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Studies on Acetanilide-hydrolyzing Esterase of Rat Liver
Microsomes. I. Solubilization, Purification,
and Intramicrosomal Localization

Teruaki Akao

SUMMARY

An acetanilide-hydrolyzing esterase of rat liver microsomes was found to be easily solubilized from the membranes by treatments with phospholipase A (pH 6.8), aqueous acetone, low concentrations of detergents, alkali, and sonic oscillation. Most of the microsomal enzymes such as cytochrome b₅ and NADPH-cytochrome c reductase were not solubilized by these treatments. Trypsin digestion, on the other hand, failed to liberate the esterase from the membranes, although this treatment was very effective in solubilizing cytochrome b₅ and NADPH-cytochrome c reductase.

The esterase was extracted from the acetone powder of trypsin-treated rat liver microsomes and purified about 50-fold by Sephadex G-150 gel filtration and DEAE-Sephadex chromatography. A rabbit antiserum was prepared against the purified esterase. The antibody thus prepared did not inhibit the acetanilide-hydrolyzing activity of the purified enzyme, but precipitated this activity efficiently. However, evidence was obtained to indicate that the antibody could not react with the esterase in untreated microsomal vesicles. This finding suggested that the enzyme in microsomes is not located at the outside surface of the vesicular membranes.

These results are discussed in relation to the binding of the esterase to the endoplasmic reticulum.

INTRODUCTION

Recent studies on the turnover of two enzymes of rat liver microsomes, *i.e.* NADPH-cytochrome c reductase and cytochrome b₅, showed that these proteins have different half-lives in vivo (1). This finding suggested that individual proteins of the microsomal membranes are turning over independently. However, in order to establish the validity of this concept, it was desirable to determine the turnover rates for several more proteins associated with the microsomal membranes. In a search for proteins suitable for such studies, we became aware of a report by Krisch et al. (2) on the purification of an acetanilide-hydrolyzing esterase from pig liver microsomes. Judged from the degree of purification and high recovery attained by these workers, we thought it feasible to develop for this enzyme a small-scale purification method, which is a prerequisite for the study of its turnover in rats.

In the present study we examined the solubilization of the esterase from rat liver microsomes by various treatments and established a procedure for its purification to a satisfactory purity. An antibody to the purified esterase was prepared and shown to precipitate the solubilized enzyme. However, the antibody could not interact with the enzyme associated with microsomal vesicles. Based on the observations reported in this paper, we conclude that the esterase is loosely bound to the inside surface of the microsomal vesicles. This localization of the esterase is similar to that of nucleoside diphosphatase (3) and is in contrast to those of cytochrome b₅, NADPH-cytochrome c reductase and NADH-cytochrome b₅ reductase which are

situated at the outside surface of the membranes (4,5). The application of the purification method developed to the study of turnover in vivo of the esterase is reported in the accompanying paper (6).

MATERIALS AND METHODS

Reagents and Biochemicals -- NADH, NADPH, trypsin, and cabbage phospholipase D were purchased from Sigma Chemical Company. Nagarse was purchased from Nagase Industrial Company. Yeast cytochrome c was a generous gift from Sankyo Company. Phospholipase C purified from Clostridium perfringens was kindly supplied from Dr. J. Hase of Toyama University. Crude venom of the snake Trimeresurus flavoviridis obtained by courtesy of Dr. A. Ohsaka was heated as described previously (19) and used as phospholipase A preparation. This preparation was free of protease activities. Emalgen 900 series of detergents (nonylphenyl polyoxyethylenes) were kindly donated from Kao-Atlas Company.

Microsomal Preparation and Subcellular Fractionation -- Liver microsomes were prepared from Sprague-Dawley rats and washed as described previously (7). Separation of rough and smooth microsomes from the postmitochondrial supernatant of a 20 % liver homogenate in 0.88 M sucrose was carried out as described previously (8). Liver homogenates in 0.25 M sucrose were fractionated into the nuclear, mitochondrial, lysosomal, microsomal, and soluble fractions as described previously (21).

Solubilization Treatments -- Details of the conditions employed for solubilization of microsomal components are described in legends for figures and tables. In each experiments, the microsomal suspension that had been treated with a solubilizing agent was centrifuged at $105,000 \times g$ for 90 min, and the components recovered in the supernatant were regarded as have been solubilized. The enzyme activities of the treated suspension were also assayed to determine the degrees of inactivation

during the treatment. Solubilization percent of an enzyme was expressed based on the activity remaining after the treatment.

Enzyme Assays -- The acetanilide-hydrolyzing activity of the esterase was determined by measuring the formation of aniline by a modification of the method of Krisch (2). The reaction mixture contained, in a final volume of 1.0 ml, 10 μ mole of acetanilide, 0.1 M Tris-HCl buffer (pH 8.5), and enzyme. Incubation was carried out at 37° for 10 min, and the reaction was stopped by adding 1.0ml of 0.2 N HCl. To this mixture were added 0.25 ml of 0.2 % sodium nitrite, and 5 min later 0.25 ml of 1 % ammonium sulfate, and 3 min later 0.5 ml of 30 % sodium acetate. Immediately after the addition of sodium acetate, 0.25 ml of 0.2% N-(1-naphthyl)-ethylenediamine dihydrochloride was added and the mixture was allowed to stand for 10 min. Then 1.0 ml of 50 % trichloroacetic acid (TCA) and 1.5 ml of ethanol were added, and the color of the mixture was measured at 558 nm. One unit of the esterase was defined as the amount hydrolyzing one μ mole of acetanilide per min under the above assay conditions. The activities of NADH-ferricyanide reductase (9), NADH-cytochrome c reductase (10), NADPH-cytochrome c reductase (7), and gulonolactone dehydrogenase (11) were assayed by the published methods.

Analytical Methods -- Protein was determined by the method of Lowry et al. (12), using bovine serum albumin as the standard. Cytochrome b₅ was determined as described previously (13). Phospholipid was extracted from TCA-precipitated microsomes with ethanol-ether (3:1 by volume) according to the method of Volkin and Cohn (14). Phospholipid phosphorus was deter-

mined by Allen's method (15), and the value obtained was multiplied by 25 to obtain the amount of phospholipid. Microsomal phospholipid fraction was separated into its components by thin-layer chromatography as described by M. Noda (16). The liberation of choline from microsomes by phospholipase C and phospholipase D was estimated by the method of H. Staudinger (17). RNA was determined by the orcinol method of Mejbaum (18).

Purification of Esterase -- A suspension of washed microsomes in 0.1 M phosphate buffer, pH 7.5, (10 mg of protein per ml) was digested with 0.03 % trypsin at 4° overnight to solubilize about 25 % of the microsomal proteins including cytochrome b₅ and NADPH-cytochrome c reductase (1,4). The mixture was centrifuged at 105,000 × g for 90 min, and the suspension of the pellet retaining the esterase activity in the same buffer (30 mg of protein per ml) was treated with 9 vol. of acetone. The treated pellet was then dried with diethyl ether. The dried powder was homogenized with ca 20 vol. of 10 mM potassium phosphate buffer, pH 7.5, and the suspension was centrifuged at 105,000 × g for 60 min to obtain a clear supernatant. Most (70-90 %) of the esterase activity as well as about 20 % of the protein were usually extracted by this treatment. Nagarse was added to supernatant to a final concentration of 0.003 %, and the mixture was incubated at 4° overnight. After adding NaCl to 0.1 M, the esterase in the solution was precipitated by acetone fractionation (0 to 60 %). The precipitate collected by centrifugation at 6,000 × g for 10 min was dissolved in a small amount of 40 mM potassium phosphate buffer, pH 7.5, and then centrifuged as above to remove insoluble materials. The

solution was subjected to gel filtration through a Sephadex G-150 column (1.5 × 80 cm) which had been equilibrated with 40 mM potassium phosphate buffer, pH 7.5; elution was conducted with the same buffer. The esterase activity was eluted in two forms having a peak and the shoulder. The peak containing the dimeric form of esterase was eluted slightly behind the void volume, and the shoulder containing the monomeric form was eluted thereafter. For further purification, only the fractions corresponding to the peak (containing more than 150 munits per mg of protein) were combined and applied to a DEAE-Sephadex A-50 column (1 × 5 cm) equilibrated with 40 mM potassium phosphate buffer, pH 7.5. Immediately inactive proteins were eluted with 50 mM phosphate buffer of the same pH. The esterase was then eluted by increasing the buffer concentration to 80 mM. The fractions thus eluted having almost the same specific esterase activities were combined and used as the purified preparation.

Preparation of Antiserum to Purified Esterase -- Two mg of the purified esterase preparation obtained as described above in 1.0 ml of 80 mM potassium phosphate buffer, pH 7.5, were mixed with 1.0 ml of Freund's complete adjuvant (Difco Company), and the mixture was injected subcutaneously to an albino rabbit weighing 2.5 Kg. Four weeks after the first injection, 1.0 mg of the purified enzyme was injected intravenously to the same animal. One week after the second injection, blood was collected from the ear vein of the immunized animal. The γ -globulin fraction was prepared from the blood by the conventional ammonium sulfate fractionation method. The γ -globulin fraction was also obtained from a control rabbit.

RESULTS

Esterase as a Microsomal Component -- Fig. 1 shows the distribution of the acetanilide-hydrolyzing activity among subcellular fractions of rat liver. This distribution pattern is similar to those of typical microsomal enzymes such as glucose-6-phosphatase and aniline hydroxylase (20,21) and confirms the microsomal localization of the esterase reported for pork liver (2). However, the subcellular fractions studied in Fig.1 were not washed, and therefore the possibility still remained that the enzyme had been adsorbed to microsomes during the fractionation procedure. That this was not the case was shown by the observation that washing of the microsomes with 1.0 and 2.0 M NaCl resulted in no loss of the esterase activity. It was thus established that the esterase is a true microsomal component but is not an adsorbed artifact. As shown in Table I, the esterase activity was present in both rough and smooth microsomes, and the specific esterase activities in the two submicrosomal fractions were almost identical with each other when expressed per mg of phospholipid. Since the phospholipid content can be regarded as proportional to the amount of membranes, it was concluded that the esterase is bound to the microsomal membranes but not to ribosomes.

Esterase Solubilization by Detergents -- A series of non-ionic detergents, having nonylphenyl group as hydrophobic part and differing lengths of polyoxyethylene side chain as hydrophilic part, were tested, each at a concentration of 2.5 mM, to solubilize the esterase and some other enzymes from the smooth subfraction of liver microsomes. The results are shown in Fig.2,

where the hydrophile-lipophile balance (HLB) values of the detergents used are plotted against the degrees of enzyme solubilization. Detergents with higher HLB values represent those containing more hydrophilic groups in the molecule. It was found that treatment of microsomes with the detergents having HLB values lower than about 17 caused efficient solubilization of the esterase. This solubilization behavior of the esterase was markedly different from the other two microsomal enzymes studied, i.e. gulonolactone dehydrogenase and NADH-ferricyanide reductase. The latter enzyme was not solubilized to a significant extent with all the detergents tested at 2.5 mM. Then the effect of detergent concentration on the solubilization of esterase was studied using two of the detergents, Emalgen 911 and Emalgen 920, having HLB values of 13.7 and 15.5, respectively. As shown in Fig.3, the esterase was efficiently solubilized at the detergent concentration range of 1-2 mM, where gulonolactone dehydrogenase and NADPH-cytochrome c reductase were practically not solubilized at all. It should be noted that the detergent treatments of microsomes were usually accompanied by considerable inactivation of the esterase.

Alkali Treatment of Microsomes -- The esterase was solubilized by treatment of microsomes with NH_4OH as in the case of nucleoside diphosphatase (22). The microsomal suspension was adjusted to pH 10.8 by the addition, with vigorous stirring, of 28 % NH_4OH and was then immediately brought back to pH 6.5 with glacial acetic acid. Prolonged treatment of the microsomal suspension at the alkaline pH resulted in a pronounced loss of the enzyme activity. The suspension was then centrifuged for

90 min at 105,000 × g. It was thus found that 50-65 % of the total activity of the suspension was recovered in the supernatant together with about 20 % of the protein. This treatment, however, failed to release NADPH-cytochrome c reductase, NADH-ferricyanide reductase, and cytochrome b₅ from the microsome membranes.

Sonication of Microsomes -- Treatment of microsomes with sonic oscillation at 30° also solubilized esterase, though at 0° the solubilization was much less. None of the other microsomal enzymes tested were released from microsomes by the sonic treatment both at 30° and 0°, although about 15 % of protein was solubilized.

Organic Solvent Treatments of Microsomes -- Lyophilized microsomes were treated with organic solvents; the treatment was carried out at temperatures below 0° using an ice-salt mixture. The treatment with 90 % acetone removed most phospholipid from microsomes, whereas 100 % acetone and 100 % diethylether removed phospholipid only partially (Table II). After the treatment, the organic solvent used was completely removed by evacuation. The microsomal powder thus obtained was then homogenized with 50 mM Tris-HCl (pH 7.5), and the suspension was centrifuged at 105,000 × g for 90 min. It was thus found that almost all the esterase activity of the powder prepared with 90 % acetone was recovered in the supernatant. The enzyme was, however, only partially extracted from the powders prepared with 100 % acetone and 100 % diethylether (Table II). The other microsomal enzymes assayed (cytochrome b₅ and NADPH-cytochrome c reductase) were not solubilized by any of the

organic solvent treatments.

Treatments with Phospholipases -- Since the results of organic solvent treatments suggested that removal of phospholipid is related to the release of the esterase, it was of interest to examine the actions of phospholipases on microsomes. Thus, microsomes were treated with snake venom phospholipase A at pH 6.8, where lysophospholipid produced by the hydrolysis of membrane lipid exerts no appreciable detergent action. The action of 0.001 % phospholipase A overnight at 0° caused the decomposition of 50-60 % of the microsomal phospholipid as revealed by thin-layer chromatographic analysis of phospholipid (Table III). This treatment also resulted in about 50 % inactivation of esterase activity. However, the remaining activity was quantitatively recovered in the 105,000 × g supernatant. The phospholipase A treatment could not liberate NADH-ferricyanide reductase, NADPH-cytochrome c reductase, and cytochrome b₅ from microsomes. In contrast to phospholipase A, neither C. perfringens phospholipase C nor cabbage phospholipase D caused appreciable solubilization of the esterase even when almost all the membrane phospholipid was attacked by the lipolytic enzymes (Fig. 4). It was also observed that the decomposition of all the phospholipid in microsomes by these enzymes was accompanied by about 50 % inactivation of the esterase. The treatments with phospholipases C and D also failed to solubilize the other microsomal enzymes such as NADH-ferricyanide reductase.

Protease Treatments -- Despite the fact that the esterase was readily solubilized by treatments with organic solvents, low concentrations of non-ionic detergents, etc., it was not solu-

bilized by the actions of proteases on microsomes. In confirmation of previous findings (1,4), both NADPH-cytochrome c reductase and cytochrome b₅ were very efficiently solubilized by incubating microsomes at 4° overnight with low concentrations of trypsin, but little solubilization of the esterase was achieved even at the highest concentration (0.1 %) of trypsin used (Fig. 5). Similar results were obtained when nagarse, papain, pepsin, and a fungal acid protease were employed instead of trypsin. These results suggested that the mode of linkage of the esterase to the microsomal membranes is entirely different from those of cytochrome b₅ and NADPH-cytochrome c reductase.

Purification of Esterase -- Based on the solubilization behavior of the esterase described above, attempts were made to purify the enzyme from liver microsomes and a purification procedure as described under Materials and Methods was finally adopted. In brief, this procedure consisted of 1) trypsin digestion of microsomes, 2) treatment of the trypsin-treated microsomes with 90 % acetone, 3) extraction of the acetone-dried powder with phosphate buffer, 4) gel filtration of the extract through Sephadex G-150, and 5) chromatography on a small column of DEAE-Sephadex A-50. As shown in Fig. 6, Sephadex G-150 gel filtration revealed the presence of two forms of the esterase activity, i.e. a peak eluted slightly behind the void volume containing the dimeric form of the esterase and the shoulder containing the monomeric form. Only the peak was used for the next step, because it was very difficult to purify from the shoulder to a satisfactory extent by the subsequent chromatographic step. This is the reason why a very poor recovery of

the enzyme was obtained in the gel filtration step (see Table IV). Fig. 7 shows the elution pattern obtained in the final step of DEAE-Sephadex chromatography. Because of the lability of the enzyme at this step, a small column and stepwise elution had to be used to minimize the time required to obtain separation. The peak eluted by 80 mM phosphate buffer contained most of the esterase activity. Two or three tubes of this peak, showing almost the same specific activities, were combined and used as the purified enzyme preparation.

As can be seen from Table IV, the esterase was purified about 50-fold with an overall yield of 4-5%. When subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (23), the purified preparation showed one major protein band together with a few impurity protein bands (Fig. 8); the molecular weight of the major protein component was roughly estimated to be 60,000-65,000.

Immunological Studies on Esterase Localization -- To determine the localization of the esterase in microsomal vesicles a rabbit antiserum was prepared against the purified esterase preparation. The γ -globulin fraction of the anti-serum did not inhibit the acetanilide-hydrolyzing activity of partially purified preparations of the esterase, but it precipitated the activity from solution (Fig. 9). The γ -globulin fraction of normal rabbit serum, on the other hand, did not precipitate the esterase activity. Graded amounts of the anti-esterase γ -globulin fraction (21.4 mg of protein per ml) was incubated with a sufficient amount of microsomes (198 munits esterase) at 30° for 30 min, and then the mixture was centrifuged at 105,000 \times g

for 90 min to remove the microsomal vesicles. If the antibody reacted with the esterase in the microsomal vesicles during the incubation, the antibody titer of the supernatant should have been reduced considerably. However, the capacity of the supernatant to precipitate the partially purified esterase was the same as that of the untreated anti-esterase globulin fraction. It was thus concluded that the esterase of microsomal vesicles did not react with the externally added antibody.

DISCUSSION

As reported by Krisch (2) for pig liver, acetanilide hydrolyzing esterase of rat liver is also recovered in microsomal fraction upon cell fractionation. The observation that this enzyme cannot be removed from microsomes by washing with salt solutions of high ionic strength excludes the possibility that this localization is an artifact arising from translocation and readsorption. The equal distribution of the enzyme between rough and smooth microsomes indicates that it is not associated with ribosomes. Instead, the parallelism between the esterase activity and phospholipid content in the two submicrosomal fractions suggests that this enzyme is attached to the membrane portion of microsomes. Although it is difficult to exclude the possibility that the esterase is located in the space enclosed by the vesicular membrane, it may be tentatively concluded that the enzyme is bound to the membrane itself. This conclusion receives a further support from the finding, to be reported in the accompanying paper (6), that the esterase level in liver microsomes remains almost unchanged even 4 hr after injection of cycloheximide to rats. Since most of the proteins present in the lumen of the endoplasmic reticulum of hepatocytes can be regarded as secretory proteins, it is quite unlikely that these proteins stay in the lumen for such a long time after cessation of their new synthesis by cycloheximide.

The solubilization behavior of the esterase from microsomes indicates that the mode of its attachment to the membrane is rather unique. The esterase can be readily solubilized by treatments with sonic oscillation, alkali, aqueous acetone, and low

concentrations of neutral detergents. Since all these treatments do not cause significant solubilization of most of the known microsomal enzymes, the linkage of the esterase to the membrane seems to be unusually weak. Phospholipase A, when acted on microsomes at pH 6.8, also causes little liberation of proteins. Yet the esterase is efficiently solubilized by this treatment. The efficient release of the esterase by such agents as aqueous acetone and phospholipase A may be taken as evidence for the involvement of phospholipids in the binding of the esterase to the microsomal membrane. This possibility is especially of interest in view of recent reports that mitochondrial NADH dehydrogenase (24) and monoamine oxidase (25) are bound to the membranes through acidic phospholipids. However, both phospholipases C and D are ineffective in solubilizing the esterase. An interesting, but not yet proven, possibility is that the fatty acid chains, but not the hydrophilic moiety, of membrane phospholipids play an important role in the binding to the esterase.

Despite the ease with which the esterase is liberated by various treatments, protease digestion of microsomes neither solubilizes nor inactivates the enzyme. This treatment, however, causes efficient solubilization of both NADPH-cytochrome c reductase and cytochrome b₅ together with a sizable amount of membrane proteins (1,4). Based mainly on the fact that the vesicular structure of microsomes is still preserved even after the protease digestion, it has been concluded that both the reductase and cytochrome are located at the outer surface of the microsomal membrane (4). Studies with antibodies to these

proteins have also supported this conclusion (5). In view of these observations, the resistance of the microsomal esterase to proteolysis suggests that the enzyme is not exposed to the outside medium surrounding the microsomal vesicles. The validity of this concept is more clearly supported by the demonstration that an antibody to the esterase fails to react with the enzyme bound to microsomes.

Two possibilities should, therefore, be considered regarding the intramicrosomal location of the esterase; 1) it is buried in the hydrophobic matrix of the membrane, and 2) it is anchored to the inner surface of the vesicular membrane. Of these two alternatives, the first possibility seems to be unlikely because the purified esterase is considerably hydrophilic and freely soluble in water. The observations that the esterase is easily solubilized by treatments which do not release most of the typical microsomal proteins are also against the first possibility. It may, therefore, be concluded that the esterase is attached by a very weak linkage to the inner surface of the vesicular membrane. It is likely that the esterase is readily liberated from microsomes when the vesicular structure is ruptured by sonication, aqueous acetone, alkaline pH, and so forth. Protease digestion is, however, incapable of releasing the esterase because this treatment does not destroy the vesicular structure (4). The localization of the enzyme at the inner surface of the membrane is also compatible with our preliminary observations that the purified ¹⁴C-labeled esterase does not exchange with the microsomal bound enzyme and that the purified enzyme is not rebound to microsomes from which the

esterase has been removed by sonication. The mode of attachment of the esterase to microsomes discussed above seems to resemble that of nucleoside diphosphatase which is also easily solubilizable by alkali or low concentrations of detergents but is resistant to proteolytic digestion (3).

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FIGURE LEGENDS

Fig. 1. Subcellular distribution of acetanilide hydrolyzing esterase in rat liver. Cell fractionation was carried out as described previously (21). The ordinate indicates the specific activity of the esterase per mg of protein. The subcellular fractions are shown in the abscissae by their relative protein contents in the order of their isolation. N, Mt, Ly, Ms and S represent the nuclear, mitochondrial, lysosomal, microsomal and soluble fractions, respectively.

Fig. 2. Solubilization of enzymes and protein from liver microsomes with polyoxyethylene nonylphenylethers having different HLB values. Smooth microsomes suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 2.5 mM detergent (10 mg of protein per ml) were incubated at 0°C for 30 min, and then the suspension was centrifuged at $105,000 \times g$ for 90 min. Percentages of the esterase (acetanilide hydrolase), gulonolactone dehydrogenase, and NADH-ferricyanide reductase activities as well as microsomal protein solubilized in the supernatant were determined.

Fig. 3. Effects of concentrations of two nonionic detergents, Emalgen 911 (HLB, 13.7) and Emalgen 920 (HLB, 15.5), on the solubilization of the esterase, gulonolactone dehydrogenase, and NADPH-cytochrome c reductase from liver microsomes. The detergents used were both polyoxyethylene nonylphenylethers. The experimental conditions were the same as in Fig. 2, except that the indicated detergents were used at different concentrations.

Fig. 4. Solubilization and inactivation of the microsomal esterase by phospholipases C and D. Phospholipase C digestion was conducted at 30°C for 30 min, and the reaction mixture contained liver microsomes (10 mg of protein per ml), 0.05 M borate buffer, pH 7.5, 5 mM CaCl₂, and various amounts of C. perfringens phospholipase C. The reaction mixture for phospholipase D digestion contained liver microsomes (10 mg of protein per ml), 0.1 M acetate buffer, pH 5.6, 0.5 mM CaCl₂, and various amounts of cabbage phospholipase D. The reaction was run at 30°C for 60 min. Liver microsomes were prepared from rats which had been injected with 5 µCi of ¹⁴C-labeled choline 30 min prior to sacrifice. The amount of phospholipids decomposed by digestion was estimated from the radioactivity recovered in the supernatant (105,000 × g for 90 min) of the reaction mixture.

Fig. 5. Tryptic solubilization of the esterase, cytochrome b₅, and NADPH-cytochrome c reductase from liver microsomes. Liver microsomes suspended in 0.1 M phosphate buffer, pH 7.5 (10 mg of protein per ml), were digested at 4°C overnight with indicated concentration of trypsin. The suspension was centrifuged at 105,000 × g for 90 min. The contents of the enzymes and protein solubilized in the supernatant were determined.

Fig. 6. Sephadex G-150 gel filtration of esterase. Gel filtration procedures used were as described in the Materials and Methods. Flow rate was 40 ml per hour, with the elute being collected in 4.0 ml fractions.

Fig. 7. DEAE-Sephadex A-50 chromatography of esterase.

Chromatographic procedures were carried out as described in the Materials and Methods.

Fig. 8. Polyacrylamide gel electrophoresis of the purified esterase preparation. Electrophoresis was carried out at room temperature according to the method of Weber and Osborn (23). A current of 7 mA per tube was applied for 5 hr, and 5 % gel was used. After staining with Coomassie brilliant blue, the gel was destained with 7 % acetic acid - 25 % methanol solution.

Fig. 9. Precipitation of purified esterase by anti-esterase antibody and failure of microsomes to adsorb the antibody. To a solution containing 220 milliunits of partially purified esterase in 0.25 M sucrose - 20 mM phosphate buffer, pH 7.5 was added indicated amount of the γ -globulin fraction of rabbit anti-esterase serum or that of control serum. The mixture was incubated at 30°C for 30 min and then centrifuged at 3,000 \times g for 20 min to precipitate the antigen-antibody complex. The esterase activity remaining in the supernatant was measured (o—o). In another experiment, indicated amount of anti-globulin was first incubated with microsomes having 198 milliunits of the esterase activity. After incubation at 30°C for 30 min, the mixture was centrifuged at 105,000 \times g for 90 min to sediment the added microsomes. To the supernatant thus obtained were added 220 milliunits of partially purified esterase as above. The mixture was then incubated and centrifuged and the esterase activity remaining in the final supernatant was measured as above (x-----x).

Table I

Distribution of Esterase between Rough
and Smooth Microsomes

Rough and smooth microsomes were prepared as described in
the Materials and Methods.

Fraction	RNA	Phospholipid(PL)	Esterase	
	$\mu\text{g}/\text{mg prot.}$	$\mu\text{g}/\text{mg prot.}$	$\text{munits}/\text{mg prot.}$	$\text{munits}/\text{mg PL}$
Smooth microsomes	16	404	9.1	22.5
Rough microsomes	253	302	5.9	19.6

Table II

Solubilization of Esterase from Microsomes Treated with 90 % Acetone, 100 % Acetone, and 100 % Diethylether

Lyophilized microsomes were treated by the organic solvents to give the dried powder of microsomes. The powder was extracted with Tris-HCl buffer (pH 7.5) as described in the text, and esterase activity in supernatant and whole suspension was assayed. The percentage of phospholipid remained in microsomal powder after solvent treatment is also shown in the Table.

Treatment	Protein Sol.(%)	PL in residue (%)	Esterase Sol.(%)
Buffer*	11.3	100	15.9
90% Acetone	19.3	19.7	97.7
100% Acetone	16.6	83.5	55.0
100% Ether	15.1	77.6	41.0

*, 10 mM potassium phosphate, pH 7.5 was used for the extraction.

Table III

Solubilization of Esterase by Phospholipase A.

Washed microsomes were digested overnight at 0° in 0.1 M Tris-HCl (pH 6.8) containing 0.1 mM CaCl₂ with indicated amount of snake venom phospholipase A. The suspension was centrifuged at 105,000 × g for 90 min. The phospholipid fraction of the reaction mixture was subjected to thin-layer chromatography and phosphatidyl choline and phosphatidyl ethanolamine separated were determined from phospholipid-phosphorus obtained by Allen's procedure (15).

PLase A concentration (%)	Esterase (%)		Phospholipid decomposed (%)	
	Activity	Solubilization	Phosphatidyl- choline	Phosphatidyl- ethanolamine
0	100	5.9	-	-
0.001	45.2	97.1	64.0	54.5

Table IV
A Summary of Purification of Esterase
from Rat Liver Microsomes

	Total protein mg	Total activity units	Specific activity munits/mg prot.
Washed microsomes	2820	48.3	17.2
Extract from acetone powder	541	30.5	56.3
G-150 filtration	31.4	5.5	175
DEAE-Sephadex	3.0	2.3	770

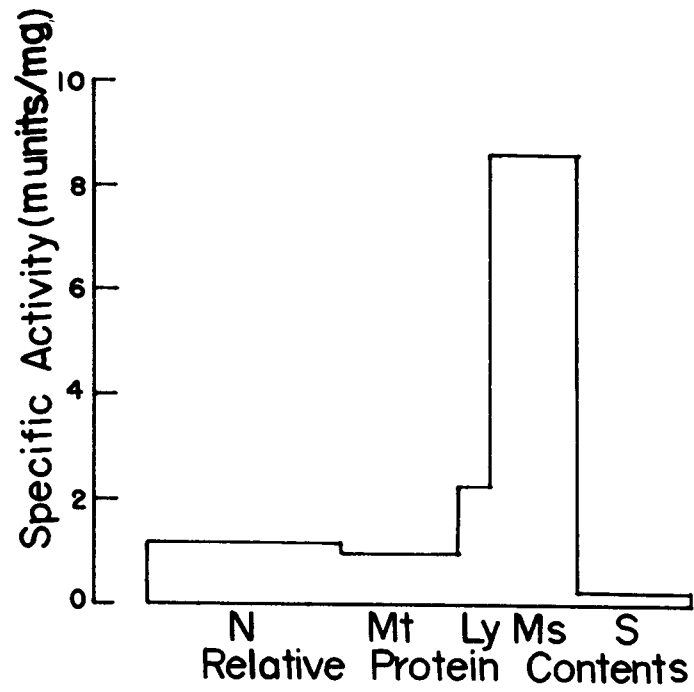


Fig. 1. Subcellular Distribution of Acetanilide-hydrolyzing Esterase in Rat Liver.

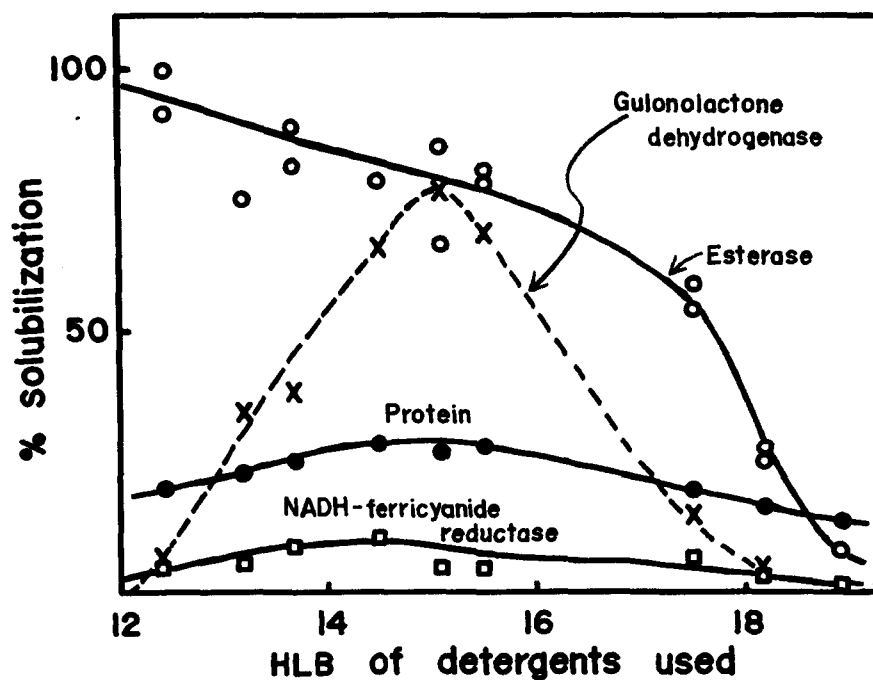


Fig. 2. Solubilization of Enzymes and Protein from Liver Microsomes with Polyoxyethylene Nonylphenylethers Having Different HLB Values.

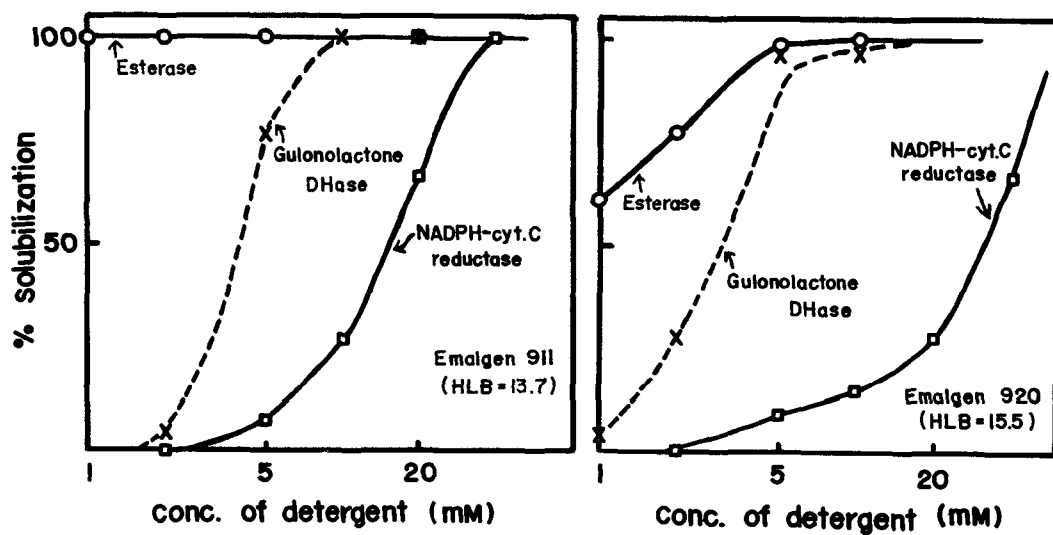


Fig.3. Effects of Concentrations of Two Nonionic Detergents, Emalgen 911 (HLB, 13.7) and Emalgen 920 (HLB, 15.5), on the Solubilization of the Esterase, Gulonolactone Dehydrogenase, and NADPH-cytochrome c Reductase from Liver Microsomes.

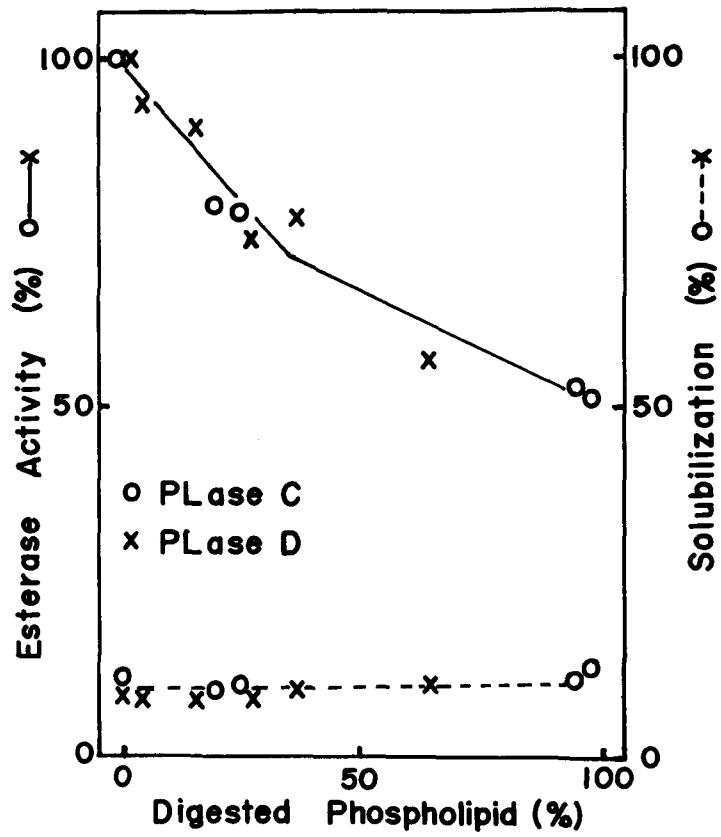


Fig. 4. Solubilization and Inactivation of the Microsomal Esterase by Phospholipases C and D.

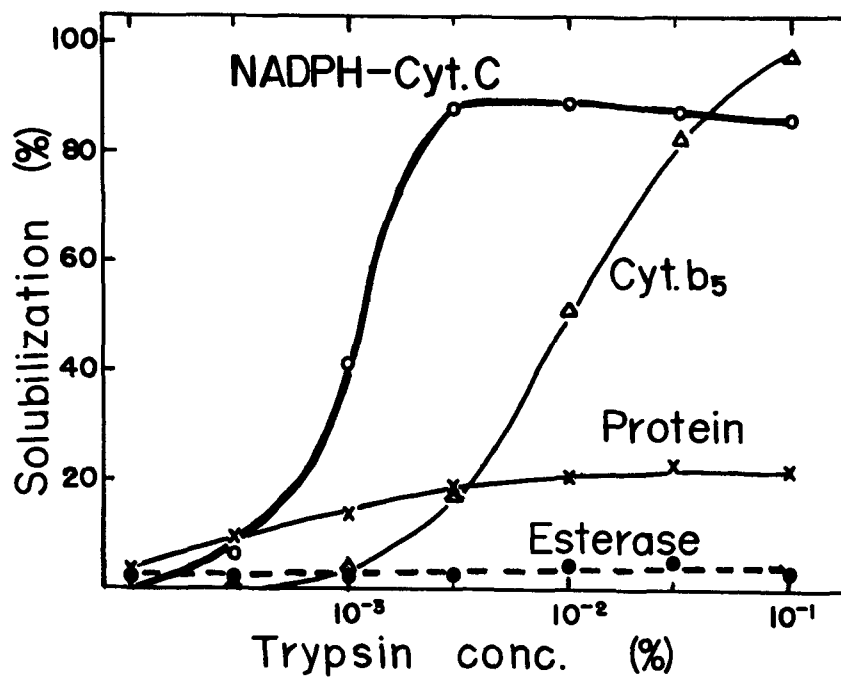


Fig.5. Tryptic Solubilization of Esterase, Cytochrome b₅, and NADPH-cytochrome c Reductase from Liver Microsomes.

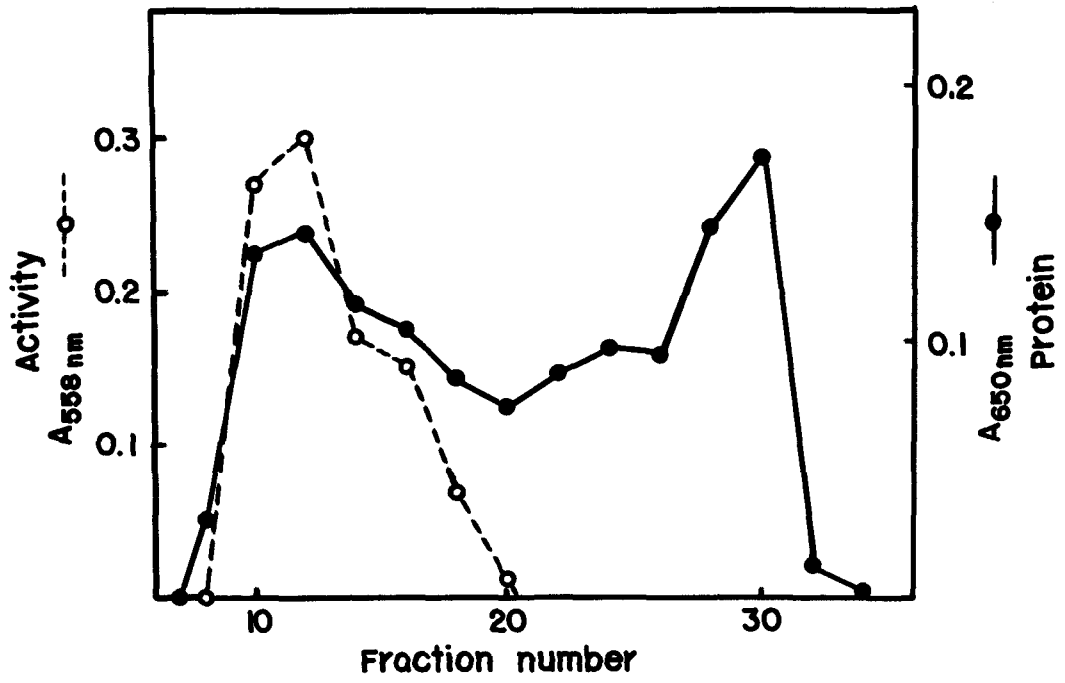


Fig.6. Sephadex G-150 Gel Filtration of Esterase.

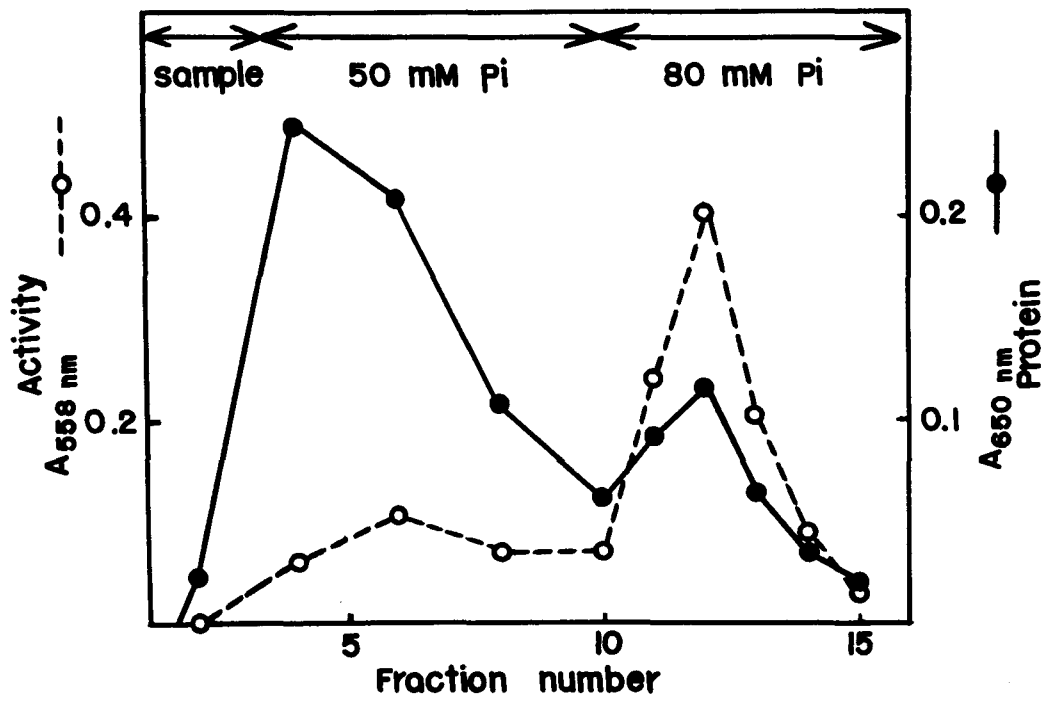


Fig.7. DEAE Sephadex A-50 Chromatography of the Esterase.

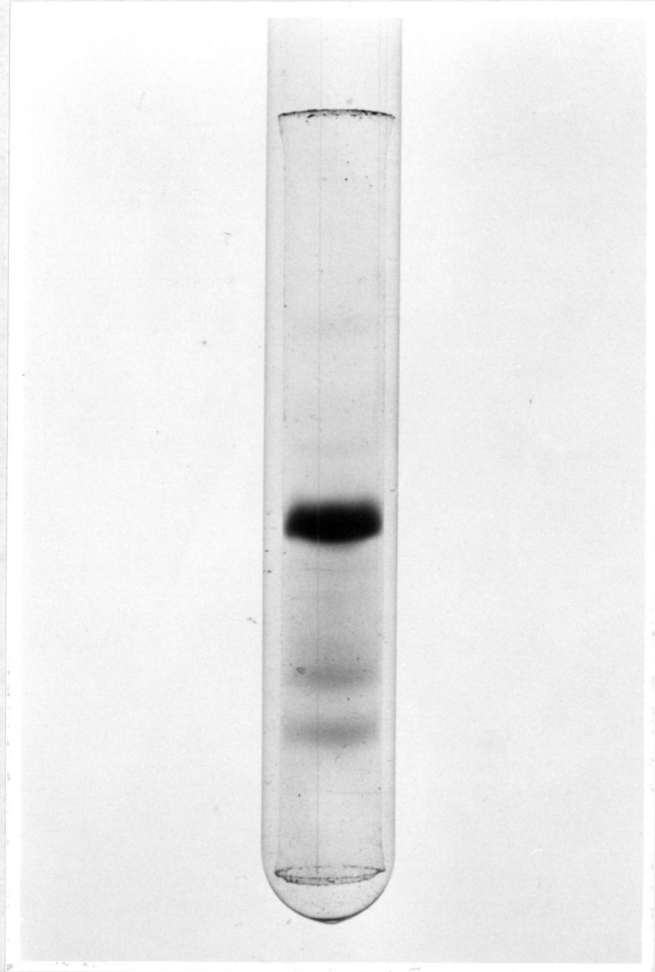


Fig.8. Polyacrylamide Gel Electrophoresis of the Purified Esterase Preparation.

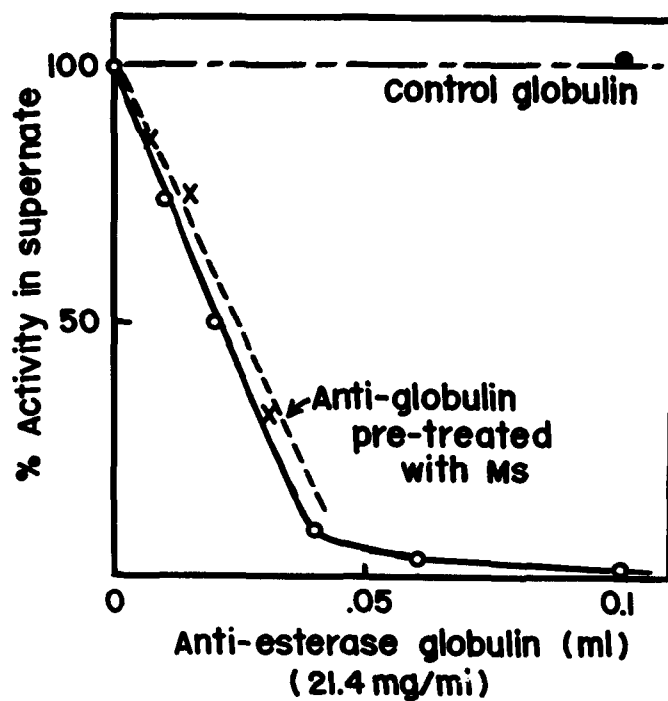


Fig.9. Precipitation of Purified Esterase by Anti-esterase Antibody and Failure of Microsomes to Adsorb the Antibody.

Studies on Acetanilide-hydrolyzing Esterase of Rat Liver
Microsomes. II. Turnover Studies.

Teruaki Akao

SUMMARY

The apparent rate of turnover in vivo of acetanilide-hydrolyzing esterase of rat liver microsomes was measured by isolating the enzyme at intervals after injection of ^{14}C -labelled leucine to the animals. The incorporation of the label into the enzyme was very rapid reaching a maximum 30 min after the injection. However, about 80 % of the newly synthesized enzyme disappeared rapidly within 90 min after the incorporation had reached the maximum. The degradation thereafter was very slow and a half life of about 4 days was determined for this slow phase. Since the esterase activity in liver microsomes was not lowered to any measurable extent even 4 hr after injection of cycloheximide to rats, it was suggested that the esterase undergoing the rapid degradation did not contribute significantly to the total esterase content in liver microsomes. Thus, it could be concluded that most of the esterase is turning over with a half life of 4 days. This finding, together with previous data on other microsomal proteins, provide additional evidence in favor of the independent turnover of microsomal membrane components.

INTRODUCTION

Omura et al. (1) have shown that two protein components, i.e. NADPH-cytochrome c reductase and cytochrome b₅, of the endoplasmic reticulum membranes of rat hepatocytes undergo turnover in vivo at significantly different rates. Subsequent work on other microsomal enzymes has also suggested that the individual components of the microsomal membranes are turning over independently (1,3-5). However, the number of the proteins so far studied in this respect is still limited. Therefore, it is necessary to extend this type of study to several more protein components of the microsomal membranes. In the accompanying paper (2), we reported that acetanilide-hydrolyzing esterase of rat liver microsomes can be highly purified by a relatively simple procedure starting from a small amount of material. Furthermore, we also showed that the esterase is different from most of the microsomal proteins including NADPH-cytochrome c reductase and cytochrome b₅, in the intramicrosomal localization and the mode of attachment to the membranes. It seemed therefore of interest to measure its turnover rate in vivo and compare it with those of other microsomal enzymes whose turnover rates have already been determined (1,3-6).

In this communication, we report that most of the esterase is turning over at a rate (half life, 4 days) which are different from those determined for other microsomal proteins. It is also reported that about 80 % of the newly synthesized esterase is rapidly lost from liver microsomes within about 2 hr, although the fate of this rapidly disappearing fraction of the esterase is not clear.

MATERIAL AND METHODS

Treatment of Animals -- Male Sprague-Dawley rats weighing 160 to 190 g were used. They were kept on a diet containing 15 % casein for about two weeks before initiation of the experiments. The rats were fasted for 24 hr before the injection of radioactive leucine ($1\text{-}^{14}\text{C}$ -DL-leucine or $1\text{-}^{14}\text{C}$ -L-leucine) which was given intravenously through the caudal vein as a solution in isotonic saline. The exact amount of the amino acid given to the animals is described below for each experiment.

Preparation of Hepatic Microsomes -- Liver microsomes were prepared from rats and washed as described previously (3).

Purification of Microsomal Enzymes -- Solubilization and purification of NADPH-cytochrome c reductase and cytochrome b₅ from liver microsomes were performed as reported previously (3). Acetanilide-hydrolyzing esterase was solubilized and purified by the small-scale procedure developed in the accompanying paper (2). This small-scale purification method yielded the esterase preparation having higher specific activities than previously described large-scale purification methods. The esterase preparations having 800-1000 munits per mg protein were used to measure its radioactivities. This value is comparable with that obtained by Krisch et al. for the purified esterase from pig kidney microsomes (18).

Analytical Methods -- NADPH-cytochrome c reductase and cytochrome b₅ in both microsomes and purified enzyme preparations were assayed as described previously (3). Esterase

were assayed using acetanilide as the substrate according to the procedure of Akao and Omura (2). Protein was determined by method of Lowry et al. (7) using bovine serum albumin as the standard.

The radioactivities of microsomal protein and purified enzyme preparations were determined as described previously (1). The radioactivities of gel pieces slice from polyacrylamide gel discs were determined by liquid scintillation counting in a toluene-Triton X-100 (5:3, by volume) solution.

Disc electrophoresis in polyacrylamide gel was performed at 4° according to Davis (8) using 7.5 % polyacrylamide gel (pH 8.9) or at room temperature according to Weber and Osborn (9) using 5.0 % polyacrylamide gel containing 0.1 % sodium dodecylsulfate (pH 7.2) as the separation gel. After electrophoresis, the gels were stained with Coomassie brilliant blue and then destained with 7 % acetic - 25 % methanol solution.

Reagents and Biochemicals -- NADPH and trypsin were obtained from Sigma Chemical Company. Nagarse was purchased from Nagase Industrial Company. 1-¹⁴C-DL-leucine (25 mCi per mmole) and 1-¹⁴C-L-leucine (40 mCi per mmole) were purchased from Dai-ichi Pure Chemicals Company and Radiochemical Centre, respectively. Yeast (Candida krusei) cytochrome c was a gift from Sankyo Company and further purified before use (10). Rabbit anti-serum to rat albumin was kindly supplied by Dr. K. Ogata of Niigata University.

RESULTS AND DISCUSSION

The time course of decay in vivo of specific radioactivity of the esterase in rat liver microsomes after injection of $1\text{-}^{14}\text{C}$ -DL-leucine (5 μCi per 100 g of body weight) to the animals is shown in Fig. 1, together with those for cytochrome \underline{b}_5 and total microsomal protein. The data on cytochrome \underline{b}_5 , which could be purified simultaneously with the esterase from the same microsomal preparation, were used as a marker of reutilization of the isotope under the conditions employed. This was possible because apparent half lives have already been determined for this cytochrome using both $1\text{-}^{14}\text{C}$ -DL-leucine and guanido- ^{14}C -L-arginine (1,3), the latter being known to be the least reutilizable amino acid precursor (11,12,13). As can be seen, the specific radioactivity of the esterase measured 3 hr after the injection was unusually high, deviating considerably from the line drawn with the other three points obtained 2, 4, and 6 days after the injection. This suggests that the decay in vivo of the esterase is apparently biphasic, consisting of a rapid phase and a slow phase. No such biphasicity was detected for cytochrome \underline{b}_5 and total microsomal protein.

From the data of Fig. 1, half lives of 4, 4.5-5, and 2.5-3 days were determined for the degradation of the esterase (slow phase), cytochrome \underline{b}_5 , and total microsomal protein, respectively. The values obtained for cytochrome \underline{b}_5 and total microsomal protein are in good agreement with those reported previously using $1\text{-}^{14}\text{C}$ -DL-leucine (1,3) and some 20 % longer than those determined with guanido- ^{14}C -L-arginine (3). The half life of 4 days obtained for the slow phase of the esterase

degradation is considerably longer than the value of 50-60 hr obtained by Krisch et al. (20) by an indirect method. At any rate, these data, together with half lives obtained previously for some other microsomal proteins, e.g. 2.5 days for NADPH-cytochrome c reductase (1,3), 1-1.5 day for nucleoside diphosphatase (5), and 18 days for NADH glycohydrolase (4), reinforce the previously proposed view (1,3,4,5) that protein components of the endoplasmic reticulum membranes undergo degradation in vivo independently from one another under the steady state.

Having determined the half life of the esterase at its slow degradation phase, we then proceeded to explore the unusual biphasic decay of the enzyme. For this purpose, it was necessary to follow the change in specific radioactivity of the enzyme during short periods after injection of a labelled amino acid. $1-^{14}\text{C}$ -DL-leucine used in the above experiment is not suitable for such short term experiments, because Negishi and Omura (15) have recently found ^{that} the D-isomer of the labelled amino acid is slowly converted in vivo to the L-isomer and gradually incorporated into the tissue proteins. This indicates that a short pulse labelling cannot be obtained by injection of ^{14}C -DL-leucine. Therefore, we used $1-^{14}\text{C}$ -L-leucine as the label in the following experiments.

Fig. 2 shows the time courses of the changes in specific radioactivities of the esterase, NADPH-cytochrome c reductase, and total microsomal protein during the initial 3 hr period following the injection of ^{14}C -L-leucine to rats. NADPH-cytochrome c reductase was included in this experiment, since

the behavior of this enzyme during the short period after the ^{14}C -L-leucine has been determined (15). As can be seen, the label was rapidly incorporated into the esterase and incorporation reached a maximum about 30 min after the injection. However, after this point there was a rapid decrease in the specific radioactivity of the enzyme until it was lowered to about 20 % of the peak value within 90 to 120 min. A half life of about 30 min could be obtained for this phase. After this phase, however, the loss of the enzyme was very slow as described above. In confirmation of previous work (15), the patterns obtained for NADPH-cytochrome c reductase and total microsomal protein were qualitatively similar to that obtained for the esterase. These findings suggest that about 80 % of the esterase newly synthesized and bound to the microsomal membranes are removed or degraded.

Before discussing the significance of the observations described above, one must be cautious about the possibility that contamination of the purified enzymes by newly synthesized serum proteins is responsible for the phenomenon. Since these proteins, notably albumin, are synthesized on rough microsomes and secreted into blood stream within short periods (e.g. about 20 min in the case of albumin) (21), contamination of the purified enzymes by these proteins can be expected to affect the results of turnover studies of the type described above. In the case of NADPH-cytochrome c reductase, the purification method employed has been shown to yield the enzyme in a homogeneous state (22) and thus excludes the possibility of contamination by secretory proteins. However, the

esterase purified was not completely pure, as judged from the presence of several minor protein bands in polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (Fig. 3). It was, however, clear that the major band represented the esterase, because it corresponded to a molecular weight of about 62,000, a value which is identical with that reported for the esterase obtained from pig and bovine liver microsomes in a seemingly homogeneous state (17).

We therefore checked if the time course shown in Fig. 2 for the esterase actually reflects the turnover of the enzyme itself but not the contaminating minor proteins. For this purpose, the same amount (60 μ g) of the esterase preparations purified from rats that had been killed 30 and 120 min after injection of 14 C-L-leucine were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. After staining with Coomassie brilliant blue and destaining, the gel columns were cut into slices at 2 mm intervals and the gel slices were dissolved in 30 % H_2O_2 , heated at 60°C for 6 hr (16), and then counted. The results thus obtained are illustrated in Fig. 4. It was thus found that the radioactivity associated with the major band corresponding the esterase decreased dramatically at 120 min to about 20 % of that obtained at 30 min. This indicates that the time course of turnover shown in Fig. 2 actually represents the behavior of the esterase at least qualitatively.

It was further found that more than 70 % of the radioactivity of the purified esterase preparation was not precipitated by rabbit antiserum to rat albumin, and that ethanol

extraction of the preparation after trichloroacetic acid precipitation, a procedure which removes albumin (19), did not decrease the radioactivity of the preparation significantly. These observations lend further support to the view that the rapid loss of radioactivity observed during 30 and 120 min after injection of labelled leucine is not due to contamination by secretory proteins, confirming that this initial disappearance of the newly synthesized protein from microsomes represents the actual behavior of the esterase in vivo. Similar behavior has recently been reported for NADPH-cytochrome c reductase (15) and was confirmed in this study.

At present we have no explanations for the fate of the esterase which is removed from microsomes shortly after its synthesis and incorporation into the membranes. Three possibilities should be explored in future in this respect. First, the enzyme is degraded into amino acids for certain reasons. Secondly, it is secreted into blood stream. Thirdly, it is transferred to some other subcellular organelles which are not recovered in the microsomal fraction. The third possibility seems, however, least likely in view of the clearly microsomal localization of the esterase on fractionation of liver homogenates (2). The second possibility is also unlikely, because we could not detect the esterase activity in rat serum. However, the possibility cannot be ruled out that the esterase is glycosylated before secretion and this process leads to the loss of the esterase activity.

As mentioned above, the radioactivity of D-leucine can be incorporated slowly into liver microsomal proteins, but this

incorporation does not last longer than about 2 hr (15). This fact validates the use of ^{14}C -DL-leucine as a label in the long-term experiments shown in Fig. 1. Moreover, when the esterase preparation, purified from rats that had been killed $\frac{1}{2}$ hr after injection of ^{14}C -DL-leucine, was subjected to disc electrophoresis in the presence of sodium dodecylsulfate, the major protein band corresponding to the esterase contained more than 70 % of the total radioactivity. It can, therefore, be concluded from the data of Fig. 1 that the slow degradation having a half life of 4 days is also inherent to the esterase itself.

Finally, we examined the change in the esterase level in liver microsomes after cycloheximide was injected to the animals to inhibit protein synthesis. The dose of cycloheximide used (0.2 mg per 100 g of body weight) has been shown to inhibit about 85 % of the incorporation of labelled leucine into microsomal proteins (15). As shown in Table I, the specific activity of the esterase in isolated microsomes remained essentially unchanged even 4 hr after the injection. Both the NADPH- and NADH-cytochrome c reductase activities were also unaffected, and the recovery of microsomal protein from 1 g of wet liver was also constant. This finding clearly indicated that the esterase which disappears rapidly after synthesis does not contribute significantly to the content of the esterase in microsomes, since inhibition of its synthesis by cycloheximide should cause a rapid decrease in the esterase content if the rapidly turning over portion of the esterase constitutes a considerable part of the microsomal esterase under the steady state.

In summary, the work presented in this paper reveals two interesting facets regarding the dynamic behavior of the endoplasmic reticulum membranes in vivo. First, this work provides another support to the concept that protein components of the endoplasmic reticulum membranes are turning over independently from one another. Secondly, considerable portions of the esterase as well as NADPH-cytochrome c reductase, which have been newly synthesized and incorporated into the membranes, are lost rapidly from the membranes by an unknown mechanism. More work in future is obviously needed to elucidate the significance of these findings in the membrane biology.

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Fig.1. Time courses of decay in vivo of specific radioactivities of the esterase, cytochrome b₅, and total protein of washed liver microsomes. 1-¹⁴C-DL-leucine (5 μ Ci per 100 g of body weight) was injected intravenously through the caudal vein to each rat. At each time point, three rats were killed and microsomes, esterase and cytochrome b₅ were prepared from pooled livers and counted. Arrows denote half lives of the individual components determined graphically.

Fig.2. Changes in specific radioactivities of the esterase, NADPH-cytochrome c reductase, and total protein of washed liver microsomes during 3 hr after a single injection of ¹⁴C-L-leucine. ¹⁴C-L-leucine (3 μ Ci per 100 g of body weight) was injected intravenously to each rat at time 0. The animals were starved for 24 hr before the injection, and given no food except water during the experiment. The values obtained 10, 30, 60, and 120 min after the injection are the means of three independent determinations. Pooled livers from three rats were used for each time point.

Fig.3. Polyacrylamide gel electrophoresis pattern of the purified esterase. The purified esterase was analyzed by disc electrophoresis using column (5 \times 50 mm) of 5 % polyacrylamide gel containing 0.1 % SDS, pH 7.2 (left) and 7.5 % polyacrylamide gel of pH 8.5 (right) as the separation gel. About 60 μ g of enzyme protein was dissolved in a solution containing 1 % SDS, 4 M urea, and 1 % mercaptoethanol and applied to the left gel. Electrophoresis was carried out at room temperature with a current of 7 mA per column as described by Weber and Osborn (9). The same amount of enzyme was dialyzed 0.05 M Tris-

glycine buffer, pH 8.5, and applied to the right gel. Electrophoresis was carried out at 4°C with a current of 3 mA per column according to Davis (8).

Fig.4. Distribution of ^{14}C in polyacrylamide gel electrophoreograms of the esterase which was purified 30 and 120 min after ^{14}C -L-leucine injection. Ten male rats weighing 160 to 180 g were injected intravenously with ^{14}C -L-leucine (5 μCi per 100 g of body weight), and five rats each were killed 30 and 120 min after the injection and the esterase was purified from their liver microsomes. The esterase purified at each time point was subjected to disc electrophoresis in 5 % polyacrylamide gel containing 0.1 % SDS, pH 7.2. After staining with Coomassie brilliant blue and destaining, the gel was cut into 2 mm slices and each slice was dissolved in 30 % H_2O_2 , heated at 60°C for 6 hr, and then count. Slice number 10 corresponded to the major protein band representing the esterase.

Table I. Effects of cycloheximide injection on the levels of the esterase, NADPH-, and NADH-cytochrome c reductase activities in rat liver microsomes.

Cycloheximide (0.2 mg per 100 g of body weight) was injected intravenously through the caudal vein into each rat. At each time point three rats were killed and microsomes were prepared from pooled livers. The animals were starved for 24 hours before the injection, and given no food except water during the experiment.

Hours after cycloheximide injection	NADPH-cytochrome c reductase units/mg prot.	NADH-cytochrome c reductase units/mg prot.	Esterase munits/mg prot.	Microsomal protein per g liver mg
0	0.127	0.55	16.7	14.6
1	0.142	0.56	19.8	14.0
2	0.125	0.61	19.6	12.7
4	0.160	0.90	13.5	13.6

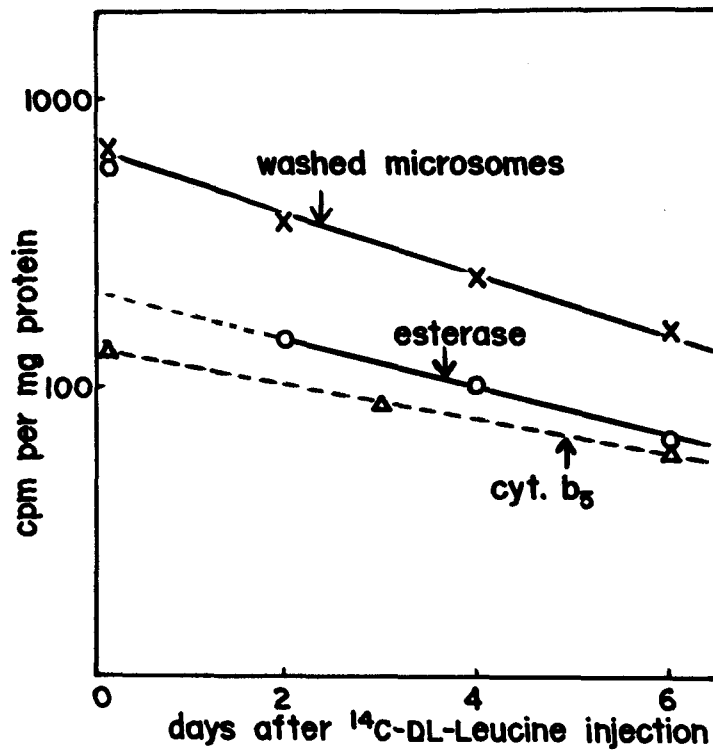


Fig.1. Time Courses of Decay in vivo of Specific Radioactivities of the Esterase, Cytochrome b₅, and Total Protein of Washed Liver Microsomes.

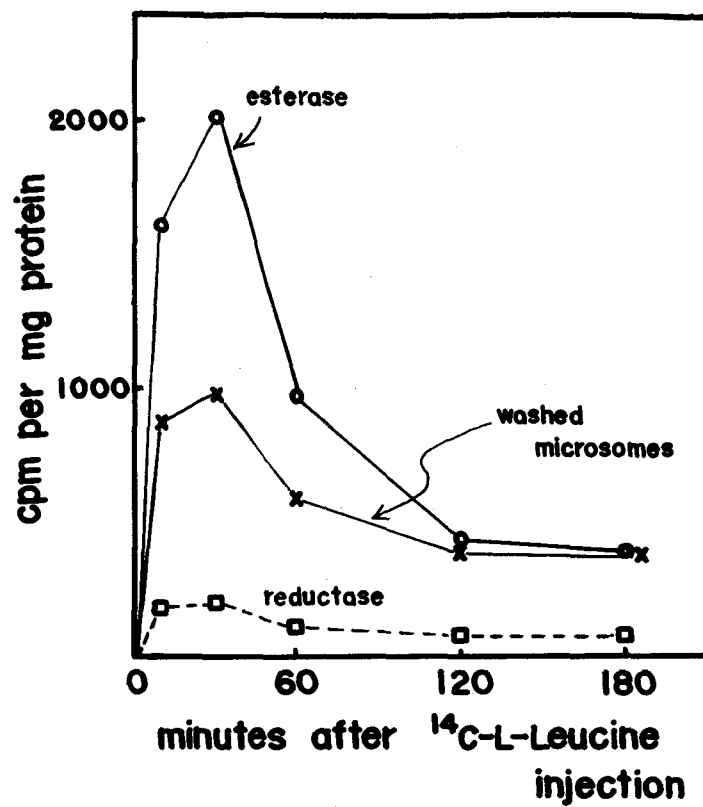


Fig.2. Changes in Specific Radioactivities of the Esterase, NADPH-cytochrome c Reductase, and Total Protein of Washed Liver Microsomes during 3 hr after a Single Injection of ^{14}C -L-leucine.

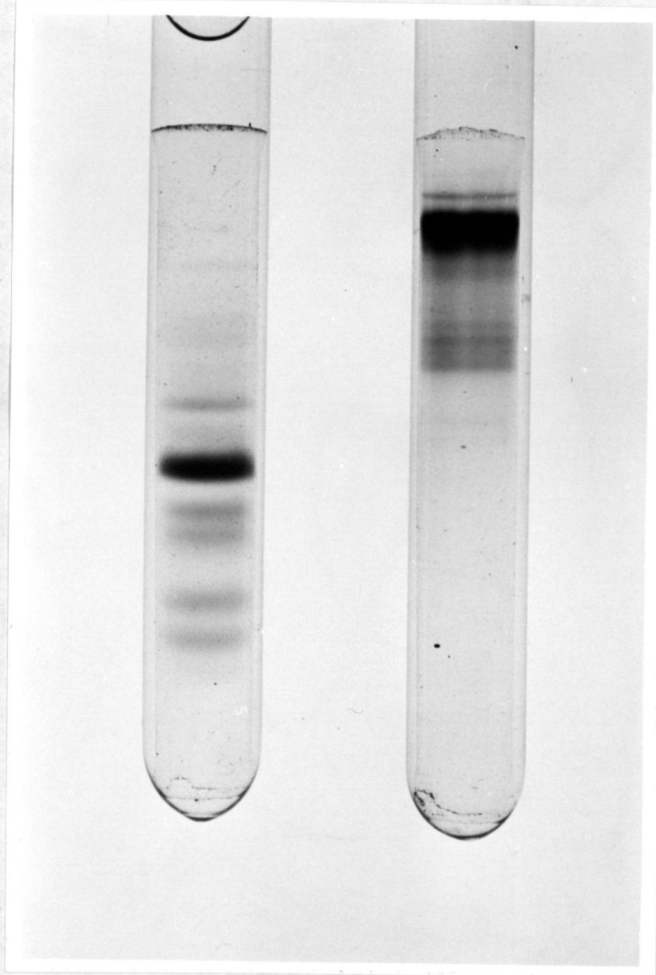


Fig.3. Polyacrylamide Gel Electrophoresis Pattern of the Purified Esterase.

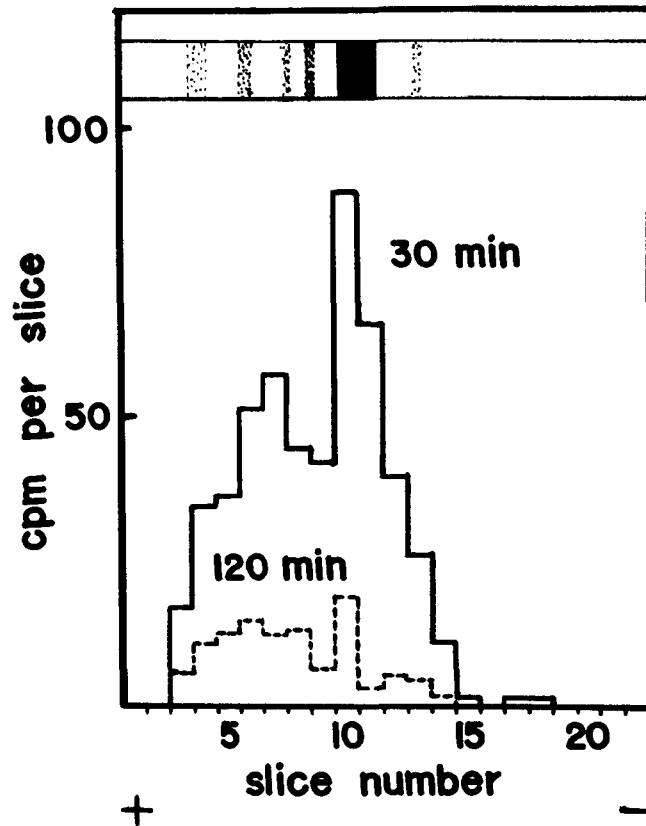


Fig.4. Distribution of ^{14}C in Polyacrylamide Gel Electrophoreograms of the Esterase Which was Purified 30 and 120 min after ^{14}C -L-leucine Injection.