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ISOLATION AND PARTIAL CHARACTERIZATION OF THE RAT LIVER LIGANDOSOME FRACTION

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The subcellular distribution in rat liver of non-latent and latent NADH pyrophosphatase was determined by analytical sucrose density gradient centrifugation. Non-latent NADH pyrophosphatase activity was distributed similarly to the plasma membrane marker, 5′-nucleotidase. However, latent NADH pyrophosphatase was found at the low density region of the gradient, similar to the distribution of galactosyl transferase, a Golgi marker. A population of membranes, corresponding to those from the low density region, was prepared by discontinuous sucrose gradient centrifugation. Radiolabelled insulin was used, to monitor the involvement of these membranes in ligand internalization. The membrane perturbant, digitonin, was used to effect a partial separation between membranes bearing NADH pyrophosphatase and those bearing galactosyl transferase. The mechanism by which this separation is effected has been investigated and it was shown that, although digitonin caused a loss of enzyme latency, the density shift was not due to this effect. The partially purified ligandosome-rich fraction was characterized by enzymic and ultrastructural analysis. A novel EM cytochemical stain for NADH pyrophosphatase identified a vesicular fraction distinct from Golgi lamellae.

Introduction

Analytical subcellular fractionation of liver homogenates by sucrose density gradient centrifugation has shown that insulin and glucagon are taken up by rat liver and translocated to membranes of modal density, 1.12 $g \cdot cm^{-3}$. In addition to elements of the Golgi complex, this density fraction contains membranes rich in NADH pyrophosphatase. These latter membranes (ligandosomes) have been implicated in the subcellular processing of hormones and can be resolved from the bulk of galactosyl transferase-containing membranes by density gradient centrifugation after treatment with digitonin [1,2].

In the present study rat liver homogenates were subjected to discontinuous gradient centrifugation to prepare a low density membrane fraction. Golgi-rich and ligandosome-rich subfractions were isolated by further discontinuous density gradient centrifugation after treatment of the low density membrane fraction with digitonin. The use of cholesterol-binding reagents indicates that the NADH pyrophosphatase is contained in cholesterol-rich vesicles distinct from those involved in the coated pit and coated vesicle model [3,4].

Materials and Methods

Tissue preparation. Non-fasted male Sprague-Dawley rats (200–250 g) were killed by cervical dislocation. Portions of liver, 2 g, were minced at scissor points, homogenised in 10 ml of 0.25 mol/l sucrose containing 1 mmol/l Na_2 EDTA (pH 7.2) and 22 mmol/l ethanol with 10 strokes each of a

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loose fitting and of a tight-fitting pestle in a 15 ml Dounce homogeniser (Kontes Glass Co., Vineland, NJ, U.S.A.) and filtered through a 50 μm mesh filter.

Hepatic portal vein injections. Sprague-Dawley rats (250–400 g) were anaesthetised by intraperitoneal injection of phenobarbitone (50 mg/kg). ^{125}Iodo[A^{14}]tyrosyl-insulin (40 pmol/l, 0.5 μCi) was injected into the portal vein and subsequently the liver was excised and homogenised as above.

Analytical subcellular fractionation. Analytical subcellular fractionation of rat liver homogenates was carried out on a Beaufay automatic zonal rotor, as described by Smith and Peters [1].

Preparative subcellular fractionation. Preparative centrifugation was carried out in a 3 × 25 ml swing out rotor of a Superspeed 65 Centrifuge (MSE Ltd., Crawley, Sussex). Sucrose solutions for the first centrifugation step contained 22 mmol/l ethanol and 1 mmol/l Na_2 EDTA, and for the second centrifugation step, 10 mmol/l imidazole-HCl (pH 7.4). In the first step liver homogenate, 2.5 ml was layered onto a discontinuous gradient of 5 ml sucrose-EDTA solution, $\rho = 1.09$ g · cm^{-3} and 10 ml of sucrose-EDTA solution $\rho = 1.14$ g · cm^{-3}. After centrifugation at 100 000 × g for 1 h, the upper and lower interfaces were aspirated in a volume of 2.5 ml and the pellet resuspended in sucrose-EDTA solution.

In the second step the lower interface, $1.09 < \rho < 1.14$ g · cm^{-3}, was treated with an equal volume of ice-cold aqueous digitonin, 0.2 mg/ml, added dropwise with constant mixing. Aliquots of this mixture, 4 ml, were layered onto a second gradient of 5 ml of sucrose-imidazole solution $\rho = 1.17$ g · cm^{-3} and 10 ml of sucrose-imidazole solution $\rho = 1.25$ g · cm^{-3}. After centrifugation for 4 h at 100 000 × g, the upper (Golgi-rich) and lower (ligandosome-rich) interfaces were aspirated separately in volumes of 3 ml. In some experiments, [G-^{3}H]digoxin was added to a final activity of 1 μCi/mg digitonin.

Enzyme and protein assays. NADH pyrophosphatase was assayed by the method of Skidmore and Trams [5]. All other enzymes were assayed as previously described [1]. Protein was assayed by a modification of the method of Lowry [6] and by the fluorescent assay of Bohler et al. [7] with bovine serum albumin as standard.

Distribution of ^{125}I in fractions. Fractions from the first and second preparative spins were prepared from rats which had received ^{125}I-insulin by hepatic portal injection. Total ^{125}I and trichloroacetic acid-precipitable ^{125}I were counted on a Beckman 7000 Gamma Counter. To determine trichloroacetic acid-precipitable radiolabel, 25 μg/ml bovine serum albumin, and 1 μg/ml Triton X-100 were added to the sample, followed by an equal volume of ice cold 20% (w/v) trichloroacetic acid. After centrifugation for 15 min at 1500 × g in an MSE Coolspin centrifuge, radioactivity in the pellet was counted.

Effects of perturbants on latent enzyme activity. Aliquots of the lower band ($1.09 < \rho < 1.14$ g · cm^{-3} from the first preparative centrifugation step were mixed with an equal volume of cold aqueous solutions of digitonin, digoxin in 5% ethanol, or filipin in 5% dimethyl sulphoxide. Enzymic analysis of these fractions was then carried out as previously described, except that assay buffers were isotonic (0.25 mol/l) in sucrose. Enzyme activities of the treated membranes were compared with untreated membranes incubated in isotonic assay buffer, with and without 0.1% (w/v) Triton X-100. The method allows a titration of the amount of perturbant needed to disrupt the vesicles.

Electron microscopy

(a) Negative staining. Samples of membrane preparations were dried onto formvar carbon-coated copper grids and viewed under the electron microscope following negative staining with 2% potassium phosphotungstate (pH 7.0).

(b) Positive staining. Samples of membrane preparations were pelleted in an Eppendorf centrifuge and fixed in 3% glutaraldehyde containing 0.1 mol/l sodium cacodylate (pH 7.4) and 5.0% sucrose for 30 min at 0°C. After 3 washes of 30 min in cacodylate buffer, the fractions were post-fixed in 1% osmium tetroxide in cacodylate buffer, dehydrated through a graded series of acetones and embedded in Spurr resin with epoxypropane as the transitional solvent.

Ultrathin sections were cut on a Reichert OM U3 ultratome with a diamond knife. Sections were counterstained with uranyl acetate and lead citrate

and examined in a Phillips EM300 electron microscope operating at 60 kV.

(c) Cytochemistry for NADH pyrophosphatase activity. Samples of the lower band after the first centrifugation step were fixed in 1% glutaraldehyde, and washed as above. The washed pellet was resuspended in incubation medium containing 100 mM Tris-maleate buffer (pH 9.0), 150 mM sucrose, 20 mM $MgCl_2$, 2 mM alkaline lead citrate and 1 mg/ml alkaline phosphatase. After incubation at 37°C for 30 to 60 min, the reaction was terminated by a brief wash in cacodylate buffer, and then post fixed, dehydrated and sectioned as above. Sections were examined under the electron microscope without counterstaining.

Materials

Filipin was a gift from Upjohn Co. Digitonin, digoxin and alkaline phosphatase (calf, Type 1S) were purchased from Sigma London Chemical Company Ltd., Poole, Dorset. Reagents for electron microscopy were obtained from TAAB Laboratories Ltd., Reading. ^{125}I-Insulin labelled specifically at the A-14 tyrosine was prepared after Jorgensen and Larsen [8].

Results

Analytical subcellular fractionation

Rat liver homogenate was subjected to analytical sucrose density centrifugation. The distributions of total and of non-latent NADH pyrophosphatase were determined. The distribution of latent NADH pyrophosphatase was calculated by difference. Fig. 1 compares the distribution of latent and non-latent NADH pyrophosphatase with that of the plasma membrane marker 5′-nucleotidase and the Golgi marker galactosyl transferase. The non-latent component of NADH pyrophosphatase shows a broad distribution similar to 5′-nucleotidase, and is consistent with location to the plasma membrane. About 30% of the NADH pyrophosphatase is latent, and distributed similarly to the galactosyl transferase in the fractions of low equilibrium density.

Characterisation of the low density membrane fraction (Table I)

Rat liver homogenate was fractionated on a discontinuous sucrose gradient, as described in Materials and Methods, and the three fractions were assayed for marker enzyme content. Fraction II, which contains 6% of the homogenate protein, was 6-fold enriched in galactosyl transferase and 11-fold enriched in latent NADH pyrophosphatase and was largely free from contamination by soluble, mitochondrial and lysosomal components. The specific activities of the endoplasmic reticulum marker, neutral α-glucosidase, and of the plasma membrane marker, 5′-nucleotidase, were similar to those in the homogenate. The fraction is 7-fold enriched in endocytosed insulin but 10-fold depleted in the lysosomal marker *N*-acetyl-β-glucosaminidase.

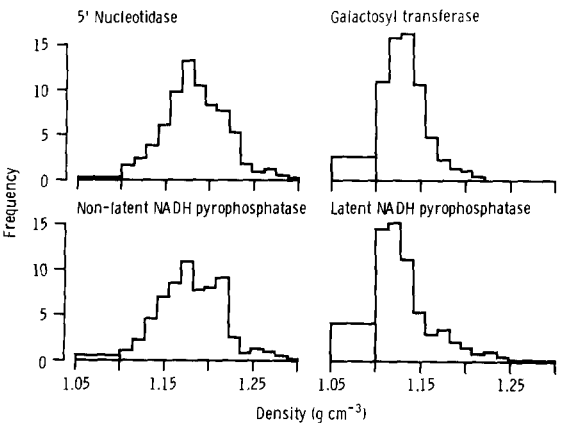

Fig. 1. Distribution of 5′-nucleotidase, galactosyl transferase, latent NADH pyrophosphatase and non-latent NADH pyrophosphatase in subcellular fractions from rat liver homogenate assayed immediately after collection. The results are averaged from two gradients. Recoveries of enzyme activity were: 5′-nucleotidase (93%); galactosyl transferase (130%); non-latent NADH pyrophosphatase (47%); latent NADH pyrophosphatase (60%).

Action of membrane perturbants on low density membranes

Low density membranes, corresponding to fraction II described above, were aspirated into a volume of 2.5 ml. After treatment with digitonin, digoxin or filipin, aliquots were taken either for estimation of the latency of enzyme activities, or were layered onto a second gradient and subfractionated as described in Materials and Methods. Fig. 2 shows the effect of digitonin on the latency of NADH pyrophosphatase, 5′-nucleotidase and

TABLE I

ENZYMIC ANALYSIS OF DISCONTINUOUS SUCROSE DENSITY GRADIENT FRACTIONS

Results show mean ± S.D. for four experiments of specific (homogenate) and relative specific (fraction) activities. Percent distribution of recovered activity shown between parentheses. The specific activities are given as μmol of substrate converted per minute per mg of protein.

Enzyme	EC No.	Homogenate activity (mU/mg protein)	Fraction I (density $\rho < 1.09$ g·cm^{-3})	Fraction II (density 1.09 < $\rho < 1.14$ g·cm^{-3})	Fraction III (density $\rho > 1.14$ g·cm^{-3})	Recovery (%)
Total NADH pyrophosphatase	3.6.1.9	128 ± 3	0.027 ± 0.012 (2)	1.83 ± 0.39 (17)	0.88 ± 0.04 (81)	55 ± 1
Latent NADH pyrophosphatase		25.6 ± 11.6	0.090 ± 0.012 (3)	10.7 ± 2.7 (76)	0.30 ± 0.13 (21)	74 ± 3
Galactosyl transferase	2.4.1.38	0.006 ± 0.0009	0.090 ± 0.004 (7)	6.10 ± 0.46 (72)	0.18 ± 0.04 (21)	44 ± 4
5′-Nucleotidase	3.1.3.5	42.8 ± 15.3	0.052 ± 0.022 (3)	1.48 ± 0.32 (11)	1.27 ± 0.20 (85)	76 ± 12
Neutral α-glucosidase	3.2.1.20	1.57 ± 0.007	0.35 ± 0.06 (17)	1.05 ± 0.5 (12)	1.14 ± 0.02 (71)	76 ± 4
N-Acetyl-β-glucosaminidase	3.2.1.30	1.44 ± 0.35	0.125 ± 0.035 (3)	0.09 ± 0.08 (4)	2.12 ± 0.62 (93)	119 ± 40
Succinate dehydrogenase	1.3.99.1	25.9 ± 3.5	0.082 ± 0.058 (3)	0.047 ± 0.033 (< 1.0)	1.92 ± 0.22 (97)	102 ± 12
Lactate dehydrogenase	1.1.1.27	163 ± 10	2.06 ± 0.38 (94)	0.27 ± 0.09 (2)	0.060 ± 0.030 (4)	79 ± 8
Trichloroacetic acid-precipitable ^{125}I			0.17 ± 0.04 (10)	6.71 ± 0.76 (59)	0.37 ± 0.11 (31)	60 ± 7
Protein (Lowry assay)		165 mg protein/ g wet weight	(38 ± 4.2)	(6 ± 1.0)	(56 ± 1.0)	91 ± 1

galactosyl transferase activities. Enzyme activity, at increasing digitonin concentrations, is given as a percentage of activity expressed in 0.1% Triton, when all the membranes are disrupted. NADH pyrophosphatase has a high latency (75%) and this latency is lost in the digitonin range 10–80 μmol/mg protein: 5′-nucleotidase is largely non-latent. In contrast, galactosyl transferase is mainly latent, but this remains largely unaffected by concentrations of digitonin sufficient to disrupt all the vesicles containing NADH pyrophosphatase.

The three reagents digitonin, digoxin and filipin were compared in their action on the latency of NADH pyrophosphatase activity (Fig. 3) and in their ability to cause a density shift of NADH pyrophosphatase-containing membranes (Table II). The data show that digitonin disrupts the vesicles and causes a concomitant density shift. Filipin causes loss of latency of NADH pyrophosphatase at concentrations equimolar with digitonin, but does not cause a density shift. Digoxin neither destroys latency nor causes a shift in equilibrium density. In experiments where [^{3}H]digoxin was added in tracer amounts, the label remained unassociated with the membranes and was recovered in the sample layer.

Enzymatic characterisation of the ligandosome membrane preparation

Low density membranes, treated with digitonin (80 μmol/mg protein), were subfractionated on a sucrose-imidazole gradient as described in Materi-

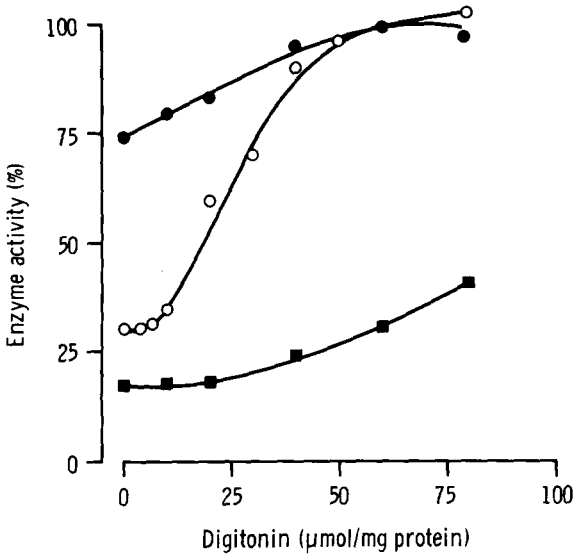

Fig. 2. The effect of digitonin on the latency of 5′-nucleotidase (●), NADH pyrophosphatase (○) and galactosyl transferase (■) activities. Aliquots of low density membrane fraction, prepared as described in Materials and Methods, were treated with solutions of digitonin then assayed under conditions which preserve the integrity of the untreated membranes. Enzyme activity is given as a percentage of the total activity expressed in the presence of 0.1%(w/v) Triton. The data is representative of two experiments giving similar results.

als and Methods. Low density membranes (fraction II) were compared in composition with the Golgi-rich subfraction (IIA), $\rho < 1.17$ g · cm^{-3} and

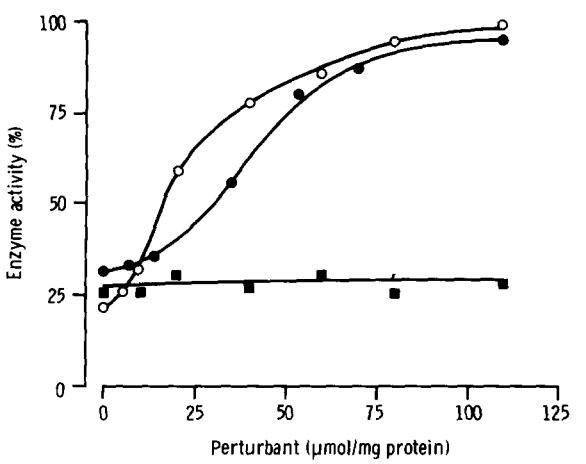

Fig. 3. Comparison of the action of digitonin (○), filipin (●) and digoxin (■) on the latency of NADH pyrophosphatase in low density membrane fraction. Enzyme activity (%) is defined as in Fig. 2. The data is representative of two experiments giving similar results.

TABLE II

ACTION OF PERTURBANTS ON THE DENSITY OF NADH PYROPHOSPHATASE-CONTAINING VESICLES

The distribution of NADH pyrophosphatase between the upper interface, $\rho < 1.17$ g·cm^{-3} and the lower interface, $1.17 < \rho < 1.25$ g·cm^{-3}, after treatment with various membrane perturbants was estimated as described in Methods.

Perturbant	Concentration (μmol/mg protein)	Distribution (%)	
		Upper	Lower
None	0	95	5
Digitonin	20	67	33
Digitonin	40	59	41
Digitonin	80	12	88
Filipin	80	91	9
Digoxin	80	94	6

the ligandosome rich subfraction (IIB), $1.17 < \rho < 1.25$ g · cm^{-3} (Table III). The majority of NADH pyrophosphatase is present in fraction IIB, with only 7% remaining in fraction IIA. The variability in the enrichment of the enzyme in fraction IIB (7-fold) reflects the variability in the protein measured in this fraction (8 ± 3%). Some galactosyl transferase (25%) is recovered in this fraction, and is enriched over the low density fraction. The percentage recovery of galactosyl transferase in this fraction corresponds with the percentage loss of latency of the enzyme after digitonin treatment (Fig. 2). There is no detectable succinate dehydrogenase in fraction IIB and the bulk of neutral α-glucosidase and *N*-acetyl-β-glucosaminidase are removed. The fraction is slightly enriched in 5′-nucleotidase, although recoveries of this enzyme are low.

A small enrichment (1.5-fold), of ^{125}I-insulin was found in the ligandosome fraction, while the Golgi fraction was depleted. A low recovery (36%) of the ^{125}I-insulin was found in the membrane fractions, the remainder in the soluble fraction.

Electron microscopy

Effect of digitonin on membrane profile. The low density fraction from the first preparative centrifugation was examined after positive staining (Fig. 4a) and negative staining (Fig. 4b). Both techniques show the stacked lamellae of the Golgi complexes, along with small filled vesicles and large empty

TABLE III

ENZYMIC ANALYSIS OF DISCONTINUOUS SUCROSE DENSITY GRADIENT FRACTIONS

Results show the means ± S.D. for six experiments of specific (fraction II) and relative specific (fractions IIA and IIB) activities. Percentage distribution shown between parentheses. The specific activities are given as μmol of substrate converted per min per mg of protein.

Enzyme	Fraction II (density 1.09–1.14, $g \cdot cm^{-3}$)	Fraction IIA (density $< 1.17\ g \cdot cm^{-3}$)	Fraction IIB (density 1.17–1.25 $g \cdot cm^{-3}$)	Recovery (%)	Enrichment over homogenate (*n*-fold)
(Latent) NADH pyrophosphatase	90.2 ±10.1	0.020±0.09 (17)	7.0 ±2.4 (68)	64 ±12	75
Galactosyl transferase	0.026± 0.014	0.95 ±0.17 (73)	2.92±0.10 (27)	88 ± 3	18
5′-Nucleotidase	36.0 ± 7.0	0.11 ±0.22 (26)	2.64±0.74 (74)	27 ± 4	4
Neutral α-glucosidase	3.27 ± 1.20	1.00 ±0.07 (94)	0.54±0.28 (6)	69 ± 4	0.6
N-Acetyl-β-glucosaminidase	1.04 ± 0.33	0.92 ±0.05 (87)	1.10±0.35 (13)	69 ± 4	0.1
Trichloroacetic acid-precipitated ^{125}I		0.35 ±0.07 (62)	1.50±0.60 (38)	36 ±12	10
Protein (fluorescamine assay)	10.1 mg/g	(86.7 ±2.8)	(13.3 ±4.8)	73.8± 5.6	–

vacuoles. After treatment with digitonin at concentrations sufficient to effect a separation of Golgi and ligandosome membranes, considerable disruption with disorder and fragmentation of the elements and a loss of definition of the surrounding medium is seen by positive (Fig. 4c) and negative staining (Fig. 4d).

Morphology of the ligandosome fractions. Digitonin-treated low density membranes were subfractionated as described in Materials and Methods to yield a ligandosome-rich fraction. Electron microscopy of positively (Fig. 5a) and negatively (Fig. 5b) stained fractions show a collection of heterogeneous vesicles, membrane ghosts and short lamellae.

Cytochemistry for NADH pyrophosphatase. Cytochemical staining of low density membranes (Fig. 6) shows that NADH pyrophosphatase is mainly localised to the interior of a heterogeneous population of empty vesicles some of which are in apposition to Golgi structures. There is very little labelling of the Golgi lamellae, though some label is associated with lipoprotein-packed vesicles.

Discussion

The intracellular fate of internalised hormones is still unresolved. However, the Golgi apparatus has been reported to be their primary destination [9,10]. Smith and colleagues showed that insulin [2] and glucagon [11] were translocated in rat liver, to a population of membranes having the same equilibrium density, in sucrose gradients, as the Golgi apparatus. Treatment with digitonin resolved the membrane-bound hormones from the bulk of the galactosyl transferase-containing elements, suggesting that they were associated with vesicles rich in NADH pyrophosphatase which were termed 'ligandosomes'. The present study has achieved a partial purification and characterisation of these vesicles.

Analytical subcellular fractionation of rat liver shows a differential distribution of latent and non-latent NADH pyrophosphatase, consistent with a dual localization to low density vesicles and plasma membrane sheets, respectively. The classical plasma membrane marker, 5′-nucleotidase

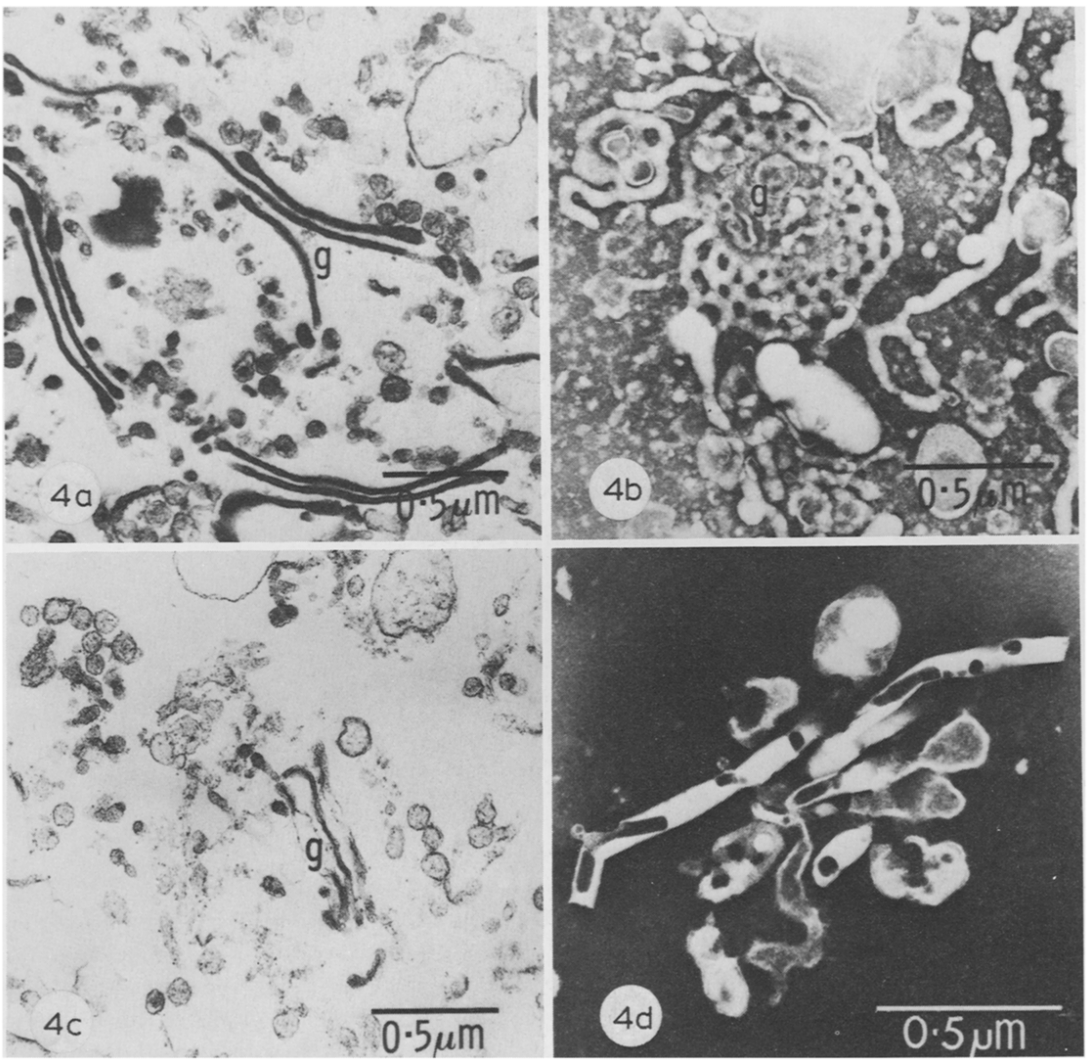

Fig. 4. (a) Electron micrograph of a thin section of the low density fraction stained with uranyl acetate and lead citrate, showing Golgi lamellae (g) along with large and small vesicles (×3200).
(b) Electron micrograph of the low density fraction negatively stained with phosphotungstic acid (PTA) (pH 6.3), showing a Golgi complex (g) as well as large and small vesicular structures (×37800).
(c) Electron micrograph of a thin section of digitonin treated low density fraction stained with uranyl acetate and lead citrate (×32000).
(d) Electron micrograph of digitonin-treated low density membrane fraction negatively stained with PTA (pH 6.3) (×51000). 4c and 4d both show a disruption of the Golgi (g) and a loss of definition of the membranes.

seems to be excluded from these vesicles, since the rather low activity present in the low density membranes shows a much lower latency than that of NADH pyrophosphatase.

The distribution of total NADH pyrophosphatase differs from that described by Smith and Peters [2]. The difference is probably due to the lability of the non-latent (plasma membrane) form of the enzyme in sucrose-EDTA solutions. In the present studies, a half-life of less than 1 h at 4°C was observed, and subsequently all assays were carried out immediately after fractionation.

Analytical fractionation shows the vesicles to have an equilibrium density of 1.09–1.14 $g \cdot cm^{-3}$,

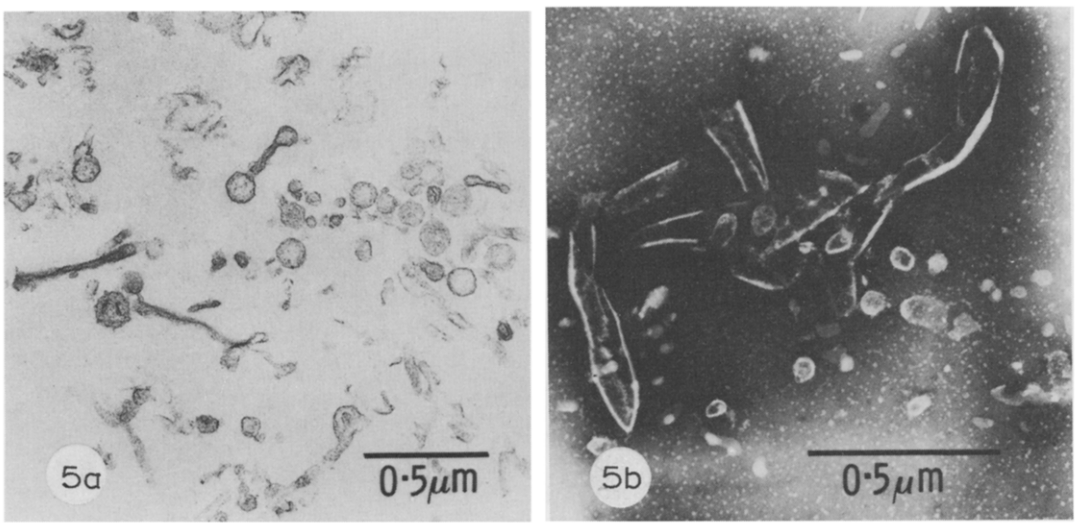

Fig. 5. (a) Electron micrograph of a thin section of the ligandosome-rich fraction stained with uranyl acetate and lead citrate showing short lamellae and a collection of vesicles (×32000).
(b) Electron micrograph of the ligandosome rich fraction stained with PTA (pH 6.3) showing short lamellae and a collection of vesicles (×50000).

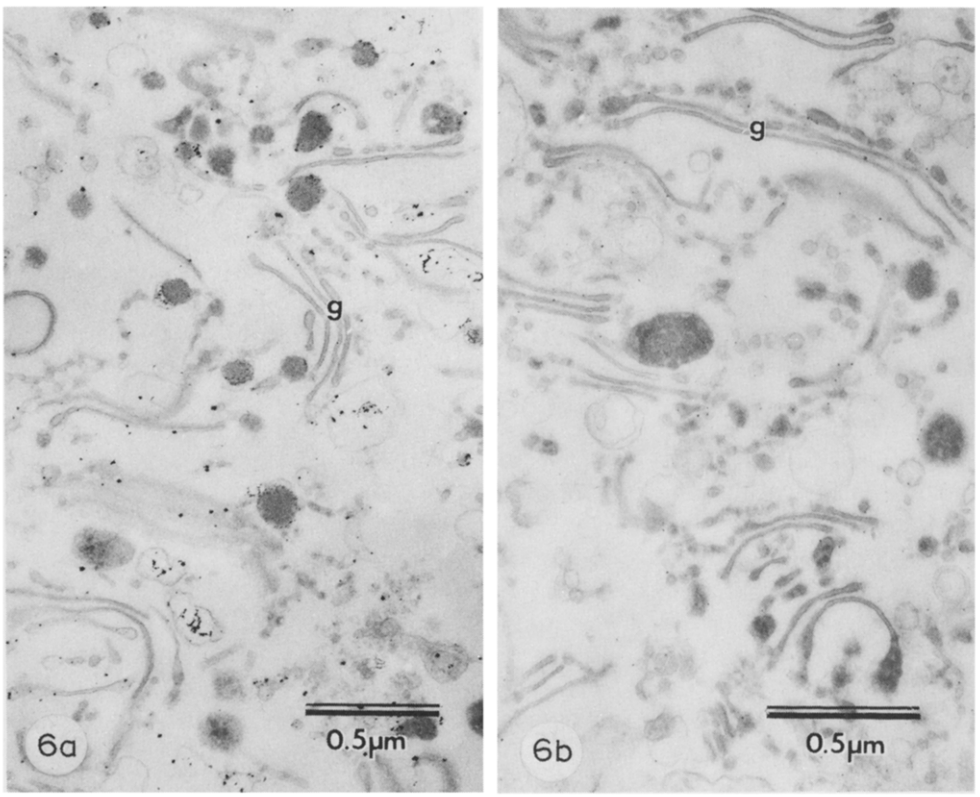

Fig. 6. (a) Electron micrograph of a thin section of the low density fraction that had been incubated for the localization of NADH pyrophosphatase. Lead phosphate precipitate can be seen in association with vesicular structures and not the Golgi (g) stacks (×44500).
(b) Incubation for NADH pyrophosphatase with alkaline phosphatase omitted. Lead deposition is not seen. Similarly incubation of membranes omitting NADH, and incubation of boiled membranes with the complete substrate mixture gave no lead precipitate.

and these density boundaries were employed for preparative centrifugation on discontinuous sucrose gradients. This gave an 11-fold enrichment of latent NADH pyrophosphatase. These membranes were treated with digitonin and centrifuged on a second discontinuous gradient with density steps of 1.17 and 1.25 $g \cdot cm^{-3}$. Amounts of digitonin sufficient to cause a density shift were found to cause concomitantly, a loss of latency of NADH pyrophosphatase activity. Sucrose solutions containing 10 mM imidazole-HCl buffer (pH 7.4), were required to preserve NADH pyrophosphatase activity in this second centrifugation step. The density interface $1.17 < \rho < 1.25$ $g \cdot cm^{-3}$, contained about 0.04% of the homogenate protein, and on the basis of latent NADH pyrophosphatase, represents a 75-fold purification of ligandosome membranes.

In addition to enzyme markers, the distribution of in vivo-administered ^{125}I-insulin was followed. The studies confirm previous work [2,11] showing that rat liver rapidly translocates insulin to a population of low density vesicles quite distinct from the lysosomes. The enrichment of ^{125}I-insulin in this fraction (7-fold), is comparable to that of galactosyl transferase and NADH pyrophosphatase. After digitonin treatment and the second centrifugation step, 65% of the insulin had dissociated from the membranes and was recovered in the soluble fraction. The Golgi fraction was depleted in insulin and the label present in this layer could be accounted for by soluble contamination. The ligandosome layer was slightly enriched in insulin (1.5-fold). However the association was not as marked as that found in analytical studies. Presumably, the longer time for preparative fractionation, (4–5 h), as opposed to analytical studies (30 min) results in greater dissociation.

These studies confirm that digitonin increases the equilibrium density of NADH pyrophosphatase containing low density vesicles from rat liver. However, since the amounts of digitonin required to cause an optimal density shift also led to a loss of latency of NADH pyrophosphatase, it initially seemed possible that digitonin was acting as a detergent making the vesicles permeable to sucrose. The experiments with digoxin and filipin strongly argue against this possibility. Digoxin is a homologue of digitonin having three rather than five sugar residues attached to the steroid ring. Digoxin does not destroy the latency of NADH pyrophosphatase, cause a density shift, or bind to the membranes. Filipin, a polyene antibiotic, increases the permeability of membrane, by interaction with cholesterol [12,13]. Despite having different detergent properties, filipin destroys the latency of NADH pyrophosphatase with very similar stoichiometry to digitonin. However, it does not cause a density shift showing that increasing the permeability of the vesicles to sucrose is not, in itself, sufficient to give a density shift. These results suggest that digitonin does cause a density increase by specific interaction with the ligandosome membrane.

Filipin-cholesterol complexes can be observed by electron microscopy in freeze-fractured membranes. This technique has suggested that cholesterol is excluded from the coated pit-receptosome pathway, [14]. However, our studies indicate the presence of a cholesterol-rich pathway for ligand internalization in rat liver.

There are three possible explanations for the presence of NADH pyrophosphatase in intracellular membranes. Firstly, the intracellular enzyme may be nascent enzyme en route to the plasma membrane. In this context, Howell and Palade [10] have detected alkaline phosphodiesterase I activity, an alternative expression of NADH pyrophosphatase in highly purified rat liver Golgi vesicles. However, the cytochemical studies suggest that NADH pyrophosphatase is not greatly enriched in the VLDL-containing vesicles. Secondly, NADH pyrophosphatase may exist in two completely separate pools on the plasma membrane and in low density vesicles. Detailed studies of the properties of the enzyme from the two locations would be of value. Thirdly, the intracellular enzyme may derive from plasma membrane internalization during endocytosis. This would imply highly selective incorporation of certain plasma membrane markers into the endosome, since 5′-nucleotidase is nonlatent in these fractions, despite having the same plasma membrane topology as NADH pyrophosphatase [13,16]. This is consistent with previous studies showing that antibodies to 5′-nucleotidase and to NADH pyrophosphatase are themselves internalised by different routes by perfused rat liver [17].

This study attempts to prepare ligandosome membranes from rat liver, using digitonin to resolve them from Golgi membranes. However, it is apparent both from latency studies and from electron microscopy that digitonin causes extensive membrane damage at concentrations sufficient to effect this separation, and this membrane damage permits the release of endocytosed insulin. Also, although the bulk of Golgi membranes were not shifted by digitonin, the ligandosome fraction is 2-fold enriched in galactosyl transferase. Polyacrylamide gel electrophoresis revealed similar polypeptide compositions of the Golgi and ligandosome fractions (data not shown). It is possible that the lipoprotein filled vesicles, in which Bergeron and co-workers report endocytosed insulin to be concentrated [18], would have shifted with digitonin, although the damage accrueing from digitonin treatment and subsequent ultracentrifugation makes this impossible to determine ultrastructurally.

Clearly, it is necessary to attempt to resolve Golgi and ligandosome membranes in the absence of digitonin for the following reasons. Firstly, if ligandosome membranes are to be analysed for comparison with other cell membranes, they should be as free from damage as possible. Secondly, in defining the ligandosome, Smith and Peters [2] used digitonin to suggest that polypeptide hormones are internalized to a structure distinct from the Golgi apparatus. Thirdly, the association between NADH pyrophosphatase and internalized hormone suggested by these workers remains to be established. The present study indicates that digitonin may act by liberating the various components of the native Golgi complex, the ligand-bearing and NADH pyrophosphatase-bearing membranes being amongst them.

One approach to determining whether the NADH pyrophosphatase-bearing membrane is a distinct entity or a domain of the Golgi apparatus is by EM cytochemistry of the low density membrane fraction for NADH pyrophosphatase. The results confirm the localization of the enzyme to the lumen of a heterogeneous population of vesicles, some of which contain lipoproteins, and some of which appear in intimate association with Golgi lamellae.

In further experiments, the low density membranes were preincubated at 25°C for 1 h in 5 mM EDTA (pH 7.4) prior to cytochemistry. This procedure inactivated 90% of the non-latent pyrophosphatase activity, leaving the latent activity unchanged (data not shown). Although the morphology of the Golgi lamellae was altered by this treatment, a similar density of cytochemical labelling for NADH pyrophosphatase was observed, and was again localized to the various vesicular structures. This range of vesicular structures is similar to those in which Bergeron and co-workers [19,18] localize insulin by autoradiography, although the cytochemical labelling appears denser in the empty vacuoles, while the hormones appear concentrated in the secretory elements.

Further studies will be directed towards investigating the possible relationship of the ligandosome with the Golgi apparatus, to prepare ligandosome membranes without the use of digitonin perturbation and to characterize this pathway of endocytosis.

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