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DEACETYLATION OF PHENACETIN BY LIVER ESTERASE

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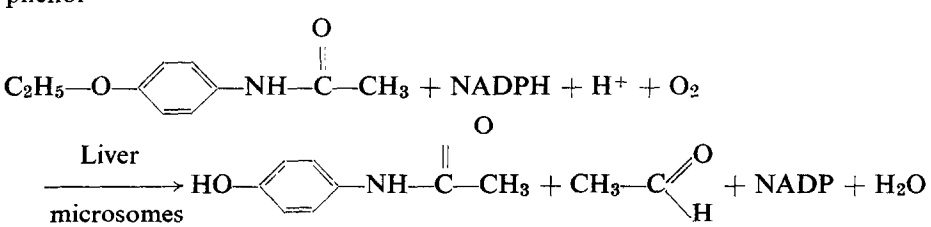
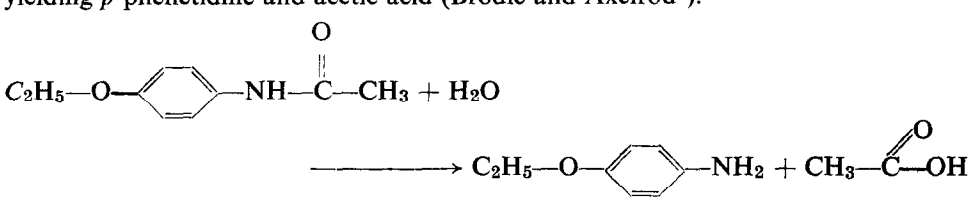
Abstract—Microsomal liver esterase cleaves the amide bond of the phenacetin molecule *in vitro* yielding *p*-phenetidine and acetic acid as reaction products. Some kinetic data of this reaction are reported. N-acetyl-*p*-aminobenzoic acid, being more polar than acetanilide, is hydrolysed about 1000 times slower by the isolated enzyme.

RECENTLY we reported on the isolation of a new hydrolytic enzyme from hog liver microsomes.[1] In column chromatography, electrophoresis and ultracentrifugation experiments this enzyme behaved essentially as an homogeneous protein[2]. Its molecular weight is 174,000.[3] Studies on the substrate specificity revealed that not only carboxylic esters, i.e. tributyrin, procaine, L-tyrosine ethyl ester, are attacked but also amide bonds of certain aromatic compounds, i.e. acetanilide, xylocaine, monoethylglycine-2,6-xylidide, and L-leucyl-β-naphthylamide. Generally, the turnover numbers of amide substrates were found to be considerably lower than those of carboxylic esters. For the isolated enzyme the name "microsomal liver esterase" is proposed. It is possibly identical with the long-known liver ali-esterase (E.C.3.1.1.1. Carboxylic ester hydrolase). This question is, however, difficult to decide because so far most preparations of liver ali-esterase are unsatisfactorily defined as far as homogeneity and substrate specificity are concerned.[4–6]

The main pathway of phenacetin metabolism in man and higher animals leads in an oxidative NADPH dependent reaction to the formation of N-acetyl-p-aminophenol[7–9]



It is known, however, that phenacetin is also deacetylated *in vivo* to a small extent yielding *p*-phenetidine and acetic acid (Brodie and Axelrod[7]).



* Part of this work was done by Miss E. Bernhammer for a M.D. thesis at the university of Giessen.

The formation of methemoglobin following administration of phenacetin is attributed to this side-reaction. *p*-phenetidine is believed to be a precursor of the actual methemoglobin forming compound.[7] Up to now little is known about the enzyme catalyzing this deacetylation reaction. In 1950 Bray *et al.*[10] reported on the deacetylation of several acetamido-compounds *in vitro* by extracts from liver and kidney without further purifying the enzymes involved.

Considering the fact that the amide bond of acetanilide is readily hydrolyzed by microsomal liver esterase it was of interest to study the metabolism of phenacetin *in vitro* by this enzyme. The results of such studies are presented in this paper.

METHODS

1. *Microsomal liver esterase*

The enzyme was isolated according to our previous prescription up to step VI.[1] In several cases the enzyme preparation was rechromatographed on a DEAE sephadex A 50 column.

2. *Determination of* p-*phenetidine*; *standard test*

p-phenetidine was determined by the method of Brodie and Axelrod.[7] The procedure was modified in the following manner:

Substrate solution: 89·5 mg phenacetin + 9·6 ml acetonitrile are made up to 100 ml with 0·1 M tris· HCl buffer pH 8·6. Acetonitrile had to be used as a solvent because phenacetin is only slightly water-soluble. Control experiments showed that a final concentration of 4·8% acetonitrile inhibits microsomal liver esterase (tested towards acetanilide as a substrate) about 10–20%.

0·5 ml substrate solution and 0·1–0·4 ml enzyme solution (according to activity; about 100 μg protein) are made up in shaking tubes to a volume of 1 ml with 0·1 M tris buffer pH 8·6. The final phenacetin concentration in the experiments is $2{\cdot}5 \times 10^{-3}$ M. The samples are incubated 15 min at 37°. The reaction is stopped by putting the shaking tubes into ice and immediate extraction with 6 ml isoamylalcohol/benzene (1·5/100; v/v). The glass-stoppered tubes are shaken for three min. A 4 ml aliquot of the benzene phase is taken and transferred to another shaking tube containing 2·0 ml N HCl. The samples are shaken for another three min. Then the organic layer is carefully removed by suction. A 1 ml aliquot of the aqueous phase is taken and pipetted into a test tube. At this step it is essential that contamination of the aqueous 1 ml aliquot with benzene is carefully avoided.

Diazotization: 0·5 ml 0·2% sodium nitrite are added and the samples put into ice. After 20 min the tubes are taken out of the ice and 0·5 ml 1% ammonium sulfamate is added. After another 3 min 0·1 ml 10% resublimed α-naphthol in 96% ethanol and 1·0 ml 4 N NaOH are pipetted into the tubes and the samples are left in ice for 10 min. Subsequently fine gas bubbles are removed by shaking and optical density is read in 5 mm cuvettes at a wavelength of 510 mμ. The color is stable up to 30 min. In each series of determinations a substrate blank and 3 standards of *p*-phenetidine (0·1, 0·2 and 0·3 μMoles) are run through the procedure.

3. *Identification of* p-*phenetidine*

10 ml phenacetin (substrate solution, see above) + 8 ml tris buffer pH 8·6 + 2 ml enzyme (2–4 mg protein) are incubated 90 min at 37°. 2 × 0·1 ml of the incubation

mixture are removed and run through the quantitative procedure (see 2). The remaining 19·8 ml are stopped by addition of 2 ml 5 N HCl and transferred into a separatory funnel. The acidified mixture is extracted twice with 100 ml ether in order to remove excess phenacetin. (This step is omitted in the experiments for thin-layer chromatography). The ether phase is discarded and the aqueous layer neutralized by addition of 5 N (finally 1 N) NaOH up to a pH of 7·5–8·0. Then the *p*-phenetidine is extracted twice by shaking with 60 ml distilled benzene for 3 min. The combined benzene phases are treated with anhydrous sodium sulfate, filtered and evaporated to dryness in a rotary evaporator (water pump vacuum; t $\approx$ 50°). For thin-layer chromatography the residue is dissolved in a small amount of acetone-ether (1 + 1) and 0·1–0·2 ml of the extract are applied to a thin-layer plate (silicagel HF_{254} "E. Merck"/ Darmstadt). The solvent systems are given in the legend of Fig. 2. The plates are viewed under a UV-lamp (254 mμ) and the spots are marked. Within a few days the *p*-phenetidine spots become brownish by autoxidation.

UV-spectrum: The spot with the mobility of *p*-phenetidine is scraped off the plate with a spatula and eluted with 2·5 ml 96% ethanol. The UV-spectrum of the ethanolic extract was recorded in a Zeiss-spectrophotometer, model PM Q II. A blank spot of a thin-layer plate treated the same way served as a blank.

IR-spectrum: For IR spectroscopy the extract was not chromatographed because of the interfering background from the HF plate. In this case the benzene extract is concentrated to a volume of 5–10 ml and 80–100 mg KBr are added. The residual benzene is evaporated to dryness (rotary evaporator). Then a tablet is pressed and an IR spectrum recorded using a Perkin-Elmer spectrophotometer, model 221. As reference compound *p*-phenetidine (40 μmoles + 0·48 ml acetonitrile in 10 ml 0·1 M tris buffer pH 8·6) is run through the whole extraction procedure. This is important because there are some losses during the procedure and some minor bands of authentic *p*-phenetidine become slightly modified.

4. *Determination of* p-*aminobenzoic acid*

In the experiments with N-acetyl-*p*-aminobenzoic acid as a substrate the reaction was followed by measuring the *p*-aminobenzoic acid formed. *p*-Aminobenzoic acid is diazotized according to the method described previously for the acetanilide standard test.[1] The azo dye formed, however, is not benzene soluble and therefore has to be extracted with n-butanol. In each experiment standard amounts of *p*-aminobenzoid acid (0·1, 0·2 and 0·3 μMoles) are run through the procedure.

RESULTS

1. *Reaction rate as a function of enzyme concentration*

The deacetylation of phenacetine was followed by determination of the *p*-phenetidine formed. The primary amino group of *p*-phenetidine was diazotized and coupled with α-naphthol to a pink azo dye (see Methods). Control experiments revealed that the reaction product *p*-phenetidine incubated 15 min at 37° in the presence of enzyme was not further metabolized under these conditions. The recovery was 100 $\pm$ 3%. In Fig. 1 the reaction rate is plotted versus the enzyme concentration of microsomal liver esterase. There is a linear relationship between the amount of *p*-phenetidine formed in 15 min and the enzyme concentration (up to about 200 μg of protein/ml). No deacetylation occurred when phenacetin was incubated in the presence of chymotrypsin, trypsin, serum albumen and γ-globulin (about 200 μg of protein each).

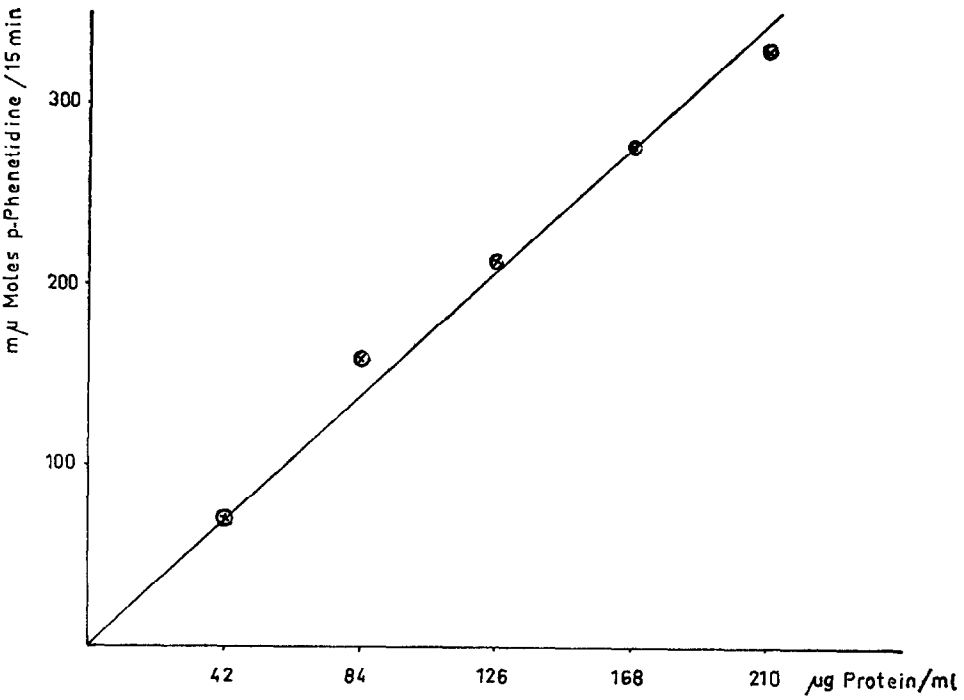


FIG. 1. Effect of enzyme concentration on reaction velocity.

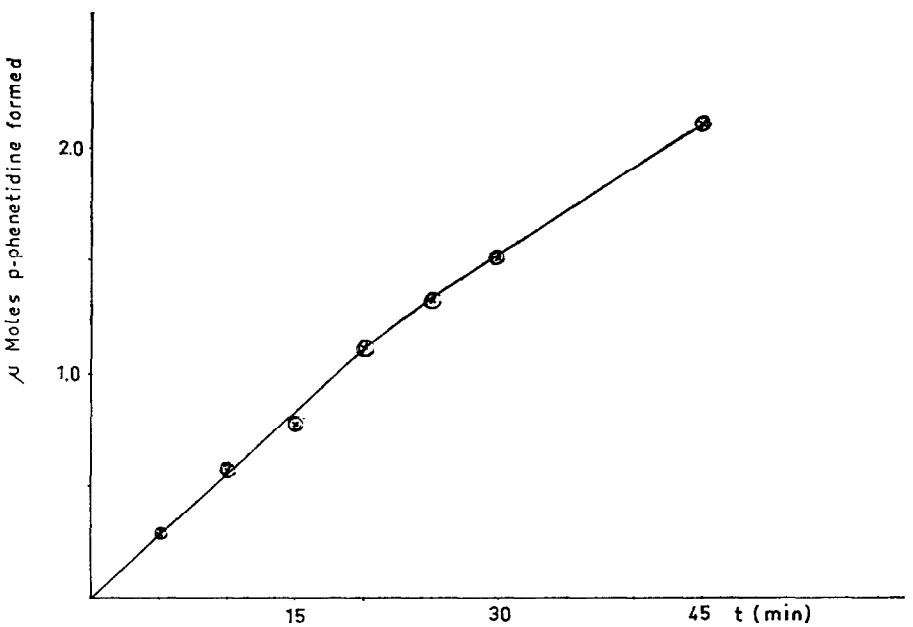


FIG. 2. Enzymatic formation of p-phenetidine as a function of time
7·5 ml phenacetin (substrate solution, see Methods) + 6·7 ml tris · HCl buffer pH 8·6 + 0·8 ml enzyme (step V (1), containing 3·25 mg protein) are incubated at 37°. At the times indicated 1·0 ml aliquots are taken and analysed for *p*-phenetidine.

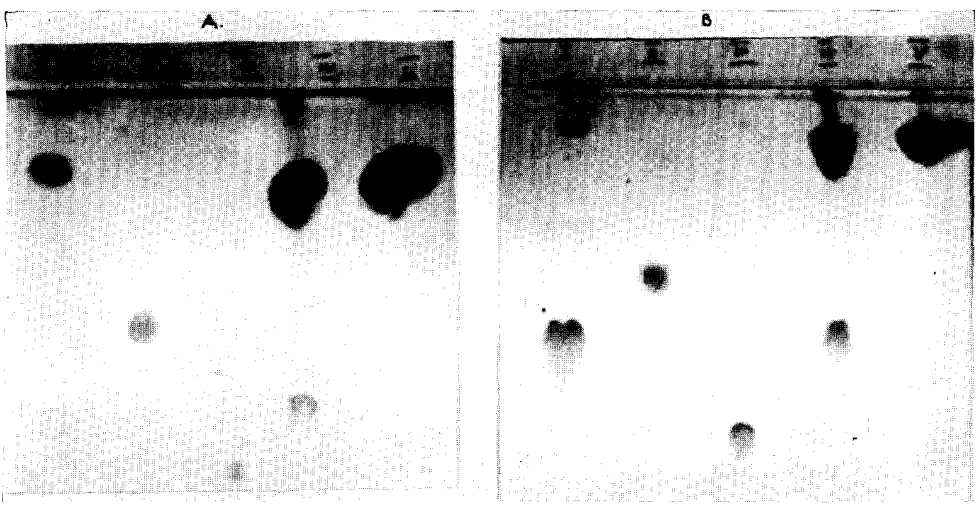


FIG. 3. Thin-layer chromatography of phenacetin and *p*-phenetidine.

I = phenacetin + *p*-phenetidine (the latter being the slower moving compound), II = N-acetyl-*p*-aminophenol, III = *p*-aminophenol (of all reference substances 0·1 μMoles were applied), IV = extract of reaction mixture, native enzyme, V = control experiment, boiled enzyme. Solvent systems: A chloroform 42·5: toluene 15: glacial acetic acid 42·5 (v/v). B benzene 79: methanol 14: glacial acetic acid 7 (v/v).

The plates were coated with a 200–300 μ thin layer of fluorescent silicagel HF_{254} according to Stahl (E. Merck, Darmstadt/Germany). Following chromatography the plates were photographed using a u.v. lamp of 254 mμ.

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2. *Formation of* p-*phenetidine as a function of time*

The time course of the reaction is shown in Fig. 2. The curve proceeds linearly up to about 20 min and then starts to flatten off. It can be assumed that by incubating the samples 15 min initial velocities are measured.

3. *Identification of* p-*phenetidine*

(a) Thin-layer chromatography: In a preparative experiment the *p*-phenetidine formed by the enzymatic reaction was extracted with benzene and subjected to thin-layer chromatography in two different solvent systems. The results are shown in Fig. 3. It can be seen that by incubation of phenacetin with microsomal liver esterase a product is formed which migrates in both solvent systems like authentic *p*-phenetidine (V). In a control experiment with boiled enzyme no *p*-phenetidine can be detected but only unmetabolized phenacetin (VI). There is no indication of a cleavage of the phenol ether bond of the phenacetin molecule which would result in the formation of N-acetyl-*p*-aminophenol or *p*-aminophenol, respectively. This is not surprising because it is well known that the phenol ether bond is split by a firmly structure bound microsomal enzyme system requiring the presence of molecular oxygen and NADPH.

(b) UV-spectra: The spot corresponding to *p*-phenetidine was eluted from the thin-layer plate and a u.v.-spectrum recorded (Fig. 4). There is good agreement between

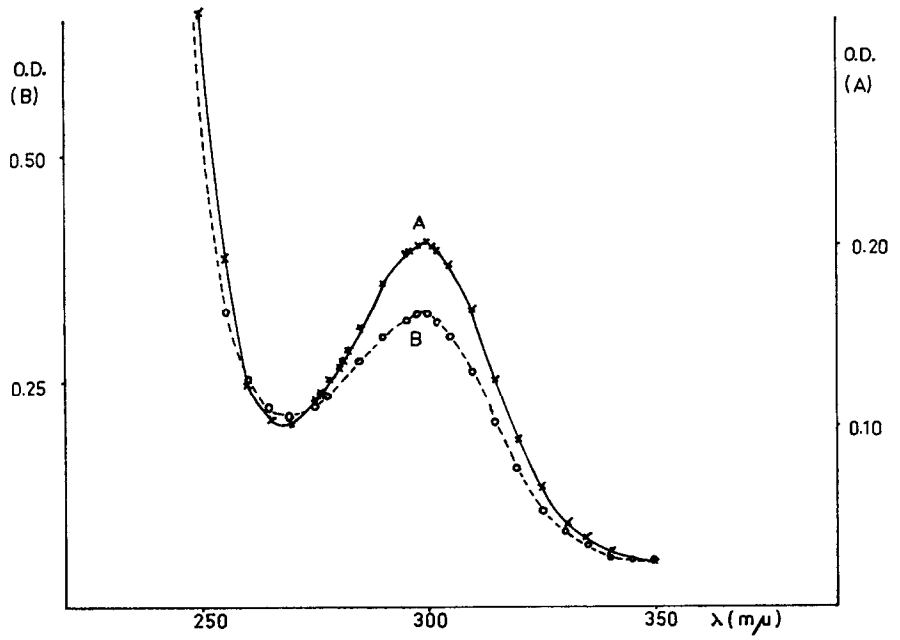

FIG. 4. UV-spectra of *p*-phenetidine following thin-layer chromatography and elution with 96% ethanol

A = *p*-phenetidine, B = substance isolated from the enzymatic reaction mixture.

the spectrum of the substance formed enzymatically and authentic *p*-phenetidine run through the same procedure. It should be noted, however, that there is a shift in the absorption maximum of authentic *p*-phenetidine when subjected to thin-layer chromatography as compared to the spectrum of non-chromatographed *p*-phenetidine in

ethanol. This is probably due to a secondary degradation of *p*-phenetidine during thin layer chromatography.

(c) IR-spectra: In addition the *p*-phenetidine formed enzymatically from phenacetin was identified by its IR-spectrum. The IR-spectra of authentic *p*-phenetidine (after having been run through the extraction procedure) and *p*-phenetidine extracted from the enzymatic reaction mixture were identical.

4. *Turnover numbers*

The activities of four different enzyme preparations using phenacetin as a substrate were determined. The following values were found: 101, 127, 129 and 154 mμMoles *p*-phenetidine formed/min × mg protein. As reported recently the molecular weight of microsomal liver esterase is 174,000.[3] From these figures a turnover number of 22·3 ± 3·8 is calculated assuming one active site per enzyme molecule. (This figure is

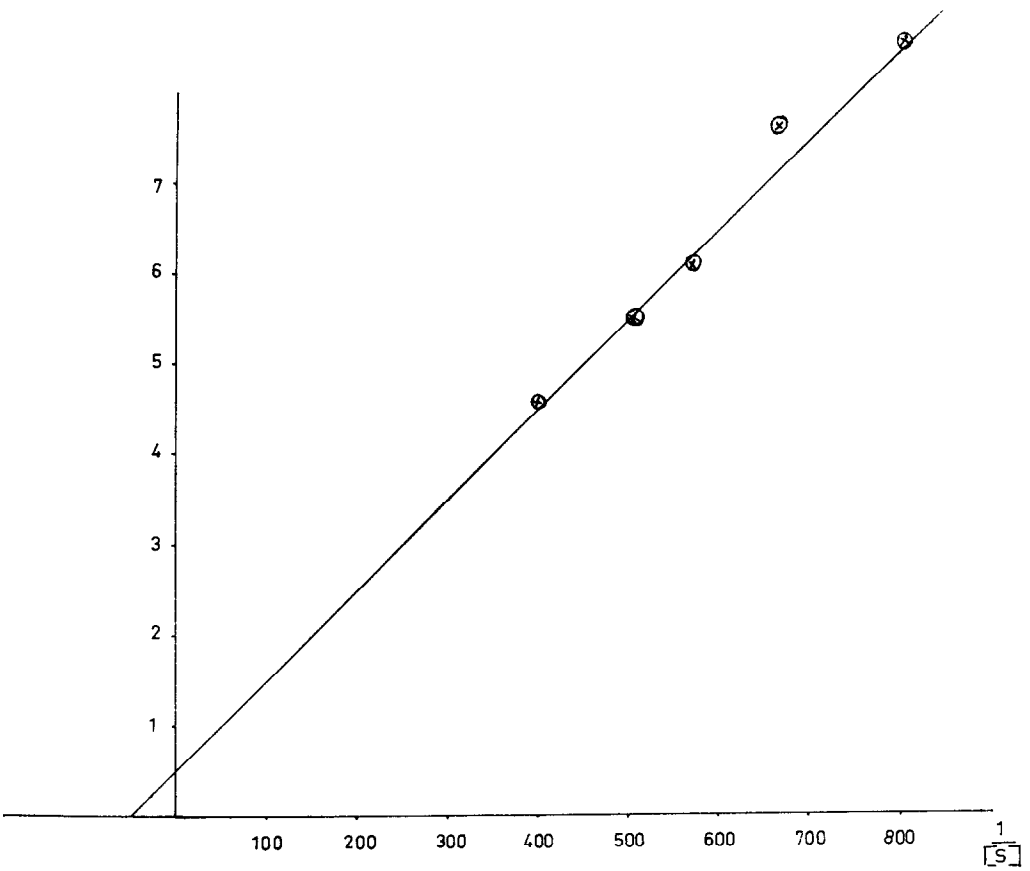

FIG. 5. Effect of phenacetin concentration on reaction velocity. Lineweaver-Burk diagram.

probably 10–20% too low due to the inhibitory effect of acetonitrile used as a solvent for phenacetin, see Methods). Experiments to determine the number of active sites by inhibitor studies with organophosphorus compounds are now under way in our laboratory. It is striking that the turnover number of 22 Moles phenacetin metabolized

per minute by one Mole of enzyme is rather low. This turnover number is in the same order of magnitude as those for acetanilide and other amide substrates.[2] This lends further support to the view that esters and not amide derivatives are the favored substrates for microsomal liver esterase.

5. *Michaelis constant*

We have studied the dependency of the reaction velocity on the concentration of phenacetin. The values obtained were plotted in the double reciprocal way according to Lineweaver and Burk[11] (Fig. 5). The Michaelis constant was found to be

$$K_M \text{ (phenacetin)} = 2 \times 10^{-2}\text{M}$$

This comparatively high figure indicates a rather small affinity of microsomal liver esterase towards phenacetin as a substrate.

6. *Influence of several inhibitors*

Microsomal liver esterase exhibits a characteristic inhibition pattern when incubated in the presence of several inhibitors such as organophosphorus compounds (Diisopropyl fluorophosphate, paraoxon). Table 1 shows the influence of a number of inhibitors on the enzymatic deacetylation of phenacetin. It can be seen that the reaction is inhibited by a number of typical liver esterase inhibitors, such as paraoxon, quinine and atoxyl. The extent of inhibition is similar to the data reported previously using acetanilide as a substrate.[2] Another potent inhibitor is physostigmine salicylate.

TABLE 1. EFFECT OF SEVERAL INHIBITORS ON THE DEACETYLATION OF PHENACETIN BY MICROSOMAL LIVER ESTERASE

Inhibitor	Concentration (M)	Activity (per cent of uninhibited reaction)
Paraoxon	10^{-5}	1
(Diethyl-*p*-nitrophenyl phosphate)	10^{-6}	52
Physostigmine salicylate	10^{-4}	3
	10^{-5}	6
	10^{-6}	44
Quinine · HCl	10^{-3}	33
	10^{-4}	79
Atoxyl	10^{-2}	9
(Sodium arsanilate)	10^{-3}	22
SKF 525-A	$0{\cdot}5 \times 10^{-4}$	87
Diethylaminoethyl diphenylpropyl-acetate · HCl)	10^{-5}	83

The values given represent means of 3 or 4 experiments.

This compound in a concentration of 10^{-5} M causes almost complete inhibition of microsomal liver esterase. There is no significant difference between the inhibitory effects of physostigmine salicylate and sulfate.

Another interesting inhibitor is SKF 525-A (β-diethylaminoethyl diphenylpropyl-acetate · HCl). This compound inhibits most NADPH-dependent oxidative microsomal reactions and was considered by many authors to be a "microsome inhibitor".

The action of SKF 525-A is, however, not restricted to microsomal reactions. In 1959 Netter[12] described the non-competitive inhibition of human plasma procaine esterase by SKF 525-A. Recently Netter and Seidel[13] found differences in the inhibition pattern of procaine esterases in serum and liver microsomes by SKF 525-A and several other phenylsubstituted acetic acid derivatives. They concluded that plasma procaine esterase is probably not derived from procaine esterase in liver microsomes. As can ben seen from Table 1 SKF 525-A has only a slight inhibitory effect on the deacetylation of phenacetin by microsomal liver esterase.

7 *Hydrolysis of N-acetyl-p-aminobenzoic acid*

All substrates for microsomal liver esterase studied so far are more or less lipid soluble compounds. Peptides and proteins are not attacked.[2] It seemed of interest to increase the polarity of the acetanilide molecule by introducing a carboxyl group in *p*- position and compare the turnover numbers of the enzyme towards N-acetyl-*p*-aminobenzoic acid with those towards acetanilide. When N-acetyl-*p*-aminobenzoic acid was incubated in the presence of enzyme an activity 0·45 mμMoles *p*-aminobenzoic acid formed/min × mg protein was found. When tested using acetanilide as a substrate the same enzyme preparation had an activity of 391 mμMoles aniline formed/min × mg protein. The corresponding turnover numbers are 0·078 for N-acetyl-*p*-aminobenzoic acid and 68 for acetanilide. From these figures follows that N-acetyl-*p*-aminobenzoic acid is an extremely poor substrate. In agreement to our expectations acetanilide being less polar and considerably less water soluble is hydrolyzed almost 1000 times faster by microsomal liver esterase than N-acetyl-p-aminobenzoic acid. One might speculate that the carboxyl group of glutamic acid situated next to the serine in the active centre of liver esterase[14] prevents binding of negatively charged substrates.

DISCUSSION

According to our results phenacetin is deacetylated *in vitro* by a highly-purified esterase from hog liver microsomes. Rat liver microsomes also split the amide bond of the phenacetin molecule.[15] The extent of deacetylation *in vivo* varies in different species.[8] It would be interesting to correlate the data on the *in vivo* deacetylation with the esterase activities of liver microsomes *in vitro* in various species. Probably the liver is not the only tissue by which phenacetin is deacetylated. In preliminary experiments p-phenetidine was formed by hog kidney microsomes too. Studies to further characterize microsomal esterases in tissues other than liver are now under way in our laboratory. Another important question is whether there exist more than only one esterase in microsomes.

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