Biochemical Pharmacology, Vol. 30, No. 15, pp. 2051–2056, 1981.
Printed in Great Britain.

0006-2952/81/152051-06 $02.00/0

CHARACTERIZATION OF STEROID HORMONE ESTER HYDROLYZING ENZYMES IN LIVER MICROSOMES

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(*Received* 30 *July* 1980; *accepted* 16 *December* 1980)

Abstract—To clarify the characteristics of steroid hormone ester hydrolyzing enzymes, optimum pH, species differences, tissue distribution, hepatic subcellular distribution, and the effects of several enzyme inhibitors were determined with steroid hemisuccinate and acetate esters as charged and uncharged substrates respectively. Kinetic experiments were also performed. The optimum pH for charged and uncharged esters, respectively, was pH 5.5 and 8.0. Diethyl-*p*-nitrophenylphosphate (E-600) and diisopropylfluorophosphate (DFP) (10^{-5} M) completely inhibited esterase activities for all substrates. Bis-*p*-nitrophenylphosphate (BNPP) and phenylmethylsulfonyl fluoride (PMSF) (10^{-5} M) inhibited 80 and 95 per cent, respectively of hemisuccinate esterase activities, but only 20 per cent or less of acetate esterase activities. $HgCl_2$ and *p*-chloromercuribenzoate (PCMB) (10^{-4} M) inhibited approximately 80 per cent or more of steroid acetate esterase activities. The K_m values for acetate and hemisuccinate esterases were almost the same (0.3 to 0.7 mM), but three different V_{max} values were obtained. These results indicate the presence of two or more steroid hormone ester hydrolyzing enzymes.

Many steroid esters are used therapeutically and are known to be rapidly hydrolyzed in the body. Schöttler and Krisch [1] investigated enzymatic hydrolysis of twenty-two steroid hormone esters by a highly purified non-specific carboxylesterase (EC 3.1.1.1) from pig liver microsomes and found that charged steroid hormone esters, such as prednisolone-21-hemisuccinate and HC-Suc‡ could not be hydrolyzed by this carboxylesterase. In addition, Krisch [2] also reported that carboxylesterase catalyzes the hydrolysis of only uncharged carboxylic esters.

In recent years, Satoh *et al.* have reported on the microsomal esterase/amidase responsible for drug metabolism, demonstrating that procaine, as well as isocarboxazid, is hydrolyzed by non-specific carboxylesterases of hepatic microsomes from various species [3, 4]. Later, three enzymes were purified from hepatic microsomes of guinea pigs [5] and rats [6].

In preliminary experiments [7], it was demonstrated that charged steroid hormone esters such as MP-Suc and HC-Suc are hydrolyzed by a non-specific carboxylesterase. Gratzl *et al.* [8] also demonstrated the possibilities of hydrolysis of charged esters by a carboxylesterase.

The present study was undertaken to clarify the characteristics of steroid hormone ester hydrolyzing enzymes (steroid esterase) in comparison with those of non-specific carboxylesterases, using charged and uncharged steroid hormone esters as substrates. Optimum pH, tissue distribution, subcellular distribution, effects of several enzyme inhibitors, species differences, and kinetic parameters for steroid esterases are presented in the study.

MATERIALS AND METHODS

Chemicals. Methylprednisolone, MP-Suc, HC-Suc, and fluoxymesterone were obtained from The Upjohn Co. (Kalamazoo, MI, U.S.A.). Hydrocortisone, cortisone, C-Ac, MP-Ac, HC-Ac, NEM, and PCMB were purchased from the Wako Pure Chemical Industry Co. (Tokyo, Japan) and BNPP from Nakarai Chemicals (Kyoto, Japan). PMSF and E-600 were obtained from the Sigma Chemical Co. (St. Louis MI, U.S.A.), DFP from Fluka AG (Switzerland), and ASA and salicylic acid from the Kanto Chemical Co. (Tokyo, Japan). All other reagents were of the highest grade commercially available.

Enzyme preparation. Male animals were used in the present study. Sprague–Dawley rats (300–350 g), Hartley guinea pigs (400–450 g), ICR mice (30–35 g), golden hamsters (60–80 g), Japanese white rabbits (2.0–2.5 kg), a pig, and a cow were used. The livers of all animals except the pig and cow were perfused with 1.15% KCl solution for removal of blood. Tissues were homogenized with 4 vol. of ice-cold 1.15% KCl solution in a Potter–Elvehjem-type homogenizer with a teflon pestle. Subcellular fractionation was carried out by differential centrifugation according to the method of de Duve *et al.* [9]. Sucrose (0.25 M) was employed for only the subcellular distribution experiment. Protein concentrations were determined by the method of Lowry *et al.* [10]. The protein standard was crystalline bovine serum albumin.

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‡ Abbreviations: HC-Suc, hydrocortisone-21-hemisuccinate; MP-Suc, methylprednisolone-21-hemisuccinate; MP-Ac, methylprednisolone-21-acetate; HC-Ac, hydrocortisone-21-acetate; C-Ac, cortisone-21-acetate; ASA, acetylsalicylic acid; DFP, diisopropylfluorophosphate; E-600, diethyl-*p*-nitrophenylphosphate; BNPP, bis-*p*-nitrophenylphosphate; PMSF, phenylmethylsulfonyl fluoride; PCMB, *p*-chloromercuribenzoate; and NEM, *N*-ethylmaleimide.

Assay procedures for steroid esterase activities. MP-Suc and HC-Suc were dissolved in distilled water to a concentration of 1×10^{-2} M. MP-Ac, HC-Ac, and C-Ac were dissolved in dimethylformamide (5×10^{-2} M). When steroid hemisuccinates were used as substrates, an incubation mixture, containing 50 μmoles of citrate phosphate buffer (pH 5.5) and aliquots of enzymes in a total volume of 0.9 ml, was preincubated for 15 min at 37° and then incubated for another 15 min at 37° with substrate. When steroid acetates were used as substrates, the incubation mixture was prepared with 50 μmoles of Tris–HCl buffer (pH 8.0) and aliquots of enzymes. After preincubation for 5 min at 28°, substrate was added and then the mixture was incubated for 5 min at 28°. The reaction was stopped by addition of 1 ml of 10% trichloroacetic acid (TCA) in acetonitrile containing fluoxymesterone (2.5 μmoles/ml). After centrifugation, the supernatant fraction was assayed by high-pressure liquid chromatography (h.p.l.c.).

Assay procedures for ASA deacetylase activities. ASA deacetylase activity was determined by the ultraviolet absorption method [11]. An incubation mixture containing 50 μmoles of phosphate buffer (pH 7.4), 1 μmole of substrate, and aliquots of enzymes in a total volume of 1 ml was incubated for 5 min at 28°. The reaction was stopped by addition of 1 ml of 10% TCA. Following addition of 2 ml of distilled water, the reaction mixture was centrifuged for 5 min at 1500 *g*. The absorbance of the deacetylated product (salicylic acid) in the supernatant fraction was measured at 304 nm. The molar absorption coefficient for salicylic acid was 3500 M^{-1} cm^{-1}.

High-pressure liquid chromatography analysis procedures. Conditions for h.p.l.c. were as follows. Hydrolyzed products (free steroid) were determined with a high pressure liquid chromatograph equipped with a 254 nm wavelength ultraviolet detector (Hitachi-635A) and a 4 mm i.d. × 25 cm length column packed with Nucleosil C-8 (7 μm). The mixture of acetonitrile–0.05 M phosphate buffer, pH 7.4 (50 : 50, v/v), was used as the mobile phase. The flow rate was 1 ml/min.

Effects of inhibitors on steroid esterase and ASA deacetylase activities. E-600 and DFP each were dissolved in ethyl alcohol to a concentration of 5×10^{-2} M, and the solutions were diluted with distilled water (1×10^{-3} M to 1×10^{-5} M). BNPP and PMSF were dissolved in distilled water (4×10^{-4} M to 4×10^{-5} M). PCMB dissolved in a small volume of 0.5 N sodium hydroxide was diluted with distilled water (1×10^{-2} M to 1×10^{-5} M). The sodium hydroxide concentration in the incubation mixture was finally less than 0.0025 N. An inhibitor was added to the incubation mixture. After preincubation of the mixture with no substrate for 15 min at 28° or 37°, the mixture was incubated with substrate.

Optimum pH of steroid esterases in rat liver microsomes. To determine optimum pH, citrate–phosphate buffers, Tris–HCl buffers, and carbonate buffers were employed in ranges of pH 3.0 to 7.5, 7.0 to 8.5, and 8.5 to 9.5 respectively.

RESULTS

Methylprednisolone, hydrocortisone, cortisone and fluoxymesterone were well separated under the conditions employed. The correlation coefficient for each calibration curve was 0.9999 or more.

Steroid acetate esters were dissolved in dimethylformamide, and 20 μl of each solution was added to an incubation mixture. In preliminary experiments, 2% dimethylformamide had no effect on steroid esterase activities.

The pH-activity curves of liver microsomal enzymes for charged and uncharged steroid esters are shown in Fig. 1. When steroid acetate esters were used as the substrate, the optimum pH was between 8.0 and 8.5. The optimum pH for steroid hemisuccinate esters was 5.5.

Figure 2 shows species differences of steroid esterase in hepatic microsomes. High activities for steroid acetate esters were observed in hamster, cow, and pig hepatic microsomes. Activities for hemisuccinate esters were high in mouse, hamster, cow, and pig hepatic microsomes. The rat showed the lowest

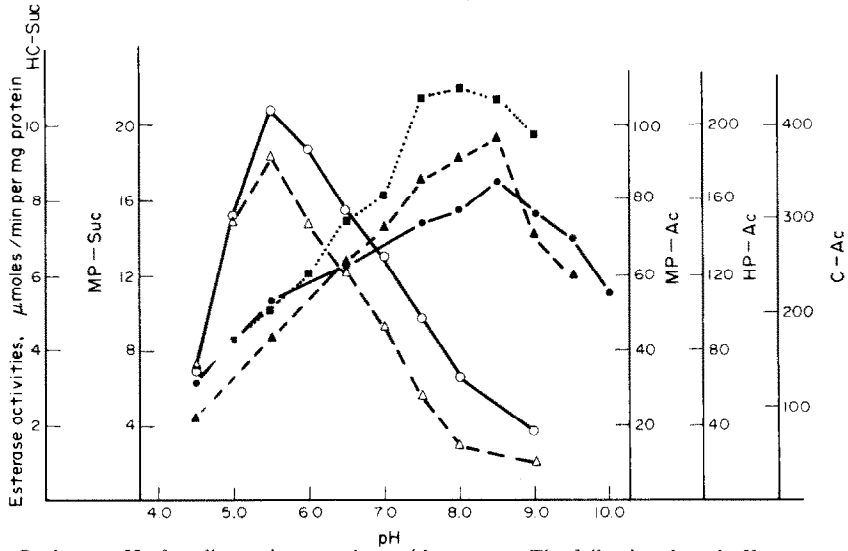

Fig. 1. Optimum pH of rat liver microsomal steroid esterases. The following three buffer systems were used: citrate–phosphate buffers (pH 4.0 to 7.5), Tris–HCl buffers (pH 7.0 to 9.5), and carbonate buffers (pH 8.5 to 10.0). MP-Suc (○), HC-Suc (△), MP-Ac (●), HC-Ac (▲) and C-Ac (■) were used as substrates.

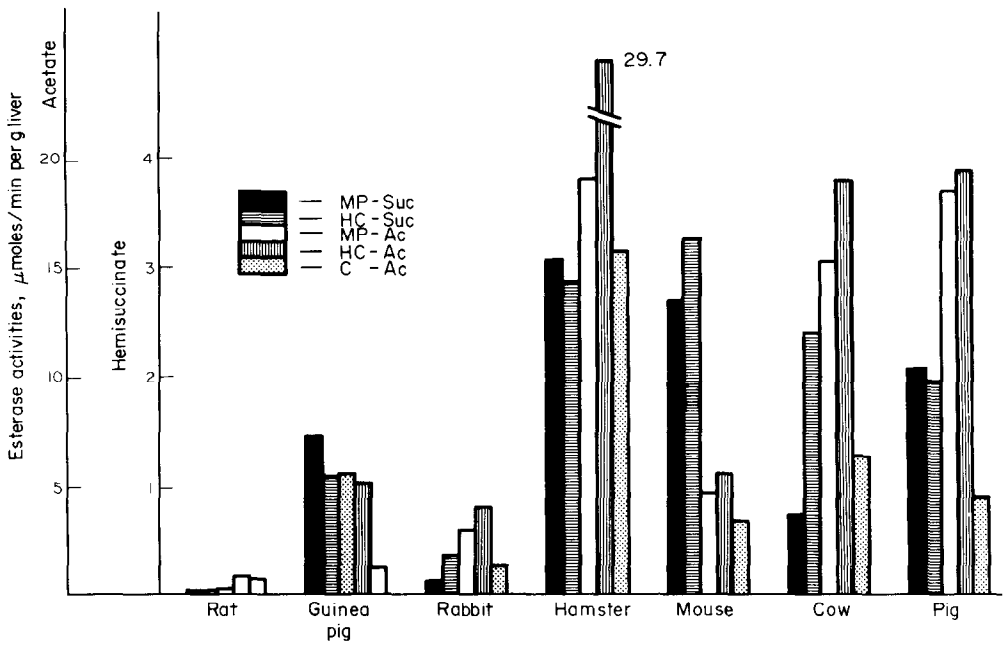

Fig. 2. Hepatic microsomal steroid esterase activities in various species. Appropriate amounts of microsomes of various species were incubated at 37° for 15 min at pH 5.5 for hemisuccinate esters and at 28° for 5 min at pH 8.0 for acetate esters. MP-Suc, HC-Suc, MP-Ac, HC-Ac, and C-Ac were used as substrates. Each microsomal fraction was prepared from the homogenate of five rat livers, five guinea pig livers, five hamster livers, ten mouse livers, one cow liver, and one pig liver. Results are the means of two determinations.

activities for all substrates. ASA deacetylase activity was high in guinea pig, mouse, and rat hepatic microsomes, but it was very low in the hamster, rabbit, and cow.

Table 1 shows the steroid esterase activities in homogenates of various rat organs. The duodenum and small intestine showed the highest activities for hemisuccinate esters. The highest activities for acetate esters were observed in the liver, duodenum, and small intestine. Activity in the pancreas was also higher than in other organs. Differences in distribution of steroid esterase activities in various organs were observed with different substrates. Unexpectedly, activities in serum and kidney were very low for all steroid esters.

Subcellular localization of steroid esterase and ASA deacetylase activities in rat liver is shown in Fig. 3. Subcellular distribution of steroid esterase activity was almost identical for all substrates. Approximately 60 per cent of total activity was recovered in the microsomal fraction and 35 per cent of total activity was detected in the cell debris plus nuclear fraction. Relatively low activities were detected in mitochondrial, lysosomal, and cytoplasmic fractions. ASA deacetylase activity was located principally in microsomal and nuclear fractions (34.7 and 38.5 per cent respectively); however, a relatively high activity was detected in the cytoplasmic fraction.

The results of inhibition experiments are shown

Table 1. Tissue distribution of steroid esterase activities in rats*

Tissues	Steroid esterase activities [nmoles·min^{-1}·(g tissue)$^{-1}$ or (ml serum)$^{-1}$]				
	MP-Suc	HC-Suc	MP-Ac	HC-Ac	C-Ac
Serum	26 ± 5	22 ± 7	99 ± 3	40 ± 12	114 ± 37
Brain	5 ± 1	8 ± 2	292 ± 46	72 ± 14	43 ± 11
Liver	352 ± 29	350 ± 33	5123 ± 987	8934 ± 1187	6529 ± 737
Kidney	216 ± 27	333 ± 19	413 ± 13	714 ± 163	450 ± 45
Adrenal gland†	40	21	156	524	340
Stomach	97 ± 6	109 ± 5	445 ± 28	342 ± 19	86 ± 23
Duodenum	1208 ± 172	1679 ± 246	4652 ± 726	9462 ± 626	3322 ± 723
Small intestine	1672 ± 122	2122 ± 176	4027 ± 436	7778 ± 754	4877 ± 704
Large intestine	221 ± 28	248 ± 40	117 ± 24	255 ± 30	348 ± 120
Testicle	128 ± 1	20 ± 2	134 ± 13	318 ± 62	42 ± 5
Pancreas	99 ± 6	45 ± 4	2625 ± 305	1151 ± 131	145 ± 54

* Hemisuccinate esters were incubated at pH 5.5, and acetate esters at pH 8.0. Values are the means ± S.E. of five rats.

† The homogenate was prepared from five rat adrenal glands.

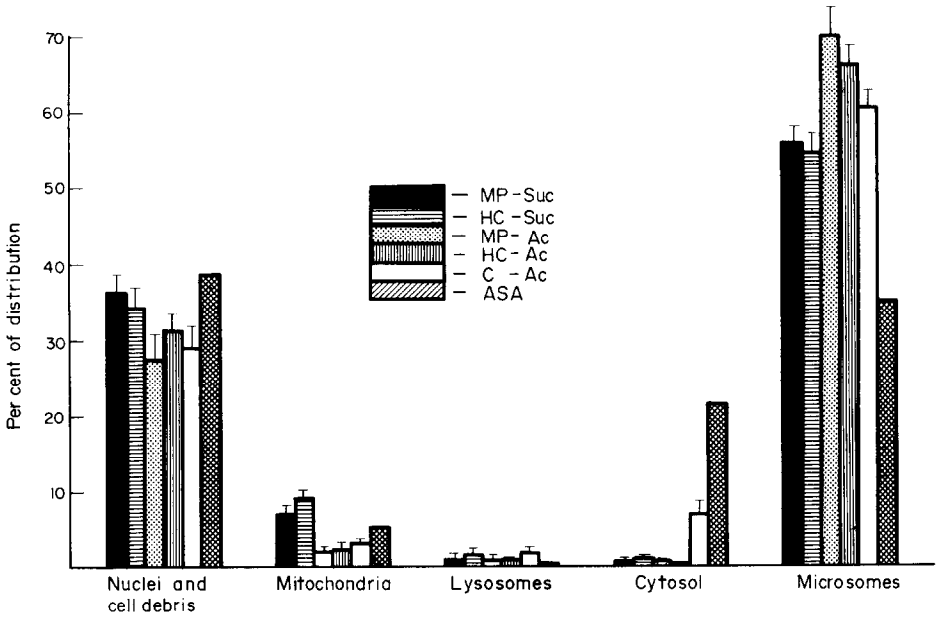

Fig. 3. Subcellular distribution of steroid esterase and ASA deacetylase activities in rat liver. Appropriate amounts of enzyme samples were incubated at 37° for 15 min (pH 5.5) for hemisuccinate esters and at 28° for 5 min (pH 8.0) for acetate esters. Results are means ± S.E. of five determinations except for ASA deacetylase (mean of two determinations).

in Fig. 4. DFP, E-600, PMSF and BNPP were used as serine function inhibitors and $HgCl_2$, PCMB and NEM as SH function inhibitors. The most striking effects were obtained by organophosphorus compounds such as DFP and E-600 at a concentration of 1×10^{-5} M. Since steroid esterase activities were inhibited completely by DFP and E-600 in all substrates, the steroid esterases can be called B-esterase (carboxylesterase) as classified by Aldridge [12]. ASA deacetylase activity was also inhibited completely by 1×10^{-5} M DFP and E-600. The other serine function inhibitors, PMSF and BNPP, inhibited steroid hemisuccinate esterase activities (80 and 95 per cent respectively) at a concentration of 1×10^{-5} M, but they did not significantly inhibit steroid acetate esterases (20 per cent or less). PMSF and BNPP also inhibited ASA deacetylase (15 and 40 per cent respectively). The inhibitory effects of sulfhydryl reagents on steroid esterases, i.e. $HgCl_2$, PCMB and NEM, were conflicting. $HgCl_2$ and PCMB at a concentration of 1×10^{-4} M inhibited approximately 80 per cent or more of steroid acetate esterase activities, but 50 per cent or less of hemisuccinate esterase activities. NEM (1×10^{-3} M) did

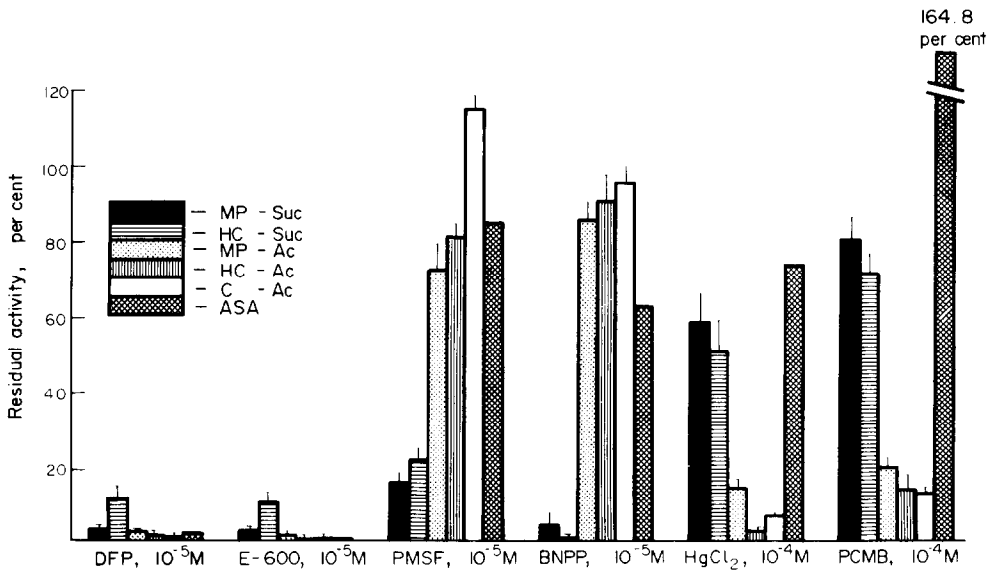

Fig. 4. Effects of several inhibitors on steroid esterase and ASA deacetylase activities in rat liver microsomes. Hemisuccinate esters were incubated at 37° for 15 min (pH 5.5), acetate esters at 28° for 5 min (pH 8.0) and ASA at 28° for 5 min (pH 7.4). Before addition of substrates, incubation mixtures with inhibitors were preincubated for 15 min. Results are means ± S.E. of at least four determinations except for ASA deacetylase (mean of two determinations).

Table 2. Kinetic parameters for steroid esterases in rat liver microsomes*

Substrates	K_m (mM)	V_{max} [nmoles·min^{-1}·(mg protein)$^{-1}$]
Hemisuccinate esters		
Methylprednisolone	0.520	7.38
Hydrocortisone	0.368	7.38
Acetate esters		
Methylprednisolone	0.302	142.05
Hydrocortisone	0.488	143.27
Cortisone	0.702	531.92

* Data were obtained by Lineweaver–Burk plots using 0.1 to 2.0 mM concentrations of substrates. Hemisuccinate esters were assayed in 50 mM citrate–phosphate buffer (pH 5.5) and acetate esters in 50 mM Tris–HCl buffer (pH 8.0).

not affect the activities of all steroid esters. Inhibition of ASA deacetylase by SH function inhibitors contrasted sharply to the inhibition of steroid esterases.

The K_m values for acetate and hemisuccinate esters did not vary significantly, although the V_{max} values for the hemisuccinate esters of methylprednisolone and hydrocortisone, the acetate esters of methylprednisolone and hydrocortisone, and the acetate ester of cortisone were, respectively, 7.38, 142, and 531 nmoles·min^{-1}·(mg protein)$^{-1}$ (Table 2).

DISCUSSION

The multiplicity of carboxylesterases in mammalian tissues has been well established [13–16]. Several studies on the enzymatic hydrolysis of steroid hormone esters were performed using homogenates of various tissues [17–19]. These studies, however, did not present detailed characteristics of these enzymes. Recently, Schöttler and Krisch [1] reported that charged steroid esters were not hydrolyzed by a highly purified carboxylesterase. We found, however, that charged steroid esters were hydrolyzed by the carboxylesterase [7]. It was necessary to investigate the characteristics of steroid ester hydrolyzing enzymes, referred to as steroid esterases in this paper, to explain the contradictions presented in these papers.

The optimum pH of microsomal enzymes for steroid acetate esters was in an alkaline range (pH 8.0 to 8.5). This range approximates that for common substrates for carboxylesterase [20–22]. The optimum pH for hemisuccinate esters was 5.5. These data suggest that hydrolysis reactions with charged and uncharged steroid esters are catalyzed by different enzymes. The results presented by Schöttler and Krisch [1] showing that charged esters were not hydrolyzed by a carboxylesterase, may have been due to the alkaline conditions employed in their experiments. Generally, the dissociation rates of compounds at a particular pH are important factors affecting the hydrolysis rates when charged esters are used for substrates. But, the fact that hydrolysis of oxazepam hemisuccinate [23] was carried out under alkaline conditions shows that the characteristics of these enzymes themselves, and not their dissociation constants, determine the hydrolysis rates.

Species differences in the hydrolysis of acetate and hemisuccinate esters were observed. The greatest hydrolysis of acetate esters was exhibited in the hamster, cow and pig. When hemisuccinate esters were used as substrates, the greatest hydrolytic activity was observed in the hamster and mouse. These differences in the behaviors of steroid esterases for acetate and hemisuccinate esters suggest that these enzymes themselves are fundamentally different.

In spite of the unusual optimum pH for hemisuccinate ester hydrolyzing activities, subcellular distribution of the enzyme activities was similar to that of other substrates of carboxylesterases [20, 24–26] and steroid acetate esters: the highest activities were observed in the microsomal fraction. Relatively high steroid esterase activity was observed in the nuclear fraction. Haugen and Suttie [25] reported that 70 per cent of carboxylesterase activity was recovered in the rat liver microsomal fraction by washing the pellets from the initial centrifugation of homogenate with 0.25 M sucrose, but the recovery without washing was 45 per cent. Ljungquist and Augstinsson [20] also reported that about 40 per cent of the carboxylesterase activity was found in the nuclear fraction. Their results might explain why the nuclear fraction exhibited so much steroid esterase activity.

Steroid esterases for hemisuccinates were inhibited completely by BNPP and PMSF (10^{-5} M). Hydrolysis of acetate esters was also inhibited, but less than for hemisuccinates. In contrast, SH inhibitors, such as PCMB and $HgCl_2$, inhibited hydrolysis of acetate esters more than hemisuccinate esters. ASA deacetylase activity was inhibited approximately 90 per cent by NEM (10^{-3} M) and activated approximately 65 per cent by PCMB (10^{-4} M). The varied effects of these inhibitors suggest that hydrolysis is catalyzed by different enzymes.

Gratzl *et al.* [8] suggested that carboxylesterases hydrolyzing carboxylumbelliferone acetate and carboxyl-*p*-nitrophenyl acetate were inhibited by a charged inhibitor, BNPP, and that these enzymes are located on the outer surface of the microsomal membrane. This suggestion and the fact that enzymes hydrolyzing charged esters are present in membrane fractions should be considered in future studies of enzyme localization in membranes.

The studies on kinetic parameters confirmed the multiplicity of steroid esterases. The possibilities of the presence of two steroid esterases, an esterase hydrolyzing hemisuccinate ester and an esterase hydrolyzing acetate ester, were reinforced. Kinetic studies also demonstrated the possibility of another steroid esterase for acetate esters.

Arndt and Krisch [13] reported five chromatographically different carboxylesterases of rat liver microsomes. They succeeded in purifying two carboxylesterases, E_1 [16] and E_a [22]. The behaviors of these esterases toward PCMB and $HgCl_2$ were similar to those of steroid esterases. In the present study, it was not determined whether the steroid esterases were identical to carboxylesterase E_1 or E_a.

Further studies on the differences in the characteristics of several molecular species of carboxylesterases and steroid esterases of hepatic microsomes are now underway in our laboratories.

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