

Isolation from the microsomal fraction of rat liver of a subfraction highly enriched in uncoated endocytic vesicles with high H^+ -ATPase activity and a 50 kDa phosphoprotein

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A subcellular fraction, highly enriched in uncoated vesicles (UCV) with high H^+ -ATPase (EC 3.6.1.34) activity, was isolated from the crude microsomal fraction of rat liver homogenates by discontinuous sucrose gradient centrifugation. The UCV fraction, recovered at the interface of sucrose density 1.08 and 1.10 g/ml, was shown morphologically to be a mixture of small, smooth-surfaced univesicular and a few multivesicular structures. A permeable anion (e.g. chloride) was required for internal acidification, indicating an electro-neutral proton pump. Specific inhibitors of anion transport (pyridoxal 5'-phosphate and 4-acetamide-4'-isothiocyanostilbene-2,2'-disulfonic acid) totally inhibit proton translocation. The proton pump activity was insensitive to oligomycin, but was completely inhibited by about 5 μ M of the tridentate bathophenanthroline chelate of Fe(II). The activity was also inhibited 100% by low concentrations of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, the proton conduction inhibitor *N,N'*-dicyclohexylcarbodiimide and the ionophore monensin. The UCV fraction contained 2 proteins of M_r 50000 (major) and 54000 (minor) which were phosphorylated by an endogenous cyclic nucleotide- and Ca^{2+} -independent protein kinase.

Endocytic vesicle Proton pump H^+ -ATPase Protein phosphorylation Rat liver

1. INTRODUCTION

Mammalian parenchymal liver cells endocytose macromolecules by receptor-mediated endocytosis [1–9]. Thus, asialoglycoproteins, which are recognized by their galactose terminal oligosaccharide chains [1], are internalized into coated vesicles [4],

and the uncoated endocytic vesicles sequentially formed [4] either fuse with lysosomes, where at least a major part of the degradation takes place [2,4], or interact with the Golgi apparatus as part of the recycling process [4,5], e.g. of transferrin and its receptor [5,6]. These processes have been extensively studied in recent years, and among the proteins studied so far, asialofetuin [3,6], transferrin [5] and asialotransferrin [6–9] should be particularly mentioned. Although acidification (pH $\approx$ 5.0) of endocytic vesicles has been demonstrated to be an early event, e.g. in fibroblasts [10,11], this has not been shown in isolated hepatocytes [12]. It has been found that the endocytic vesicles of rat liver hepatocytes reveal a pronounced morpholo-

Abbreviations: H^+ -ATPase, H^+ -transporting adenosine triphosphatase (EC 3.6.1.34); DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ER, endoplasmic reticulum; NEM, *N*-ethylmaleimide; Δ pH, pH difference across the membrane; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); TPA, 12-*O*-tetradecanoylphorbol-13-acetate

gical heterogeneity [4,8] and a distribution within a broad range of densities [6–9,12] which may explain why these organelles are so difficult to isolate in a pure state from parenchymal liver cells. Since such an isolation is of considerable interest to obtain more information about the endocytotic pathway in these cells, further investigations were undertaken to establish a procedure for the rapid isolation of uncoated vesicles (UCV) with well preserved functional properties. Since it is generally considered that endocytic vesicles have a low internal pH, generated by a membrane-bound H^+ -transporting ATPase (EC 3.6.1.34) [10,11,13], we have used the proton pump activity as the main marker enzyme of UCV in our fractionation studies. Furthermore, it has recently been observed that clathrin-coated vesicles from bovine brain [14–16], bovine adrenal gland [16] and rat liver [14–16] contain a 50 kDa protein which is phosphorylated by a cyclic nucleotide- and Ca^{2+} -independent protein kinase, and its possible use as a marker also for UCV was studied.

2. MATERIALS AND METHODS

2.1. Chemicals

SDS was purchased from ICN Chemicals, (Poole, England). Molecular mass standard protein kit, ATP, acridine orange, 4-acetamide-4'-isothiocyanostilbene-2,2'-disulfonic acid, calmodulin, dithiothreitol, pyridoxal 5'-phosphate, TPA, and monensin were obtained from Sigma (MO) and FCCP was a product of Boehringer (Mannheim, FRG). Phosphatidylserine was from Pharmacia (Uppsala, Sweden). Essentially fatty acid free bovine serum albumin was obtained from Miles Biochemicals (IN). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2000 Ci/mol) was purchased from the Radiochemical Centre (Amersham, England). The tridentate bathophenanthroline chelate of Fe(II) ($\text{BPh}_3\text{Fe(II)}$) was prepared as described in [17].

2.2. Animals and preparation of a microsomal fraction

Male albino rats (Wistar strain, purchased from Møllegaard, Denmark) of 150 g body wt, fed ordinary pellet diet, were fasted for 4 h, stunned and decapitated. The livers were homogenized (2

strokes at 460 rpm) and the crude microsomal fraction prepared as in [18] using 0.25 M sucrose, 5 mM Hepes buffer, 1 mM dithiothreitol and 0.2 mM EDTA as the homogenization medium; pH 7.2. A centrifugal effect of $\int_0^t (\text{rpm})^2 dt = 4 \times 10^9 \text{ min}^{-1}$ was used to sediment the large granule fraction, including a minor lysosomal population with an s value of about 4000 S [18,19].

The crude microsomal fraction (pellet) was resuspended in the homogenization medium and further subfractionated by discontinuous sucrose gradient centrifugation (fig.1) with 7.5 mM Pipes buffer, pH 7.0, containing 1 mM dithiothreitol. The fractions recovered from the gradient were stored in liquid nitrogen, and each aliquot was thawed only once at 25°C, immediately before use.

2.3. Assay of ATPase and proton pump activity

The total Mg^{2+} -ATPase activity was assayed at 25°C as in [20]. The rate at which the pH gradient was generated across vesicular membranes, was measured with acridine orange as a probe [20,21]. The standard incubation medium contained 7.5 mM Pipes buffer (pH 7.0), 50 mM KCl, 0.5 mM dithiothreitol, 5 μg oligomycin, 3 μM acridine orange and the subcellular fraction to be tested (approx. 50 μg protein). Kinetic constants were calculated as in [20].

2.4. Protein phosphorylation

Protein phosphorylation by endogenous kinase(s) was carried out at 25°C in a basal incubation medium containing 150 mM Hepes-NaOH (pH 6.8), 60 mM NaF, 30 mM Mg^{2+} -acetate and 60 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2 $\mu\text{Ci/ml}$). Further additions are given in table 2. The phosphorylated proteins were visualized by SDS-polyacrylamide gel electrophoresis (7.5% acrylamide) [22] and autoradiography [23].

2.5. Assay of marker enzymes and other analytical methods

NADH:glyoxylate oxidoreductase activity was assayed as in [24]. Galactosyl transferase was measured using *N*-acetylglucosamine as acceptor [25]. NADPH:cytochrome *c* oxidoreductase activity was assayed as in [26]. Protein was determined according to Bradford [27] using bovine serum albumin as a standard.

2.6. Morphological analysis

Selected subcellular fractions were processed for transmission electron microscopy as described in [28]. The final magnification was 23 750.

3. RESULTS

3.1. Selection of conditions for density gradient centrifugation

In previous studies on receptor mediated uptake of radiolabelled asialotransferrin in rat liver hepatocytes, an early labelling of particles with a median equilibrium density of $1.11 \text{ g} \cdot \text{ml}^{-1}$ was observed on isopycnic equilibrium centrifugation [6–9]. Fractions collected from such gradients have been found to consist of a mixture of univesicular and multivesicular structures [8], and are considered to represent endocytic vesicles from which the clathrin coat is released. Endocytic vesicles are therefore present in the crude microsomal fraction [3] as obtained by classical differential centrifugation [29].

3.2. Distribution of marker enzymes after discontinuous gradient centrifugation of the crude microsomal fraction

Two discontinuous sucrose gradients were designed to isolate uncoated endocytic vesicles relatively free from other subcellular elements (fig.1). The total microsomal fraction [18] was resuspended in 0.25 M sucrose medium and applied to 2 alternative discontinuous sucrose gradients (fig.1a,

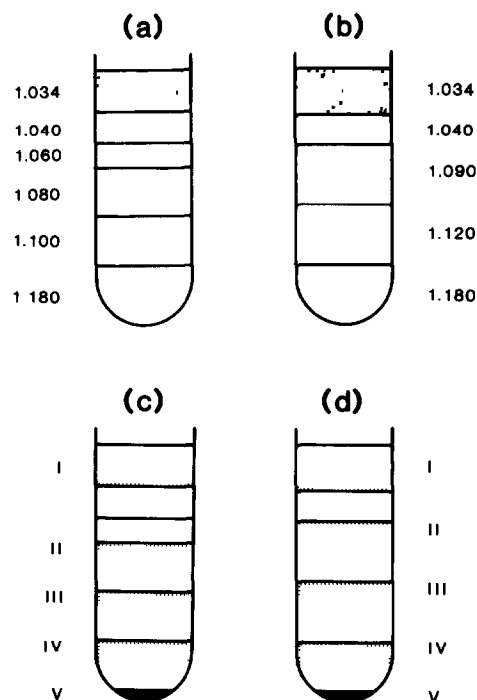


Fig.1. Outline of the procedure to fractionate uncoated endocytic vesicles by discontinuous gradient centrifugation (for experimental details, see section 2). (a and b) Initial state: The crude microsomal fraction was suspended in 5 ml of medium, pH 7.0 and layered over the gradient. (c and d) After centrifugation at a time integral of $\int_0^t \omega^2 dt = 6.6 \times 10^{10} \text{ s}^{-1}$ ($t = 240 \text{ min}$). Fraction I, suspending medium; fraction II, fraction enriched in galactosyltransferase activity; fraction III, uncoated endocytic vesicles; fraction IV, smooth ER; and fraction V, pellet of rough ER.

Table 1

Protein content and relative specific activities of some marker enzymes in representative subfractions of rat liver microsomes obtained by gradient centrifugation (fig.1c)

Fraction number	Protein (mg)	Mg^{2+} -ATPase	H^{+} -ATPase	NAD(P)H: acceptor oxidoreductase		Galactosyl transferase
				NADH: glyoxylate	NADPH: cytochrome c	
II	2.80	n.d.	0	1.00 ^c	0.14	1.00 ^e
III	3.33	1.00 ^a	1.00 ^b	0.87	0.67	0
IV	45.12	0.73	0.03	0.13	1.00 ^d	0
V	19.60	n.d.	0	0.15	0.88	0

The absolute enzyme activities were: ^a $71 \text{ U} \cdot \text{mg}^{-1}$ protein; ^b see fig.2, trace a; ^c $1.30 \text{ U} \cdot \text{mg}^{-1}$ protein; ^d $0.111 \text{ U} \cdot \text{mg}^{-1}$ protein; ^e $0.157\text{--}0.369 \text{ mU} \cdot \text{mg}^{-1}$ protein ($n = 3$) n.d., not determined

b). After centrifugation to equilibrium 4 main sub-fractions were obtained in either case (fig.1c,d), i.e. a pellet of mainly rough ER, a major band of smooth ER at the interface $1.10/1.18 \text{ g} \cdot \text{ml}^{-1}$ (fig.1c) or $1.12/1.18 \text{ g} \cdot \text{ml}^{-1}$ (fig.1d), a minor band of UCV at the interface $1.08/1.10 \text{ g} \cdot \text{ml}^{-1}$ (fig.1c)

or $1.09/1.12 \text{ g} \cdot \text{ml}^{-1}$ (fig.1d), and a minor band at the interface $1.06/1.08 \text{ g} \cdot \text{ml}^{-1}$ (fig.1c) or $1.04/1.09 \text{ g} \cdot \text{ml}^{-1}$ (fig.1d) enriched in galactosyl transferase activity. The UCV fraction of gradient 1 (fig.1c) was found to represent 0.41–0.51 mg protein/g wet wt liver ($n = 4$). The proton trans-

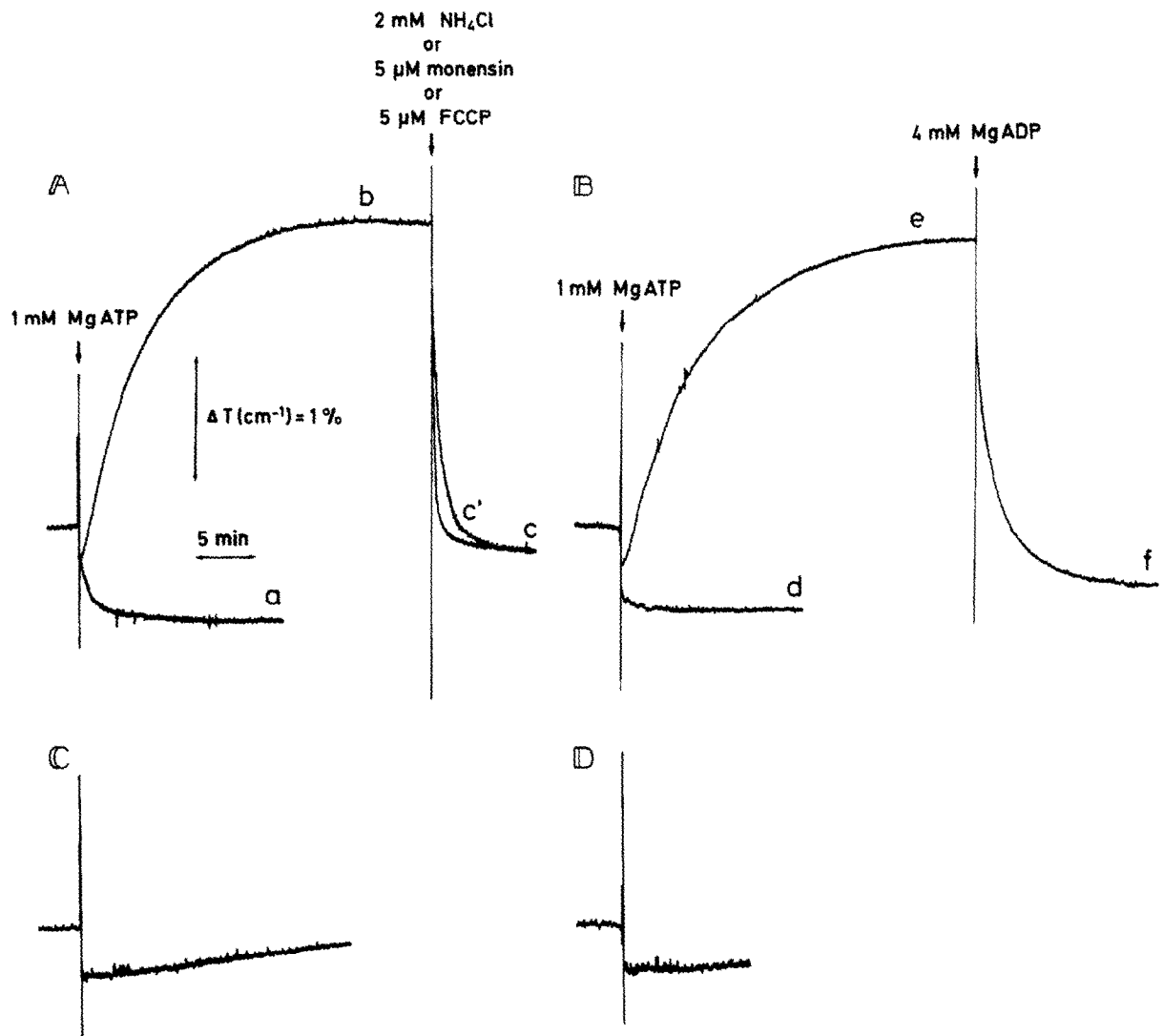


Fig.2. (A) The time course for the change in absorbance of acridine orange induced by 1.0 mM MgATP in uncoated endocytic vesicles in the absence (trace a) and presence (trace b) of 50 mM KCl. The effect of 2 mM NH₄Cl or 5 μM monensin (trace c) and 5 μM FCCP (trace c') is also shown. (B) Complete inhibition of the proton pump by 750 μM pyridoxal 5'-phosphate and 25 μM 4-acetamide-4'-isothiocyanostilbene-2,2'-disulfonic acid (trace d) and reversal of the pH gradient by MgADP (trace f). (C,D) The time course for the acridine orange response induced by 1.0 mM MgATP in the smooth ER fraction (C) and in the fraction enriched in galactosyltransferase activity (D). The protein content per ml reaction mixture was 44 (UCV), 52 (smooth ER) and 53 μg (fraction enriched in galactosyltransferase activity).

locating ATPase activity was rather selectively enriched in the 2 fractions recovered at the 1.08/1.10 and 1.09/1.12 $\text{g} \cdot \text{ml}^{-1}$ density interface in gradient 1 (fig.2) and 2, respectively. However, the contamination of the UCV fraction by smooth ER was higher in gradient 2 (not shown) than in 1 (based on marker enzymes and SDS-polyacrylamide gel electrophoresis), and gradient 1 was selected as a standard procedure for preparation of UCV; the results obtained for some marker enzyme assays are shown in table 1.

3.3. Mg^{2+} -ATPase activity

The overall Mg^{2+} -ATPase activity in the UCV fractions obtained from gradient 1 was found to be $71 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein at 20°C , as compared to $52 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein for the smooth ER (table 1).

3.4. Proton pump activity of the H^+ -ATPase and changes in the steady-state proton electrochemical potential

Although absolute measurements of steady-state pH gradients are preferentially obtained by e.g. isotope distribution methods [30], measurement of initial rates for the generation of the pH gradient ($\Delta\text{pH}/\Delta t$) is preferred for kinetic studies [20,21].

As shown in fig.2, a stable base-line was obtained within 5 min when the UCV were incubated in the standard incubation medium containing acridine orange. However, no proton pump activity could be measured on the addition of 1 mM MgATP in the absence of a permeable anion, notably Cl^- (fig.2A, trace a). On the other hand, in the presence of 50 mM KCl (standard medium) a pH gradient (acid inside) was generated, reaching its half-maximal value about 3.7 min after addition of MgATP (fig.2A, trace b) and a maximum value after approx. 22 min. If NH_4Cl or monensin was added at this point, the pH gradient was immediately dissipated (fig.2A, trace c), whereas the addition of the protonophore FCCP caused a slower decay of the gradient (fig.2A, trace c'). The dependence on anion transport was further substantiated by the 100% inhibition of proton translocation by about $700 \mu\text{M}$ pyridoxal 5'-phosphate or by $25 \mu\text{M}$ 4-acetamide-4'-isothiocyanostilbene-2,2'-disulfonic acid (fig.2B, trace d), which did not affect the overall Mg^{2+} -ATPase activity; half-maximal inhibition of the proton pump was ob-

tained at about 350 and $3.5 \mu\text{M}$ of the 2 inhibitors. The activity was also completely inhibited by about $5 \mu\text{M}$ $\text{BPh}_3\text{Fe(II)}$.

A progressive increase in the rate of proton pump activity was observed over the concentration range of MgATP tested. In one preparation, with a notably high specific proton pump activity, the K_m value for MgATP was found to be $122 \pm 10 \mu\text{M}$ (mean $\pm$ SE).

In contrast, only a very low proton pump activity was observed in the smooth ER fraction (fig. 2C), and none in the rough ER fraction or in the fraction enriched in galactosyltransferase activity (fig.2D).

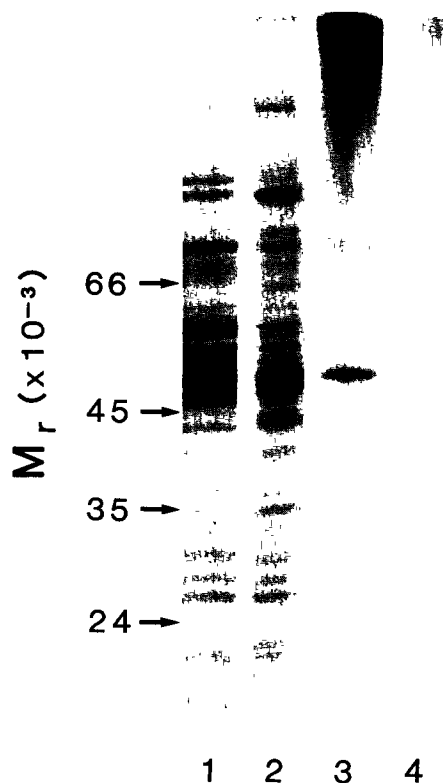


Fig.3. Analysis of phosphorylated proteins by SDS-polyacrylamide gel electrophoresis. The smooth ER (lanes 1 and 4) and the UCV (lanes 2 and 3) fractions ($20 \mu\text{g}$ protein) were incubated in the standard phosphorylating system (see section 2). After 10 min incubation and 25°C , the reaction mixture was analyzed by SDS gel electrophoresis. The gels were stained with Coomassie blue (lanes 1 and 2) and subjected to radioautography (lanes 3 and 4). The positions of standard M_r markers are indicated on the left.

3.5. Reversibility of the H^+ -ATPase

From fig.2B, trace f it is seen that steady-state energization is reversed by the addition of 4 mM MgADP, indicating that the H^+ -ATPase is reversible. This conclusion is further supported by the finding that the steady-state energization was found to be determined by the external phosphorylation potential (not shown).

3.6. Gel electrophoresis and autoradiography

SDS-polyacrylamide gel electrophoresis of the UCV fraction (fig.3, lane 2) revealed a polypeptide pattern different from that of smooth ER (fig.3, lane 1), but with some common bands. It should be particularly noted that the UCV fraction did not contain a prominent band corresponding to an M_r of about 180 000, the M_r of the clathrin subunit [31]. When incubated in the presence of [γ - 32 P] ATP 2 proteins of M_r 50 000 (major component) and 54 000 (minor component) were phosphorylated in the UCV fraction (fig.3, lane 3), but not in the smooth ER fraction (fig.3, lane 4). The phosphorylation of the 50 kDa protein did not seem to be affected by added cAMP, Ca^{2+} + calmodulin, and Ca^{2+} + phospholipid (table 2). On the other hand, the phosphorylation of a minor protein component of M_r about 100 000 was increased by cAMP (not shown).

3.7. Morphological results

The membrane fraction recovered at the interface 1.08/1.10 g·ml of gradient 1 (fig.1c) was shown morphologically to be a mixture of small smooth-surfaced univesicular and a few multivesicular structures (not shown), i.e. similar to that previously reported for a 'light' endosomal fraction isolated from hepatocytes [8].

4. DISCUSSION

It is well established that isolated clathrin-coated vesicles of bovine brain [32-34] contain an ATP-dependent proton pump, and a similar function has recently been assigned to endocytic vesicles of parenchymal liver cells [35]. Such a proton pump accounts for the rapid acidification (pH < 6.0) of the newly formed endocytic vesicle in receptor-mediated endocytosis [10,11,13].

In this study a simple procedure is described for the rapid isolation of a subcellular fraction, highly enriched in UCV with well preserved functional properties, from a crude microsomal fraction of rat liver. The preparations recovered at the interface of sucrose density 1.08 and 1.10 g·ml⁻¹, i.e. close to the median equilibrium density (1.11 g·ml⁻¹) of the early labelling after receptor-mediated uptake of asialotransferrin in isolated

Table 2

Effect of cAMP, Ca^{2+} + calmodulin and Ca^{2+} phospholipid on the phosphorylation of a 50 kDa protein component in the uncoated vesicle fraction of gradient 1

Phosphorylating conditions	Radioactivity in the 50 kDa protein (%) ^a
Standard incubation medium:	
+ EGTA (140 μ M) + EDTA (40 μ M)	100 ^b
+ EGTA (140 μ M) + EDTA (40 μ M) + cAMP (25 μ M)	121
+ Ca^{2+} (0.5 mM) + calmodulin (100 nM)	86
+ Ca^{2+} (0.5 mM) + PS (7.5 μ g/ml)	83
+ Ca^{2+} (0.5 mM) + TPA (25 μ g/ml)	53

For experimental conditions, see section 2; incubation time 10 min

^aThe numbers represent the means ($n = 3$) of the integrated values (arbitrary units) obtained by scanning of the radioautograms (see fig.3)

^bReference value

hepatocytes [6], represent light endosomes [6] and were relatively free from contamination by smooth ER and Golgi elements (fig.3 and table 1). Of particular interest is the finding that the H^+ -ATPase activity was selectively enriched in the UCV fraction (fig.2 and table 1). This is in contrast to a recent study by Rees-Jones and Al-Awqati [36] in which the existence of an H^+ -transporting ATPase in the rough and smooth microsomal membrane fractions was proposed. It should be noted, however, that the resolution of their discontinuous density gradient was less efficient than that of the gradients used in this study, and that rather high concentrations of KCl and $MgCl_2$, which both favour aggregation of subcellular particles [29], were present in the gradient medium. It should also be mentioned that the crude microsomal fraction prepared by our procedure, removed quantitatively even the lysosomal particles of low s value (i.e. about 4000 S) [18,19].

It has been observed that clathrin-coated vesicles from bovine brain [14–16], bovine adrenal gland [16] and rat liver [14–16] contain a protein of M_r 50 000 which is rather selectively phosphorylated by an endogenous cyclic nucleotide- and Ca^{2+} -independent protein kinase. Since the 50 kDa polypeptide co-purified with clathrin, it was proposed to constitute a component of a regulatory system present in coated vesicles [16]. The vesicular fraction isolated by our procedure contains no clathrin of M_r 180 000 detectable by SDS-polyacrylamide gel electrophoresis (fig.3, lane 2). However, the endogenous protein kinase of the UCV fraction phosphorylated 2 proteins of M_r 50 000 (major component) and 54 000 (minor component), i.e. similar to that described for coated vesicles [14–16]. Furthermore, the phosphorylation of the 50 kDa protein in UCV was independent of cyclic nucleotides and Ca^{2+} (table 2). This finding suggests that the 50 kDa phosphoprotein is not a unique component of clathrin-coated vesicles as suggested [16], but has a more general distribution among vesicular structures of the endocytic pathway. The physiological function and mechanism of this phosphorylation reaction remains to be defined by future work.

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REFERENCES

- [1] Ashwell, G. and Hartford, J. (1982) *Annu. Rev. Biochem.* 51, 531–554.
- [2] Gregoriadis, G. (1975) in: *Lysosomes in Biology and Pathology*, (Dingle, J.T. and Dean, R.T. eds) vol. 4, pp. 265–294, North-Holland, Amsterdam.
- [3] Tolleshaug, H., Berg, T., Nilsson, M. and Norum, K.R. (1977) *Biochim. Biophys. Acta* 499, 73–84.
- [4] Wall, D.A., Wilson, G. and Hubbard, A.L. (1980) *Cell* 21, 79–93.
- [5] Stein, B.S., Bensch, K.G. and Sussman, H.H. (1984) *J. Biol. Chem.* 259, 14762–14772.
- [6] Tolleshaug, H. (1984) *Biochim. Biophys. Acta* 803, 182–190.
- [7] Debanne, M.T. and Regoeczi, E. (1981) *J. Biol. Chem.* 256, 11266–11272.
- [8] Debanne, M.T., Evans, W.H., Flint, N. and Regoeczi, E. (1982) *Nature* 298, 398–400.
- [9] Tolleshaug, H., Chindemi, P. and Regoeczi, E. (1981) *J. Biol. Chem.* 256, 6526–6528.
- [10] Tycko, B. and Maxfield, F.R. (1982) *Cell* 28, 643–651.
- [11] Tycko, B., Di Paola, M., Yamashiro, D.J., Fluss, S. and Maxfield, F.R. (1983) *Ann. NY Acad. Sci.* 421, 424–433.
- [12] Berg, T., Blomhoff, R., Naess, L., Tolleshaug, H. and Drevon, C.A. (1983) *Exp. Cell Res.* 148, 319–330.
- [13] Yamashiro, D.J., Fluss, S.R. and Maxfield, F.R. (1983) *J. Cell Biol.* 97, 929–934.
- [14] Pauloin, A., Bernier, I. and Jolles, P. (1982) *Nature* 298, 574–576.
- [15] Pfeffer, S.R., Drubin, D.G. and Kelly, R.B. (1983) *J. Biol. Chem.* 258, 40–47.
- [16] Pauloin, A., Loeb, J. and Jolles, P. (1984) *Biochim. Biophys. Acta* 799, 238–245.
- [17] Phelps, D.C., Nordenbrand, K., Nelson, B.D. and Ernster, L. (1975) *Biochem. Biophys. Res. Commun.* 63, 1005–1012.
- [18] Berge, R.K., Osmundsen, H. and Flatmark, T. (1984) *Eur. J. Biochem.* 141, 637–644.
- [19] Tangerås, A. (1983) *Biochim. Biophys. Acta* 757, 59–68.
- [20] Flatmark, T., Grønberg, M., Husebye, E.S. and Berge, S.V. (1985) *FEBS Lett.* 182, 25–30.

- [21] Husebye, E.S. and Flatmark, T. (1984) *J. Biol. Chem.* 259, 15276–15276.
- [22] Laemmli, U.K. (1970) *Nature* 227, 686–685.
- [23] Døskeland, A.P., Schworer, C.M., Døskeland, S.O., Chrisman, T.D., Soderling, T.R., Corbin, J.D. and Flatmark, T. (1984) *Eur. J. Biochem.* 145, 31–37.
- [24] Crane, F.L. and Low, H. (1976) *FEBS Lett.* 68, 153–156.
- [25] Hymes, P. and Mullinax, F. (1984) *Anal. Biochem.* 139, 68–72.
- [26] Tolbert, N.E. (1974) *Methods Enzymol.* 31, 734–746.
- [27] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [28] Shio, H. and Lazarow, P.B. (1981) *J. Histochem. Cytochem.* 29, 1263–1272.
- [29] De Pierre, J.W. and Dallner, G. (1975) *Biochim. Biophys. Acta* 415, 411–472.
- [30] Nicholls, D. (1982) *Bioenergetics*, pp. 190, Academic Press, London.
- [31] Pearse, B.M.F. (1975) *J. Mol. Biol.* 97, 93–98.
- [32] Stone, D.K., Xie, X.-S. and Racker, E. (1983) *J. Biol. Chem.* 258, 4059–4062.
- [33] Stone, D.K., Xie, X.-S. and Racker, E. (1984) *J. Biol. Chem.* 259, 2701–2703.
- [34] Forgac, M. and Cantley, L. (1984) *J. Biol. Chem.* 259, 8101–8105.
- [35] Saermark, T., Flint, N. and Evans, W.H. (1985) *Biochem. J.* 225, 51–58.
- [36] Rees-Jones, R. and Al-Awqati, Q. (1984) *Biochemistry* 23, 2236–2240.