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ESTERASE XXVII

PURIFICATION AND CHARACTERIZATION OF ESTERASE-9A OF MOUSE KIDNEY

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Summary

Esterase-9A, which appears electrophoretically as a triplet of the bands III-50, III-40 and III-30, was isolated from the kidneys of male NMRI-mice by isoelectrofocusing and refocusing followed by repeated molecular sieve chromography. The overall purification was approx. 250 fold and each of the three bands was isolated separately. The band of the triplet nearest to the cathode, III-50, changed in vitro into the satellite bands III-40 and III-30 and, further, into the band III-22 not observed before in the homogenate. It is assumed that the band III-50 represents the original gene product. The molecular weight (45 000) of the band III-50 is identical with those of III-40 and III-30, as measured by analytical electrophoresis, whereas the molecular weight obtained by thin-layer chromatography was 51 000. There were no obvious signs that esterase-9 was composed of subunits. The K_m constant for 4-nitrophenyl proprionate was identical for each of three bands. The esterase-9A is the first testosterone-dependent isozyme of the mouse carboxylesterase (carboxylic-ester hydrolase, EC 3.1.1.1) system which has been isolated.

Introduction

At least 7 esterases (all of which seem to be isozymes of the carboxylesterase (carboxylic-ester hydrolase, EC 3.1.1.1)) are governed by genes located within a narrow distance on chromosome 8 of the mouse [1] and esterase-9A is one we have studied previously [2]. Two alleles of the locus Es-9 are known: Es-9^b (coding for esterase-9B) in strain SK/Cam and perhaps *Mus musculus* molossinus and Es-9^a (coding for esterase-9A) in NMRI, C_3H, C_{57} Bl/10Sn and additionally in many other strains of laboratory mice [3]. Esterase-9A appears

electrophoretically as a triplet of bands 50, 40 and 30 in zone III, according to the nomenclature of Wienker et al. [4]. In the kidney, esterase-9 is testosterone-dependent [5,6] and is one of the most prominent kidney esterases.

Esterase-9A has not been biochemically investigated until now. In the present work, we are dealing with a method for the isolation and first characterization of this genetically well-defined enzyme, as well as the biochemical interrelationship of the three electrophoretic bands III-50, III-40 and III-30.

Experimental methods

Esterase assay. The esterase activity was measured by recording the hydrolysis of 4-nitrophenyl propionate at 405 nm [7] for 1—2 min (final substrate concentration 0.22 mmol/l 4-nitrophenyl propionate, 0.066 mol/l phosphate buffer, pH 7.3, 25°C. The enzyme activity is expressed as 1 unit = 1 μmol substrate split/min.

Protein. The protein content of the fractions was determined by the relationship of the absorbance at 260 and 280 nm, [8], or according to Lowry et al. [9].

Polyacrylamide gel electrophoresis. Electrophoresis for identification purposes was performed in cylindrical 7% polyacrylamide gels (90 × 5 mm), using a short spacer gel. Separation gel buffer was 75.6 mmol/l tris, 12 mmol/l HCl, pH 8.9; electrode buffer was 4.95 mmol/l tris, 34.4 mmol/l glycine, 0.01% bromophenol blue, pH 8.3 [10]. The gel was loaded with 2—10 munits of enzyme activity. Following electrophoresis, the gels were immediately stained for esterase activity using α-naphthyl acetate as substrate and Fast Red TR as diazonium salt. Protein staining was performed with Coomassie brilliant blue G 250 [11].

Molecular weight determinations. For molecular weight determinations in polyacrylamide gel [12], the gel concentrations were varied between 6 and 10%. The following calibration proteins were used: Lactate dehydrogenase I (M_r = 137 000) from pig heart; bovine serum albumin (M_r = 67 000); *Bacillus subtilis* α-amylase (M_r = 45 000) (all from Serva, Heidelberg). The gel load was about 10 μg protein and the molecular weights were calculated according to Thorun [13].

The molecular weight determination by thin-layer chromatography was accomplished according to Andrews [14] using 20 × 20 cm plates with a 0.6 mm Sephadex G 200 Superfine layer. Equilibration was performed overnight and development was for 4—5 h until the cytochrome *c* spots had migrated for 5 cm.

Chymotrypsinogen, ovalbumin and bovine serum albumin (Boehringer) served as further calibration proteins. The esterase spots were stained with α-naphthyl acetate/Fast Blue BB, and the calibration proteins were detected with diazotized sulfanilic acid.

Production of antibody. Small aliquots from a mixture of 0.5 ml Freund's complete adjuvant and 0.5 ml esterase-9A (7 U, 96 μg protein) were injected intradermally into a rabbit at several sites. The procedure was repeated 15 days later. Blood samples of about 0.5 ml were collected from the rabbit 2 weeks later. After clotting, the blood was centrifuged and the serum stored at 4°C.

Immunodiffusion studies. Double immunodiffusion [15] was done in 1% agar gel (0.666 mol/l phosphate buffer (pH 7.3), 0.04% NaN_3). The samples and the antiserum were allowed to diffuse at 4°C for 20 h. After washing with 3% NaCl for 20 h the gel was stained for esterase with α-naphthyl acetate/Fast Red TR.

Results

Purification of esterase-9A

Step 1 — Homogenization. 6 g kidneys of 25 adult male NMRI mice (3 months old, from the departmental animal house) were decapsulated and homogenized in 9 vol. of 0.2% aqueous Triton X-100 (Potter S, 1000 rev./min, 10 complete strokes in an ice water bath). After being at room temperature for 20 min, the homogenate was centrifuged (60 min at 100 000 × *g*) and the pellet discarded. The supernatant was concentrated by ultrafiltration (Amicon 202 cell, Diaflo PM 10 membrane). The concentrated protein solution had a specific esterase activity of 0.9 units/mg protein. The specific 9A-activity was estimated to be about 0.225—0.27 units/mg protein (Table I).

Step 2 — First isoelectric focusing. 60 ml concentrated solution (containing a total esterase activity of 2900 units) were applied, with the less-dense solution, to an isoelectric focusing column (column I; LKB Instrument Co, 440 ml capacity) with pH 5—7 gradient provided by 2% Ampholine carrier ampholytes. A linear 0—40% sucrose gradient was performed for stabilization. A run usually lasted about 75 h, starting at 350 V, 4.75 mA and ending at 700 V, 2.8 mA. After the completion of the run, 3-ml fractions were collected. The esterase activity was focused at five peaks, A_0—E_0 (Fig. 1), containing 75% of the column load (53% B_0, 15% C_0, 5% D_0, 2% $A_0 + E_0$). The composition of esterase bands of the single peaks was established by analytical polyacrylamide electrophoresis (Fig. 2). Peak A_0 (pH 3,85—3.95) contained band 10 of zone II. Probably, the esterase is identical with the genetically well-known esterase-1B

TABLE I

PURIFICATION OF ESTERASE 9A FROM MOUSE KIDNEY

Step	Event	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg protein)	Yield (%)		Purification (-fold)
						Kidney esterase	Esterase 9A	
1	supernatant (ultrafiltrated)	60	2900	3200	0.9	100		
	estimated 9A content		580	3200	0.225—0.27	25—30	100	1
2	peak D_0	18	160	15	10.7	5.5	18—22	40—47
3	peak D_1 fraction 14—17	10	130	5.6	23.3	4.5	15—18	85—100
4	gel filtration G 100, fraction 15—18	10	87	1.75	50	3	10—12	185—220
5	gel filtration Ac A 44, fraction 19—23	12	72	1.1—1.2	60—65	2.5	8.3—10	240—265

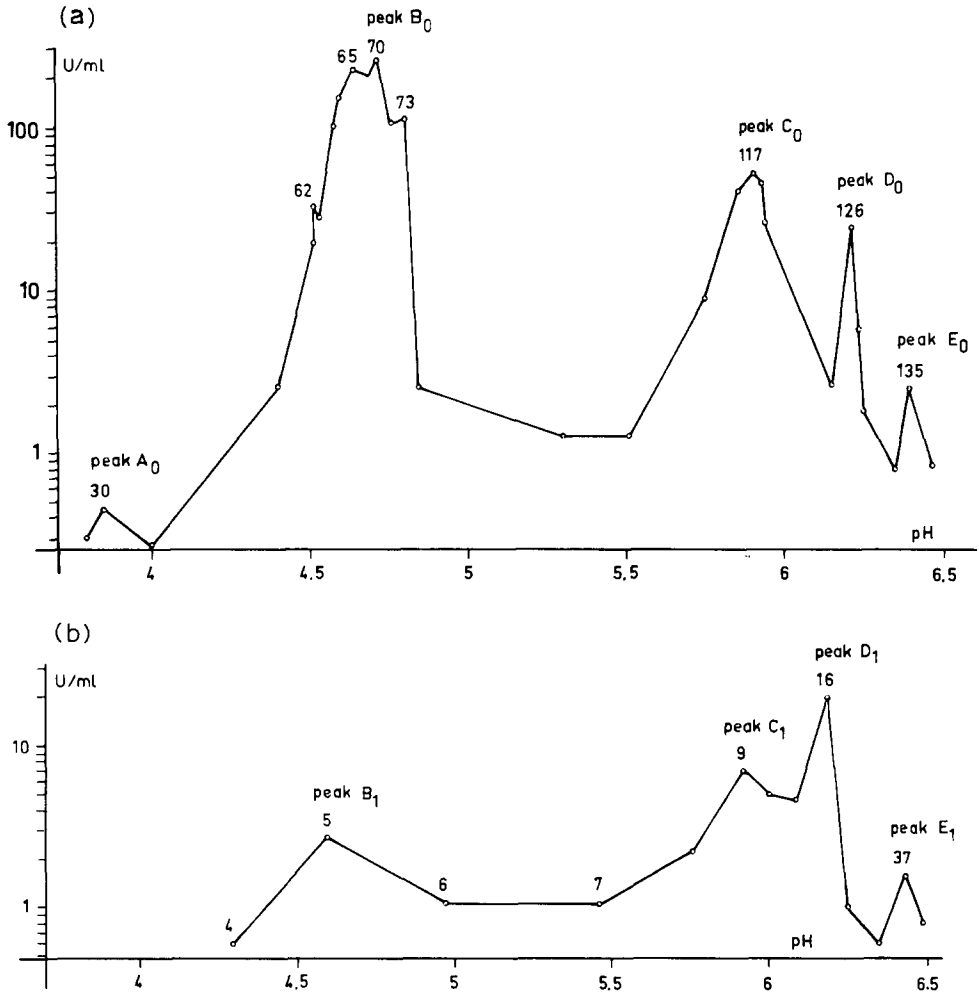

Fig. 1. Purification of mouse esterase-9A. Esterase activity towards 4-nitrophenyl propionate after fractionation by isoelectric focusing and refocusing respectively. (a) fractionation of kidney homogenate containing 2900 units (column I, 440 ml capacity, 3-ml fractions, 2% Ampholine, pH 5—7). (b) fractionation after refocusing combined fravtions 124—129 of peak D_0 containing 160 units. Column II (110 ml capacity, 2 ml fractions, 0.4% Ampholine, pH 5—7).

[16—18]. Among kidney esterases, the band II-10 is characterized by its low isoelectric point (Table II). Since band II-10 moves more slowly than the esterase-2B in 7% polyacrylamide gel, it must have a higher molecular weight.

Peak B_0 (pH 4.4—4.95) contained four esterase bands of the zone I (Fig. 2). The band I-30 was the main component and is identical with the genetically-defined esterase-2B [19]. Of the four bands, band I-30 was nearest the cathode and showed the highest isoelectric point of the zone I esterases.

Peak C_0 (pH 5.8—5.95) was made up of the triplet III-50, III-40 and III-30 and was contaminated by some bands of zone IV. The triplet is governed by one gene designated as Es-9 [2].

Peak D_0 (pH 6.1—6.2) consisted almost entirely of band III-50.

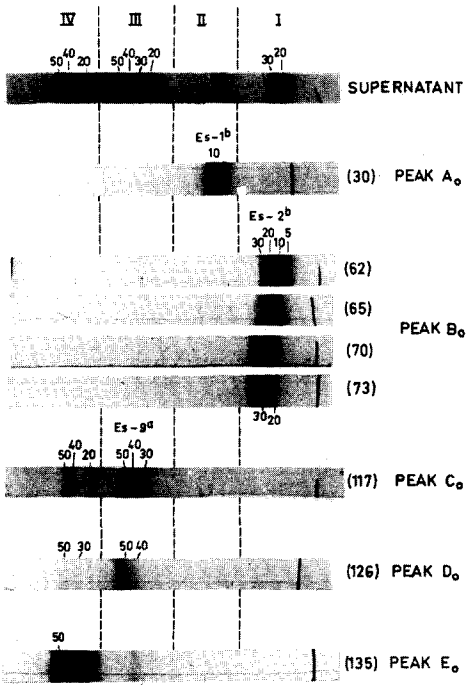

Fig. 2. Esterase bands of representative fractions (number in parenthesis) of the five peaks A_0, B_0, C_0, D_0 and E_0. Above, the original column charge (kidney supernatant) for comparison. The anode is at the right side as in the following figures.

Peaks C_0 and D_0 contained together about 18—22% of the original activity of the kidney homogenate. Only the esterases of peak D_0 were further purified, which accounts for the small overall yield of our esterase-9 preparation. Peak E_0 (pH 6.3—6.5) contained mainly zone IV esterases. Since, hitherto, they have not been genetically characterized clearly enough, they are not dealt with further here.

Step 3 — Repeat of isoelectric focusing. Combined fractions 124—129 (pH 6.14—6.23) of peak D_0 with a volume of 18 ml were refocused (column II,

TABLE II

ESTIMATION OF THE KINETIC CONSTANT k FOR THE (IRREVERSIBLE) INHIBITION REACTION OF ESTERASE-9A WITH BIS-*p*-NITROPHENYL PHOSPHATE

b_0 = concentration of bisnitrophenyl phosphate, a_0 = concentration of esterase-9A.

Expt. No.	b_0/a_0	k ($l \cdot mmol^{-1} \cdot min^{-1}$)	Range of measurement	
			% turnover	Time (min)
1	12.4	66.0 ± 17	65	12
2	6.12	65.5 ± 9	90	20
3	9.4	66.3 ± 13	80	15
4	4.59	58.2 ± 7	76	17
mean		64 ± 11.8		

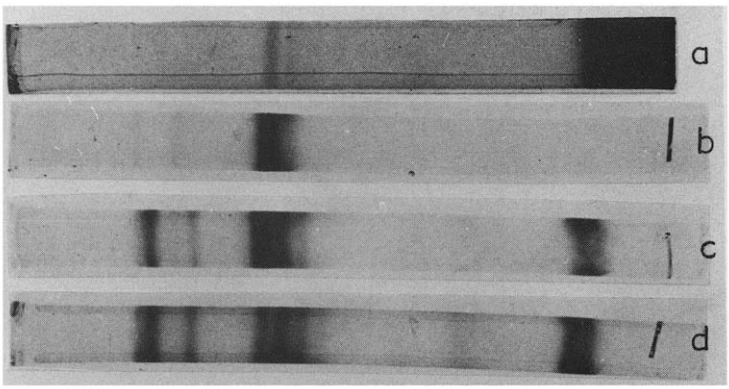

Fig. 3. Fraction 16 of peak D_1 stained for protein (a), and esterase (b). Besides a slight contamination by zone IV esterases (not to be seen in the photograph) fraction 16 (b) contains mainly band III-50 as revealed by mixing (c) fraction 16 with crude kidney supernatant (d). With Coomassie blue only band III-50 is stained, accompanied by weak steaning of III-40 (a).

LKB instrument Co, 110 ml capacity) as described above. The ampholine carrier was adjusted to 0.4% by addition of the required amount of 11% ampholyte (pH 5.9—6.3). 60 × 2 ml fractions were collected. Four peaks (B_1, C_1, D_1, and E_1) were again obtained, which exactly corresponded with the peaks B_0, C_0, and E_0 due to their pH (Fig. 1b). Analytical electrophoresis of some representative fractions of B_1, C_1, D_1 and E_1 revealed a total accordance of the bands of this series with the corresponding bands of the peaks B_0, C_0, D_0 and E_0. Peak D_1 (fractions 14—17, pH 6.1—6.2) with an overall volume of 10 ml contained mainly band III-50 (Fig. 3).

Step 4 — Gel filtration on Sephadex G-100. Four fractions of peak D_1 (fractions 14—17, pH 6.1—6.2) were combined and concentrated to a volume of 0.5 ml by ultrafiltration. The esterase activity of the concentrate was found to be 100 units and it was applied to a Sephadex G-100 column (90 × 1 cm) equilibrated with 20 mmol/l phosphate buffer (pH 6.1). At a flow rate of 0.03 ml/min, the eluate was collected in 2.5 ml portions. The main activity was found in fractions 15—18 with the highest specific activity (50 units/mg protein) in fraction 16. Fractions 15—18 contained two bands in zone III, while zone IV

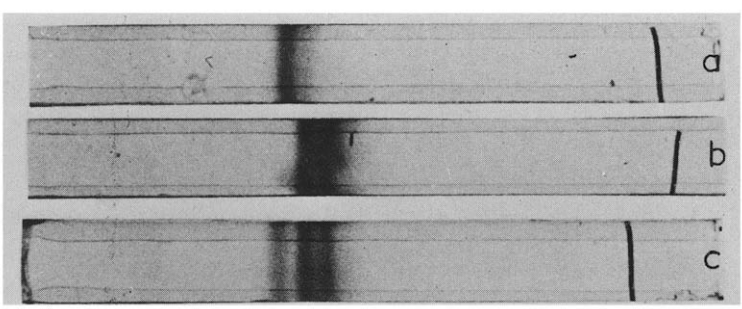

Fig. 4. Rearrangement of band III-50 to III-40 and III-30 during Sephadex G-100 chromatography. Column charge III-50 (a) was mixed with column eluant (b). The mixture (c) shows clearly three bands indicating for a nearly total rearrangement of III-50.

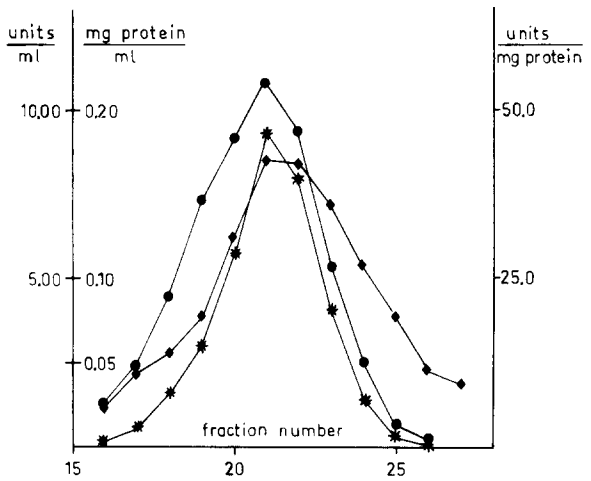

Fig. 5. Sephacryl gel filtration of the esterase-9 peak from the Sephadex column. A single protein peak ♦———♦ was found which corresponded to the esterase peak *———*. The specific activity ●———● of the purest fraction amounted to about 55 units/mg protein.

bands could no longer be detected. As could be established by analytical polyacrylamide gel electrophoresis, the two zone III bands turned out to be III-40 and III-30 (Fig. 4), whereas the column load contained only III-50. Therefore, it is assumed that band III-50 was converted to III-40 and III-30 during Sephadex chromatography.

Step 5 — Repeated gel filtration on Sephacryl gel AC A 44. Four fractions (15—18) of the Sephadex column (step 4) were combined, concentrated to 0.5 ml by ultrafiltration and applied to a second molecular sieve column (83 × 1 cm) filled with Sephacryl Ac A 44 in 20 mmol/l phosphate buffer (pH 6.1). At a flow rate of 0.03 ml/min, the eluate was collected in 2.4-ml fractions. A peak with esterase-9A activity was found in fraction 21 (Fig. 5). The specific activity was about 60 units/mg protein. The peak contained pure esterase-9A bands, mainly band III-40.

Immunoprecipitation tests for purity of esterase-9A

Immunoelectrophoresis of kidney supernatant followed by staining for esterase activity revealed that only the zone III bands reacted with 9A-antibody. No precipitate was found in the esterase-2 or in the cathodal regions.

Double immunodiffusion in Ouchterlony plates showed a single precipitin line which was confluent with purified esterase-9A and kidney supernatant (Fig. 6).

Properties of mouse esterase-9A

Rearrangement of 9A bands. Following ultrafiltration of an ampholine-containing fraction of III-50, this band diminished, whereas III-40 and III-30 increase (Fig. 7). This change does not take place suddenly, but takes several hours, the procedure being dependent on temperature (Fig. 7). Band III-30 is not the end product of this rearrangement for it is converted further to band III-22, hitherto not observed in kidney homogenates. The change of III-50 is

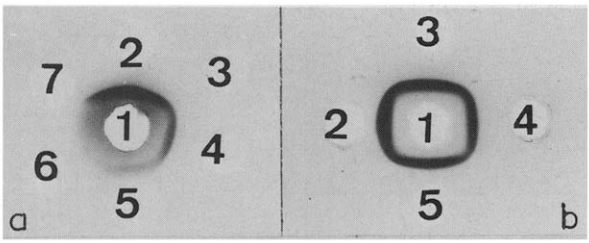

Fig. 6. (a) Immunodiffusion of esterase-9A. (1) 10 µl rabbit 9A-antiserum, (2—7) esterase-9A, 35, 17.5, 8.75, 4.325, 2.162, 1.08 mU, respectively. The precipitate was stained for esterase. (b) Immunodiffusion of esterase-9A (1) 10 µl rabbit 9A-antiserum, dilution 1 : 4. (2) 10 µl (male) kidney supernatant, dilution 1 : 4, (3) 10 µl purified esterase-9A, 23 mU. (4) 10 µl (male) kidney supernatant, dilution 1 : 8 (approx. 20 mU). (5) 10 µl purified esterase-9A, dilution 1 : 8, 11.5 mU. The precipitation line of esterase-9A is confluent with that of kidney supernatant.

also pH-dependent. The higher the pH, the faster III-40 and III-30 develop, while III-50 decreases. If ultrafiltration of a fraction rich in III-50 is accomplished at pH 4.0, no change can be observed after 24 h at 4°C while the same fraction at pH 6.1 is obviously changed. A reverse change of III-40 or III-30 into III-50 could not be observed under any conditions, thus indicating an irreversible process. Therefore, it is assumed that III-40 and III-30 are artifacts.

When a kidney was homogenized at pH 5.0 and the supernatant left standing overnight, the main component in zone III was band III-50. The kidney supernatant of the same animal homogenized at pH 6.0, showed III-50 as well as III-40 in zone III. Following homogenization at pH 7.3, all other conditions being the same, band III-40 is the most prominent and band III-50 stains only weakly.

Molecular weight of zone III esterases. Preliminary measurements in polyacrylamide gels had revealed a molecular weight of 45 000 for band III-50 (Trefzer, J., unpublished data). Using a preparation containing III-40 as well as III-30 and III-20, it was established that the bands had similar molecular

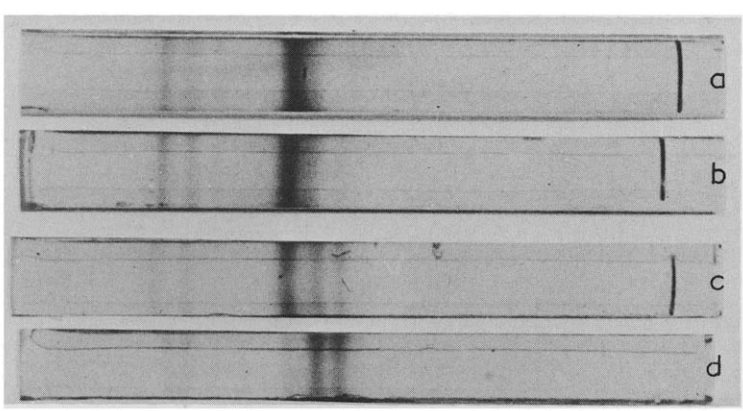

Fig. 7. Influence of storing, temperature, ampholine, and/or sucrose upon the rearrangement velocity of band III-50. Fraction 17 of peak D_1 containing band III-50 together with ampholine and sucrose after storing one day at 4°C (a) and at 25°C (b). The same fraction following ultrafiltration after storage for 1 day at 4°C (c) and at 25°C (d). It can be seen that ultrafiltration, as well as temperature, favour the rearrangement.

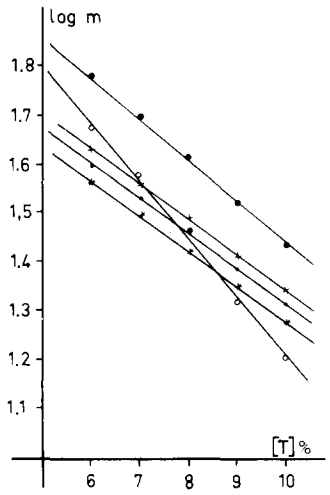

Fig. 8. Plot of the logarithm of the electrophoretic mobility m of three esterase bands (III-40 +———+, III-30 ○———○, III-20 ∗———∗ and three calibration proteins, (serum albumin ●———●, α-amylase .———., (the same as III-30), and lactate dehydrogenase ○———○) versus gel density T for estimation of the retardation constant K.

weights amounting to approx. 45 000 (Fig. 8). Thus, the conversion of the bands could not have been accomplished by the loss of relatively large molecular fragments. A similar molecular weight was obtained by thin layer gel chromatography [20] on Sephadex G-200 Superfine.

For esterase-9A, a molecular weight of 51 000 ± 3000 was calculated.

Inhibition by bis-p-nitrophenyl phosphate. Four kinetic measurements were done utilizing 440 µl buffered enzyme solution (66 mmol/l phosphate, pH 7.3) with 0.15—0.40 units of esterase activity (corresponding to $0.045—0.135 \cdot 10^{-9}$ mol esterase-9) which were mixed with 10 µl bisnitrophenyl phosphate to different final concentrations, b_0 from $1.2—2.4 \cdot 10^{-6}$ mol/l. Immediately thereafter, the enzyme activity was measured in a 10 µl aliquot. The measurement of further aliquots was continued during the next 20 min. Within 20 min, the activity drops down to 10—20% of the original value (Fig. 9). The velocity

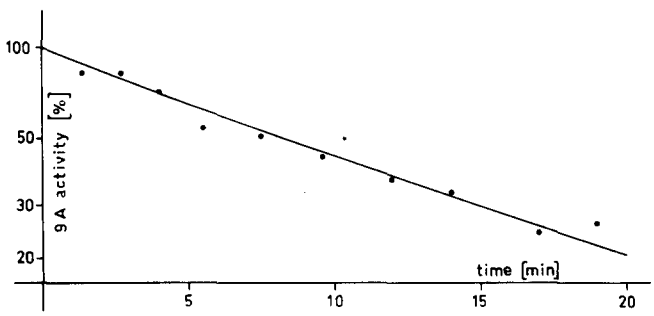

Fig. 9. Time slope of inhibition of esterase-9A by bisnitrophenyl phosphate. The solid line was computed and represents the theoretical course of a bimolecular process for $b_0 = 1.51 \cdot 10^{-6}$ mol/l, $a_0 = 3.0 \cdot 10^{-7}$ mol/l, $k = 58.2 \cdot \text{mmol}^{-1} \cdot \text{min}^{-1}$. The experimental data are in good agreement with the computed curve (compare Table II).

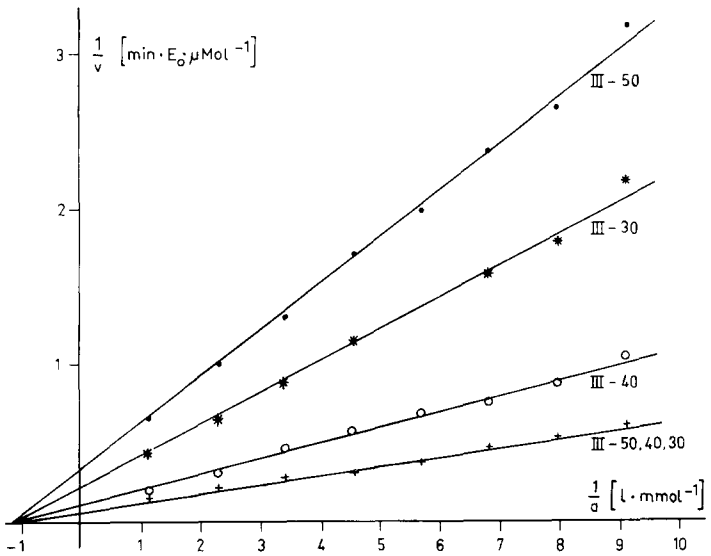

Fig. 10. Lineweaver-Burk plots of three different fractions of a refocusing column which contained band III-50, III-40, or band III-30 together with ampholine. A fourth fraction, after ultrafiltration, contained the three bands III-50, III-40 and III-30 together. The preparations show each the same K_m constant of $0.9 \cdot 10^{-3}$ mol/l. The different V values are due to different enzyme concentrations. Substrate was nitrophenyl propionate.

constant, k, for the bimolecular reaction was computed by the equation

$$b_0 - a_0 = a\left(\frac{b_0}{a_0} e^{kt(b_0 - a_0)} - 1\right)$$

a = concentration of active enzyme, a_0 = concentration of active enzyme at t = 0, t = time, b_0 = concentration of bisnitrophenyl phosphate. The mean of four experiments resulted in $k = 64 \pm 11.8$ l · mmol^{-1} · min^{-1} (Table II).

Isoelectric points. The results of different focusing and refocusing runs were all in good agreement as is shown by the small standard deviation. The isoelectric points (Table III) are valid for a concomitant ampholine concentration between 0.4 and 2%.

K_m constants with nitrophenyl propionate. The initial velocities, v, of nitrophenyl propionate hydrolysis at pH 7.3 were measured with different esterase-

TABLE III

ISOELECTRIC POINTS OF ZONE III ESTERASES IN DIFFERENT FOCUSING AND REFOCUSING EXPERIMENTS (AMPHOLINE CONCENTRATION APPROX. 0.4%)

For comparison the isoelectric points of some other bands. n = number of measurements, S.D.M. = standard deviation of the mean.

	III-50	III-40	III-30	I-30	I-20	I-10	II-10
n	14	11	3	1	1	1	1
pH	6.182	5.905	5.65	4.77	4.69	4.63	3.85
S.D.M.	0.006	0.009	0.028				

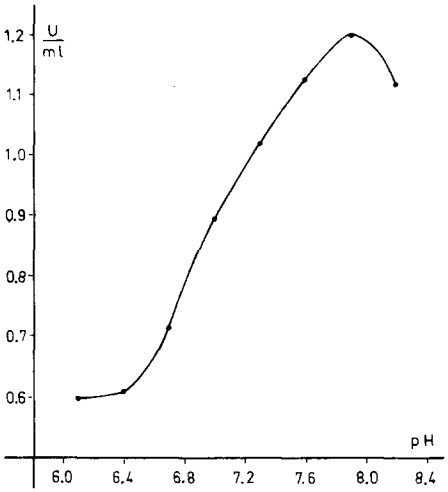

Fig. 11. Esterase activity for nitrophenyl propionate of band III-50 versus pH. A maximum reaction velocity was found at about pH 7.9.

9A fractions, each containing predominantly one band, namely III-30, III-40, or III-50. A fourth fraction was also tested, which originally contained only band III-50, but which had partially converted into III-40 and III-30, following ultrafiltration (Fig. 10). The substrate concentration, a, varied from 0.1—1 mmol/l. As shown by Lineweaver-Burk plots [21], straight lines were obtained with each fraction which had an identical intercept at the abscissa. This means that the K_m constant towards nitrophenyl propionate of each of the esterase-9A bands is the same, and amounts to $0.9 \cdot 10^{-3}$ mol/l. With respect to the specific activity of about 62 units/mg protein and a molecular weight of 45 000 the turnover number for nitrophenyl propionate of the purified esterase-9A amounts to at least 230 per s at 25°C at pH 7.3.

pH optimum. Employing nitrophenyl propionate as substrate, the reaction velocity was measured in 0.066 mol/l phosphate buffer between, pH 6.1—8.2. A single peak was observed with maximum activity at about pH 7.8 (Fig. 11). Esterase activity against other substrates: all other substrates applied were cleaved quite well by esterase-9A. Beside α-naphthyl acetate, the chromogenic substrates, α-napthyl butyrate, 5-bromoindoxyl acetate, 8-hydroxyquinoline acetate and butyrate, as well as the fluorogenic substrates methylumbelliferyl acetate and butyrate [22] and fluorescein diacetate [23] are useful for detection of 9A-esterase in electrophoretic gels.

Discussion

In this study, we have examined the isolation of the first genetically defined esterase, which is hormone dependent. This esterase occurs in many mouse organs (including liver, kidney, and skeletal muscle) and appears electrophoretically in the form of the three bands in zone III, namely III-50, III-40, and III-III-30. The esterase-9 seems to be identical with the testosterone dependent kidney esterase of Shaw and Koen [24], the bands III-30$^+$, III-20$^+$, and III-10$^+$

of Ruddle and Harrington [25] and the bands E-13 and E-14 of Komeda [26].

The interrelationship between the three bands III-50, III-40, and III-30 is now obvious. It was demonstrated that the more slowly migrating bands spontaneously change into the faster ones by a gradual, irreversible process: If we start with III-50, III-40 and III-30 arise at room temperature within a few hours, if kept at pH 6—7.

The latter changes further into a new band not observed before in homogenates and designated III-22. At pH 5 no change occurs. A reversal of these changes could not be observed between pH 4.8 and 7.5, although it was carefully investigated.

Considering the temperature dependence of this process, we conclude that it is not only a matter of conformational change, but an alteration of the primary structure of the esterase molecule itself perhaps caused enzymatically. The suggestion of an enzymic process is supported by the observation that the intensity of the esterase-9 bands in mouse duodenum strongly depends on contamination with pancreas juice, according to the varying functional state of the pancreas [25,27]. Differences of molecular weight of the three bands could not be detected. Hydrolysis of small fragments of the esterase molecule is probably the main cause of lowering of the isoelectric point. Using the periodate reaction there was no definite proof that esterase-9A contains carbohydrate groups which have been observed in other esterases or lipases [28—32]. Therefore, either a proteolytic cleavage of terminal amino acids or a gradual deamidation of glutamine or asparagine moieties of the protein molecule is assumed to occur. Since esterases are capable of hydrolyzing certain carbonic acid amides [33], a deamidation could be performed by the esterase-9 itself. Yet, the change of bands occurs also in the presence of 10^{-4} mol/l E 600, which is known to completely inhibit the esterase-9 (v. Deimling, O., unpublished data). We cannot rule out a small contamination of our esterase-9A either with an aminopeptidase (which is abundant in mouse kidney) or with an exopeptidase, without being directly demonstrable.

The molecular weight was estimated to be about 45 000 (electrophoresis) and 51 000 (gel chromatography). Liver also contains esterase-9. Tegelström [34] described, in liver, a band no. 6 which we think is identical with our esterase-9. The molecular weight of the earlier preparation was 44 000 when determined by gel chromatography and 20 000 by ultracentrifugation. However, we did not succeed in obtaining any evidence for the existance of subunits.

Esterase-9A is strongly inhibited by bisnitrophenyl phosphate, which is an anionic organophosphate and was introduced by Heymann and Krisch [35]. Since cholinesterases are resistant to bisnitrophenyl phosphate, it is thereby confirmed that the esterase-9 is a carboxylesterase (EC 3.1.1.1). The velocity constant, k, for the bimolecular inhibition reaction was estimated to 64 $l \cdot mmol^{-1} \cdot min^{-1}$. This constant seems to be a suitable criterion for further characterization of carboxylesterase isozymes since the corresponding constants for other mouse isozymes of the chromosome-8 cluster, e.g. esterase-2, esterase-1 differ by several times (unpublished data).

In the house mouse, at least seven isozymes of the carboxylesterase are known to exist, as revealed by genetic analysis [1,23]. The corresponding loci, Es-1, Es-2, Es-5, Es-6, Es-9, Es-11 (and Es-7; Chapman, V., unpublished data),

which are clustered within a narrow distance on chromosome 8, may have developed by repeated gene duplication followed by divergent evolution. The investigation of esterase-9A is the first step in characterising these isozymes. Thus, the comparative investigation of mouse esterases which arise from the same gene cluster is a unique opportunity to study the divergent shifts of perhaps topological, catalytic or regulatory properties within a system of tightly related proteins.

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