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GLYCOSIDASE ACTIVITY OF BOVINE LIVER PLASMA MEMBRANES

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SUMMARY

1. Plasma membranes were isolated on a large scale from bovine liver by a modified method of NEVILLE[1]. They were characterized with respect to contamination by endoplasmic reticulum, lysosomes, and mitochondria.

2. The membranes were found to contain glycosidases capable of hydrolyzing uridine diphosphogalactose (UDP-Gal) and uridine diphosphoglucose as well as *o*-nitrophenyl α- or β-galactosides.

3. The hydrolysis of UDP-Gal using a ^{14}C-labeled substrate in the galactose moiety allowed a rapid estimation of the amount of plasma membrane contaminating microsomal fractions. Smooth microsomes were contaminated about 14 % with plasma membranes. This value agreed with estimates based on the content of Mg^{2+}-stimulated ATPase and of 5'-nucleotidase. Rough microsomes contained about 5 % plasma membranes using this criterion. Purified nuclei and mitochondria from bovine liver showed negligible amounts of this activity.

4. The optimum pH of the enzyme was 7.2 using cacodylate buffer. Mg^{2+} stimulated the activity slightly whereas Mn^{2+} was inhibitory. The K_m for UDP-Gal was 0.33 mM.

INTRODUCTION

In the course of studying glycosyl transfer reactions in bovine liver subcellular fractions, we found that a significant amount of uridine diphosphogalactose (UDP-Gal) was broken down regardless of the presence of an added acceptor for galactose. Since the activity was found primarily in smooth rather than in rough microsomes, it appeared that the activity was due to contamination of the smooth microsomes with plasma membranes or Golgi vesicles. Therefore, we investigated the ability of highly purified plasma membranes from bovine liver to decompose glycosides. It was found that plasma membranes catalyzed the breakdown of both α- and β-glucosides and galactosides and that the small amount of activity found in smooth microsomes was probably due to plasma-membrane contamination.

Abbreviations: UDP-Gal, uridine diphosphogalactose; UDP-Glc, uridine diphosphoglucose; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

MATERIALS AND METHODS

Large scale preparation of plasma membranes

Bovine liver was obtained from the slaughterhouse, packed in ice and used within 1 h after the death of the animal. Plasma membranes were prepared from bovine liver using a modification of the method of NEVILLE[1].

All operations are carried out at 4°. Connective tissue is trimmed from the liver which then is ground in a small meat grinder. Eight 18-g portions of the mince are each suspended in 18 ml of 1 mM $NaHCO_3$ and transferred to a 50-ml Potter–Elvehjem homogenizer. Two full strokes at 1000 rev./min with a teflon pestle (measured clearance of 0.020 inch) are used to homogenize each portion of liver. The eight portions are suspended in 3600 ml of 1 mM $NaHCO_3$ and stirred for 3 min. The mixture is filtered first through two layers and then through four layers of cheesecloth. The filtrate is centrifuged at 2800 rev./min (2000 × *g*) for 11 min in six 650-ml plastic bottles (IEC No. 2936) using a MSE-6L centrifuge equipped with a No. 59116 blood bag angle rotor. The supernatants are carefully decanted and the bottles drained briefly. Lipid adhering to the upper wall of each bottle is wiped away, and the contents are suspended by swirling with 35 ml of 1 mM $NaHCO_3$. Occasionally a dark ring is observed in the pellet. This ring is not suspended by gentle swirling and is discarded. The pellet from each bottle is homogenized with one stroke of an "A" size pestle in a Dounce homogenizer. The contents of six homogenizers are diluted with 3.2 l of 1 mM $NaHCO_3$ and are stirred for 3 min. The resulting suspension is centrifuged at 2500 rev./min for 11 min (1500 × *g*). The membranes are suspended with a Dounce homogenizer as before using washes of 10–15 ml of 1 mM $NaHCO_3$. Two loads are made concurrently: one is suspended while the second is centrifuged. The two batches of crude membranes are combined (final volume about 140 ml), and 63 % sucrose (300 ml) is added to the suspension before the final concentration is adjusted to 44.1 ± 0.1 % using a Bausch and Lomb Abbe-3L refractometer. The suspension is divided among 15 tubes (34–36 ml each), is overlaid with 18 ml of 42.2 % sucrose and is centrifuged 2 h at 25000 rev./min in a Spinco SW-25.2 rotor. The plasma membrane is recovered in the scum at the upper surface of the suspension using a broad spatula and is resuspended in 60 ml of 1 mM $NaHCO_3$. 4.5 ml are layered on each of three 250-ml MSE glass bottles containing a 25-ml cushion of 50 % sucrose overlaid with 200 ml of a linear gradient between 37 and 7.2 % sucrose. The bottles are centrifuged 45 min at 2700 rev./min in the MSE 6L centrifuge and are allowed to come to rest without using the brake. The cushion and the pink upper 2/3 of the gradient are discarded, and the membranes, which appear as flocculant aggregates in the lower 1/3 of the gradient and at the interface, are collected. The membranes are diluted with an equal volume of 1 mM $NaHCO_3$ and are centrifuged at 30000 rev./min for 10 min in a Spinco No. 30 rotor. They are resuspended finally in 12 ml of 1 mM $NaHCO_3$. The yield of membranes is 80–140 mg protein.

Preparation of microsomes

All operations are carried out at 4°, and all centrifugations are made using a Spinco preparative ultracentrifuge. Microsomes are prepared from bovine liver by a modification of the method of DALLNER[2]. The liver is trimmed of connective tissue and ground in a small meat grinder. 200 g of the mince are suspended in 600 ml of

0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) containing 0.25 M sucrose and adjusted to pH 7.55 with KOH. The mixture is homogenized thoroughly with a Potter–Elvehjem homogenizer using a teflon pestle with a clearance of 0.020 inch. The entire mixture is homogenized again with two full strokes using a pestle with a clearance of 0.012 inch. The final pH of the homogenate is adjusted to 7.1, and the mixture is centrifuged for 15 min at 15000 rev./min in a No. 30 rotor. The supernatant is poured through four layers of cheese-cloth, and the pellet is discarded. The supernatant is centrifuged for 45 min at 60000 rev./min in a No. 60 titanium rotor, and after discarding the supernatant, any lipid adhering to the walls of the tubes is wiped away. The pellets are suspended in 250 ml of 0.25 M sucrose (pH 7.5) and are homogenized with a pestle of 0.012 inch clearance. The mixture is centrifuged for 10 min at 18000 rev./min in a No. 30 rotor, and any dark-brown button is discarded. The remainder of the pellet and the supernatant are combined, resuspended as before and diluted to a final volume of 250 ml with 0.25 M sucrose (pH 7.5). 10 ml of this fraction are retained as the total microsome fraction. To the remaining 240 ml are added 3.6 ml of 1.0 M CsCl. After 15 ml of this mixture are carefully layered over 20 ml of 1.3 M sucrose containing 15 mM CsCl, the tubes are centrifuged for 1.75 h at 60000 rev./min in a No. 60 titanium rotor. The membranes at the interface (smooth membranes) are collected, diluted with an equal volume of cold distilled water and recovered by centrifuging for 45 min at 60000 rev./min in a No. 60 titanium rotor. The pellets are finally suspended in 0.25 M sucrose; the pellet from the centrifugation in CsCl (rough endoplasmic reticulum) is also suspended in 0.25 M sucrose. The yield from 200 g of wet weight of liver is 250–500 mg of smooth membranes and 60–80 mg of rough membrane protein.

Preparation of nuclei and mitochondria

Beef liver mitochondria were prepared as described previously[3]. Nuclei were prepared by a modification of the method of CHAUVEAU *et al.*[4].

Enzymic assays

α- and β-galactosidase activity was measured according to HUGHES AND JEANLOZ[5] except that 25 mM sodium cacodylate buffer (pH 7.2) was used instead of a phosphate–citrate buffer (pH 6.3) as recommended by these authors. In addition, the assay mixture was centrifuged 5 min in a clinical centrifuge after adding 0.2 M carbonate–bicarbonate buffer (pH 10.8) to prevent turbidity due to the plasma membranes.

When galactosidase or glucosidase activity was determined using nucleotide bound ^{14}C-labeled sugars, the following procedure was used. The assay mixture contained the following (μmoles in 75 μl): sodium cacodylate, 6; $MgCl_2$, 1; 2-mercaptoethanol, 3; UDP-Gal or uridine diphosphoglucose (UDP-Glc) uniformly ^{14}C-labeled in the sugar moiety (specific activity $3 \cdot 10^6$ counts/min per μmole), 0.15; and approx. 50 μg of protein. Incubation was for 1 h at 37° with shaking. At this time, 17 μl of 0.3 M EDTA were added, and the mixture was chilled to stop the reaction. The reaction mixture was then passed through a column (0.5 cm × 2.0 cm) of Dowex 2 in the Cl^- form; the column had previously been washed with distilled water. Unreacted UDP-Gal remained bound to the column, while free galactose (or glucose in the case of UDP-Glc) was washed directly into a tared planchet using two washes with 0.5 ml

of distilled water. The planchets were dried using an infrared lamp, and the radioactivity was determined using a Nuclear-Chicago gas-flow counter Model No. 4312 equipped with a micromil window. The dried planchets were weighed, and corrections were made for self-absorption. For each set of assays, a control sample without enzyme was included to correct for nonenzymic hydrolysis or for contamination of the substrate with free radioactive sugar. Under these assay conditions, the amount of hydrolysis was linear with time and with the quantity of plasma-membrane protein added.

Succinate–cytochrome *c* reductase, NADH–cytochrome *c* reductase, and Mg^{2+}-stimulated ATPase activities were determined as described previously[6]. 5'-Nucleotidase was determined according to MICHELL AND HAWTHORNE[7], glucose-6-phosphatase according to SWANSON[8] and acid phosphatase according to WATTIAUX AND DEDUVE[9]. The inorganic phosphate released from glucose-6-phosphatase and acid phosphatase was measured using the method of CHEN *et al.*[10]. For ATPase and 5'-nucleotidase, the Fiske–SubbaRow method as modified by KING[11] was used.

Chemical analyses

UDP-Glc and UDP-Gal, both uniformly ^{14}C-labeled in the glycoside residue were obtained from New England Nuclear Corp. *o*-Nitrophenyl α- and β-galactosides were obtained from Mann.

Protein was determined by the method of LOWRY *et al.*[12] and phosphorus by the method of CHEN *et al.*[10].

The product of the enzymic reaction was characterized in two ways. (1) High-voltage electrophoresis was carried out using a Savant pressure-plate type electrophoresis unit. After the incubation and the addition of EDTA, the entire reaction mixture was applied as a band to Whatman 3 MM paper saturated with 1 % sodium borate. Electrophoresis was run at 2500 V for 1 h using 1 % sodium borate in the reservoirs. Water at 5° was circulated continuously through one plate during the run. Each band was cut into 1-cm wide strips which were placed directly into scintillation bottles. 15 ml of toluene containing 4 g of 2,5-diphenyloxazole *plus* 50 mg of 1,4-bis-(5-phenyloxazolyl-2)benzene per l were added. Samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. (2) Chromatography of the reaction products was carried out on DEAE-cellulose paper (Whatman No. DE 81) sheets. The entire incubation mixture after addition of EDTA was applied as a bar 1 inch wide at the origin. The developing solvent was *n*-butanol–*n*-propanol–water (3:1:1, by vol.), and chromatography was continued for 48 h. At this time, the paper was air-dried overnight, and the radioactivity distribution was determined as described for the electrophoretic separation. Sugars were visualized after chromatography by spraying with aniline phthalate (260 mg per 10 ml water-saturated *n*-butanol) and by heating for 5–10 min at 105°.

Electron microscopy

The procedures used for fixation, dehydration, embedding and negative staining have been described previously[13], except that Araldite (CIBA No. 502) was substituted for Epon as an embedding medium. All micrographs were taken with a Hitachi 11B electron microscope.

RESULTS

The enzymic profile found for isolated bovine liver plasma membranes is compared to smooth and rough microsomes in Table I. The low values for acid phosphatase and succinate–cytochrome *c* reductase indicate less than 1 % contamination of the cell membrane preparation with lysosomes or mitochondria. The somewhat

TABLE I

COMPARISON OF PLASMA MEMBRANES AND SMOOTH AND ROUGH MICROSOMES OF BOVINE LIVER

All assays carried out at 32° except glucose-6-phosphatase and acid phosphatase which were at 37°.

Assay	*Plasma membranes*	*Rough microsomes*	*Smooth microsomes*
μg P per mg protein	17.1	34.7	40.0
ATPase*	2.5	0.074	0.390
Glucose-6-phosphatase*	0.048	0.30	0.27
5′-Nucleotidase*	0.81	0.029	0.074
Succinate–cytochrome *c* reductase**	0.0034	0.005	0.009
NADH–cytochrome *c* reductase**,***	0.087	2.3	2.4
Acid phosphatase*	0.0039	0.026	0.022
mg RNA per mg protein	—	0.308	0.068

* μmoles P_i released per min per mg protein.
** μmoles cytochrome *c* reduced per min per mg protein.
*** 4 μg rotenone added in 4 μl ethanol (or 8 μg antimycin added in 8 μl ethanol) per 1 ml assay mixture.

higher and variable amounts of glucose-6-phosphatase found indicate a maximum value for the microsomal contamination of 10–16 %. The rotenone or amytal insensitive NADH–cytochrome *c* reductase values indicate less contamination; however, the level of activity found in liver microsomes decreases on storage and may not be a reliable quantitative marker. The plasma membranes have high ATPase and 5′-nucleotidase activities. The ATPase is not Na^+ and K^+ stimulated and is ouabain insensitive.

Fig. 1A shows an electron micrograph of purified plasma membranes. A high frequency of desmosomes and clusters of bile canaliculi are evident throughout. A high magnification of a desmosome is shown in Fig. 1B. Fig. 1C shows a portion of the plasma membrane negatively stained with phosphotungstic acid. The surface of the membrane appears studded with particles about 50 Å in diameter. Similar particles which appear to contain leucyl-β-naphthylamidase activity, have been described by EMMELOT *et al.*[14] in rat liver plasma membranes.

Table II summarizes the assay results of various bovine liver cell fractions for galactosidase activity using UDP-[^{14}C]Gal as the substrate. It can be seen that a 10–15 % contamination of smooth microsomes with plasma membrane is sufficient to account for the activity found in this fraction. The same level of contamination is indicated by the ATPase and 5′-nucleotidase activities of smooth microsomes (*cf.* Table I).

The reaction product was identified as free galactose using high-voltage electro-

phoresis of the reaction mixture in 1% sodium borate. Free galactose moved as a distinct band just behind unreacted UDP-Gal. Since some overlapping of the two occurred, better quantitation could be obtained using chromatography on DEAE-cellulose paper. Unreacted UDP-Gal remained close to the origin. Carrier galactose, added to the reaction mixture and located with the aniline phthalate spray, migrated

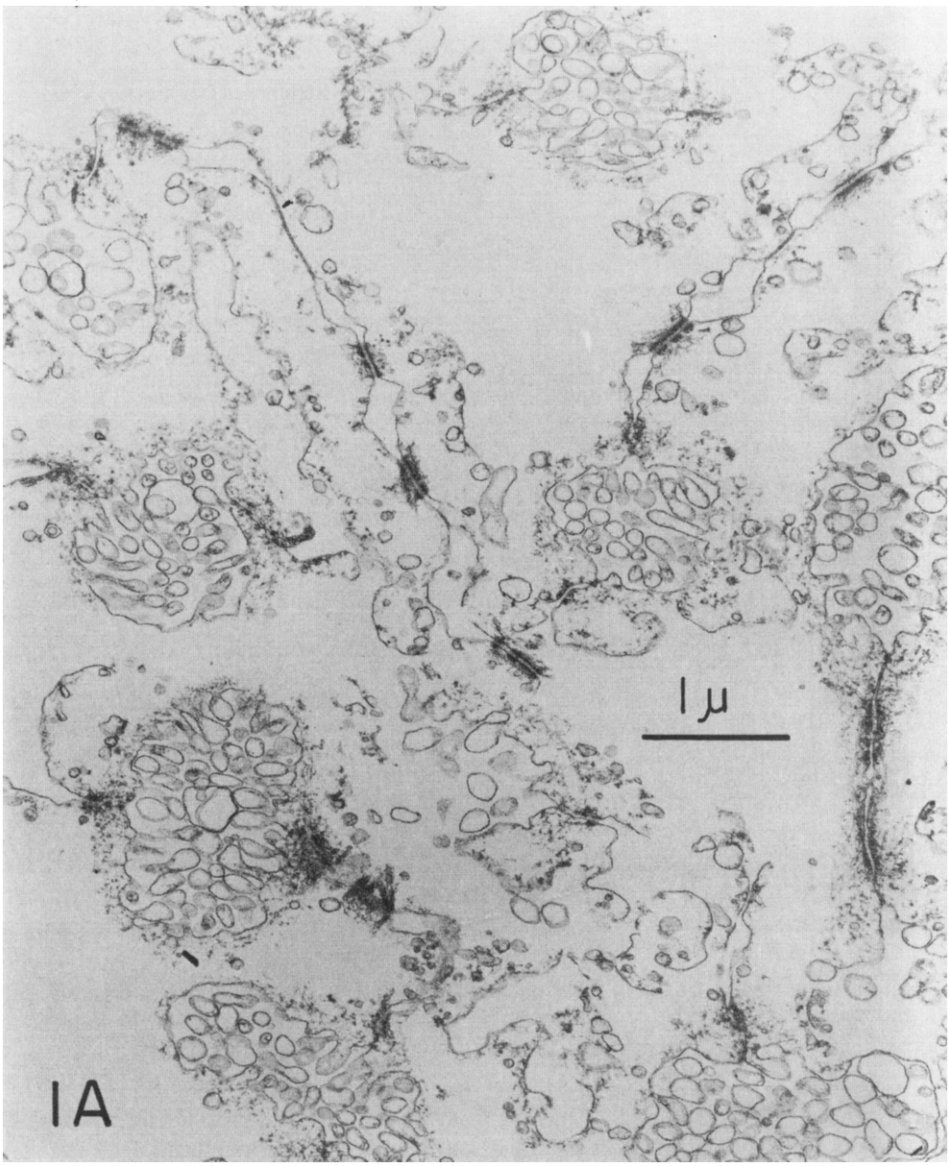
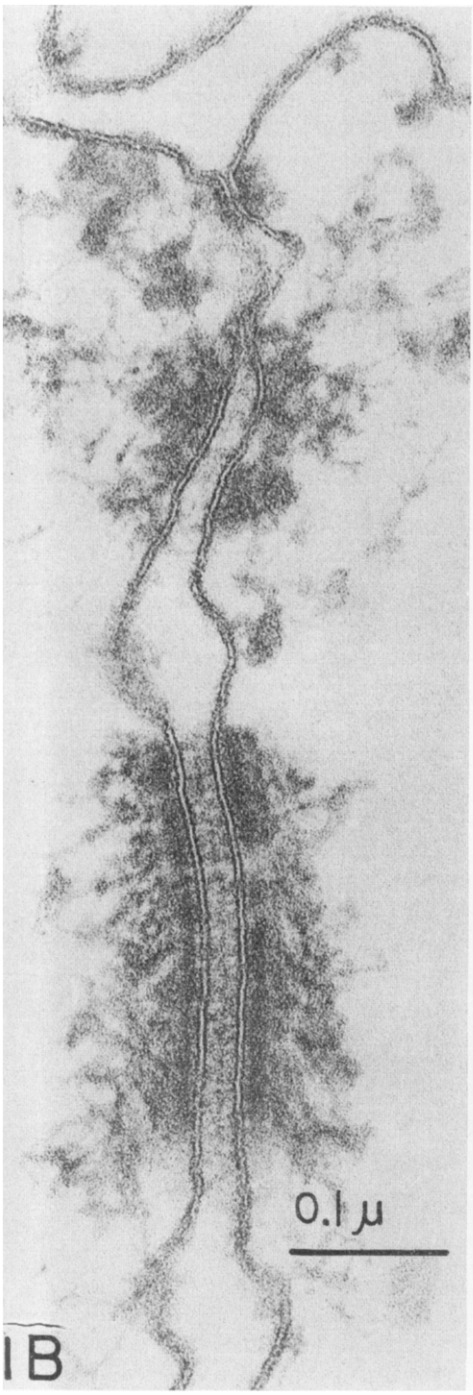
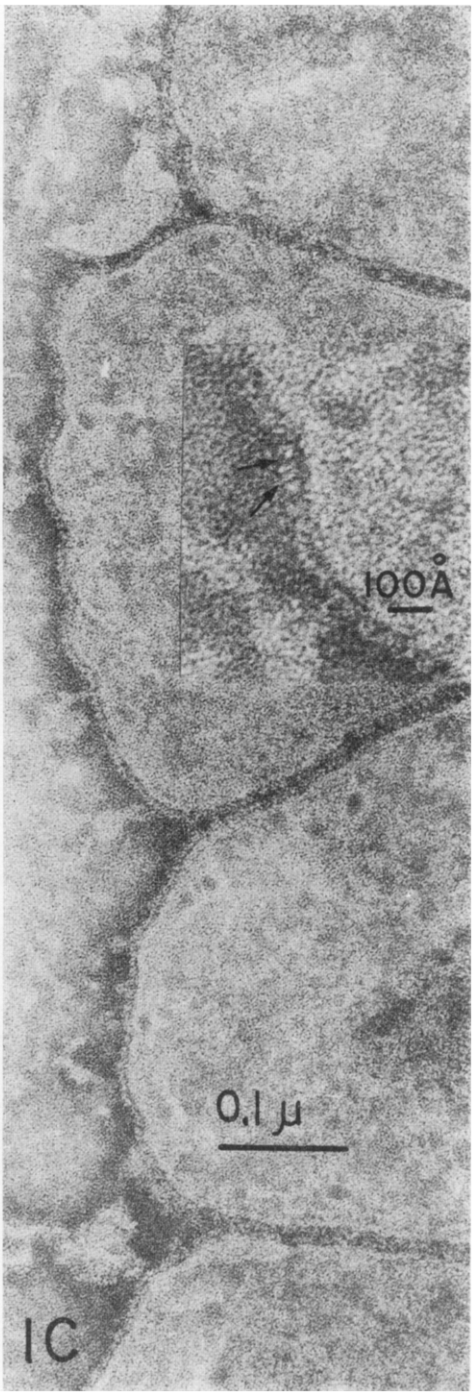


Fig. 1. Isolated bovine liver plasma membranes. A. Low magnification (× 17 500) showing prevalence of bile canaliculi and desmosomes. B. High magnification of desmosome (× 200 000). C. Negatively stained preparation using 2% potassium phosphotungstate (× 160 000) showing small knob-like protrusions about 50 Å in diameter. A higher magnification of the membrane is shown in the insert (× 400 000). Arrows indicate the knobs arrayed on the edge of the membrane.


0.1 μ
1B


100 Å
0.1 μ
1C

together with the product and was clearly separated from disaccharides such as maltose which migrated much more slowly. The specific activity of plasma membranes determined chromatographically corresponded well with the results using Dowex 2 to remove unreacted substrate.

TABLE II

GALACTOSIDASE ACTIVITY OF BOVINE LIVER CELL FRACTIONS USING UDP-[^{14}C]Gal AS SUBSTRATE

Rate expressed in nmoles [^{14}C]galactose released per h per mg protein at 37°.

Fraction	*Rate*
Total homogenate	9.8
Rough microsomes	18.2
Smooth microsomes	56.9
Mitochondria	0.1
Nuclei	0.0
Plasma membranes	407

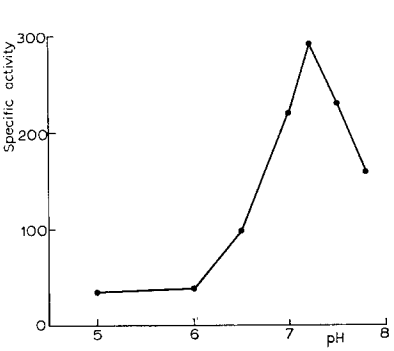


Fig. 2. The effect of pH on the hydrolysis of UDP-[^{14}C]Gal by plasma membranes. 3 μl of 0.2 M cacodylate buffer, adjusted to the desired pH with HCl or NaOH, were added to the assay mixture. Final volume of the assay mixture was 75 μl. Specific activity is expressed as nmoles [^{14}C]galactose released per h per mg protein at 37°.

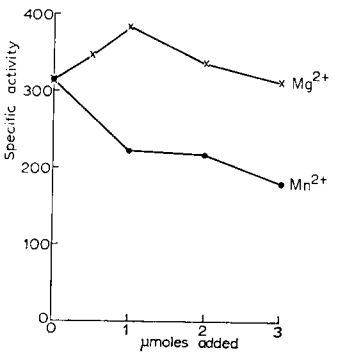


Fig. 3. The effect of added $MgCl_2$ or $MnCl_2$ on the hydrolysis of UDP-[^{14}C]Gal by plasma membranes (pH 7.2). The final volume of the assay mixture was 75 μl. Specific activity is expressed as nmoles [^{14}C]galactose released per h per mg protein at 37°.

TABLE III

SPECIFICITY OF GLYCOSIDASE ACTIVITY OF PLASMA MEMBRANES

Specific activity: nmoles sugar released per h per mg protein at 37° (pH 7.2). The same preparation of plasma membranes was used for all assays.

Substrate	*Specific activity*
UDP-[^{14}C]Gal	387*
UDP-[^{14}C]Glc	331*
o-Nitrophenyl α-galactoside	156**
o-Nitrophenyl β-galactoside	135**

* Assayed using Dowex 2 method.
** Assayed measuring free nitrophenol formed (details in text).

The pH optimum of the reaction is shown in Fig. 2. The enzyme activity shows a sharp maximum at pH 7.2, and then rapidly declines above pH 7.8. When 0.2 M phosphate buffer was used in place of 0.2 M sodium cacodylate, it inhibited the activity between pH 7 and 8.

Fig. 3 summarizes the effects of adding Mg^{2+} or Mn^{2+} to the assay mixture. Some stimulation occurs when Mg^{2+} is added; the optimal amount is 1 μmole per 75 μl of assay mixture (13 mM). Mn^{2+} does not substitute for Mg^{2+} but actually appears inhibitory. Addition of 5 μmoles EDTA to the assay mixture prior to incubation completely abolishes the galactosidase activity of the membranes. The highly inhibitory effect of 0.2 M phosphate buffer may be related to its ability to sequester all the Mg^{2+} present in the assay mixture.

The K_m for UDP-Gal was found to be 0.33 mM.

Table III shows that the membranes are capable of hydrolyzing both α-glucosides and galactosides involving a UDP group linked to the sugar. When *o*-nitrophenyl galactosides are used as substrates, under the conditions found optimum for hydrolyzing UDP-Gal, the activity is significantly lower than for sugar nucleotides, although both α- and β-galactosides were hydrolyzed to some degree. The lack of specificity may be due to the presence of more than one enzyme in the membrane.

DISCUSSION

Homogenization of the tissue is a key step in the preparation of plasma membranes. Bovine liver presents special problems, since this tissue is much tougher than rat liver. The original homogenization method recommended by NEVILLE[1] using eight vigorous strokes with a Dounce homogenizer is not applicable to bovine liver. The use of a small meat grinder followed by homogenization with a loose homogenizer at low speed mashes the tissue sufficiently for the preparation of a reasonable yield of plasma membranes. Similar procedures have been used in our laboratory to prepare other cell organelles from this tissue namely nuclei, mitochondria and microsomes and Golgi apparatus. The variables which must be controlled are the clearances of the pestles, the number of complete passes used and the rev./min of the pestle. Since the homogenization steps are done on small portions of the suspension at one time, they are more time-consuming in a large-scale preparation and are carried out entirely in the cold room.

This study is the first report on the preparation of plasma membranes from bovine tissue. Recently, a procedure has been published for the large-scale preparation of rat liver plasma membranes using zonal centrifugation[15]. It is difficult to compare our preparation to that of these authors since the purity of their preparation is not fully documented. The yield of membranes in our procedure is lower. This is probably due both to the large amount of connective tissue found in bovine liver as well as to the larger number of fractionation steps used in our procedure to obtain a preparation of high purity.

The localization of an enzyme in a given organelle by fractionation procedures has at least two prerequisites: an assurance (1) that the preparation is relatively pure and (2) that small amounts of contaminating materials cannot be responsible for the observed activity. The preparation of plasma membranes obtained using our procedure is of reasonably high purity as determined qualitatively by electron microscopy and

quantitatively by the presence of marker enzymes for contaminating organelles. The marker enzymes are succinate–cytochrome *c* reductase for mitochondria[16] and glucose-6-phosphatase and rotenone-insensitive NADH–cytochrome *c* reductase for the rough or smooth endoplasmic reticulum[17]. The specific activity of these enzymes in purified plasma membranes as compared to purified mitochondrial and microsomal fractions indicates that the main contaminants of the plasma membranes are microsomes (10–16 % based on glucose-6-phosphatase assays). It has been claimed that in rat liver, there is a small amount of smooth endoplasmic reticulum which has no glucose-6-phosphatase activity[2]; however, we have not yet assessed whether such a fraction exists in bovine liver. Contamination of the preparations with lysosomes, measured by the level of acid phosphatase activity, appears to be negligible. Contamination with nuclei was not determined, but purified nuclei did not contain glycosidase activity. Golgi membranes were not detectable in the preparation. The evidence for this will be documented in a forthcoming publication[18].

Of the enzymes used as markers for plasma membranes, only 5′-nucleotidase and Mg^{2+}-stimulated ATPase[19] were useful. The latter is not a unique marker for plasma membrane, since mitochondria also contain a high level of this activity. A high specific activity of Mg^{2+}-stimulated ATPase and 5′-nucleotidase in the plasma membrane preparations was always correlated with lower amounts of contamination with endoplasmic reticulum and mitochondria and with high specific glycosidase activity. The significantly higher activities of all three plasma-membrane enzymes in smooth microsomes as opposed to rough microsomes is a good indication that plasma membranes tend to contaminate this fraction to a greater degree.

The presence of 5′-nucleotidase in microsomal and in plasma-membrane preparations of rat liver has been interpreted by some workers to indicate that this enzyme is actually localized in more than one membrane of the cell[20]. The specific activity of 5′-nucleotidase found in microsomes in both rat liver and beef liver is 10–20 % of that found in purified plasma membranes. It is our view that microsomes are contaminated with plasma membranes to that extent since bovine liver smooth microsomes always appear to have about twice as high an amount of plasma membrane enzymes as do rough microsomes, and cytochemical evidence supports the localization of 5′-nucleotidase in plasma membranes of rat liver, particularly in bile canaliculi[21].

It was not possible to measure the nucleoside triphosphate pyrophosphohydrolase described by LIEBERMAN *et al.*[22] as present in plasma membranes of rat liver. When assayed as described by these authors, our preparation released approx. 10 times as much inorganic phosphate as pyrophosphate from ATP.

The glycosidase activity reported here appears to be another very characteristic activity which can be used either to follow the purification of plasma membranes from liver homogenates or to assess the degree of contamination of a fraction with plasma membranes. It should be pointed out, however, that the reaction product must be confirmed to be free galactose in the assay using UDP-[^{14}C]Gal, since other products may also pass through Dowex 2. For example, kidney rough and smooth microsomes catalyze a rapid reaction under these conditions in which the products appear to be oligomers of galactose (B. FLEISCHER, unpublished observation).

The galactosidase activity of the purified membranes was 40-fold greater than that observed in the total homogenate. It is probable that some of the activity in the homogenate is due to the presence of lysosomal enzymes. Partially purified

lysosomal fractions have been shown to contain β-glycosidases which hydrolyze *o*-phenyl-β-D-galactoside (pH 5)[23,24]. β-Galactosidase released from ox liver by autolysis has been shown to have a broad plateau of activity (pH 5–7) in a phosphate–citrate buffer[25]. This enzyme is probably lysosomal in origin, and the high activity in phosphate buffer indicates that it is distinct from the activity reported here.

Recent reports have indicated the presence of a glycosyl transferase localized in HeLa cell membranes[26]. The enzyme is capable of catalyzing the transfer of glucose from UDP-Glc to galactose in collagen. It is not possible at the present time to say whether the enzyme described here is purely a hydrolytic enzyme or whether it would behave as a transferase if the correct receptor were used.

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REFERENCES

1 D. M. NEVILLE, JR., *Biochim. Biophys. Acta*, 133 (1967) 168.
2 G. DÄLLNER, *Acta Pathol. Microbiol. Scand., Suppl.*, 166 (1963) 1.
3 S. FLEISCHER, G. ROUSER, B. FLEISCHER, A. CASU AND G. KRITCHEVSKY, *J. Lipid Res.*, 8 (1967) 170.
4 J. CHAUVEAU, Y. MOULÉ AND CH. ROUILLER, *Exptl. Cell Res.*, 11 (1956) 317.
5 R. C. HUGHES AND R. W. JEANLOZ, *Biochemistry*, 3 (1964) 1535.
6 S. FLEISCHER AND B. FLEISCHER, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 406.
7 R. H. MICHELL AND J. N. HAWTHORNE, *Biochem. Biophys. Res. Commun.*, 21 (1965) 333.
8 M. A. SWANSON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic Press, New York, 1955, p. 541.
9 R. WATTIAUX AND C. DEDUVE, *Biochem. J.*, 63 (1956) 606.
10 P. S. CHEN, T. Y. TORIBARA AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756.
11 E. J. KING, *Biochem. J.*, 26 (1932) 292.
12 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
13 S. FLEISCHER, B. FLEISCHER AND W. STOECKENIUS, *J. Cell Biol.*, 32 (1967) 193.
14 P. EMMELOT, A. VISSER AND E. L. BENEDETTI, *Biochim. Biophys. Acta*, 150 (1968) 364.
15 C. PFLEGER, N. G. ANDERSON AND F. SNYDER, *Biochemistry*, 7 (1968) 2826.
16 C. DEDUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX AND F. APPELMANS, *Biochem. J.*, 60 (1955) 604.
17 L. ERNSTER, P. SIEKEVITZ AND G. PALADE, *J. Cell Biol.*, 15 (1962) 541.
18 B. FLEISCHER, S. FLEISCHER AND H. OZAWA, *J. Cell Biol.*, in the press.
19 P. EMMELOT AND C. J. BOS, *Biochim. Biophys. Acta*, 120 (1966) 369.
20 C. C. WIDNELL AND J. C. UNKELESS, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 1050.
21 E. REID, in D. B. RODYN, *Enzyme Cytology*, Academic Press, New York, 1967, p. 321.
22 I. LIEBERMAN, A. I. LANSING AND W. E. LYNCH, *J. Biol. Chem.*, 242 (1967) 736.
23 O. Z. SELLINGER, H. BEAUFAY, P. JACQUES, A. DOYEN AND C. DEDUVE, *Biochem. J.*, 74 (1960) 450.
24 S. MAHADEVAN AND A. L. TAPPEL, *Arch. Biochem. Biophys.*, 128 (1968) 129.
25 G. A. LENNY, A. MCALLAN AND A. J. HAY, *Biochem. J.*, 82 (1962) 225.
26 A. HAGOPIAN, H. B. BOSMANN AND E. H. EYLAR, *Arch. Biochem. Biophys.*, 128 (1968) 387.