoh, *Biochem. Pharmac.* **24**, 1517 (1975).
8. S. Kaur and B. Ali, *Toxic. appl. Pharmac.* **70**, 156 (1983).
9. M. Järvinen, R. S. S. Santti and V. K. Hopsu-Havu, *Biochem. Pharmac.* **20**, 2971 (1971).
10. E. Heymann, in *Metabolic Basis of Detoxication* (Eds. W. B. Jacoby, J. R. Bend and J. Caldwell), p. 229. Academic Press, New York (1982).
11. K. Moroi and T. Satoh, *Biochem. Pharmac.* **24**, 1436 (1975).
12. U. Nousianen and O. Hänninen, *Acta pharmac. tox.* **49**, 77 (1981).
13. S. Kaur and B. Ali, *Biochem. Pharmac.* **32**, 3479 (1983).
14. W. S. Schwark and D. J. Ecobichon, *Can. J. Physiol. Pharmac.* **46**, 207 (1968).
15. R. M. Lee and B. H. Livett, *Biochem. Pharmac.* **16**, 1757 (1967).
16. B. Ali and S. Kaur, *Biochem. Pharmac.* **31**, 3683 (1982).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).
19. K. Hattori, M. Kamio, E. Najakima, T. Oshima, T. Satoh and H. Kitagawa, *Biochem. Pharmac.* **30**, 2051 (1981).
20. M. Salmona, C. Saronio, R. Bianchi, F. Marcucci and E. Mussini, *J. pharm. Sci.* **63**, 222 (1974).
21. T. Satoh and K. Moroi, *Jap. J. Pharmac.* **27**, 233 (1977).
22. W. N. Aldridge, *Biochem. J.* **53**, 110 (1953).