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STUDIES ON PLASMA MEMBRANES
XIII. Co^{2+}-ACTIVATED AMINOPEPTIDASE(S) IN THE GLOBULAR UNITS LOCALLY COATING RAT-LIVER PLASMA MEMBRANES

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SUMMARY

1. Isolated rat-liver plasma membranes, freed from protein soluble in 0.15 M NaCl, hydrolyzed leucyl-β-naphthylamide, leucinamide, leucylglycine, leucylglycylglycine and glycylglycylglycine, but not glycylglycine and glutathione.

2. Peptidase activity was inhibited by EDTA, activated by Co^{2+}, thiol independent, and optimal around pH 8.0. In these and other respects, the plasma-membrane enzyme activity resembled the particulate rat-kidney aminopeptidase described by FELGENHAUER AND GLENNER[1]. Some indications of enzyme diversity with respect to various of the hydrolyzed peptides were obtained.

3. The aminopeptidase activity, as shown for all substrates, was completely released from the membranes by papain (EC 3.4.4.10); trypsin (EC 3.4.4.4) was ineffective. Other membrane enzymes were not (lipase, EC 3.1.1.3) or only partially (arylesterase, EC 3.1.1.2) released by papain.

4. The gel-filtration profiles of the papain-released aminopeptidase activity were similar for all substrates, but differed markedly from that of the arylesterase activity released from the plasma membranes under similar conditions, while the latter closely resembled the profile of the microsomal esterase activity released by papain.

5. On account of these and other criteria established previously, it is concluded that the aminopeptidase(s), in contrast to arylesterase, lipase, alkaline glycerolphosphatase (EC 3.1.3.1) and other plasma-membrane enzymes, is (are) associated with the 50–60-Å globular knobs which coat the liver-plasma membranes lining the bile spaces. The globular units are functionally specialized particles covering plasma membranes at areas of transport, and each particle very probably contains but one enzyme molecule.

INTRODUCTION

In a previous paper of this series[2], the view has been presented that the enzymic hydrolysis of leucyl-β-naphthylamide displayed by isolated rat-liver plasma membranes is associated with the 50–60-Å globular knobs present on these membranes. The evidence was as follows: (1) the enzyme activity was completely released from

the membranes by papain but not at all by trypsin; (2) the globular units were no longer detectable on membranes which had been pretreated with papain, while they remained present on trypsin-treated membranes; (3) particles resembling the globular units were abundantly present in the supernatant of the papain-treated membranes, and fractions derived therefrom, which showed enriched specific enzyme activities. From these results (1–3) combined with (4) the finding that the globular units were restricted to certain of the isolated plasma-membrane sheets, (5) the histochemical presence of the enzyme activity along the plasma membranes lining the bile spaces (canaliculi) *in situ*, and (6) the abundant presence of bile-space-like structures in the isolated membrane preparations, it was concluded that the plasma-membrane enzyme hydrolyzing leucyl-β-naphthylamide was exclusively present in, and served as a "marker"-enzyme for the globular knobs coating the bile-space-lining membranes.

Following the finding that leucinamide, leucylglycine, leucylglycylglycine and triglycine were also hydrolyzed by the plasma membranes, an attempt was made in the present investigation to characterize these enzymic activities and to trace their location. Enzyme properties were studied by the usual procedures. Enzyme localization in the 50–60-Å globular knobs was established using the criteria that the hydrolytic activity towards all four substrates should be completely released from the membranes by papain, resistant against trypsin, and present in the globule- and leucyl-β-naphthylamide hydrolase-rich fractions derived from the papain-treated membranes by gel filtration.

MATERIALS AND METHODS

Plasma membranes were isolated[3] from the livers of adult rats, mostly males from the inbred strain R-Amsterdam. In all experiments the isolated membranes were first freed from their saline-soluble proteins by suspension in 0.9 % NaCl overnight at 0°, followed by washing and resuspension in 1 mM $NaHCO_3$ (pH 7.5). Mild digestion with activated papain (concn. 1:500 with respect to membrane protein by wt.) for 10–15 min at 37° was carried out as described[2]. Membranes were treated with trypsin (1:200) for 10–15 min at 37° in 0.1 M phosphate buffer (pH 7.0). Soluble fraction and sediment were obtained from the papain and trypsin-treated membranes by centrifugation for 1 h at 32000 rev./min in a Spinco rotor 40 holding 3-ml adaptors. Trypsin and papain (both from Sigma) did not hydrolyze any of the substrates used in the present experiments. L-Leucyl-β-naphthylamide (0.37 mM) was incubated with membrane samples corresponding to 50–100 μg protein, measured according to the method of LOWRY *et al.*[4], routinely in phosphate buffer(pH 7.0) for 10–15 min at 37°, and the resulting β-naphthylamine was measured according to the method of GOLDBARG AND RUTTENBURG[5]. 5 mM L-leucinamide, L-leucylglycine, L-leucylglycylglycine (all from Sigma) and triglycine (British Drug Houses) were incubated for 45–60 min 37° with membranes (200–400 μg protein) in 0.04 M Tris–HCl buffer (pH 8.0). The use of other buffers and pH's are indicated in the text. The hydrolysis of the substrates was estimated by the ninhydrin method described by SPIES[7], or with 2,4,6-trinitrobenzene-1-sulfonic acid according to the method of SATAKE *et al.*[8] (*e.g.* for K_m value of triglycine hydrolysis). Leucinamide hydrolysis was also determined (*e.g.* for K_m value of leucinamide hydrolysis, and in the substrate competition experiments) by a modification of the microdiffusion method of CONWAY[9] measuring ammonia spectrophotometrical-

ly[10]. The effect of $CoCl_2$, $MgCl_2$, $MnSO_4$, $ZnSO_4$ and $CdSO_4$ on the peptidase activities was studied under various conditions mentioned in the text. Assay of arylesterase activity using α-naphthyl caprylate was performed according to NACHLAS AND SELIGMAN[11], that with *p*-nitrophenyl acetate as substrate according to the directions of HUGGINS AND LAPIDES[22].

Leucylglycylglycine, leucine, glycylglycine and glycine and the components in the reaction medium following incubation of membranes with leucylglycylglycine were identified by three-layer chromatography on DC-Fertigplatten Cellulose (Merck) using propanol-2–formic acid–H_2O (80:4:20, by vol.) for 3.5 h. Detection by ninhydrin. Puromycin (3 mM) inhibition of leucyl-β-naphthylamide hydrolysis by plasma membranes was studied in either 0.05 M Tris–HCl buffer (pH 7.4) using 135 μg membrane protein, incubation for 15 min with or without 30 min preincubation of membranes with puromycin at 37°, or in 0.1 M Tris–maleate buffer (pH 8.0) using 275 μg membrane protein preincubated for 60 min with puromycin, and incubation for 2 min. Triglycine and leucinamide hydrolysis by 275 μg membrane protein was assayed in both buffers incubated with the corresponding substrate for 60 min in the presence of puromycin.

For the gelfiltration experiments plasma membranes and microsomes were isolated[2] from 1 mM $NaHCO_3$ homogenates of liver, suspended for 45 min at room temperature in 0.9 % NaCl, spun down and washed. The sediment was resuspended in 1 mM $NaHCO_3$ to yield about 2 mg protein per ml and incubated for 10 min at 37° with activated papain (4 μg/ml). After chilling the preparations were subjected to centrifugation for 1 h at 32000 rev./min in an adapted Spinco rotor 40. 3 ml of the supernatant containing 1.7–2.3 mg protein was applied to a Sephadex G-200 column (37 cm × 2 cm), and eluted with 1 mM $NaHCO_3$ (or otherwise as indicated in the text). Fractions of 4 ml were collected at 15 ml/h. The void volume (40–44 ml) was measured with Blue Dextran 2000.

For the lipase assay, membranes corresponding to 340 μg protein, and the sediment (390 μg protein) and soluble fraction (100 μg protein) resulting from centrifugation of papain-treated membranes suspended in 0.2 or 0.3 ml 1 mM $NaHCO_3$, were added to 0.1 ml 0.1 M ammonium acetate buffer (pH 5.0) containing 10 % serum albumin and 0.2 ml of a 1 % ^3H-labelled triolein (3 μC) emulsion and made up to a final volume of 0.5 ml with bidistilled water, if required. Glyceryl-tri (oleate-9,10-T(n)), product of the Radiochemical Center (Amersham), was diluted to the appropriate specific activity with unlabelled triolein and the water suspension was subjected to ultrasonic oscillation for 10 min to yield a stable suspension. Incubation was carried out at 37° for 2 h. A control *minus* membranes was included. Incubation was stopped by addition of 2.5 ml of DOLE'S extraction mixture[14] containing 10 mg oleic acid per ml, the mixture was mechanically agitated by a Vortex apparatus for 30 sec, left standing for 10 min, followed by addition of 1 ml heptane and 1.5 ml water and 30 sec agitation. The upper heptane layer was separated and one aliquot was used for determination of total radioactivity whereas a second aliquot was used for separation on a column (28 cm × 0.53 cm) containing Florisil (6 g, containing 7 % water). Elution according to CHINO AND GILBERT[15] with 50 ml 15 % ether in hexane (to yield triglycerides), followed by 30 ml of 50 % ether in hexane (diglycerides), 2 % methanol in ether (monoglycerides) and 4 % acetic acid in ether (free fatty acids). Fractions of 7.5 ml were mixed with 10 ml BRAY'S solution[16] and counted in a Packard Tricarb

liquid scintillation spectrometer Model 4312. The radioactivity put on the column was fully recovered. From the drop in the triglyceride counts the hydrolysis of triolein was calculated as μg disappeared per mg membrane protein per h.

The papain-released globular knobs were negatively stained with phosphotungstate at low temperature and examined under the electron microscope (Philips EM 200) as described previously[2].

Analytical ultracentrifugation was performed in a Spinco Model E centrifuge equipped with absorption optics and an automatic scanning device, previously developed in this laboratory[17]; $A_{280\,nm}$ and the differentiated absorbance were recorded directly.

RESULTS

Peptide hydrolysis by plasma membranes

Specific enzyme activities

In 40 mM Tris–HCl buffer (pH 8.0) the plasma membranes hydrolyzed 4.50 $\pm$ 0.52 μmoles leucinamide, 1.41 $\pm$ 0.16 μmoles leucylglycine, 11.3 $\pm$ 1.2 μmoles leucylglycylglycine and 8.36 $\pm$ 0.94 μmoles triglycine per mg protein (0.15 M NaCl-insoluble) per h at 37° in six experiments. With the amounts of membranes (corresponding to 200–400 μg protein) used in these experiments, hydrolysis of the substrates (5 mM), except leucinamide, proceeded linearly during the 45–60-min incubation periods. With membranes corresponding to 400 μg protein, leucinamide hydrolysis deviated from linearity after 30 min of incubation. The initial reaction rate for this substrate amounted to 6.5 $\pm$ 0.7 μmoles split per mg protein per h at 37°. The rate of triglycine hydrolysis was directly proportional to membrane concentration (50–300 μg protein incubated during 20 min) and proceeded linearly up to 70 min, using 200 μg membrane protein.

Thin layer chromatography of the medium in which the membranes had been allowed to hydrolyze leucylglycylglycine for 20 and 60 min showed that leucine and glycylglycine had been formed, no glycine could be detected. Glycylglycine and glutathione were not hydrolyzed by the membranes.

K_m values

These were determined for triglycine, leucinamide and leucyl-β-naphthylamide hydrolysis by membranes suspended in 40 mM Tris–HCl (pH 7.6). The K_m for triglycine (200 μg membrane protein, 60 min incubation) and leucinamide (400 μg protein, 15 min) amounted to 1.8–2.0 and around $2.8 \cdot 10^{-3}$ M, respectively, as determined from substrate concentrations ranging from $1.5 \cdot 10^{-3}$ to $7.5 \cdot 10^{-3}$ M. As illustrated in Fig. 1 for triglycine hydrolysis, marked enzyme inhibition was observed for substrate concentrations higher than the latter value. The K_m value of leucyl-β-naphthylamide hydrolysis (50 μg protein, 15 min) was about $1.6 \cdot 10^{-4}$ M with substrate concentrations from $0.5 \cdot 10^{-4}$ to 10^{-3} M. No substrate inhibition was observed up to $2.5 \cdot 10^{-3}$ M, at higher concentration leucyl-β-naphthylamide became insoluble at pH 7.6.

The pH dependence and effect of buffer

The pH dependence was studied by incubating the membranes for 15 min in 0.1 M Tris–maleate buffers with leucyl-β-naphthylamide, triglycine or leucinamide. In all cases optimal activity was found at pH 8.0. Hydrolysis of triglycine at pH's 5.0, 6.0, 7.0 and 9.0 proceeded at respectively 11, 14, 89 and 75 % of the rate observed

at pH 8.0. The corresponding values for leucinamide were 17, 54, 83 and 90 % and for leucyl-β-naphthylamide 22, 80, 98 and 70 %. The solubility of the latter compound is limited at alkaline pH.

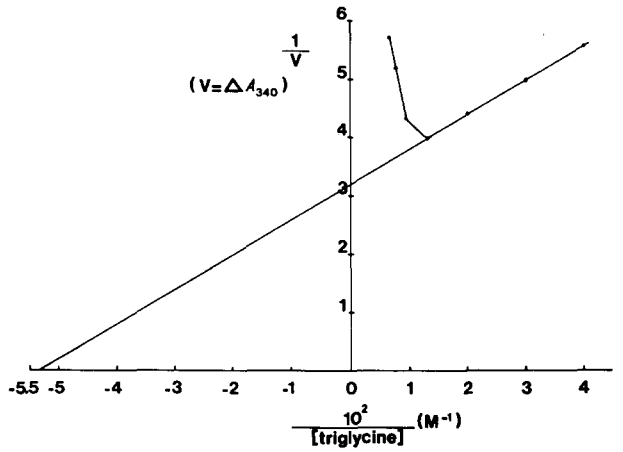

Fig. 1. Lineweaver–Burk plot for determination of K_m of triglycine hydrolysis. Note the autoinhibition at triglycine concentrations higher than 7.5 mM.

In view of some experiments reported below, the effect of 40 mM Tris–HCl and 0.1 M phosphate buffer (pH 7.0) on the enzymic hydrolysis of triglycine and leucinamide was studied. In Tris, respectively phosphate buffer of pH 7.0 leucinamide was split at 75–80 and 66–72 (70 ± 2) %, and triglycine at 79–88 and 53–63 (56 ± 4) % of the corresponding rates obtained in Tris buffer (pH 8.0). Phosphate buffer (pH 7.0) appears to be somewhat inhibitory to triglycine hydrolysis, a finding previously reported by ELLIS[18] for L-lysyl-*p*-nitroanilide hydrolysis by a thiol-activated aminopeptidase of the pituitary.

Thiol dependence

Mammalian tissues may contain various aminopeptidases, *e.g.* one that is activated by Mg^{2+} or Mn^{2+}, inhibited by EDTA and unaffected by SH-reagents, and another that is activated by thiols and inhibited by SH-reagents[18]. In the present experiments with plasma membranes the hydrolysis of leucyl-β-naphthylamide, leucylglycine, leucinamide and triglycine was not (significantly) affected by either *p*-hydroxy-mercuribenzoate (0.125 mM) or 2-mercaptoethanol (10 mM) following 60 min preincubation of membranes with these compounds.

Puromycin inhibition

Under the conditions described under MATERIALS AND METHODS, 3 mM puromycin inhibited leucyl-β-naphthylamide hydrolysis for about 50 %, while the hydrolysis of triglycine was inhibited for 0–14 %, and that of leucinamide for 19–21 %.

Metal dependence, inhibition and stimulation

For studying the effects of divalent cations and EDTA on triglycine, leucinamide and leucyl-β-naphthylamide hydrolysis by the plasma membranes, the latter were preincubated with Mg^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+} (1 mM) or EDTA (1–2.5 mM) for 1 h at 37° in twice-distilled water or 1 mM $NaHCO_3$ prior to addition of buffer and substrate, incubation and enzyme assay. As shown in Table I, Mg^{2+} was without effect,

TABLE I

EFFECT OF DIVALENT CATIONS AND EDTA ON PEPTIDE HYDROLYSIS BY PLASMA MEMBRANES

Relative activities (as % of controls preincubated in the absence of EDTA and cations) are illustrated for at least four experiments. Assay of leucyl-β-naphthylamide hydrolase activity in phosphate buffer (pH 7.0), that of triglycine and leucylamide in Tris buffer (pH 8.0); further conditions as described in the text.

Addition (*1 mM*)	*Relative activities*		
	Leucyl-β-naphthylamide	*Triglycine*	*Leucinamide*
None	100	100	100
EDTA	0	0	0– 10
Mg^{2+}	95–105	95–105	90–100
Cd^{2+}	0– 10	0	0
Co^{2+}	142–181	55– 68	55– 70
Mn^{2+}	71–100	41– 70	42– 61
Zn^{2+}	85–100	0	0

but Cd^{2+} and EDTA abolished all three peptide hydrolase activities; the latter finding indicated that these activities, including that acting on triglycine, were dependent on enzyme-bound metals. Zn^{2+} abolished triglycine and leucinamide splitting, but had hardly if any inhibitory effect on the hydrolysis of leucyl-β-naphthylamide; an analogous though less pronounced trend was observed for Mn^{2+}. Co^{2+} activated leucyl-β-naphthylamide, but moderately inhibited both triglycine and leucinamide hydrolysis. The differential ion effects would suggest that leucyl-β-naphthylamide hydrolysis by the membranes was catalyzed by an enzyme different from that (those) hydrolyzing triglycine and leucinamide. The splitting of leucyl-β-naphthylamide and leucylglycine by human serum preparations has also been found[19] to be differentially

TABLE II

EFFECT OF Co^{2+}, Zn^{2+} AND Mn^{2+} ON PEPTIDE HYDROLYSIS BY PLASMA MEMBRANES IN TRIS OR PHOSPHATE BUFFER (pH 7.0)

A typical experiment, and the average relative activities obtained in three experiments are listed.

Addition (*1 mM*)	*μmoles substrate hydrolyzed per mg protein per h* (*relative activities*)		
	Triglycine	*Leucinamide*	*Leucyl-β-naphthylamide*
Tris buffer			
None	7.2 (100)	4.6 (100)	4.0 (100)
Co^{2+}	5.1 (87 ± 9)	4.3 (99 ± 5)	3.8 (99 ± 5)
Zn^{2+}	0.6 (15 ± 7)	0.0 (0)	0.2 (5 ± 1)
Mn^{2+}	5.6 (82 ± 4)	3.2 (80 ± 10)	2.9 (77 ± 2)
Phosphate buffer			
None	4.9 (100)	4.4 (100)	4.4 (100)
Co^{2+}	6.5 (122 ± 10)	8.0 (186 ± 5)	6.1 (145 ± 9)
Zn^{2+}	5.3 (104 ± 4)	4.2 (96 ± 1)	4.1 (95 ± 3)
Mn^{2+}	5.5 (103 ± 9)	4.1 (97 ± 3)	4.0 (85 ± 6)

affected by Co^{2+} and Zn^{2+}, *viz.* in a manner similar to the present results. However, in both cases phosphate buffer of pH 7.0 was used for the assay of leucyl-β-naphthylamide hydrolysis, whereas Tris buffer of pH 8.0 (our experiments) or 8.6 (FLEISHER *et al.*[19]) was applied for the assay of the other substrates mentioned. We, therefore, investigated the effect of the Tris·HCl (40 mM) and phosphate (0.1 M) buffers on peptide hydrolysis in the presence of Mn^{2+}, Co^{2+} and Zn^{2+} in comparative experiments at pH 7.0. In these experiments buffer was present during the 1-h preincubation of the membranes with the metal ions. It then appeared that the differential effects obtained under the former conditions were due to the different buffers used. As shown in Table II, Zn^{2+} abolished the hydrolysis of the three substrates by the membranes suspended in Tris buffer, but had no effect in phosphate buffer. Mn^{2+} was weakly inhibitory in Tris and without effect in phosphate buffer. Co^{2+} was without effect in Tris, but stimulatory in phosphate buffer. The stimulation by Co^{2+} of the leucinamide hydrolysis by the membranes was significantly higher than that obtained with the other two substrates; its significance has not been explored further. Also in these experiments (*cf.* above) was phosphate buffer (pH 7.0) found to be inhibitory (about 30%) to triglycine hydrolysis as compared with Tris buffer, whereas no such effect was observed for the other two substrates. This finding could indicate a difference in binding-strength of an intrinsic metal to enzymes. Finally, in Table III, two experiments are illustrated in which the membranes had been preincubated for 1 h in 40 mM Tris–HCl buffer (pH 7.6) in the presence of 1 mM EDTA to deplete endogenous ions, followed by incubation of aliquots of the suspensions with the substrates in the presence of either 1 mM Co^{2+} or Mn^{2+} or Mg^{2+}. The hydrolysis of leucylglycine, leucinamide, triglycine and leucyl-β-naphthylamide by this preparation was activated by the ions in the order $Co^{2+} > Mn^{2+} > Mg^{2+}$, the latter being almost inactive. In these experiments the rates of peptide splitting were lower than those recorded in the previous series, probably as a result of the preincubation with EDTA. This chelating agent damaged the membranes (fragmentation and change in unit-membrane structure)[23] and solubilized considerable amounts of proteins.

The combined results show that under proper conditions cobalt ions are the most or only effective metal ions in activating the enzymic hydrolysis of the four substrates by plasma membranes.

TABLE III

EFFECT OF Co^{2+}, Mn^{2+} AND Mg^{2+} ON PEPTIDE HYDROLYSIS BY PLASMA MEMBRANES PREINCUBATED WITH EDTA

Tris–HCl buffer (pH 7.6). Preincubated with 1 mM EDTA for 60 min at 37°. Values between parentheses obtained in 0.1 M phosphate buffer (pH 7.6).

Addition (1 mM)	*μmoles substrate split per mg protein per h*			
	Leucylglycine	*Leucinamide*	*Leucyl-β-naphthylamide*	*Triglycine*
None	0.0	0.0	0.0	0.0
Mg^{2+}	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.8 ± 0.4
Mn^{2+}	0.6 ± 0.3	1.6 ± 0.0	0.9 ± 0.1	2.2 ± 0.1
Co^{2+}	0.8 ± 0.2	2.8 ± 0.1	1.8 ± 0.2	3.8 ± 0.4
	(0.7 ± 0.2)	(3.2 ± 0.4)	(3.4 ± 0.3)	(4.4 ± 0.2)

Substrate competition experiments

The following experiments were carried out in an attempt to differentiate between enzyme activities, *i.e.* whether one or more enzymes were acting on the various peptides. If an excess of one convertible substrate does not (markedly) inhibit the enzymic conversion of a second, K_m values taken into consideration, it is likely that different enzymes are concerned. By contrast, inhibition in such a system can be interpreted in two ways: competition for the active site of one enzyme may or may not be accompanied by enzymic conversion of the competing substrate. The simultaneous presence of triglycine (3.7 mM) in 10-fold excess to leucyl-β-naphthylamide did not inhibit the splitting of the latter substrate by membranes incubated in 0.1 M phosphate or 40 mM Tris–HCl buffer (pH 7.0) (Table IV). In 0.1 M Tris–maleate buffer (pH 8.0) hydrolysis of leucyl-β-naphthylamide (0.37 mM) was inhibited for only 14 and 19% by, respectively, 5 and 10 mM triglycine. It should be noted (*cf.* Fig. 1) that the latter triglycine concentration (being 27-fold that of leucyl-β-naphthylamide) was auto-inhibitory. These results may give some support to the assumption that leucyl-β-naphthylamide and triglycine are hydrolyzed by different enzymes.

TABLE IV

EFFECT OF PEPTIDES ON THE HYDROLYSIS OF LEUCYL-β-NAPHTHYLAMIDE BY PLASMA MEMBRANES IN PHOSPHATE OR TRIS BUFFER (pH 7.0)

Three experiments; leucyl-β-naphthylamide: 0.37 mM.

Addition (3.7 mM)	*Relative leucyl-β-naphthylamidase activity*	
	Phosphate buffer	*Tris buffer*
None	100	100
Triglycine	99 ± 3	98 ± 2
Leucinamide	80 ± 2	76 ± 2
Leucylglycine	45 ± 9	—
Leucine	55 ± 5	—

Under the former conditions leucyl-β-naphthylamide hydrolysis was weakly inhibited by leucinamide, and moderately so by either leucylglycine or leucine (Table IV). Human serum leucyl-β-naphthylamidase activity[19] and the classic leucine aminopeptidase[20] (EC 3.4.1.1.) are also inhibited by leucine, which might indicate that the inhibition of the plasma-membrane leucyl-β-naphthylamide hydrolase by leucylglycine and leucinamide could be of similar nature, *i.e.* by the hydrophobic leucine residue *per se* of the peptides, and not necessarily involved their hydrolysis by the same enzyme.

In similar experiments it was found that triglycine (10–25 mM), present in 2–5-fold higher concentration than leucinamide (5 mM), inhibited the hydrolysis of the latter substrate in Tris–HCl or –maleate buffers of pH 7.6 and/or 8.0 for 0–15% as measured by the release of ammonia. This result may also indicate that triglycine and leucinamide are hydrolyzed by different enzymes, especially if one considers the approximately similar K_m's and the autoinhibition of triglycine hydrolysis by the substrate concentration used. In contrast, under similar conditions leucinamide hydrolysis was completely inhibited by the presence of 25 mM leucylglycylglycine.

Effect of bile duct ligation on peptidase activities of plasma membranes

Bile duct ligation for 72 h has previously been found[3] to increase the alkaline glycerolphosphatase (EC 3.1.3.1) and to decrease the adenosine triphosphatase (EC 3.6.1.3) activities of the subsequently isolated liver plasma membranes. The enzymes are concentrated in the plasma membranes lining the bile canaliculi, but they are not associated with the globular knobs coating these membrane areas (ref. 2, and below). As shown in Table V for a typical experiment, bile duct ligation notably inhibited the leucyl-β-naphthylamide, leucinamide and triglycine hydrolase activities of the membranes which are associated with the globular knobs as demonstrated in the next sections.

TABLE V

EFFECT OF BILE DUCT LIGATION ON PEPTIDE HYDROLASE ACTIVITIES OF PLASMA MEMBRANES

Animals were sacrificed 72 h after bile duct ligation.

Substrate	*μmoles substrate hydrolyzed per mg protein per h by plasma membranes*		*Inhibition* (%)
	Untreated rats	*Ligated rats*	
Leucyl-β-naphthylamide	3.3	1.2	67
Leucinamide	5.4	2.5	54
Triglycine	7.8	3.6	54

Release of peptidase activity from plasma membranes by papain

As shown previously[2], the leucyl-β-naphthylamide hydrolase activity was completely released from liver plasma membranes without loss of activity by mild treat-

TABLE VI

RELEASE OF ENZYMES FROM ISOLATED PLASMA MEMBRANES BY PAPAIN

Plasma membranes were incubated with activated papain (500:1, w/w) for 10 min at 37° followed by centrifugation for 60 min at 32000 rev./min in a Spinco Rotor 40 to yield a supernatant (*b*) and residual membranes (*c*). Typical experiments; results of five experiments with *p*-nitrophenyl acetate.

Enzyme substrate	*μmoles substrate converted per mg protein per h by membranes*			*Percentage recovery of activity after papain treatment*	
	Untreated	*Papain-treated*		*Supernatant*	*Supernatant + sediment*
		Supernatant	*Sediment*		
	(*a*)	(*b*)	(*c*)	($b + c = 100$)	($a = 100$)
Leucyl-β-naphthylamide	3.91	11.34	0.20	97	100
Leucinamide	4.19	11.21	0.78	88	104
Leucylglycine	1.23	3.96	0.0	100	109
Leucylglycylglycine	11.4	22.8	1.3	92	80
Triglycine	7.51	22.50	1.52	86	92
β-Glycerol phosphate	3.3	0.12	4.9	1	105
α-Naphthyl caprylate	13.7	12.3	17.8	30	115
p-Nitrophenyl acetate	2.46 ± 0.48	4.58 ± 0.58	2.53 ± 0.47	56.4 ± 4.3	122.1 ± 7.6

ment of the membranes with papain, all activity arriving in the supernatant following high-speed centrifugation. Similarly, in the present experiments the triglycine, leucinamide, leucylglycine and leucylglycylglycine hydrolase activities were (almost) completely released from the membranes by papain treatment (Table VI). For none of these substrates had trypsine any such effect, since their hydrolysis remained membrane-bound following high-speed centrifugation.

Papain did not release other enzymes such as the 'uninduced' alkaline glycerol-phosphatase of plasma membranes isolated from the livers of male R-rats (for lipase, compare below).

Arylesterase activities, papain sensitivity

In view of the finding that peptidases may show esterolytic activity and that hog-liver microsomal esterase exhibits leucyl-β-naphthylamidase activity[21,22], the hydrolysis by plasma membranes of *p*-nitrophenyl acetate and α-naphthyl caprylate as substrates for arylesterase activity (EC 3.1.1.2) was studied in the above context. Esterase could be differentiated from the peptidase activities by differences in (a) effect of EDTA and Cd^{2+}, (b) extent of papain-mediated enzyme release, and (c) gel-filtration profiles of the released enzyme activities.

In contrast to the abolishment of peptide hydrolysis by EDTA, the chelating agent did not affect the esterase activities of the plasma membranes under similar experimental conditions. One mM Cd^{2+} which inhibited peptide hydrolysis for 90–100 %, inhibited the esterase activities for 50 % only.

The release of esterase activity from the membranes by papain was studied in particular with *p*-nitrophenyl acetate as substrate. As shown in Table VI, from 50 to 60 % of the esterase activity was released by papain, this percentage being significantly less than that obtained for the peptide hydrolase activities under similar conditions. In all five experiments the recovery of esterase activity in the supernatant and membrane sediment following papain treatment and high-speed centrifugation, was significantly more than 100 %. Probably there occurs a slight stimulation of enzyme activity as a result of its release. In two other experiments carried out with α-naphthyl caprylate as substrate some 30 % of the esterase activity was released by papain.

Gel-filtration profiles of peptidase and arylesterase activities released from plasma membranes and microsomal membranes by papain

Papain released the 50–60-Å globular knobs from liver plasma membranes as shown electron microscopically[2]. On gel filtration over Sephadex G-200 (present experiments; previously[2] Bio Gel P100 was used with similar results), these particles, separated from the residual membrane by high-speed centrifugation, were eluted directly after the void volume (measured with Blue Dextran 2000) simultaneously with the protein and leucyl-β-naphthylamidase peaks (Fig. 2). Triglycine and leucinamide hydrolase activities showed similar elution profiles, as illustrated in Fig. 2 for the papain-released triglycine hydrolase activity. Small mutual differences which were occasionally observed were very presumably due to the relative insensitivity of the assay method used to measure triglycine hydrolysis.

However, in comparison with the peptidase activity, the papain-released esterase activity of the plasma membranes was retarded on the column as its peak activity was eluted two or three 4-ml fractions later (Fig. 3). In addition, the esterase profiles

were either asymmetric (showing at least one shoulder in the case of α-naphthyl caprylate hydrolysis) or consisted of two peaks (*p*-nitrophenyl acetate hydrolysis, this latter difference being presumably only due to the sampling procedure employed). It is evident that the esterase activity is contained in (much) smaller material of heterogeneous nature as compared with the apparently more homogeneous nature of the peptidase containing globular knobs.

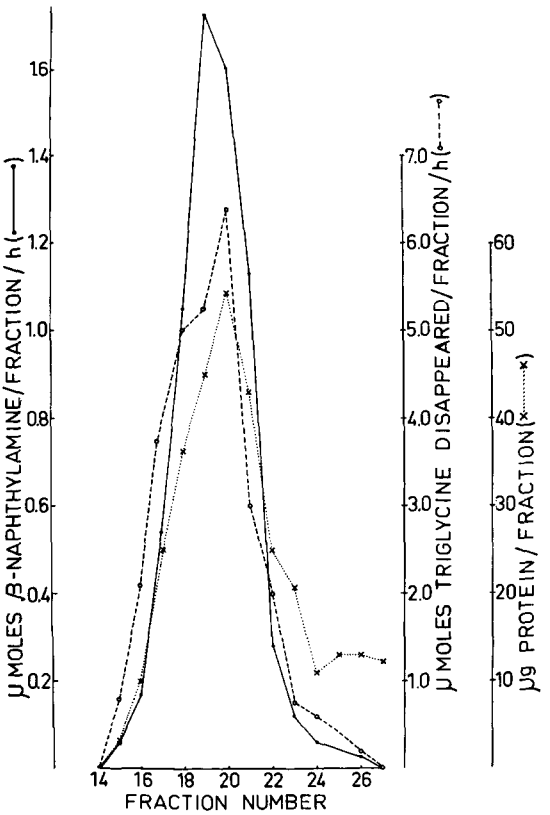

Fig. 2. Gelfiltration (Sephadex G-200) profiles of leucyl-β-naphthylamide and triglycine hydrolase activities released from isolated rat-liver plasma membranes by papain.

It is of interest that the esterase activities which were released by papain from liver microsomes, using similar conditions as applied to the plasma membranes, yielded gel-elution profiles (Fig. 4) which resembled in detail those obtained with the plasma-membrane esterase activities. Therefore the possibility that part of the latter activity was present in a distinct species of globular knobs is not very likely since microsomal membranes do not contain such particles[23].

Size estimation of globular knobs

The elution profiles of the peptide hydrolase activities from the various gels used in the present and previous investigations, *i.e.* Bio Gel P100 and P200, Sephadex G-100 and G-200, and Sepharose 6B (exclusion limit for protein at $4 \cdot 10^6$ mol.wt.) were similar, using preparations suspended in and eluted with either 1 M $NaHCO_3$,

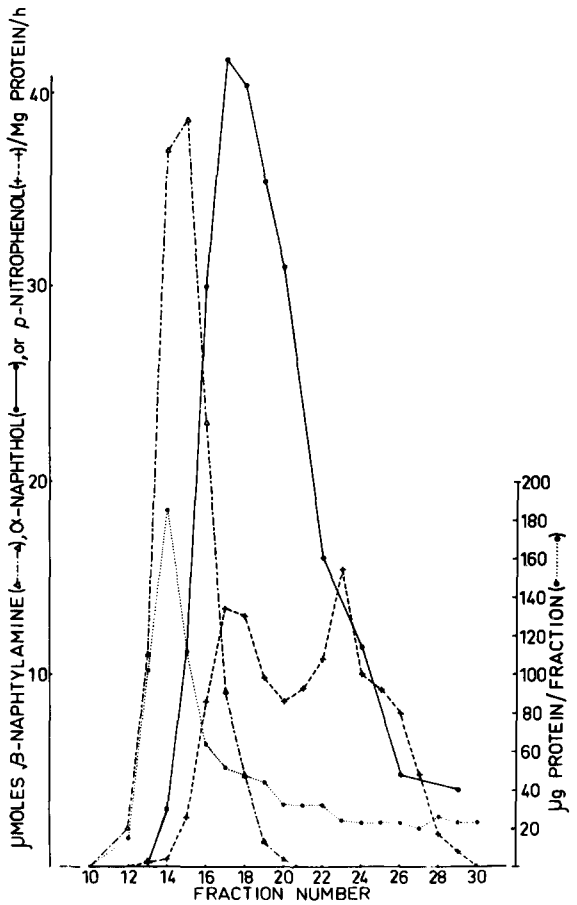

Fig. 3. Gelfiltration (Sephadex G-200) profiles of leucyl-β-naphthylamide and arylester hydrolase activities released from isolated rat-liver plasma membranes by papain. Arylesterase measured with α-naphthyl caprylate and *p*-nitrophenyl acetate as substrates.

0.1 % NaCl or 0.1 M phosphate buffer (pH 7.0) with or without 0.01 % sodium dodecyl sulfate. The globular knobs have a diameter of 50–60 Å, and when considered as 60-Å protein spheres of specific density 1.3, an approximate particle weight of about 90000 can be calculated. The finding that the peptidase activities (and globular knobs) passed the various gels in a similar manner would therefore indicate that the particles aggregated at some time during the experiment. Evidence for this has been obtained electron microscopically following negative staining of the gel-eluate fractions with phosphotungstate[2]. Since aggregation in this case could be an artefact of specimen preparation for electron microscopy, the supernatant of the papain-treated membranes was studied in the analytical ultracentrifuge both before and after passage over Sepharose 6B. (In this experiment the elution profiles of the triglycine and leucyl-β-naphthylamide hydrolase activities coincided exactly; the activity starting to emerge in Fraction 10–Fraction 9 containing the last Blue Dextran 2000-, peak activity in Fraction 11, and activity approaching zero in Fraction 19.) The freshly

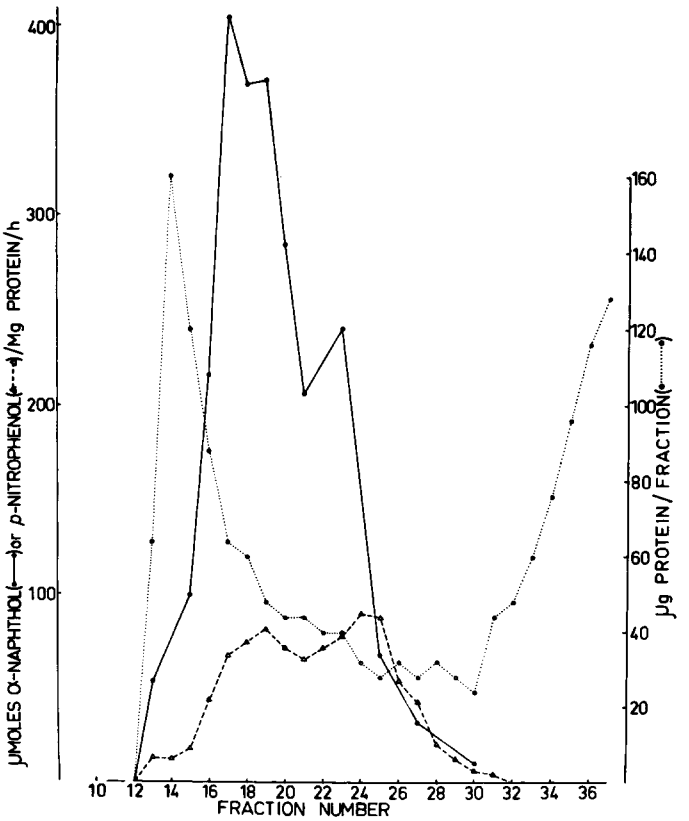

Fig. 4. Gelfiltration (Sephadex G-200) profiles of arylester hydrolase activity released from isolated rat-liver microsomes by papain. Arylesterase measured with α-naphthyl caprylate and *p*-nitrophenyl acetate as substrates.

prepared particle fraction before gel passage showed an average sedimentation coefficient ($s_{w,20}$) of 5 S corresponding to an approximate particle weight of 80000. In contrast, the gel-eluate fractions (11–16) exhibited an average sedimentation coefficient of about 15 S, corresponding to a particle weight of at least $0.5 \cdot 10^6$. These results show that the globular knobs aggregated on contact with the gels.

Lipase activity

Using [^{14}C]palmitic acid-labelled chylomycrons, lipase (EC 3.1.1.3) activity has previously been demonstrated in rat-liver plasma-membrane preparations by HIGGINS AND GREEN[24]. Also in our hands did plasma membranes hydrolyze triolein, *i.e.* glyceryl-tri(oleate-9,10-T(n)), about four times faster at pH 5.0 than at neutral pH. From the results of a typical experiment, described under MATERIALS AND METHODS and listed in Table VII, it was calculated that plasma membranes hydrolyzed 267 μg triolein per mg protein per h, the sedimentable membranes following papain digestion and centrifugation hydrolyzed 330 μg triolein per mg protein per h, whereas the supernatant of papain-treated membranes hydrolyzed only 58 μg triolein per mg protein per h. As can be seen in Table VII the latter value is, of necessity, not very

TABLE VII

LIPASE ACTIVITY OF FRESH AND PAPAIN-TREATED PLASMA MEMBRANES

See MATERIALS AND METHODS.

Enzyme source (*μg protein*)	*Radioactivity* (%) *contained in*				*μg triglycine disappeared per mg protein per h*
	Tri-glyceride	*Di-glyceride*	*Mono-glyceride*	*Free fatty acids*	
None	92.62	3.85	0.56	2.95	
Membranes (340 μg)	82.94	7.76	1.17	8.12	267
Papain-treated membranes					
Sediment (390 μg)	78.70	10.36	1.45	9.47	330
Supernatant (100 μg)	92.01	4.46	0.61	2.90	57.5

reliable, but in any case the lipase activity of the supernatant was insignificant. Of the lipase activity of the intact membranes 104 % was recovered in the sediment *plus* supernatant of the papain-treated membranes, and 95 % of the latter activity (sediment *plus* supernatant = 100) was sedimentable, *i.e.* membrane bound. It follows that lipase is not associated with the globular knobs.

DISCUSSION

Location of the peptidase activity

On account of the criteria established in the introduction of this paper, the present results leave little doubt that the 50–60-Å globular knobs, which coat liver plasma membranes lining bile spaces, are capable of hydrolyzing not only leucyl-β-naphthylamide but also leucinamide, leucylglycine, leucylglycylglycine and triglycine. Other enzymes such as the alkaline glycerolphosphatase, which is concentrated in the same membrane areas, and lipase, are not released by papain, and consequently are not present in the globular knobs, but are embedded in the membrane element *per se*. The esterase activity of the plasma membranes was partly released by papain, but it was demonstrated that this activity was not identical with the peptidase activity. Although the latter experiments were not intended to study the authenticity of the plasma-membrane esterase, the results on gelfiltration of the papain-released esterase activities suggested a close similarity between plasma-membrane and microsomal-membrane enzymes. This finding may indicate either that the esterase activity of plasma membranes is due to microsomal contamination (discussed in refs. 44 and 45), or that this enzyme is integrated with both types of membranes in a similar manner. The presence of another enzyme, inosine diphosphatase, has by electron histochemical examination of liver been demonstrated[25] in both plasma membranes and endoplasmic reticulum membranes. Esterase has been supposed[26] to be present in the surface membranes of intestinal mucosa cells, and has recently been demonstrated[27] on restricted areas of the cell surface of lung cells *in situ*. Lysosomal membranes also contain esterase activity[28], and similarity between these membranes, which are partly derived from plasma membranes, and plasma membranes has been noted[29].

Enzyme characterization

On account of salient differences in properties, the plasma-membrane peptidase activity was not identical with the following more or less well documented enzymes and enzyme activities: dipeptidase[30], aminotripeptidase[31] (EC 3.4.1.3), the classic leucine aminopeptidase[20] (EC 3.4.1.1), thiol-dependent aminopeptidase[18], arylamidases[32–35], and the lysosomal enzymes[36] of liver and kidney hydrolyzing leucyl-β-naphthylamide.

Inspection of the literature, however, revealed a close similarity between the plasma-membrane peptidase activity and the particle-bound aminopeptidase of rat kidney described by FELGENHAUER AND GLENNER[1]. Both were metal dependent, activated by Co^{2+} (with Mg^{2+} and Mn^{2+} being far less or not active), not dependent on thiol groups for activity, and showed a pH optimum in the alkaline range, similar K_m values for leucyl-β-naphthylamide and leucinamide, and the same relative rates of substrate hydrolysis (Leu–Gly–Gly > Leu–NH_2 > Leu–β-naphthylamide > Leu–Gly), and lacked esterolytic activity.

Apart from the similarity in many conditions of hydrolysis of the various peptides by the plasma membranes, some diversity was also noted: (a) phosphate buffer (pH 7.0) was somewhat inhibitory to triglycine hydrolysis but not to the other substrates, (b) puromycin caused a pronounced inhibition of leucyl-β-naphthylamide hydrolysis only, (c) excess triglycine failed to reduce the hydrolysis of leucyl-β-naphthylamide and leucinamide to any significant extent. (These effects were not studied by FELGENHAUER AND GLENNER[1].) These differences may one lead to postulate that more than one enzyme in the plasma membranes was catalyzing the splitting of the various substrates; *e.g.* differences in puromycin inhibition of the leucine aminopeptidase and arylamidases have been reported[35].

Because of (a) the impossibility to list the plasma-membrane activities in such categories and (b) the similarities in reaction conditions otherwise found, we prefer the view that — if diversity in the reactions mentioned reflects diversity in enzymes —, a number (three?) of closely related enzymes could be present rather than separate classes of enzymes. Evidence for two isozymes of the Co^{2+}-activated kidney aminopeptidase has been obtained[1].

In view of the mol. wt. recorded for various peptidases (80000–250000)[37–39] and assuming that the plasma-membrane enzymes fall in the same order of magnitude, it further follows (*cf.* section on size estimation of globular knobs) that one globular knob cannot contain more than one enzyme molecule. Hence, in case of enzyme diversity, various populations of globular knobs exist, each functionally specialized. The brush border membranes of intestinal mucosa cells contain similar globular knobs[40], and EICHHOLZ[41] has shown that the hydrolases acting on leucyl-β-naphthylamide and leucylglycine are differentially released by papain; enzyme localization in the globular knobs and enzyme properties were not studied[41] (*cf.*, however, ref. 42 for the presence of leucyl-β-naphthylamide hydrolase in these globular knobs). The Co^{2+}-activated kidney aminopeptidase also appears to be associated with the brush border of the proximal tubuli[1]. The presence of globular knobs on the plasma membranes lining these areas has also been reported[46]. Parenthetically, since upon isolation plasma-membrane fragments may arrive in the microsomal fraction, the finding of Co^{2+}-activated 'arylamidase' activity in that fraction (liver, kidney[36]) may be due to contamination.

It may be a general property of plasma membranes specialized for transport to be locally differentiated (at bile canaliculi and brush borders) by possessing 50–60-Å globular knobs containing Co^{2+}-activated aminopeptidase(s). Accordingly, a role in protein catabolism is indicated, and the functional capacity of the kidney enzyme has been suggested[1] to be that of an aminopolypeptidase. Preliminary experiments in this laboratory have failed to show that rat-liver plasma membranes hydrolyze haemoglobin or the hide-bound azo dye Azocoll, this latter substrate being slowly hydrolyzed by erythrocyte membranes[43]. It is also worth mentioning that the plasma membranes isolated from rat hepatoma-484 hydrolyze the various peptides at about the same rates as liver plasma membranes do, but that the hepatoma is devoid of bile canaliculi and its isolated plasma membranes do not contain the globular knobs[2].

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