BBA 75126

# STUDIES ON PLASMA MEMBRANES

## VII. A LEUCYL-β-NAPHTHYLAMIDASE-CONTAINING REPEATING UNIT ON THE SURFACE OF ISOLATED LIVER AND HEPATOMA PLASMA MEMBRANES

P. EMMELOT, A. VISSER AND E. L. BENEDETTI

*Departments of Biochemistry and Electron Microscopy, Antoni van Leeuwenhoek-Huis: the Netherlands Cancer Institute, Amsterdam (The Netherlands)*

(Received November 9th, 1967)

SUMMARY

1. Plasma membranes isolated from rat and mouse liver and hepatomas displayed L-leucyl-β-naphthylamidase (EC 3.4.1.1) activity, of which a minor part was soluble in 0.15 M NaCl.

2. Papain (EC 3.4.4.10) released the leucyl-β-naphthylamidase (almost) completely from rat- and mouse-liver and mouse-hepatoma membranes but only partly from rat-hepatoma membranes. Trypsin (EC 3.4.4.4) was ineffective. From the supernatant of the papain-treated rat-liver membranes a fraction was obtained by gel filtration showing a 10-fold enriched leucyl-β-naphthylamidase activity. Seven other enzymes associated with liver plasma membranes were not released from the membranes by papain to any significant extent.

3. The 50–60 Å globular knobs, previously demonstrated by electron microscopy on the surface of liver plasma membranes, were released from the membranes by papain but not by trypsin. Particles resembling the globular knobs could be demonstrated in leucyl-β-naphthylamidase-containing fractions derived from the supernatant of papain-treated liver membranes. Rat-hepatoma, in contrast to mouse-hepatoma plasma membranes, did not contain the globular units.

4. It is concluded that leucyl-β-naphthylamidase is located in the globular knobs which represent a structurally and functionally specialized repeating unit that locally coats the plasma membrane surface, *i.e.* that lining the bile spaces of liver and those differentiated liver tumors which still contain the latter structures.

## INTRODUCTION

The electron microscopic examination of negatively stained plasma membranes isolated from rat liver has revealed the presence of globular units, 50–60 Å in diameter on the average, on the membrane surface[1]. Membranes containing similar particles have been observed in rat-liver homogenate[2]. Since the globular units were restricted to certain sheets of the isolated plasma membrane or parts thereof, BENEDETTI AND

EMMELOT[1] concluded that the globules were either readily detached by the techniques applied *in vitro*, or naturally restricted to some specialized region of the plasma membrane such as that lining the bile canaliculi and their microvilli. Similar globular units have also been found on the microvilli of brush borders isolated from the small intestine epithelium of the hamster[3] and rabbit[4]. As reported in abstract by ODA AND SEKI[5] and more recently in more detail by JOHNSON[66], these units could be isolated from the latter type of membranes. The isolated particles contained invertase (EC 3.2.1.26) and leucine aminopeptidase (EC 3.4.1.1) activity, which had earlier been demonstrated in various membrane preparations of the intestinal mucosa enriched in plasma membranes and in isolated brush borders[7–9]. Leucyl-β-naphthylamidase has also been demonstrated histochemically to be associated with the intestinal brush borders and the plasma membranes lining rat-liver bile canaliculi[10,11]. Plasma membranes isolated from the livers of the guinea pig[12] and the rat[13] also contained the latter enzyme. In the present investigation the isolation and enzymic assay of the globular units from isolated liver plasma membranes of the mouse and the rat have been undertaken. Since our work is aimed at a comparative investigation of normal and neoplastic cell membranes in order to delineate the role of the plasma membrane in carcinogenesis and malignancy[14–17], plasma membranes isolated from transplanted rat and mouse hepatomas were included in the present study.

## MATERIALS AND METHODS

Plasma membranes were isolated from rat liver (strain R-Amsterdam), mouse liver (CBA), the transplanted hepatocellular rat hepatoma-484 (originally induced by 4′-dimethylaminoazobenzene in a female R-rat), and the transplanted mouse hepatomas 143066 and 4189 (these had arisen spontaneously in old CBA males); for more details on the hepatomas see ref. 15. The livers and mouse hepatomas were homogenized in 1 mM $NaHCO_3$ (pH 7.5) and the rat hepatoma-484 in 1 mM $NaHCO_3$ containing 2 mM $CaCl_2$ (ref. 16). Isolation of the plasma membranes was carried out as described previously[18]. The isolated plasma membranes were washed once with 1 mM $NaHCO_3$ prior to their use. Trypsin, applied as indicated in the text, was obtained from Sigma Chemical Co.

A fresh papain solution was prepared for each experiment. 0.0625 ml of a papain suspension (Sigma) containing 16 mg papain per ml was added to 0.05 ml 2 M NaCN and allowed to stand for 3 min at room temperature followed by dilution of the solution with twice-distilled water to 10 ml to give a final concentration of 1 μg papain per 0.01 ml. The desired aliquot of the papain solution was added to the isolated plasma membranes suspended in ice-cold 1 mM $NaHCO_3$ (about 1 mg membrane protein per ml) to give a papain concentration of 1:25, 1:50, 1:200, 1:500 or 1:1000 in respect to the membrane protein. The mixture was incubated for 10 min at 37°, rapidly cooled in ice and centrifuged for 90 min at 45000 × *g* (32000 rev./min, Spinco rotor 40) at 4° to give a supernatant and a sediment containing the residual membranes. The supernatant, the sediment (suspended in a volume of 1 mM $NaHCO_3$ equal to that in which the membranes had been present originally), and the untreated membranes (kept at 0° in 1 mM $NaHCO_3$) were used for enzyme assay. Leucyl-β-naphthylamidase was measured according to the method of GOLDBARG AND RUTENBURG[19], and inosine diphosphatase (IDPase; EC 3.6.1.6) according to that of PLAUT[20];

the assays of the other enzymes have been reported previously[18,21]. Protein was measured by the Folin method.

The supernatant of the papain-treated rat-liver membranes, obtained as indicated above, was layered on Bio-Gel P100 (45 cm × 2 cm), the gel was eluted with a solution containing 0.01 M NaCl and 1 mM $NaHCO_3$ in the cold room and 1-ml aliquots were collected for leucyl-β-naphthylamidase assay. Application of Bio-Gel P200 and 70 cm × 1 cm columns gave similar results. Void volume was measured with Blue Dextran 2000 (Pharmacia, Uppsala, Sweden).

The plasma membranes and fractions were negatively stained with phosphotungstate at low temperature and examined under the electron microscope (Philips EM 200) as described previously[1].

## RESULTS

### L-*Leucyl-β-naphthylamidase activity of isolated plasma membranes*

The specific leucyl-β-naphthylamidase activity of isolated rat-liver plasma membranes amounted to 3.9 ± 0.2 μmoles β-naphthylamine formed from L-leucyl-β-naphthylamide per mg membrane protein per h at 37°. Some 15 % of the total enzyme activity of the membranes was soluble in 0.15 M NaCl; the remaining part of the

TABLE I

DISTRIBUTION OF LEUCYL-β-NAPHTHYLAMIDASE ACTIVITY OF RAT-LIVER AND RAT-HEPATOMA PLASMA MEMBRANES OVER THE SALINE-SOLUBLE AND -INSOLUBLE MEMBRANE PORTIONS

The membranes were suspended in 0.15 M NaCl and kept for 1 h at room temperature, followed by centrifugation for 15 min at 1500 × *g* and one washing with saline in order to obtain the saline-soluble and -insoluble portions.

| *Plasma membrane fraction* | *μmoles β-naphthylamine* | | *Percent of total enzyme activity* |
|---|---|---|---|
| | *Per mg protein per h* | *Per fraction derived from 1 mg membrane protein* | |
| Rat-liver membranes | 3.6 | (3.6) | (100) |
| saline-insoluble portion (75)* | 4.1 | 3.1 | 85 |
| saline-soluble portion (25)* | 2.2 | 0.55 | 15 |
| Rat-hepatoma-484 membranes | 5.3 | (5.3) | (100) |
| saline-insoluble portion (79)* | 6.2 | 4.9 | 93 |
| saline-soluble portion (21)* | 1.8 | 0.38 | 7 |

* Percent of total membrane protein.

activity was recovered in the saline-insoluble membrane portion (Table I). The specific leucyl-β-naphthylamidase activities displayed by mouse-liver and rat-hepatoma plasma membranes were of the same order of magnitude (Tables I and III) as that found for rat-liver membranes, whereas the mouse-hepatoma membranes tended to show somewhat higher activities. Also from these membranes only a minor amount of the enzyme was made soluble by physiological saline (*e.g.*, 7–17 % in the case of the rat-hepatoma plasma membranes). Previous experiments[15,16,22,23] have shown that the positively charged protein released from isolated liver plasma membranes

TABLE II

EFFECT OF NaCN AND/OR PAPAIN ON THE LEUCYL-β-NAPHTHYLAMIDASE ACTIVITY OF PLASMA MEMBRANES

Rat-liver plasma membranes and the saline-soluble and -insoluble portions of rat-hepatoma plasma membranes were preincubated at 37° (30 and 10 min, respectively) in 1 mM $NaHCO_3$ with and without NaCN (5 mM) and/or papain (1:50 or 1:25, respectively). The preparations were then cooled to 0° and aliquots were assayed for leucyl-β-naphthylamidase.

| *Plasma membranes* | *μmoles β-naphthylamine/mg protein per h* | | |
|---|---|---|---|
| | *Controls* | *NaCN* | *Papain plus NaCN* |
| Rat liver | 3.8 | 3.6 | 3.1 |
| Rat hepatoma-484 | | | |
| saline-soluble portion | 1.7 | 2.8 | 2.1 |
| saline-insoluble portion | 4.2 | 4.2 | 3.9 |

TABLE III

RELEASE OF LEUCYL-β-NAPHTHYLAMIDASE FROM ISOLATED PLASMA MEMBRANES BY PAPAIN

Plasma membranes were incubated with papain in the concentrations indicated for 10 min at 37°, followed by centrifugation for 90 min at 45000 × *g* in the cold to yield a supernatant (b) and the residual membranes (c).

| *Source of plasma membranes (papain concn.)* | *μmoles β-naphthylamine/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) |
| Rat liver | | | | | |
| (1:50) | 4.1 | 7.2 | 0.03 | 99.8 | 90 |
| (1:200) | | 6.5 | 0.03 | 99.8 | 90 |
| (1:500) | 4.1 | 14.5 | 0.93 | 87 | 101 |
| (1:1000) | | 14.5 | 1.87 | 70 | 97 |
| (1:500)* | 4.2 | 13.4 | 1.7 | 79 | 119 |
| Rat hepatoma-484 | | | | | |
| (1:200 | 3.4 | 5.4 | 1.9 | 56 | 83 |
| (1:500) | | 4.2 | 2.7 | 41 | 92 |
| Mouse liver | | | | | |
| (1:200) | 3.4 | 8.6 | 0.85 | 86 | 98 |
| (1:500) | | 14.7 | 1.5 | 72 | 118 |
| (1:500) | 3.0 | 7.3 | 0.89 | 78 | 93 |
| Mouse hepatoma-143066 | | | | | |
| (1:200) | 5.55 | 9.5 | 0.66 | 93 | 97 |
| (1:500) | | 12.75 | 3.1 | 67 | 104 |
| Mouse hepatoma-4189 | | | | | |
| (1:500) | 5.4 | 10.2 | 2.7 | 61 | 91 |

* In this experiment saline-insoluble membranes were used.

by physiological saline originates for the greater part, if not exclusively, from the cytoplasm and intercellular fluid, becoming electrostatically linked to the negatively charged plasma membranes proper (*i.e.* the saline-insoluble membrane portion) under the hypotonic conditions of tissue homogenization and membrane isolation. The specific leucyl-β-naphthylamidase activity of the soluble cell fraction (105000 × *g* supernatant derived from homogenates used for plasma membrane isolation) amounted to 0.51, 0.52, 0.20, and 0.21 μmole β-naphthylamine from leucyl-β-naphthylamide per mg protein per h for rat liver, rat hepatoma-484, mouse liver and mouse hepatoma-143066, respectively.

*Effect of trypsin on the leucyl-β-naphthylamidase of rat-liver plasma membranes*

Trypsin incubated with rat-liver membranes in a ratio of 1:50 (based on membrane protein weight) did not inactivate the membrane-bound leucyl-β-naphthylamidase activity to any significant extent. After preincubation of the membranes with trypsin for 30 min in 1 mM $NaHCO_3$ (pH 7.5), the specific leucyl-β-amidase activity of the trypsin-treated membranes amounted to 3.4 μmoles β-naphthylamine, as against 3.8 μmoles for the control membranes. In a similar experiment carried out with saline-insoluble membranes treated with trypsin for 45 min at 37° in 0.1 M phosphate buffer (pH 7.0), the specific enzyme activity amounted to 5.05 μmoles β-naphthylamine released as compared with 5.85 μmoles for control membranes exposed to buffer only. In an experiment in which trypsin (1:200) was incubated with plasma membranes for 10 min at 37° in the latter phosphate buffer, 90% of the total leucyl-β-naphthylamidase activity was recovered in the supernatant and residual membranes obtained after centrifugation for 90 min at 45000 × *g*. Only 11% of leucyl-β-amidase activity was present in the supernatant, which contained 51% of the membrane protein, this amount of enzyme being of the same order of magnitude as that released by the phosphate buffer alone. Similar results were obtained with trypsin at 1:50, which released 55–65% of the membrane protein. It can be concluded that trypsin did not release the leucyl-β-naphthylamidase from the plasma membranes.

*Release of leucyl-β-naphthylamidase from plasma membranes by papain*

*Rat-liver plasma membranes.* Papain was free of leucyl-β-naphthylamidase activity. Under the extreme conditions mentioned in Table II, papain caused little inactivation of the leucyl-β-naphthylamidase activity of the plasma membranes. As shown by the experiment with rat-hepatoma membranes, NaCN (used to activate papain) increased the leucyl-β-naphthylamidase of the saline-soluble membrane protein but not that of the saline-insoluble membranes; the soluble enzyme is sensitive (*cf.* ref. 20) to traces of heavy metals which apparently are removed by cyanide. The saline-soluble membrane protein suffered more loss (26%) of leucyl-β-naphthylamidase activity by the papain treatment than did the saline-insoluble membranes (10%).

After incubation of rat-liver plasma membranes with papain (1:50 and 1:200) for 10 min at 37° in 1 mM $NaHCO_3$ followed by centrifugation for 90 min at 45000 × *g* in the cold to obtain the residual membranes and supernatant, the latter fraction contained all the leucyl-β-naphthylamidase activity while the residual membranes were essentially free of enzyme activity (Table III). Thus, papain very effectively released the dipeptidase from the membranes. When the concentration of papain

relative to the membrane protein was reduced to 1:500 and 1:1000, less protein was released and the specific leucyl-β-naphthylamidase activity of the supernatant of the papain-treated membranes was further increased (Table III). These results show that by lowering the papain concentration from 1:200 to 1:500, a preferential release of the leucyl-β-naphthylamidase as compared with enzymatically inactive protein is obtained. However, the lower concentrations of papain did not release all the dipeptidase activity from the membranes.

The supernatant obtained after papain digestion (1:500) of rat-liver plasma membranes and centrifugation for 90 min at 45000 × *g* was used for further fractionation of the leucyl-β-naphthylamidase over Bio-Gel P100. As shown in Fig. 1, fractions with a specific enzyme activity of up to 30.9 μmoles β-naphthylamine, representing a 10-fold enrichment of the enzyme as compared with the control membranes of this experiment, were collected. About 90% of the total enzyme activity applied to the column was recovered. A similar result was obtained with the 45000 × *g*

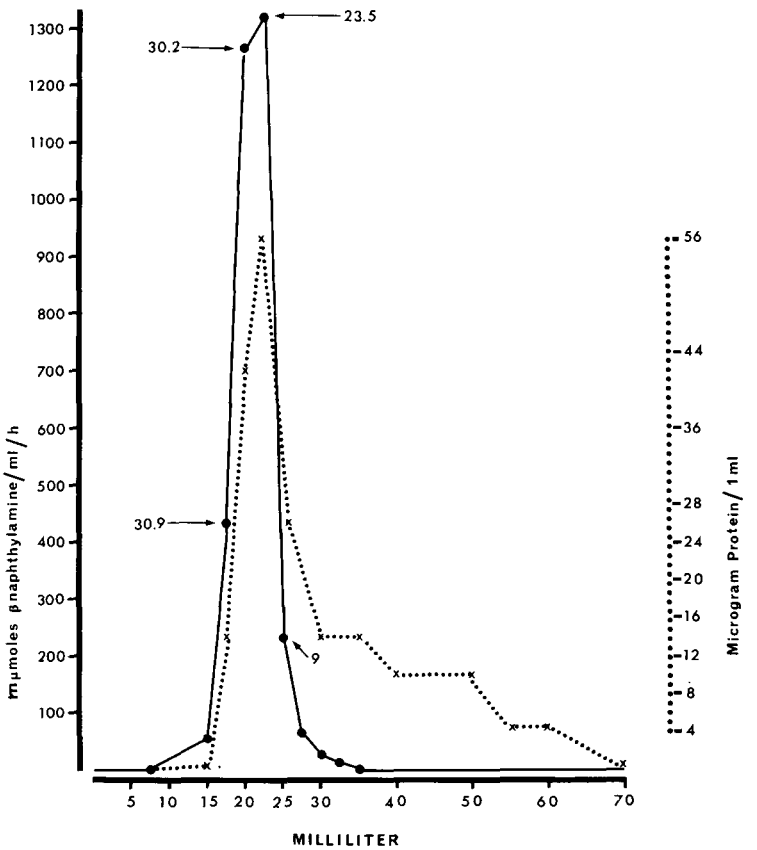



Fig. 1. Gel filtration of leucyl-β-naphthylamidase activity released from isolated rat-liver plasma membranes by papain. 2 ml of the 45000 × *g* supernatant from papain-treated membranes (1:500), containing 1.24 mg protein in 1 mM $NaHCO_3$, were applied to the top of a column containing Bio-Gel P100 (45 cm × 2 cm), previously washed with a solution containing 1 mM $NaHCO_3$ and 10 mM NaCl, and eluted with the latter medium at 4°. Enzyme activity appeared just after the void volume which is not illustrated in the figure. The specific enzyme activities in μmoles β-naphthylamine released per mg protein per h are indicated for 4 fractions.

supernatant of papain-treated membranes (1:600) which had previously been stripped of their saline-soluble proteins. In this experiment the gel-filtrate fraction with the highest specific leucyl-β-naphthylamidase activity (35.6 μmoles β-naphthylamine) showed a more than 3-fold enrichment as compared with the 45000 × *g* supernatant (11.2 μmoles β-naphthylamine) applied to the column.

*Mouse-liver, mouse-hepatoma and rat-hepatoma plasma membranes.* As shown in Table III, the release of leucyl-β-naphthyl-amidase activity from mouse-liver and mouse-hepatoma plasma membranes by papain (1:200 or 1:500) was similar to that observed for the rat-liver plasma membranes. However, in the case of the rat-hepatoma plasma membranes distinctly less of the dipeptidase activity was released by papain, *e.g.* in the experiment illustrated in Table III the release was about half that from rat-liver plasma membranes. In two other experiments (not illustrated) only about 20% of the enzyme activity was released by papain (1:200).

*Effect of papain on other enzymes of rat-liver plasma membranes*

5'-Nucleotidase (EC 3.1.3.5), inosine diphosphatase (EC 3.6.1.6), ATPase (EC 3.6.1.3) and alkaline glycerolphosphatase (EC 3.1.3.1) have, like leucyl-β-naphthyl-amidase, been shown by (electron) histochemistry to be associated with the plasma membranes lining the liver bile canaliculi[10,11,24,25]. Unlike the latter enzyme, however, the former enzymes and ($Na^+$-$K^+$)-ATPase, esterase (EC 3.1.1.2) and alkaline phosphodiesterase (EC 3.1.4.1) were not at all, or only to a very slight extent, released by papain (1:200 or 1:500) from rat-liver plasma membranes (Table IV). The opposite behavior of the leucyl-β-naphthylamidase in response to papain is compatible with

TABLE IV

EFFECT OF PAPAIN ON SOME ENZYMES OF RAT-LIVER PLASMA MEMBRANES

For the assay of the 5'-nucleotidase (substrate: AMP) and esterase (substrate: α-naphthyl caprylate) the membranes were incubated with papain in a concentration of 1:200 in respect to membrane protein; papain at 1:50 gave similar results. In the remaining experiments papain at 1:500 was used. The substrate of the alkaline phosphodiesterase was bis-(*p*-nitrophenyl) phosphate. The alkaline phosphatase (substrate: β-glycerol phosphate) was assayed in plasma membranes isolated 96 h after bile duct ligation (i), and 26 h after subtotal hepatectomy (ii). Papain was allowed to interact with the membranes for 10 or 15 min at 37°; *cf.* Table III.

| *Enzyme* | *μmoles product/mg protein per h* | | | *Percentage recovery after papain treatment* | |
|---|---|---|---|---|---|
| | *Untreated membranes* | *Papain-treated membranes* | | *Supernatant* | *Supernatant + sediment* |
| | | *Supernatant* | *Sediment* | | |
| | (*a*) | (*b*) | (*c*) | (*b* + *c* = 100) | (*a* = 100) |
| 5'-Nucleotidase | 63.2 | 0.0 | 160.1 | 0 | 100 |
| Esterase | 18.4 | 4.2 | 39.2 | 16 | 87 |
| IDPase | 31.0 | 6.75 | 49.9 | 8 | 91 |
| ATPase | 84.2 | 13.2 | 125.9 | 6 | 71 |
| ($Na^+$-$K^+$)-ATPase | 12.1 | 2.7 | 23.1 | 9 | 89 |
| Phosphodiesterase alkaline | 6.5 | 0.84 | 9.9 | 5 | 86 |
| Glycerolphosphatase alkaline | | | | | |
| (i) | 6.8 | 0.1 | 12.1 | 0.7 | 97 |
| (ii) | 12.8 | 1.5 | 18.0 | 5 | 79 |

the view that the dipeptidase is located in a specialized membrane structure of which the other enzymes do not form part.

*Electron microscopic observations*

Negative staining of isolated rat-liver plasma membranes has revealed[1] the presence of 50–60 Å globular knobs on the surface of a certain number of these mem-

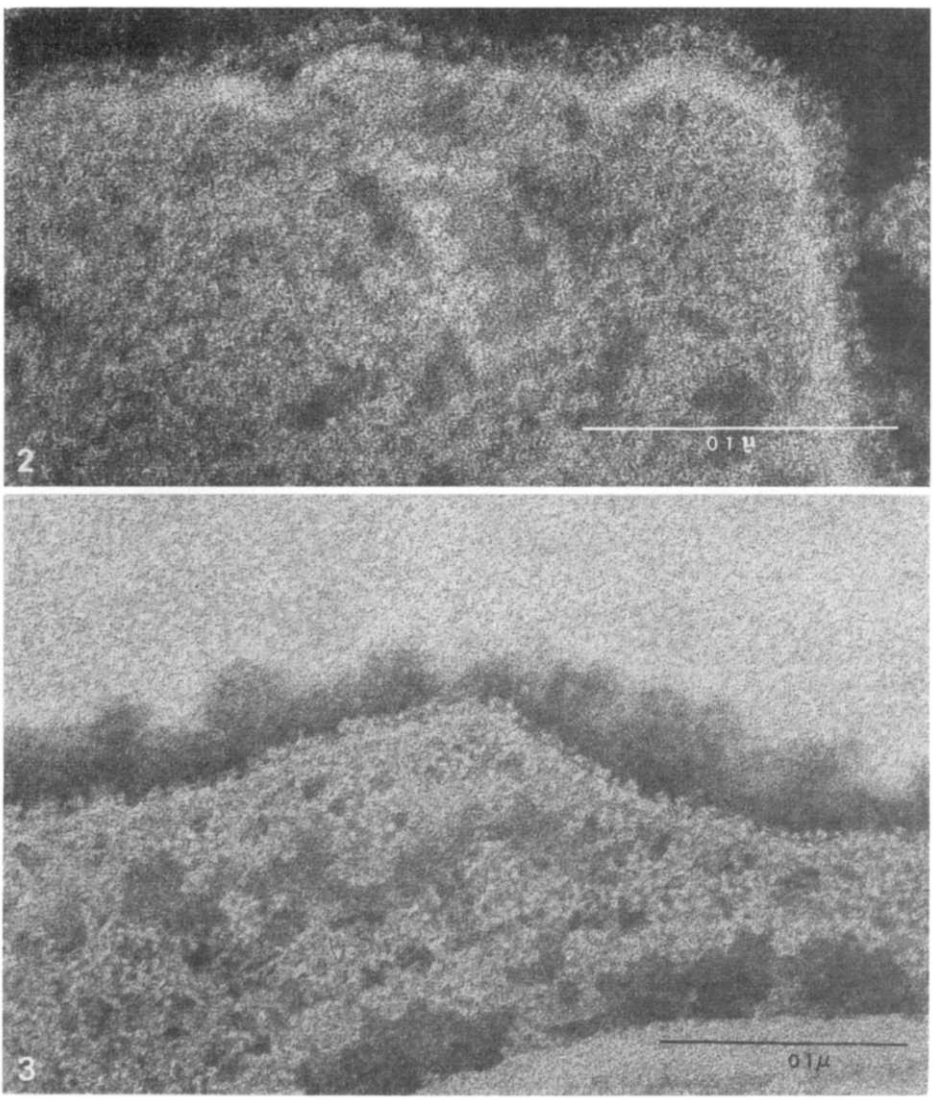
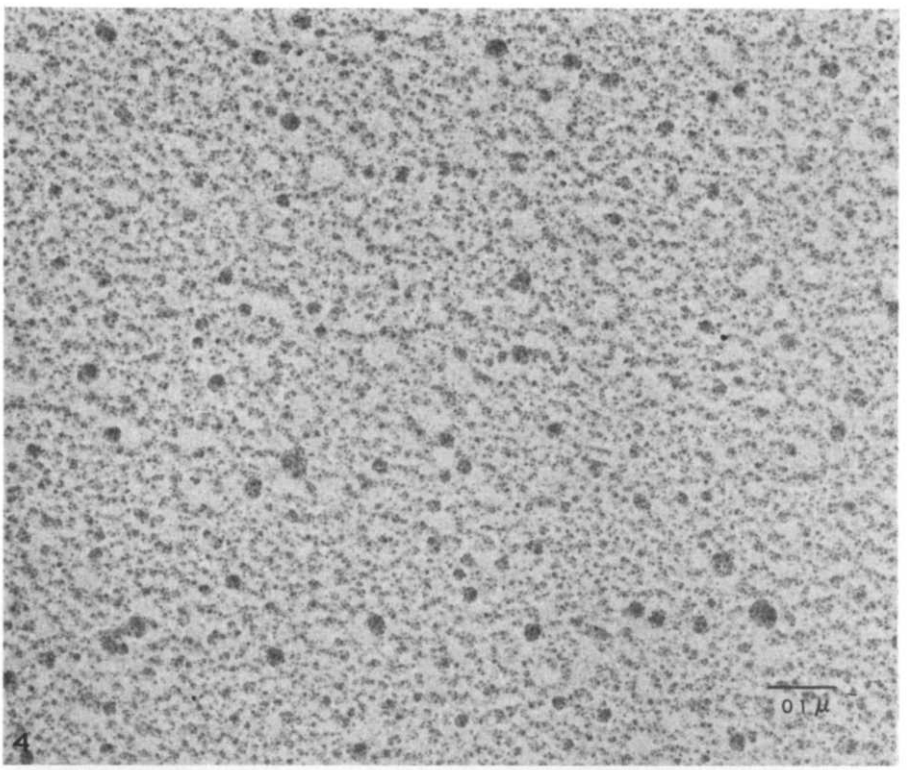



Fig. 2. (Top) Isolated mouse-liver plasma membranes negatively stained with phosphotungstate (2%, pH 7.2) at 4°. On the surface and especially on the edge of the membrane sheet globular knobs of about 60 Å width are visible.

Fig. 3. (Bottom) Isolated rat-liver plasma membranes treated with trypsin (1:50, in phosphate buffer, 45 min), washed once with twice-distilled water and negatively stained at 4°. On the surface and edge of the membrane sheet many globular knobs are visible.



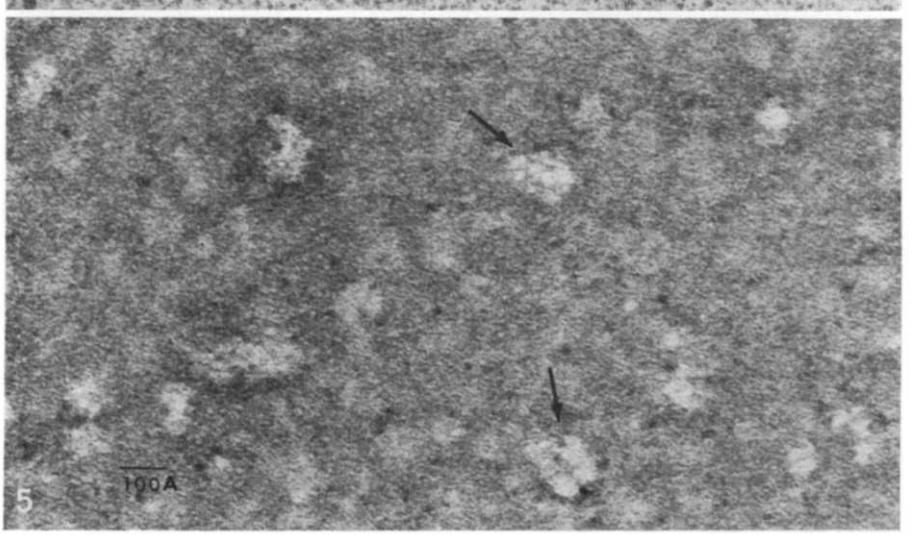




Fig. 4. (Top) Particles isolated by centrifugation for 18 h at 105000 × *g* of a supernatant obtained from rat-liver plasma membranes treated with papain (1:100) by centrifuging for 90 min at 45000 × *g*. The particles were spread on carbon film and shadowed with platinum and carbon while rotating.

Fig. 5. (Bottom) Particles present in the gel-filtrate fraction of Fig. 1 (17th ml), negatively stained with phosphotungstate at 4°. The particles vary in shape and size while some (arrow) appear to consist of smaller subunits. Aggregation is suggested by the appearance of some of the particles shown on the left side of the micrograph.

branes or parts thereof. Similar units were present on the plasma membranes isolated from mouse liver (Fig. 2) and mouse hepatoma (see also ref. 15).

After incubation of rat-liver plasma membranes with trypsin, which did not release the leucyl-β-naphthylamidase activity, the globular units were still present on the membrane surface (Fig. 3). This was not the case after treatment of the liver plasma membranes with papain (1:50 or 1:200), these membranes being now entirely devoid of the globular units. Particles, resembling the globular units *in situ*, could be demonstrated in (i) the supernatant of the papain-treated membranes following centrifugation for 90 min at 45000 × *g*, (ii) the pellet* obtained from the latter supernatant by centrifugation at 105000 × *g* for 18 h (Fig. 4), and (iii) the gel-filtrate fractions showing high dipeptidase activity (Fig. 5). Figs. 4 and 5 show that the isolated particles vary in shape and size. This may be due to (i) the effect of papain, (ii) aggregation resulting from the salt treatment of the particles during gel filtration (Fig. 5, left; extensive aggregation of particles has been observed in some of these fractions) or the drying of the preparation during the negative staining, and (iii) some variability in shape and size of the particles *in situ* (*cf.* micrographs in refs. 1, 15, 22 and 23, and also Fig. 2). Some of the globular knobs of isolated rat-liver plasma membranes are detached from the membranes during negative staining and lie free in the phosphotungstate film[1]. The diameter of the latter particles amounts to some 100 Å, probably as a result of their flattening[22].

On none of the plasma membranes isolated from the rat hepatoma-484 could the globular knobs be detected[15]. The surface of these membranes was in many areas coated by a fluffy layer (illustrated in ref. 15) that might obscure the presence of the globular knobs. After treatment of the membranes with trypsin the fluffy layer was removed but no globular knobs became visible. Hence it can be concluded that the rat-hepatoma membranes do not contain these units.

## DISCUSSION

The association of leucyl-β-naphthylamidase with the 50–60 Å globular units present on isolated liver plasma membranes has been established by the following criteria. (i) The enzyme is released from the membranes by papain but not by trypsin; (ii) the globular units are no longer detectable on the membranes which have been treated with papain while they remain present on trypsin-treated membranes; (iii) particles resembling the globular units are abundantly present in the supernatant of the papain-treated membranes and fractions derived therefrom which contain leucyl-β-naphthylamidase activity. These results, together with the finding that the globular knobs are apparently restricted to certain plasma membrane sheets or parts thereof, allow one to conclude that the globular units represent a structurally and functionally specialized type of repeating unit that locally coats the plasma membrane surface. The site of location of this repeating unit is very probably the plasma membrane lining the bile spaces (canaliculi) since the marker-enzyme has been demonstrated[10,11] histochemically to be associated with this region of the plasma membranes *in situ*,

* Although the specific leucyl-β-naphthylamidase activity of this pellet was higher than that exhibited by the residual membranes and the 105000 × *g* supernatant, an as yet unexplained loss of enzyme activity in the 105000 × *g* pellet and supernatant occurred.

while the isolated liver plasma membranes are also rich in bile spaces[14,15,18,22,23]. If it is assumed that the gel-filtrate fractions showing the highest specific leucyl-β-naphthylamidase activity contain only globular knobs, it can be concluded that some 10% of the total membrane protein (including the saline-soluble protein) is present in these units. This estimate is too high if other high-molecular weight breakdown products are released by papain from the membranes since these products would also be excluded from the gel.

It has further been demonstrated that the plasma membranes isolated from the differentiated (and slow growing) mouse hepatomas 4189 and 143066 contained the globular units in which leucyl-β-naphthylamidase was concentrated and that these hepatomas also possessed bile spaces[15]. By contrast, plasma membranes isolated from the more anaplastic (less differentiated and faster growing) rat-hepatoma-484 did not contain the globular units and this hepatoma also lacked bile spaces[15]. These differences provide additional, although indirect, support for the conclusion that the globular units are present on the plasma membranes lining the bile spaces. Although the rat-hepatoma plasma membranes lacked the globular units, these membranes still contained leucyl-β-naphthylamidase activity which in the other plasma membranes was concentrated in the globular units. It was shown, however, that papain released relatively much less of the dipeptidase activity from the rat-hepatoma plasma membranes than it did in the case of the rat-liver plasma membranes. Thus, although the hepatoma leucyl-β-naphthylamidase is still present in a form that is partly sensitive to papain, the association of the enzyme with the non-differentiated plasma membranes of the rat hepatoma appears to differ from that with the rat- and mouse-liver and mouse-hepatoma plasma membranes.

The specific leucyl-β-naphthylamidase activity of the mouse- and rat-liver plasma membranes is smaller than that exhibited by plasma membranes isolated by the same method from guinea-pig liver, as reported by COLEMAN AND FINEAN[12]. In contrast, the 5′-nucleotidase activity of the latter membranes is smaller than that shown by rat-liver plasma membranes[18,21]. These differences are very likely to be species dependent, since previous experiments in this laboratory have also revealed marked differences in the specific activities of the 5′-nucleotidase and alkaline glycerophosphatase between the plasma membranes isolated from rat and mouse liver[15].

## REFERENCES


1 E. L. BENEDETTI AND P. EMMELOT, *J. Cell Biol.*, 26 (1965) 299.
2 N. P. CUNNINGHAM AND F. L. CRANE, *Exptl. Cell Res.*, 44 (1966) 31.
3 J. OVERTON, A. EICHHOLZ AND R. K. CRANE, *J. Cell Biol.*, 26 (1965) 693.
4 T. ODA AND S. SEKI, *J. Electronmicroscopy, Tokyo*, 14 (1965) 210.
5 T. ODA AND S. SEKI, *Proc. 6th Intern. Congr. Electron Microscopy, Kyoto, 1966*, Biol. vol., p. 387.
6 C. F. JOHNSON, *Science*, 155 (1967) 1670.
7 G. HÜBSCHER, G. R. WEST AND D. N. BRINDLEY, *Biochem. J.*, 97 (1965) 629.
8 J. W. PORTEUS AND B. CLARK, *Biochem. J.*, 96 (1965) 159.
9 J. H. HOLT AND D. MILLER, *Biochim. Biophys. Acta*, 58 (1962) 239.
10 M. M. NACHLAS, B. MONIS, D. ROSENBLATT AND A. M. SELIGMAN, *J. Biophys. Biochem. Cytol.*, 7 (1960) 261.
11 B. MONIS, *Nature*, 208 (1965) 587.
12 R. COLEMAN AND J. B. FINEAN, *Biochim. Biophys. Acta*, 125 (1966) 197.
13 P. EMMELOT AND E. L. BENEDETTI, *Abstr. 7th Intern. Congr. Biochemistry, Tokyo, 1967*, p. 195.
14 P. EMMELOT, E. L. BENEDETTI AND PH. RÜMKE, in P. BUFFA, *From Molecule to Cell*, C.N.R., Rome, 1964, p. 253.

15 P. EMMELOT AND E. L. BENEDETTI, *Carcinogenesis: A Broad Critique, M. D. Anderson Hospital and Tumor Inst. Symp.*, University of Texas, Williams and Wilkins, Baltimore, Md., 1967, p. 471.
16 P. EMMELOT AND C. J. BOS, *Biochim. Biophys. Acta*, 121 (1966) 434.
17 E. L. BENEDETTI AND P. EMMELOT, *J. Cell Sci.*, 2 (1967) 499.
18 P. EMMELOT, C. J. BOS, E. L. BENEDETTI AND PH. RÜMKE, *Biochim. Biophys. Acta*, 90 (1964) 126.
19 J. A. GOLDBARG AND A. M. RUTENBURG, *Cancer*, 11 (1958) 283.
20 G. W. E. PLAUT, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. VI, Academic Press, New York, 1963, p. 231.
21 P. EMMELOT AND C. J. BOS, *Biochim. Biophys. Acta*, 120 (1966) 369.
22 E. L. BENEDETTI AND P. EMMELOT, in F. HAGUENAU AND A. J. DALTON, *The Membranes*, Academic Press, New York, 1967, in the press.
23 P. EMMELOT AND E. L. BENEDETTI, in H. PEETERS, *Protides of the Biological Fluids, 15th Colloquium, Bruges*, Elsevier, Amsterdam, 1967, p. 315.
24 A. B. NOVIKOFF AND M. HEUS, *J. Biol. Chem.*, 238 (1963) 710.
25 A. B. NOVIKOFF, E. ESSNER, S. GOLDFISCHER AND M. HEUS, *Symp. Intern. Soc. Cell Biol.*, 1 (1962) 149.