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PARTIAL PURIFICATION AND CHARACTERIZATION OF TWO ENZYMES FROM GUINEA-PIG LIVER MICROSOMES THAT HYDROLYZE CARCINOGENIC AMIDES 2-ACETYLAMINOFLUORENE AND *N*-HYDROXY-2-ACETYLAMINOFLUORENE

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Abstract—Two enzymes capable of deacylating carcinogenic compounds *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF) and 2-acetylaminofluorene (AAF) were liberated with sonication from guinea pig liver microsomes. The enzymes were separated by using fractionation with ammonium sulfate, chromatography on Sephadex and hydroxylapatite or DEAE-cellulose. The Enzyme I (mol. wt. 200,000) hydrolyzed *N*-hydroxy-AAF 265 times faster than AAF, while Enzyme II (mol. wt. 41,000) hydrolyzed AAF about 1·4 times faster than *N*-hydroxy-AAF. Both enzymes hydrolyzed the acylamido derivatives of fluorene and naphthalene faster than those of benzene. Substitution of the acyl hydrogen atoms enhanced markedly the hydrolysis rates. Both enzymes hydrolyzed readily ester substrates, e.g. tyrosine ethyl ester and 1-naphthyl acetate, and were inhibited by the organic phosphorus compounds.

THE CARCINOGENICITY of 2-acetylaminofluorene (AAF) is dependent on the maintenance of adequate levels of *N*-hydroxy-AAF or of further carcinogenic derivates in the tissues. Guinea-pigs are found resistant to the carcinogenic action of AAF[1,2] and Irving[3] did not find any evidence of *N*-hydroxylation of AAF by guinea-pig liver microsomes, whereas he had previously found hydroxylation by rabbit microsomes.[4] The hypothesis that the failure of the guinea-pig to *N*-hydroxylate aromatic amines was the cause of the resistance to the carcinogenic action of AAF was supported by the finding of Miller *et al.*[2] that *N*-hydroxy-AF and *N*-hydroxy-AAF produce sarcomas at the site of injection in guinea-pigs as well as in other animals. Kiese *et al.*[5] observed, however, that guinea-pig liver microsomes *N*-hydroxylate 2-aminofluorene rather rapidly; they also found *N*-hydroxy-AF in the urine of guinea pigs injected intraperitoneally with AAF. The rapid deacylation of *N*-hydroxy-AAF to *N*-hydroxy-AF by guinea-pig liver may explain the rapid elimination of *N*-hydroxy-AAF; thus adequate levels of the active carcinogen cannot be maintained in guinea-pig tissues.[6,7]

Irving[7] found that the deacylation rate of *N*-hydroxy-AAF by guinea-pig liver microsomes was about 20-fold the deacylation rate of the same substrate by rabbit liver microsomes and 75-fold that by rat liver microsomes. The rate of deacylation of *N*-hydroxy-AAF was 17-times the deacylation rate of either AAF or acetanilide by guinea-pig liver microsomes. This suggested that *N*-hydroxylation enhanced the deacylation of the substrate by the enzymes of this species. These findings initiated our effort to analyse the pattern of enzymes acting in guinea-pig liver microsomes. This report deals with a partial purification of the enzymes and their characterization with

respect to the effect of aromatic ring, acyl moiety and *N*-hydroxylation on the deacylation activity of the microsomal enzymes.

MATERIALS AND METHODS

Substrates and chemicals

The substrates used and their sources were as given earlier.[8] *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF) was synthetized as described by Miller *et al.*[9] by using 2-nitrofluorene (Fluka AG) as the starting compound. The slightly yellowish product was crystallized from benzene and gave a melting point of 145–147° (values given earlier: 144–146°). 2-Acetylaminofluorene (AAF) was from Nutr. Biochem. Corp. The other chemicals used were *pro analysi* grade and obtained from E. Merck AG, unless otherwise stated.

Assay methods

Most substrates were first dissolved in methanol and then diluted with distilled water (or diluted NaOH) to obtain a neutral stock solution of suitable concentration. The substrates were incubated in the presence of enzyme and buffer in a water bath at 37° for a pre-determined time (from 5 min to several hours). The details of the incubation conditions for each substrate and the methods used for estimation of the enzymatic hydrolysis of the substrates have been published earlier.[8] All assays were carried out in duplicate. In control experiments either enzyme or substrate was omitted from the incubation medium. For standards, free reaction products were used under identical conditions.

The hydrolysis of *N*-hydroxy-AAF was measured colorimetrically according to the method of Irving[7] based on the observation of Boyland and Nery[10] that trisodium pentacyanoamine ferrate forms with arylhydroxylamine a stable and coloured complex. The tubes containing 1 ml of 0·05 M Na-phosphate buffer, pH 6·5, 0·5 ml of sodium pentacyanoamine ferrate solution (4 mg/ml) and 0·5 ml of enzyme solution were preincubated at 37° for 5 min, after which the reaction was started by adding 2 μmoles of *N*-hydroxy-AAF in 0·1 ml of ethanol. Incubation time was 15–30 min and the enzymatic reaction was stopped by adding 2 ml of ethanol. Absorbancies were measured after 30 min at 575 nm (tubes were centrifuged at 3000 *g* for 10 min if necessary) and the amount of the liberated *N*-hydroxy-2-aminofluorene (*N*-hydroxy-AF) was calculated. A 10 μg/ml solution of *N*-hydroxy-AF had an absorbance of 0·265 at 575 nm for a 1-cm optical path. The reaction of *N*-hydroxy-AF with trisodium pentacyanoamine ferrate was independent of pH in the range of pH 4–9.[7]

The hydrolysis rate of AAF was measured as presented earlier.[8] One ml of M tris–HCl buffer, pH 7·0, 0·5 ml of enzyme solution and 0·5 ml of 1 mM AAF-solution (22·3 mg AAF in 50 ml of methanol diluted with 50 ml of water) were pipetted into tubes which were transferred into a 37° water bath for 15–60 min. After incubation, the reaction was terminated by 2 ml of *p*-dimethylaminobenzaldehyde solution (50 mg *p*-dimethylaminobenzaldehyde/5 ml ethanol diluted with 5 ml of 1 M acetate–HCl buffer, pH 1·4). The tubes were allowed to stand for 30 min at room temperature and the absorbances were measured at 460 nm. The unit of enzyme activity was defined as the amount of enzyme which hydrolyzed 1 nmole AAF or *N*-hydroxy-AAF in 1 min under the conditions mentioned above.

Formation of peptides was studied according to Benöhr and Krisch.[11] For incubation 3 ml of 0·05 M ammonium acetate buffer, pH 7·0, 0·5 ml of 10 mM tyrosine ethyl ester in 0·05 M ammonium acetate, pH 7·0, and 1·5 ml of enzyme solution were pipetted into tubes. Incubation was carried out at 37° for 30–60 min after which the samples were rapidly frozen and lyophilized. The residue was dissolved in 2 ml of 80% ethanol. The precipitate was removed by centrifugation at 3000 *g* for 10 min and the supernatant was applied onto a thin-layer of silica gel (Kieselgel G, Merck AG). Tyrosine, tyrosyl-tyrosine and tyrosine-ethyl ester (Nutr. Biochem. Comp.) dissolved in 80% ethanol were used as standards. The chromatograms were developed with butanol–acetic acid–water (60:20:20, by vol.) or *n*-propanol–ammonia (70:30, v/v). The spots were visualized by spraying with ninhydrin.

Experimental animals and tissue preparation

The animals used were guinea-pigs (weight 350–700 g, age 3–5 months) and Wistar rats (weight 180–200 g, age 3–4 months) of both sexes, fed with ordinary laboratory food and water *ad lib*. After the animals were decapitated, the livers were removed, dissected into pieces, washed with deionized water at +5° and weighted. The pieces were homogenized in 0·25 M sucrose solution (1:10, w/v) using a Potter–Elvehjem teflon homogenizer. The homogenate was centrifuged at 7000 *g* for 25 min. The sediment was discarded and the supernatant centrifuged at 85,000 *g* for 60 min. The sediment was suspended into half of the original volume of 0·25 M sucrose and recentrifuged at 105,000 *g* for 40 min. The microsomal sediment was suspended into one-third of the original volume of 10 mM Na-phosphate buffer, pH 7·0, containing 2-mercaptoethanol (2·5 mM). This material was used for further studies. A microsomal fraction was obtained similarly also from guinea-pig kidney and brain as well as from rat liver.

Determination of protein

Protein concentration was determined according to Lowry *et al.*[12] with albumin (dried Bovine Albumin Fract. V n.o. 132, Poviet Producten) as standard.

Purification procedures

I. *Solubilization of the enzymes.* The microsomal suspension was treated with ultrasound (Raytheon Ultrasonic Disintegrator, 250 W) for 8 min followed by centrifugation at 105,000 *g* for 40 min. The sonication time was a compromise between solubilization and inactivation of the enzymes; activity of the supernatant towards *N*-hydroxy-AAF increased up to a 12-min treatment while the activity towards AAF began to decrease after a 6-min treatment. Mercaptoethanol in the microsomal suspension (2·5 mM) enhanced the yield considerably, especially the activity towards AAF.

II. *Precipitation with ammonium sulphate.* Crystalline ammonium sulphate was added up to 50 per cent saturation with continuous agitation at 5° and after 20 min the precipitate was removed by centrifugation at 7000 *g* for 20 min and the sediment was discarded. The supernatant solution was saturated up to 70 per cent, the sediment was collected by centrifugation and dissolved in a small volume of 0·01 M sodium phosphate buffer, pH 7·0 containing 2·5 mM 2-mercaptoethanol.

III. *Gel filtration on Sephadex G-100.* The solution was passed through a column (3·5 × 65 cm) of Sephadex G-100 (Pharmacia, Uppsala). The column was equilibrated

with 0·01 M sodium phosphate buffer, pH 7·0, containing 2-mercaptoethanol (2·5 mM) and the same buffer was used as eluent. The flow rate was 40 ml/hr and the hydrostatic pressure was 0·5 m. One hundred (5 ml) fractions were collected. The enzymatic activity towards *N*-hydroxy-AAF and AAF as well as the protein concentration in the fractions were determined. The result is seen in Fig. 1. The fractions

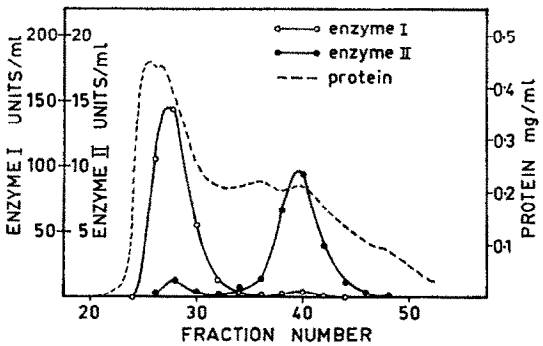

FIG. 1. Sephadex G-100 gel filtration of 50–70% ammonium sulphate fraction. Fractions 25–30 (Enzyme I) and fractions 36–43 (Enzyme II) were pooled. Test conditions are given in Materials and Methods.

25–30 and 36–43 were pooled. The first enzyme eluted was called Enzyme I and the second Enzyme II.

IV(a) *Chromatography on hydroxylapatite.* Enzyme I solution was subjected at room temperature to chromatography on a column (2·0 × 3·0 cm) of hydroxylapatite (Bio-Gel HT, Bio-Rad) equilibrated with 0·01 M sodium phosphate buffer, pH 7·0, with 2-mercaptoethanol (2·5 mM). The same buffer served also as the first eluent. The proteins were eluted stepwise with 0·1 M, 0·125 M, 0·150 M and 0·2 M phosphate buffer, pH 7·0, with 2-mercaptoethanol, by using about 40 ml of each. The enzyme activities towards *N*-hydroxy-AAF as well as protein concentrations in the 10 ml fractions were determined and the results are presented in Fig. 2. Fractions 12–19 were pooled.

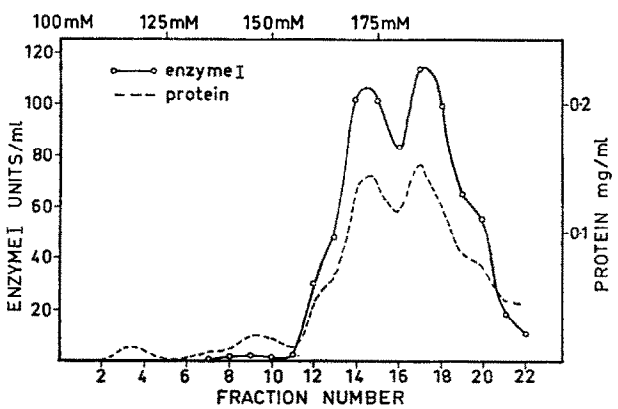

FIG. 2. Hydroxylapatite chromatography of Enzyme I pooled after gel filtration on Sephadex G-100. Fractions 12–19 were pooled. The test conditions are given in Materials and Methods.

IV(b) *Chromatography on DEAE-cellulose.* Enzyme II solution obtained in step 3, i.e. in gel filtration on Sephadex G-100, was subjected directly to chromatography on DEAE-cellulose (Whatman DE 11), pretreated according to Himmelhoch and Peterson.[13] The cellulose column (2·0 × 25·0) was equilibrated with 0·01 M sodium phosphate buffer, pH 7·0, containing 2-mercaptoethanol (2·5 mM). A linear gradient of NaCl (0–0·4 M) in the equilibration buffer was used in the elution. The elution volume was 400 ml, the flow rate 60 ml/hr, and the fraction volume 5 ml. The result is presented in Fig. 3. The enzyme activities towards AAF as well as protein concentrations in the fractions were determined.

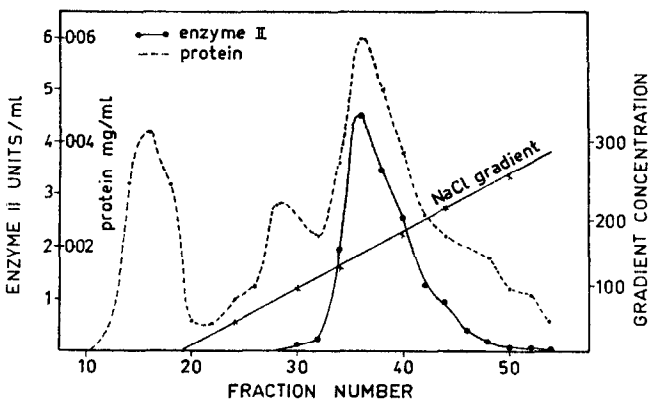

FIG. 3. DEAE-cellulose chromatography of Enzyme II pooled after gel filtration on Sephadex G-100. Fractions 33–42 were pooled. The test conditions are given in Materials and Methods.

The active fractions 33–42 were combined.

V. *Desalting by Sephadex G-25 gel filtration.* The preparations in step IV (IVa and IVb) were passed through a column (2·0 × 25·0 cm) of Sephadex G-25 equilibrated with 0·01 M sodium phosphate buffer, pH 7·0, and the same buffer was used as eluent. The fractions containing the enzyme were pooled.

The whole procedure was carried out at 5°. The numerical data obtained throughout the procedure are summarized in Table 1. Enzyme I was enriched 42-fold and Enzyme II 85-fold over the activity present in the purified microsomal suspension. The ratio of the *N*-hydroxy-AAF hydrolysis rate to that of AAF was 290 in Enzyme I and 0·82 in Enzyme II preparation. Enzyme I was fairly labile; 8 days at +5° caused a 50 per cent inactivation while over 75 per cent was left of Enzyme II activity. 2-Mercaptoethanol (2·5 mM) and Clealand's reagent (dithiothreitol, Calbiochem) (1 mM) prevented inactivation almost completely; after 8 days over 90 per cent of activity was left. No inactivation of either enzyme preparation was found at −20° during 8 days.

Determination of molecular weights

The estimation of molecular weights was based on gel filtration;[14] Sephadex G-200 was used with the following proteins of known molecular weight as references: thyroglubulin (Bovine, Sigma, mol. wt. 650,000), gamma-globulin fraction III (Bovine, NBC, mol. wt 70,000), albumin (Bovine serum, Sigma, mol. wt. 70,000) and trypsine

TABLE 1. SUMMARY OF THE NUMERICAL DATA THROUGHOUT THE PURIFICATION PROCEDURE DESCRIBED IN THE TEXT

Purification step	Volume (ml)	Total protein (mg)	Total activity N-OH-AAF (U)	Total activity AAF (U)	Specific activity N-OH-AFF (U/mg)	Specific activity AAF (U/mg)	Recovery N-OH-AAF	Recovery AAF	Purification coefficient N-OH-AAF	Purification coefficient AAF
Microsome suspension	195	624	14·800	720	24	1·2	100	100	1	1
I. Solubilized microsomes	160	176	11·800	300	67	1·7	80	42	2·8	1·4
II. 50–70% ammonium-sulphate fraction	10	43	9·800	224	230	5·2	61	31	9·6	4·3
III. Sephadex G-100 chromatography, pooled preparations	33 (40)	12·6 (7·6)	6·080 (192)	33 (196)	480 (25)	2·5 (25·8)	41 (1)	5 (27)	20·0 (1·0)	2·1 (22)
IVa. Hydroxylapatite chromatography, pooled preparation	41	3·4	3·450	12	1000	3·5	23	2	41·7	2·9
IVb. DEAE-cellulose chromatography, pooled preparation	53	1·4	117	143	84	102	1	20	3·5	85

In brackets are data of the later eluted peak of Sephadex G-100 gel filtration.

(Bovine pancrease type I, Sigma, mol. wt. 23,800). All of these proteins were dissolved in water at a concentration of 1 mg/0·5 ml and used immediately. The void volume was determined by using Blue Dextran (Pharmacia AB, mol. wt, 2,000,000). The column (2 × 55 cm) was equilibrated with 0·01 M sodium buffer, pH 7·0, containing 2-mercaptoethanol (2·5 mM).

RESULTS

Effect of enzyme concentration

The dependence of the *N*-hydroxy-AAF hydrolysis by Enzyme I on time and enzyme concentration is shown in Figs. 4a and b. The corresponding plots of AAF hydrolysis by Enzyme II are shown in Figs. 5a and b.

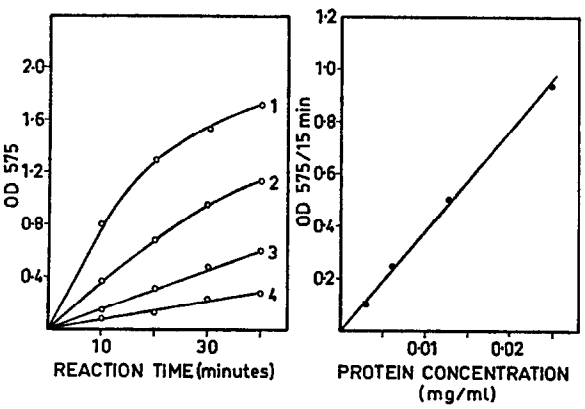

FIG. 4. Effect of reaction time and enzyme concentration on the hydrolysis of *N*-hydroxy-AAF by the purified and desalted Enzyme I. The protein concentration in experiment 1 was 0·025 mg/ml. In experiments 2–4 the enzyme was diluted to 1/2, 1/4 and 1/8.

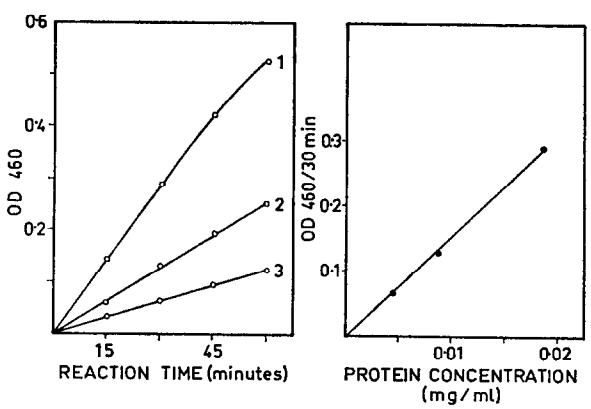

FIG. 5. Effect of reaction time and enzyme concentration on the hydrolysis of AAF by the purified and desalted Enzyme II. The protein concentration in experiment 1 was 0·19 mg/ml. In experiments 2–3 the enzyme solution was diluted to 1/2 and 1/4.

Effect of substrate concentration

This was studied with *N*-hydroxy-AAF as substrate for Enzyme I and AAF for Enzyme II and analysing the data according to Lineweaver and Burk.[15] In the former case K_m was $0{\cdot}44 \times 10^{-3}$ M and in the latter case K_m was $0{\cdot}375 \times 10^{-3}$ M. Ethanol concentration in the incubation medium was 5% (v/v) in the former and 12·5% in the latter case. The highest substrate concentration in the former case was 1 mM and 0·25 mM in the latter.

Effect of pH

Dependence on pH of the hydrolysis of *N*-hydroxy-AAF, AAF and chloracetanilide by the two enzyme preparations was determined in 0·1 M Michaelis barbital–acetate[16] and Britton–Robinson buffer.[17] The result is presented in Fig. 6. All these substrates

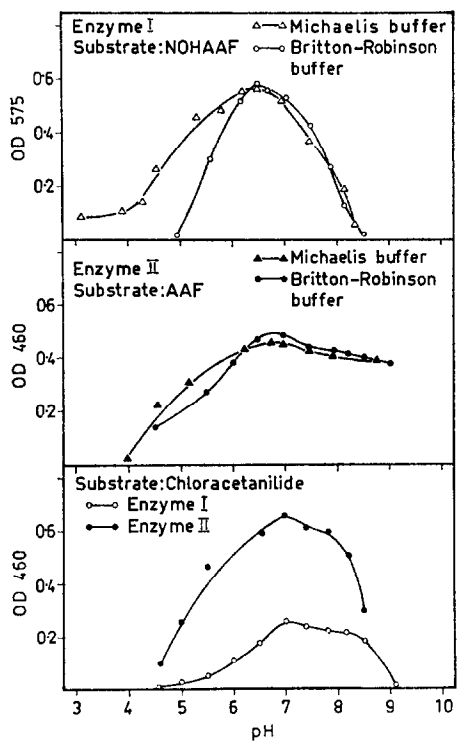

FIG. 6. Effect of pH on the hydrolysis of *N*-hydroxy-AAF, AAF and chloracetanilide by the purified Enzyme I and Enzyme II.

were hydrolyzed by Enzyme I optimally at pH 6·5 in Michaelis barbital–acetate buffer and at pH 6·7 in Britton–Robinson buffer. The pH optimum for Enzyme II was 6·5–7·0 in Michaelis barbital–acetate buffer and 6·75–7·0 in Britton–Robinson buffer.

Effect of modifying substances

The effect of some metal ions, thiol reagents and organophosphorus compounds on the enzyme activity was tested without preincubation of the enzyme in the presence of

TABLE 2. EFFECT OF VARIOUS MODIFIER SUBSTANCES ON THE HYDROLYSIS OF *N*-OH-AAF BY ENZYME I AND AAF BY ENZYME II

Modifier	Concentration (M)	Inhibition (%) Enzyme I N-OH-AAF as substrate	Enzyme II AAF as substrate
EDTA	10^{-3}	0	0
	10^{-4}	0	0
Iodoacetic acid	10^{-3}	31	13
	10^{-4}	33	12
Iodoacetamide	10^{-3}	28	0
	10^{-4}	6	0
Arsenate	10^{-3}	4	54
	10^{-4}	0	26
p-Chloromercuribenzoate	10^{-5}	31	76
	10^{-6}	0	12
Diethyl *p*-nitrophenyl phosphate	10^{-5}	98	95
	10^{-6}	95	73
NaF	10^{-3}	84	32
	10^{-4}	42	0
KCN	10^{-3}	2	0
	10^{-4}	2	0
Hydroxylamine	10^{-3}	4	3
	10^{-4}	2	1
$CuCl_2$	10^{-3}	48	94
	10^{-4}	37	22
$ZnCl_2$	10^{-3}	27	61
	10^{-4}	26	28
$HgCl_2$	10^{-3}	94	91
	10^{-4}	91	26
$FeCl_2$	10^{-3}	73	0
	10^{-4}	29	0
Pb acetate	10^{-3}	31	0
	10^{-4}	1	0
$CaCl_2$	10^{-3}	52	0
	10^{-4}	4	0
Mercaptoethanol	10^{-3}	2	0
	10^{-4}	0	0

the modifier. The results are given in Table 2. E600 (diethyl *p*-nitrophenyl phosphate) inhibited both enzymes at very low concentrations. A clear inhibition was also produced by iodoacetic acid, iodoacetamide, *p*-chlormercuribenzoate, mercuric chloride and sodium fluoride. No marked differences between the two enzymes were noticed.

Substrate specificity

The hydrolysis rate of several substrates by Enzyme I and Enzyme II is recorded in Table 3. The hydrolysis rates relative to that of AAF (indicated as 1·0) are also included.

Both enzyme preparations hydrolyzed tyrosine ethyl ester but no formation of tyrosyl-tyrosine or tyrosyl-tyrosine ethyl ester could be observed.

TABLE 3. ABSOLUTE AND RELATIVE HYDROLYSIS RATES OF SEVERAL SUBSTRATES BY THE PURIFIED ENZYMES

Substrate	Absolute hydrolysis rate (U/ml)		Relative hydrolysis rate*	
	Enzyme I	Enzyme II	Enzyme I	Enzyme II
Acetyl-1-naphthylamide	8·7	150	2·0	1·08
Acetyl-2-naphthylamide	22	31	5·1	0·21
Formyl-1-naphthylamide	228	156	53	1·04
Formyl-2-naphthylamide	244	146	57	0·97
Chloroacetyl-1-naphthylamide	21,000	26,200	488	175
Trifluoroacetyl-1-naphthylamide	19,700	39,600	4580	264
Leucyl-2-naphthylamide	48	81	11	0·74
1-Naphthyl acetate	97	59	22·6	0·39
2-Naphthyl acetate	9	8·7	2·1	0·058
Acetanilide	3·1	19	0·72	0·127
Chloroacetanilide	660	700	154	4·7
Trifluoroacetanilide	16,200	23,600	3770	157
Formanilide	304	390	71	2·6
p-Nitroacetanilide	48	114	11·2	0·76
p-Nitrophenyl acetate	1270	970	295	6·5
p-Acetamidobenzoic acid	28	210	6·5	1·4
Procain HCl	129	16	3	0·11
p-Ethoxyacetanilide		0	3	0
Acetyl-anthranilic acid	3·3	36	0·77	0·24
Chloroacetyl anthranilic acid	18·2	440	4·2	2·9
Formyl-anthranilic acid	11	108	2·56	0·72
N-formyl-L-kynurenine	9	48	2·1	0·32
Monoethylglysinxylidide	0	0	0	0
Tyrosinethylester	69	4760	16	31·7
Acetamide	0	0	0	0
N-OH-AAF	1140	103	265	0·69
AAF	4·3	150	1	1

* The hydrolysis rates are relative to that of AAF which has been recorded as 1. Details of assay methods are given in Material and Methods.

Molecular weights

To obtain a rough estimate of the molecular size of the enzyme proteins a 1 ml sample of each with reference substances was passed through a Sephadex G-200 column and the enzyme hydrolysis of *N*-hydroxy-AAF and AAF was estimated. The calculation of the molecular weight was performed according to the method of Whitaker.[14] The approximate molecular weight of 200,000 for Enzyme I and 41,000 for Enzyme II was obtained.

Distribution of the enzymes in various microsomal fractions

The hydrolytic activity of the microsomal fractions of guinea-pig liver, kidney and brain as well as of rat brain was assayed with *N*-hydroxy-AAF and AAF as substrates. The results are given in Table 4 in which also the results of Irving[7] are reproduced. The hydrolytic activity of the guinea-pig liver microsomes toward *N*-hydroxy-AAF

was about 10-fold that of the rat liver preparation. Our findings on the guinea-pig liver microsomes are concordant with that of Irving but we found a faster hydrolysis rate by the rat liver microsomes than did Irving. The activity towards *N*-hydroxy-AAF was lower in guinea-pig kidney and brain than in liver and the *N*-hydroxy-AAF:AAF ratio in hydrolysis rates was lower in these organs.

TABLE 4. THE RATE OF DEACETYLATION OF *N*-HYDROXY-AAF AND AAF BY MICROSOMES OF GUINEA-PIG AND RAT. FOR COMPARISON THE DATA OF IRVING (1966) ON RAT AND GUINEA-PIG LIVER MICROSOMES ARE REPRODUCED

Tissue	Enzyme activity (μmoles/hr/g)		Irving (1966)	
	N-hydroxy-AAF	AAF	*N*-hydroxy-AAF	AAF
Guinea-pig liver microsomes	2·94	0·15	3·48	0·21
Guinea-pig kidney microsomes	1·0	0·3		
Guinea-pig brain microsomes	0·42	0·052		
Rat liver microsomes	0·25	0·097	0·047	0·067

DISCUSSION

In this work we were able to separate two enzymes capable of deacylating *N*-hydroxy-AAF and AAF from the guinea-pig liver microsomes. Both microsomal enzymes had closely similar characteristics although some distinct differences were demonstrated, too.

The Enzyme I with the higher molecular weight (mol. wt. 200,000) hydrolyzed *N*-hydroxy-AAF 265 times faster than AAF, while Enzyme II with lower molecular weight (mol. wt. 41,000) hydrolyzed AAF about 1·4 times faster than *N*-hydroxy-AAF. The first enzyme thus clearly prefers the substrate with *N*-hydroxy groups to that of usual amide type. As far as the aromatic ring of the acetamido compound is concerned, both enzymes seem to prefer fluorene to benzene as substrate. The hydrolysis rate of naphthylamine derivatives appears to be about the same order of magnitude as the hydrolysis of aminofluorene derivatives. Substitution of the hydrogen atoms in the acyl group by electronegative halogen atoms enhances markedly the hydrolysis rate: the more substituted, the higher the hydrolysis rate by both enzymes. This phenomenon has been found true in connection with some other amidases but, on the other hand, is not true in the case of formamidase.[8] The hydroxy-AAF enzyme (Enzyme I) appeared to prefer formyl derivatives to acetyl derivatives. Substitution in the para-position of the benzene ring appeared to increase markedly the hydrolysis rate, e.g. *p*-acetamidobenzoic acid was hydrolyzed ten times faster than acetyl-anthranilic acid.

The fact that the microsomes of guinea-pig liver hydrolyzed *N*-hydroxy-AAF about 20-fold faster than AAF means that both enzymes probably are at least partially in an active state in the microsomes. The comparative ratios, 3·3 in guinea-pig kidney, 8·1 in guinea-pig brain and 2·6 in rat liver, would suggest either that both enzymes are present also in these tissues in different proportions or that some additional enzymes with different substrate specificity characteristics are to be found. The latter possibility would agree with the findings of Weisburger[18] and Irving[7] that rat liver microsomes split acetanilide considerably faster than AAF.

The inhibition caused by the compounds known to react with SH-groups does not allow us to conclude whether SH-groups are essential for the activity of the enzymes. The effect of SH-reagents, heavy metal ions and sodium fluoride did not show, however, any marked differences between the two enzymes. Both enzymes were inhibited by the organic phosphorus compound, diethyl *p*-nitrophenyl phosphate (E 600), at low concentrations, a fact concordant with the findings of Irving.[7] This finding reveals that both of the enzymes are so-called esterase-type enzymes. They also hydrolyze easily ester substrates, e.g. tyrosine ethyl ester, *p*-nitrophenyl acetate and 1-naphthyl acetate.

Several E 600 sensitive esterases have been purified from liver microsomes. From hog liver and kidney Krisch[19,20] purified a carboxylesterase which may be identical to that described by Hollunger.[21] More recently Benöhr and Krisch[11] purified an esterase from bovine liver microsomes which differed somewhat from the hog enzymes. It is difficult to decide whether those enzymes should be considered identical with the present ones although all these enzymes appear to hydrolyze both ester and amide substrates. It should be noted, however, that neither of the enzymes purified by us was capable of forming tyrosyltyrosine as did the enzyme purified by Benöhr and Krisch.

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