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IDENTIFICATION OF THE CHLORAMPHENICOL-HYDROLYZING ENZYME OF GUINEA PIG LIVER AS ONE OF THE NONSPECIFIC CARBOXYLESTERASES

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Abstract—Guinea pig liver has the highest chloramphenicol-hydrolyzing capacity among the livers of various mammals. The enzyme responsible for the hydrolysis of the amide-bond in chloramphenicol is one of the isoenzymes of the microsomal nonspecific carboxylesterases. This isoenzyme is related to the well-known acetanilide-hydrolyzing carboxylesterases/amidases of pig and rat liver. The guinea pig liver enzyme is purified 24-fold starting with microsomes. The purified enzyme is essentially free from other proteins except other carboxylesterase isoenzymes with similar properties. The chloramphenicol-hydrolyzing esterase has an apparent molecular weight of about 180,000, a subunit weight of 60,000 and a pH optimum at 8.5. It also hydrolyzes methyl butyrate and acetanilide and it is completely inhibited by diethyl-4-nitrophenyl phosphate.

Two assay procedures for the enzymatic chloramphenicol hydrolysis are described: a thin-layer chromatographic assay using radioactive chloramphenicol and a colorimetric assay utilizing the reaction of the liberated amine with trinitrobenzenesulfonic acid.

Because of its toxic side effects the antibiotic chloramphenicol is seldom used in human medicine. However, there are many veterinary applications for this low-priced and effective drug. More or less legally, it is even used as an animal food-additive, e.g. in hog-raising. Therefore, it is very likely that low amounts of chloramphenicol and its metabolites are being consumed by men, and all aspects of the metabolism of this drug should be known.

Although the hydrolysis of chloramphenicol is only a minor metabolic pathway [1] it deserves investigation, because the product of hydrolysis, 1-(4-nitrophenyl)-2-aminopropane-1,3-diol, has specific physiological effects on smooth muscles [2]. It has been reported that mouse liver microsomes catalyze the hydrolysis of chloramphenicol [3], but nothing is known about the enzymes involved in this hydrolysis in mammals. An isolation of the chloramphenicol-hydrolyzing enzyme should reveal its nature and should answer the question whether this enzyme is related to one of the isoenzymes of liver carboxylesterases.

We choose the guinea pig for this study on the identification of the hydrolytic enzyme, because cavian liver turned out to have relatively high activity. In contrast to rat or the large domestic animals, very little was known about hydrolytic enzymes, especially the carboxylesterases/amidases, from guinea pig liver. Chow and Ecobichon [4] proved the existence of a number of carboxylesterase isoenzymes in this organ and estimated their M_r by gel filtration. Only in two cases a purification of cavian liver hydrolases has been reported: Järvinen *et al.* [5] isolated two enzymes with M_r of 200,000 and 41,000 that hydrolyzed carcinogenic acid amides. A microsomal isocarboxazid-hydrolase with M_r of 210,000 has been isolated by Satoh and Moroi [6]. It could be possible that the chloramphenicol-hydrolyzing enzyme was identical with one of these isolated hydrolases which probably belonged to the group of nonspecific carboxylesterases/amidases.

MATERIALS AND METHODS

Liver cell fractions

Adult guinea pigs (no inbred strain) of both sexes were used for the enzyme isolation. The animals were killed by decapitation after a 24 hr-period of starving, and the fresh livers were homogenized in ice-cold 0.25 M sucrose with a glass homogenizer and Teflon pestle so that 1 g of fresh liver corresponded to 4 ml of homogenate. Microsomes and other cell fractions were obtained by differential centrifugation at pH 7.4 [7]. All particulate fractions were resuspended in a buffer pH 7.4 containing 0.25 M sucrose, 1 mM EDTA and 20 mM Tris–HCl so that 1 ml corresponded to 1 g of fresh liver. The liver microsomes of other animals were obtained in a similar manner (see Table 1 for the strains used).

Reagents

Radioactive chloramphenicol (dichloroactyl-1,2-[^{14}C]) and Omnifluor were purchased from N.E.N. (Dreieich, West Germany). Unlabeled chloramphenicol and its hydrolysis product D-threo-1-(4-nitrophenyl)-2-aminopropane-1,3-diol were obtained from Boehringer (Mannheim, West Germany). Sephadex gels were from Pharmacia (Freiburg, West Germany), Ampholine from L.K.B. (Gräfelfing, West Germany), and silica gel G from Merck (Darmstadt, West Germany).

Enzymatic hydrolysis of chloramphenicol

Procedure A. Enzyme in 0.1 M phosphate buffer

pH 8.5 was incubated for 30 min at 30° with labeled chloramphenicol (final concentration 0.5 mM, 2.4 Ci/mole). Samples of 20 µl of the incubation mixture were applied to thin-layer plates with silica gel G and chromatographed with toluene/2-chloroethanol/25% ammonia in water/pyridine (50:30:30:15, v/v). The radioactivity on the plates was localized with a TLC-scanner of Berthold (Wildbad, West Germany). In this solvent chloramphenicol had an R_f value of 0.93 and dichloroacetic acid of 0.13. The radioactive spots were scraped into counting vials and extracted with 15 ml scintillation cocktail (100 g naphthalene and 4 g Omnifluor per litre of dioxane). The measured radioactivity (liquid scintillation counter Packard 3380) of the dichloroacetic acid spots was linear with the amount of acid in the range of 0.3–3 nmoles per spot. A blank without enzyme was run with each series, the radioactivity of its dichloroacetic acid-containing spot was counted even if nothing could be detected with the TLC-scanner. See [8] for a detailed description of this assay procedure.

Procedure B. An assay procedure without radioactivity could be used for the partially purified enzyme solutions. Enzyme in 1 ml 0.1 M phosphate buffer pH 8.5 was incubated for 30 min at 37° (30°) with unlabeled chloramphenicol (final concentration 2 mM). The reaction was stopped by addition of 100 µl 3M trichloroacetic acid. After 10 min of vigorous shaking the proteins were removed by centrifugation (1 min at 12,000 rpm). Of the clear supernatant, 900 µl was mixed with 900 µl of 0.5 M borate buffer pH 10.5 and 200 µl of 10 mM trinitrobenzene sulfonic acid in ethanol and incubated for 40 min at 40°. The final pH of the mixture was 9.0–9.3. The absorbance of this solution at 405 nm was compared to that of standards with 10 or 20 µM nitrophenyl-aminopropanediol that underwent the same procedure. A blank without substrate, another one without enzyme, and a reagent blank were run with each series of assays. The absorbance at 405 nm was linear with the nitrophenyl-aminopropanediol concentration up to 0.15 mM (absorbance of about 1 at 1 cm optical path length).

Other enzyme assays and protein determination

The hydrolysis of methyl butyrate (20 mM) was monitored by the pH-stat method using the autotitrator TTT 11 (Radiometer, Copenhagen, Denmark) with automatic burette filled with 40 mM NaOH. The liberation of nitrophenol from 4-nitrophenyl acetate [9] and of aniline from acetanilide [10] was determined spectrophotometrically. The protein concentration was determined with a modified biuret procedure [11].

Enzyme purification

Unless stated otherwise, all purification steps were performed at 0–4°. The carboxylesterases from guinea pig liver microsomes were solubilized by mild autolysis (1 hr at 37°) using the procedure described for the solubilization of pig and ox liver esterases [7]. However, only the second procedure described in [7], starting with microsomes obtained by ultracentrifugation could successfully be applied with guinea pig liver. After fractionation with ammonium sulfate [7] the bulk of chloramphenicol-, acetanilide- and methyl butyrate-hydrolyzing activities was found in the fraction of 50–70% saturation. The sediment after 70% ammonium sulfate saturation was redissolved in 5 mM phosphate buffer pH 8.0 and applied to a gel chromatography column (Sephadex G200, 2.6 × 90 cm), equilibrated and eluted with the same buffer. The three hydrolase activities were found in a single peak around an elution volume of 280 ml (fraction I). A small second range of acetanilide-hydrolyzing activity, peaking at 380 ml was discarded (fraction II). The main hydrolase-containing eluate of this gel column was directly applied to an anion exchange column (DEAE-Sephadex A50, 2.6 × 30 cm, equilibrated with 60 mM NaCl in 5 mM phosphate, pH 8.0). This column was eluted with a concave NaCl-gradient formed in a three-chamber gradient mixer filled with 2 × 300 ml of 60 mM NaCl and 1 × 300 ml of 460 mM NaCl in the same buffer. The three hydrolase peaks (fractions A, B and C; Fig. 3) of the eluate were separately isolated and rechromatographed on Sephadex G200 (same conditions as above).

Dodecyl sulfate electrophoresis

The M_r of the enzyme subunits was determined by dodecylsulfate electrophoresis in cylindrical acrylamide gels (6%) according to Weber and Osborn [12]. Ovalbumin and bovine serum albumin served as marker proteins.

Isoelectric focusing

Preparative isolectric focusing of the purified esterase fractions after rechromatography on Sephadex G200 (see above) was performed using the apparatus type 8100 (440 ml) of LKB (Gräfelfing, West Germany) and 1% Ampholine (LKB, pH 4–6) according to the procedure described by the manufacturer.

RESULTS AND DISCUSSION

Chloramphenicol-hydrolyzing activity in livers of various vertebrates

We assayed the capacity of various vertebrate liver homogenates to hydrolyze chloramphenicol. Except

Table 1. The hydrolysis of chloramphenicol by liver homogenates and microsomes of various species

Species	Strain	No. of animals	Specific activity (**) pmol/(min mg) Homogenate	Microsomes
Guinea pig	Mixed	6	72 (26)	288 (63)
Rabbit	White New Zealander	5	48 (18)	206 (67)
Mouse (*)	Black C57	6	43 (20)	141 (38)
Rat (*)	Wistar	9	<10	33 (6)
Pig	Mixed	5	12	31 (11)
Ox	Mixed	5	<10	20 (3)
Cat	Mixed	4	<10	12 (2)
Frog	Rana esculenta	4	<10	<10

* Inbred strain from Zentralinstitut für Versuchstierzucht, Hannover, West Germany.

** Mean values and standard deviation (in brackets).

in guinea pig, rabbit and mouse, the homogenate activities were so low, that an exact quantitation would have been too costly, even with the assay procedure using radioactive substrate (Table 1). However, in all species the specific chloramphenicol-hydrolyzing activity of the microsomes was higher than that of the liver homogenate. Therefore, we used this cell fraction for a comparative study. Both the homogenate and the microsomal fraction of guinea pig liver had the highest activities among the species investigated (Table 1). It has been reported that the esterolytic activities of guinea pig liver microsomes with tributyryl-glycerol or procaine as substrates were also higher than those of other mammals [13]. This might indicate that guinea pig liver is especially rich in carboxylesterases (EC 3.1.1.1) and that one of the esterase isoenzymes might be responsible for the hydrolysis of chloramphenicol. Our investigations described below confirmed both assumptions.

Subcellular distribution of esterolytic and amide-hydrolyzing activities in guinea pig liver

The distribution of the chloramphenicol-hydrolyzing enzyme in cell fractions obtained from guinea pig liver by differential centrifugation is shown in Fig. 1A (solid lines). This distribution pattern is typical for enzymes of the endoplasmic reticulum. A parallel investigation of the cell fractions with three substrates of carboxylesterase (EC 3.1.1.1), namely methyl butyrate, 4-nitrophenyl acetate (Fig. 1B), and acetanilide (Fig. 1A) [13] revealed a close similarity in the subcellular distribution of all activities. (Note that the scales of parts A and B of Fig. 1 differ by 4 orders of magnitude).

The similar distribution of the activities suggested again that the chloramphenicol-hydrolyzing enzyme might be identical to one of the carboxylesterase isoenzymes. The solubilization of both membrane-bound activities by mild autolysis also paralleled

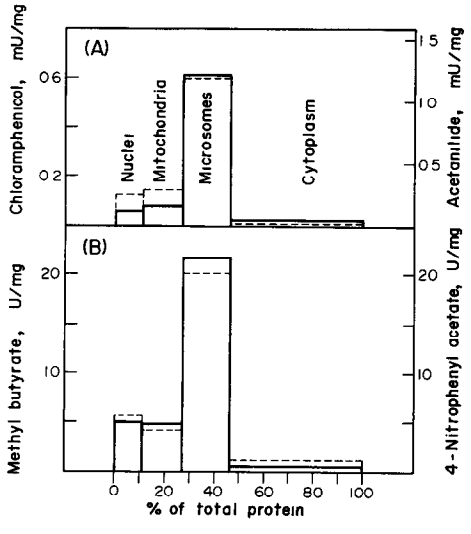

Fig. 1. Distribution of chloramphenicol-hydrolyzing activity and carboxylesterase in cell fractions of guinea pig liver. (A) Solid lines: chloramphenicol hydrolysis; broken lines: acetanilide hydrolysis. (B) Hydrolysis of methyl butyrate (solid) and 4-nitrophenyl acetate (broken lines).

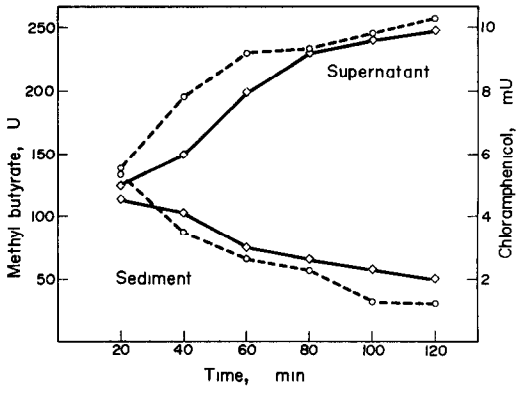

Fig. 2. Solubilization of methyl butyrate- and chloramphenicol-hydrolyzing activity from liver microsomes by autolysis. The microsomal suspension was incubated at 37° (pH 8.5). Samples were taken every 20 min, centrifuged at 105,000 g_{max}, and the hydrolytic activity was assayed in the supernatant and the resuspended sediment. O---O, methyl butyrate; ◇——◇, chloramphenicol.

each other, (Fig. 2). Therefore, we decided to assay both the hydrolysis of chloramphenicol and the carboxylesterase activities during the enzyme purification procedure.

Purification of the cavian liver carboxylesterases and identification of the chloramphenicol-hydrolyzing enzyme

We tried several procedures for the solubilization of the membrane-bound hydrolase activities from the microsomes. It was not possible to apply the combined heat treatment and acid precipitation that has been used for the solubilization of pig liver carboxylesterases [7]. However, under mild autolysis conditions (Fig. 2) the bulk of esterase and chloramphenicol-hydrolase passed into the soluble fraction. Ultrasonication as proposed by Järvinen *et al.* [5] for the solubilization of an acetylaminofluorene-hydrolase from guinea pig liver was also effective in this case, however, the specific activities of the solubilisates were slightly lower.

The further purification of the cavian liver hydrolases was performed by conventional procedures that are summarized in Table 2. During the enzyme isolation experiments it became evident that the chloramphenicol-hydrolyzing activity paralleled that for acetanilide, but not that for methyl butyrate (Table 2, Fig. 3). However, this was not true for a second acetanilide-hydrolyzing peak obtained in the gel filtration step, (fraction II in Table 2). This peak in the lower M_r range showed a relatively low chloramphenicol-hydrolyzing activity and was not further characterized. The parallelism of acetanilide- and chloramphenicol-hydrolysis became especially evident in the elution profile of the chromatography on DEAE-Sephadex (Fig. 3). The three enzymatically active fractions (A, B and C) of this chromatography were treated separately in our further investigations. They were eluted as single sharp peaks upon rechromatography on Sephadex G200.

The three purified enzyme fractions (A, B and C;

Table 2. Purification of the hydrolase activities from cavian liver

Fraction	Protein (mg)	Total activity			Specific activity		
		Chloramphenicol (mU)	Acetanilide (mU)	Methyl butyrate (U)	Chloramphenicol (mU/mg)	Acetanilide (mU/mg)	Methyl butyrate (U/mg)
Microsomes	9040	3530	6150	75900	0.39	0.68	8.4
Solubilisate	2550	3050	5110	66100	1.20	2.00	25.9
Salt precipitate	1250	2860	4810	63900	2.29	3.85	51.2
First gel filtration							
Fraction I	602	2468	3640	52400	4.1	6.05	87.0
Fraction II	259	202	1020	1600	0.78	3.95	6.1
DEAE-Sephadex							
Fraction A	135	460	650	25300	3.4	4.8	187
Fraction B	84	510	730	6700	6.1	8.7	80
Fraction C	123	1120	1650	5550	9.1	13.4	45
Second gel filtration							
Fraction A	76	270	430	18700	3.6	5.7	246
Fraction B	36	280	440	5290	7.9	12.3	147
Fraction C	77	710	1190	3870	9.2	15.5	50

Values from a representative single experiment.

Table 2) were completely inhibited by 10^{-4} M paraoxon (15 min at 30°, pH 8.0). The same applied to the chloramphenicol-hydrolyzing activity of liver microsomes from all species described in Table 1. Therefore, all enzyme activities described here were of the serine-hydrolase type and could be classified as B-esterases [13].

Dodecylsulfate electrophoresis of the three purified fractions in the presence of mercaptoethanol (Fig. 4) revealed the presence of two types of subunits that occurred in varying amounts in all of the three fractions: fraction A mainly contained the faster migrating subunit, whereas fraction C consisted mainly of the slower moving unit. The intermediate fraction B contained similar amounts of both, plus some faster moving impurities. We assumed that the two subunit-types in the M_r range of 60,000 represented two major types of carboxylesterase isoenzymes (compare next paragraph). Although these isoenzymes could not completely be separated from each other (Fig. 4) only traces of

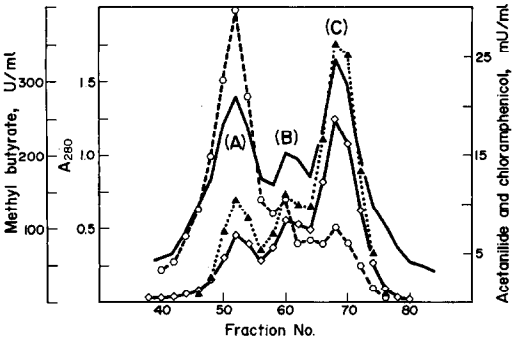

Fig. 3. Separation of the liver amidases and esterases on DEAE-Sephadex A50. ——A_{280}. Hydrolysis of ▷---◁, methyl butyrate; ▲····▲, acetanilide; and ◇——◇, chloramphenicol.

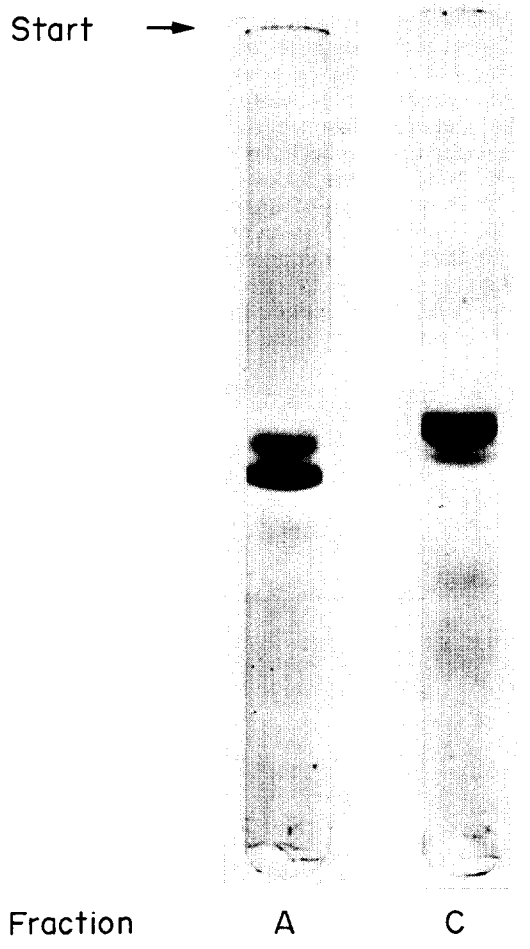

Fig. 4. Dodecyl sulfate electrophoresis of the purified enzyme fractions A and C. Gel stained with Coomassie brilliant blue.

other proteins were present in the purified enzyme fractions A and C.

This was confirmed by an active site titration [9, 13] of the highly purified fractions A and C with paraoxon: both fractions have a purity [13] of >90%, i.e. the fractions contained less than 10% of proteins other than serine-type carboxylesterases. Fraction B had a purity of about 75%. These titrations were based on the assumptions that each subunit of M_r 60,000 bore one active site and that the biuret protein estimation with a factor of 1.91 [14] gave correct values. Both assumptions have been found to be true for pig liver carboxylesterases [14]. We concluded that the main component of fraction C was the chloramphenicol-hydrolyzing carboxylesterase/amidase.

Chloramphenicol was an effective competitive inhibitor both for the acetanilide-hydrolyzing (Fig. 5B, K_i = 0.18 mM) and the methyl butyrate-hydrolyzing activity of fraction C. The apparent K_m values of fraction C were 0.2 mM with chloramphenicol (Fig. 5A), 2.2 mM with acetanilide, and 2.4 mM with methyl butyrate as substrates.

Two other groups isolated guinea pig liver hydrolases that might be identical with those described here. An 'isocarboxacid-hydrolase' [6] with M_r of about 200,000 hydrolyzed various acid-amides including penicilline-derivatives, but not acetanilide.

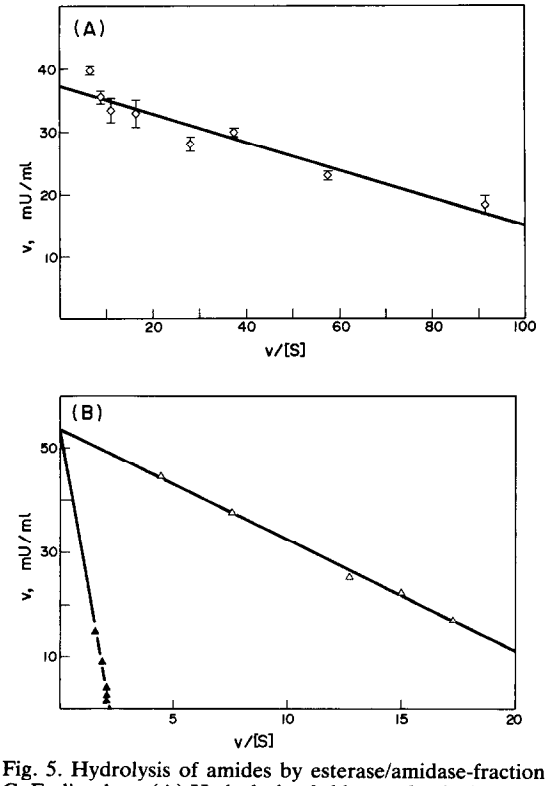

Fig. 5. Hydrolysis of amides by esterase/amidase-fraction C, Eadie-plots. (A) Hydrolysis of chloramphenicol at concentrations between 0.2 and 6 mM, mean values of four series. The marks above and below the symbols indicate the standard deviation of v. (B) Hydrolysis of acetanilide at concentrations between 1 and 10 mM. △, without chloramphenicol; ▲, in the presence of 1.6 mM chloramphenicol.

Therefore, this enzyme might be identical with the main component of our fraction A, the 'aliesterase'-type isoenzyme (see below). It is known that this type of liver carboxylesterase hydrolyzed various aromatic [13] or aliphatic [15] amides, but had very little or no activity towards acetanilide [13, 16–18]. In contrast, the acetylaminofluorene-hydrolyzing enzyme of M_r 200,000 that was described by Järvinen *et al.* [5] might be identical to the chloramphenicol hydrolase described here, because both preparations cleaved acetanilide and were sensitive to inhibition by organophosphates.

Comparison of the cavian liver carboxylesterases/amidases with carboxylesterases from rat and pig liver

Guinea pig liver, as compared to the livers of other species [13], was especially rich in microsomal carboxylesterases. This could be concluded from the high specific activities of the microsomes, and from the only 23–30 fold purification necessary for a separation of the non-esterase proteins (Table 2).

A complete characterization of the guinea pig liver carboxylesterases was not the aim of this investigation. However, some important general features of these enzymes should be compared to those of liver esterases of other species.

The substrates methyl butyrate and acetanilide have been used for the discrimination of the main carboxylesterase isoenzymes of rat [16, 17] and pig [19] liver. While methyl butyrate was a very good substrate for the 'aliesterase'-type isoenzyme, i.e. esterase pI 6.0 of rat [17] or isoenzyme V of pig liver [19], acetanilide was specific for another type. The main component of the purified fraction A from cavian liver seemed to be homologous to the 'aliesterase'-type enzyme, whereas the chloramphenicol-hydrolyzing enzyme, fraction C, corresponded to isoenzyme I of pig [19] or esterase pI 5.6 [17] of rat liver. Both types of carboxylesterase

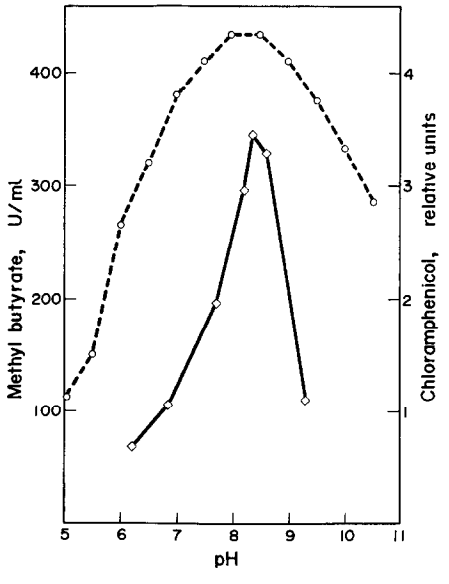

Fig. 6. pH Dependency of the hydrolysis of chloramphenicol, ◇——◇; and methyl butyrate, O---O, by guinea pig liver microsomes.

split a wide variety of carboxylic esters and amides, including monoglycerides of long chain fatty acids [17].

Both in rat and in pig liver the acetanilide-hydrolyzing carboxylesterase, i.e. isoenzyme I of pig liver or esterase pI 5.6 of rat liver, had a slightly lower mobility in dodecyl sulfate electrophoresis as compared to the 'aliesterase'-type [17, 19]. This rule held also for the main carboxylesterase isoenzymes of guinea pig liver (Fig. 4).

The molecular size of the guinea pig carboxylesterases was very similar to that of pig liver esterase, which had been described as a trimeric molecule of M_r 180,000 [13]. This could be concluded from the elution profiles of the Sephadex-G200 column used for the rechromatography of the enzyme fractions A, B and C (last step of the purification procedure): after calibration of the column with highly purified pig liver carboxylesterase [7] and bovine serum albumin the guinea pig esterases exhibited the same elution volume as the pig esterase. After dissociation in dodecyl sulfate the cavian carboxylesterases behaved as proteins of M_r 60,000–65,000 in the polyacrylamide electrophoresis as had previously been shown for liver carboxylesterases from many other species [13]. Thus, the cavian esterases probably were also trimeric molecules.

The pH optima of the purified esterases were in the range of pH 8–9. It is remarkable that the pH profile for the hydrolysis of chloramphenicol by microsomes was much steeper than that obtained with methyl butyrate (Fig. 6). This could be explained by the assumption that several enzymes with slightly differing pH optima were responsible for the cleavage of methyl butyrate, whereas only a single enzyme split chloramphenicol. A similar effect observed in pig liver microsomes [18] could be proven to be caused by such a heterogeneity.

In general the heterogeneity of the guinea pig liver carboxylesterases appeared to be very similar to that of pig liver [18, 19]. Preparative isoelectric focussing of the purified fractions A, B or C indicated the existence of at least four different esterase forms with isoelectric points in the range of pH 4.6–5.0. Figure 7 depicts an elution profile of a focussing column of fraction B as an example. All of these forms consisted of at least one of the subunit types shown in Fig. 4. A further separation and characterization of the individual forms was not attempted. It is possible that these forms were oligomers, (e.g. AAA, CCC) and oligomeric hybrids (AAC, ACC) of the two subunit types, as has been proposed for pig liver carboxylesterase [18, 19].

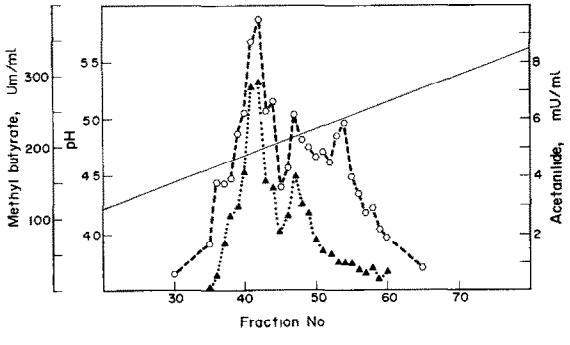

Fig. 7. Preparative isoelectric focussing of esterase fraction B with ampholyte pH 4–6. O---O, hydrolysis of methyl butyrate; ▲····▲ chloramphenicol; ———, pH.

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REFERENCES

1. A. J. Glazko, *Antimicrob. Agents Chemother.* 655 (1966).
2. S. Banerjee, C. Mitra and A. K. Mukherjee, *J. Pharm. Sci.* **66**, 1239 (1977).
3. D. L. Hill and R. F. Struck, *Biochem. Pharmac.* **22**, 127 (1973).
4. A. Y. K. Chow and D. J. Ecobichon, *Biochem. Pharmac.* **22**, 689 (1973).
5. M. Järvinen, R. S. S. Santti and V. K. Hopsu-Havu, *Biochem. Pharmac.* **20**, 2971 (1971).
6. T. Satoh and K. Moroi, *Biochem. Pharmac.* **21**, 3111 (1972).
7. E. Heymann, W. Junge, K. Krisch and G. Marcussen-Wulff, *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 155 (1974).
8. D. Kuhn, Dissertation, University of Kiel (1980).
9. K. Krisch, *Biochim. biophys. Acta* **122**, 265 (1966).
10. K. Krisch, *Biochem. Z.* **337**, 531 (1963).
11. J. Alt, K. Krisch and P. Hirsch, *J. gen. Microbiol.* **87**, 260 (1975).
12. K. Weber and M. Osborn, *J. biol. Chem.* **244**, 4406 (1969).
13. E. Heymann, in *Enzymatic Basis of Detoxication* (Ed. W. B. Jacoby), Vol. 2, pp. 291–323. Academic Press, New York (1980).
14. M. Kunert and E. Heymann, *FEBS Lett.* **49**, 292 (1975).
15. E. Heymann, unpublished.
16. R. Arndt, E. Heymann, W. Junge and K. Krisch, *Eur. J. Biochem.* **36**, 120 (1973).
17. R. Mentlein, S. Heiland and E. Heymann, *Archs Biochem. Biophys.* **200**, 547 (1980).
18. W. Junge and E. Heymann, *Eur. J. Biochem.* **95**, 519 (1979).
19. E. Heymann and W. Junge, *Eur. J. Biochem.* **95**, 509 (1979).