Studies on the Classification of the Enzymes Hydrolysing Ester-form Drugs in Liver Microsomes

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running title: Classification of Microsomal Esterases

INTRODUCTION

The drug-metabolizing enzymes catalyzing such reactions as oxidation, reduction, conjugation and hydrolysis have been reported to be localized mainly in liver microsomes (1,2). Microsomes may also be considered as hydrolytic particles (3). The enzymes hydrolyzing ester-form drugs, e.g. aspirin (4,5,6), procaine (7), cocaine (8,9,10), atropine (8,11,12) and choline-esters (13,14) have also been shown to be in the liver and some other tissues, but little is known about the properties and intracellular localization of these esterases as compared with cholinesterase which is closely related to synaptic transmission. It appears of interest to investigate drug-metablizing enzymes in liver microsomes further to elucidate their pharmacological actions and mechanisms of detoxication.

As a fundamental classification of esterases, Aldridge (15) showed that 10^{-5}M organo-phosphorous compounds inhibit many enzymes possessing carboxylic esterase activity while other esterases are unaffected. He has named the latter A-type and the former B-type esterase. Neither A- nor B-type are sensitive to 10^{-5}M eserine. Cholinesterase is inhibited completely both by 10^{-6}M organophosphates and by 10^{-5}M eserine. Subsequently A- and B-types and cholinesterase in many vertebrate plasmata were separated electrophoretically by Goutier (16) and Augustinsson (17). Microsomal esterases have not been examined with this technique.

The present paper describes experiments on the

classification of esterases, especially drug-hydrolyzing enzymes, in liver microsomes of some rodents. The microsomes "solubilized" with sodium deoxycholate were fractionated chromatographically or electrophoretically, and the sensitivities of the esterases in each fraction to inhibitors and their kinetics were investigated.

EXPERIMENTAL

Substrates and inhibitors

The substrates used in this study were phenyl acetate (PhAc), phenyl butyrate (PhBu), tributyrin (TrBu), acetylcholine hydrochloride (AcCh), butyrylcholine iodide (BuCh), polyoxyethylene sorbitan monolaurate (Tween 20), aspirin and hydrochlorides of cocaine, procaine, hexylcaine and tetracaine, which were obtained commercially.

The esterase inhibitors used as selective inhibitors were eserine sulfate and diisopropyl fluorophosphate (DFP, in propylene glycol).

Measurements of enzyme activities

Determination of esterase activity for location of enzyme peaks was carried out on all chromatographic or electrophoretic fractions by incubating suitable aliquots of each fraction with substrate for 5 to 30 minutes at 37°C and pH 7.5. Esterase activities toward aspirin and procaine were estimated spectrophotometrically in phosphate buffer, pH 7.5 by the method of Hofstee (18) and of Brodie (19), respectively while those toward the other esters was estimated in bicarbonate-CO_2 buffer manometrically (20). In these spectrophotometric methods the measurement of esterase activity was not influenced by the presence of the other esters used in this study, so that kinetic analysis of esterase inhibition was possible. Suspensions of substrate, such as PhAc, PhBu or TrBu, were prepared by dissolving these materials in small amounts of methanol according to the method of Aldridge (15). The substrates

were added in the buffer solution at final concentrations of 0.01 M in the reaction mixture. All the reaction mixtures were composed of 0.5 ml of a enzyme aliquot and 2.5 ml of the buffer containing substrate and Ca^{++} and Mg^{++} ions.

In experiments on inhibitors, esterase activity was estimated after incubation of the enzyme solution with 10^{-5}M DFP or 10^{-5}M eserine at 37°C, pH 7.5, for 30 minutes prior to addition of substrate.

In experiments on pH-dependence the following buffers were used to cover the range required: 0.1 M sodium acetate for pH 4.0-6.5; 0.1 M sodium phosphate for pH 6.5-9.0; 0.1 M sodium borate for values above pH 9.0.

Enzyme preparation

All steps of the preparations were carred out at 0-3°C. Livers of rabbit, rat and guinea-pig weighing about 70, 7 and 5 g, respectively, were perfused with physiological saline, and microsomal fractions of the liver cells were isolated from the supernatant obtained by centrifugation at 250,000 g-min in 0.25 M sucrose medium by centrifugation at 3,000,000 g-min using a Martin christ omega type ultracentrifuge according to the method of De Duve (21). After discarding the soluble fraction, the microsomal pellet was homogenized with 0.5 per cent sodium deoxycholate (DOC) in approximately four volumes of buffer as described by Palade (22). After standing the mixture at 0°C for 15 minutes, the

homogenized microsomes were centrifuged at 6,600,000 g-min and then the slightly yellowish solubilized microsome preparation was carefully sucked off. To remove DOC, the samples were dialyzed against veronal buffer (pH 8.4, I=0.1) or Tris phosphate buffer (pH 8.0, 0.005 M) before electrophoresis and chromatography, respectively.

Separation of esterases

The preparative column electrophoretic apparatus used for this study was built in our laboratory. Potato starch in veronal buffer was poured into a vertical glass column (2 X 60 cm) to a height of 40 c m. The upper end of the tube was fitted with a rubber support and connected by a KCl-agar bridge with the cathode chamber containing KCl solution. The lower end of the column was fitted with a glass filter and dipped into a veronal buffer chamber to which the current was carried from the anode chamber containing KCl solution through the agar bridge. Platinum-sheet electrodes were fixed in both chambers. About 2.5 ml of the sample which had been dialyzed against veronal buffer was introduced into the column and the zone containing the sample was allowed to move 2 to 3 cm below the surface of the starch. The electrophoresis was carried out at 300 volts and 23 to 25 milliamperes, for 30 to 36 hours in a cold room (0-1°C). After electrophoresis, a rubber stopper with a glass capillary tube was introduced into the lower end of the tube and the liquid in the column was displaced by the same veronal buffer at a flow rate

of 1 ml per 8 minutes. Fractions of 0.7 ml were collected.

Chromatography was carried out on a 1.2 X 30 cm column of DEAE-cellulose. The adsorbent in 0.005 M Tris phosphate buffer was adjusted to pH 8.0 with phophoric acid, and then equilibrated with Tris phosphate buffer for 24 hours. About 2 ml of the solubilized microsome sample was applied to the column, then the column was washed with 70 ml of this buffer. A 500 ml round bottomed flask, containing 250 ml of 0.005 M Tris phosphate buffer, pH 8.0, served as a mixing chamber. A 500 ml reservoir flask was filled with 250 ml of the same buffer, containing 0.5 M sodium chloride. Linear gradient elution was carried out with increasing sodium chloride concentration at a flow rate of 1 ml per 2 minutes and fractions of 7 ml were collected.

RESULTS

Distribution of esterase activity in the liver cell.

Table 1 shows that most esterase activities toward aspirin and procaine are localized in the microsomal fraction.

Various esterase activities of solubilized microsomes

The hydrolysis rates of various substrates by solubilized microsomes are given in Table 2. Activities are expressed as μmoles of acid liberated per 1 mg of protein per 10 minutes, and corrected for nonenzymic hydrolysis. PhAc and TrBu were decomposed very fast. Activities toward cholinesters or local anesthetic esters varied from species to species.

Chromatographic separation of microsomal esterases

Seven main esterase peaks were eluted from the chromatogram. B-type esterase(s) for PhBu and TrBu was eluted first, but the separations of A- and B-type esterase and of A-type and cholinesterase were not complete.

Electrophoretic separation of microsomal esterases

When solubilized microsomes of rodent liver were fractionated by electrophoresis, quite good separations of A- and B-type esterase and cholinesterase were obtained. Figs. 1, 2 and 3 show the substrate specificities and sensitivities to inhibitors of the fractions obtained by electrophoresis of guinea-pig, rabbit and rat preparations.

The peaks of esterase activity represented as A, B and C, refer to A- and B-type esterases and cholinesterase, respectively. Esterases which were not inhibited more than 50 per cent by 10^{-5}M DFP were classified as A-type.

Guinea-pig (Fig. 1) The solubilized microsome fraction from guinea-pig was separated into six main esterase fractions, designated as A_1, (A_2+C_2), A_3, A_4, B_1, and C_1. PhAc, TrBu, Tween 20 and BuCh were hydrolyzed by at least two enzymes, and PhBu by three. The first esterase fraction (A_1) split only PhBu. The A-type esterase was separated into four fractions, and cholinesterase into two, one (A_2+C_2) of which showed the properties of both cholinesterase with BuCh and of A-type esterase with all the substrates tested. The hydrolyses of aspirin and the local anesthetic esters by this fraction were not affected by 10^{-5}M DFP. TrBu and Tween 20 were hydrolyzed by at least two A-type esterases. On electrophoresis, the B-type esterase fraction (B_1) migrated slowest of the esterases and remained as one fraction. Cholinesterase was relatively hard to separate from the A-type esterases.

Rabbit (Fig. 2) The rabbit sample was also separated into six main esterase peaks, i.e. A_1, A_2, A_3, B_1, A_4 and B_2 in order of electromobility. PhAc and TrBu were split by three esterase fractions (A_2, B_1 and A_4), PhBu by four (A_1, A_3, A_4 and B_1), Tween 20 by at least two (A_2 and

A_3), cocaine by three (A_1, A_2 and B_1), and aspirin by two (A_2 and A_4). The esterase fractions acting on TrBu could not be clearly separated.

Rat (Fig. 3) The sample from rat liver formed six main esterase fractions on electrophoresis. Three A-type peaks appeared followed by three B-type peaks. PhAc and TrBu were hydrolyzed by at least two esterase fractions (A_2 and B_1), PhBu by four (A_1, A_3, B_2 and B_3), Tween 20 by two (A_1 and B_1) and aspirin by one (B_1).

Schematic representation of the electrophoretic fractions of microsomal esterases

Fig. 4 shows a schematic representation of the distribution of esterase among the fractions of the solubilized microsome preparations obtained from the experiments shown in Figs. 1, 2 and 3. The shaded squares are proportional to the area of each esterase peak, which were measured with an Amsler planimeter. Generally the first fraction (A_1 in each species) had a high esterase activity with PhBu. The cholinesterase activity of the guinea-pig sample was measurable after electrophoresis, but those of rabbit and rat were too low to be detected. The C-fractions of the guinea-pig sample followed the first A-type peak (A_1). In all three species a relatively non-specific esterase peak appeared in the middle part of the microsomal protein separated by electrophoresis.

PhAc, TrBu and Tween 20 were hydrolyzed by two or three esterase fractions, and PhBu by three or four. With the guinea-pig sample aspirin, procaine, hexylcaine and tetracaine were split most by a rather non-specific esterase(s) in fraction (A_2+C_2), which was insensitive to both DFP and eserine at concentrations of 10^{-5}M. The solubilized microsomes of guinea-pig and rabbit had a relatively high A-type esterase activity, whereas that of rat had high B-type activity.

Identification of procainesterase and esterases for various other ester-form drugs

Since the esterase activities to procaine, hexylcaine, tetracaine and aspirin which were all insensitive to DFP were found in peak (A_2+C_2) of guinea-pig microsomes (Fig. 1) and the optimum pH range of procainesterase and aspirinesterase in this fraction was pH 7.0 to 8.5 (Fig. 5), the identity of these esterases were analyzed further by the Lineweaver and Burk equation (23). Seven different concentrations of procaine, ranging from 10^{-4}M to 2 X 10^{-3}M, were used as substrate with fraction (A_2+C_2), and constant concentrations of procaine, hexylcaine or aspirin were added. Incubation was carried out for 15 minutes at 37°C. The hydrolysis of procaine by esterase(s) in fraction (A_2+C_2) was inhibited by hexylcaine, tetracaine and aspirin (Fig. 6). These inhibitions were competitive. Acetylcholine did not inhibit procainesterase. The converse, namely the inhibition of the aspirinesterase of fraction

(A_2+C_2) by procaine, could be demonstrated by this method, and this inhibition was also competitive (Fig. 7).

DISCUSSION

Many hydrolytic enzymes are localized in microsomes, such as aliesterase (24), cholinesterase (14), alkaline phosphatase (25) and vitamin A esterase (26). In the present work (Table 1), esterases toward aspirin and procaine were also found in the microsomes.

For electrophoretic or chromatographic studies the microsomal enzymes must be in a soluble form, so some esterases which were not easily extractable may not have been demonstrated. From Tables 1 and 2, however, it was calculated that about 80 per cent of the activity of the aspirinesterase in the solubilized microsome preparation of rabbit liver cells is recovered when 85 per cent (22) of the microsomal protein is obtained. Ramachandran et al. (30) reported that the rates of hydrolysis of acetylcholine and butyrylcholine by a microsomal DOC-soluble fraction were 10 to 18 times higher than those of the insoluble fraction in terms of specific activity.

Aldridge found two types of esterases (i.e. A- and B-type) other than cholinesterase hydrolyzing p-nitrophenyl acetate, propionate and butyrate in the sera of rabbit, rat and horse (15). Aldridge's method of classification of esterases on the basis of the effect of inhibitors seems to be very clearcut and fundamental. The nomenclature of such esterases as aliesterase, arylesterase and azolesterase is not thought to be adequate, since overlap of substrate specificities of hydrolytic enzymes can be shown (34). The separation of esterases

has already been attempted by many investigators. Augustinsson (17) studied the esterases in mammalian plasmata of 12 species using as substrates a series of aliphatic, aromatic, heterocyclic and choline esters to study the substrate specificities of the various esterase components obtained by electrophoresis and their sensitivities to certain selective inhibitors. It was demonstrated that on electrophoresis A-type esterase (arylesterase) usually migrated with or close to the albumin, while B-type esterase (aliesterase) moved in the α_1-globulin region, and cholinesterase between α_2- and β-globulin.

Since it has been suggested that serum esterases are probably produced in the liver (27), studies on the liver esterases have also been made. Markert et al. (28) reported that aqueous extracts of mouse liver and of 32 other tissues could be separated electrophoretically into more than 10 different esterase zones by the zymogram technique. Moreover, one of the esterase bands was cholinesterase, since it was inhibited by 10^{-4}M eserine, but no analysis was made of its sensitivity to organo-phosphate. Comparing esterases in human liver with those in serum by the zymogram technique, Ecobichon et al. (13) obtained three esterase zones from liver and two from serum with naphthyl- and cholinesters as substrates. They concluded that liver esterases were markedly different from serum esterases, both in behavior on electrophoresis and in their substrate specificities. In their work micro-

somal esterase was probably not detected because the enzyme preparation was an aqueous extract.

In the present work the following results were obtained with rodent liver: 1) Microsomal esterases were separated into at least five or six esterase peaks by zone electrophoresis. 2) Most of the A-type esterases migrated faster toward the anode than the B-type on electrophoresis. 3) The esterases in a middle part of the microsomal protein fractionated on electrophoresis had a non-specific esterase activity toward most esters and ester-form drugs. Microsomal esterases, as well as serum esterases, have also been shown to be composed of several esterase proteins differing in electromobility and sensitivity to inhibitors. Some esters (e.g. phenyl acetate and butyrate) were hydrolyzed by several enzymes which differed electrophoretically. Therefore the liver microsomes seem to contain the isozyme of esterase.

Serum albumin and α- and β-globulin had been shown by perfusion experiments to be produced in liver by Miller et al. (29). In hepatic diseases esterase activity is low and tends to rise as the patient recovers (27). Thus it appears of interest to compare liver esterase with that in serum, since most serum enzymes may come from liver cells. Fig. 8 shows by arrows the distribution of each type of esterase in serum and solubilized microsome preparations of liver from guinea-pigs after electrophoresis. With regard to electromobility the esterase peaks of the liver microsome preparation corresponded very poorly with

those of the serum. The same results have been reported on the esterases toward α-naphthyl acetate (13) and procaine (2) in human liver and serum. There was little albumin in the protein component of the solubilized liver microsome preparation (Fig. 8). It has been reported that more than one esterase migrated with the albumin of the plasmata of many species (17), but no such esterase with a large mobility was observed in the microsomal samples. The reason why the electromobilities of esterases of solubilized microsomes and of serum did not correspond, may be that serum esterases are markedly different from microsomal esterases, or that esterase protein is modified when liver esterase is released from liver cells into the blood stream. It may be denied that there is a possibility that esterase protein is partially affected by treatment with DOC, since data has been obtained showing that esterases of microsomes when solubilized by sonic oscillation showed the same electrophoretic properties as those obtained by treatment with DOC.

Ramachandran et al. using solubilized microsomes and other solubilized cell fractions from rat liver, found four esterase peaks and corresponding radioactive peaks after fractionation by DEAE-cellulose chromatography and incubation with $DF^{32}P$. They concluded that most esterase peaks took up radioactivity and that there were no radioactive peaks without esterase activity (30,31). It was found that the esterase activity of each peak was not proportional to its radioactivity. From the fact

that in the present investigation three A-type and three B-type esterases were separated by electrophoresis from the microsomal preparation of rat liver, it may be considered that the difference in the capacities of esterases to bind $DF^{32}P$ is due to their sensitivities to DFP. After incubation with $DF^{32}P$ single radioactive peptides could be obtained from a number of esterases by proteolysis, and Oosterbaan *et al.* suggested that the DFP-binding site on the enzyme is concerned with the activation of the enzyme substrate complex, whereas another site, with a configuration and amino acid composition which varies from enzyme to enzyme, is responsible for binding the substrate and differences in this site cause differences in specificity (32). These esterases must be B-type, since they combine with DFP.

In guinea-pigs high activity of an enzyme(s) hydrolyzing aspirin and ester-form anesthetics was localized in liver microsomes and this activity has also been reported to be present human and horse tissues (7). This esterase was contained in one of the esterase peaks of the guinea-pig sample, and was of the A-type. From kinetic analyses it was thought that aspirin, procaine, hexylcaine and tetracaine might compete in binding with an active center of one of the esterases in liver microsomes. It is interesting that these drugs were decomposed by the same enzyme. If aspirin and procaine which both have weak analgesic actions are administered together, synergism of the analgesic action of the two compounds can be shown.

The dissociation constant of procaine and the "procainesterase" of fraction (A_2+C_2) was about 1.2×10^{-3}. Kalow has shown that the Km of procainesterase in human serum is 6×10^{-6}, and he demonstrated that this enzyme and benzoylcholinesterase were identical by kinetic analysis (33). Therefore the guinea-pig liver microsomal esterase toward procaine seems to be different from human serum procainesterase. So far, cocaine has been found to be attacked markedly only by rabbit liver microsomes.

SUMMARY

Liver microsomes from guinea-pig, rabbit and rat were solubilized by treatment with DOC. The DOC-soluble proteins of the liver microsomes were fractionated by DEAE-cellulose chromatography or preparative column electrophoresis. The esterase activity of each fraction was examined using aliphatic, aromatic and choline esters and some esters of drugs as substrates.

The esterase groups in solubilized liver microsomes, which were classified by their sensitivities to inhibition by 10^{-5}M DFP and 10^{-5}M eserine, could be further separated by electrophoresis. At least five or six esterases were found in the microsomes of rodent liver which differed in electromobility, substrate specificity and sensitivity to inhibitors. Cholinesterase activity toward butyrylcholine and acetylcholine was not present in rabbit or rat liver in any measurable amount after electrophoresis. The electromobilities of A-type esterases were higher than those of B-type esterases. The esterases in the middle part of the microsomal protein fractionated by electrophoresis had relatively non-specific activity toward most of the esters so far studied. Liver microsomes were composed of several different kinds of esterase proteins. Microsomal esterases could be separated better by zone electrophoresis than by DEAE-cellulose chromatography.

Evidence was presented that the esterases toward aspirin, procaine, hexylcaine and tetracaine in one of the microsomal esterase fractions of guinea-pig liver may be a single enzyme.

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Table 1. Distribution of esterase activity in rabbit liver cell fractions. The activity is expressed as μmoles of acid liberated per 1 mg of protein per 10 min.

substrate / fraction	aspirin	procaine
Homogenate	0.68	3.93 ($\times 10^{-2}$)
Nuclei	0.35	1.81
Mitochondria	0.28	0.54
Microsomes	1.22	7.28
Supernatant	0.13	0.69

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Table 2. Enzymic hydrolysis of various esters by solubilized microsomes from rodent liver. The activity is expressed as in Table 1.

Species	PhAc	PhBu	AcCh	BuCh	Tween20	TrBu	Aspirin	Procaine	Hexyl-caine	Tetra-caine	Cocaine
Rabbit	15.60	0.62	0.27	0.00	0.20	22.30	1.16	0.07	0.05	0.00	0.21
Rat	12.30	0.83	0.22	0.26	2.44	22.40	1.33	0.06	0.05	0.00	0.00
Guinea pig	54.00	1.56	0.00	1.04	0.55	46.40	2.72	0.16	0.62	0.14	0.00

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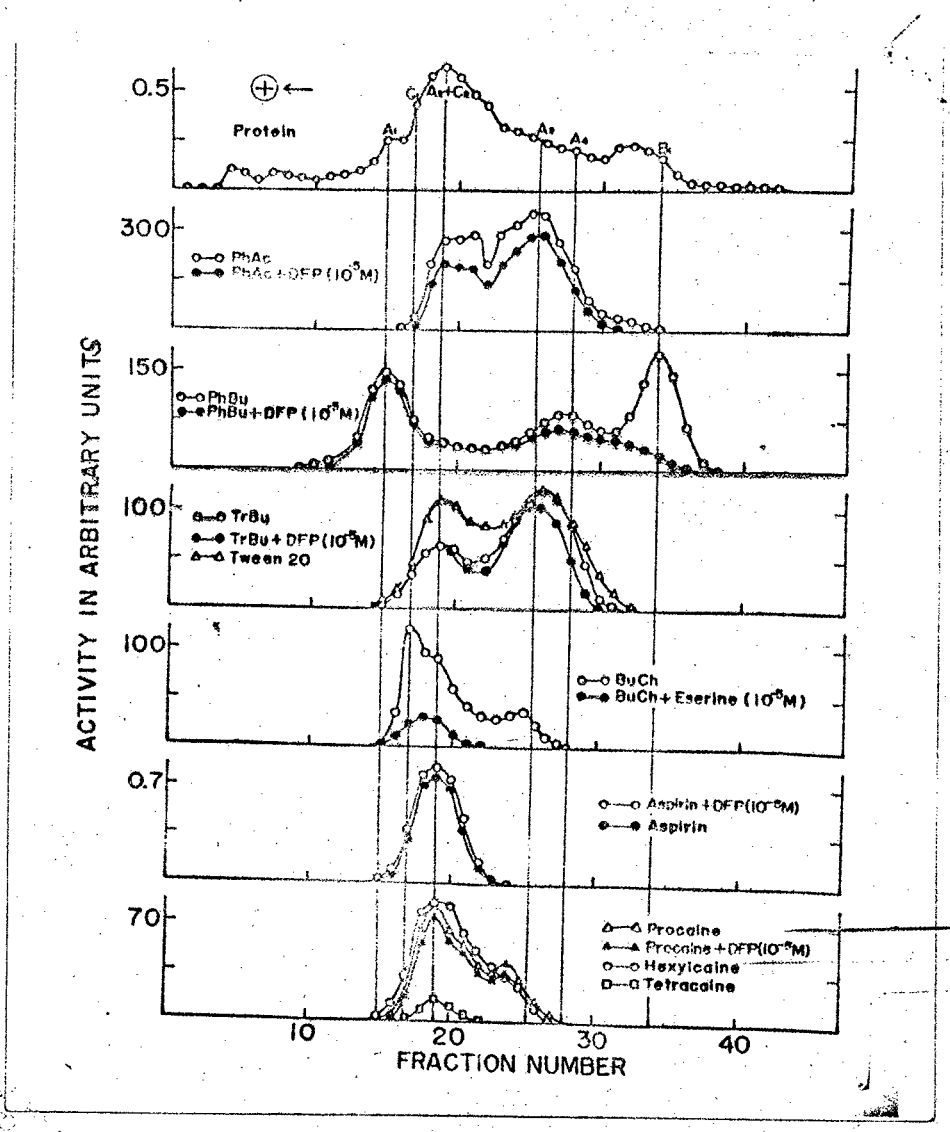

Fig. 1. Distribution of protein and esterase activity of solubilized microsomes from guinea-pig liver after column electrophoresis. Ordinates: relative protein content based on absorbancy and esterase activity in arbitrary units. Abscissa: fraction number.

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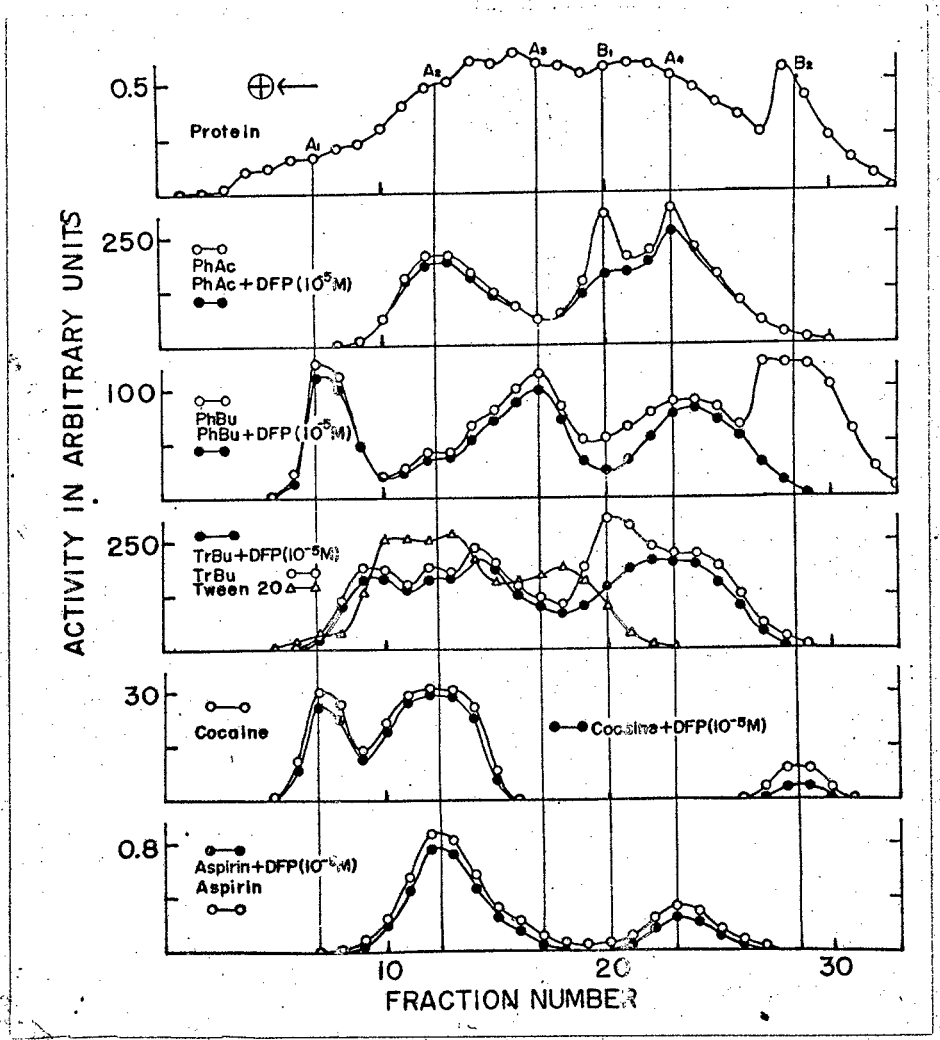

Fig. 2. Distribution of protein and esterase activity of solubilized microsomes from rabbit liver after column electrophoresis. Ordinates and abscissa are as in Fig. 1.

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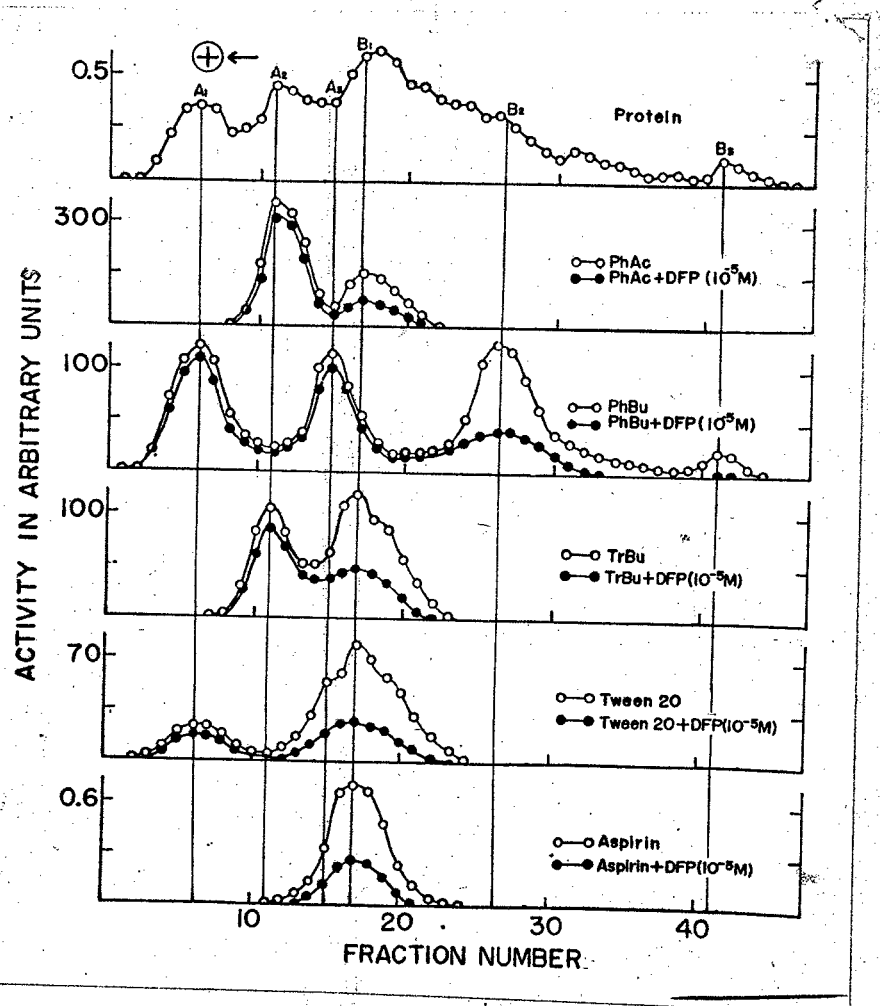

Fig. 3. Distribution of protein and esterase activity of solubilized microsomes from rat liver after column electrophoresis. Ordinates and abscissa are as in Fig. 1.

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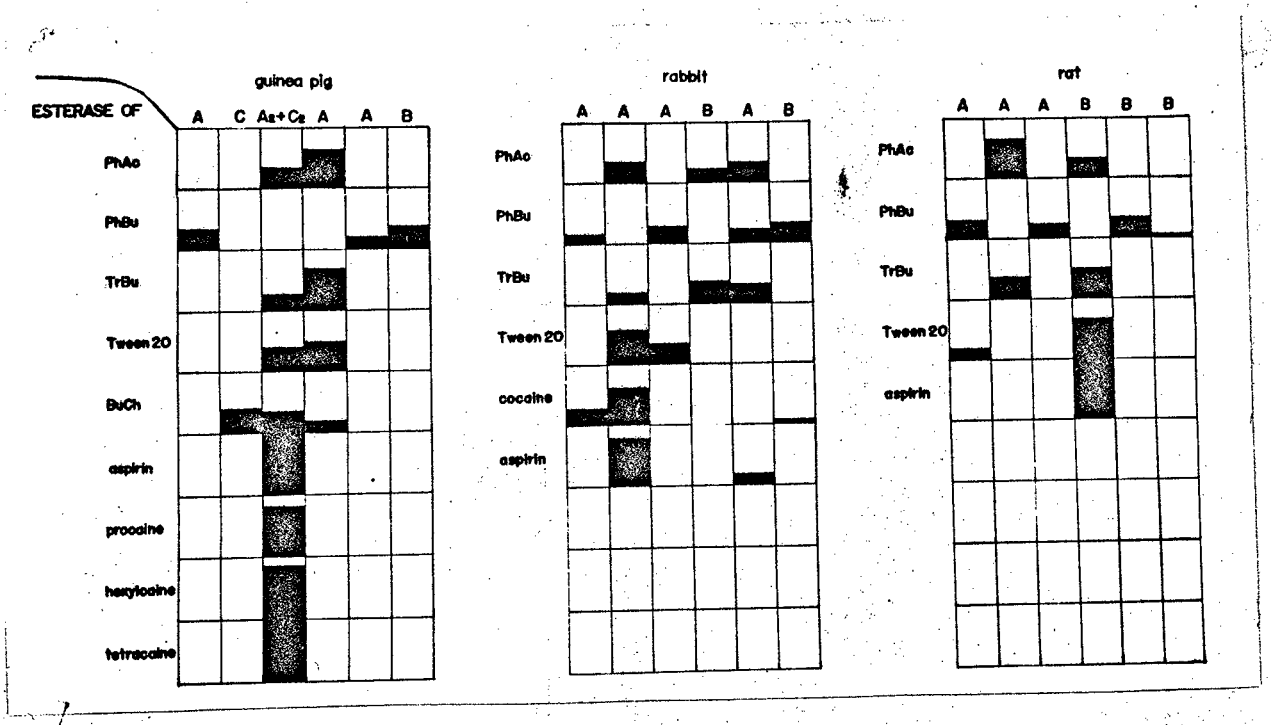

Fig. 4. Schematic representation of distribution of main esterase fractions obtained by electrophoresis. Ordinates: substrate. Abscissa: type of esterase. The anodes are on the left.

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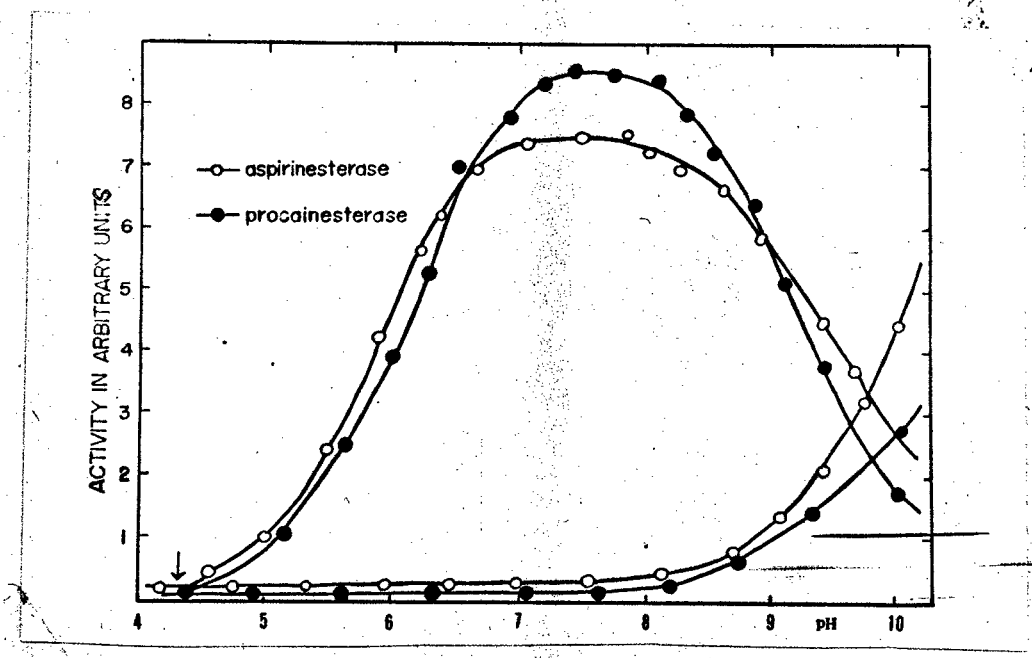

Fig. 5. Effect of pH on aspirinesterase and procainesterase in fraction (A_2+C_2). Ordinate: esterase activity in arbitrary units. Abscissa: pH. The lower two curves represent nonenzymic hydrolysis.

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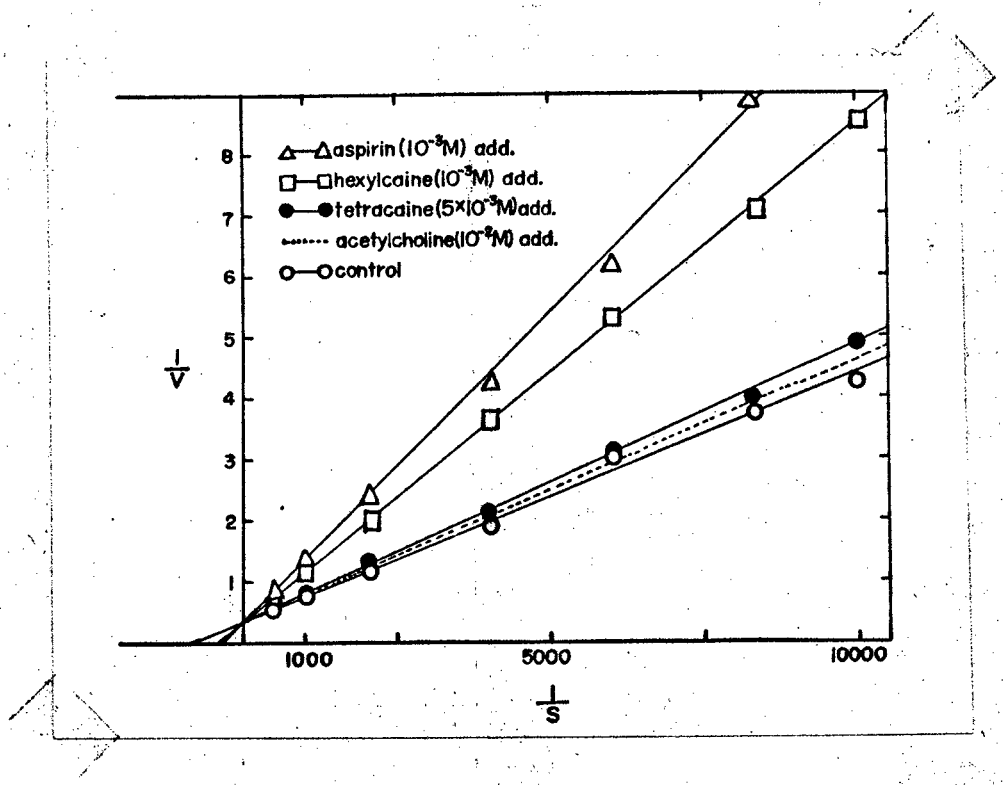

Fig. 6. Effect of aspirin, hexylcaine, tetracaine and acetylcholine on procainesterase in fraction (A_2+C_2). Ordinate: reciprocal of procainesterase activity. Abscissa: reciprocal of substrate concentration.

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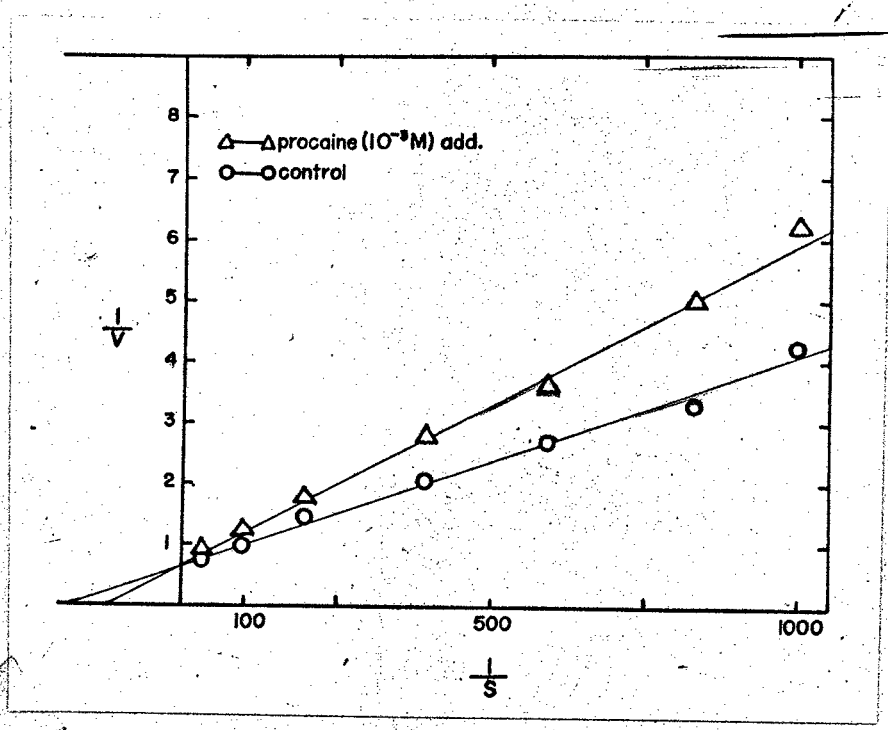

Fig. 7. Effect of procaine on aspirinesterase in fraction (A_2+C_2). Ordinate: reciprocal of aspirinesterase activity. Abscissa: reciprocal of substrate concentration.

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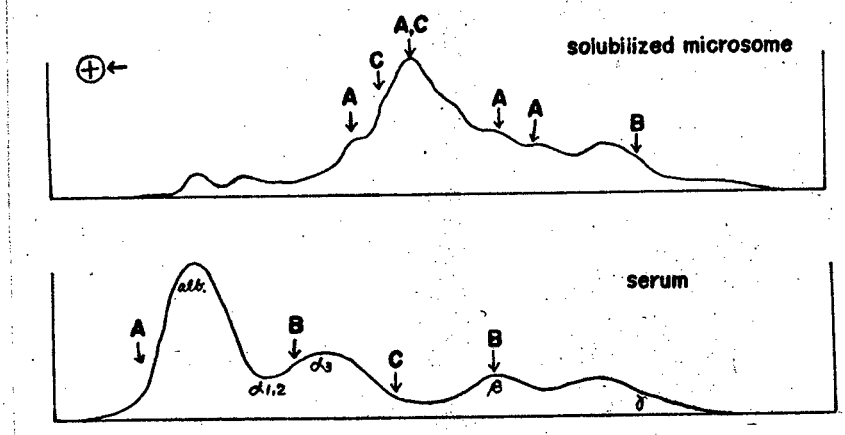

Fig. 8. Distribution of each type of esterase in serum and solubilized microsomes from guinea-pig after column electrophoresis.