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CHARACTERIZATION OF THE ESTERASES OF GUINEA PIG LIVER AND KIDNEY

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Abstract—The esterases of cavian liver and kidney were separated electrophoretically into 12 and 14 distinct, tissue-specific bands, respectively. Based on substrate specificity and subcellular fractionation studies, the tissue esterases were identified as carboxylesterases (aliesterases) of microsomal origin. There was no evidence of arylesterase or cholinesterase activity in either tissue. Inhibition studies revealed the presence of two types of carboxylesterases in each tissue, one type being sensitive to organophosphorus esters, the second being resistant. Gel filtration and molecular weight estimation on a Sephadex G-200 column yielded two distinct molecular forms of hepatic enzymes having mol. wt. of 240,000 and 56,000, respectively. The higher molecular weight form could be dissociated into units of 56,000 mol. wt by a combination of acidic pH and high salt concentration. One molecular form was observed for renal esterases, the molecular weight being of the order of 56,000. The presence of electrophoretically different multiple forms of activity in the fractions isolated by gel filtration suggested the existence of charge isomers as well as readily formed aggregates.

THE TERM esterase is commonly used to designate enzymes which catalyze the hydrolysis of an ester of a carboxylic acid. However, the distinction between various types of such hydrolytic enzymes is not always clear-cut. On the basis of sensitivity to organophosphorus compounds, Aldridge[1] divided tissue esterases into two classes, the A-type which was insensitive and the B-type (including cholinesterase; ChE*) which was sensitive to inhibition by organophosphates. Augustinsson[2,3] classified the esterases into three distinct categories on the basis of substrate specificity plus inhibitor sensitivity. The arylesterases (ArE) were resistant to inhibition by organophosphates,[4] activated by calcium ions,[4,5] inhibited by inorganic and organic mercurials and EDTA[6] and preferentially hydrolyzed short chain aromatic esters.[3,7,9] Aliesterases (AliE) showed broad overlapping substrate specificity toward short chain and longer chain (aromatic and aliphatic) esters[2,3] and, while unaffected by divalent ions, were inhibited by organophosphorus esters and high concentrations of physostigmine (eserine).[3,7,9] Cholinesterases were identified by their ability to hydrolyze choline esters at a higher

* The following abbreviations have been used: ArE, arylesterase (EC 3.1.1.2); AliE, aliesterase or carboxylesterase (EC 3.1.1.1); ChE, cholinesterase (EC 3.1.1.8); BuTCh, butyrylthiocholine iodide; α-NA, alpha-naphthyl acetate; α-NP, alpha-naphthyl propionate; α-NB, alpha-naphthyl butyrate; *p*-NPA, *p*-nitrophenyl acetate; *p*-NPP, *p*-nitrophenyl propionate; *p*-NPB, *p*-nitrophenyl butyrate; DFP, diisopropylfluorophosphate; E600, paraoxon, diethyl-*p*-nitrophenyl phosphate; DDVP, dichlorvos, *O,O*-dimethyl-2,2-dichlorovinyl phosphate; *p*-HMB, *p*-hydroxymercuribenzoate; *p*-CMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetetraacetic acid disodium salt; PMS phenazine methosulphate.

rate than other esters and by inhibition with low concentrations of eserine and organophosphorus esters.[3] An unusual type of esterase found in hog kidney, termed C-esterase, was resistant to organophosphate inhibition, but unlike ArE, could be activated by organic mercurials (phenylmercuric acetate, *p*-CMB).[10]

Attempts have been made to characterize cavian tissue esterases by both physical and enzymatic studies. Starch gel electrophoretic patterns of cavian liver and kidney extracts have revealed multiple bands of esterase activity.[11,12] Holmes and Masters[13] reported the presence of ArE, AliE and ChE in the liver and kidney of guinea pig. As part of a study to characterize the tissue esterases of different mammalian species, cavian liver and kidney were studied. The present paper deals with the application of techniques including electrophoresis, differential centrifugation of subcellular particles, spectrophotometric and titrimetric assays with specific and nonspecific substrates in the presence and absence of inhibitors, and gel filtration to characterize the hepatic and renal esterases of guinea pigs.

METHODS

Chemicals. The substrates used in this study (α-naphthyl and *p*-nitrophenyl esters, D-glucose 6-phosphate, disodium salt) were purchased from Sigma Chemical Co., St. Louis, Mo. Succinic acid was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. Diisopropylfluorophosphate (DFP) and butyrylthiocholine iodide (BuTCh) were purchased from Mann Research Laboratories, Inc., New York, N.Y., the paraxon (E600) from K K Laboratories, Plainview, N.Y., while the dichlorvos (DDVP) was a gift of the Shell Oil Company of Canada. The EDTA and 2,6-dichlorophenolindophenol were purchased from British Drug Houses, Toronto, Ont. The *p*-hydroxymercuribenzoate (*p*-HMB), *p*-chloromercuribenzoate (*p*-CMB) and phenazine methosulphate (PMS) were purchased from Sigma Chemical Co.

Tissue extracts. Male and female guinea pigs weighing between 350 and 400 g were used in all experiments. The animals were killed by a blow on the head and samples of liver and kidney were removed, minced and washed thoroughly with 0·9% saline to remove residual blood. The tissues were homogenized at 0° with a Potter–Elvehjem glass homogenizer and motor-driven Teflon pestle, using sufficient distilled water to produce a final homogenate concentration of 20% (w/v). After freezing and thawing, the homogenates were centrifuged at 0° for 1 hr at 11,000 *g*. The sediments were discarded and the supernatants either used at once or stored at −20°. For the substrate specificicity and inhibition studies of the tissue esterases, a pooled extract prepared from four male and four female animals was used.

Subcellular fractionation. Samples of liver and kidney obtained from guinea pigs of both sexes were minced and thoroughly washed with 0·25 M sucrose containing 10^{-3} M EDTA. The tissues were then homogenized in sufficient 0·25 M sucrose solution to make up to a 20 per cent homogenate. The various subcellular fractions were prepared from the homogenates by differential centrifugation according to the method of Booth and Boyland,[14] as described in detail by Schwark and Ecobichon.[15] Three particulate fractions, namely nuclei and cell debris (N), mitochondrial (Mt) and microsomal (Mc), were successively isolated by sedimentation at 600 *g* for 1 hr, 20,000 *g* for 10 min and 90,000 *g* for 30 min, respectively. At each fractionation step, the pellet was separated from the supernatant fraction, washed twice by resuspension

in a known volume of 0·25 M sucrose and recentrifuged. The washings were combined with each supernatant fraction (stored at 0°) and then processed for the isolation of the next subcellular fraction. The final supernatant obtained after separation of the microsomes was called the cell sap (CS) fraction. All procedures were carried out at 0°. Mitochondria were disrupted either by freezing and thawing or by resuspending the mitochondrial pellet in 1·0% Triton X-100 for 10 min prior to centrifuging it at 20,000 *g* for 10 min.[16] The resulting pellet was discarded and the supernatant used for assays.

Electrophoresis and staining. The tissue extracts were separated by vertical zone electrophoresis in starch gel according to the method of Smithies.[17,18] The gels were prepared with 16 per cent starch in 0·02 M borate buffer, pH 8·4. Electrophoresis was carried out for 18–20 hr at room temperature using a constant current of 20 mA. After electrophoresis, the gels were suitably sliced and stained with substrates to localize the esterase activity according to the methods described by Ecobichon and Kalow.[9]

Protein determination. The protein concentration in tissue extracts was determined spectrophotometrically using the method of Waddell.[19] With subcellular fractions obtained from the liver and kidney homogenates, protein concentration was measured by the method of Lowry *et al.*[20] using bovine serum albumin as the standard.

Enzyme assays. Esterase activity was determined quantitatively using the spectrophotometric and titrimetric techniques described by Ecobichon.[21] The spectrophotometric technique measures the change in absorbance due to the enzymatic hydrolysis of substrate in a Unicam SP500 spectrophotometer equipped with a thermostated cell chamber and a Coleman model 165 recorder. Aliesterase activity was determined using the acetate, proprionate or butyrate esters of α-naphthol or *p*-nitrophenol.[21] Initial rates of hydrolysis of various esters studied at 25° and pH 7·4 were related to prepared standard curves and the total activity expressed as micromoles substrate hydrolyzed per minute per milligram of protein. The values were corrected for spontaneous hydrolysis of the substrates. The apparent K_m and V_{max} were calculated from the double-reciprocal plots of replicate experiments. Cholinesterase activity was measured using the method of Ellman *et al.*[22] at pH 8·4 with BuTCh at a substrate concentration of 5×10^{-4} M.

Esterase activity was determined titrimetrically under nitrogen using a Radiometer pH-stat titrimeter, as described previously.[21] The enzymatic hydrolysis of α-naphthyl acetate (α-NA) and *p*-nitrophenyl acetate (*p*-NPA) was carried out in a reaction vessel at 25°, the change in pH due to the formation of carboxylic acid being continuously back-titrated to pH 7·4 with 0·01 N NaOH. The rates of hydrolysis were corrected for spontaneous hydrolysis of the substrates.

To detect the presence of ArE, the technique described by Aldridge[23,24] and Ecobichon[21] was employed. Aliquots of tissue extract were dialyzed overnight at 4° against 0·06 M phosphate buffer, pH 7·4, containing 10^{-4} M EDTA. The dialyzed samples were incubated in the presence of 5×10^{-4} M divalent cations (Ca^{2+} Co^{2+}, Mg^{2+}, Mn^{2+}) for 5 min, following which the residual activity was assayed using a homologous series of *p*-nitrophenyl esters (*p*-NPA, *p*-NPP, *p*-NPB) as substrates. The enzymatic hydrolysis of E600 by hepatic and renal extracts was investigated, recording the release of *p*-nitrophenol from 10^{-3} M or 5×10^{-4} M substrate at 345 nm.[21]

Succinate dehydrogenase activity was determined on the subcellular fractions using the method described by Arrigoni and Singer[25] with 0·02 M succinic acid as the substrate. The change in absorbance was measured spectrophotometrically on a Unicam SP800 spectrophotometer at 600 nm using PMS as the immediate electron acceptor and 2,6-dichlorophenolindophenol as the terminal electron acceptor. All assays were carried out at 25° and pH 7·6.

Glucose 6-phosphatase was determined by the method of de Duve *et al.*,[26] which involved incubation of the enzyme with glucose 6-phosphate for 30 min at 37°. The amount of inorganic phosphate released was determined by the method described by Chen *et al.*,[27] measuring the intensity of the blue complex at 820 nm.

Inhibition studies. The sensitivity of the hepatic and renal esterases to organophosphates (DFP, DDVP and E600) was determined by incubating aliquots of the enzyme preparations with different concentrations of inhibitors at pH 7·4 for 5 min at 25°. The residual activity was determined using 10^{-3} M α-NA as the substrate. The per cent inhibition was calculated as a fraction of the control esterase activity.

Gel chromatography. Sephadex G-200 (Pharmacia of Canada Ltd., Montreal, Quebec) was hydrated by suspension in excess 0·025 M phosphate buffer, pH 7·0, containing 0·02% sodium azide. A column (2·5 × 40 cm) was packed as described previously to yield a bed length of approximately 35 cm.[28] The flow rate (12·5 ml/hr) was controlled by an Isco model 310 metering pump using the upward-flow technique. Molecular weights were repeatedly determined, applying pure globular proteins in 0·5–1·0-ml vol. (5–10 mg/ml) to the column. Aliquots (0·5–1·0 ml, containing 15–25 mg protein) of hepatic and renal extracts were applied in similar fashion to the calibrated columns. The elution volumes (V_e) of Blue Dextran, lactate dehydrogenase, bovine serum albumin, ovalbumin, chymotrypsinogen A, cytochrome *c* and AliE activity were detected as described previously.[28,29] Using the calibration curves for pure globular proteins (plots of V_e, V_e/V_o or K_{av} against log mol. wt), the molecular weights of the eluted hepatic and renal AliE were estimated.

RESULTS

Figure 1 shows representative starch gel esterase patterns of guinea pig hepatic and renal extracts (11,000 *g* supernatants) prepared from three animals, the enzymatic activity being stained with α-NA. Twelve distinct hepatic esterase bands were localized in the gel, whereas 14 renal esterase bands were observed. Identical patterns were detected using α-naphthyl butyrate (α-NB) as the substrate. Distinct tissue-specific patterns were observed for the liver and kidney extracts, but no significant differences were observed in the esterase patterns in corresponding adult male and female tissues.

The electrophoretically separated esterase bands detected with α-naphthyl esters did not correspond in position in the gel with any visible protein bands detected with Amido Black 10B. No hydrolysis of BuTCh was observed by the esterases of either tissue.

The protein concentration in extracts of adult cavian liver (18·38 ± 4·24 mg/ml; mean of 14 animals ± S.D.) was about twice that in the kidney (10·06 ± 2·17 mg/ml), while the hepatic esterase activity (0·30 ± 0·14 μmole/min/mg) was about 6-fold higher than that of the renal tissue (0·05 ± 0·01 μmole/min/mg). No significant difference was observed between male and female animals for protein content or specific activity toward α-NA.

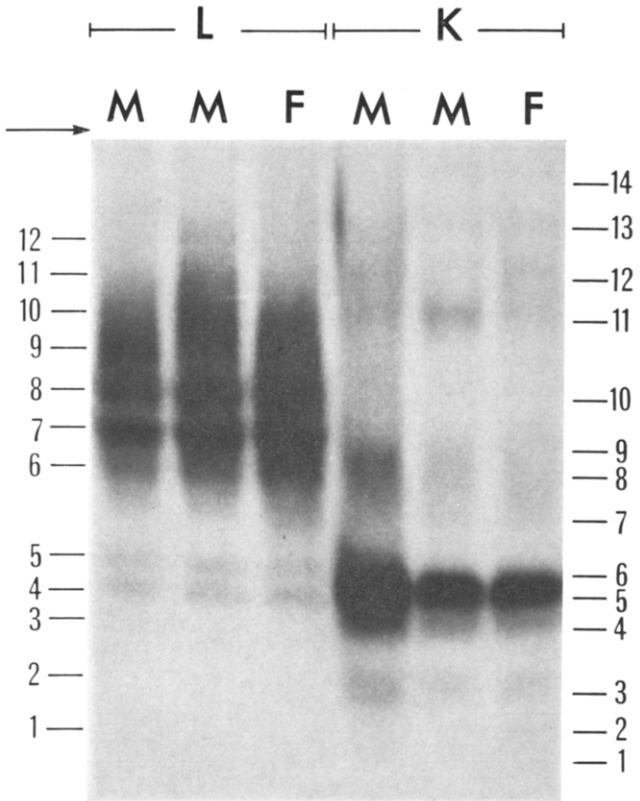

FIG. 1. Esterases of liver (L) and kidney (K) extracts (11,000 *g* supernatant) from two males (M) and a female (F) adult guinea pig following electrophoresis in starch gel at pH 8·4. The esterase activity was localized with α-NA. The arrow indicates the point of sample application. The direction of migration was toward the bottom of the photograph.

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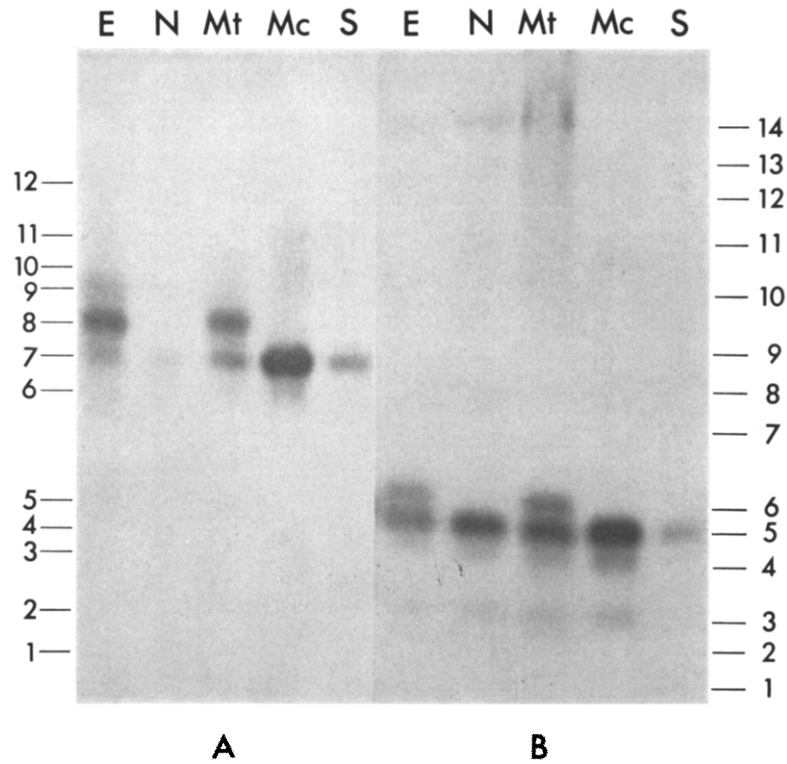

FIG. 3. Starch gel esterase patterns of nuclei and cell debris (N), mitochondria (Mt), microsomal (Mc) and cell sap fractions (S) prepared by differential centrifugation of hepatic (A) and renal (B) homogenates according to the method of Schwark and Ecobichon.[15] The hepatic and renal extract prepared in the usual manner (E) served for comparison. The esterase activity was localized with α-NA. See text for the preparation of the fractions used.

The optimal pH determined spectrophotometrically and titrimetrically for the hydrolysis of α-NA and *p*-NPA by hepatic and renal esterases was pH 7·8, though the pH of the tissue extracts was usually 7·4. Table 1 shows the V_{max} and K_m values

TABLE 1. KINETIC DATA OF THE HYDROLYSIS OF VARIOUS SUBSTRATES BY HEPATIC AND RENAL ESTERASES*

Substrate	Hepatic esterases		Renal esterases	
	V_{max}	K_m apparent ($\times 10^{-4}$ M)	V_{max}	K_m apparent ($\times 10^{-4}$ M)
α-Naphthyl acetate	0·42	1·57	0·05	1·60
α-Naphthyl propionate	0·87	0·89	0·06	0·94
p-Nitrophenyl acetate	0·13	0·75	0·01	3·88
p-Nitrophenyl propionate	0·34	1·15	0·04	1·86
p-Nitrophenyl butyrate	0·48	1·57	0·04	0·72

* Activity expressed as micromoles per minute per milligram of protein.

apparent for the hydrolysis of α-naphthyl and *p*-nitrophenyl esters at pH 7·4 by hepatic and renal esterases. While longer chain esters of α-naphthol were available these could not be employed because of difficulty with solubility and the fact that the amount of organic solvent required to maintain solution caused partial denaturation of the esterase protein. The hepatic esterases hydrolyzed α-naphthyl propionate (α-NP) at a rate 2-fold higher than that for α-NA, though little difference was observed in the rates of hydrolysis by renal esterases. Using esters of *p*-nitrophenol, the hepatic esterases hydrolyzed the esters in a decreasing order of *p*-NPB > *p*-NPP > *p*-NPA. The renal esterases hydrolyzed *p*-NPB and *p*-NPP at the same rate, though much more rapidly than *p*-NPA. The hepatic and renal extracts did not hydrolyze BuTCh.

The sensitivity of hepatic and renal esterases to inhibition by DFP, DDVP and E600 is shown in Fig. 2. With the hepatic esterases, the inhibition curves produced by DFP, DDVP and E600 were biphasic. Phase I, between 5×10^{-10} and 1×10^{-6} M, showed a progressive increase in per cent inhibition, the I_{50} being approximately 2×10^{-8} M for all three organo-phosphates. Between 1×10^{-6} and 5×10^{-5} M, there was a plateau at approximately 80 per cent inhibition followed by phase II, a more gradual inhibition as the inhibitor concentration was increased, the I_{50} being approximately 1×10^{-4} M. At an inhibitor concentration of 10^{-3} M, there was complete blockade of hydrolysis of α-NA. On exposure to DFP and E600, the renal esterases were more resistant than the hepatic esterases, the I_{50} for phase I being 1×10^{-7} M. However, the plateau region (75 per cent inhibition) was observed between $2{\cdot}5 \times 10^{-6}$ and $3{\cdot}5 \times 10^{-5}$ M inhibitor concentration. The renal esterases appeared to be more resistant to inhibition by DFP and E600 than did the hepatic enzymes, the I_{50} for phase II being approximately 8×10^{-4} M. Complete inhibition was not observed at 10^{-3} M inhibitor concentration. Dimethyl dichlorovinyl phosphate (DDVP) produced a somewhat different inhibition curve. No plateau was observed and the inhibition assumed a completely different slope above 10^{-5} M inhibitor concentration.

Dialysis against EDTA had no effect on the hydrolysis of *p*-NPA, *p*-NPP or *p*-NPB

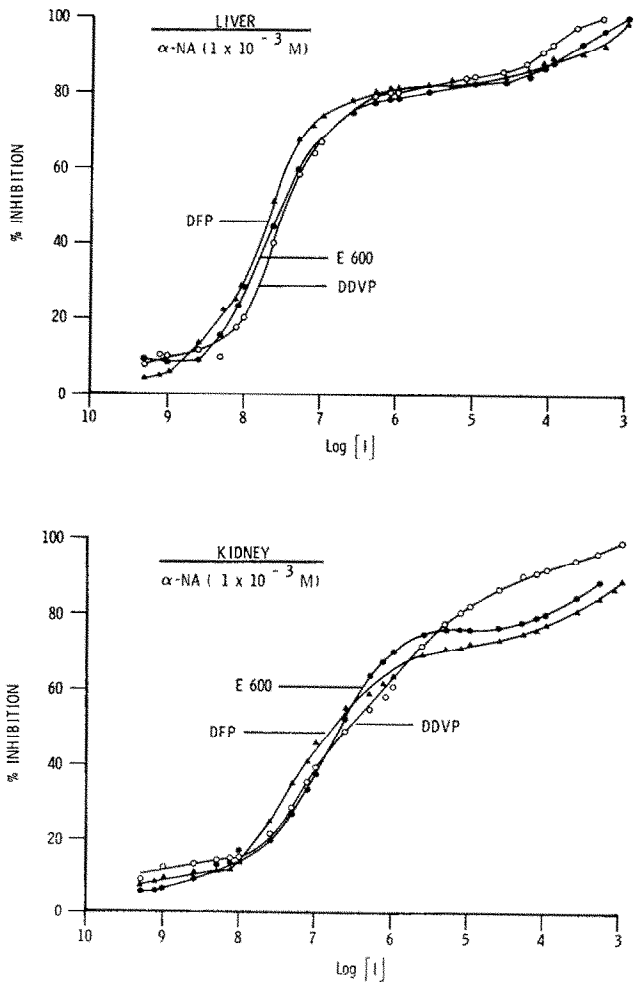

FIG. 2. Inhibition of hepatic and renal esterases by DFP, E600 and DDVP. Aliquots (0·05 ml) of hepatic and renal extracts were incubated with different concentrations of the inhibitor for 5 min before the residual activity was determined with 10^{-3} M α-NA. Each point is the mean of duplicate determinations.

by either hepatic or renal esterases. Prior incubation of 0·05-ml aliquots of the EDTA-dialyzed hepatic and renal extracts with divalent cations (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}) neither inhibited nor activated the hydrolysis of the three esters. While *p*-HMB at 10^{-4} M had no effect on the hydrolysis of the *p*-nitrophenyl esters by hepatic esterases, it inhibited the activity of the renal esterases by about 50 per cent. With the hepatic esterases, 10^{-4} M DFP completely abolished the hydrolysis of these substrates, but approximately 30 per cent of the renal esterase activity was resistant to inhibition by this concentration of the inhibitor.

Aqueous extracts (11,000 *g* supernatants) of hepatic and renal tissue used *per se* or dialyzed against phosphate buffer, pH 7·4, containing 10^{-4} M EDTA and assayed

in the presence or absence of 5×10^{-4} M $CaCl_2$ produced no detectable formation of *p*-nitrophenol with E600 as the substrate.

Bergmann *et al.*[10] showed that hog kidney contained an esterase (C-esterase) which was resistant to inhibition by DFP, hydrolyzed *p*-NPA, and was activated by *p*-CMB and phenylmercuric acetate. The possibility that the organo-phosphate-resistant cavian renal esterase activity might be due to a similar enzyme was investigated. Parallel experiments with hog and cavian kidney tissue extracts were carried out to demonstrate the presence of C-esterase. When an aliquot of hog renal extract was incubated with 10^{-4} M DFP at 25° for 10 min, the residual activity was 7 per cent of the uninhibited activity. Further incubation with 10^{-4} M DFP at 4° for 15 hr resulted in a further 14·5 per cent reduction in activity (6 per cent of original activity). When hog renal extract was incubated with DFP together with 10^{-5} M *p*-CMB or 10^{-5} M *p*-HMB for 15 hr at 4°, the residual activities were both 150 per cent of the activity in the absence of mercurials, indicating activation of hog renal enzymes by mercurials (9 per cent of original activity). Treating aliquots of guinea pig renal extract in an identical manner showed that after 10 min DFP treatment at 25°, the residual activity was 28 per cent of the uninhibited control. Further incubation with the same concentration of DFP for 15 hr at 4° caused a further 47 per cent inhibition of the residual activity (15 per cent of original activity). Incubation with *p*-CMB and *p*-HMB resulted in a further 53·4 per cent inhibition (7 per cent of original activity) rather than activation as was observed with the hog renal esterases.

Figure 3 shows the starch gel esterase patterns of the subcellular fractions [nuclei and cell debris (N), mitochondrial (Mt), microsomal (Mc) and cell sap fractions (S)] prepared by differential centrifugation of hepatic (A) and renal (B) homogenates. Hepatic and renal extracts (E) prepared in the usual manner served for comparison. In the case of the liver, E and Mc were diluted 5-fold with saline while fractions N, Mt and S were used *per se*. The esterase activity in E was composed of the slow-migrating bands (bands 6–12) and a rapidly migrating band (band 5). The rapidly migrating esterase bands (bands 1–4) were weakly stained with α-NA. The microsomal fraction showed the highest activity in comparison to the other subcellular fractions. The esterase activity in Mc was localized in bands 4, 6, 7, 10 and 11. The mitochondrial esterase pattern was comparable to that of E. Very little activity was found in the N and S fractions; that present was localized in bands 4 and 7. With the guinea pig kidney, E was diluted 3-fold; N, Mc and S were used *per se*, whereas Mt was concentrated 10-fold by dialysis against Carbowax 4000.[7] Due to the dilution of E, some of the esterase bands (bands 1, 2, 6–11) were weakly stained. As with the liver, the renal microsomal fraction had the highest activity compared to other subcellular fractions and it had the same number of bands as E, though band 6 was very weakly stained. The esterase pattern of N was comparable to that of Mc, though some of the bands in N were less intensely stained. The mitochondrial fraction had an esterase pattern comparable to that of E. Very little activity was observed in S, that present being localized in bands 3, 5 and 14.

The results of quantitative determination of esterase activity in the subcellular fractions of liver and kidney expressed as a percentage of the total activity are presented in Table 2. The hepatic microsomal fraction contained the bulk of the esterase activity (81·5 per cent), being 8-fold higher than the activity in the cell sap fraction. Little esterase activity was found in the nuclei-debris and the mitochondrial fractions

TABLE 2. INTRACELLULAR DISTRIBUTION OF THE TOTAL ESTERASE ACTIVITY IN ADULT GUINEA PIG LIVER AND KIDNEY*

Fraction	Activity as percentage of total activity	
	Liver	Kidney
Nuclei and cell debris	3·38 ± 1·42	9·92 ± 2·74
Mitochondria	5·07 ± 1·24	4·65 ± 3·08
Microsomes	81·51 ± 3·11	59·42 ± 6·09
Cell sap	10·02 ± 1·32	25·90 ± 2·20

* The results for the liver fractions were the mean ± the standard deviation of eight individual experiments (using the fresh tissues of four male and four female guinea pigs), while those for the kidney were from two male and two female guinea pigs. Esterase activity was determined using 10^{-3} M α-NA, and the total activity was the sum of the esterase activity in all the fractions from each tissue.

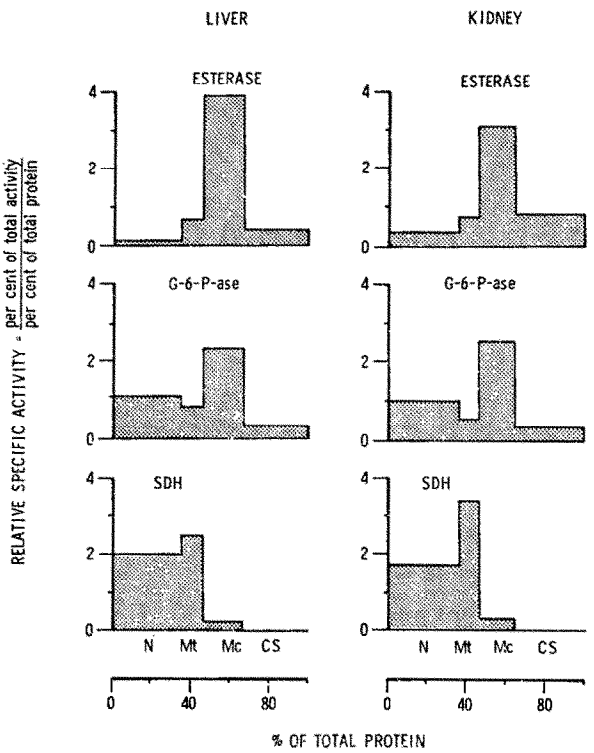

FIG. 4. Subcellular distribution pattern of esterases, glucose 6-phosphatase (G-6-P-ase) and succinate dehydrogenase (SDH) in the nuclei-debris (N), mitochondrial (Mt), microsomal (Mc) and cell sap (CS) fractions of liver and kidney from adult guinea pigs. See text for details of preparation. The data are presented in the manner proposed by de Duve *et al.*[26] Glucose 6-phosphatase and succinate dehydrogenase served as reference enzymes for microsomal and mitochondrial particles, respectively. The esterase activity was determined using α-NA at a cuvette concentration of 1×10^{-3} M.

of the liver. The subcellular distribution of the renal esterase activity appeared to be different from that in the liver. Although peak activity (59·4 per cent) was observed in the microsomal fraction, it was only 2-fold higher than that in the cell sap fraction. The activity in the nuclei-debris fraction of the kidney was much higher than that in the liver, while the mitochondrial fraction in the two tissues had about the same percentage of the total esterase activity.

Figure 4 shows the intracellular distribution patterns of AliE, glucose 6-phosphatase and succinate dehydrogenase activity in cavian liver and kidney presented in the manner proposed by de Duve *et al.*[26] Glucose 6-phosphatase and succinate dehydrogenase were assayed in all the fractions to serve as reference enzymes for microsomes and mitochondria respectively.[26] The subcellular distribution of hepatic and renal esterase activity resembled closely that observed for the microsomal enzyme, glucose 6-phosphatase, peak esterase activity being observed in the microsomal fraction.

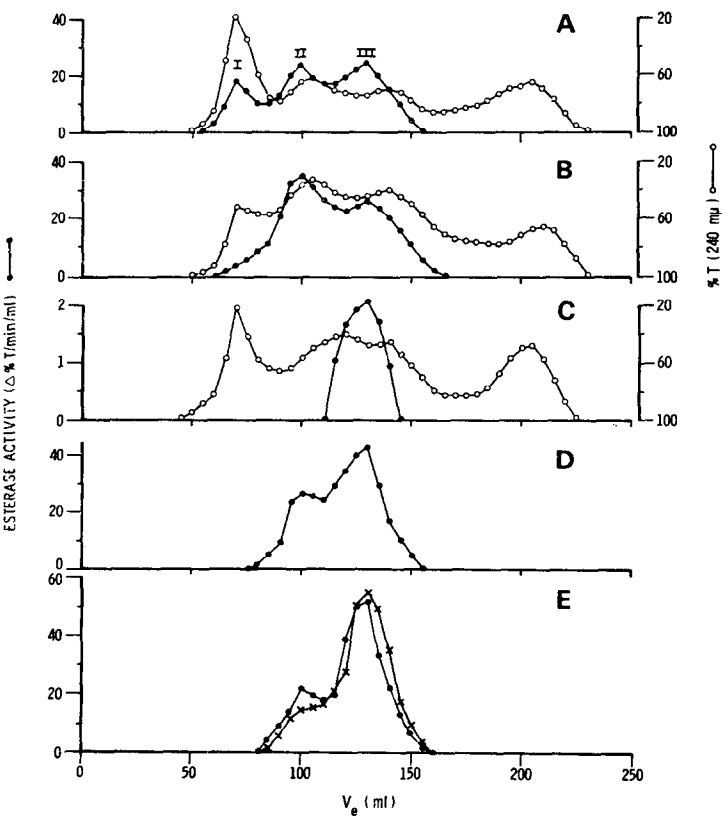

FIG. 5. Protein (○) and esterase (●) elution patterns of 0·5-ml aliquots of hepatic 11,000 *g* supernatant A, 4-fold concentrated hepatic 105,000 *g* supernatant B, and renal 11,000 *g* supernatant C following gel filtration on a 2·5 × 40 cm column of Sephadex G-200 equilibrated with 0·025 M phosphate buffer, pH 7·0. In D, the esterase elution of a 0·5-ml aliquot of hepatic 105,000 *g* supernatant incubated with 0·5 M NaCl for 24 hr at 25° and applied to the same column now equilibrated with buffer containing 0·5 M NaCl is shown. In E, the hepatic 105,000 *g* supernatant was incubated with 0·01 M sodium acetate, pH 4·5, for 40 min (●) and 120 min (×) prior to the addition of solid NaCl to give a 0·5 M concentration. The mixture was adjusted to pH 7·5 and applied immediately to the column used in D.

Figure 5 shows the elution patterns of the proteins and esterases of extracts of cavian liver and kidney fractionated on a 2·5 × 40 cm Sephadex G-200 column equilibrated with 0·025 M phosphate buffer, pH 7·0. With a 0·5-ml aliquot of the 11,000 *g* supernatant of the hepatic extract, three peaks of esterase activity were detected (Fig. 5A). Peak I was eluted with the void volume (V_o = 70 ml determined with Blue Dextran), while peaks II and III had elution volumes (V_e) of 100 ml and 130 ml, respectively. The elution of peak I with the void volume for the column suggests that the hepatic extract (11,000 *g* supernatant) contained either extremely large aggregates or insoluble particles. Past experience has demonstrated that particulate matter tends to become trapped in the nylon support (10 micron mesh) of the flow adapter and causes a reduction of the flow rate. This did not occur in the present study. After centrifugation at 105,000 *g* and concentration of the supernatant solution by dialysis against Carbowax 4000,[7] an aliquot (0·5 ml) of the soluble hepatic esterases was resolved into two peaks (II and III) having the same elution volumes as before (Fig. 5B). The AliE activity of the renal extract (11,000 *g* supernatant) was resolved into a single peak having an elution volume (V_e = 130 ml) identical to that of hepatic peak III (Fig. 5, B and C). No peak of activity was observed which moved with the void volume. On electrophoretic separation, hepatic peaks I and II were composed of the slow-migrating bands (bands 6–12). Hepatic peak III contained both slow- and fast-migrating bands (bands 1–5). Electrophoretic separation and staining of the renal peak revealed the presence of all bands (bands 1–14).

Cytoplasmic AliE extracted from porcine, equine and human liver have been shown to aggregate on storage, on concentration, and during extract preparation, the aggregates being completely dissociated by a combination of acidic pH and high salt concentration.[29,30] Since the presence of a high molecular weight cavian hepatic enzyme was detected in extracts by gel filtration, experiments were performed in an attempt to dissociate the complex. When an aliquot of the 105,000 *g* supernatant was incubated for 24 hr with 0·5 M sodium chloride and a 0·5-ml vol. was reapplied to the same Sephadex G-200 column equilibrated with buffer containing 0·5 M NaCl, the pattern in Fig. 5D was observed. Peak II was reduced in height and accompanied by a proportionate increase in the height of peak III. In the second experiment, aliquots of the 105,000 *g* supernatant were diluted with an equal volume of 0·01 M sodium acetate, the pH was adjusted to 4·5, and the solutions were incubated for 40 and 120 min at 25°. After incubation, the samples were treated with sufficient solid sodium chloride to yield a final concentration of 0·5 M, and pH was quickly adjusted to 7·5, and 0·5-ml aliquots were applied to the same Sephadex G-200 column. The results presented in Fig. 5E show a marked reduction in the quantity of high molecular weight enzyme (peak II) and a concomitant increase in the quantity of the low molecular weight form. Complete dissociation was not achieved, even incubating in the acidic salt medium for 120 min.

To estimate the molecular weight of the hepatic and renal esterases, pure globular proteins of known molecular weights were used to calibrate a 2·5 × 40 cm Sephadex G-200 column prepared as above. Figure 6 is a representative plot of the ratio of V_e/V_o against the molecular weights of the pure proteins. The molecular weights of the two hepatic esterase peaks (II and III) were estimated to be 240,000 and 56,000 respectively. The single peak of renal AliE activity has a molecular weight identical to that of the hepatic peak III.

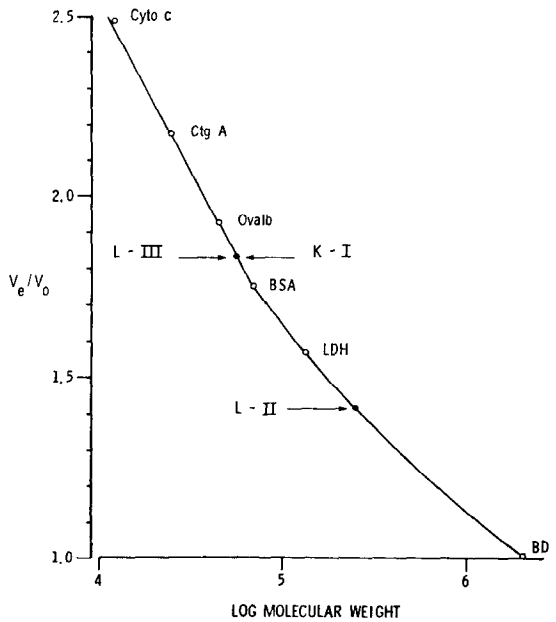

FIG. 6. Relationship between V_e/V_o and log molecular weight for purified globular proteins separated on a 2·5 × 40 cm column of Sephadex G-200 equilibrated with 0·025 M phosphate buffer, pH 7·0. The V_e/V_o of the hepatic esterase peak II (L-II) and peak III (L-III) and of the single renal esterase peak (K-I) are indicated by arrows. The purified standard proteins are abbreviated as follows: Cyto *c*, horse heart cytochrome *c*; Ctg A, bovine pancreatic chymotrypsinogen A; Ovalb, ovalbumin; BSA, bovine serum albumin; LDH, rabbit muscle lactate dehydrogenase; BD, Blue Dextran 2000

DISCUSSION

On the basis of the electrophoretic separation and staining with α-NA and α-NB (Fig. 1), the substrate specificity (Table 1), inhibition sensitivity and the subcellular distribution (Table 2 and Fig. 4), it would appear that the predominant hepatic and renal esterases of the guinea pig are of microsomal origin and belong to the highly heterogeneous AliE group of enzymes.[2,3] Distinct tissue-specific patterns were observed. The results are in agreement with other reports indicating that the hepatic esterases are microsomal in origin in the guinea pig as well as in other species.[31–33] The renal esterases showed a somewhat different distribution, though the highest activity was still associated with the microsomal fraction.

The biphasic character of the inhibition curves (Fig. 2) suggested the presence of at least two types of AliE in cavian tissue. The type I AliE, found in both liver and kidney, was sensitive to low concentrations of organophosphorus esters. The type II hepatic AliE was inhibited only by high concentrations of organo-phosphates. A different second type of AliE was present in the kidney, being very resistant to inhibition by organophosphates. Similar results have been obtained by Ljungquist and Augustinsson[33] with rat hepatic microsomal esterases. The affinity of organophosphorus esters for the active sites of the aggregated form might vary considerably. While this could account for observations with the hepatic enzyme, it does not

adequately explain the similar observations with the renal esterases when aggregated forms of enzyme were not in existence. A possible explanation was recently proposed by Greenzaid and Jencks,[34] who reported the presence of two different active sites in purified hog hepatic AliE. One active site, catalyzing the hydrolysis of aromatic and alkyl esters, was sensitive to organo-phosphorus esters. A second site, only catalyzing the hydrolysis of larger esters (substituted phenyl and naphthyl esters), was resistant to such inhibitors. The routine use of such esters in our study possibly explains the biphasic inhibition curves, the inhibition of α-NA hydrolysis at the first site occurring before inhibition at the second active site. The possibility of an organo-phosphate-resistant C-esterase similar to that reported by Bergmann *et al.*[10] was eliminated by the different characteristic properties of the cavian renal enzyme(s) and that found in hog kidney.

The absence of ArE and ChE in extracts of cavian liver and kidney contradicts the observations of Holmes and Masters,[13] who reported substantial amounts of both enzymes in addition to AliE in both tissues. The presence of 37 per cent (hepatic) and 19 per cent (renal) ChE would have been easily detected by our spectrophotometric assay and gel-staining technique. The different observations may have resulted from improper preparation of the tissue extract, since it has been demonstrated that thorough washing of the minced tissue with 0·9% saline prior to homogenization effectively removes trapped blood from the capillaries and markedly reduces the ChE activity of the extract.[9,35] While Holmes and Masters[13] found ArE activity in cavian liver (1·3 per cent) and kidney (13·6 per cent), the spectrophotometric results demonstrating the absence of inhibition by EDTA and organic mercurials and activation by divalent cations and the absence of hydrolysis of E600 strongly suggest that ArE activity was not present in the tissue extracts. We have already reported the absence of ArE in the plasma of this particular strain of guinea pig.[36] The inhibition studies of Holmes and Masters[13] were carried out in acrylamide gel, incubating the gel with inhibitor for 10 min prior to staining with naphthyl esters. It has been our experience with similar studies in starch gel that such a short incubation time results in incomplete penetration of the gel by the inhibitor and only partial inhibition of the enzymes present. Subsequent staining results in a faint band of activity which could easily be misinterpreted as indicating the presence of a resistant enzyme. Such difficulties are avoided by sensitive spectrophotometric techniques.

Kingsbury and Masters,[37] using a polyacrylamide disk electrophoretic technique, estimated the guinea pig hepatic AliE to be of the order of 80,000 mol. wt. Using a Sephadex G-200 column calibrated with pure globular proteins, two distinct molecular forms of hepatic AliE were detected, having mol. wt of 240,000 and 56,000 respectively. The behavior of the higher molecular weight form suggests that it is a tetrameric aggregate held together by electrostatic forces, which dissociates into units of the order of 56,000 mol. wt when exposed to an acidic medium and high salt concentration. Similar observations have been made for porcine, human and equine hepatic AliE.[29,30] While aggregation is somewhat dependent upon protein concentration, the concentrated cavian renal AliE failed to polymerize, only the unit having a mol. wt of 56,000 being detected. The presence of multiple forms of activity in the hepatic (peak III) and renal (peak I) fractions separated by gel filtration suggests that, while these molecules are of similar weight, they possess a slightly different negative charge on the surface of the protein. The existence of charge isomers as well

as aggregates which could reform prior to or during electrophoresis probably accounts for the multiple bands observed in the starch gels.

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